Characterisation of arbuscular mycorrhizal fungal communities associated with *Hieracium lepidulum* in Central Otago, New Zealand

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Abstract

Alien plants cost the New Zealand economy over $1 billion per annum in lost revenue and control measures, and can modify native plant communities to the detriment of endemic biodiversity. *Hieracium lepidulum* has invaded several regions of New Zealand and is found in high densities among the hills in Central Otago. The roles of microbes are increasingly included in theoretical models of plant invasion, and this study investigates the diversity and spatial structure of a group of ubiquitous organisms, the arbuscular mycorrhizal fungi (AMF), growing in symbiosis with *Hieracium lepidulum*.

Three AMF-specific molecular primer sets were tested to determine their relative sensitivity and specificity for detecting AMF in cultures established from field collected propagules. The optimal primer set was then used to characterise the AMF community associated with *H. lepidulum* in modified subalpine grassland. The fungi from 30 plant individuals within a $1.8 \times 1.8$ m plot were characterised using restriction fragment length polymorphism (RFLP) analysis and cloning. AMF communities colonising individual plants were found to be diverse, uncorrelated with root biomass, and possess significant phylogenetic structure. Nine phylogenetically distinct taxa were defined, with no plant individual possessing more than seven taxa, despite one AMF taxon comprising over 67% of total abundances. Spatial analysis found evidence of significant positive spatial autocorrelation in the identities of AMF colonising neighbouring *H. lepidulum* up to 0.5 m. Spatial clustering was also detected in the distributions of *H. lepidulum* individuals at similar scales, potentially indicating common mechanisms structuring both host and symbiont distributions.

Phylogenetic analysis of the sequence data found evidence that the detected AMF taxa were potentially endemic and widespread generalists, indicating that the success of *H. lepidulum* as an invader is not likely to be the result of facilitation by coinvasive AMF.
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Chapter 1: Arbuscular mycorrhizal fungi and plant invasions

Introduction

The invasion of exotic species is one of the greatest drivers of biodiversity loss worldwide, posing serious challenges for conservation and economic aspirations (Heywood 1989; Sala et al. 2000; Pimentel et al. 2005). Plant invaders drive changes in plant community structure and modify ecosystem processes at several trophic levels (Evans et al. 2001; Mitchell et al. 2006). Understanding the factors that control plant invasions is vital for the formulation of models to predict and effectively manage invasion.

Arbuscular mycorrhizal fungi (AMF) are a functionally important component of plant communities that directly influence belowground interactions between plants, including competition for nutrients and water, through the formation of hyphal networks (van der Heijden et al. 1998c; Fitter 2005; Finlay 2008). Invasive plants have been shown to interact with AM fungal networks, affect their structure, and in some cases drive feedback which promotes further invasion (Reinhart et al. 2003; Callaway et al. 2004; Vogelsang & Bever 2009). Little is known about the distribution, diversity and structures of AMF communities in New Zealand. This thesis aims to investigate these aspects of an AMF community associated with an invasive plant species Hieracium lepidulum (Stenstr.) Omang (Asteraceae) in a New Zealand subalpine grassland ecosystem.

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi are an ancient group of obligate phytotrophs comprising the phylum Glomeromycota (Schübler et al. 2001a). An arbuscule is a “tree-like” fungal structure within a root cortex cell, and the term mycorrhiza is derived from the Greek “mycos” and “rhiza”, literally translated as “fungus-root”. The name refers to the symbiotic relationship where fungal structures form within the roots of plants and hyphae propagate into the surrounding soil in the form of extraradical mycelium. By colonising plant roots, AMF obtain photosynthetically derived hexose which they utilise as their primary carbon source. Evidence of Gomeromycota-like organisms have been found in fossilised plant roots over 400 million years old (Remy et al. 1994; Redecker et al. 2000; Parniske 2008), and it is believed that AMF may have been instrumental in the establishment of land plants, although more recent discoveries also implicate other fungal groups (Bidartondo et al. 2011). AMF are
present in almost every terrestrial ecosystem on earth; it is estimated that up to 90% of plant species are associated with AMF, including members from every major clade within the plant kingdom. The prevalence of the AMF symbiosis further supports the hypothesis that these organisms have had a long history of coevolution with land plants (Helgason & Fitter 2005).

AMF symbiosis
Water and vital macronutrients like phosphorus (P) are often present in limiting quantities in natural environments (i.e., Chapin et al. 1987; Raghothama & Karthikeyan 2005). By extending several centimetres from the plant root, mycorrhizal hyphae are able to access and liberate nutrients that are physically or physiologically unavailable to the plant roots (Ollson et al. 2002; Finlay 2008). The fungal mycelia act as extensions of the host plant’s root system, effectively expanding the soil surface available for exploitation of resources, with hyphal length typically in the order of 1500 cm hyphae cm$^{-3}$ (Ollson et al. 2002). AM hyphae are also able to obtain nutrients by accelerating decomposition and lysing soil microbes (Hodge et al. 2001). In many cases plants do not interact directly with the soil environment, rather they may interact indirectly with soil via their AMF (Smith & Read 2008). Phosphate crosses the fungal plasma membrane and is converted into polyphosphate, bound within vesicles (Javot et al. 2007). Polyphosphate is transported back through the fungal hyphae to the host plant, and through this mechanism AMF are capable of providing up to 90% of their host plant’s phosphorus requirements (van der Heijden et al. 2008). Uncolonised plants have been shown to respond to P gradients in soil with the localised stimulation of root growth and it is believed the AMF exploit this response; AMF colonisation sites may be perceived by the plant as areas of high P, which stimulates an influx of sugar to the root cell (Helgason & Fitter 2009). Polyphosphate molecules are also believed act as transport vehicles for other nutrients, most notably nitrogen in the form of arginine (Govindarajulu et al. 2005). The AMF symbioses comes at a cost to the plant host, with studies demonstrating that the carbon allocation to the AMF can be as much as 20% of net host photosynthate (Johnson et al. 2002). Despite the cost, the benefits to the host plant can be significant, including improved growth (Mosse et al. 1969), protection from pathogens and herbivores (Toussaint 2007; Koricheva et al. 2009) and enhanced drought tolerance (Auge 2001, 2004). Estimates put the carbon cost of hyphae at one to two orders of magnitude below the carbon cost of roots (Helgason & Fitter 2005), which may partially explain the persistence and ubiquity of the symbioses in terrestrial systems.
AMF evolution and development

As obligate symbionts of plants AMF have maintained the ability to form symbioses with most plant species, leading to the Glomeromycota being characterised as generalist organisms. It is becoming evident that some combinations of host and fungus are more compatible than others (van der Heijden et al. 1998c; Klironomos 2003), and that AMF can be adapted to substrate, with local AMF conferring greater benefits to plants in droughty, saline and polluted environments (e.g. Juniper & Abbott 1993; Ruiz-Lozano 2003; Christie et al. 2004). Anthropogenic modification of the environment has been shown to alter the function of AMF assemblages; soil amendments that alleviate nutrient deficiencies can cause AMF to parasitise their host (Bethlenfalvay et al. 1982; Koide 1991). The position of the AMF symbiosis along a parasite-mutualist gradient is therefore contingent on both the biotic and abiotic environment (Crush 1975; Johnson et al. 1997).

In most environments, the root systems of a mycorrhizal host are colonised by several AMF strains, which simultaneously associate with several hosts. Laboratory experiments have indicated that mutual recognition of good “reciprocators” can lead to the formation of distinct suites of AMF associating with particular plant species (Davison et al. 2011; Kiers et al. 2011). This phenomenon has also been observed in the field, with several studies demonstrating that co-occurring species can possess distinct AMF communities (Sykorova et al. 2007; Davison et al. 2011). Geographical isolation has also been shown to influence the symbiosis with local AMF-host combinations showing more extreme growth responses (both positive and negative) than AMF-host combinations of mixed origin (Klironomos 2003).

Evolutionary theory views mutualisms as “reciprocal exploitations”, and predicts that mutualisms should eventually develop into exploitative or parasitic associations due to the selection of “cheating” (Foster & Wenseleers 2006). Two species forming a mutualism can be considered to face the “Prisoner’s Dilemma”, where both members of the symbiosis can benefit from cooperation, but each benefits most by adapting to cheat their partner (Trivers 1971). AMF are believed to be largely asexual organisms (although see Croll & Sanders 2009; Helgason & Fitter 2009), and as a result should be susceptible to the accumulation of deleterious mutations within the fungal genome, a process known as Muller’s Ratchet (Muller 1964; Felsenstein 1974). The asexual mode should result in the AM mutualism being particularly susceptible to cheating by an exploitative host. It has been demonstrated that both cheating within symbioses and Muller’s Ratchet have resulted in the decline mutualistic and asexual groups throughout history (Foster & Wenseleers 2006). The asexual spores of the
Glomeromycota differ from typical eukaryote propagules by possessing multiple nuclei and multiple genomes (Kuhn et al. 2001; Rosendahl & Stukenbrock 2004). These nuclei arrive within the developing spore via cytoplasmic streaming from the mycelium, and it is hypothesised that AMF may avoid Muller’s Ratchet by some selective mechanism operating at the scale of individual hyphae or nuclei (Hijri & Sanders 2005; Jany & Pawlowska 2010). Other aspects of genetic and spatial organisation are also believed to play a role (Sanders 2002; Pawlowska & Taylor 2004; Yamaura et al. 2004; Croll et al. 2009). It is clear that knowledge of both genetic and ecological aspects of these organisms is required to understand the apparent evolutionary stability of the 400 million year AM symbiosis (Kiers & van der Heijden 2006; Jany & Pawlowska 2010; Kiers et al. 2011).

AMF dispersal

Two forms of infective propagules are recognised in AMF, the hypogeous asexual glomerospore (sensu Goto & Maia 2006), and the extraradical hyphae. Wind dispersal of glomerospores has been shown to be important in disturbed arid environments (Warner et al. 1987), and evidence of invertebrate and mammalian dispersal of glomerospores has been found in several ecosystems, with vectors including rodents and the Australian brushtail possum (Trichosurus vulpecula) (Cowan 1989; Janos et al. 1995; Mangan & Adler 2002). In the New Zealand context, the potential for avifauna as dispersal vectors may also be significant (Johnston 2009).

In many systems, the proliferation of AMF extraradicle hyphae is thought to be the dominant pathway for infection of new host plants (Klironomos & Hart 2002). Inter- and intra-specific root systems can become connected via common mycorrhizal networks (CMN), which have been shown to translocate both plant and fungus-derived nutrition (He et al. 2003; van der Heijden & Horton 2009). These networks are hypothesised to be the pathway whereby AMF facilitate the maintenance of plant diversity (Grime et al. 1987; Marler et al. 1999); there is evidence that some host plants improve their fitness at the expense of other plants within the community (Crush 1976; van der Heijden et al. 2008).

Global AMF diversity

To date the Glomeromycota comprises approximately 210 described species (Helgason & Fitter 2009), in 16 genera (www.amf-phylogeny.com) with broad biogeographic distributions. The bulk of data concerning the identity and distribution of AMF have been
gained through morphotyping spores grown in controlled environments (Öpik et al. 2010). The adoption of molecular methods has shown that morphotyping provides poor resolution of species, and is prone to error resulting from environmentally triggered spore phenotype plasticity (Stockinger et al. 2009). The adoption of molecular methods has also resulted in a burgeoning database of AMF sequences from environmental samples, unconnected with any cultures or morphological specimens. As of 1st June 2012, there are nearly 20,000 environmental AMF sequences available on GenBank unattributed to any species (URL: http://www.ncbi.nlm.nih.gov/genbank). While these data are highly suggestive of as-yet uncharacterised diversity, it is difficult to determine the significance of this number in terms of AMF species diversity (Öpik et al. 2006). The most commonly targeted genetic regions include fragments of the nuclear small ribosomal subunit (18S rSSU), and to a lesser extent, the internal transcribed spacer region (ITS) and large subunit (25S rLSU) (Öpik et al. 2010). The multinucleate spores and hyphae of AMF contain multiple genomes, and may express many copies of the gene regions targeted by molecular primers. Efforts to reconcile morphological and phylogenetic species concepts are being made, but are limited by the small number of reference strains that have been successfully isolated and maintained in culture.

AMF diversity in New Zealand

Compared with other kingdoms of life, there is relatively poor understanding of fungal diversity and function in New Zealand’s ecosystems; only a third of New Zealand’s predicted fungal diversity has been discovered, and fungi contain the greatest proportion of “data deficient” species (Buchanan et al. 2012). Morphological studies from the 1970s underpin our understanding of AMF species diversity in New Zealand (e.g. Hall 1977; Johnson 1977; Baylis 1978). There are currently 38 species in the Glomeromycota recognised within New Zealand, two of which are considered naturalised, with the rest having “unknown” or “uncertain” status (Buchanan 2012). Of these, five species had unique spore morphologies found in samples taken from coastal Otago forests (Hall 1977), while the remainder represent spores extracted from field and culture soils that were similar in morphology to those described in the treatise on North American AMF spore morphology by Gerdemann and Trappe (1974). Data concerning AMF molecular diversity from New Zealand have become available primarily via three published studies (Russell et al. 2002;
Russell & Simon 2005; Bidartondo et al. 2011). Each of these studies has revealed phylogenetically distinct sequences that may represent AMF taxa endemic to New Zealand.

Experimental, descriptive and taxonomic investigations of AMF in New Zealand are primarily hindered by the lack of AMF culture collections, and up until recently, the relative high cost of and technical limitations of DNA-based methods. As already discussed, there are important ecological motivations for improving the state of knowledge of AMF in New Zealand. There are also important economic motivations, including the roles of AMF in soil carbon cycling, erosion prevention and as nutrient vectors in agricultural systems (Schwartz et al. 2006; Bonfante & Genre 2010).

**AMF and invasion**

It is clear that many factors are involved in determining whether a plant species becomes invasive, with copious literature investigating the roles of allelopathy, reproductive ability, novel biotic and abiotic conditions, and the escape from specialist herbivores and pathogens (e.g. Torchin et al. 2003; Blair & Wolfe 2004; Mitchell et al. 2006). The known influences of AMF on the relative fitness of individuals within plant communities have led to investigations into the role of AMF in some plant invasions. These include the positive reinforcement of invasive species by enhanced nutrient uptake, protection against root pathogens, and negative feedback on native species through reallocation of soil resources. Isotope studies have demonstrated that dominant plants may subsidise subdominant plants within a community through unequal investment in CMNs, and potentially by the transmission of carbon from one plant to another (Grime et al. 1987).

Glasshouse experiments have shown that the identity of both host and AMF influence the outcomes of the symbiotic relationship in one-to-one situations (van der Heijden et al. 1998b; Helgason et al. 2002), indicating that relative strength of AMF-conferred growth enhancement of invasive plants may vary, depending on the associated AMF. However, studies investigating the AMF communities on invasive plants have found that both facultative and obligately mycorrhizal invasive plant species gain competitive benefit from a broad range of local AMF species (Bever et al. 2001; Pringle & Bever 2008). Marler et al. (1999) found that the exotic spotted knapweed, *Centaurea maculosa* (Asteraceae), grew significantly larger in the presence of native species when local AMF species were also present. Invasive plant species tend to be generalists and it has been hypothesised by Pringle...
et al. (2009) that generalist plants with broad distributions are likely to associate with generalist AMF species.

It has been postulated that in some circumstances plant invasion may be facilitated by coinvasive AMF species (Pringle et al. 2009). AMF are able to be transported in soil, and countries like New Zealand have probably gained many new species of AMF over the two centuries of European colonisation. Introduction of new AMF species may still be occurring, with commercially produced innocula available for importation from several foreign sources. The disruption of indigenous and agricultural systems by exotic cryptic fungal species has already been demonstrated in New Zealand. The colonisation of wilding pine trees (Pinus spp.) by exotic ectomycorrhizal species has facilitated their invasion into agricultural and indigenous vegetation, with huge implications for indigenous plant communities (Dickie et al. 2010).

Until recently, the cryptic lifestyle of AMF has prevented the collection of large scale data on distributions and abundances that are needed to test general hypotheses (Fitter 2005; Öpik et al. 2006; Öpik et al. 2010), however recent studies using next generation sequencing methods are beginning to elucidate the identity of AMF associated with invasive plants over broad geographic regions (Moora et al. 2011).

Hieracium lepidulum

New Zealand’s tussock grasslands, which are managed extensively for high country grazing and conservation values, face threats from several exotic plant invaders. Of particular concern in subalpine habitats is Hieracium lepidulum, one of a number related species that are invasive in New Zealand (Wiser & Allen 2000; Chapman et al. 2004), and elsewhere (Davis 1977; Connor 1992). In its native continental Europe, H. lepidulum generally constitutes a subdominant portion of the plant communities of subalpine and alpine meadows. In New Zealand H. lepidulum can grow at very high densities, and forms almost monospecific patches of several hundred square metres in some locations.

H. lepidulum distribution

H. lepidulum is found in Taranaki in the North Island, and in Nelson, Marlborough, Canterbury and Otago in the South Island. It is a generalist species, found in many habitats including grasslands, scrub, Nothofagus and plantation forests. It is most common in the high country grasslands of Central Otago and Canterbury, particularly in areas that are grazed or
which have been retired from grazing (Rose et al. 1995). *H. lepidulum* is a triploid, diplosporous apomict (Gadella 1992); all seeds produced are likely to be genetically identical to the parent plant. A recent analysis of *H. lepidulum* at several sites in Canterbury and Central Otago led the authors to conclude that the gene flow among populations was very low and that each population was likely to have been colonised by a single dispersal event (Chapman et al. 2004). The analysis also predicted that the Pisa Range in Central Otago was the most likely founding population, probably as a contaminant of pasture seed in the late 19th century (Chapman et al. 2004). Invasion by *H. lepidulum* has negative impacts for both economic and conservation values in Central Otago, including reductions in the relative abundance of palatable species (Scott 1993) and indigenous biodiversity (Rose et al. 2004; Rose & Frampton 2007).

**Mechanisms underlying *H. lepidulum* invasion**

Previous studies of the mechanisms underlying *H. lepidulum* invasion have been unable to fully elucidate how it becomes such an aggressive invader of indigenous communities. In glasshouse trials of competitive ability *H. lepidulum* was found to outcompete native species only under fertile situations, a result that is inconsistent with the field observations of *H. lepidulum* invasion and proliferation in predominantly low nutrient habitats (Radford et al. 2010). In contrast, in field situations, the addition of nutrients results in a significant decrease in *H. lepidulum* cover and density (Radford et al. 2010), and management of *H. lepidulum* and related species can include the application of fertilisers, which favours the growth of more desirable, usually pasture species (Walker et al. 2003). A combination of both herbivore selection and a relatively high resilience to herbivory have been suggested as important mechanism for the success of *H. lepidulum* (Radford et al. 2007), a hypothesis consistent with experimental results and the proliferation of *H. lepidulum* in areas that have experienced historical grazing pressure (Rose et al. 1995). However, studies have also observed *H. lepidulum* invasion into ungrazed vegetation, suggesting that there are other mechanisms influencing *H. lepidulum* success (Rose & Frampton 2007).

**H. lepidulum** and AMF

*Hieracium* species have been shown to depend upon AMF symbionts to maintain their relative abundance in experimental mesocosms (van der Heijden et al. 1998c), and studies
have been conducted to investigate the role of AMF in explaining the invasion of *H. lepidulum* in New Zealand. Significantly greater establishment, cover and biomass has been reported in plants inoculated with field soil extracts, and high abundances of AMF structures associated with the treatment support the hypothesis that the positive growth responses were mediated by AMF (Downs & Radford 2005). The results from two more recent studies are less conclusive. Roberts *et al.* (2009) found no significant change in the competitive ability of *H. lepidulum* when inoculated with AMF spores and Spence *et al.* (2011) found that *H. lepidulum* inoculated with field soils from a *Nothofagus* forest experienced a depression in growth over a 60 day period. In both of these studies methodological bias, including the length of the growing period and the lack of specificity in the AMF treatment, may limit the utility of these results for broader generalisations.

**Thesis structure**

Chapter Two: The Culture and Detection of AMF

**Research Topic One: AMF trap culture**

Known intra- and inter-specific variability in the effects of AMF on plant growth indicates that the use of bulk inocula from field soils may mask complexity within the AMF-host symbiosis in experimental situations. Little is known about the indigenous AMF of New Zealand, and no studies have yet attempted to determine the identity of AMF strains associated with *H. lepidulum*. Furthermore, a lack of cultured specimens of New Zealand glomeromycetes prevents the study of these organisms in controlled environments. In order to investigate the potential of single spore trap cultures as a method of cultivating AMF in a glasshouse environment, the second chapter of this thesis details the isolation and inoculation of trap cultures with AMF associated with *H. lepidulum* in two invaded subalpine plant communities – an indigenous *Chionochloa* tussock grassland and a modified short *Festuca* grassland pasture system.

**Research Topic Two: Molecular detection of AMF**

Despite the recognised complexity and as yet unresolved nature of genetic organisation within AMF species, molecular tools provide an avenue to better understand both the distribution and diversity of AMF in environmental samples. When applied to single spores, or cultures derived from single spores, molecular methods can assist with the attribution of
ribosomal gene diversity to individuals and species. This information can then assist with the more authoritative attribution of genetic diversity from environmental samples to taxonomic diversity. In order to optimise DNA-based detection of AMF from trap culture and environmental samples, Chapter Two will document the experimental process of PCR primer selection, optimisation, and sensitivity, and discuss the implications and potential pitfalls of the chosen methods.

**Research Questions**

1) Is single spore trap culture a viable method for the isolation of AMF associated with *H. lepidulum*?

2) Are the currently available AMF-specific molecular primers equally sensitive in their ability to amplify AMF DNA from soil, spores and root samples?

Chapter Three: Spatial and genetic diversity of AMF associated with *H. lepidulum*

**Research Topic Three: Molecular diversity of AMF from environmental samples**

The development of AMF–specific primers allows AMF diversity from environmental samples to be studied with better resolution than was previously available using morphology-based methods. The molecular methods developed in Chapter Two will be applied to a comprehensive study of the AMF diversity associated with all members of a population of *H. lepidulum* from a spatially restricted area. Chapter Three will also discuss the relative merits of restriction fragment length polymorphisms (RFLPs) to discriminate AMF taxa, the impacts of sampling intensity on detected diversity, and the interpretation of diversity metrics based on genes from multigenomic organisms. Phylogenetic methods will be used to compare the detected sequences with published AMF sequences from New Zealand and abroad.

**Research Topic Four: Spatial diversity in AMF communities**

It has been shown in several studies that a large sampling effort is required to ensure that all AMF taxa within a site are detected. It has also been demonstrated that spatial structure in AMF communities occurs at very small scales (<1 m), if at all (Wolfe et al. 2007; Mummey & Rillig 2008). In Chapter Three, the distribution of the diversity of the AMF community
amongst the plant individuals will be investigated, as will the presence and scales of spatial autocorrelation in the AMF communities.

**Research Questions**

1) What is the molecular diversity associated with *H. lepidulum* at the sample location?
2) Do RFLP-based methods provide sufficient resolution to differentiate AMF taxa?
3) Can phylogenetic methods determine the likely origin of AMF associated with *H. lepidulum*?
4) Are all *H. lepidulum* individuals within the study site associated with the same suite of AMF?
5) Is there any evidence of spatial autocorrelation in the identities of AMF associated with *H. lepidulum*?
6) What are the appropriate levels of replication needed to obtain representative data on AMF community composition at the study site?
7) Is there any evidence of spatial clustering of *H. lepidulum* individuals within the site?

**Chapter Four: Conclusions and experimental recommendations**

The final chapter of this thesis will bring together the findings from the second and third chapters in the context of the current understanding of AMF diversity, and in terms of their role in the invasion of *H. lepidulum* in New Zealand. The chapter will also outline recommendations for further research into AMF in New Zealand, with a focus on questions and methodologies that could shed further light on the relationship between the taxonomy of this cryptic, yet ecologically significant group, and their potential roles in invasion of indigenous plant communities.
Chapter 2: Establishment and detection of arbuscular mycorrhizal fungi in trap culture

Introduction

The recognition of individuals and species is a relatively straightforward matter for sexual organisms — the biological species concept applies, and individuals are typically both physically and genetically distinct. Asexual species can generally be defined using a genetic species concept, and individuals defined on the basis of physically distinct ramets. However, in the case of arbuscular mycorrhizal fungi (AMF), both the physical and genetic extents of individuals are difficult to determine, and as yet no operational species concept exists (Taylor et al. 2000; Rosendahl 2008).

In terms of physical extent, some estimates put the length of AMF extraradical mycelia on the order of metres per cubic centimetre of soil (Olsson et al. 2002), and the biomass of living and dead arbuscular mycorrhizal (AM) hyphae at over 50% of total soil microbial biomass (Read & Perez-Moreno 2003). The AM mycelia are also capable of forming anastomoses between hyphae belonging to the same isolate (Giovannetti et al. 2001), and single isolates are able to colonise many different plant species. In natural systems these anastomoses could produce an “infinite hyphal network” connecting together different plants over large areas (Giovannetti et al. 2004). This physiognomy, as well as the microscopic size and morphologically uninformative nature of both intra- and extra-radical AM hyphae, makes the physical differentiation of AMF impossible on the basis of vegetative structures (Whitcomb & Stutz 2007).

AMF species are known to possess multiple copies of ribosomal (Sanders et al. 1995; Lloyd-Macgilp et al. 1996; Kuhn et al. 2001), protein coding (Viera & Glenn 1990; Hijri & Sanders 2005; Hijri et al. 2007) and non-coding regions (Pawlowska & Taylor 2004). Some studies have indicated that the intra-specific genetic variation may be greater than inter-specific variation, and that species from separate genera may possess identical copies of some genes (Clapp et al. 1999). Whether the AM genomic diversity is contained within identical nuclei with high ploidy (homokaryotic) or whether nuclei contain distinct genomes (heterokaryotic) remains contentious (Kuhn et al. 2001; Rosendahl & Stukenbrock 2004). The coenocytic nature of AM hyphae allows nuclei to freely stream throughout the “individual” (Giovannetti et al. 2001), making it possible for local differentiation in genotype expression, possibly in response to local conditions (Helgason & Fitter 2009). An operational
species concept for AMF requires that the “full range and frequency of sequence variation [...] be obtained for each discrete taxon” (Clapp et al. 1999). However, such a detailed analysis may reveal that the premise of “discrete taxa” does not apply to AMF (Bachmann 1998). For example, it has been suggested that ancient asexual lineages may in fact represent “microspecies”, with each individual “propagating its own genetic heritage” (Coyne & Orr 2004).

Trap culture

Prior to the widespread adoption of molecular methods for diversity studies, total community diversity of AMF was estimated by differentiating AM spores isolated from field or glasshouse soils. This led to conclusions that AMF sporulation could be seasonal, and that some AMF taxa were rarely represented by spores in field soils (Gemma et al. 1989; Sanders 1990). Trap culture is the method whereby individual spores, groups of spores, or other infective AM propagules are inoculated onto a receptive host plant and grown in a controlled environment. Trap culture of field soils, particularly successive rounds using the same media, has been shown to induce sporulation of both recalcitrant and prolific AMF (Smith & Read 2008). Single AMF spores can then be inoculated onto trap plants, allowing the production of infective propagules from a single AMF strain. Single spore trap culture has advantages over other methods of producing AMF inoculum, such as bulk soil and multispore culture, including a reduction in contamination by fungi which parasitise AMF (Brundrett 1991). Although it is a clear simplification of the ecological processes occurring in functional ecosystems, cultures initiated from a single multinucleate spore also provide opportunities to study aspects of intra-“individual” genetic organisation, genomic inheritance to asexual progeny, and functional roles in symbiosis.

There is a large disparity between number of AMF taxa that have been described from morphological specimens, and those that have only ever been recorded as DNA fragments from environmental samples. For every species for which morphological data are available there are over 50 unique “uncultured” sequences available on GenBank (URL: http://www.ncbi.nlm.nih.gov/genbank), and there is evidence of many more to be discovered, particularly in Oceania, where relatively few studies have been carried out (Öpik et al. 2010). It is difficult to interpret this large body of environmental sequence data in an ecological context for two major reasons. The first is the lack of phylogenetic resolution afforded by short (usually ribosomal) DNA fragments, and the second, and perhaps more significant, is
the general lack of understanding about the intra- and inter-specific genetic organisation of the Glomeromycota (Gamper et al. 2009; Öpik et al. 2010). These issues have led several workers to state that the only way to definitively characterise the genetic diversity within species of AMF is through their establishment in culture (Redecker et al. 2003; Gamper et al. 2009; Öpik et al. 2010).

Influence of AMF on *Hieracium lepidulum* growth and competitive ability

By investing photosynthetically derived carbon into AM fungal hyphae, host plants are able to acquire nutrients at a lower carbon cost than by producing roots (Helgason & Fitter 2005). Individuals which invest fewer resources into growth can expend more on other structures and processes, such as reproduction and defence (Berta et al. 1993; Vance et al. 2003; Bennett et al. 2006), and plant species that are able to do this have been shown to be both superior competitors and successful invaders (Blossey & Notzold 1995).

It is possible that the invasion by *H. lepidulum* is facilitated through an association with AMF and both bulk soil trap culture and multi-spore trap culture have been used to inoculate seedlings of *Hieracium lepidulum* in previous studies (Downs & Radford 2005; Roberts et al. 2009; Spence et al. 2011). *H. lepidulum* is colonised by AMF in the field and can be colonised in a glasshouse situation, however the effects of AMF colonisation on *H. lepidulum* growth and competitive ability are inconsistent. Host response has been shown to vary between studies, with some showing enhanced growth, and others showing no effect or suppressed growth. Inoculation of *Pilosella* species, which are closely related to *Hieracium*, with single spore culture AMF strains has produced significant and positive growth responses in glasshouse trials (van der Heijden et al. 1998b).

**Research Questions**

Trap culture establishment

Several sources of bias have been identified to account for the inconsistencies in *H. lepidulum* growth response to inoculation, including the roles of other soil organisms, soil disturbance, AMF selection biased towards sporulating species, and the potential for AMF related benefits to be restricted to a specific stage in the plant life cycle. The relative benefit of an AM symbiosis has been shown to depend on the identity of the AMF strain involved. It may be that interactions between AMF strains and other soil organisms within bulk inoculum are responsible for the variation observed in host response (e.g. van der Heijden et al. 1998a).
In order to test hypotheses of functional diversity within AMF it is necessary to isolate and compare the relative effects of individual AMF strains on a plant host phenotype. *H. lepidulum* has invaded a wide range of vegetation types, and this study seeks to determine whether the AMF colonising *H. lepidulum* vary between *Chionochloa* dominated tussock grassland and a modified *Festuca* grassland in subalpine Central Otago. These two vegetation types were chosen to represent a range of anthropogenic disturbance and plant communities, both of which could be expected to influence the diversity and composition of the AMF colonising *H. lepidulum*. This study uses a single spore culture method to investigate the isolation of monospecific AMF cultures from *H. lepidulum* in its invaded range. Verification of successful trap culture was carried out using a combination of visual inspection of trap cultured roots for AM structures, and AMF specific primers.

**Molecular detection**

Several attempts have been made to design DNA primers specific to the Glomeromycota, and while some have had widespread use, all suffer from major interpretive and methodological limitations, including lack of specificity and bias. The commonly used gene regions for ecological studies include the rDNA genes (Clapp *et al.* 1995; Rosendahl & Stukenbrock 2004), particularly the region of the small subunit (18S SSU rDNA) targeted by the AM1 & NS31 primers (Helgason *et al.* 1998). More recently, two new primer sets have been developed; the ~750 bp 18S SSU rDNA gene fragment primers (Lee *et al.* 2008), and the ~1800 bp SSU–ITS–LSU fragment nested primer set (Krüger *et al.* 2009) (Figure 1). The authors of these primer sets claim that they can be used to amplify all subgroups of AMF while excluding all other organisms. This study aims to optimise the polymerase chain reaction (PCR) protocols for the three primer sets mentioned above, and determine which primer set is best suited for detection of AMF both in trap culture, and for environmental studies, in terms of sensitivity, specificity and phylogenetic resolution.
Methods

Site description

Two mountain ranges were chosen as field sites in Central Otago: the Obelisk access road on the Old Man Range and Locharburn Station on the Pisa Range. These sites were chosen because of good road access and in the case of Locharburn station, and because access had previously been granted to researchers from Otago. The sites were similar in aspect and elevation, and complementary in terms of representing land use and dominant vegetation in subalpine Central Otago. The Old Man Range site has a diverse range of herbs and grasses dominated by narrow leaved snow tussock (*Chionochola rigida* (Raoul) Zotov), and, to a lesser extent, exotic grasses *Anthoxanthum odoratum* L. and *Agrostis capillaris* L. The Locharburn site has had a history of disturbance associated with sheep and cattle farming for over 100 years, as well as pre-European burning. At this site, refugia of *Podocarpus cunninghamii* Colenso. remain on rock outcrops, but the dominant plants are *Festuca matthewsii* (Hack.) Cheeseman, exotic agricultural species *Anthoxanthum odoratum* and *Agrostis capillaris*, with locally abundant *Aciphylla aurea* W. R. B Oliver and *Discaria toumatou* Raoul. Both sites have been invaded by *Hieracium lepidulum*, although the populations at Locharburn are much larger in extent and relative dominance than the Old Man Range populations, which tend to consist of small patches or individuals. It is thought that the Pisa Range may have been the point of entry for *H. lepidulum*, possibly through contaminated pasture seed (Chapman *et al.* 2004).
Propagule collection

Ten kilograms of soil and roots were collected from hapazardly selected *H. lepidulum* individuals to a depth of 10 cm at both field sites. The soil was transported and stored in large sealed low-density polyethylene (LDPE) bags until it reached Dunedin, where it was processed within 24 hours. The samples from each site were pooled together and passed through a 2 cm sieve. Five replicate 100 g sub-samples were taken from each pool and used for spore isolation and seedling inoculation in single spore trap culture as detailed below.

Soil samples collected from the two field sites were also used to establish bulk soil trap cultures, which were grown in parallel with the single spore culture. The bulk soil trap culture were composed of bulk soil, containing AMF spores, hyphae and root fragments. The homogenised soil from each site was combined with an equal volume of autoclaved horticultural sand and potted up in sterile 1.7 L pots, with 12 replicate pots for each site. Approximately 50 surface-sterilised *Trifolium repens* cv. Huia seed were then broadcast sown in each pot. The pots were grown on in a glasshouse with no added nutrients and watered as required with tap water. After 3 months of growth, 100 g of mixed soil and roots were taken from 3 replicates from each site and pooled for extraction of spores. Extracted spores that were obviously free of damage or parasitism were subsequently used for a second round of single spore culture.

Spore isolation

AMF spores were isolated from the field soil in 10 batches using a modified wet sieving method (INVAM, URL: http://invam.caf.wvu.edu) In each batch, 100 g of soil was suspended in 1.5 L of tap water and left for 2–3 hours to let the heavier fractions settle. Due to the presence of lipid bodies within the AMF spores they were expected to float in the water column or be trapped in the meniscus and froth at the water surface (H. Ridgeway, pers. comm. 2010). The liquid fraction was decanted through two stacked sieves of decreasing pore size: 500 µm and 32 µm; the first sieve to catch large pieces of floating organic debris and AMF sporocarps, and the second, smaller sieve to capture AMF spores and fine particles. The fine particles were then washed with tap water, at low pressure to avoid disrupting the spores.

A 20/60% sucrose density gradient was set up in 50 mL centrifuge tubes. Half a teaspoon of the particles from the 32 µm sieve were added to 6 tubes which were centrifuged for 1 minute at 1000 g. A 50 mL syringe with a 100 mm length of low density polyethylene
tube was used to collect the particles suspended in solution and trapped at the sucrose gradient interface. The particles from the 6 replicate tubes were pooled together in a 32 µm sieve and rinsed for 1–2 minutes under tap water and then transferred to a sterile 50 mL centrifuge tube with 20 mL distilled water. Cleaned spores were maintained at 4°C in distilled water for no more than 7 days prior to inoculation.

Figure 2. a), b): Microscope images of a single spore and multiple AMF spores. c) An AMF spore inoculated on to germinating *T. repens* radicle.
Inoculation

*T. repens* seeds were surface-sterilised following the method of Triplett and Barta (1987) and germinated over two days in a sterile Petri dish. Under a dissecting microscope, spores were transferred to a watchglass containing distilled water and cleaned of soil debris with a fine paint brush. Spores were then transferred individually onto the emergent radicle of a surface sterilised *T. repens* seed with a 10 µL micropipette (Figure 2 c). Taking care not to dislodge the spore, the germinating seeds were then carefully planted into sterile 1 mL plastic pipette tips filled with a 5:4:3 mixture of horticultural sand: unfertilised potting mix: pumice screened to <0.5 mm and steam-sterilised for 1 hour at 121°C (Ridgway *et al.* 2006). The clover seedlings were grown for 2 weeks and then the entire pipette tip was transplanted into 1.7 L pots containing 1.5 L of an unscreened mixture of the same media as detailed above, which was also steam-sterilised for 1 hour (Figure 3). Each pot was inoculated with 50 mL of 1 µm soil filtrate from the original field site in order to introduce non-AMF soil microbes. The lower 5 mm was cut off each pipette tip to allow roots to emerge into the pot.

![Figure 3. T. repens inoculated with AMF spore in a 1 mL pipette tip transplanted into 1.7 L pot containing sterile medium.](image)

Additional surface-sterilised clover seeds were broadcast sown into the pots to provide addition root colonisation habitat for the AMF. For each site, 40 apparently viable spores were transferred onto individual clover radicles, making a combined total of 80 pots with 5 uninoculated controls. In order to obtain spores from recalcitrant species that may not have
been present in the field soils a further 40 single spore culture pots from each site were inoculated using spores isolated from bulk soil trap culture after 3 months of growth in culture, for a total of 160 culture attempts. All pots were watered as required with tap water. Trap culture pots were kept 0.3 m apart throughout the experiment to minimise cross contamination.

Detection
After a minimum of 4 months growth, root samples were taken from two positions within randomly selected single spore culture pots: from the circumference, where fine roots were bound together, and in the centre of the root ball at the point of emergence from the pipette tip. Ten centimetres of roots, composed of five 2 cm sections of various diameters, were taken from each location for each pot. Scalpels and forceps were flame-sterilised between each single spore culture pot to avoid cross contamination. The roots were washed in tap water to remove soils particles, placed in biopsy cassettes and cleared by immersion in 10% KOH at 90°C for 15 minutes. The roots were then rinsed 3 times in tap water, and then immersed in a solution of 5% v/v acetic acid with 0.05% v/v Acme black ink overnight. The roots were rinsed of excess ink solution and left overnight in fresh water to improve visibility of fungal structures. The cleared and stained root fragments were systematically screened under 40× magnification transmission light microscopy. The detection of AMF structures, including non-septate hyphae, arbuscules and vesicles, were considered evidence of culture success. Clover roots grown in field soil and the online AM reference database INVAM (URL: http://invam.caf.wvu.edu) were used as positive controls for the staining methods, and as a reference for structures associated with AMF colonisation. A total of 40 inoculated root systems were harvested for staining and microscopic assessment.

Molecular methods
Morphological studies of root fragments are time consuming, and the large number of inoculated pots used in the present study meant that a faster method for screening culture was desirable. The extreme sensitivity of molecular techniques and the existence of AMF-specific primer sets provided an opportunity to test for the presence of AMF simultaneously in several single spore culture pots by pooling together root samples, and extracting and amplifying the pooled root DNA. However, prior to this the relative ability of each primer set to detect AM fungal DNA was tested. Positive controls that were known to contain AM fungal DNA were
extracted from environmental samples of *H. lepidulum*, including some from a previous study of *H. lepidulum* AMF community composition (D. Lyttle unpubl.), and bulk soil trap culture *T. repens*. The primers were the commonly used AM1–NS31 pair (Helgason *et al.* 1998), the “improved” AMF specific primer pair AmL1–AmL2 (Lee *et al.* 2008), and the nested primer set designed by Krüger *et al.* (2009). Upon successful amplification with positive controls, the primer sets were tested to compare their ability to detect the presence of AMF from single spore culture. Sample DNA was isolated using three different methods: single spore culture soil, single spore culture roots and single spores isolated from bulk soil trap culture.

### Soil DNA extraction

Ten gram soil samples were taken from the top 50 mm of single spore trap culture substrate, predominantly under the host root systems. The 20 samples were taken randomly, 10 from each of the sample sites (Old Man and Locharburn). DNA was extracted from each of the pots in 250 mg subsamples using the MO BIO PowerSoil® DNA Isolation Kit following the manufacturer’s “centrifuge” protocol, with a final elution volume of 30 µL.

### Root DNA extraction

Root samples were taken using the same root extraction method described in the morphological study. The roots were sealed in LDPE bags on ice for no more than 4 hours. The roots were then washed 3 times under tap water, and agitated by hand to remove soil particles, followed by a further two washes with distilled water under a dissecting microscope to remove any remaining particles. The roots were then patted dry on paper towels, placed in paper bags, and dried in a 50 °C oven for 16 hours. Samples were thoroughly disrupted in liquid nitrogen using an acid washed mortar and pestle. Upon disruption, 20 mg of homogenous powdered tissue was immediately loaded into a 1.5 mL microcentrifuge tube and suspended in 400 µL Qiagen lysis buffer AP1. DNA was then extracted following the Qiagen DNeasy plant mini protocol, with a final elution volume of 50 µL. For the culture screening experiments, the 20 mg of disrupted root tissue was taken from 10 pooled samples.

### Spore DNA extraction

Three AMF spore DNA extraction attempts were made with spores that were isolated from two bulk soil trap culture pots, both containing soil originating from Locharburn Station. The spores were isolated using the spore isolation method described above. Single cleaned spores
were transferred onto the wall of a 1.5 mL microcentrifuge tube with a fine paintbrush and crushed with a flame sterilised needle. The disrupted spore was then spun down by centrifuge, and suspended in 200 µL lysis buffer AP1 (Qiagen). DNA was then extracted following the Qiagen DNeasy plant mini protocol, with a final elution volume of 30 µL.

**PCR conditions and equipment**

Unless stated otherwise, all PCR mastermixes were composed of 17 µL Thermoprime® Reddymix containing 1.25 U Thermoprime Plus DNA polymerase, 75 mM Tri-HC (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% (v/v) Tween® 20, and 0.2 mM of each nucleotide, 10 pmol of both forward and reverse primers, and 1 µL DNA template for a total PCR reaction volume of 20 µL. All PCR reactions were carried out using a thermocycler (Eppendorf MastercyclerGradient), and the protocols used are listed below (Table 1). PCR optimisation led to the inclusion of 0.02 µL 10% (w/v) bovine serum albumin (BSA) in the AmL1-AmL2 PCR mastermix. The PCR products were loaded on 1% agarose with 1% TAE buffer with ethidium bromide staining (1 µg mL⁻¹). The gels were visualised by gel electrophoresis under ultra-violet transillumination using a 1 kb plus ladder (Invitrogen) as a standard.

**Table 1. PCR thermocycling conditions for each primer pair used in this study**

<table>
<thead>
<tr>
<th></th>
<th>AM1-NS31</th>
<th>NS1-NS4</th>
<th>AmL1-AmL2</th>
<th>SSUmAf–LSUmAr</th>
<th>SSUmCf–LSUmBr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial denaturation</strong></td>
<td>3 min @ 94°C</td>
<td>3 min @ 94°C</td>
<td>3 min @ 94°C</td>
<td>5 min @ 99°C</td>
<td>5 min @ 99°C</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>1 min @ 94°C</td>
<td>1 min @ 94°C</td>
<td>30 sec @ 94°C</td>
<td>30 sec @ 94°C</td>
<td>10 sec @ 99°C</td>
</tr>
<tr>
<td></td>
<td>35 ×</td>
<td></td>
<td>30 ×</td>
<td>30 ×</td>
<td>40 ×</td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
<td>1 min @ 58°C</td>
<td>1 min @ 40°C</td>
<td>40 sec @ 58°C</td>
<td>30 sec @ 60°C</td>
<td>30 sec @ 60°C</td>
</tr>
<tr>
<td></td>
<td>30 ×</td>
<td>30 ×</td>
<td>30 ×</td>
<td>40 ×</td>
<td>30 ×</td>
</tr>
<tr>
<td><strong>Extension</strong></td>
<td>1 min @ 72°C</td>
<td>1 min @ 72°C</td>
<td>30 sec @ 72°C</td>
<td>1 min @ 72°C</td>
<td>1 min @ 72°C</td>
</tr>
<tr>
<td><strong>Final extension</strong></td>
<td>10 min @ 72°C</td>
<td>10 min @ 72°C</td>
<td>5 min @ 72°C</td>
<td>10 min @ 72°C</td>
<td>10 min @ 72°C</td>
</tr>
</tbody>
</table>
Cloning

PCR products were cloned into pCR®2.1-TOPO® TA vector and transformed into chemically competent *Escherichia coli* Top10 to the manufacturer’s instructions (Invitrogen). Transformed cells were screened using blue/white colony screening on 90 mm plates containing Luria-Bertani (LB) agar, 50 µg mL\(^{-1}\) kanamycin and coated with 40 mg mL\(^{-1}\) X-gal. Thirty positive transformants from each sample were transferred onto patch plates. Using the same PCR protocol as was used to produce the amplicons, the 30 clones from each sample were then amplified using 1 µL of diluted transformed cells as template. Transformants that failed to amplify, or that produced bands of a size other than expected, were excluded from subsequent analysis.

RFLP typing

Restriction fragment length polymorphism (RFLP) analysis was used to screen the cloned rDNA fragments. The restriction enzyme *Hinfl* (Roche Applied Science, Mannheim, Germany) was used in 25 µL reactions comprising 5 µL PCR product, 1 U enzyme, 2.5 µL 10× concentration SuRE/Cut™ buffer A (Roche Applied Science, Mannheim, Germany) in 16.5 µL MilliQ H\(_2\)O. Reactions were digested for 4 hours at 37 °C. 10 µL of each reaction was analysed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and visualised by UV transillumination using a 1 kb plus ladder as standard.

Sequencing

Sequencing reactions were either performed on plasmid purifications of the transformed cells using a Qiagen minprep plasmid purification kit, and primers directed against the M13f and M13r promoter regions, or, if direct sequencing was carried out, with the primer set used to amplify the DNA. Sequencing was carried out by the Genetics Analysis Service, Department of Anatomy, University of Otago, using dye terminator sequencing chemistry (BigDye Terminator v. 3.1, Applied Biosystems) on an ABI 3730xl DNA Analyser (Applied Biosystems). Sequence assemblies and alignments were performed using the software GENEIOUS v5.1 (Drummond *et al.* 2010), and sequence identity was verified using BLAST searches of the NCBI database (URL: http://www.ncbi.nlm.nih.gov).
Results

Trap culture

Of the 160 single spore trap cultures initiated, none of the 40 randomly selected samples screened using microscopic methods contained detectable levels of AMF after 4 months of growth. No obvious differences in growth or biomass between the inoculated and uninoculated *T. repens* occur over this period. However as the plants were not destructively harvested so empirical comparisons of biomass were not made. This was a precautionary approach to avoid destroying trap cultures which had successfully been colonised by AMF. All plants were relatively small and showed signs of nutrient deficiency, particularly phosphorus as evidenced by chlorosis of the older leaves. Clover roots grown in field soils were infected with non-septate hyphae, with both arbuscules and vesicles observable (Figure 4). Spore densities were comparable to those present in the field soils, however no attempt was made to quantify this, or to qualify the differences in spore morphology of spores used to inoculate the single spore culture. Of the remaining 120 single spore culture samples, 20 were tested using soil DNA, 15 were tested using root DNA, and the remaining 85 were tested using pooled root DNA of 10 or fewer samples, as detailed below.

Figure 4. Microscopic images of stained AMF structures within cleared *T. repens* roots from bulk soil trap culture. Non-septate hyphae and vesicles as visible as dark regions in the micrographs
AM1-NS31 primers

The AM1–NS31 primer pair successfully produced amplicons of the predicted size for three DNA extractions known to contain AM fungal DNA. Amplification was only successful in the positive control samples that had been diluted 1:10 or 1:100. DNA from *T. reprens* single spore culture roots did successfully amplify, however multiple bands were present, including amplicons of the predicted size, indicating the presence of non-specific amplification, possibly in addition to amplification of AM fungal DNA (Figure 5).

Figure 5. Electrophoresis gel showing amplicons of the predicted size from the positive controls (lanes 1-12, three samples, each at four dilutions), and non-specific amplification from five *T. repens* single spore culture root DNA samples.

The positive control OMR2 was amplified across a gradient of primer annealing temperatures at multiple dilutions in order to increase the stringency of the primer binding and reduce the amplification of non-target DNA (not shown). Amplification of the positive control ceased at 64°C, and this annealing temperature was used in a high stringency PCR to amplify four single spore culture root DNA samples. However, multiple bands were still evident in the single spore culture DNA samples even at this higher annealing temperature, although they included bands of the expected size (Figure 6).
Figure 6. Electrophoresis gel showing a positive control (OMR3, lanes 1-3) and four single spore culture root DNA samples amplified using the AM1-NS31 primer pair using an annealing temperature of 64°C. Arrows mark bands excised for purification and sequencing.

Bands representing amplicons of the expected size were excised from the gel, purified and sequenced to determine their identity. BLAST searches of the excised bands revealed that they were *T. repens* 18S rSSU DNA, indicating non-specific amplification, whereas the positive control was confirmed to be from Glomeromycota (Table 2).

Table 2. Top BLAST search results from direct sequencing of AM1-NS31 amplicons showing sample DNA origin, and accession number, organism and % similarity of closest match. The “best match” was based on the highest bit-score result for each sample, and both bit score and percent identity are given for each match. SSC = single spore culture.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>Band</th>
<th>Best Match</th>
<th>Identities</th>
<th>Sequence organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMR2</td>
<td>+ive control</td>
<td>A</td>
<td>AF437708</td>
<td>758.701 (98.9%)</td>
<td>Glomus sp. Glo3 isolate 20</td>
</tr>
<tr>
<td>OMS9r</td>
<td>SSC (root)</td>
<td>B</td>
<td>EF023302</td>
<td>489.99 (96.8%)</td>
<td><em>Phaseoleae</em> environmental sample clone</td>
</tr>
<tr>
<td>OMS9r</td>
<td>SSC (root)</td>
<td>C</td>
<td>EF023302</td>
<td>489.99 (96.8%)</td>
<td><em>Phaseoleae</em> environmental sample clone</td>
</tr>
<tr>
<td>OMS4r</td>
<td>SSC (root)</td>
<td>D</td>
<td>EF023302</td>
<td>489.99 (96.8%)</td>
<td><em>Phaseoleae</em> environmental sample clone</td>
</tr>
<tr>
<td>OMS4r</td>
<td>SSC (root)</td>
<td>E</td>
<td>EF023450</td>
<td>475.57 (96.0%)</td>
<td><em>Phaseoleae</em> environmental sample clone</td>
</tr>
</tbody>
</table>

AmL1-AmL2 primers

Samples which contained AM fungal DNA (positive controls OMR3, OMR2, 34R, 35R) showed only faint amplification using the AmL–AmL2 primer pair across a range of template concentrations when the “field protocol” described in Lee *et al* (2008) was used. As plant and soil derived compounds can inhibit PCR, it was hypothesised that amplification using the universal eukaryotic rDNA primers NS1–NS4 would produce a template containing the sample rDNA, including AM fungal DNA, while excluding any inhibitory compounds, a
technique used successfully by Lee et al. (2008) during their design of the primers. Amplification using NS1–NS4 was successful, and subsequent nested amplification of the amplicons at 1:100 dilution with the AmL–AmL2 primer pair yielded amplicons of the expected size (Figure 7, lane 3). The nested approach was also applied to DNA extractions from AMF culture attempts with unknown AMF status. As with the positive controls, there was no amplification of any of these samples when the AmL1–AmL2 primers were used following the “field protocol”, however pre-amplification with NS1–NS4 primers produced a template which could be successfully amplified using the AMF specific primers (Figure 7, lanes 4-15).

Figure 7. Electrophoresis gel showing amplicons from AmL1-AmL2 primer pair as nested primers, with the template DNA being 1 µL amplicons diluted 1:100 from a PCR using universal eukaryotic primers NS1-NS4. The gel shows amplicons from five samples, with the first three lanes displaying the positive control (OMR3), and the remaining twelve displaying four individual single spore culture root DNA extractions (OMS20, OMS13, OMS6 and OMS7). The three lanes for each sample correspond to the template dilutions in the NS1–NS4 PCR, from left to right: undiluted, 1:10 and 1:100. The final lane is the negative control.

The nested approach was also used to target AM fungal DNA from single spore culture soil and bulk soil trap culture spore DNA extractions. No amplicons resulted from the spore DNA template, but nested PCR using the AmL1-AmL2 primers produced amplicons of the expected size for the soil DNA samples (Figure 8).
Figure 8. Electrophoresis gel showing amplicons from AmL1-AmL2 primer pair as nested primers, with the template DNA being 1 µL amplicons diluted 1:100 from a PCR using universal eukaryotic primers NS1-NS4. Lanes 1-3 show the absence of amplification for the AMF spore DNA samples, while lanes 4-15 show the predominantly positive amplification of eleven single spore culture soil samples. The final lane is the negative control.

Cloning and sequencing was used to determine the identity of the putatively AM fungal amplicons from root and soil DNA extractions. Representative samples were selected on the basis of a single band of the expected size from the nested AmL1-AmL2 PCR. Thirty positive clones from five samples were screened using RFLP analysis with the restriction enzyme HinfI, and three unique RFLP-types from each sample were selected for sequencing. BLAST searches of the sequence data revealed that all three of the clones from the positive control samples belong within Glomeromycota. However the clones from the putatively mycorrhizal single spore culture roots were revealed to be from the host plant (Trifolium sp.) and associated basidiomycetes (Sistotrema spp.), while the single spore culture soil clones included algae and bryophyte rRNA gene fragments (Table 3). Due to the non-specific amplification resulting from nested PCR of single spore culture samples and the inability to visually differentiate non-specific amplification using gel electrophoresis, an effort was made to optimise PCR conditions for the AmL1-AmL2 primer pair so that nested PCR was not needed. The addition of 0.02 µL 10% (w/v) BSA to each reaction resulted in successful amplification of positive controls under PCR thermocycling conditions which otherwise produced only weak amplification (Figure 9). When single spore culture samples which had been shown to lack AM fungal DNA were amplified in the presence of BSA no non-specific amplification was detected. Furthermore, the optimised protocol was able to successfully
amplify AM fungal DNA from positive controls over a wide range of template dilutions (Figure 10).

Table 3. Top BLAST search results from cloned AmL1-AmL2 amplicons. Sequences were obtained from clones (numbered) or direct sequencing of PCR product (ds). The “best match” was based on the highest bit-score result for each sample, and both bit score and percent identity are given for each match. SSC = single spore culture.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clone</th>
<th>Origin</th>
<th>Best Match</th>
<th>Identities</th>
<th>Sequence organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMR3</td>
<td>4</td>
<td>HIElep (root)</td>
<td>AJ699061</td>
<td>1337.58 (99.9%)</td>
<td>Glomus environmental</td>
</tr>
<tr>
<td>OMR3</td>
<td>1</td>
<td>HIElep (root)</td>
<td>JF414174</td>
<td>1355.62 (99.9%)</td>
<td>Glomeromycota sp. MIB 8384</td>
</tr>
<tr>
<td>OMR3</td>
<td>3</td>
<td>HIElep (root)</td>
<td>JF14191</td>
<td>1324.96 (99.3%)</td>
<td>Glomeromycota sp. MIB 8392</td>
</tr>
<tr>
<td>OMS6</td>
<td>8</td>
<td>SSC (root)</td>
<td>DQ898712</td>
<td>1332.17 (99.9%)</td>
<td>Sistotrema brinkmannii</td>
</tr>
<tr>
<td>OMS6</td>
<td>7</td>
<td>SSC (root)</td>
<td>EF024316</td>
<td>1260.04 (98.0%)</td>
<td>Phaseoleae environmental</td>
</tr>
<tr>
<td>OMS6</td>
<td>5</td>
<td>SSC (root)</td>
<td>DQ898718</td>
<td>1323.16 (99.7%)</td>
<td>Sistotrema farinacium</td>
</tr>
<tr>
<td>OMS13</td>
<td>10</td>
<td>SSC (root)</td>
<td>EF23623</td>
<td>1323.16 (99.7%)</td>
<td>Phaseoleae environmental</td>
</tr>
<tr>
<td>OMS13</td>
<td>4</td>
<td>SSC (root)</td>
<td>EF023244</td>
<td>1243.81 (97.2%)</td>
<td>Phaseoleae environmental</td>
</tr>
<tr>
<td>OMS13</td>
<td>3</td>
<td>SSC (root)</td>
<td>DQ898712</td>
<td>1227.58 (96.9%)</td>
<td>Sistotrema brinkmannii</td>
</tr>
<tr>
<td>LBS6</td>
<td>2</td>
<td>SSC (soil)</td>
<td>HQ246325</td>
<td>1296.11 (98.9%)</td>
<td>Bractracoccus cohaerens</td>
</tr>
<tr>
<td>LBS6</td>
<td>1</td>
<td>SSC (soil)</td>
<td>EF024801</td>
<td>1076.09 (92.9%)</td>
<td>Chlamydomonas rosea</td>
</tr>
<tr>
<td>LBS6</td>
<td>2</td>
<td>SSC (soil)</td>
<td>X80980</td>
<td>1319.55 (99.9%)</td>
<td>Thaumatomonidida environmental</td>
</tr>
<tr>
<td>LBS6</td>
<td>1</td>
<td>SSC (soil)</td>
<td>EU091862</td>
<td>1341.19 (100%)</td>
<td>Leptobryum pyriforme</td>
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<tr>
<td>OMS23</td>
<td>4</td>
<td>SSC (soil)</td>
<td>X80980</td>
<td>1348.4 (99.8%)</td>
<td>Uncultured Banisveld eukaryote clone</td>
</tr>
</tbody>
</table>

Figure 9. A comparison of amplification success of positive control samples using the AmL1-AmL2 primer pairs with (right) and without BSA, with all other PCR conditions unchanged. Lanes 1-4 correspond to the same samples in each gel, with lane 5 being a negative control.
SSUmAf–LSUmAr, SSUmCf–LSUmBr primers
Positive controls were unable to be amplified using the SSUmAf–LSUmAr primers over a range of template concentrations 1, 1:10, 1:100, 1:500, and a range of annealing temperatures (55–60°C). Amplification was not improved by hot start PCR. Despite a lack of detectable amplification via transillumination, the results of the SSUmAf–LSUmAr reaction were used as template (undiluted, 1:10, 1:100) for nested PCR using SSUmCf–LSUmBr primers following the method of Kruger et al. (2009). Similarly, nested PCR failed to produce any detectable amplification.

Molecular screening of single spore culture
The AmL1-AmL2 primer pair were considered to be the most appropriate for screening single spore culture for two reasons: they were the only AMF-specific primers that were able to amplify positive samples while avoiding non-specific amplification in negative samples, and the primer pair was shown to amplify the target region across a wide range of template concentrations (Table 4). The root samples from the remaining 85 single spore culture samples were pooled into 9 samples. DNA was extracted from the pooled root samples and these extractions were used as template for PCR using the optimised protocol either undiluted
and diluted 1:10. None of the 12 reactions showed any amplification at either template dilution, indicating an absence of AM fungal DNA from all pooled root samples.

Table 4. Summary of PCR amplification success of primers for the detection of AM fungal DNA from positive control and culture samples. Positive (+) and negative (-) amplification results are tabulated. SSC = single spore culture, BSC = bulk soil trap culture.

<table>
<thead>
<tr>
<th></th>
<th>AM1-NS31</th>
<th>NS1-NS4</th>
<th>AmL1-AmL2 (nested)</th>
<th>AmL1–AmL2</th>
<th>SSUmAf-LSUmAr</th>
<th>SSUmCf-LSUmBr</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ ive control</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ (w/ BSA)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SSC root</td>
<td>+ (non-AMF)</td>
<td>+</td>
<td>+ (non-AMF)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SSC soil</td>
<td>+ (non-AMF)</td>
<td>+</td>
<td>+ (non-AMF)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSC spore</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Discussion**

**Trap culture**

This study has demonstrated that it is possible to grow and detect AMF from both of the field sites under glasshouse conditions; ten randomly selected bait plants extracted from 24 bulk soil trap culture found to have mycorrhizal structures when studied by light microscopy. However, despite high replication in culture attempts, no AMF structures or AM fungal DNA were detectable in any of the 160 trap cultures inoculated with single spores after 4 months of growth. Previous studies that have investigated AMF diversity using trap culture methods have reported successful colonisation rates of 5% (Hall 1977). This statistic may be illustrative of the large numbers of variables involved in attempting to recreate AMF symbioses in a glasshouse environment. The large number of replicates undertaken in this study was an attempt to ensure successful colonisation in at least some cultures despite this recognised poor success rate. Hall (1977) cites low viability of field collected spores as the primary cause for low colonisation success, as well as the considerable time required for some associations to establish (over a year in some cases). The use of glasshouse propagated bulk soil trap culture spores was an effort to produce spores of a higher quality (i.e., less parasitised) than field collected spores, however with no successful colonisation there can be no definitive conclusion regarding the influence of spore origin or parasitism on culture success. Other potential causes of culture failure may include spore dormancy. Other workers have suggested that AMF propagules can remain dormant for several months, and that a cool treatment is required for successful germination (Gemma & Koske 1988; Tommerup 1992).
Alternatively, it may be that spore germination was successful but colonisation was too localised to be detected using either of the methods employed in this study.

Several factors have been shown to influence culture success, including sterilisation methods, medium type, nutrient availability and host identity. Biocides, particularly Benomyl, have been used to sterilise soils of AM in experimental studies. Concerns of residual toxicity prevented their use in this study. Heat based methods have been compared unfavourably to sterilisation by fumigation or exposure to gamma radiation due to heat induced release of “nutrient flushes” and heavy metals (Trevors 1996). Despite this, in the absence of a gamma source, autoclaving was selected over fumigation to avoid the potential of residual chemical toxicity in the medium. Particle size and bulk density have been shown to significantly influence the production of spores from trap culture, but the medium combination used in this study had previously been found to work (Ridgway et al. 2006). Fertilisation of the trap cultures was avoided, as it is recommended only under signs of extreme nutrient deficiency (chlorosis of young leaves), and nutrient poor conditions favour the establishment of AMF (INVAM, URL: http://invam.caf.wvu.edu). It is unlikely that the choice of *T. repens* as host plant influenced the outcomes of the inoculation attempts, as other authors have reported successful use of *T. repens* (Liu & Wang 2003; Ridgway et al. 2006). In this study, AMF structures and DNA were successfully detected in the roots of *T. repens* from bulk soil trap culture. In studies where single spore cultures have been successfully initiated (using a different host), seedlings were grown for 10–12 days before inoculation (Morton et al. 1993), indicating that the developmental stage of the host roots may be a critical aspect of AMF colonisation not taken into account in this study.

Many methods for extracting spores from soil samples advocate the homogenisation of the soil samples (i.e., (Klironomos et al. 1993)). It is unlikely, therefore, that the process of pooling the soil samples used in this study had any significant negative impact on the viability of the AMF spores. Furthermore, it is considered that the potential benefits from pooling soil samples outweighed any disadvantages. Pooled soil samples could be expected to contain a greater diversity of AMF spores, and therefore a greater range of infectivity. The major disadvantage from pooling soil samples is the loss of spatial resolution, however the spatial distribution of AMF spores was outside the scope of this study.
Molecular methods

The AMF-specific primers tested in this study varied in their ability to amplify positive controls. Amplification success depended on the PCR protocol, and on the concentration of AM fungal DNA present within the template. The AM1–NS31 primers can successfully amplify positive controls, but they were sensitive to template concentration. Undiluted positive controls could not be amplified, suggesting inhibition either from too much DNA, or by some other substance within the DNA extractions. Under PCR conditions where AM fungal DNA is successfully amplified from positive samples, the primer pair was also found to amplify non-target DNA from negative samples, some fragments of which presented as amplicons of the expected size (Figures 4-6). This non-specific binding has been reported from AM1-NS31 in previous studies (Douhan et al. 2005), as has the inability of the AM1–NS31 primers to detect some basal AM groups (Clapp et al. 1995; Helgason et al. 1999). As pooled root DNA samples could be expected to amplify even in the absence of AM fungal DNA the AM1–NS31 primers were considered inappropriate for screening trap cultures.

The AmL1-AmL2 primers were the most successful of the three primer sets tested, being able to amplify AM fungal DNA from environmental root DNA template, and function as nested primers with the NS1-NS4 primer pair. However, when the AmL1-AmL2 primers are used in nested PCR with template lacking AM fungal DNA amplification of vascular plant, fungal and invertebrate rDNA occurred. Furthermore, the amplicons resulting from this non-specific binding were the same size as was expected for AMF gene fragments. Nested PCR using the NS1-NS4 primers was carried out by Lee et al. (2008) to amplify AM fungal DNA from spores and AMF roots. It was also used to test the specificity of the primers against non-Glomalean DNA using template from non-AMF roots and leaves, including T. repens which did not amplify. Lee et al. (2008) reported only very faint amplification of two plant species (Sorghum bicolour and Lilium tigrinum) even under low stringency conditions (annealing at 50 °C). The experiments in this chapter have shown non-specific amplification at least six distinct organisms not reported in their publication, including Basidiomycetes (Sistotrema brinkmannii, Sistotrema farinaceum), algae (Bracteacoccus cohaerens, Chlamydomonas rosae) and a bryophyte (Leptobryum pyriforme). The authors stated that the AmL1–AmL2 primer pair were a solution to the issues of non-specificity detected in the AM1–NS31 primer pair, and that they could be confidently used to characterise environmental samples using non-sequence based approaches such as terminal RFLP. However this study has demonstrated that care must be taken when using non-
sequence based approaches, even when the “improved” AMF primer set is used, as non-specific binding is possible.

The addition of BSA to the mastermix resulted in amplification success with the AmL1-AmL2 primers over a broad range of template concentrations, with no evidence of non-specific binding. The specific action whereby BSA promotes amplification is poorly understood, however several studies of AMF from environmental samples have reported its utility as a PCR additive to improve Taq function (Pivato et al. 2007; Davison et al. 2011). It is recommended that future studies investigating AMF using molecular methods include BSA addition as part of the PCR optimisation process.

The positive control samples could not be amplified using either the SSUmAf-LSUmAr or the SSUmCf-LSUmBr primer pairs, even when a nested approach was used under a range of PCR conditions. Other workers have also reported difficulty with this primer set (H. Ridgway pers. comm. 2011). In their published paper Krüger et al. (2009) state their use of Phusion High-Fidelity DNA polymerase 2x mastermix (Finnzymes, Espoo, Finland) for all of their reactions, however it is unlikely that the polymerase type used in this study is responsible for the lack of amplification (D. Orlovich, pers. comm. 2012). BSA was not used as a PCR additive with these primers, and future studies may find that this will improve their amplification success.

DNA origin

DNA extraction from single spores is routinely reported in molecular studies of AMF, but there are several methods used for disrupting and extracting DNA. The methods used in this study have been successfully used elsewhere (Raab et al. 2005), but others have reported successful amplification of DNA from single spores crushed directly into the PCR mastermix (Lee et al. 2008). The lack of amplification by any of the primer pairs used in this study probably indicates a lack of template DNA, however this was not tested using gel electrophoresis, and more attempts are required to optimise this methodology.

The failure of soil DNA extractions to produce AM fungal DNA in this study is attributed to the failure of the single spore culture to produce AMF structures. The presence of 18S rDNA from other eukaryotic organisms in the sequenced clones shows that DNA was successfully extracted from the soil samples. In the absence of positive amplification of AM fungal DNA this study cannot definitively critique the use of soil DNA samples, although the
relatively high cost of soil DNA extraction kits makes a less attractive approach as a method for screening trap cultures.

The most successful DNA extraction method in this study was the use of dried root tissue. Root DNA based methods are preferable for AMF screening as they are less likely to contain contaminants which can inhibit PCR success, although this study did find evidence of PCR inhibition in undiluted root DNA samples in the absence of BSA. This may be the result of the relatively high proportion of host DNA to AM fungal DNA. Comparisons of the influence of root tissue preservation methods have found that the drying process had a negative effect on AM fungal DNA integrity. Other preservation methods which maintain the hydration of the sample (i.e. freezing) produce DNA of a quality that was comparable to fresh tissue samples (Bainard et al. 2010). Therefore, freezing should be the preferred method of preservation in future studies using molecular based methods to screen trap cultures or isolate AM fungal DNA from environmental samples.

Conclusion

The production of monospecific AMF inoculum is a key step in investigating the functional roles of AMF, including their potential role in the invasion of indigenous vegetation by exotic plant pests. In this study, AMF propagules were successfully extracted from fresh field soils, and from field soil which had been maintained in a glasshouse situation for 3 months, but no attempts to inoculate seedlings with single spores were successful. In the absence of successful cultures no comparisons can be made regarding the identities of AMF from the two field sites. Further studies may investigate culture conditions, including host age at inoculation, the use of pre-germinated AMF spores, and alternative culture media and may thereby improve culture success and allow these questions of functional and genetic diversity to be readdressed. This study has identified a labour saving method for screening multiple culture attempts simultaneously, without the need for labour intensive microscope-based observations of root colonisation. However, under certain PCR conditions evidence of non-specific amplification was found that could not be differentiated from positive amplification, suggesting that care should be taken when using AMF-specific primers for the verification of AMF presence in samples with little or no AM-fungal DNA.
Chapter 3: Spatial and phylogenetic diversity of arbuscular mycorrhizal fungi associated with *Hieracium lepidulum*

**Introduction**

Both biotic and abiotic processes can be reflected in the spatial structures on biological communities (Smith & Wilson 2002). The structures themselves are important components of biodiversity, and a key goal of ecology is the attribution of mechanisms to structures in nature. The organisms that comprise natural communities differ in scale, with each experiencing diverse and scale-dependent interactions with the biotic and abiotic environment. As a result, the processes that produce spatial structures tend also to be scale dependent (Legendre 1993).

At the largest scales, spatial structures can be attributed to the interactions between organisms and large scale processes (such as orogenesis, vicariance and climate). At these scales the abiotic environment drives local similarity by filtering adaptive traits. At smaller scales, particularly within homogenous environments, the distribution of species and individuals are more likely to be determined by biotic factors, such as dispersal, competition and predator-prey interactions (Allen & Starr 1982; Legendre & Fortin 1989). The study of the spatial structures at small scales can therefore indicate the types and strength of biotic processes acting within a community. The scales and extents of spatial structures within communities can be measured by testing the observed distributions against a null model (Legendre 1993).

One such indicator of spatial structures is spatial autocorrelation (SAc), which is a measure of whether nearby localities are be more (or less) similar than expected by random chance. Spatial autocorrelation often occurs because nearby localities are influenced by the same “generating process” (Legendre & Fortin 1989). Epidemics or natural disasters are examples of large scale ecological processes that could be described by SAc, however many smaller scale ecological processes can also produce spatial autocorrelation, generally in the form of patches or gradients. *A priori* knowledge of spatial heterogeneity within a given system is important when testing experimental hypotheses: samples taken from within the scales at which SAc is operating will not be independent and classical statistical tests will reject the null hypothesis more often than is warranted due to violation of underlying assumptions of independence.
The spatial structure and diversity of arbuscular mycorrhizal fungi (AMF) in natural systems have been relatively unstudied (Chaudhary et al. 2008; Dumbrell et al. 2010). Early studies of AMF distributions found evidence of spatial structure at sub-metre scales (Klironomos & Moutoglis 1999; Carvalho et al. 2003), however these studies used the proxy of spore abundance, which is known to vary seasonally and in response to the plant host identity (Bever et al. 1996). There is also evidence that neither the correlation between spore abundance and extraradical hyphal biomass, nor the morphological species concept, are sufficiently resolved to provide meaningful measures of diversity and abundance (Clapp et al. 1995). More recent studies of AMF community structure have utilised deoxyribonucleic acid (DNA)-based molecular methods. Using AMF-specific primers, typically targeting the 18S small subunit (SSU) ribosomal DNA (rDNA) genes, AM fungal DNA can be amplified from environmental DNA samples, typically extracted from either soil or roots. Studies using DNA-based methods to study AMF spatial structure are few, but indicate that the strength of spatial autocorrelation within AMF communities depends on the habitat type under consideration and the scale at which the study is conducted. Significant positive spatial autocorrelation has been found in an AMF community in a temperate grassland environment (Mummey & Rillig 2008). Using 18S rDNA-based methods on soil samples AMF community composition was found spatially structured at scales of < 50 cm, and that extraradicle hyphal lengths were spatially structured at < 30 cm (Mummey & Rillig 2008). Glomus 25S large subunit (LSU) rDNA ribotypes isolated from Hieracium pilosella roots along parallel transects were found to have extents ranging from several centimetres up to ten metres (Rosendahl & Stuken brock 2004). Conversely, no evidence of spatial structure was found in AMF community composition in a calcareous wetland community using 18S rDNA-based methods (Wolfe et al. 2007). The understanding of how AMF communities are structured in nature is important for developing theoretical models to explain the distributions of plants within plant communities, and to understand how AMF can facilitate plant species coexistence.

AMF facilitation of plant species coexistence is recognised as an important factor contributing to the maintenance of diversity and productivity in terrestrial ecosystems (Grime et al. 1987; van der Heijden et al. 1998a; Wagg et al. 2011). However, the relative importance of AMF facilitation for exotic plant invaders remains unclear. Until recently it was commonly believed that AMF were “a swarm of interchangeable types” that could associate equally well with any host plant (Smith & Read 2008). It is increasingly evident that AMF can form distinct communities in the soil associated with specific plant hosts.
(Bever et al. 1996; Eom et al. 2000; Davison et al. 2011). Several case studies have found co-occurring plant species that possess different AMF communities, indicating that some mechanism for preferential association between plant and fungal taxa is operating within plant communities (Helgason et al. 2002; Vandenboornhuyse et al. 2003; Öpik et al. 2008). It is therefore conceivable that invasive plants that form mycorrhizal associations will modify AMF communities within an invaded range, with potential impacts on native biodiversity and implications for more widespread invasion. Invasive plants have previously been implicated as drivers of feedback within the soil microbial communities influencing the “trajectory of invasion” (Vitousek & Walker 1989; Bever et al. 1997; Stylinski & Allen 1999; Vogelsang & Bever 2009).

AMF research in New Zealand is still in its infancy; little is known about the composition and structure of mycorrhizal communities, the number and distribution of AMF species, or the relative specificity and identity of AMF associated with particular plant species. It is unlikely that a single mechanism underlies the success of *H. lepidulum* as an invader of subalpine Central Otago, but research into relative growth performance has identified several avenues for research (Radford et al. 2007), including the potential of AMF facilitation. The purpose of this study is to determine the composition and sub-metre spatial structure of arbuscular mycorrhizal fungi growing on the roots of the invasive forb *Hieracium lepidulum*, and to investigate the potential of AMF facilitated invasion.

*H. lepidulum* forms associations with AMF in the field, but neither the identity of the AMF species involved, nor their functional significance have been determined (Downs & Radford 2005; Roberts et al. 2009). There are two hypothetical scenarios regarding the identity of AMF associated with *H. lepidulum* in its invaded range. It is possible that European AMF species were introduced by colonial New Zealanders via some vector (e.g., bulk soil or contaminated machinery), and that these pre-adapted AMF have facilitated the invasion of *H. lepidulum* into new habitats. Alternatively, *H. lepidulum* may associate with generalist native AMF that are able to form a symbiosis with the exotic plant. An invader whose persistence is facilitated by a specific suite of mutualists is expected to have little variation in AMF community composition between individuals within a homogeneous environment, whereas a generalist suite of AMF symbionts could vary in identity between individuals. This study will use 18S rDNA-based methods to characterise the diversity of AMF taxa associated with *H. lepidulum* individuals within a field site in Central Otago, New Zealand. Phylogenetic analysis will be used to distinguish molecular operational taxonomic
units (MOTUs) and to determine the relationships of the sequences with described species and environmental samples from New Zealand and abroad.

It has been demonstrated that comprehensive sampling of AMF diversity requires intensive sampling effort (Klironomos et al. 1999; Whitcomb & Stutz 2007). In this study, the sampling effort is focussed on exhaustive sampling of the root systems of *H. lepidulum* individuals within a 1.8 × 1.8 m plot, with records made of the physical location of each individual plant. In addition to providing sufficient replication to ensure that the majority of AMF taxa associated with *H. lepidulum* are sampled, this approach also allows the spatial structure of the AMF community to be measured. Determination of AMF spatial structure provides a snapshot of the distributions of individual ribotypes and phylogenetically defined molecular operational taxonomic units (MOTU) at one point in time.

Invasive species can cause positive feedback by modifying the local environment to produce conditions favourable for establishment of their progeny. Kilometre-scale spatial autocorrelation in *Hieracium* spp. cover has previously been observed (Duncan et al. 1997), and this study seeks determine whether spatial autocorrelation is also present in the distributions of *H. lepidulum* individuals at the sub-metre scale. By measuring the positions of individuals within a 10 × 2 m area, this study will reveal whether there is evidence of positive feedback (clustering) by comparing the distributions of plant individuals within a community against a null model of random distribution. The rectangular shape of the sampling area was chosen to make the mapping of the *H. lepidulum* individuals with a total station more systematic, thereby reducing the likelihood of missed individuals, and to reduce trampling of the vegetation.

**Study site**

The Pisa Range is thought to be the point of origin of *Hieracium lepidulum* into the country, probably via contaminated pasture seed from Europe (Chapman et al. 2003). *H. lepidulum* is a prevalent species on the Pisa Range, particularly on Locharburn Station where it has been present for at least 20 years (Chapman et al. 2004). Locharburn Station comprises 4250 ha of low-production sheep and beef grazing on the southern slopes of the Pisa Range. The Station boundaries extend from 300 to 1100 m asl and are bordered by Pisa Station to the west and Locharburn scenic reserve to the east. Site characteristics include low phosphate retention and a cool mesic soil temperature regime (Landcare Research 2011). The underling schist is
exposed in increasingly larger tors towards the upper reaches of the station, and the pale, schist-containing soils are of the Typic Argillic Pallic type. Median annual rainfall for the site is between 700 and 750 mm per annum. The tors appear to have facilitated the persistence of several indigenous tree species, including *Podocarpus cunninghamii* Colenso, *Kunzea ericoides* (A.Rich.) Joy Thomps., and *Phyllocladus alpinus* Hook.f.

The proportion of *H. lepidulum* in the plant communities in the Locharburn station varies with altitude. At 800 masl, the communities are less heavily grazed, tend to include higher indigenous diversity than the lower slopes and *H. lepidulum* is present in relatively low abundances. The plant communities at this elevation are dominated by matagauri (*Discaria toumatou* Raoul), browntop (*Agrostis capillaris* L.), sweet vernal (*Anthoxanthum odoratum* L.) and *Balbinella angustifolia* (Cockayne & Laing) L.B.Moore, with patchily distributed *Aciphylla aurea* W.R.B.Oliv. and *Melicytus alpinus* (Kirk) Garn.-Jones. On the slopes above 800–1000 m *H. lepidulum* forms low meadows with a varying proportions and composition of native and exotic herbs and grasses, predominantly *Poa colensoi* Hook.f., *Leucopogon fraseri* A.Cunn ex DC. and *B. angustifolia*. *H. lepidulum* senesces over winter and can produce several rosettes of leaves from a single, persistent root system. At many of these sites the density of *H. lepidulum* is too high to differentiate between individual plants without disturbing the root systems. The sample site is located -44° 51' 8.26" S, +169° 16' 43.01" E (NZGD 2000) at a height of 800 m asl.

**Methods**

**Sample collection**

In order to ensure that replicate samples were not inadvertently taken from the same individual, samples were taken from a community with obvious spatial separation of *H. lepidulum* individuals. This method was also most amenable for determining the spatial pattern of the plants. The site choice of site was also influenced by the assumption that low sparse vegetation should have correspondingly shallow roots and therefore be easier to sample. To reduce the influence of environmental factors the *H. lepidulum* individuals were sampled from a site with apparently homogenous environmental conditions, although this was not empirically tested. Site selection, therefore, was based upon the density of *H. lepidulum* individuals and the physiognomy, homogeneity and diversity of site vegetation.

Individual plants were marked with tags and counted until a roughly symmetrical area of thirty-six *H. lepidulum* individuals was demarcated. This sampling technique was used to
ensure that all individuals in an area were included and that the interpretation of the spatial structure was not confounded by gaps of unsampled individuals.

A total station (Leica T410) was used to measure the spatial coordinates of each of the marked *H. lepidulum* plants within the sample plot, as well as the location of all individual *H. lepidulum* rosettes from a 10 × 2 m transect within the same community. The coordinate data were managed using Leica Geo Office Tools software.

Extraction of the samples was limited by the practical restrictions on representative sampling in soil environments, namely the fragility and unknown extent of the belowground root structures. A compromise between representation and practicality was reached by excavating a symmetrical 15 × 15 × 15 cm mass of soil centred on the plant. For the purposes of this study, the AMF colonising the roots contained within these soil masses represent the fungal diversity of each plant sample. Plants with neighbours < 15 cm away necessitated the excavation of larger clods with overlapping sample extents to accommodate the arbitrary 15 cm spatial grain. Samples were stored in plastic bags and the spades were scrubbed and rinsed between individuals to avoid soil cross-contamination. The samples were stored at ambient temperature for 5 hours in transit and then stored at 4°C until processed (no longer than 48 hours).

Sample processing

*H. lepidulum* plants and root systems were manually extracted from the soil by manipulation in tap water, with particular care taken to keep the root system intact. Samples were processed individually, with the extracted individual plants transferred to plastic bags and stored on ice. At this stage some neighbouring samples were pooled as it became evident that they constituted single individuals. This reduced the sample count from 36 to 30, which is the minimum sample number recommended to detect spatial autocorrelation using the Mantel test (Legendre & Fortin 1989). Other plant species found within the plant community were similarly extracted, identified and washed in tap water. Subsamples of these species’ root systems were preserved in 50 mL centrifuge tubes containing 70% (v/v) ethanol prior to staining to determine their mycorrhizal status.

Within 24 hours of being removed from soil, the *H. lepidulum* roots were excised from the aboveground structures and washed three times in distilled water to remove all remaining soil particles and unattached root fragments. Any root fragments that were not attached were discarded. The wet weight of the belowground samples were measured,
however the aboveground structures were not weighed for biomass data because of obvious grazing. The number of culms and rosettes per plant were counted as a proxy for aboveground biomass. The washed roots from each sample were then patted dry on paper towels and cut into ~ 2 cm fragments. Root fragments were taken from each sample to from five replicate 100 mg subsamples. The samples were transferred into sterile 1.5 mL microcentrifuge tubes and stored at −18 °C. If insufficient root material was available for five subsamples then fewer subsamples of the same size were taken.

DNA extraction
The sample tubes were immersed in liquid nitrogen and maintained below freezing point while sterile micropestles were used to grind each sample into a fine powder. While still frozen, 500 µL of lysis buffer (Genomic DNA Mini Kit (Plant), Genaid) was added and allowed to thaw while mixing with the micropestle. DNA was extracted from the samples using the Geneaid Plant Mini Kit following the manufacturer’s instruction, with a final elution volume of 30 µL.

PCR conditions
An ~800 bp region of AMF 18S rRNA (SSU) gene was selectively amplified using the AMF specific primer pair AmL1–AmL2 designed by Lee et al., (2008). These primers have been shown to amplify AMF DNA, including basal groups Ambisporaceae and Paraglomaceae, making them more representative than the hitherto most commonly used AMF specific primers AM1 and NS31 (Simon et al. 1992; Helgason et al. 1998; Lee et al. 2008).

Polymerase chain reactions (PCR) were set up in 20 µL volumes comprising: 1 µL DNA template, 10 pmol of each primer, 0.02 µL of 10% (w/v) bovine serum albumin (BSA), and 17 µL of 1.1 x concentration Thermoprime® Reddymix containing 1.25 U Thermoprime® Plus DNA polymerase, 75 mM Tri-HC (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% (v/v) Tween® 20, and 0.2 mM of each nucleotide.

PCR conditions were modified from the “field sample” protocol of Lee et al., (2008), with the initial denaturing step of 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C for 40 seconds and 72°C for 30 seconds with a final extension step of 5 minutes. Reactions were run on 1% agarose gels stained with ethidium bromide and visualised by UV transillumination.
Cloning

Replicate PCR reactions were pooled to produce a representative sample of rDNA fragments from each of the 30 sampled plant root systems. This method of pooling replicate samples has been shown to detect similar levels of AMF diversity to cloning each replicate individually (Renker et al. 2006). There was no attempt to control for variable amplification success in the subsamples, which were pooled in equal volumes. Replicates that failed to amplify were discarded.

The pooled PCR products were cloned into pCR®2.1-TOPO® TA vector and transformed into chemically competent Escherichia coli Top10 following the manufacturer’s instructions (Invitrogen). Transformed cells were screened using blue/white colony screening on 90 mm plates containing Luria-Bertani (LB) agar, 50 µg mL\(^{-1}\) kanamycin and coated with 40 mg mL\(^{-1}\) X-gal.

Positive transformants were picked from each of the 30 cloning reactions and transferred to patch plates. Using the same PCR protocol as described above, between 47 and 70 transformants from each sample were then amplified using 1 µL of diluted transformed cells as template. Transformants that failed to amplify, or that produced bands different to the expected size were excluded from subsequent analysis.

RFLP typing

Restriction fragment length polymorphism (RFLP) analysis was used to screen the cloned rDNA fragments. In total, 1418 amplicons were individually digested with restriction enzymes \textit{Hind}I, \textit{Alu}I, and \textit{Sau}3AI (Roche Applied Science, Mannheim, Germany) in 12.5 µL reactions comprising 2.5 µL PCR product, 0.5 U enzyme, 1.25 µL of 10× concentration SuRE/Cut™ buffer A (Roche Applied Science, Mannheim, Germany) in 8.25 µL autoclaved MilliQ H\(_2\)O. Reactions were digested for 4 hours at 37 °C. A 10 µL aliquot of each reaction was analysed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and visualised by UV transillumination using a 1 kb plus ladder as standard. Observed banding patterns were recorded and categorised using a library of banding-pattern images that were compiled as the samples were processed.

Sequencing

An attempt was made to sequence every unique RFLP type. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen). Sequencing reactions were performed by the Genetics
Analysis Service, Department of Anatomy, University of Otago, using dye terminator sequencing chemistry (BigDye Terminator v. 3.1, Applied Biosystems) with vector primers directed against the M13f and M13r promoter regions. Reactions were sequenced on an ABI 3730xl DNA Analyser (Applied Biosystems). The software GENEIOUS v5.1 (Drummond et al. 2010) was used to simulate the restriction digestion of sequenced DNA fragments, in order to verify concordance between RFLP-types and sequence data.

Staining and quantifying AMF infection
To determine the AMF status of *H. lepidulum* and the other plant species found in the study site, roots from arbitrarily selected individuals were washed and cut into 2 cm fragments, placed in biopsy cassettes (SimPort) and cleared in 10% w/v KOH at 90°C for 10–20 minutes. Thick-rooted species required longer clearing time than thin rooted-species. The samples were then rinsed 3 times in tap water and placed in 1 % HCl for 30 minutes to acidify the roots and improve staining. The samples were stained in 1:1:1 water:glycerine:lactic acid (v/v/v) with 0.05% w/v trypan blue for 12–16 hours. After staining, the root samples were stored in water at 4 °C for at least 48 hours to allow excess stain to leach from the plant tissue. AMF infection rates were quantified for each plant using 10 root fragments that were mounted in parallel on slides and scored at 200 × magnification using the modified line-intersect method (Tennant 1975).

Phylogenetic analysis
The RFLP abundance data and associated sequence data were used to determine the diversity and relative abundance of AMF ribotypes. In GENEIOUS, the software CLUSTALW2 (Larkin et al. 2007) was used to align the sequenced 18S SSU rRNA gene fragments with sequence data from an AMF phylogeny (Schüßler et al. 2001a; Krüger et al. 2012), published sequences from two New Zealand studies (Russell & Simon 2005; Bidartondo et al. 2011), and the closest BLAST matches from the NCBI database (URL: http://www.ncbi.nlm.nih.gov). PAUP* v. 4.0b10 (Swofford 2003) was used to construct a neighbour-joining tree using a general time-reversible model of nucleotide substitution rates, with equal rates of variation. *Endogone pisiformis* was used as the outgroup. PAUP* was also used to construct a maximum parsimony bootstrap tree. The bootstrap support was calculated using a heuristic search and random addition of sequences. The analysis was terminated after 10,000 replicates and the bootstrap replicates were summarised by a 50% majority rule tree. The bootstrap values were
then transposed onto the neighbour joining tree. Following the methods employed by most AMF community studies using molecular methods, the taxonomic units were defined by well supported clades of ≥97% sequence identity.

Functions in the R package PICANTE (Kembel et al. 2011) were used to assess aspects of community phylogenetic structure. The function \(pd\) was used to calculate Faith’s phylogenetic diversity, for correlation with root biomass and above ground structures (Faith 1992). The function `comm.phylo.cor()` was used to measure community phylogenetic structure, using the Jaccard index of co-occurrence, with a null model of phylogeny tip label shuffling (`sample.taxa.labels`), on a neighbour joining tree constructed from representative samples from each MOTU.

**Diversity estimation**

Non-parametric estimators are useful when it is difficult to determine whether scarcity in the dataset is the result of natural scarcity, or could be an artefact of the sampling method (Chao 2004). The Chao method (Chao 1987) is a non-parametric estimator that uses the proportion of rare taxa in the site to predict the number of unsampled taxa:

\[
S_p = S_o + \frac{a_1(a_1-1)}{2(a_2+1)},
\]

where \(S_p\) is the predicted species richness, \(S_o\) is the total number of observed species and \(a_1\) and \(a_2\) are the number of species detected only once or twice in the collection respectively. This bias-corrected form of the Chao equation provides richness estimates unaffected by cases where \(a_2\) is zero (Chao 2004). The statistical analyses for this study were performed in R version 2.13.2 (R Development Core Team 2011b).

A value of Chao and its associated standard deviation was obtained by using the R function `specpool()` in the package VEGAN (Oksanen et al. 2011). Because of the sensitivity of this method to rare species, these extrapolations are highly dependent on the taxonomic resolution used in this study. Analysis using higher similarity phylogenetic clusters would produce correspondingly higher extrapolated taxonomic diversity of the site as more singletons would be represented in the data. Chao diversity was estimated at each number of samples, using the R function `poolaccum()`, in the package VEGAN to show how the estimated diversity was influenced by sampling effort.
Community analysis

Accumulation curves were constructed in order to assess the rate of taxon discovery at the site and to visually represent estimated total diversity. The curves were produced by random permutation of taxon incidence between samples. The analysis was performed using the function `specaccum()` in the package `VEGAN`. In order to determine how the number of clones screened per sample influenced the detection of diversity within the site, the data were resampled under progressively more intensive regimes. Clone identities were resampled from each individual plant to simulate differing levels of sampling effort, with 100 permutations at each level. The analysis was performed using the function `balanced.specaccum()` from the R package `BiodiversityR` (Kindt & Coe 2005). Self-starting species-area models for the species accumulation distribution were constructed using their respective functions in the package `VEGAN`. Three species-area models were constructed:

Arrhenius: \[ k \times n^2, \]

Gleason: \[ \sqrt{k} + z \times \log n, \]

and Gitay: \[ (k + z \times \log n)^2, \]

where \( n \) = number of \( H. \ lepidulum \) individuals sampled and \( k \) and \( z \) are estimated model parameters (\( k \) = the expected number of species in a unit and \( z \) = steepness of the species-area curve). These models were tested using nonlinear least-squares regression with the `nls()` function in the package `stats` (R Development Core Team 2011a) with the relative best fit to the observed data being ascertained using the Akaike information criterion (AIC) (Akaike 1974). The best-fitting model was then extrapolated over a range of more intensive sampling regimes.

In order to compare the relative abundances of the MOTUs present in the site, the clone frequency data were used to construct dominance/diversity plot. The distributions were fitted against four commonly used models: broken-stick (Null model), geometric (preemption), general lognormal, Zipf and Zipf-Mandelbrot. The R function `radfit()`, in the package `VEGAN`, was used to fit the models to the data, and find the best fitting model according to the (AIC).
Spatial analysis

The spatial structure of the AMF community was tested using Mantel tests (Mantel 1967) comparing the AMF taxa (defined by either RFLP or MOTU) between plant root systems at varying distances. Dissimilarity matrices of AMF data were constructed for taxon incidence (Jaccard index) and standardised abundance (Bray-Curtis index). Following Sturges’s rule (Sturges 1926), a matrix of Euclidean distance between samples was binned into equally sized “distance classes”, which contained a variable numbers of sample pairs. This method produces correlograms which are easier to draw and interpret (Legendre & Fortin 1989). The two largest distance classes were excluded from further analysis because they contained too few pairs to be statistically valid (Legendre & Fortin 1989). A correlation coefficient was calculated to test for an overall spatial structure in the data using function `mantel()` in the R package VEGAN (Oksanen et al. 2011). In order to test for statistically significant correlation between samples within defined distance classes, correlograms were constructed with the R function `mantel.correlog()` in VEGAN. The correlograms indicate the scale and magnitude of the autocorrelation present in the community data. Progressive Bonferroni correction was applied to account for multiple significance testing. An underlying assumption of this analysis is stationarity i.e., that the site has a fixed diversity with no immigration or emigration (Legendre & Fortin 1989).

Point pattern analysis was applied to test the distribution of *H. lepidulum* individuals within the 10×2 m quadrat against a null model of random (Poisson) distribution using Ripley’s K-function (Ripley 1977) `Kest()` in SPATSTAT (Baddeley & Turner 2005), with confidence intervals produced by `varblock()`. An isotropic correction was applied to account for bias resulting from the unobserved points outside the sampling area (Ripley 1988).

Results

Validation of extraction methods

AM fungal DNA was successfully extracted and amplified from all but one of the 147 subsamples of *H. lepidulum* roots. Due to a scarcity of root material from 3 plants, and a single failed amplification, 4 of the 30 plant samples were represented by 4 (as opposed to 5) pooled subsamples. Amplification strength was variable both within and between samples, potentially indicating variation in DNA template concentrations between samples (Fig. 1).
This may reflect AMF biomass within the roots, however amplification strength cannot be quantified using this PCR method.

Figure 1. Electrophoresis gel showing products from the amplification of 4 samples (28, 14, 29 and 34), with subsamples indicated by the letters a–e. The 1 Kb Plus ladder shows that the amplicons are the expected size (~750 bp), although they vary in brightness between and within samples.

Validation of cloning methods

Between 45 and 64 positive transformants were successfully reamplified from each sample using the AML1 and AML2 primer pair. Putative non-AMF DNA fragments of ~500 and ~1000 bp occurred in 1.7% of positive transformants (Fig. 2) and these were excluded from subsequent analysis on the basis that they were likely to represent non-specific amplification.

Figure 2. Electrophoresis gel showing an example of the variation in amplicon size produced from cloned DNA fragments from sample 14. Clones 19 and 27 were excluded from the RFLP analysis.
**RFLP analysis**

The positive transformants were differentiated using RFLP analysis. The restriction enzymes differed in their ability to detect variation between the clones, with *Hinf*I finding the most diversity (18 unique RFLPs), and *Alu*I (14 unique RFLPs) finding the least (Fig. 3). Both *Hinf*I and *Sau*3AI were able to discriminate between several of the most abundant RFLP-types, whereas *Alu*I predominantly produced only 2 banding patterns (Fig. 4. a–c). Considered together, the analysis of 1421 clones from 30 plants found 53 unique RFLP-types (Appendix 1, Supporting data 1-3).

![Figure 3. Three RFLP-type accumulation curves, with 95% confidence intervals, each demonstrating the total diversity and rate of discovery of new RFLP types by each of the endonucleases used in this study: *Hinf*I (blue), *Sau*3AI (red), and *Alu*I (green).]
Figure 4. An electrophoresis gel demonstrating the variation in discriminatory ability shown by the three endonucleases *HinfI* (top), *Sau3AI* (middle), and *AluI* (bottom), on the same 9 sequences (sample 1, clones 19-27).

Figure 5. Dominance/diversity plot of RFLP abundance (note log scale). The distribution of abundance data shows extremely high relative abundances of the most common taxa and a large number of “singleton” RFLP-types, which were only detected once at the site.
The RFLP analysis revealed a highly uneven community of AMF within the roots of *H. lepidulum*, with the majority of the clones (892/1421) found to be of a single RFLP-type (type 633), and only one other RFLP-type that was represented by more than 10% of the clones (type 111). A large proportion (68%) of the total RFLP diversity was comprised of RFLP-types that were detected only once at the site (Fig. 5). These singleton RFLP-types were not confined to particular samples, but distributed throughout the site.

Phylogenetic analysis of sequence data

Diversity measured by phylogenetic analysis was lower than diversity detected by the RFLP analysis due to high sequence similarity between several RFLP types. Attempts to sequence all unique RFLP types were made, however the majority of the clones representing singleton RFLP types could not be sequenced. As a result, some putatively rare taxa were not represented in the later analyses. SSU fragment sequences were produced for 69% (36/52) of RFLP patterns found, representing 98.9% (1405/1421) of all colonies analysed. Highly supported terminal clades were used to define phylogenetic clusters, following the method of Helgason *et al.* (1999). Retrospective comparison of RFLP-type diversity measures shows a degree of consistency between the RFLP banding patterns and the clusters defined by parsimony analysis, however some of the RFLP-types could not be reproduced *in silico* using virtual restriction digestions of the sequence DNA, potentially indicating further uncharacterised diversity. The RFLP-types whose sequences constituted single phylogenetic clusters generally differed by a single restriction enzyme RFLP, usually *Hinfl*.

In order to determine the phylogenetic relationships between the RFLP-types detected in this study and elsewhere a neighbour joining tree annotated with bootstrap values from a maximum parsimony bootstrap analysis was constructed (Figure 6). The tree includes described AMF taxa sequences from an AMF rDNA phylogeny (Schüßler *et al.* 2001b), AM fungal rDNA sequences from two published New Zealand studies, and the most similar sequences from around world as defined by a BLAST algorithm search (Altschul *et al.* 1990) against the NCBI and MAAJAM databases (URL: http://blast.nebi.nlm.nih.gov; http://maarjam.botany.ut.ee) were also included (Table 2).
Table 2. Top BLAST search results of representative sequences from the MOTUs found in *H. lepidulum* root systems, showing NCBI accession number and percent similarity.

<table>
<thead>
<tr>
<th>MOTU</th>
<th>Best Match</th>
<th>Identities</th>
<th>Host Plant</th>
<th>Collection Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlomA02</td>
<td>AJ699069</td>
<td>751/753 (99.7%)</td>
<td><em>Marchantia foliacea</em></td>
<td>New Zealand</td>
</tr>
<tr>
<td>GlomA04</td>
<td>JF414177</td>
<td>751/751 (100%)</td>
<td><em>Marchantia foliacea</em></td>
<td>New Zealand</td>
</tr>
<tr>
<td>GlomA06</td>
<td>JF414176</td>
<td>743/754 (98.5%)</td>
<td><em>Tmesipteris tannensis</em></td>
<td>New Zealand</td>
</tr>
<tr>
<td>GlomA05</td>
<td>JF414191</td>
<td>751/754 (99.6%)</td>
<td><em>Symphyogyna subsimplex</em></td>
<td>New Zealand</td>
</tr>
<tr>
<td>GlomA03</td>
<td>JF414180</td>
<td>749/753 (99.5%)</td>
<td><em>Phaeoceros carolinianus</em></td>
<td>New Zealand</td>
</tr>
<tr>
<td>GlomB01</td>
<td>FN869808</td>
<td>758/760 (99.7%)</td>
<td><em>Psoralea bituminosa</em></td>
<td>Spain</td>
</tr>
<tr>
<td>Acau02</td>
<td>FN825900</td>
<td>750/755 (99.3%)</td>
<td><em>Plantago lanceolata</em></td>
<td>Scotland</td>
</tr>
<tr>
<td>Acau01</td>
<td>EU332730</td>
<td>726/728 (99.7%)</td>
<td><em>Miscanthus sinensis</em></td>
<td>South Korea</td>
</tr>
<tr>
<td>GlomA01</td>
<td>FR848612</td>
<td>745/753 (98.9%)</td>
<td><em>Glycine max</em></td>
<td>United Kingdom</td>
</tr>
</tbody>
</table>

Backbone support of the 50% parsimony bootstrap tree was high at family level, with Glomeraceae and Diversisporaceae emerging as well-supported sister clades. The genus *Rhizophagus* was monophyletic, with 73% bootstrap support, and was nested within a larger, well-supported (100% bootstrap support) clade containing sequences from environmental samples. The *Rhizophagus* clade contained a single sequence (GlomA01) from this study, which was detected only once at the site, and was most closest related to an environmental sample from soybean (*Glycine max*) roots from the United Kingdom (Table 2). The most abundant phylotype detected in this study (GlomA02) formed a clade with environmental sequences from New Zealand, Ecuador and the USA. Sister to this clade was a well supported (91%) monophyletic group containing GlomA03, along with a New Zealand environmental sample from the bryophyte *Phaeoceros carolinianus*. The second most common phylotype from this study (GlomA04) formed a well supported monophyletic group with environmental samples of New Zealand origin. This was nested within a strongly supported (98% bootstrap support) clade of environmental samples from China and the USA, which has an unresolved placement within Glomales, and no closely related, named species. Sequences of *Glomus macrocarpum* were monophyletic within a strongly supported (98% bootstrap support) clade of environmental samples. The placement of the phytotype GlomA05 was poorly resolved within this clade, although there was weak support (72% bootstrap support) of a relationship with New Zealand environmental samples. The forth most common phylotype (GlomA06) formed a well supported monophyletic group (95% bootstrap support), containing only sequences from this study, nested within environmental samples from Ecuador. A clade containing sequences of the genus *Claroideoglomus* was well supported (98% bootstrap support), however the relationships between sequences from described species and the environmental samples was poorly resolved. GlomB01 formed a
polytomy with environmental samples from New Zealand, Spain and the United Kingdom. Two sequences were placed within the clade (100% bootstrap support) containing sequences for Acaulosporaceae. Acau01 was placed within a strongly supported clade (100% bootstrap support) containing sequences for *Acaulospora longula* and environmental samples from South Korea, while the placement of Acau02 within Acaulosporaceae was poorly resolved.

On the basis of this phylogeny, nine AM fungal MOTUs from at least four genera are proposed: *Rhizophagus*, *Funneliformis*, *Claroideoglomus* (Glomeraeaceae) and *Acaulospora* (Acaulosporaceae) (Schüßler et al. 2001a; Krüger et al. 2012), with evidence of another strongly supported taxonomic group not characterised by any type specimens (GlomA04), but which were identical to sequences obtained from the thallus of the liverwort *Marchantia foliacea* from a New Zealand podocarp/broadleaf forest.
Figure 6. Neighbour-joining phylogenetic tree based on concatenated ribosomal small subunit (18S SSU) gene fragments of Glomeromycota with 50% majority rule bootstrap values and outgroup (Endogone pisiformis). Sequence data include AM fungal DNA isolated from *Hirvicoccus lepidulum* root samples (orange labels), with the closest matches according to a BLAST algorithm search of the NCBI database, an AFM rDNA phylogeny from Schüßler et al. (2001), and all known fungal rDNA SSU sequences from New Zealand (green labels). Branches with less than 50% bootstrapping support were not labelled. Well supported clades of ≥ 97% similar sequences have been labelled to indicate molecular operational taxonomic units (MOTUs) in this study, and the phylogenetic tree has been divided to indicate order and genus level distinctions.
Despite repeated efforts to sequence them, 19 unique RFLP patterns remain unidentified. It is unknown to what extent these represent artifacts introduced via polymerase errors, mutational hotspots, cloning of heteroduplexes or chimeras (Patel et al. 1996; Speksnijder et al. 2001), or whether some or all of them represent rare taxa. The 19 unidentified RFLP types account for 1.3% of the total clones screened in this study.

**Plant-level variation in AMF communities**

Individuals plants differed in the number of AM fungal MOTUs detected in their root systems (Figure 7). While both the richness and evenness of the AMF communities varied between individual plants, it is not possible to test for variation within each plant as the replicate root DNA extractions were pooled together. It can be seen that AMF were detected in the roots of every plant within the community, however no individual possessed all nine MOTUs detected at the site. Of the 30 plants sampled, the most prevalent AMF taxon, GlomA02 (67% of site clone abundances), was present 28 of the 30 plant samples. The two samples which did not possess GlomA02 were the only samples where GlomB01 was detected. Sequences placed within Glomeraceae were the most prevalent at the site, colonising between 67–87% of individual plant root systems, apart from GlomA01, which was detected only once. The members of Acaulospora were comparatively rare at the site: Acau02 colonised 33% of samples and Acau01 was detected only once.

![Figure 7. Histogram showing the frequency of which a given number of AM fungal MOTUs were detected in individual H. lepidulum root systems](image_url)

There was a significant negative correlation ($p = 0.03$) between MOTU co-occurrence and phylogenetic distance within the site, indicating that the communities were less closely related then expected at random (phylogenetic overdispersion). No significant correlation was
found between AMF Shannon or phylogenetic diversity and the size of the host root system. Similarly, the proportion of total root system sampled for DNA extraction was not significantly correlated with AMF diversity.

**Rank abundance analysis**

MOTUs defined by strongly supported phylogenetic groups may be more representative of biological species than RFLP-based diversity measures. Under a niche-based model of species coexistence the relative abundance data represent the relative proportions of resources utilised by each taxa. In order to determine the relative abundances of the MOTUs within the site, and to test the distribution against model distributions, a dominance/diversity plot was constructed (Figure 8).

![Dominance/diversity plot](image)

**Fig 8.** Dominance/diversity plot of site MOTU relative abundance distribution overlaid with model distributions. The lognormal distribution (bold green line) was the best model fit of the observed data (AIC = 100.9).

Ecological interpretation of dominance/diversity plots is underpinned by an assumption that the relative abundance of each phylotypes is directly proportional to the size of the niche it occupies. Under the null model, the niche space is sequentially divided into successive niches, with the probability of division proportional to the size of the niche – the “broken stick” model of MacArthur (Macarthur 1957). The Akaike information criterion (AIC) found that of the models tested, the rank abundance data is best fitted by a log-normal distribution. From an ecological perspective, this distribution can be produced by a conceptually similar mechanism to the broken stick model, except that the probability of niche division is
independent of niche size (Wilson 1991). Several other ecological and statistical processes are also recognised to produce log normal distributions.

Extrapolation of site diversity

The MOTU data were used to produce a taxon accumulation curve for the site (Fig. 9). When a sampled population is static, without immigration or emigration, repeated sampling will reach an asymptote of total population richness. It can be seen from the shape of the observed species accumulation curve (S) that after 10 host plants were sampled the new phylotypes were detected at a fairly stable rate.

Figure 9. Observed species accumulation curve (S) and Chao estimates of total AMF phylotype richness for the sampled site, including 95% confidence intervals.

Alongside the taxon accumulation curve is the extrapolated accumulation curve based on the Chao estimator. This estimator is a representation of taxon accumulation of the observed taxa, as well as taxa which were probably present within the site but not detected. Unlike the observed taxon accumulation curve, the Chao diversity estimate is an indication of the estimated sampled and unsampled diversity at a given sampling effort. Due to the strong influence of rare species (singletons and doubletons) on the Chao estimate of total site diversity, the size of the statistic is highly dependent on how taxonomic units are defined. When the taxonomic units comprise unique RFLP-types (n = 56), of which the majority were singletons, the site diversity estimate is 379 taxa. When MOTUs are used (n= 9) the site diversity is estimated to be 11 taxa (Fig. 9).
Spatial analysis

In order to detect spatial structure in the AMF communities, and to determine how spatial structure was influenced by taxonomic resolution, Mantel tests were calculated using RFLP data, and MOTUs defined by either 97% or 98% sequence similarity (Table 3).

Table 3. A comparison of the size and global significance of the Mantel test of spatial structure in the AMF community data using either incidence or abundance based similarity indices. * indicates significant values, ** indicates highly significant values

<table>
<thead>
<tr>
<th>Taxonomic unit(s)</th>
<th>Mantel statistic (r)</th>
<th>Incidence data</th>
<th>Abundance data</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP types</td>
<td>0.30**</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>RFLP types (ex singletons)</td>
<td>0.28**</td>
<td>0.16*</td>
<td></td>
</tr>
<tr>
<td>98% similar MOTUs</td>
<td>0.26**</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>97% similar MOTUs</td>
<td>0.063</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

The distributions of both RFLP types and 98% similar MOTUs showed highly significant positive spatial autocorrelation when incidence data were used. Where clone frequency was included, the spatial autocorrelation decreased, and remained significant only for RFLP types in the absence of singletons. It would be expected that the removal of the RFLP singletons would have a negligible effect on size or global significance of the Mantel statistic (r), as it was calculated using rank similarity based indices. The merging of MOTUs at the 97% similarity level resulted communities defined by six, rather than nine distinct MOTUs due to the collapse of GlomA01, GlomA02 and GlomA03 into a one MOTU, and GlomA05 and GlomA06 into another. When the AMF communities were defined by 97% sequence similarity, no significant spatial autocorrelation was detected.

A correlogram at 98% MOTU similarity was constructed to determine the spatial extent of spatial autocorrelation in the AMF communities (Figure 10). The first and third distance classes (< 0.1, 0.27-0.44 m respectively) both contain pairs of samples which are significantly more similar than expected at random. The significant similarity found in the smallest distance classes may be expected purely on the basis of the sampling methodology, as sample pairs within this distance classes were taken within overlapping sampling extents.
Figure 10. Correlogram showing the correlation over distance of Jaccard similarity in the AMF phylotypes colonising *H. lepidulum* individuals, using 98% sequence similarity to define MOTUs. Filled squares indicate significant deviations from the null model after correction of \( \alpha \) for multiple significance testing.

The third distance class, between 0.27-0.44 m contained 142 sample pairs, and the AMF communities between pairs of samples in this distance class were significantly more similar than expected at random. The second distance class spanned from 0.1–0.27 m and contained 90 pairs of samples, and there was evidence of positive but not significant spatial autocorrelation.

The point at which the correlogram initially intersects zero can be interpreted as representative the physical extents of the structures under consideration. Under this interpretation the patches in this study are estimated to have an extent of ~0.55 m. No significant SAc was detected in the larger distance classes and it is outside the scope of this study to describe the influence of distances of more than a metre on AMF community similarity. The shape of the correlogram is indicative of the spatial structures under consideration, but as different spatial structures can produce similar correlograms it is important that it is considered alongside a map showing species distributions (Legendre 1993); this is shown in Figure 11. Similar AMF assemblages can be seen for many neighbouring samples, e.g. samples 1 and 3 have 100% overlap in AMF taxa detected, and the only instance of the taxon *Glom*B01 at the site. While there is evidence that neighbouring plants to have similar AMF suites, it appears that the scale of the AMF patches depends on
the particular taxon under consideration. The two abundant, widespread taxa *Glom*A02 and *Glom*A04 occur in almost all samples, and probably extend beyond the boundaries of this study. On this basis it could be predicted that these taxa form patches of > 1 m. However, neither sample 1 nor 3 possess the most common MOTU at the site, indicating that the distributions of locally abundant species are spatially restricted. *Acau*02 appears to follow a less patchy distribution than other MOTUs, having numerous incidences within the site, but the autocorrelation appears to be anisometric, following a roughly north-south distribution, rather than an isometric patch. The distribution of the least common species appears to be patchy at very small scales, with single (i.e., *Glom*A01, *Acau*01) or few (*Glom*B01) neighbouring incidences.

Stepwise removal of individual AMF MOTUs showed that no single MOTU was responsible for the observed spatial signal. However, the removal of the second most abundant MOTU, *Glom*A04, improved the spatial structure in the AMF community. This phenomenon may be attributable to the absence of *Glom*A04 in two samples, 10 and 14, despite their having several neighbours within close proximity which possess the phylotype. It is likely that the widespread presence of *Acaulopora* MOTUs are largely responsible for the detected signal of phylogenetic overdispersion in the AMF communities.
Spatial structure of *H. lepidulum* individuals

Significant and positive evidence of clustering was found in the distribution of 379 *H. lepidulum* individuals recorded within the 10 × 2 m transect. The observed distribution was tested against the distribution expected under complete random spatial organisation using Ripley’s K function. The K function for the null model (Poisson distribution) was calculated for 99 model runs to determine an upper and lower envelope of K across all distances (r). Deviations that fall outside these envelopes at any point in the distribution are considered significant. The K values of *H. lepidulum* within the study site were consistently and significantly larger than expected, indicating the presence of spatial clustering of *H.*
lepidulum at these scales relative to a null model of complete spatial randomness (Figure 12). Individuals within the transect were 10 cm away from their nearest neighbour on average. The maximum distance from an individual to its nearest neighbour was 45 cm.

Figure 12. Ripley’s K-function showing the estimated number of additional H. lepidulum (K) within distance r of a typical random point. The red curve and bounding shaded area shows the distribution expected under complete spatial randomness with the upper and lower estimates from 100 model runs. This distribution shows significant clustering for all values of r.

AMF status of conspecifics

Most of the plant species growing within the plant community were found to be colonised by AMF with the exception of three species (Carex breviculmis, Rumex acetosella, and Scleranthus uniflorus) that belong to predominantly “non-mycorrhizal” families (Harley & Harley 1990). The roots of S. uniflorus possessed extensive fine root hairs up to 400 µm in length, and of a similar diameter to the intraradical hyphae detected in mycorrhizal species (Fig 13. e). The determination of mycorrhizal status from some species present at the site was confounded by root pigmentation, and no AMF data were collected for these species (namely Geranium sessifolium and Leucopogon fraseri). The relative abundance of fungal structures varied by plant species. The plant species with the highest proportions of hyphal colonisation (61%) was Pimelea oreophila, but this was represented by only a single individual with 23 observable root sections. The genus Pimelea (Thymeleaceae) is known to form mycorrhizal
associations (Brundrett & Abbott 1991), and while no arbuscules were found, several vesicles with the “irregular” characteristics of Acaulospora species were detected (Fig 11 b). Kellaria villosa, also a member of Thymeleaceae, possessed the ellipsoid, thin walled vesicles considered typical of Glomus mycorrhizae, as did Celmisia gracilenta (Asteraceae) (Fig 13. f) and Wahlenbergia albomarginata (Campanulaceae) (Fig 13. c). All species at the site belonging to Asteraceae (with the exception of Raoulia subsericea) had relatively high levels of mycorrhizal colonisation, with fungal structures present in >50% of root intersections observed (Table 4).

Table 4. Proportions and types of AM fungal structures detected as quantified by the root intersection method on stained root samples from the Locharburn study site. N/A values indicate roots which could not be sufficiently cleared to visualise AM fungal structures.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>observations</th>
<th>no structure</th>
<th>hyphae</th>
<th>arbuscules</th>
<th>vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisotome brevistylis</td>
<td>43</td>
<td>72%</td>
<td>28%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Anthosachne solandri</td>
<td>35</td>
<td>51%</td>
<td>20%</td>
<td>14%</td>
<td>14%</td>
</tr>
<tr>
<td>Anthoxanthum odoratum</td>
<td>56</td>
<td>57%</td>
<td>13%</td>
<td>29%</td>
<td>2%</td>
</tr>
<tr>
<td>Bulbinella angustifolia</td>
<td>31</td>
<td>68%</td>
<td>10%</td>
<td>19%</td>
<td>3%</td>
</tr>
<tr>
<td>Carex breviculmis</td>
<td>45</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Celmisia gracilenta</td>
<td>36</td>
<td>44%</td>
<td>14%</td>
<td>25%</td>
<td>17%</td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>57</td>
<td>61%</td>
<td>9%</td>
<td>26%</td>
<td>4%</td>
</tr>
<tr>
<td>Geranium sessiliflorum</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Hieracium lepidulum</td>
<td>36</td>
<td>47%</td>
<td>31%</td>
<td>19%</td>
<td>3%</td>
</tr>
<tr>
<td>Hypochaeris radicata</td>
<td>38</td>
<td>26%</td>
<td>42%</td>
<td>18%</td>
<td>13%</td>
</tr>
<tr>
<td>Kellaria villosus</td>
<td>30</td>
<td>73%</td>
<td>23%</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>Leptinella pectinata subsp. villosa</td>
<td>46</td>
<td>78%</td>
<td>13%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Leucopogon fraseri</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Pilosella officinarum</td>
<td>33</td>
<td>36%</td>
<td>30%</td>
<td>27%</td>
<td>6%</td>
</tr>
<tr>
<td>Pimelea oreophila</td>
<td>23</td>
<td>30%</td>
<td>61%</td>
<td>0%</td>
<td>9%</td>
</tr>
<tr>
<td>Poa colensoi</td>
<td>68</td>
<td>96%</td>
<td>3%</td>
<td>1%</td>
<td>0%</td>
</tr>
<tr>
<td>Poa hesperia</td>
<td>128</td>
<td>80%</td>
<td>12%</td>
<td>5%</td>
<td>2%</td>
</tr>
<tr>
<td>Raoulia subsericea</td>
<td>33</td>
<td>85%</td>
<td>15%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Rumex acetosella</td>
<td>32</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Scleranthus uniflorus</td>
<td>44</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Trifolium dubium</td>
<td>30</td>
<td>57%</td>
<td>20%</td>
<td>20%</td>
<td>3%</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>33</td>
<td>79%</td>
<td>15%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>Wahlenbergia albomarginata</td>
<td>30</td>
<td>77%</td>
<td>20%</td>
<td>0%</td>
<td>3%</td>
</tr>
</tbody>
</table>
Figure 13. Micrographs of plant roots extracted from the study site, which have been cleared and stained for endogenous AMF structures. *Kellaria villosa* (a), *Pimelia oreophila* (b), *Wahlenbergia albomarginata* (c), *Hieracium lepidulum* (d), *Scleranthus uniflorus* (e), *Celmisia gracilenta* (f). Scale bars = 100 µm
Effect of sampling effort

In order to determine the optimal level of clone screening, clones within samples were randomly resampled at different levels of sampling effort, and pooled to reveal how this influenced the total MOTU diversity measured at the site (Fig. 14). The number of taxa detected at each sampling intensity were fitted to an Arrhenius model which was extrapolated to show the estimated diversity up to 100 clones per sample. Depending upon the diversity estimator used, this analysis shows that 66–88% of the described site diversity is likely to be detected at a sampling intensity of 20 clones per sample — equivalent to 45% of the screening effort used in this study. The predicted taxon accumulation curve shows that a much greater sampling effort would be required to ensure representatives of all taxa were detected using these field sampling and DNA extraction methods.

![Graph showing the effect of clone screening effort per sample on the total site MOTU diversity detected](image)

**Figure 14.** The effect of clone screening effort per sample on the total site MOTU diversity detected. The dashed line indicates the extrapolated diversity at sampling sizes >45 clones modelled using the Arrhenius species-area relationship.
Discussion

Key findings
This study has identified nine phylogenetically supported AMF taxa associated with *Hieracium lepidulum* in an invaded plant community and shown that the composition, richness and evenness of AMF suites varies between individuals within a population. Only one of the detected AMF MOTUs was closely related to a described taxon, *Acaulospora longula*. Many of the detected sequences form monophyletic groups with sequences previously detected in New Zealand, indicating the existence of potentially endemic, but geographically widespread AMF taxa. Furthermore, this study has demonstrated that AMF communities associated with *H. lepidulum* plants are positively autocorrelated at scales below 50 cm.

AMF community structure

When the AMF are considered using clone abundance data, the phylotype *GlomA02* is clearly dominant at the site comprising more than 60% of clones screened, indicating a fungus or group of closely related fungi which are particularly well adapted to both the soil at the site and colonisation of *H. lepidulum*. Overdominance, where one or two species account for more than 40% of the total abundance, generally characterises impoverished or unstable communities, and is unusual in communities of larger organisms with a lognormal distribution (Whitaker 1975; Dumbrell *et al.* 2010). However in a meta-analysis 32 studies Dumbrell *et al.* (2010) found overdominance to be a general trait of AMF communities in plant roots, with the most abundant taxon occupying, on average, 40% of the total abundance. The meta-analysis also found that the most abundant taxon tended to belong within *Glomus* group A, comprising *Rhizophagus, Funneliformis* and *Glomus* — a pattern which was also found in this study. Relative to other members of the Glomeromycota, members of Glomeraceae generally produce the majority of fungal biomass within the plant root, whereas Acaulosporaceae has more equal distribution of fungal biomass inside and outside of the host (Hart & Reader 2002; Powell *et al.* 2009). It has been proposed that the extensive colonisation patterns of the Glomeraceae may reflect a functional role in the protection of roots from infection by soil pathogens (Newsham *et al.* 1995). This may partially explain the tendency for members of Glomeraceae to dominate molecular studies based on root samples (Santos-Gonzalez *et al.* 2007).
The number of AMF taxa detected in the roots of *H. lepidulum* individuals varied, ranging from two to seven taxa, with no individual possessing all taxa detected at the site, and no relationship between host biomass and AMF diversity. This was surprising, considering that increased root biomass should provide more root surface areas for AMF colonisation. Phylogenetic analysis revealed that taxa sharing host root systems were significantly less related than expected. From a niche-based assembly perspective this phylogenetic overdispersion may represent competitive interactions between AMF taxa, or environmental filtering of convergent traits (Webb *et al.* 2002). If phylogenetic overdispersion is considered in the context of the dominance distribution of the community, it may be that rare AMF are a functionally important component, rather than a random assemblage. Evidence of phylogenetic trait conservatism has been found in other AMF studies (Maherali & Klironomos 2007), and while this study seems to support those findings, further investigations of AMF in natural communities needs to be conducted to determine whether phylogenetic overdispersion is a general characteristic of AMF communities.

**Phylogenetic analysis**

A pattern of monophyly in groups of NZ sequences may represent indicate endemic taxa, with four of the MOTUs (i.e., *Glom*A03, *Glom*A04, *Glom*A05 and *Glom*A06) found in this study being most closely related to sequences of undescribed AMF isolated from New Zealand bryophytes in podocarp/broadleaf forests (Russell & Simon 2005; Bidartondo *et al.* 2011). The majority of studies which have investigated molecular diversity of AMF have found unique taxa and around 50% of taxa detected are found only at one site, even when those taxa are locally abundant (Öpik *et al.* 2006). It is unclear whether this statistic is indicative of high diversity globally, or is a reflection of how few molecular studies have been carried out thus far (Helgason *et al.* 2002; Fitter 2005). The “exceptionally” low diversity of AMF that were found to associate with *Marchantia* was interpreted as indicating highly specific symbiotic relationships (Russell & Simon 2005). The low taxonomic resolution of the AmL1-AmL2 primers may produce monophyletic groups that are not necessarily indicative of species-level relationships; however the presence of several shared ribotypes between an exotic asterad in a dry grassland habitat and a thalloid liverwort in a podocarp/broadleaf forest indicates the presence of geographically widespread generalist AMF taxa. The ecological implications of such widespread AMF taxa are considerable, potentially indicating that the podocarp/broadleaf systems are also vulnerable to *H. lepidulum*.
invasion and that there may be some mechanism for AMF gene flow between these habitats. Within the Locharburn study site, further investigation of AMF molecular diversity at other loci may reveal that some of the MOTUs defined in this study represent several AMF taxa. This may help to explain the extremely high relative abundance of some MOTUs.

In an experimental study of AMF communities on the palm *Trachycarpus fortunei*, introductions of the palm to several sites across Europe resulted in colonisation by a relatively conserved suite of AMF (Moora *et al.* 2011). The authors concluded that the detected AMF taxa represented generalists with global distributions whose life strategy was particularly amenable to *T. fortunei*, which is also a generalist species. A broader hypothesis from the latter authors’ findings is that widely distributed generalist AMF taxa are most likely to colonise invading plant species, particularly if they also generalists (Pringle *et al.* 2009; Moora *et al.* 2011). Further investigations of AMF associated with *H. lepidulum* and other species may reveal whether this hypothesis is supported in the context of plant invasions in New Zealand.

Evidence against the proposed endemic AMF taxa includes the position of foreign sequences within the NZ clades, such as the North American environmental sequence within *Glom*A02 (Figure 6). A similar situation was found when a Canadian AMF culture sequence emerged within a phylogeny of *Rhizopagus intraradices* isolated from a Swiss agricultural system (Koch *et al.* 2004). The workers concluded that it represented either gene flow between the land masses, or that small scale diversity may account for diversity on a much larger scale. However, more data are required before definitive conclusions are able to be drawn.

Comparison of diversity measures
Under the current taxonomic delineations, the nine taxa detected in this study represent at least four distinct genera in two families. However several distinct RFLP-types, which are likely to represent rare taxa, remain unknown as they could not be sequenced. Furthermore, there were several RFLP types which could not be reproduced using *in silico* endonuclease digestions of the sequence data, potentially indicating preferential amplification or cloning bias. The probability of there being unsampled taxa within the study site is also supported by the Chao diversity estimate, based on the proportion of rare taxa detected in the site, which predicted at least 2 undetected taxa. The MOTU rank abundance data based on clone frequencies indicates that the AMF community is heavily dominated a few extremely
common species, and it is likely that any further species remaining within the community are very rare and colonise only single plant hosts.

Spatial analysis

All of the attempts to characterise the spatial structure of AMF communities to date have used systematic sampling of the soil environment, with variable results. This is the first study that has attempted to characterise the sub-metre spatial structure of a suite of AMF associated with a particular plant species. This study has shown that the AMF suites of *H. lepidulum* individuals < 0.5 m distant are significantly more similar than would be expected if AMF were randomly assorted among plants. It is likely that the similarity of AMF suites is at least partially explained by the extents and interactions of the roots systems of plant individuals. The Mantel correlogram indicates that plants up to 0.2 m apart have significantly more similar AMF suites than expect at random, consistent with the hypothesis of hyphal dispersion as the dominant mechanism for colonisation of plants, however it would be expected that individuals < 0.15 m apart would be similar as they represent overlapping sample extents. The detection of similarity in these overlapping samples could be considered indirect support of the accuracy of the sampling methods used in this study, but not explicit evidence of AMF spatial structure *per se*. A correlogram of positive spatial autocorrelation is expected to show a peak of significant autocorrelation at the smallest distance classes, with the size of the correlation coefficient declining as the distance between pairs of host individuals increases. It may be that the variation in the magnitude and significance of the detected spatial structure in the first three distance classes is the result of taxon-specific variation in patch size (Figure 10). The reduced signal of spatial structure in the Mantel statistic when clone abundances were used may be due to the variation in amplification success between samples. It is likely that the relative abundances of clones from each sample are representative of taxon relative abundance (Suzuki *et al.* 1998; Dumbrell *et al.* 2011), but variation in the absolute abundances of the initial AMF biomass within each root system requires that the clone abundances be scaled to reflect this.

It can be seen from the map of AMF spatial distributions (Fig. 11), that the AMF community at the site was dominated by common taxa (predominantly within *Glomus* group A) represented in the majority of host plants. It has been shown that AMF species are able to form anastomoses between individuals possessing the same genotype (Giovannetti *et al.* 2003). Although there is no direct evidence to support the hypothesis, the detected spatial
distribution of the phylotypes, particularly the most dominant phylotypes, is consistent with what would be expected from common mycorrhizal networks connecting the host root systems belowground. An alternate and less parsimonious explanation would be that phylotypes form discrete patches around each host plant, and independently develop AMF suites from the local species pool. Conversely the rare taxa show stronger evidence of patchy spatial restriction, having only single instances (i.e. GlomA01 and Acau01) or two nearby instances (i.e., GlomB01, samples 1 and 3, Fig. 10). These localised fungi, which appear less able to develop and maintain hyphal networks with $H. \text{lepidulum}$, may represent less beneficial symbionts that the host plant has discriminated against (Bever et al. 2009; Powell et al. 2009).

It has been demonstrated that plant hosts are able to discriminate between AMF and preferentially allocate carbon to the taxa that provide the greatest reciprocal P increase, and vice versa (Bever et al. 2009; Kiers et al. 2011). Furthermore, it has shown that $H. \text{lepidulum}$ expends a relatively high proportion of energy on investment in belowground structures under nutrient poor conditions (Radford et al. 2007). This physiological adaptation may give $H. \text{lepidulum}$ a relatively large influence on the composition of the AMF community. The facilitation may not contingent upon the identity of the AMF taxa per se, rather that it is a physiological adaptation of $H. \text{lepidulum}$ that allows it to associate with local AMF which are particularly suited to the local environment, and maximise the benefits from these symbiosis, however this hypothesis would require more work, and is outside the scope of this thesis. In future studies, temporal and spatial replication could show how the identity of the dominant taxa fluctuates over space and time, which will help elucidate the roles and scales of positive feedback within AMF communities. Further investigations are also necessary to determine other factors that influence the distribution of $H. \text{lepidulum}$ and AMF within the study site. The observed spatial structures are probably partially explained by spatially structured environmental variables, such as soil physico-chemical properties and the distributions of mycorrhizal plant species, and by pure spatial effects (Legendre et al. 2005).
Conclusion

This study represents the first time that molecular methods have been used to measure AMF community composition of *Hieracium lepidulum*, and the first time that molecular methods have been used to characterise AMF diversity and spatial structure within a NZ grassland community. *H. lepidulum* has been shown to associate with at least nine distinct fungal taxa from four genera, several of which are highly similar to AMF sequences previously amplified from New Zealand vegetation. The organisation of the fungal taxa within the site has been shown to be spatially non-random, with nearby plants tending to possess more similar suites than expected. At the site, the AMF community is dominated by a single taxon, the abundance and phylogenetic placement of which is consistent with findings from previous molecular surveys from diverse ecosystems. Further investigations of the AMF associated with *H. lepidulum* in its invaded range which include both spatial and temporal replication will help to determine the distributions and seasonal dynamics of these functionally significant soil fungi.
Chapter 4: Conclusions and experimental recommendations

The preceding chapters of this thesis have explored aspects of the interactions between the exotic plant *Hieracium lepidulum* and an ecologically important group of symbiotic fungi, the Glomeromycota, in subalpine plant communities in Central Otago, New Zealand. In the first chapter, the state of knowledge concerning these organisms, their interactions, and their distributions in the context of *H. lepidulum* invasion in New Zealand was reviewed. In Chapter Two, the potential of single spore trap culture as a method for isolating individual AMF strains for experimental manipulation, and the relative sensitivity of various AMF-specific primers for detecting their presence was investigated. In Chapter Three, the optimised PCR protocols designed in Chapter Two were used to characterise AMF ribotypes from *H. lepidulum* in a spatially explicit manner from the Locharburn field site. In this final chapter, the literature will be reviewed in the context of the findings from the two experimental chapters of this thesis, and recommendations will be made for further research into the relationship between AMF and *H. lepidulum* in New Zealand. As with many ecological studies, the results from this research prompt more questions than they provide definitive answers. In the absence of manipulative experiments we are still no closer to understanding the functional significance of the AMF symbiosis in the invasion of New Zealand indigenous vegetation by *H. lepidulum*. However, this thesis has investigated several aspects of the *H. lepidulum*–AMF symbiosis that may prove useful for informing future experimental work.

**Single spore culture screening**

The success of other workers (i.e., Koch *et al.* 2004; Avio *et al.* 2006) clearly demonstrates that the production of individual AMF strains through single spore trap culture is possible. However, this study has reinforced some of the practical limitations of AMF as experimental units, including the requirement of large numbers of culture attempts and long time frames. The phylogenetic analysis of sequences from *H. lepidulum* roots in Chapter Three shows that over 95% of the ribotypes detected form clades that are distinct in this study from any cultured species, and form monophyletic groups with “environmental samples” from around the world. Several workers have speculated that culture collections may consist predominantly of “weedy” species, which are able to be easily cultured (Helgason *et al.* 2002; Fitter 2005), and the position of the sequences from this study in the phylogeny may indicate that these are species that are difficult to establish and maintain in trap culture. Conversely,
the finding of relationships between the sequences growing on *H. lepidulum* and *Marchantia foliacea* may indicate that the AMF species concerned may be generalists, and as such they should represent species that are relatively easy to cultivate.

Due to the unknown genetic organisation of the AMF species detected in this study and of the Glomeromycota in general, it may be that some or all of the MOTUs identified in Chapter Three represent more than one AMF species. Koch *et al.* (2004) found that the rDNA sequence variation may mask significant, and ecologically relevant genetic variation at other loci. Future studies investigating the genetic diversity of cultured AMF at different loci will help to clarify the taxonomic distinctions between AMF species.

This study has identified a method for screening large quantities of single spore culture attempts that is faster and more practical than traditional root-staining based methods. This study has shown that the AmL1–AmL2 primer set is sufficiently sensitive for tens of single spore culture root samples to be pooled and simultaneously screened for the presence of AM fungal DNA. However it has been shown that the AmL1–AmL2 primers are not as specific to the Glomeromycota as previous studies have claimed. This has implications for their efficacy in the situations where AMF status is uncertain, or for studies that do not employ sequence-based characterisation methods.

**AMF spatial structure**

The intensive sampling method used in the environmental study has revealed several aspects of the AMF symbiosis that have hitherto received little attention. Prior to this study the diversity of AMF assemblages associated with *H. lepidulum* in New Zealand was unclear. At least 9 phylogenetically distinct ribotypes comprising at least 4 genera form symbioses with *H. lepidulum* at Locharburn station. Furthermore, this study has found evidence for a lognormal dominance/diversity distribution of ribotypes with one dominant taxa comprising more than 65% of taxon abundances, a distribution similar to that found in several other studies (Dumbrell *et al.* 2010). The ecological significance of this is unclear, however the similarities found among the dominance/diversity distributions of several independent studies of AMF communities may indicate the action of a common process structuring AMF communities (Dumbrell *et al.* 2010), at least within plant roots.

Future environmental studies and surveys of AMF from natural systems need to consider spatial structure within AMF communities. If, as postulated in Chapter 3, the spatial structure of plant hosts and their AM symbionts are related, future studies may show that the
scales of spatial structure in AMF communities are correlated with the spatial structure in the plant hosts, and may vary correspondingly in extent, particularly for plants with high mycorrhizal dependence.

**Phylogenetic analysis**

One of the most intriguing discoveries of this study is the phylogenetic relationship between AMF ribotypes from diverse habitats and hosts within New Zealand. There is evidence of “New Zealand-only” clades within the AMF phylogeny constructed in Chapter 3, comprising AMF ribotypes that are common to subalpine grassland and podocarp forests. Geographical isolation and environmental filtering are two large scale processes that strongly influence the distribution of plant species, and it would be expected that the associated plant symbionts would show similar distributions in response to similar environmental drivers. However, not only has this study shown evidence that AMF strains occur in very different habitat types, their isolation from widely divergent plant groups indicates that the distributions of the fungi may also be independent of host identity.

One implication of the detected ribotype distributions may be that the sequences represent generalist AMF species with widespread distributions across several habitat types in New Zealand. This hypothesis supported by the evidence of multiple ribotypes common to both habitats. Alternatively, the low resolution of the SSU rDNA (Gamper *et al.* 2009) may mean that the phylogenetic relationships observed between New Zealand sequences may reflect the distribution of some broader taxonomic grouping, perhaps at genus level. However, when it is considered that there have been only three published SSU rDNA studies of AMF in New Zealand, it is surprising that there is such a large overlap of ribotypes found among the studies. This may indicate that AMF ribotype diversity is nationally limited, which may in turn reflect a limited AMF species pool. Whereas Russell & Simon (2005) concluded that their finding of low AMF diversity from several isolates of *Marchantia foliacea* was indicative of a group of specialised AMF, this low diversity may in fact be a general characteristic of New Zealand AMF. Alternatively, as both *H. lepidulum* and *M. foliacea* could be considered generalist species, and a study has been shown that generalist plants tend to associate with generalist suites of AMF (*Moora et al.* 2011), it may be that the similarities found between the AMF colonising the hosts are a coincidence of the host life strategy. In either case, this study has found evidence that AMF ribotypes isolated from an invasive species in a New Zealand subalpine grassland are related to ribotypes from a New
Zealand podocarp forest. This finding provides evidence that the facilitation of *H. lepidulum* invasion is not via a coinvasive exotic AMF species. Conversely, the evidence indicates that the AMF involved are likely to be endemic to New Zealand.

**Testing mechanisms of *H. lepidulum* invasion**

Despite several decades of research, the factors influencing the success of *H. lepidulum* are not entirely clear. It is possible that the proximate mechanisms facilitating *H. lepidulum* invasion are not the same throughout its invaded range in New Zealand. For example, kilometre-scale autocorrelation in *Hieracium* cover was found in Otago but not in Canterbury (Duncan *et al.* 1997), a finding that was postulated to reflect the process of invasion being at an earlier stage in Otago. The relative importance of grazing on *H. lepidulum* fitness remains contentious, with contradictory evidence from several studies (e.g. Rose *et al.* 1995; Norton *et al.* 2006). In post-burning situations, grazing can help to suppress the spread of *H. lepidulum*, however it will also promote the establishment of other exotic and native unpalatable species (Mark *et al.* 2011). The relative herbivory resistance of *H. lepidulum* vegetative structures has been identified as a potentially important factor in *H. lepidulum* invasion (Radford *et al.* 2009), as has its ability to establish and persist in low-light situations (i.e., *Nothofagus* forest, *Chionochloa* tussocks). Within *Nothofagus* forest, *H. lepidulum* density is greatest at microsites of high plant diversity (Wiser *et al.* 1998; Meffin *et al.* 2010) and high AMF inoculum potential (Spence *et al.* 2011). This apparent positive diversity-invasibility relationship could be attributed to facilitation of exotic invasion by resident biodiversity, or more specifically, the AMF networks supported by the resident plant community (Spence *et al.* 2011).

Hypothetical models of *H. lepidulum* invasion have been previously published, and the following paragraphs will elaborate on some of these models in terms of AMF facilitation. Duncan *et al.* (1997) ruled out single-factor explanations for *H. lepidulum* invasion, and broadly stated that the probability of invasion is a function of two variables: (1) the suitability of the site for hawkweed establishment (susceptibility of environment) and (2) the size of the hawkweed propagules rain (strength of invasion). Here we will focus only on aspects of the susceptibility of the environment to *H. lepidulum* invasion by considering AMF facilitation as a variable which may influence community composition via interactive effects with other environmental parameters. One of the difficulties in testing hypotheses concerning the roles of AMF in invasion is the myriad functional roles that have been attributed to them,
from improved phosphate nutrition, to pathogen resistance, to a kind of plant socialism — where resources are redirected from dominant plant species to subdominant species (van der Heijden & Horton 2009). When these factors are added to the already numerous biotic and abiotic variables, experimental designs quickly become large and unwieldy.

It is likely that a more comprehensive understanding of AMF diversity and distribution in New Zealand from field sites will develop from molecular studies. Sequencing costs are dropping rapidly, with current prices at US$0.12 Mb⁻¹, down from US$100 Mb⁻¹ in 2008 and US$1000 Mb⁻¹ in 2004 (NHGRI: genome.gov/sequencingcosts). This continuing trend will facilitate the expansion of AMF research into more ecosystems and more plant species, and enable a range of questions similar to those raised in this study to be addressed.

**AMF positive diversity-invasibility model**

In a longitudinal study over six years, the cover of *H. lepidulum* was “significantly positively associated with the richness of basal herb, creeping herb and grass functional groups” at the scale of the 30 × 30 cm quadrats, but uncorrelated with any of the measured abiotic soil variables across a range of habitats (Meffin *et al.* 2010). A similar correlation was found by Wiser *et al.* (1998), and Spence *et al.* (2011) were able to show a correlation between plant diversity, AMF inoculum potential and *H. lepidulum* density. Spence *et al.* (2011)suggest that this correlation may represent the action of a positive diversity-invasibility relationship for *H. lepidulum* within their study site. A large proportion of the AMF diversity which has previously been detected in New Zealand was of found to be associated with *H. lepidulum* in this study. More data are needed to characterise AMF assemblages of *H. lepidulum* across the invaded range to determine their identity and distribution. If plant and AMF community metrics are simultaneously measured in the sampled communities it may be possible to unravel this hypothesised positive diversity-invasibility relationship and determine whether it is plant diversity that promotes *H. lepidulum* invasion, or whether particular AMF ribotypes are more important predictors of *H. lepidulum* success.

**AMF facilitation/herbivory resistance interaction model**

It may be that a combination of AMF facilitation and herbivory underlies the success of *H. lepidulum* as an invader: invasion may be facilitated by utilising the existing AMF networks within a plant community, but it is the relative resistance of *H. lepidulum* to herbivory that promotes its maintenance and eventual success within the community. It is
believed that the rosette growth form of *H. lepidulum* makes it less attractive to mammalian herbivores, particularly stock (Scott 1993). As herbivory pressure is preferentially directed at other, more easily grazed species within the community, *H. lepidulum* is able to command a higher proportion of resources from the AMF network. It has been demonstrated in glasshouse experiments that the AMF symbionts of an individual of one species can form a common mycorrhizal network with an individual of another. Isotope studies have shown that simulated grazing of one individual results in a significant transfer of nutrients to the other, resulting in an increase of biomass relative to control plants (Jakobsen 2004). It may be that similar relationships exist within plant communities containing *H. lepidulum* — common mycorrhizal networks redirect resources from the belowground structures of grazed individuals to the ungrazed individuals. Such a feedback mechanism would ensure that photosynthates remain available to the AMF network under grazing pressure, and would manifest aboveground as a proliferation of unpalatable species such as *H. lepidulum*. This hypothesis could be tested in a field situation by the simultaneous application of fungicide and grazing to experimental plots containing *H. lepidulum*. The AMF facilitation model would predict that in the absence of AMF (fungicide treatment), palatable species will persist in the community, resulting in overall lower aboveground biomass relative to grazed plots which contain AMF.

**Limiting factor release**

Mutualisms tend to be more important biotic processes shaping plant communities within low nutrient/productivity environments (Bertness & Callaway 1994) and these processes tend to facilitate the maintenance of diversity and evenness. Conversely, eutrophic conditions tend to promote competitive interactions, leading to less diverse communities dominated by one or a few species (Vitousek *et al.* 1997). It is recognised that in high nutrient soils plants are able to obtain sufficient nutrients without needing to invest in the AMF network (Collins & Foster 2009). In grassland ecosystems, it has been demonstrated that the dominant plant species following eutrophication tend to be those that show small growth responses to AMF colonisation, while the subdominant species remain mycorrhizal (Johnson *et al.* 2008). Molecular studies have demonstrated that AMF community composition can also change in response to eutrophication, potentially driving community shifts via feedback (Egerton-Warburton *et al.* 2007). Applications of phosphate fertiliser (50–100 kg ha⁻¹) and oversowing reduces the proportion of *H. lepidulum* within plant communities (Scott 1993). The addition
of fertilisers may liberate plant species from the AM symbioses, reducing the importance of the AM network as a source of nutrients for the plant community. In the resulting competition-dominated system, *H. lepidulum* would no longer be able to successfully compete with conspecifics (Radford *et al.* 2010), and would become subdominant. Furthermore, in these competition-based systems, the increased prevalence of plant species with low mycorrhizal dependency, coupled with an overall reduction in plant community dependence on AMF, may result in the suppression of AMF. As the subdominants are known to maintain their AM symbioses under eutrophic conditions, upon cessation of nutrient application there may be a proliferation of those AMF taxa which the subdominant species have maintained throughout the interim. *H. lepidulum* is known to possess phenotypic adaptations that facilitate its persistence in high country plant communities, including herbivory resistance and a relative tolerance to adverse nutrient conditions (Scott 1993; Radford *et al.* 2006). It has also been shown that in low nutrient situations *H. lepidulum* diverts a higher proportion of energy towards investment in belowground structures (Radford *et al.* 2007). As nutrient levels decline, this increased investment in belowground structures may represent greater resource availability for AMF in terms of host-derived photosynthate. As a result the influence of *H. lepidulum* on the composition and organisation of the AMF community may increase, potentially driving a switch in the plant community via positive feedback (Wilson & Agnew 1992). This model of invasion could be tested by applying different fertiliser treatments to experimental field plots containing *H. lepidulum*. Using 18S rDNA based characterisation of root and soil DNA, the AMF community composition could be compared before, during and after the treatments in both *H. lepidulum* roots, and in the soil. This model would predict a reduction in *H. lepidulum* density and soil AMF diversity resulting from fertiliser application, and a concomitant increase in *H. lepidulum* and AMF taxa associated with *H. lepidulum* as the soil nutrient levels decline after the treatment has ended. This model would also predict that the application of fungicide to a eutrophic grassland would result in the loss of subdominant species such as *H. lepidulum*. However the loss of AMF from grassland systems is likely to have significant long term negative impacts on soil stability and plant productivity.

**Conclusion**

The diversity and abundance of interactions between organisms is a mechanism thought to explain the resilience and robustness of ecosystems and ecosystem processes. A full
appreciation of the complexity underlying ecosystems may never be possible, yet technological advances are allowing increasingly detailed analyses at increasingly smaller scales. The research undertaken in this thesis has demonstrated the scales of spatial structure in arbuscular mycorrhizae colonising *Hieracium lepidulum*, and found spatial patterns of fungi and host that are consistent with facilitated invasion. It is clear that AMF play a fundamental role in the maintenance of terrestrial diversity, and that the 400 million year coevolution of AMF and terrestrial plants should inform concepts about vegetation dynamics. More research is needed to determine whether the identities and spatial structure of AMF are consistent across the invaded range of *H. lepidulum*, and to investigate the mechanisms underlying the observed spatial structures.
References


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Appendix

Supporting data contained on the attached computer disc:

S1 – RFLP gel photos

S2 – RFLP-type library

S3 – RFLP-type database