ECTOMYCORRHIZAL COMMUNITIES ASSOCIATED WITH A *PINUS RADIATA* PLANTATION IN THE NORTH ISLAND, NEW ZEALAND

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by

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Aboveground and belowground ectomycorrhizal (ECM) communities associated with different age classes of the exotic plantation species *Pinus radiata* were investigated over the course of two years in the North Island of New Zealand. ECM species were identified with a combined approach of morphological and molecular (restriction fragment length polymorphism (RFLP) and DNA sequencing) analysis.

ECM species richness and diversity of a nursery in Rotorua, and stands of different ages (1, 2, 8, 15 and 26 yrs of age at time of final assessment) in Kaingaroa Forest, were assessed above- and belowground; furthermore, the correlation between the above- and belowground ECM communities was assessed. It was found that the overall and stand specific species richness and diversity of ECM fungi associated with the exotic host tree in New Zealand were low compared to similar forests in the Northern Hemisphere but similar to other exotic plantations in the Southern Hemisphere. Over the course of this study, 18 ECM species were observed aboveground and 19 ECM species belowground. With the aid of molecular analysis the identities of *Laccaria proxima* and *Inocybe sindonia* were clarified. In the aboveground study, five species were found associated with *P. radiata* that were previously not reported with this host in New Zealand (*Inocybe sindonia, Lactarius rufus, Lycoperdon gunii, Rhizopogon pseudoroseolus* and *Wilcoxina mikolae*). Belowground, the species *Psudotomentella* sp*., P. tristis, R. luteorubescens, Tomentella* sp., *Wilcoxina mikolae* were found as new associates of *P. radiata* in New Zealand, additionally nine ECM types were found that could not be identified with molecular analysis. There was little correlation between the species fruiting and the species colonising root tips. Only seven species were found in common between the above- and belowground communities, furthermore the dominant species aboveground were not observed in the belowground ECM communities.

The influence of host age on the above- and belowground ECM communities of different age classes of *P. radiata* plantations was investigated. The aboveground species richness increased from the nursery to the oldest age group investigated (26 yrs), while diversity increased to the 15 yr old age group and decreased slightly to the oldest stand. A clear

sequence of ECM species changes was observed to be related to stand age with a growing complexity over the chronosequence. The belowground ECM communities showed a different picture and richness and diversity initially decreased from the nursery to the outplanting but increased thereafter. Belowground no change in ECM composition that was directly related to the age of the host was observed, but two distinct groups of ECM species were found – a 'young' and a 'plantation forest' group, with the respective discriminating species being *Rhizopogon rubescens* and Type unknown Basidiomycete/*Amanita muscaria*.

Another aspect of the study was the fate of the nursery ECM species in the outplanting and the arrival of non-nursery species. The ECM communities of seedlings in the nursery were investigated in 2006 and these seedlings were followed up over eight assessments in the field for one year, furthermore data from the 1-, 2 and 8 yr old plantation stands was analysed. It was found that the nursery species do survive the first year of outplanting and are dominant in the first year. The first non-nursery species occurred six months after outplanting but was only in minor abundance. Nursery ECM were dominant for two years after the seedlings were planted, and were completely replaced after seven years. *Rhizopogon rubescens* was found to be the most persistent and dominant species in the outplanting, facilitating the successful establishment of the seedlings in the plantation forest.

Key words: ectomycorrhiza, *Pinus radiata*, New Zealand, exotic plantation, nursery, diversity, succession, above- and belowground, ITS-RFLP, direct sequencing, outplanting, species displacement.

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"excuse me

but I just have to
explode

explode this body
off me

wake-up tomorrow
brand new

a little tired

but brand new"

björk – pluto

First and foremost I want to thank Sue McCurdy for placing me with this PhD and the Forest Biosecurity and Protection Group at Scion. Without you I would not be here and write these acknowledgments!

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3 ½ years later and I do feel like Björk's Pluto – just exploded, a little tired but brand new and ready for all the challenges that lie ahead of me.

Kat

March 2008

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ABBREVIATIONS

ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
ECM	Ectomycorrhiza
GenBank	collection of publicly available nucleotide sequences, produced by the National Centre for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database collaboration (INSDC)
INSD	International Nucleotide Sequence Database
ITS	Internal transcribed spacer region
PCR	Polymerase Chain Reaction method, which enables exponential amplification of target DNA
rDNA	Region of chromosomal DNA that codes for ribosomal RNA.
RFLP	Restriction Fragment Length Polymorphism. Method, in which PCR-amplified DNA is digested using restriction enzymes.
Taq	Thermostable DNA polymerase, named after thermophilic bacterium *Thermus aquaticus*
UNITE	rDNA sequence database focused on ectomycorrhizal asco- and basidiomycetes.
VAM	Vesicular – arbuscular mycorrhiza

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1 INTRODUCTION

1.1 MYCORRHIZA

In 1885 the German pathologist A. B. Frank was commissioned by royal order to investigate the growth of truffles in Prussia. Thereby he observed something "...about the nature and nutrition of plants not heretofore even slightly suspected by science..." (Frank, 2005) – Mycorrhizae (Figure 1-1).

> When one examines feeder rootlets in the soil...of any of our native oaks, beech, hornbeam, hazel or chestnut, it is evident they are generally composed of two disparate components: a core, representing the actual tree root, and an organically united mantle of fungal hyphae. This fungal mantle completely encloses the rootlet, forming a continuous cover even over the growing tip. It grows along with the root tip and behaves in every respect as an organically united, peripheral tissue belonging to the root. The entire structure is neither tree root nor fungus alone but resembles the lichen thallus, a union of two different organisms into a single, morphological organ. It can be appropriately designated as a "fungus-root" or "mycorrhiza". (Frank, 2005, p. 265)

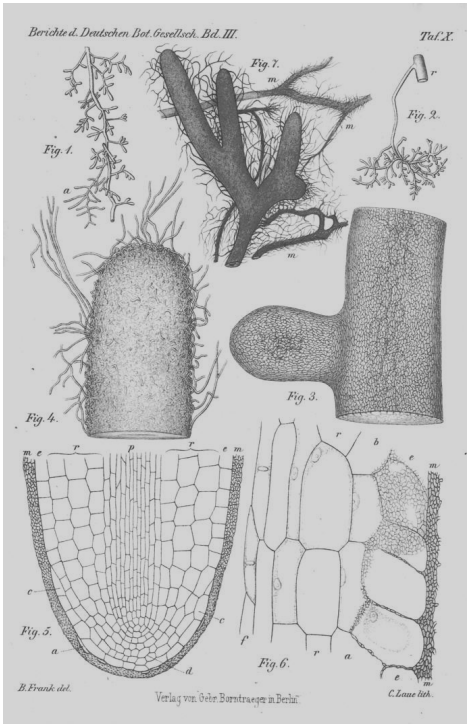

Figure 1-1: Ectomycorrhizae and their microscopic structures of *Carpinus betulus* and *Fagus sylvatica* from A. B. Frank's original paper (Frank, 1885).

Mycorrhizae[1] are symbiotic associations between the roots of a plant and a range of soil fungi (Smith & Read, 1997) and have evolved on several occasions as the norm of terrestrial plant

[1] The terms 'mycorrhizae' and 'mycorrhizas' are both used as a plural form for 'mycorrhiza', in this thesis the term 'mycorrhizae' will be used for the plural.

nutrition (Halling, 2001). The earliest evidence of mycorrhizal associations are from the early Devonian era, approximately 400 million years ago, which is congruent with fossil records of the the first land plants. In this mutually beneficial association, the fungi (mycobionts) exchange soil-derived nutrients for carbohydrates from the host plant (photobiont). It has been stated widely in the literature that around 90% of vascular plants are mycorrhizal, however, the exact percentage is more likely to be around 82% (Brundrett, 2002). The benefits that plants gain from mycorrhizal symbioses can be characterized either agronomically, by increased growth and yield, or ecologically by improved fitness. In either case, the benefit accrues primarily because mycorrhizal fungi form a critical linkage between plant roots and the soil. Mycorrhizae are, therefore, a symbiosis involving a tripartite interactions between a host plant, the mutualistic fungi and environmental factors (Brundrett, 1991).

Several different types of mycorrhizal associations exist and the general literature differentiates between at least seven forms. The main division is based on the type of cellular contact: in endomycorrhizae the fungal structures are formed within plant cells, while in ectomycorrhizae (ECM), the fungi are restricted to the inter-cellular space in cortical and epidermal layers of fine roots and do not puncture the cell membrane. Endomycorrhizal types are VAM (vesicular-arbuscular mycorrhiza, often only called arbuscular mycorrhiza), ectendo-, ericoid, arbutoid, monotropoid and orchid mycorrhiza (Brundrett, 1991; Brundrett *et al.*, 1996; Smith & Read, 1997; Brundrett, 2002).

1.1.1 ECTOMYCORRHIZAE (ECM)[2]

Ectomycorrhizae show a great morphologically variation on the terminal feeder roots of woody perennial plant species. The main diagnostic feature of ECM is the external, multi-layered, hyphal structure called the mantle (Figure 1-2 a). Size, colour and texture of this structure varies between species and different host–fungus combinations, age and soil conditions. Agerer (1987) has recognised two main types of hyphal development within the ECM mantles: pseudoparenchymatous (densely packed, highly differentiated hyphae) and plectenchymatous (loosely interwoven hyphae, linear nature of hyphae still present) (Figure 1-3). The second main diagnostic feature of ECM is the Hartig net – within the root, the fungus ramifies between the outer cells of the host, forming a complex structure, which provides a large surface area of contact between the fungus and the host, allowing the transfer or metabolites (Taylor & Alexander, 2005)(Figure 1-2 b).

[2] Ectomycorrhiza is the composite organ formed by the host plant root and ectomycorrhizal fungi (Harley & Smith, 1983).

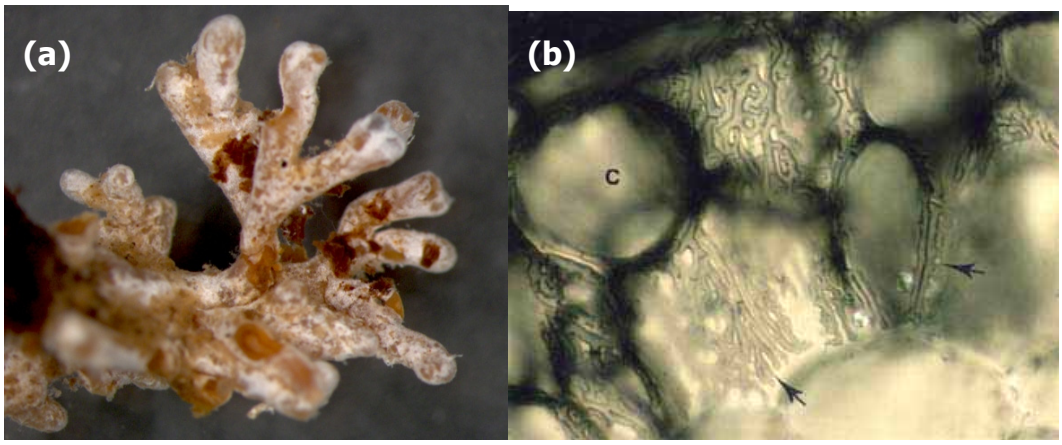

Figure 1-2: (a) Mantle of *Amanita muscaria* on *Pinus radiata*; (b) Hartig net hyphae (arrows) around cortex cells (from Brundrett *et al.* 1996).

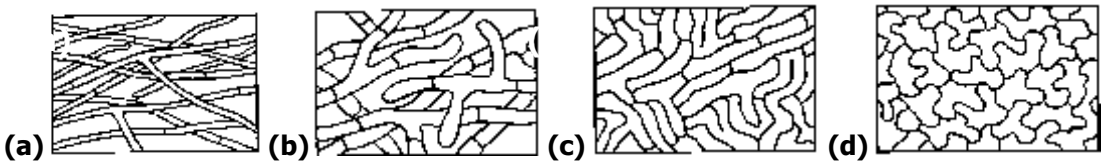

Figure 1-3: Mantle types: (a) felt plectenchymatous (b) net plectenchymatous (c) net pseudoparenchymatous (d) interlocking pseudoparenchymatous (from Goodmann *et al.* 2003).

In coniferst the fungus stimulates the formation of thickened, short, lateral roots, which have dichotomous apical mersitems and show cytodifferentiation (radial elongation, root hair decay) of root cells (Brundrett *et al.*, 1996; Agerer, 2001). These characterisitcs can also be used for morphological species identification.

The majority of ECM fungal species are homobasidiomycetes (around 95%), the remaining species being ascomycetes (4.8%) and some zygomycetes within the genus *Endogone* (Molina *et al.*, 1992). It has been estimated that about 5500 fungal species form ECM (Molina *et al.*, 1992), however, this seems to be an underestimation. The recent increase in studies of tropical forests and Australian eucalypts and their mycorrhizal associations have revealed new and previously undescribed ECM fungi (e.g. Claridge 2002, Haug *et al.* 2005). The use of molecular methods for species identification has also increased the number of ECM forming taxa. For example a study by Weiss *et al.* (2004) identified the importance of members of the *Sebacinaceae* as mycorrhizal formers, and Kõljalg *et al.* (2000) demonstrated for the first time the symbiotic nature of several resupinate thelephoroid fungi, previously thought to be saprotrophic. Considering these studies, Taylor and Alexander (2005) estimate the real size of the global community of ECM fungi to be ca. 7000 – 10,000 species.

In contrast to this fungal diversity, only approximately 3% of plant taxa form ECM while 77% form other types of mycorrhizae. However, the global importance of ECM plants is large because of their disproportionate occupancy of the land surface and economic value as timber sources. The majority of ECM hosts are woody perennials and found in generae such as Betulaceae, Pinaceae, Fagaceae and Dipterocaraceae (Smith & Read, 1997, Orlovich & Cairney, 2004), though some sedges (*Kobresia* spp.) and herbaceous *Polygonum* spp. also form ECM (Taylor & Alexander, 2005).

A single host usually forms ECM with many different fungi at the same time in the same place, and succession of different species occurs over the lifetime of a plant (Brundrett, 1991). The estimated lifespan of individual mycorrhizal tips ranges from a few months to two years, the turnover of ECM mycelia is assumed to be more rapid than that of mycorrhizal tips (Taylor & Alexander, 2005). Some ECM species can associate with multiple host species and are generalists, giving them greater access to sources of carbon (Harley & Smith, 1983), whilst some species exhibit host specificity (e.g. *Rhizopogon parksii* and *Pseudotsuga menziesii* (Horton & Bruns, 1998)).

ROLES AND BENEFITS OF ECTOMYCORRHIZA

Ectomycorrhiza is a mutualistic association between plant and fungus, which means both partners benefit from their alliance. Fungi are achlorophyllous, therefore they rely on an external source of carbon. The host plant supplies the fungus with their carbon; in some cases as much as 20% of the total carbon compounds generated by the host can be transferred to the fungus (Sylvia, 1998; Copley, 2000). ECM fungi influence and benefit the host plant in numerous ways:

Nutrient uptake: It has been demonstrated that ECM fungi improve plant nutrition by enhancing nitrogen, phosphorus and potassium uptake, amongst others (Harley & Smith, 1983). Fungal hyphae increase plant nutrient supply, as they are able to explore the soil more extensively than roots and access the nutrients from beyond the nutrient depletion zone[3] surrounding roots (Brundrett *et al.*, 1996). Furthermore, mycorrhizal hyphae release nutrients from the litter layers by the production of enzymes that mineralise organic matter and thereby make them available to plants (Halling, 2001). The nutrients are taken up by the fungal mycelium through active absorption and specific cell membrane transporters following degradation by exoenzymes, the nutrient exchange between the symbionts occurs in the Hartig net *via* cell-to-cell contact (Smith & Read, 1997). Phosphorus is generally considered to

[3] Nutrient depletion zone: develops around a root when nutrients are removed from the soil more rapidly than they can be replaced by diffusion. A poorly mobile ion such as phosphate develops a sharp and narrow depletion zone close to the root (Sylvia, 1998).

be the most important plant-growth limiting factor; the supply *via* the fungus (as $H_2PO_4^-$) results in increased plant growth, yield and general fitness (Simard *et al.*, 2002). Inorganic nitrogen can be taken up directly by plants, but ECM improve nitrogen uptake (Martin *et al.*, 2001).

Common Mycorrhizal Network (CMN): Mycorrhiza is more than an association between the immediate partners of the symbiosis. The maze of plant roots and fungal hyphae builds a mycelial pipeline which supports a number of members in a plant community and has been termed "Common Mycorrhizal Network" (CMN) (Selosse *et al.*, 2006) where mycorrhizal hyphae connect roots of plants, even of different species, to an intricate, multiway root- and mycelia-network (Newman *et al.*, 1992; Pennisi, 2004). Carbon transfer occurs between the plants through the CMN and is bidirectional in some studies (Selosse *et al.*, 2006). Mineral nutrients can also be transferred through CMNs. Some plants, such as such as *Monotropa* (Indian pipe) and some orchids (i.e. *Neottia, Corallorhiza, Epigonium*), completely rely on their fungal partner for nutrition and cease to photosynthesise, instead they "tap" into the mycelial pipeline (Newman *et al.*, 1992; Pennisi, 2004).

Water uptake: Drought periods can cause considerable stress to trees (Garbaye, 2000). Ectomycorrhizal roots are less sensitive to dry soil conditions as they are covered by the mantle and connected to an extensive network of extraradical mycelium (which extends from the mantle into the surrounding soil) with or without rhizomorphs (root like aggregation of hyphae which specialize in the uptake and transport of water and nutrients). ECM colonization also triggers lateral root formation, thickened short roots and dichotomy in the host, which can increase the root surface area up to 40-fold and hence the water uptake potential of the host plant.

Pathogens, physical and chemical defence: The ECM symbiosis can assist in protecting the host plant against root pathogens and root herbivorous soil microfauna. The fungi reduce the chance of pathogen attack through competition, production of protective antibiotics and association with antagonistic microorganisms unfavourable for pathogens and as a physical barrier to pathogens (Smith & Read, 1997; Sylvia, 1998). The overall improved general fitness of mycorrhizal plants also decreases the risk of pathogen attack (Smith & Read, 1997).

Increased shoot biomass: Increased shoot biomass of mycorrhizal *versus* non-mycorrhizal seedlings is a well reported phenomenon (Harley & Smith, 1983). This improved shoot growth increases seedling survival and competitive success.

Overall the benefits of the symbiosis for the plant are multifaceted, and combined they increase fitness, fecundity, competitive success and tolerance of environmental stresses.

ECM FUNGAL LIFECYCLES AND DISPERSAL

Fungi, like plants, show different life strategies in relation to the substrate available, environmental variables (i.e. stress, disturbance) and their competitors. The classic plant ecology concept of r- and K- selection by MacArthur and Wilson (1967) has also been applied to fungi. High population growth rate and productivity are characteristic of r-selected species. This selection type is found in pioneer and ruderal species, or species which are frequently involved in colonizing episodes (Dix & Webster, 1995; Begon *et al.*, 1998). r-selected species generally disperse *via* spores or special resting structures like sclerotia (Brundrett, 1991). K-selection refers to the carrying capacity of the environment (Dix & Webster, 1995), these species are found in stable environments with high population density. Species are bigger and have a smaller population growth (Begon *et al.*, 1998) and their primary dispersal mechanism is *via* living mycelia, which requires attachment to a living host (Simard *et al.*, 2002).

THE SOIL ENVIRONMENT – THE THIRD PARTNER

The ectomycorrhizal symbiosis is an association between a plant and a fungus where the fungal partner plays a connecting role between the plant and the soil. Thus, edaphic factors play a crucial role in the structure, diversity, abundance, and performance of ectomycorrhizal communities (Brundrett, 1991).

Soil pH: Of all parameters, soil pH is probably the most complex, since many other soil characteristics are closely linked to pH. Mycorrhizal fungi vary with regard to pH optima and changes in the soil pH will affect enzymatic capabilities of certain species, since enzymes produced by the fungi can have narrow pH optima (Erland & Taylor, 2002). ECM are acidophilic, the fungi are adapted to acidic soil conditions and many of the enzyme systems have low pH optima (pH optima are species specific, but most of them range between pH 3 - 5)(Brundrett, 1991). It has been found that increases in soil pH above pH 5 can cause a replacement of ECM by VAM fungi (Brundrett, 1991; Read, 1991). Soil acidification typically leads to a decline in diversity of ECM sporocarps and a reduction in species richness. ECM are acidophilic, but as the pH optimum of a species can be narrow, slight changes may affect the growth negatively. Large increases in soil pH that occur as a result of some forest practices, such as liming, also negatively affect ECM diversity (Erland & Taylor, 2002).

Soil fertility: ECM fungi are adapted to an environment where nitrogen and phosphorus are limited in their availability to the plant. High rates of either, or both, generally suppress ECM development (Brundrett *et al.*, 1996). Nitrogen deposition, either *via* pollution or fertilization, decreases mycorrhizal tip numbers as well as sporocarp production. 'Specialist' species (fungi with a narrow host range), especially symbionts of conifers, seem to be more adversely affected by nitrogen addition than 'generalist' species (fungi with a wide host range) (Erland & Taylor, 2002).

Soil moisture: Where soils are subject to drying out, ECM community diversity is generally lowered (Erland & Taylor, 2002). Waterlogged soil conditions are also negative for ECM communities (Brundrett *et al.*, 1996).

Temperature: The activity of ECM fungi is influenced considerably by temperature and species have specific tolerance ranges and growth optima for temperature (Brundrett, 1991). ECM are regarded as 'cold tolerant' but not 'cold loving' (Tibbett & Cairney, 2007) and the benefits provided by ECM associations are often reduced by low temperatures and may be eliminated at 5 – 10°C (Brundrett, 1991). Also the ECM feeder roots of the host tree differ in their ability to withstand colder temperatures (Castellano & Molina, 1989).

Other soil biota: Also of importance are the interactions with other organisms in the rhizosphere. These interactions can be positive, neutral, or negative on the mycorrhizal association, but enhancement in ECM formation seems to be more common (Brundrett, 1991; Sylvia, 1998). It is suggested that a community of microbes has evolved in association with ECM roots and that these are beneficial to the growth of ECM fungi. For example, specific bacteria called "mycorrhization helper bacteria" stimulate ECM formation in conifer nurseries (Brundrett, 1991; Garbaye, 1994; Sylvia, 1998). Further interactions that can occur include the interplay with mutualistic nitrogen-fixing bacteria and soil bacteria which solubilize rock phosphate (Sylvia, 1998).

Soil factors are, next to the host age, one of the major factors influencing between site differences in ECM communities. When investigating the influence of soil factors or host age on ECM diversity, the control variable must be kept as constant as possible in order to measure the response variable.

1.2 ECM SUCCESSION

1.2.1 THEORY

Succession is defined as 'the non-seasonal change of communities, involving the immigration and extinction of species, coupled with changes in the relative abundance of different species' (Crawley, 1997). It is a directional change in community structure with a continuous pattern of species populations (Begon *et al.*, 1998), ending in a stable 'climax stage' (Clements, 1916; Begon *et al.*, 1998).

Within fungal succession the primary and secondary succession has to be distinguished. In primary succession, pioneer species originate on a virgin surface, i.e. a glacier forefront, coastal sand dunes or volcanic deserts and colonise a new, previously uninhabited land. Clear successional patterns are found in these sites. ECM fungi are critical for tree establishment during primary succession as nitrogen and phosphorus are limited at this early stage (Nara *et*

al., 2003). Neighbouring forests are the source for the fungal inoculum in these virgin landscapes (Jumpponen *et al.*, 2002), the species found in primary succession are of the r-selection type, a life strategy which is characterised by a high population growth rate and productivity (Begon *et al.*, 1998). In secondary successional sites fungal inoculum from previous forests exists which changes the mechanisms of succession. In some of the initial work, the secondary mycorrhizal succession pattern has been described as a sequence of early-stage and late-stage species (Deacon *et al.*, 1983; Fleming, 1983; Last *et al.*, 1984), the former colonizing seedlings from spores and the latter dominating the roots of older trees. This hypothesis is no longer widely supported, as it oversimplifies patterns of fungal succession. ECM colonization patterns may be better described with reference to selection theory, life history strategies (Deacon *et al.*, 1983; Twieg *et al.*, 2007), competition and resource use than by temporal aspects alone (Taylor & Bruns, 1999). The driving forces behind fungal successional patterns are not fully understood but are without doubt multifaceted. It is suspected that changes in carbohydrate supply from the host tree and an increase in the litter and humus layer play a major role (Dighton & Mason, 1984). Furthermore ECM might be affected by increased internal recycling of nutrients as the trees age (Termorshuizen, 1991). Fungal competition and dispersal strategies are another factor and in recent years the influence of edaphic conditions has been stressed as being a major driver in the change of ECM species over the lifetime of a tree (Simard *et al.*, 2002).

On previously treeless sites the following successional pattern is hypothesised (Figure 1-4): 1) r-selected species (e.g. *Laccaria sp., Hebeloma sp., Inocybe sp.*) are present with young, first-generation trees, these ECM show little selectivity for their host tree; 2) fungal species diversity increases as the stand ages; 3) ECM diversity starts to decline when tree litter accumulates with a greater proportion of nitrogen in organic form. This later stage is characterised by K-selected species (e.g. *Amanita* sp., *Russula* sp.), being more host-selective, with larger, more persistent basidomata and more dependent on energy from their hosts (Pugh, 1980; Mason *et al.*, 1987; Frankland, 1998).

In recent studies it has been shown that species found in the early stage are also able to persist in mature to old growth forests (Visser, 1995; Kranabetter, 2005). Also, a decline in species diversity after canopy closure has not been observed in these studies and this change after reaching maturity may differ between host species (Visser, 1995; Bigg, 2000).

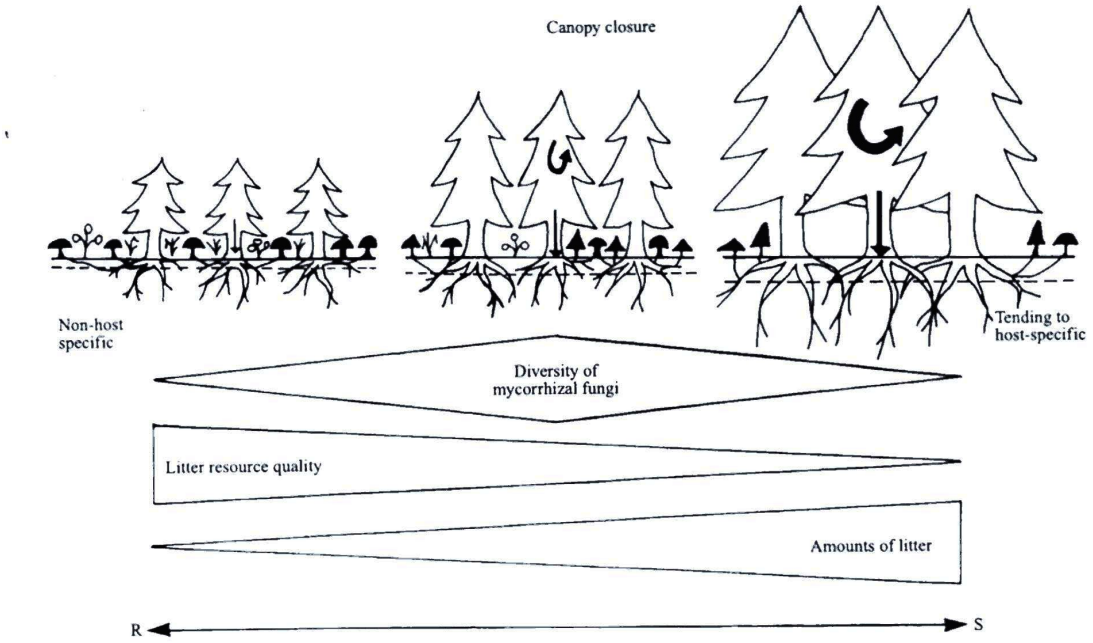

Figure 1-4: Succession of mycorrhizal fungi during forest development in relation to r- and K- selection and the litter resource (From Dighton & Mason, 1985)

1.2.2 THE DISCREPANCY BETWEEN ABOVE- AND BELOWGROUND COMMUNITIES

Until the middle of the 1990s, mycorrhizal succession work was based on aboveground (sporocarp) surveys. The recent development of molecular techniques has made the identification of symbiotic fungi colonising the root tip possible and has revealed that there is actually a poor correlation between species that fruit abundantly and those that are abundant on root tips (Horton & Bruns, 2001). This discrepancy in the above- and belowground abundance can be related to several factors such as variation in resource allocation strategies and that some species invest more in vegetative growth and competition than in reproduction. Other species are known to fruit rarely, if at all, or produce small, easily overlooked fruitbodies (Visser, 1995; Gardes & Bruns, 1996; Frankland, 1998; Peter, 2003). The seasonal shift in sporocarp production, linked with inconsistent sampling, could be another cause for the observed difference in abundance (Frankland, 1998). The pattern of resource allocation to fruit body production versus root colonisation varies among species and this needs to be considered to have a clear understanding of the ecological significance of ectomycorrhizal species diversity within a given ecosystem (Gardes & Bruns, 1996).

1.2.3 APPROACHES TO STUDY SUCCESSION

A long-term goal of community ecology is to identify spatial and temporal factors that underlie observed community structures. In the case of ECM diversity and ecology much of

the knowledge of fungal diversity results from field-based surveys of sporocarps (Horton & Bruns, 2001). A major advantage of such surveys is the potential to record and identify all fruiting ECM fungi within an area relatively quickly. However, because of the importance of exogenous environmental factors in triggering fruiting and the consequent irregularity of reproduction, such characterisation requires long term monitoring (Vogt *et al.*, 1992; Dahlberg, 2001; Durall *et al.*, 2006). Also, it is impossible to include some species in diversity assessments through sporocarp surveys as they do not produce fruiting bodies (i.e. *Cenococcum geophilum,* LoBuglio *et al.* 1996) or the fruiting bodies that they produce are inconspicuous and difficult to detect (Bruns *et al.*, 2002). Thus it is argued that it is best to assess the presence of a fungus in a site by assessing the presence in its vegetative state on the root tip. An approach to identify ECM fungi colonizing roots is 'morphotyping', which involves detailed morphological and structural descriptions of mycorrhizae (Agerer, 1987; Goodman *et al.*, 2003). This method has its drawbacks as it is time intensive, requires extensive training and experience and it often only allows grouping of samples into mycorrhizal morphotypes which do not represent single species, thus underestimating diversity (Martin & Slater, 2007).

The development of the polymerase chain reaction (PCR) and molecular tools has enabled mycorrhizal ecologists to identify ECM species directly from root tips. These methods have many advantages: they can be directed at any taxonomic level, require less time to learn and are fully reproducible in time and space (Gardes & Bruns, 1993; Dahlberg, 2001). In most cases, a combined approach employs morphological sorting of ECM root tips followed by a molecular fingerprinting method.

The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is the main target in ECM identification and ecology studies (Egger, 1995). The ITS consists of two non-coding variable regions, ITS1 and ITS2, which are located within the rDNA repeat between the highly conserved 18S, 5.8S, and 28S rRNA genes (Gardes & Bruns, 1993) (Figure 1-5). The multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or degraded DNA samples and the ITS region is often highly variable among morphologically distinct fungal species, but intraspecific variation is low (Gardes *et al.*, 1991; Gardes & Bruns, 1993). Specific primers have been designed which preferentially amplify specific fungal ITS fragments from mixtures of plant and fungal DNA (Bruns & Gardes, 1993; Gardes & Bruns, 1993).

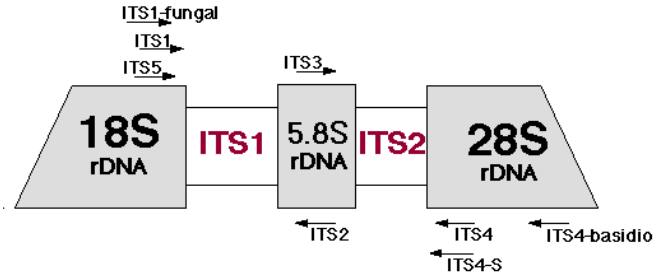

Figure 1-5: Map of location of fungal primer sites of the Internal Transcribed Spacer (ITS) region of the rDNA.

(from: http://plantbio.berkeley.edu/~bruns/picts/results/its-map.GIF).

A widely used approach for species identification, restriction fragment length polymorphism (RFLP) is performed by restriction digestion of the PCR amplified ITS region. The RFLP pattern results in a diagnostic species specific pattern of fragment sizes that can be used to identify species by matching against a database of known species (often based on sporocarp material). The RFLP is generally followed up by cloning, sequencing and sequence searching in databases such as GenBank, EMBL or UNITE. The cost and effort of sequencing has decreased in recent years and is becoming a more widely adopted approach and the databases for ECM fungal sequences are growing each year. Other molecular fingerprinting methods include LH-PCR (amplicon length heterogeneity PCR), T-RFLP (terminal restriction fragment length polymorphism) and DGGE (denaturing gradient gel electrophoresis) (Martin, 2007). T-RFLP and LH-PCR are capillary electrophoresis methods, which are more precise than RFLP, but entail a high cost for equipment and operation. DGGE is a good means to characterise ECM community variation but does not identify species.

To attain a complete as possible picture of ECM species diversity of a system it is preferable to assess both, above- and belowground species diversity due to the discrepancy in fruiting and also colonisation patterns. Furthermore aboveground collections that can be identified using morphological characteristics are needed as reference material for species identification of unknown ECM types with molecular methods.

1.3 FORESTRY

1.3.1 THE FORESTRY SECTOR IN NEW ZEALAND

The Forestry industry in New Zealand has been gaining momentum over the last 25 years and has developed to be the country's third largest export earner behind the meat and dairy industry (Taylor & Smith, 1997; NZFOA, 2006). Forestry accounts for 3.1% of New Zealand's gross domestic product and directly employs approximately 23,500 people. Forestry product

exports are worth more than NZ$3.3 billion, which accounts for 11.1 % of all New Zealand's exports (NZFOA, 2006). The country's current annual wood harvest was 21 million m^3 in 2004 and is expected to reach 40 million m^3 by 2025, equivalent to an export value of more than NZ$14 billion (Forestry Insights, 2004; NZFOA, 2006). Ninety percent of New Zealand's plantation forests are planted in *P. radiata*, the remaining ten percent is made up by *Pseudotsuga menziesii* (Douglas fir), *Cupressus macrocarpa* (macrocarpa), *C. lusitanica*, and various *Eucalyptus* spp. (MacLaren, 1993; Taylor & Smith, 1997; Burdon, 2000; Burdon, 2002).

1.3.2 DOMINANCE OF *PINUS RADIATA* IN NEW ZEALAND FORESTRY

The dominance of *P. radiata* can mainly be attributed to its growth potential in New Zealand's climate. *Pinus radiata* has a long, opportunistic growing season in this country and can grow throughout the whole year without a dormancy stage (Burdon, 2000). The species successfully combines the winter-growth capacity of many Mediterranean plants with the ordinary 'summer' growth capacity of species from cooler temperate climates (Burdon, 2000). Ideal growing conditions for *P. radiata* are warm days and cool nights, fertile soils and high rainfall spread throughout the year. It can also withstand mild frosts with -12°C being the low temperature limit. As all these conditions are combined in New Zealand's climate, *P. radiata* outgrows almost all other tree species on nearly every site, producing 20 to 25 cubic metres of wood per hectare each year and is ready to harvest in 25 to 30 years (MacLaren, 1993; Burdon, 2000; Burdon, 2002; Forestry Insights, 2004). Yet another explanation for the dominance by *P. radiata* in New Zealand is the poorer establishment of other species. In the late 1950s, Gilmoure (1958) recognised that the poor growth of *P. menziesii* was due to a lack of mycorrhizal colonisation. Many other speciality timber species, such as the stringy bark eucalypts, also perform poorly in New Zealand compared to their successful performance overseas and it is possible that this is also due to a lack of favourable mycorrhizal associations (Hall & Perley, 2006).

1.3.3 THE HOST: *PINUS RADIATA* D. DON 1836

Pinus radiata is one of the most extensively domesticated forest tree species. Approximately 4 million hectares are grown world wide, which is 500 times its natural range in California (Burdon, 2000; Burdon, 2002). The species occurs naturally in three discrete populations and covers around 4,500 ha in California (Dilworth, 2004). *Pinus radiata* has been planted on a large scale in New Zealand, south-central Chile, southern Australia and northern Spain. Other countries where the species is used as a plantation tree are Italy, Kenya, Argentina, Columbia and Ecuador (Dilworth, 2004).

Pinus radiata belongs to the subgenus Pinus, section Pinus; its common names are Monterey pine, radiata pine, insignis pine or radiata. Known variations are *Pinus radiata* var. *binata*

(Engelm.) Lemmon and *Pinus radiata* var. *cedrosensis* (J. T. Howell) Axelrod (Burdon, 2002). The evergreen tree grows to 15-30 m tall in natural stands, but can reach 55 m in exotic plantations with the final height dependent on the site conditions (Kral, 1993; Burdon, 2000; Burdon, 2002). A full botanical description of the species and its growth requirements is given by Burdon (2000, 2002), Burdon & Miller (1992) and Kral (1993).

1.3.4 ORIGIN OF *PINUS RADIATA* IN NEW ZEALAND

Although native to California, USA, the origin of Southern Hemisphere *P. radiata* is most likely England, where it's cultivation history began in 1833 (Bannister, 1973) and Australia (Weston, 1957). Early settlers imported seedlings to Canterbury and the earliest record of the plantation species in New Zealand is from the 1850's, when it was planted for shelter in treeless areas (MAF, 2004). By 1865 the species was well established and the biggest consignments of seed ever to arrive in New Zealand were probably those imported by the Government in the 1870's in connection with a land settlement scheme. New Zealand became self-sufficient for radiata pine seed from the early 1880s (Burdon & Miller, 1992).

1.3.5 SILVICULTURE IN NZ

Forest establishment is a critical phase in the forestry cycle, as success at this stage provides the basis for the whole rotation. In New Zealand, planting stock is generally produced either by seedlings, cuttings or tissue-cultured plantlets (Menzies *et al.*, 1995). Nurseries producing seedlings are more common, although containerised seedlings are gaining in popularity. Good seedling quality is important to achieve high survival rates and strong early growth in plantations. Sensible manipulation and conditioning during the time in the nursery are important factors influencing the survival rate of a tree. Inoculation with mycorrhizal fungi, fertilization and spacing of seeds can affect the morphological characteristics of the stock (Menzies *et al.*, 1995). Conditioning of the tree stock is done by techniques such as wrenching, undercutting and lateral root pruning (MacLaren, 1993; Menzies *et al.*, 1995; MAF, 2004). With healthy *P. radiata* seedlings, survival rates of 98% in the plantation are now common (MacLaren, 1993; MAF, 2004).

Pinus radiata seedlings are planted out approximately one year of age, during the winter months of May through to September. Seedling roots are trimmed before outplanting to ensure good establishment and root development, as bent or twisted roots would not provide good establishment and result in toppling at a later stage (MacLaren, 1993). Outplanting is done either manually or mechanically (MacLaren, 1993; MAF, 2004). The plantations are intensely managed with regular pruning and thinning to obtain high quality knot free clear wood (MacLaren, 1993; MacLaren & Knowles, 1995; Burdon, 2000; MAF, 2004). Pruning starts at age four and ends at eight to ten years of planting, with generally two or three prunings during this time frame. Thinning is the removal of trees from a stand that are defective, sick or diseased. It is done to reduce the number of trees to the final stocking level

and give he remaining trees room to grow. The first thinning is usually carried out at the first pruning stage and subsequent thinning may be carried out at the same time as later prunings (MacLaren, 1993; MacLaren & Knowles, 1995). A 25 to 30 year rotation is common practice in New Zealand.

1.3.6 MYCORRHIZAE IN FORESTRY

Survival and good growth of nursery grown seedlings in the plantations is increased greatly by the presence of mycorrhizal associations in both the nursery stage and in the out planting phase. Lack of mycorrhizal colonistion of root systems is a leading cause of poor plant establishment and growth in the forest. Good forest tree nursery practice and compatible inoculation programmes can ensure that seedling roots are colonised by mycorrhizal fungi, resulting in improved growth after field planting (Mikola, 1969; Molina & Trappe, 1984; Stenström & Ek, 1990; John, 1996; Smith & Read, 1997; Ortega *et al.*, 2004). Root colonisation by ECM fungi furthermore provide an effective pathogen protection in nursery beds, where seedlings are crowded together and are particularly susceptible to fungal pathogen attacks (Smith & Read, 1997).

Mycorrhizae are of great importance for the establishment of seedlings, but not all ECM fungi produce the same effect on growth and survival of a specific host plant (Molina & Trappe, 1984) due to the host specificity, succession patterns and varying effects of the symbiosis under different conditions. Therefore, it is important to have mycorrhizal fungi present in the nursery and plantation and the selection of the appropriate fungal species should be based on its position within mycorrhizal succession, its ability to stimulate the growth of the host and by the persistence of the association (Mason *et al.*, 1983; Duñabeitia *et al.*, 1996).

At present it is common practice in New Zealand to inoculate nurseries with soil or duff inoculum (sporocarps or spores) although established nurseries often do not inoculate at all, relying on natural soil population of introduced ECM. Inoculum material is usually collected from established plantations (W. Brown, personal communication, 1 September 2004). In forest conditions, where mycorrhizal roots are already present, the living hyphae are the most common source of inoculum and probably dominant over spore colonisation (Heinonsalo, 2004). Spores and sclerotia can survive in the soil for a very long time and can be activated by suitable conditions like heat shock after forest fire, increased soil moisture or stimulatory compounds in root exudates (Smith & Read, 1997). After clear cut logging, no living hyphae are available, the major source of inoculum for new tree seedlings is liekly to be sclerotia and spores. If regeneration occurs within two - three years, the remaining stumps and dying roots have been shown to support living hyphae of ECM fungi for this length of time (Hagerman *et al.*, 1999) and new seedlings can aquire mycorrhizae from these (Jones *et al.*, 2003; Simard & Durall, 2004).

1.4 ECM RESEARCH IN EXOTIC PLANTATIONS

1.4.1 NEW ZEALAND

The most significant research in New Zealand on ECM fungal diversity in exotic plantations was conducted by Chu-Chou & Grace, in the late 1970s – 90s (Chu-Chou & Grace 1979, 1980, 1983b, 1984b, 1987, 1988, 1990). Their work focused on *Pinus radiata* in the North and South Island, but also included *P. menziesii* and *Eucalyptus* spp. plantations. Methods used in these studies were sporocarp field observations, collections and pure tissue isolation of fungal symbionts from sporocarps and mycorrhizae, which were compared to each other for identification of fungal species. This research revealed low species richness in plantations, successional patterns, a variable effect of ECM species on the tree growth and a dependency of fungal communities on the soil type. The information is based mainly on sporocarp observations, and due to an inability to culture some types, many ECM species colonising the root tips remained unidentified within this research. Other research currently being conducted in this ares included a masters project investigating the effect of *Trichoderma* bio-inoculants, used to protect *P. radiata* seedlings from fungal pathogens, on ECM colonisation of seedlings (R. Minchin, unpublished, personal communication, 4 December 2007). Hall & Perley (2006) are also currently investigating the effect of nutrients, fungicides and *Rhizopogon parksii* inoculum on the mycorrhizal formation of *P. menziesii* in a nursery in the South Island.

1.4.2 OTHER COUNTRIES

ECM studies on exotic plantations in other southern hemisphere countries mainly focus on sporocarp diversity assessments or the selection of beneficial inoculant species for nursery seedlings. ECM sporocarp communities of *P. radiata* in Australia were assessed by Dunstan (1998) and Bowen (1963), and in Chile by Garrido (1986), whereas research in Spain is investigating the differential responses of various fungal species to environmental factors and their role in the mycorrhization of *P. radiata* seedlings (Duñabeitia *et al.*, 2004). A study by Baseia & Milanez (2002) investigated the diversity of *Rhizopogon* spp. associated with *P. radiata* in plantations in Brazil. Chapela *et al.* (2001) researched the ECM fungal induced soil depletion in exotic radiata pine plantations in Ecuador. Other fungal aboveground communities in exotic *Pinus* spp. plantations were studied by Barroetaveña (2005) in Argentina and by Giachini *et al.* (2004) in southern Brazil. To the knowledge of the author no study has assessed the diversity and successional patterns of both the above- and belowground communities of ECM fungi associated with an exotic *P. radiata* plantation.

1.5 AIMS AND OBJECTIVES

The ECM communities of *P. radiata* plantations in New Zealand were studied by Chu-Chou & Grace in the late 1970s – 90s (Chu-Chou & Grace 1979, 1980, 1983b, 1984b, 1987, 1988, 1990), however no further work on the ECM associates of this major forestry species in New Zealand has been conducted since. Chu-Chou & Grace's work was based on sporocarp field observations, collections and pure tissue isolation of fungal symbionts from sporocarps and mycorrhizae. Due to the difficulties associated with culturing ECM species from root tips, many ECM species colonising root tips remained unidentified within this research. Chu-Chou & Grace's research correlated well with the body of international literature (Dighton & Mason, 1984; Last *et al.*, 1987; Mason *et al.*, 1987) based on sporocarp studies that concluded that there is a succession of ECM fungi over time in the life of the host tree. This theory is based on the assumption that the first appearance of the fruiting bodies of a specific species is an indicator of the approximate time, within a couple of years, which colonization of the tree's roots has occurred. Surprisingly, the application of genetic fingerprinting in the mid 1990s, indicated that there was little correlation between species that fruit abundantly and those abundant on the roots (Dahlberg *et al.*, 1997; Smith & Read, 1997; Dahlberg, 2001; Horton & Bruns, 2001). This poses the questions – 'Is there really a succession? Or have only fruiting patterns been observed?' As it is the belowground community structure of mycorrhizal fungi that directly influences the plant nutrient acquisition, an ECM succession would provide a rational basis for the selection of fungi for nursery and forestry practices. The answer to this question also has implications for biosecurity and sustainability – can the fungi help protect against potential pathogens and are the right fungi entering the system at the right time?

The aim of this study was to expand and advance upon the earlier work in New Zealand and investigate ECM species and communities associated with *P. radiata* using new molecular techniques for simultaneous detection of ECM above- and belowground. The molecular techniques will allow more definitive assignment of ECM species, especially ECM colonising root tips. Increasing knowledge on ECM species associated with *P. radiata* in New Zealand and the change of ECM communities over time will provide a sound basis for application to forestry practises and potentially inoculation programs.

The overall objective of this work is to describe both, above- and belowground ECM communities associated with *P. radiata* in a plantation in the North Island of New Zealand at different stages of the plantation (nursery – harvest) and to observe serial changes within the ECM fungal population. The specific objectives of this thesis are:

- To identify ECM species colonising root tips using molecular methods (RFLP analysis and direct sequencing) (Chapter 3).
- To determine the overall, as well as stand specific, diversity of the above- and belowground ECM communities within different age classes of *P. radiata* plantation (Chapter 3).
- To examine the previously hypothesised succession of ECM and the influence of host age on the above- and belowground ECM communities of different age classes of *P. radiata* plantations (Chapter 4).
- To find discriminating ECM species for each age class for both above- and belowground ECM communities associated with *P. radiata* (Chapter 4).
- To determine the survival and persistence of nursery ECM species in the first year of outplanting (Chapter 5).

1.6 THESIS FORMAT

The thesis comprises six chapters, four of which are experimental chapters. As the same materials and methods were used throughout the study, a general Materials and Methods chapter has been presented in Chapter 2, details will not be repeated in the specific results chapters (Chapters 3 – 5). Chapter 7 unites the research conducted for this thesis in a concluding discussion.

2 MATERIAL AND METHODS

2.1 STUDY SITES

All research sites used in this study were monoculture *Pinus radiata* plantations located within Kaingaroa Forest, with the exception of Te Ngae nursery. Access was kindly facilitated by Kaingaroa Timberlands Ltd. Study sites were established in Kaingaroa Forest in March 2005 for the duration of the study. Kaingaroa Forest is located between Rotorua and Taupo on the Volcanic Plateau on the North Island of New Zealand (Figure 2-1).

Plots in Te Ngae Nursery were temporary, from May to July in both 2005 and 2006. Te Ngae Nursery is located on SH 30, north of Rotorua. This nursery grows *P. radiata*, *Pseudotsuga menziesii* (Mirb.) Franco and *Cupressus lusitanica* Mill. and is the main supplier for Kaingaroa Forest plantations. *Pinus radiata* is grown either from seed or cuttings, in this study only seedlings grown from seed were investigated. Seed is sown in May and seedlings are lifted the following year (W. Brown, personal communication, 8 December 2005).

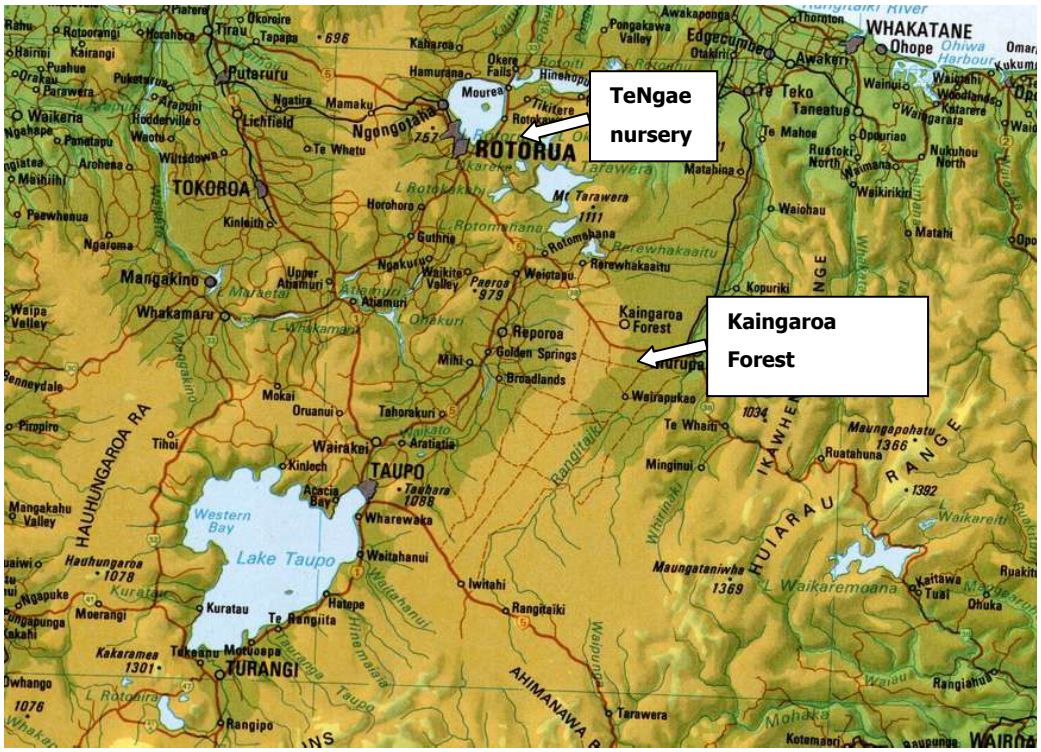

Figure 2-1: Location of Te Ngae Nursery and Kaingaroa Forest (World TopMap NZ V2.061, Map Series 1:1 Million).

Table 2-1: Study sites used with outplanting year (for plantation forest), location, site label and use during the sporocarp (SA) and soil core (SCA) assessments in 2005 and 2006.

Study site	Site label	Outplanted	Plantation age in 2006	Location	Used for
Nursery 2005	K05-N	n/a	n/a	Te Ngae Nursery	SA & SCA 2005
Nursery 2006	K06-N	n/a	n/a	Te Ngae Nursery	SA & SCA 2006
KANG 1005/7	K06F-OS	2006	<1 yr	Kaingaroa Forest	Outplanting Survey 2006/07
KANG 1068/8	K05F-1	2005	1 yr	Kaingaroa Forest	SCA 2006
KANG/92/2	K04-2	2004	2 yrs	Kaingaroa Forest	SA & SCA 2005/06
KANG/91/1	K98-8	1998	8 yrs	Kaingaroa Forest	SA & SCA 2005/06
KANG/69/1	K91-15	1991	15 yrs	Kaingaroa Forest	SA & SCA 2005/06
KANG/82/3	K80-26	1980	26 yrs	Kaingaroa Forest	SA & SCA 2005/06

n/a = not applicable; SA = sporocarp assessment; SCA = soil core assessments

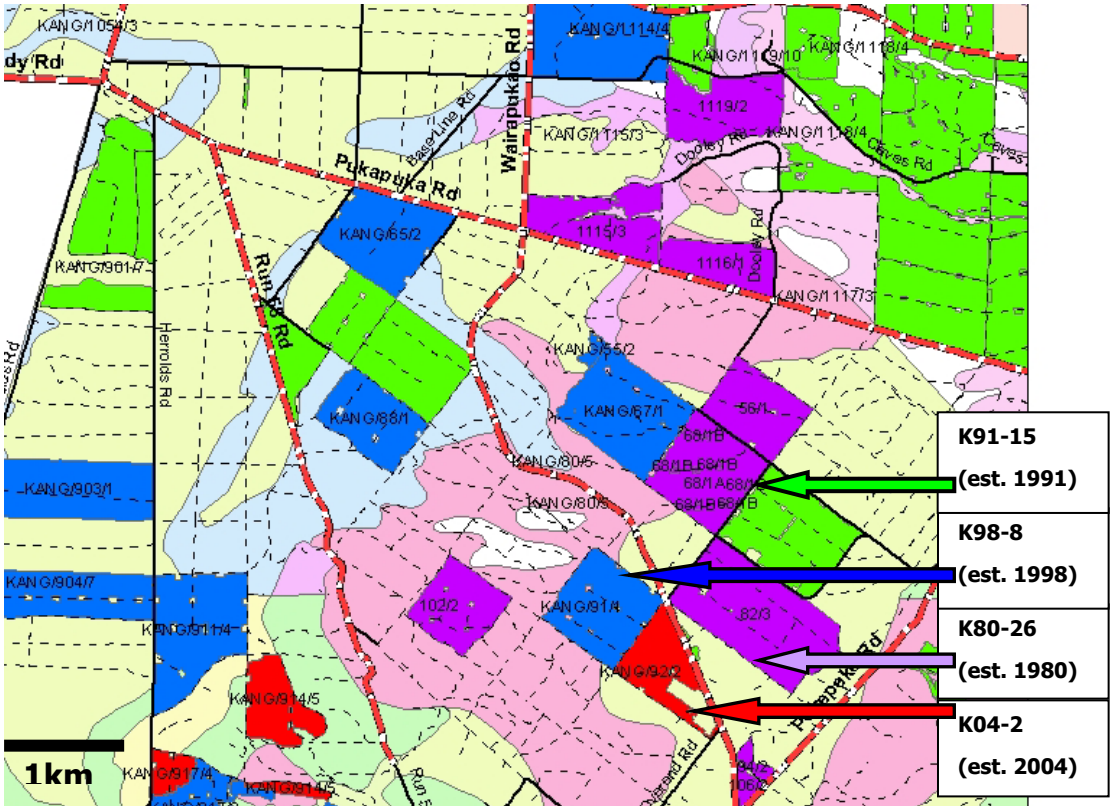

Figure 2-2: Location of study sites K04-2, K98-8, K91-15 and K80-26 within Kaingaroa Forest, Kaingaroa Timberlands Ltd, North Island, New Zealand (est.=established).

Study sites used in this research and respective site labels are listed in Table 2-1. Site labels are a combination of outplanting year and age of plantation site at the point of last assessment, 2006. The nursery sites are labelled with 'N' for identification as nursery seedlings, the site used for the outplanting survey is labelled with '-OS' as the plants are younger than one year. Study sites K04-2, K98-8, K91-15 and K80-26 in Kaingaroa Forest were located along Wairapukao Road, approximately 40 km south of Rotorua and were adjacent to each other (Figure 2-2). Study site K05F-1 was located along Northern Boundary Road; study site K06F-OS was located along No. 3 Road (Figure 2-3).

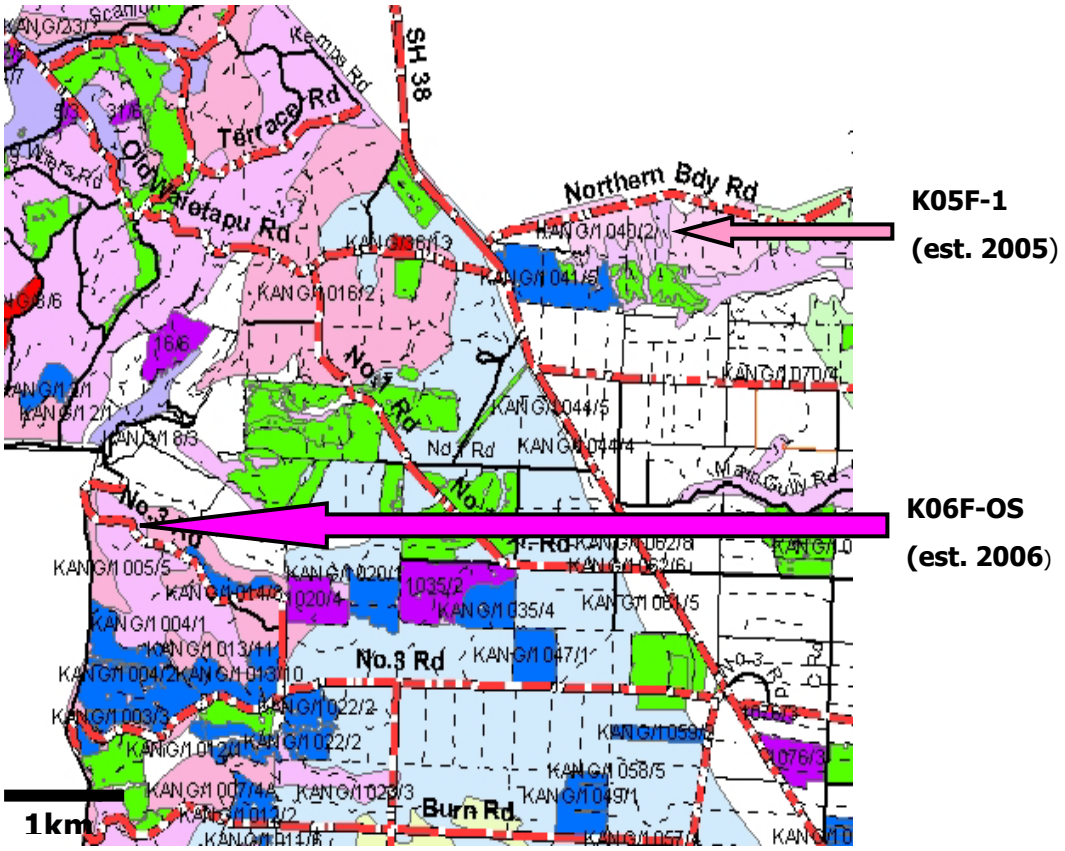

Figure 2-3: Location of study sites K05F-1 and K06F-OS within Kaingaroa Forest, Kaingaroa Timberlands Ltd, North Island, New Zealand (est.=established).

2.1.1 RATIONALE FOR STUDY SITE CHOICE

The host is the most crucial factor determining the composition of an ectomycorrhizal community, but environmental factors such as soil type, pH and microclimatic conditions play an important role in the structure, diversity, abundance and performance of mycorrhizal communities. The aim of this study is to investigate mycorrhizal communities associated with *P. radiata* with a special focus on the changes of ECM communities over different age classes of *P. radiata*. In order to focus on host age, it was important to keep external factors such as soil conditions in the study sites, which could influence species composition and diversity, as

similar as possible. Hence only study sites where factors such as geomorphology, terrain, soil type and microclimatic conditions were consistent were selected. Furthermore, only *P. radiata* stands grown from seedlings, not cuttings, were used. In the nursery stage, only seedlings grown from seed were investigated. In order to have as little variation in the source of the seedlot as possible - trees at the investigated sites were either sourced from Te Ngae Nursery, or Owhata Nursery, Rotorua. All plantation sites in Kaingaroa Forest are in the third rotation with *P. radiata,* except for site K80-26 which is in the second rotation. Additional important factors in choosing the field sites were accessibility and safety.

2.1.2 STUDY SITE DESCRIPTIONS

NURSERY STAGE

The nursery stage of the plantation trees was investigated at Te Ngae Nursery (Figure 2-4). This bare root nursery grows *P. radiata*, *P. menziesii* and *C. lusitanica*. Seedlot is lifted in May/June. Seedbeds are rotated almost every year in this nursery. Seedlings investigated in 2005 were planted on a block that was left fallow in the previous year (*P. radiata* in the year prior) whereas the seedbed in 2006 was previously planted with *P. radiata* cuttings. The soiltype in the blocks investigated was originally a sequence of loamy sand (to about 30 cm), Rotomahana mud (25 – 35 cm) and Kaharoa ash (35 – 65 cm). The soil is now well mixed due to planting and lifting of the seedlings. The soil was analysed prior to planting and treated according to recommendations (W. Brown, personal communication, 19 November 2007) with a base-dressing of Superphosphate prior to planting and two applications of Nitrophosphate following planting. The fungicide Sporetech was applied in seven – 14 day intervals depending on the weather, additionally the site was sprayed with copper in February. In 2006 the block was also treated with calcium ammonium nitrate (CAN) due to an outbreak of *Phytophthora*.

Figure 2-4: Photograph of Te Ngae nursery in 2005 (left) and 2006 (right).

K06F-OS, K05F-1 AND K04-2

These sites were all clearcut sites that were replanted in June, two years after harvest (Figure 2-5 and Figure 2-6). Remnants of blackberry (*Rubus fruticosus* agg.) were present, which re-grew in the following year. Soil type for all sites was Kaingaroa sandy loam and sites were in their third rotation. K04-2-2 is 56ha, K05F 35 ha and K06F 48 ha in size.

Figure 2-5: Photograph of study site K06F-OS.

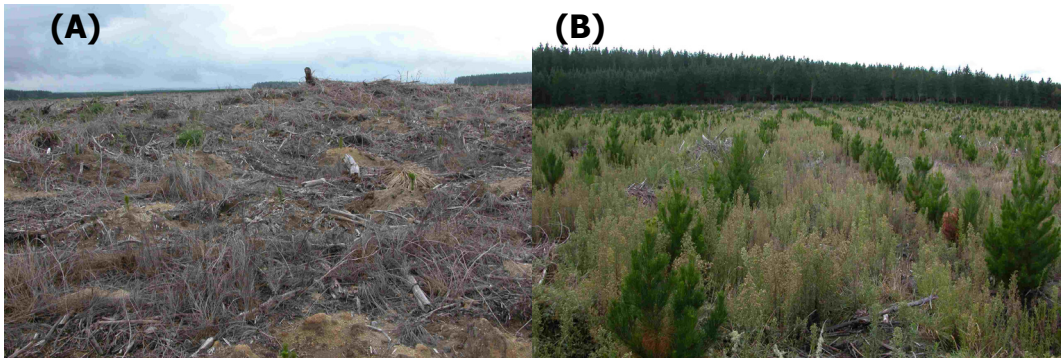

Figure 2-6: Photograph of study site K04-2 in 2005 (A) and 2006 (B).

K98-8

This stand was planted in 1998, is 83 ha in size and the trees were seven years of age at the first assessment (Figure 2-7). The site was fairly open and easily accessible. The understorey was mainly made up of pasture species and broom (*Cystisus scoparius* Linn.). The broom was dispersed in the site and grew in dense clusters. This site was pruned in early 2006, the soil type for this site is Kaingaroa sandy loam and the site is in the third rotation with *P. radiata*.

Figure 2-7: Photograph of study site K98-8.

K91-15

Planted in 1991, this site was thinned in 2002 and trees were 14 years of age at the first assessment (Figure 2-8). The understorey in the stand was made up of blackberry, bracken fern (*Pteridium esculentum* G. Forst), grasses and *Dicksonia squarrosa* G. Forst. Due to the thinning waste on the ground and the prominent growth of blackberry and bracken fern, accessibility in this site was limited and the ground cover complicated sporocarp surveying. The weeds, blackberry and bracken fern, were dense and over 1.5 m high, which made walking through some transects impossible. The soil type of the site is Kaingaroa sandy loam and it is 104 ha in size.

Figure 2-8: Photograph of study site K91-15.

K80-26

This stand, 144 ha in size, was planted in 1980 and will be harvested in 2008 (Figure 2-9). Thus this represents the oldest possible stand age for *P. radiata* in this study (25 years at time of first assessment). The soil type is Kaingaroa sandy loam and the site is in its second rotation with *P. radiata*. The understorey in this site was more diverse than the younger sites,

and included *R. fruticosus*, *P. esculentum*, *D. squarrosa* and *C. scoparius.* The accessibility in this site was good.

Figure 2-9: Photograph of study site K80-26.

2.2 SAMPLING DESIGN

At all study sites a 1 ha plot was established approximately in the middle of each stand (stand size ranging from 56 – 144 ha) to avoid an edge effect. Five permanent 100 m long transects were set up randomly within each plot (Figure 2-10). All transects were set-up in north-south orientation within the site, field sites were not thinned during the study period to avoid disturbance. At Te Ngae Nursery in 2005, the site set up for surveying was 100 m x 60 m as no more seedlings were planted in this year. In 2006 the site surveyed in the nursery was increased to 100 m x 100 m.

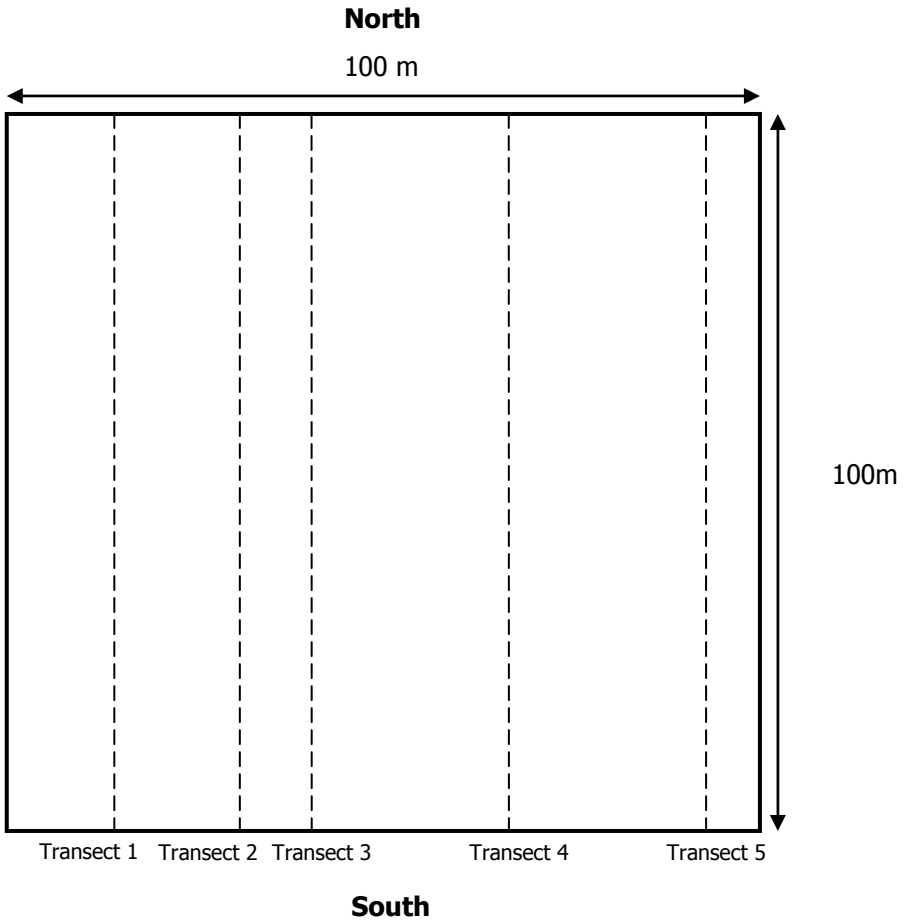

Figure 2-10: Permanent plot layout, consisting of 100 m x 100 m area roughly in the centre of each of the investigated stand with five transects for sporocarp assessments laid parallel but at a random distance to each other.

For collection of the soil cores, locations within the study site plot site were determined randomly. The location for a soil core was a combination of a randomly chosen tree row (west to east orientation within the plot) and a randomly chosen tree number within the row (north to south orientation within the plot) (Figure 2-11). Random numbers for transects and soil core location were generated with Excel (Microsoft Corp.).

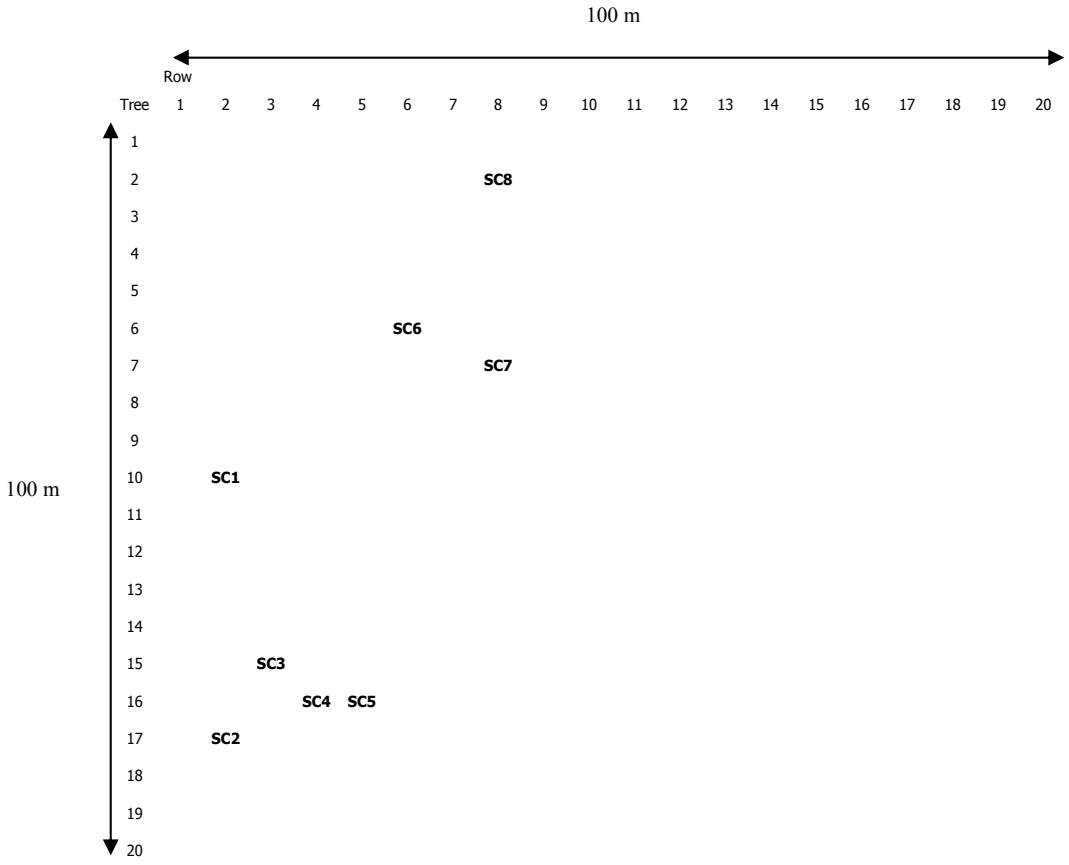

Figure 2-11: Soil core location in study site K80-26 for soil core assessment 3 as an example for random distribution of soil cores (SC 1-8). Rows and trees within the permanent plot were counted and a row and a tree within this row were randomly determined.

2.3 SPOROCARP ASSESSMENTS - SAMPLING AND LABORATORY PROCESSING

2.3.1 SPOROCARP PRESENCE AND ABUNDANCE

To investigate sporocarp diversity and abundance, sporocarp assessments were undertaken in both 2005 and 2006. Sporocarps were identified and collected at the sites during the fruiting season (dependent on climatic conditions, March – July). In 2005, the following sites were assessed: K05, K04-2, K98-8, K91-15 and K80-26. Sites were visited from April to June 2005 at fortnightly intervals; K80-26 was visited from January to June 2005 for preliminary investigations. In 2006, study sites K06, K04-2, K98-8, K91-15 and K80-26 were surveyed. Sites were visited from March to June 2006 at 3-weekly intervals due to weather conditions not being favourable for more visits (Table 2-2).

Table 2-2: Sporocarp assessments: site information, including date of site establishment, site identification label, survey period and frequency of visits, and source of seedlot used.

Site established	Site label	Sporocarp Assessment 1	Sporocarp Assessment 2	Seedling source
Nursery 2005	K05-N	April-June 05 5 visits	n/a	Te Ngae Nursery
Nursery 2006	K06-N	n/a	April-May 06 4 visits	Te Ngae Nursery
2004	K04-2	April-June 05 6 visits	March–Jun 06 4 visits	Te Ngae Nursery
1998	K98-8	April-June 05 6 visits	April-June 06 4 visits	Te Ngae Nursery
1991	K91-15	April-June 05 5 visits	April-June 06 4 visits	Owhata Nursery
1980	K80-26	January-June 05 10 visits	March–June 06 4 visits	unknown

n/a = not applicable

Sporocarps sampled were restricted to those found within approximately 2 m either side of a transect, decayed specimens were ignored. Searches for hypogeous species were kept to a minimum to avoid unnecessary disturbance. However, at sporadic locations, soil next to trees was excavated to search for hypogeous fungi. A sign of animal digging was also used as an indicator for presence of hypogeous fungi, with these areas also examined for presence of hypogeous fungi. In 2005, only presence/absence of ectomycorrhizal species was recorded. In 2006, abundance of basidiomycetes was recorded. Specimens were collected for DNA extraction, species identification and preservation at the National Forestry Mycological Herbarium (NZFRI-M; held at Ensis, Rotorua).

2.3.2 CLIMATE DATA

Climate Data was provided by Kaingaroa Timberlands from the closest meteorological station (Goudies Road) to the study sites in Kaingaroa Forest. The meteorological station recorded data in hourly intervals, for the purposes of this study only temperature, relative humidity and rainfall were analysed. The data set provided spanned from 1 January 2005 to 12 July 2006.

2.3.3 SPOROCARP IDENTIFICATION AND STORAGE

SPOROCARP PROCESSING

Specimens collected in the field were stored in paper bags at 4°C and identified and processed within one week. A collected specimen received an identification label, based on the study site collected and in numerical order of collection. For example the first sporocarp collected at study site K91-15 was labelled K91-15S1. Photographs of the specimen were taken *in situ* and in most cases further close up photos were taken on constant coloured background in the laboratory. Specimens were identified based on morphological characteristics to at least genus level and if possible to species level. Features investigated were colour, size, odour, cap and stem shape, gill abundance and attachment, veil presence or absence and other characteristics noted in the keys. Spore prints were taken by placing a cap of a specimen over white and black paper and leaving it for several hours. Spore prints were added to the voucher specimens. For identification literature used and species descriptions, see Appendix 2.

From each sporocarp a small piece of the tissue was removed with a flame sterilised scalpel for DNA extraction. The tissue was either placed in a 15 ml tube (Nalgene Nunc International, Rochester, USA) with dry silica gel (6-20 mesh, BDH Chemicals, England) and stored at -80°C until DNA extraction or used directly for DNA extraction. Sporocarp tissue was also excised from the stem or cap to attempt isolation of the fungal species into pure tissue culture. For both DNA and pure tissue culture, only fresh, clean material was used. Finally, specimens were dried in a commercial fruit dryer at 40°C for 24 – 48 hrs and lodged in the National Forestry Mycological Herbarium (NZFRI-M).

SPOROCARP PURE TISSUE CULTURE

Production of cultured specimens from sporocarps was attempted. These cultures were used for DNA extraction and as reference material. However, for some ECM species pure cultures were not obtained, as they were either unable to grow in culture due to being obligate host tree-dependent or were associated with bacteria which contaminated the cultures.

Isolation was done under sterile conditions in a laminar flow hood. Only fresh, un-infected sporocarps were used for isolation. Sporocarps were brushed free of adhering soil and fractured. Small amounts of fungal tissue were removed with flame sterilised razor blades and forceps and placed on agar in a Petri dish (9 cm diameter, Biolab Ltd, Auckland, NZ). Tissue was removed from the apex of the stem or the cap itself, in the case of puffballs and specimens of the genus *Tuberaceae*, the immature internal fertile tissue was taken (Brundrett *et al.*, 1996). Media used was Hagem media (HG), modified Melin Norkans media (MMN) and Benlate/malt extract media (BME) (Appendix 1). Initially two plates of each media were used for every isolated species (six replicates). Cultures were incubated at 20°C in the dark and checked daily for contamination and growth in the initial 2 weeks of culturing. Samples were sub-cultured every 3 months onto fresh agar, one HG and BME plate for each

sample. The MMN media was omitted in the later stages as successfully isolated species grew best on HG and BME. Cultures used for DNA extraction were sub-cultured on sterile GelAir cellophane (BIO-RAD Laboratories, Hercules CA, USA) which was placed on either HG or MBE agar plates. Mycelium was scraped off the cellophane directly. Cultures were also sub–cultured on 1.5% malt extract agar (Appendix 1) and added to the National Forestry Culture Collection (NZFS).

2.4 SOIL CORE ASSESSMENTS – SAMPLING AND LABORATORY PROCESSING

2.4.1 SOIL CORE SAMPLING

Soil core assessment was undertaken in autumn 2005 (SCA 1), summer 2005 (SCA 2) and autumn 2006 (SCA 3). The following age groups were investigated (Table 2-3): SCA1 – nursery stage and plantation forest sites established in 2004, 1998, 1991 and 1980; SCA 2 – plantation forest sites established in 2004, 1998, 1991 and 1980; SCA 3 – nursery stage and plantation forest sites established in 2005, 2004, 1998, 1991 and 1980. Seedlot source for the site established in 2005 was seedlings that were investigated at Te Ngae Nursery in 2005 (K05). A separate "Outplanting survey" (Chapter 5) was carried through from August 2006 to June 2007 on site K06F. Three seedlings each were collected monthly from June 2006 – February 2007 and once in June 2007. Seedlings were sampled randomly in a plot as described in Section 2.2.

To extract soil, a corer of 5 cm diameter and 40 cm length was used (manufactured by the Ensis workshop, Rotorua, Figure 2-12). Due to the size of seedling root systems in the nursery and the first year of outplanting, the whole seedling was collected in the nursery stage, at K04-2 and at K05F in 2005. Soil cores were stored in plastic bags at 4°C until further processing. In SCA 1 in June 2005, 20 cores each were collected from K05, K04-2, K91-15, K98-8 and K80-26 over the course of a week (Table 2-4). For this, ten trees were chosen randomly, two cores were taken in 40 and 80 cm distance from each tree. As not all of the collected cores could be processed, the number sampled was reduced for subsequent assessments. To keep the distribution of cores sampled within the site variable, it was decided to sample one core from a tree only. In a preliminary sampling, differences in amount of root tips found at 40, 60, 80 and 100 cm were assessed (data not shown). No differences were observed; hence sampling was limited to a core at 60 cm from the stem. The number of collected cores was reduced to 10 cores in SCA 2 in December 2005. In SCA 2, the same trees as in SCA 1 were used. Sites sampled were K04-2, K91-15, K98-8 and K80-26; all cores were taken within one week. Due to the increase in sites sampled in SCA3, the number of samples collected was reduced to 8 cores per site. Cores/seedlings were taken at one study site at a time and processed in the laboratory within 1 – 2 weeks. Sites sampled during SCA 3 were K06, K05F, K04-2, K98-8, K91-15 and K80-26.

Table 2-3: Soil sore assessments: site information, including date of site establishment, site identification, survey period and number of cores taken, and source of seedlot used.

Site established	Site label	Soil core assessment 1	Soil core assessment 2	Soil core assessment 3	Seedling source
Nursery 2005	K05-N	June 05 20 seedlings	n/a	n/a	Te Ngae Nursery
Nursery 2006	K06-N	n/a	n/a	May 06 8 seedlings	Te Ngae Nursery
2005	K05F-1	n/a	n/a	June 06 8 seedlings	Te Ngae Nursery 2005
2006	K06F-OS*	n/a	n/a	n/a	Te Ngae Nursery 2006
2004	K04-2	June 05 20 cores	Dec 05 10 cores	June 06 8 cores	Te Ngae Nursery
1998	K98-8	June 05 20 cores	Dec 05 10 cores	July 06 8 cores	Te Ngae Nursery
1991	K91-15	June 05 20 cores	Dec 05 10 cores	July 06 8 cores	Owhata Nursery
1980	K80-26	June 05 20 cores	Dec 05 10 cores	July 06 8 cores	unknown

* used for outplanting survey

Figure 2-12: Soil corer and hammer used for extraction of soil cores.

Table 2-4: Date and number of seedlings (K06, K05, K05F and K04-2) and soil cores sampled (K98-8, K91-15 and K80-26) from the study sites in soil core assessment (SCA) 1, 2 and 3.

Soil core assessment	K06-N	K05-N	K05F-1	K04-2	K98-8	K91-15	K80-26
SCA 1 June 2005	-	20	-	20	20	20	20
SCA 2 December 2005	-	-	-	10	10	10	10
SCA 3 May/July 2006	8	-	8	8	8	8	8

2.4.2 SOIL CORE SURVEY LABORATORY WORK

SOIL CORE PROCESSING

Soil cores were stored at 4°C after collection and processed as soon as possible. In SCA 1 and 2 all cores were collected over the course of a week and processed in the laboratory in succession. This approach was inefficient and led to extended storage of some cores, hence samples were not ideal to identify and often contaminated. This was taken into account in SCA 3. One site was sampled at a time and cores were processed within one to two weeks. The use of a new DNA extraction kit (REDExtract-N-Amp™ Plant PCR Kit (Sigma, St. Louis, Missouri, USA)) made it possible to extract DNA immediately after washing and morphotyping, which reduced contamination and also avoided the use of frozen material for DNA extraction.

For processing, cores were soaked in distilled water overnight (all of the soil was covered in water) and then washed gently with distilled water over a 2 mm sieve. The material was placed into a container and colonised root tips were removed under a dissecting microscope (Zeiss, Jena, Germany) using forceps. The roots were cleaned with fine needles and a paintbrush. ECM root tips were then categorized into 'ad hoc morphological groups'. The term 'morphological group', based on Dickie & Reich (2005), indicated a less detailed examination than a true 'morphotype' in the sense defined by (Agerer, 1987), as RFLP analysis rather than morphology was used to combine morphological groups across the sites investigated. The categorization into morphological groups was based on features such as mantle colour and texture, root branching pattern, root tip shape and morphology of mycelial strands and emanating hyphae (Agerer, 1987; Ingleby *et al.*, 1990; Goodman *et al.*, 2003). Microscopic characteristics were studied for the common types, characters investigated were: mantle type (several types of plectenchymatous and pseudoparenchymatous), hyphal junction angle, cystidia presence and type, Hartig net and anatomy of mycelial strand. Sections of colonized root tips for microscopy were cut by hand, viewed in lactic acid (85%)

and also stained with cotton blue in lactic acid (Erb & Matheis 1983; solution of 0.05 g cotton blue in 30 ml lactic acid 85 – 90%). For mantle feature observations, a piece of the mantle was peeled off of the root tip and observed in lactic acid. Also whole root tip pieces were placed in lactic acid and squashed under a cover slip. Mantle preparations were also stained with cotton blue (Ingleby *et al.*, 1990; Agerer, 1991).

The collected morphotypes received an identification label, based on the study site collected, the soil core number and numerical order of processing. For example K98-8C3T34 is a sample from site K98-8 (K), extracted from soil core 3 (SCA1) (C) and a unique morphotypes (T) within this soil core - the 34th morphotype identified in the overall study. ECM colonising root tips were quantified, each ECM tip was counted as a mycorrhiza, as this approach takes branching intensity into account (Brundrett *et al.*, 1996).

Photographs were taken with the stereomicroscope using the AxioCam MRc5 camera and Axiovision software (Zeiss) from representatives of each type per soil core and subsequently in SCA 3, for each sample used for DNA extraction. Representatives from each type per soil core were chosen randomly for DNA extraction. ECM were stored at -80°C and in FAA (Formaldehyde:ethanol 70%:acetic acid, 5:90:5). Generally, few root tips were encountered that appeared dead (shrivelled appearance, no turgor), but any tips that seemed dead were ignored and not included in the analysis.

2.5 MOLECULAR SPECIES IDENTIFICATION

2.5.1 DNA EXTRACTION

For sporocarp analysis, DNA was extracted from either fresh or frozen sporocarp material or from pure tissue culture, if available. For ECM root tip analysis, DNA was extracted from either fresh or frozen material. Within a sampled site, a minimum of two and maximum of six samples per morphotype were used (for types with 20% relative abundance or more six samples used, less than 20% relative abundance two samples were chosen).

Initially DNA was extracted using the FastDNA® Kit and the FastPrep® Instrument (Qbiogene, Inc., Valencia, CA, USA) following manufacturer's instructions. Due to amplification problems, especially with ECM root tip material, the following extraction methods were tested in the early stages of the study to improve DNA quality and amplification success. Full details of these methods are present in Appendix 3.

1. CTAB extraction method (Gardes & Bruns, 1996)
2. Modified CTAB & Phenol extraction method (Sambrook *et al.*, 1987)
3. DNeasy® Plant Mini Kit (Qiagen Inc., Hilden, Germany)

At the beginning of 2006, a novel, efficient method utilizing the plant DNA extraction kit REDExtract-N-Amp™ Plant PCR Kit (Sigma, St. Louis, Missouri, USA) was introduced (Avis *et*

al., 2003). This kit produced the best DNA for PCR amplification and was used for the remainder of the study. For Sporocarp DNA extraction, manufacturer's instructions were followed, for extraction of DNA from ECM root tips the following modifications were made to the REDExtract-N-Ampt protocol: 50 µl of extraction and dilution solution instead of the suggested 100 µl were added to the sample. Furthermore each tip was broken into at least two pieces using a pipette tip when adding the extraction solution to ensure that the solution soaked into the tissue. All protocols and details are listed in Appendix 3.

2.5.2 POLYMERASE CHAIN REACTION (PCR) PROTOCOL

The internal transcribed spacer (ITS) regions of the extracted DNA were amplified using the fungal specific primer combination of ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.*, 1990; Gardes & Bruns, 1993; Gardes & Bruns, 1996). For samples collected in 2005 (Sporocarp assessment 1), DNA was amplified in a 50 µl reaction mix containing 1 ng/µl of DNA template, 50 pmol/µl of both ITS1F (Gardes & Bruns, 1996) and ITS4 primer (White *et al.*, 1990), 1.5 U Taq DNA polymerase (Roche Applied Science, Penzberg, Germany), 10 x reaction buffer (Roche Applied Science), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, dTTP (Roche Applied Science). The thermocycling program comprised an initial denaturation step at 94°C for 85 s, followed by 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 45 s, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 2 min, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 3 min, followed by a final extension step of 72°C for 7 min (White *et al.*, 1990) on an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany).

For all samples (Sporocarp and ECM) extracted with the REDExtract-N-Amp™ Plant PCR Kit, the PCR mix supplied with the REDExtract-N-Amp™ kit was used. The mix included *Taq* enzyme, hot start *Taq*Start antibody and dNTP's, the primer concentration was 50 pmol/µl. To avoid samples solidifying, the genomic extract was diluted 1:10 for the PCR reaction. The thermocycling program for samples extracted with REDExtract-N-Amp Plant PCR Kit followed Avis *et al.* (2003) with modifications and comprised an initial denaturation step at 94°C for 85 s, followed by 14 cycles of 95°C for 35 s, 55°C for 55 s and 72°C for 45 s, 15 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 2 min, 10 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 3 min, followed by a final extension step of 72°C for 10 min on an Eppendorf Mastercycler Gradient Thermal Cycler. PCR products were separated by electrophoresis on a 1% agarose gel (Invitrogen, Carlsbad, CA, USA), run at 110 Volt for 40 min, stained for 10 min using Ethidium Bromide (12.5 µl of 1% EtBr in 500 ml distilled H_2O) and visualised under UV light (300 nm) (Sambrook *et al.*, 1987). PCR products were purified using the QIAquick® PCR purification Kit (Qiagen Inc.); in 2006 the GenElute PCR Clean-Up Kit (Sigma) was used following manufacturer's instructions. (Appendix 3).

Only PCR products consisting of a single band were used for sequencing and RFLP analysis. Where putative basidiomycete mycorrhizae produced more than one ITS PCR product, the DNA was amplified with the basidiomycete primers ITS1F and ITS4B (5'

CAGGAGACTTGTACACGGTCCAG 3') (Bruns & Gardes, 1993), before a nested re-amplification with ITS1F and ITS4 to allow subsequent comparison of RFLPs (Genney *et al.*, 2006).

2.5.3 OPTIMIZING PCR

In 2005, the initial amplification success of DNA from ECM colonising root tips was around 50%. To increase the success rate, it was attempted to either increase the quantity of the genomic extraction product or to optimize the PCR process. The optimization of the PCR was based on the following PCR reaction modifications to improve amplification outlined below:

DNA was amplified in a 50 µl reaction mix containing 1 ng/µl of DNA template, 50 pmol/µl of both ITS1F and ITS4 primer, 1.5 U Taq DNA polymerase (Roche Applied Science), 10 x reaction buffer (Roche Applied Science), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, dTTP (Roche Applied Science). The thermocycling program comprised an initial denaturation step at 94°C for 85 s, followed by 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 45 s, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 2 min, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 3 min, followed by a final extension step of 72°C for 7 min on an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany).

Magnesium concentration gradient

Several samples from different ECM types were tested with the following Mg^{2+} concentrations: 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM. All other PCR conditions were kept as described previously.

Temperature Gradient

The same samples used in the magnesium concentration gradient were used for the temperature gradient at the respective optimum magnesium concentration. The following temperatures were used: 45°C, 45.3°C, 46.4°C, 48.2°C, 50.4°C, 53°C, 55.8°C, 58.5°C, 61°C, 63.1°C, 64.7°C and 65.6°C. All other PCR conditions were kept as described previously.

DNA polymerase

The following alternative DNA Polymerases were tested: Platinum *Taq* DNA Polymerase (Invitrogen, Invitrogen, Carlsbad, CA, USA) and Ampli*Taq* Gold (Applied Biosystem, Foster City CA, USA). Both DNA polymerases require a Hotstart cycle and the PCR protocol was set up according to manufacturers instructions, using the supplied PCR Buffer.

Template concentration

The same samples used to assess the magnesium concentration and temperature gradient were used for optimizing the template concentration. Template dilutions were: undiluted, 1/10, 1/20, 1/50, 1/100, 1 ng/µl and undiluted, 1/100, 1/200, 1/500. Template volume was also varied in a second assessment – 5 µl template and 10 µl template (1/200 dilution of stock DNA) in a 25 µl reaction.

Bovine Serum Albumin

Bovine serum albumin (BSA) is a PCR additive which increases the efficiency of PCR reaction (Juen & Traugott, 2006)). One µl of 10 mg/ml BSA was added to the PCR mix described previously.

2.5.4 RFLP

RFLP patterns were generated with *Alu*I (Roche Applied Science), *Hinf*I (Roche Applied Science) and *Mbo*I (Invitrogen). PCR products were digested for 1 h at 37°C. Each reaction contained 10 µl of PCR product, 1 U of restriction enzyme (0.1 µl of 10 U/ µl enzyme), 2.5 µl of the recommended buffer, made up to 25 µl with nanopure H_2O. Digests were separated by electrophoresis on a 2% agarose gel (Invitrogen), run at 50 Volt for 100 min, stained for 10 min using ethidium bromide (12.5 µl of 1% EtBr in 500 ml distilled H_2O) and visualised under UV light (300 nm) (Sambrook *et al.*, 1987). The Bio-Rad Quantity One® Image Acquisition & Analysis Software (Bio-Rad Laboratories, Hercules CA, USA) was used to capture the gel pictures and measure the band size. Calibration was done using the Track-It™ 1 kb plus DNA ladder (Invitrogen). RFLP patterns are presented in Appendix 4.

RFLP patterns were analysed using the spreadsheet-based freeware GERM (Good-Enough RFLP Matcher, (Dickie *et al.*, 2003)) which matches unknown RFLP patterns of ECM fungi to a database of known ECM fungi (Dickie *et al.* 2003).

2.5.5 CLONING

After RFLP matching and confirmation of ECM types, a PCR generated ITS product from each ECM type was chosen for cloning. Cloning was done using the pGEM-T® Easy Vector System (Promega Corporation, Madison, USA) and XL-1 Blue MRF'KAN electroporation competent cells (grown from PCR-Script Amp Electroporation-Competent Cells Cloning Kit, Stratagene, La Jolla, CA, USA) following manufacturers instructions with modifications (Appendix 3). Positive transformants were purified by alkaline lysis, the protocol is a modification of the methods of Binboim & Doly (1979) and Ish-Horowicz & Burke (1981) in (Sambrook *et al.*, 1987) (Appendix 3). Inserts were checked by restriction digestion with *EcoR*I (Roche Applied Science) according to manufacturer's instructions (Appendix 3).

2.5.6 QUANTIFICATIONS

Concentration of extracted DNA, PCR Product and Plasmid DNA was determined on the Hoefer DyNA Quant 300 fluorometer (Amersham Biosciences, Piscataway, USA). Quantification was performed as per manufacturer's instructions. The fluorometer was calibrated with 100 ng/µl calf thymus DNA.

2.5.7 DNA SEQUENCING

Sequencing was carried out at Massey University, Palmerston North, New Zealand, using the BigDye® Terminator v3.1 & v1.1 Cycle Sequencing Kits. Sequences were edited and aligned with Sequencher version 4.7 (GeneCodes Corp. Ann ARbor, MI, USA) and identities

determined by BLAST searching (Altschul *et al.*, 1990) of GenBank (www.ncbi.nlm.nih.gov) and UNITE (http://unite.zbi.ee/; Koljalg *et al.* 2005) online nucleotide databases, using the BLASTn algorithm and with UNITE the combined UNITE + INSD function, which searches the UNITE database and the International Nucleotide Sequence Database INSD (GenBank, EMBL, DDBJ). UNITE is a Nordic-Baltic rDNA sequence database focused on ectomycorrhizal Asco- and Basidiomycetes, which holds sequences from the ITS region only. The sequences are generated from fruit bodies collected and identified by specialists and deposited in public herbaria. This database contains sequences which are not deposited in the INSD or currently in preparation by authors of the collaboration and enables a wider search within the European ECM species.

Sequences for the ITS region for all collected sporocarp species were generated from the PCR products by sequencing the PCR product in both directions using primers ITS1F and ITS4 (Gardes & Bruns, 1996). All cloned morphological ECM groups (one sample from each group) were sequenced in the forward and reverse direction using M13Forward (5'CCCAGTCACGACGTTGTAAAACG3') and M13Reverse (5'AGCGGATAACAATTTCACACAGG 3') primers.

To determine the identity of morphological ECM groups which could not be identified with RFLP matching to known ECM species the ITS sequence was BLAST searched in GenBank and UNITE search were done. For identification, a minimum of 95% sequence overlap to an existing ITS sequence of at least 450 bp in the GenBank database was required. Those samples with 97 – 100% similarity with existing species were considered a match and named to the species level. Those sequences with 96% or lower similarity to existing sequences were designated names to the genus, family or order. Unknown sequences were called Unknown 1, 2 etc. (Ashkannejhad & Horton, 2006).

2.6 STUDY LAYOUT

The data gathered was assessed to answer three main questions, each presented in a separate chapter:

ECM FUNGAL DIVERSITY – CHAPTER 3

In order to assess the above- and belowground ECM fungal diversity of species associated with different age classes of *P. radiata* in a plantation in New Zealand, all data collected during this study was included - sporocarp surveys 1 and 2 as well as soil core surveys 1, 2 and 3.

ECM CHRONOSEQUENCE COMMUNITIES - CHAPTER 4

To investigate the hypothesised change in above- and belowground ECM communities over time in a *P. radiata* plantation in New Zealand, data collected in the 2006 assessments was

used, sporocarp survey 2 and soil core survey 3, since these data sets were both based on abundance data, and data quality was good.

CHANGES IN ECM DIVERSITY FROM NURSERY TO OUTPLANTING – CHAPTER 5

To analyse the changes in ECM fungal diversity from the nursery to the early years of outplanting, data collected from soil core survey 3 (sites nursery, K05F, K04-2 and K98-8) and the outplanting survey (site K06F) was used.

2.6.1 STATISTICAL ANALYSIS

DIVERSITY ANALYSIS

To assess ectomycorrhizal diversity and characterise ECM communities, species richness, diversity, and evenness, the absolute and relative abundance of species were measured as outlined below. All diversity measures employed in this study are common in ECM ecological studies.

The following measures assess the α-diversity, the diversity of species present in a community, which is the main focus of Chapter 3.

ALPHA - DIVERSITY

Species richness measures

The simplest measure of species richness is the total number of species present in a sample (S). As this measure varies greatly with sample size and can be a crude measure of community species richness (Waite, 2000), an index of species richness was additionally calculated, which incorporates the total number of individuals and attempts to estimate species richness independently of the sample size. In this study it was chosen to use the Margalef's (1958) index because of its ease of calculation:

$$d = (S-1)/\log_e N$$

With S = total number of species, N = total individuals

To estimate the expected number of species in a sample, rarefaction and jackknife estimates were performed on S. Rarefaction compares the species richness when sample size differed and allows estimation of the number of species to be expected in community samples of an equal size (Waite, 2000). Jackknife estimation allows the mean and variance of a parameter to be estimated. This non-parametric method makes no assumptions about the underlying statistical distribution of the data (Waite, 2000).

Absolute and relative abundance

For both, aboveground and belowground data, absolute abundance was presented as the sum of the visits and soil core collections, respectively. Relative abundance values were

calculated as number of sporocarps/ECM species for each ECM taxa divided by the total number of sporocarps sampled/ECM root tips counted per study site and expressed as a percentage.

Diversity indices

A diversity index is a mathematical measure of species diversity in a community; diversity indices take the relative abundance of different species into account, therefore a diversity index not only depends on species richness but also on the evenness with which individuals are distributed among the different species. Several diversity indices have been applied in ecological studies, each index with its own strengths and weaknesses. For this study it was decided to use the Shannon and Simpson's diversity index, as these two are the most widely used indices in mycorrhizal literature, allowing comparison of this study with other studies (Giachini *et al.*, 2004; Richard *et al.*, 2004; Korkama *et al.*, 2006; Gebhardt *et al.*, 2007). The Shannon index is sensitive to changes in number and abundance of rare species whereas the Simpson index is more influenced by changes in abundant species (Waite, 2000).

Shannon index (H')

The Shannon index requires that the sample is both random and representative i.e. it contains all species, but it is less sensitive to sample size than many other diversity measures (Waite, 2000). The index is calculated as follows:

$$H' = \Sigma_i \, p_i \log(p_i)$$

With p_i = proportion of the total count arising from the i th species, $p_i = n_i/N$ where n_i is the number of individuals of species i and N is the total number of individuals in the sample. Values of H' increase with species number S, but rarely exceed 5.0. For any sample, the theoretical maximum of H' is equal to $\ln S$.

Simpson's index (1- λ')

The Simpson's index has the same pre-requirements as the Shannon index, i.e. the sample needs to be random and representative. The Simpson's index is calculated as follows:

$$1 - \lambda' = 1 - \{\Sigma_i N_i(N_i - 1)\}/\{N(N-1)\}$$

With N_i = number of individual species i

λ' is an index of dominance, with the range 0 to 1 and gives the probability that two individuals drawn randomly from a sample will belong to the same species. 1- λ' equals zero for low diversity and 1- λ' equals one at high diversity.

Species evenness (J')

This measure expresses how evenly the individuals are distributed among different species. In this study, the Pielou's evenness index was used:

$$J = H' / H'_{max} = H' / \log S$$

H'_{max} is the maximum possible value of Shannon diversity, i.e. that which would be achieved if all species were equally abundant (= log S).

BETA-DIVERSITY

The β-diversity deals with the variation in diversity from one community to the next, which is addressed in Chapter 4. The easiest way to measure β-diversity of pairs of sites is by the use of similarity coefficients, commonly used is the Sorensen index (Magurran, 1988) which is calculated as follows:

$$C_J = j/(a+b-j)$$

With j = number of species found in both sites, a = number of species in site A, b = the number of species in site B.

The index is based on presence/absence data and equals one in the case of complete similarity and zero if the sites are dissimilar and have no species in common. The disadvantage of this index is it does not take abundance of species into account and is sensitive to differences in sample size (Magurran, 1988). To further assess the β-diversity, the Shannon and Simpson diversity indices of the different communities were compared by a one-way ANOVA (see below).

Rank-abundance curves

As a means of visualising diversity, rank-abundance curves were produced, which rank species in decreasing order of their importance in terms of abundance. The species richness is viewed as the number of different species on the chart; species evenness is derived from the slope of the line. A steep slope indicates low evenness; a shallow slope indicates high evenness as the abundances of different species are similar. The rank-abundance curves can be categorized as following one of three general patterns, known as the 1. geometric series, 2. broken stick and 3. log normal type (Magurran, 1988). These models are based on competition for a food source as the driving force (resource partitioning). Some generalizations can be made about communities containing each type of diversity pattern. The 'geometric series' is typical of recently disturbed habitats or of habitats that experience harsh climatic conditions. Diversity is very low (lacking both richness and evenness), resulting from one or a few species dominating the available resources. The broken stick represents a high degree of evenness that often indicates a habitat where a single resource is being shared equitably among several species. Finally, the log normal pattern indicates a mature and varied ecosystem (Magurran, 1988).

ECM COMMUNITY ANALYSIS

To further assess the β-diversity, describe the ECM communities and determine indicator species for the age groups investigated (Chapter 4), data was analysed by hierarchical clustering and non-metric multidimensional scaling (MDS) techniques. These ordination techniques complement each other and present a complete picture of the β-diversity. Similarities and dissimilarities between communities were further analysed by comparison of average similarity within a group and average dissimilarities between groups.

Hierarchical clustering

Cluster analysis aims to find 'natural groupings' of samples such that samples within a group are more similar to each other. In this study the hierarchical clustering method based on group-average linking was used. In this ordination technique, samples are grouped and the groups themselves form clusters at lower levels of similarity. It is based on a similarity matrix and the similarity of groups is visualised in a dendrogram, using the PRIMER-E software (Clarke & Warwick, 2001).

Multidimensional scaling (MDS)

The non-metric multidimensional scaling (MDS) routine, based on a similarity matrix, constructs a dimensionless map of the analysed samples in a specified number of dimensions (two dimensions in this study) which attempts to satisfy all the conditions imposed by the rank similarity matrix. For example if sample 1 has higher similarity to sample 2 than it does to sample 3 then sample 1 will be placed closer on the map to sample 2 than to sample 3. The adequacy of the MDS representation is given in the stress value. Stress < 0.05 = excellent representation with no prospect of misinterpretation, stress < 0.1 = good ordination, stress = 0.2 potentially useful 2-dimensional picture, stress > 0.3 = ordination not acceptable. The PRIMER-E software (Clarke & Warwick, 2001) was used for this analysis.

Similarity analysis

To analyse similarity within groups, dissimilarities between groups and to determine discriminating species, the following values were calculated and analysed: average abundance, average similarity for within group, and average dissimilarity for in-between group analysis, the ratio similarity/standard deviation for within group analysis, and the ratio dissimilarity/standard deviation for in-between group analysis and percent contribution. The ratios similarity/standard deviation and dissimilarity/standard deviation are indicators for discriminating species. The standard deviation value is a measure of how consistently a species contributes to the average similarity/dissimilarity across all samples. If the ratio similarity/standard deviation or dissimilarity/standard deviation, respectively, for a species is large, then this species not only contributes greatly to the similarity/dissimilarity within a group/in-between groups, but it also does so consistently in inter-comparisons of all samples. The species would therefore be considered a good discriminating species.

STATISTICAL ANALYSIS

Values were compared with Student's t-tests and one-way ANOVA. Significant ANOVA results ($p \leq 0.05$) were followed by a Tukey's's *a posteriori* test to distinguish differences among the 'treatments' (age classes) between the communities (Magurran, 1988; Waite, 2000).

ANALYSIS PACKAGES

PRIMER

Within PRIMER (*P*lymouth *R*outines *I*n *M*ultivariate *E*cological *R*esearch, 2002 PRIMER-e Ltd), the following routines were applied:

DIVERSE: the following measures were calculated with the DIVERSE routine: total species (*S*), total individuals (N), Margalef's species richness index (*d*), Pielou's Evenness (J'), Shannon diversity index (H') and Simpson diversity index (1-λ'). **CLUSTER:** for the cluster analysis, a similarity matrix based on the Bray-Curtis similarity index was produced, data was square root transformed and standardised. The group-average method was used to plot a dendrogram. **MDS:** for the MDS ordination, a similarity matrix based on the Bray-Curtis similarity index was produced, data was square root transformed and standardised and 10 re-starts were chosen. **SIMPER:** for the SIMPER analysis, data was square root transformed. **Dominance Plot**: rank-abundance curves were produced for abundance data and visualised with the ordinary plotting method.

PAST

With PAST (PAlaeontological STatistics, version 1.67, Ryan *et al*. 1995), the Sorensen's similarity coefficient was calculated.

MINITAB

MINITAB 13.1 (2000 Minitab Inc.) was used to perform Student's t-tests, one-way ANOVA and Tukey's *a posteriori* test.

GenStat

GenStat for Windows Version 10 (VSN International Ltd) was used for the rarefaction and Jackknife estimates of species richness.

3 ECM SPECIES DIVERSITY IN A *PINUS RADIATA* PLANTATION IN NEW ZEALAND

3.1 INTRODUCTION

The diversity of ECM communities associated with a *Pinus radiata* monoculture plantation in the North Island of New Zealand is the research topic of this chapter. Diversity is defined as the richness and evenness of a group of species in a specific environment (Begon *et al.*, 1998) and is numerically described by values such as species richness, frequency, species diversity indices and evenness. It is of interest what the driving forces behind diversity are and in this present chapter the within-site (α-) diversity of both, above- and belowground ECM communities of *P. radiata* are investigated. Preceding studies of ECM fungi associated with *P. radiata* in New Zealand by Chu-Chou & Grace (1979, 1980, 1983a, 1984a, 1988, 1990) were conducted from the 1970s to mid 1990s. In their research, nurseries and plantation forests from both the North and South Island were investigated mainly by sporocarp surveys, but also root tip assessments. ECM colonising root tips were identified through morphological comparison with pure tissue cultures. This method is limited due to problems associated with culturing of ECM species, furthermore species can only be compared to cultures from sporocarps that have been collected, leaving many types unidentified. With the advent of molecular methods in the 1990s, the opportunity to identify ECM species using DNA extracted from colonized root tips arose. Using DNA fingerprinting, it has been observed that there is little correlation between the above- and belowground ECM communities, as a number of species fruit either irregularly or not at all, or form inconspicuous fruiting bodies or have been regarded as saprotrophs and not ectomycorrhizal species (Visser, 1995; Gardes & Bruns, 1996; Peter *et al.*, 2001). There have been no studies to assess the belowground ECM communities of *P. radiata* in New Zealand in using molecular methods, nor has any exotic monoculture plantation forest been assessed in this way anywhere else in the world. It was of further interest to compare diversity within the above- and belowground ECM communities and their correlation to each other. Moreover, as an expansion the continuation of the original work of Chu-Chou & Grace, using molecular identification, it was aimed to increase the national and international knowledge on ECM diversity in an exotic monoculture plantation and to contribute to the international sequence database for ECM species associated with *P. radiata.*

In order to investigate the ECM sporocarp (aboveground) and ECM fungi colonising root tips (belowground) diversity of *P. radiata* in New Zealand, two sporocarp assessments and three

soil core surveys were conducted in 2005 and 2006 at Te Ngae nursery and in four stands of varying age (2, 8, 15 and 26 yrs in 2006) in Kaingaroa Forest, North Island. The aim of this study was to assess the overall, and stand specific ECM diversity of both sporocarps and ECM fungi colonising root tips and to compare the above- and belowground species composition. A second aim was to identify ECM fungi colonising root tips to species level with the aid of RFLP analysis and direct sequencing and to add to international sequence databases.

3.2 RESULTS

3.2.1 SPOROCARP AND ECM ROOT TIP IDENTIFICATION

Sporocarp and ECM root tips were identified using a combined morphological and molecular identification process. RFLP patterns were produced (Table 3-1) as outlined in Chapter 2, bands lower than 150 bp were not clear and hence not assigned a banding size. Sequences from the internal transcribed spacer (ITS) region were BLAST searched in the GenBank and UNITE databases (Table 3-2 and Table 3-3).

SPOROCARP IDENTIFICATION

Sporocarps where first identified based on morphological features as described in Chapter 2 and then subjected to molecular identification. The ITS region of the ribosomal DNA was amplified from DNA extracted from the collected sporocarps and RFLP patterns were produced (Table 3-1 represents *in silico* RFLP patterns based on aligned sequences, Appendix 5 Table A5-1 lists RFLP patterns based on 2% agarose gel pictures). Representatives of all species (as defined by a unique ITS RFLP pattern) were direct sequenced to obtain reference sequences, confirm species identification and in several cases to clarify species identities. For increased confidence, aligned sequences were BLAST searched in the GenBank and UNITE databases (Table 3-2 and Table 3-3). The UNITE database usually confirmed the GenBank match with either the identical sequence result from the International Nucleotide Sequence Databases (INSD) or a UNITE-specific sequence (labelled with * in Table 3-3) with the same organism label, for example species *Inocybe lacera* (samples K04S3 and K91S14). Exceptions were *Inocybe* sp. 1 (*Inocybe sindonia*) and *Tricholoma* sp., in these cases the UNITE result was different (see "ECM species label clarification"). The species *Amanita muscaria* and *Scleroderma bovista* appeared to be sequences from mixed material. In these instances the sequences were aligned with the GenBank highest scoring sequence to resolve the ambiguities. The species *Amanita muscaria, Chalciporus piperatus, Inocybe lacera, Laccaria proxima, Lactarius rufus, Rhizopogon rubescens, R. luteolus, R. pseudoroseolus, Scleroderma bovista* and *Thelephora terrestris* had a maximum identity of 97% or more and therefore a species name was applied. The maximum identity for *Hebeloma* sp., *Inocybe* sp. 1, *Inocybe* sp. 2, *Suillus* sp. and *Tricholoma* sp. for the GenBank queries were below 97%; therefore, a

species name was not applied and the genus name was used. The exception was *Inocybe* sp. 1, as mentioned earlier, where the UNITE result provided a species match for this sequence.

Table 3-1: Internal transcribed spacer (ITS) – *in silico* restriction fragment length polymorphism (RFLP) generated by restriction digest with enzymes *Alu*I, *Hinf*I and *Mbo*I of ectomycorrhizal fungi from root tip (ECM) or sporocarps collected in sporocarp and soil core assessments in 2005 and 2006. Based on consent sequences.

ECM species/type	Source	Label	Uncut (bp)	*Alu*I fragment length [a] (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Amanita muscaria [b]	Sporocarp	K80S5	711	19, 105, 197, 390	8, 347, 356	60, 97, 224, 330
Amanita muscaria	ECM	K91C37T357	711	19, 104, 198, 390	8, 347, 356	60, 97, 224, 330
Chalciporus piperatus	Sporocarp	K80S25	786	59, 727	8, 62, 111, 165, 205, 235	10, 53, 60, 76, 280, 307
Hebeloma sp.	Sporocarp	K06S1	731	191, 211, 329	8, 331, 392	60, 260, 411
Hebeloma sp.	ECM	K06C5T156	733	190, 213, 330	8, 331, 394	60, 262, 411
Inocybe sp.	Sporocarp	K98S25	745	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
Inocybe sp.	ECM	K91C37T359	745	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
Inocybe lacera	Sporocarp	K04S3	722	182, 214, 326	8, 74, 258, 382	20, 60, 230, 412
Inocybe sindonia	Sporocarp	K80S33	731	144, 587	5, 8, 187, 209, 322	60, 269, 402
Laccaria proxima	Sporocarp	K04S5	730	15, 94, 96, 128, 397	8, 191, 202, 329	60, 261, 409
Lactarius rufus	Sporocarp	K80S7	763	79, 162, 522	8, 108, 264, 383	60, 106, 134, 206, 257
Rhizopogon rubescens	Sporocarp	K04S6	737	94, 251, 392	8, 11, 23, 113, 129, 210, 243	60, 62, 135, 234, 246
Rhizopogon rubescens	ECM	K06C3T138	738	94, 252, 392	8, 11, 23, 113, 129, 211, 243	60, 62, 136, 234, 246
Rhizopogon luteorubescens	ECM	K91C31T310	762	94, 668	11, 23, 112, 137, 236, 243	60, 62, 161, 234, 245
Rhizopogon pseudoroseolus	Sporocarp	K98S35	765	94, 277, 394	8, 23, 112, 144, 235, 243	60, 62, 160, 238, 245
Rhizopogon pseudoroseolus	ECM	K98C31T213	765	94, 277, 394	8, 23, 112, 144, 235, 243	62, 62, 160, 238, 245
Rhizopogon luteolus	Sporocarp	K80S64	889	67, 221, 601	7, 8, 128, 159, 228, 359	60, 62, 74, 78, 254, 361
Scleroderma bovista [b]	Sporocarp	K80S9	699	18, 59, 212, 410	8, 37, 116, 252, 286	12, 60, 124, 152, 173, 178
Suillus sp.	Sporocarp	K98S8	723	109, 614	8, 11, 23, 37, 74, 92, 124, 129, 225	60, 62, 69, 141, 157, 234
Pseudotomentella sp.	ECM	K98C35T239	721	16, 68, 75, 96, 193, 273	8, 349, 364	60, 232, 429
Pseudotomentella tristis	ECM	K91C38T363	788	19, 32, 43, 64, 69, 96, 149, 316	8, 366, 414	60, 64, 170, 234, 260
Tomentella sp.[c]	ECM	K80C37T323	700	30, 54, 67, 91, 458	8, 144, 177, 177, 194	55, 60, 363
Thelephora terrestris	Sporocarp	K91S27	701	30, 70, 601	8, 100, 241, 352	56, 60, 220, 365
Thelephora terrestris	ECM	K98C34T230	700	30, 69, 601	8, 100, 240, 352	55, 60, 220, 365
Tricholoma sp.	Sporocarp	K91S9	737	40, 77, 117, 503	8, 336, 393	60, 175, 241, 261
Tuber sp.	ECM	K06FC8T13	688	no cut site	8, 70, 101, 147, 362	49, 60, 230, 349
Wilcoxina mikolae	ECM	K04C38T193	636	no cut site	8, 161, 183, 284	49, 60, 97, 212, 218
unknown Basidiomycete	ECM	K04C35T201	722	10, 15, 94, 95, 128, 380	8, 328, 386	60, 121, 133, 408
unknown *Pezizaceae*	ECM	K06FC10T18	681	206, 232, 243	8, 9, 304, 360	60, 237, 384
unknown 8 [c]	ECM	K80C33T282	769	183, 586	93, 142, 171, 363	60, 188, 231, 290
unknown 9	ECM	K80C32T280	636	no cut site	8, 66, 241, 321	49, , 58, 60, 130, 339
unknown 10	ECM	K80C32T269	632	183, 449	66, 248, 318	49, 50, 60, 185, 288

Primer pairs ITS1F and ITS4 were used for PCR amplification of the ITS region; **a** – *Alu*I gave rise to many partial digests and was not the most robust for morphotype discrimination; **b** – These sequences appeared to be from mixed PCR products. To gain a full sequence for *in silico* RFLP analysis the sequence from the ITS1F and ITS4 primer was aligned with the GenBank result and consensus bases assigned. **c** – reverse sequence only.

Table 3-2: Results for GenBank queries from sporocarps or root tips (ECM).

Sample morphological species/ ECM morphotype	Source	Sample label	Sequence length (bp)	GenBank Accession #	Organism	GenBank score	e-value	Maximum Identity (%)
Amanita muscaria	Sporocarp	K80S5	711	AB080983	*Amanita muscaria*	1201	0.00	97
Amanita muscaria	ECM	K91C37T357a	711	AB080983	*Amanita muscaria*	1258	0.00	98
Chalciporus piperatus	Sporocarp	K80S25	786	AF335457	*Chalciporus piperatus*	1343	0.00	99
Hebeloma sp.	Sporocarp	K06S1	731	EF411103	Uncultured ECM (*Hebeloma sp.)*	1205	0.00	98
Hebeloma sp.	ECM	K06C5T156a	733	EF411103	Uncultured ECM (*Hebeloma sp.)*	1179	0.00	98
Inocybe sp. 1	Sporocarp	K80S33	731	AY751556	Cf. Inocybe sp. EC258 B207	987	0.00	92
Inocybe lacera	Sporocarp	K04S3	722	AY750157	*Inocybe lacera*	1199	0.00	99
Inocybe sp. 2	Sporocarp	K98S25	745	DQ974812	Uncultured ECM (*Inocybe*)	808	0.00	88
Inocybe sp.	ECM	K91C37T359	745	DQ974812	Uncultured ECM (*Inocybe*)	808	0.00	88
Laccaria proxima	Sporocarp	K04S5	730	DQ068958	*Laccaria proxima*	1240	0.00	99
Lactarius rufus	Sporocarp	K80S7	763	EF685089	*Lactarius rufus*	1410	0.00	100
Rhizopogon rubescens	Sporocarp	K04S6	737	AF158018	*Rhizopogon rubescens*	1112	0.00	95
Rhizopogon rubescens	ECM	K06C3T138b	738	AF158018	*Rhizopogon rubescens*	1111	0.00	95
Rhizopogon luteorubescens	ECM	K91C31T310b	762	AJ810038	*Rhizopogon luteorubescens*	1262	0.00	99
Rhizopogon sp. 1	Sporocarp	K98S35	765	AJ810042	*Rhizopogon pseudoroseolus*	1197	0.00	94
Rhizopogon pseudoroseolus	ECM	K98C31T213a	765	AJ810042	*Rhizopogon pseudoroseolus*	1252	0.00	99
Rhizopogon sp. 2	Sporocarp	K80S64	889	AF062936	*Rhizopogon luteolus*	1312	0.00	96
Scleroderma bovista	Sporocarp	K80S9	699	AB211267	*Scleroderma bovista*	1214	0.00	100
Suillus sp.	Sporocarp	K98S8	723	AY880932	Uncultured ECM (*Suillaceae*)	1221	0.00	99

Table 3-2 cont.: Results for GenBank queries of ectomycorrhizal fungi from sporocarps or root tips (ECM).

Sample morphological species/ ECM morphotype	Source	Sample label	Sequence length (bp)	GenBank Accession #	Organism	GenBank score	e-value	Maximum Identity (%)
Thelephora terrestris	Sporocarp	K91S27	701	U83486	*Thelephora terrestris*	1230	0.00	98
Thelephora terrestris	ECM	K98C34T230b	700	U83486	*Thelephora terrestris*	1230	0.00	98
Tricholoma sp.	Sporocarp	K91S9	704	AF458435	*Tricholoma ustale*	1230	0.00	96
Wilcoxina mikolae	ECM	K04C38T193a	636	DQ069000	*Wilcoxina mikolae* clone	1064	0.00	98
Pseudotomentella sp.	ECM	K98C35T239a	721	DQ377428	Uncultured ECM (*Thelephoraceae*)	1061	0.00	96
Pseudotomentella tristis	ECM	K91C38T363a	788	AJ889968	*Pseudotomentella tristis*	763	0.00	100
Tomentella sp.	ECM	K80C37T323b	700	DQ990851	uncultured ECM (*Tomentella*)	1085	0.00	95
Tuber sp.	ECM	K06FC8T13a	688	AY748861	uncultured ECM (*Tuber*)	1236	0.00	99
unknown Basidiomycete	ECM	K04C35T201a	722	AB211143	Uncultured mycorrhizal Basidiomycete	1232	0.00	98
unknown *Pezizaceae*	ECM	K06FC10T18a	681	DQ974687	Uncultured ECM *(Pezizaceae)*	1112	0.00	98
Unknown 8	ECM	K80C33T282a	769	AY641466	uncultured ECM	935	0.00	91
Unknown 9	ECM	K80C32T280a	636	AM1096	Fungal sp.	1096	0.00	99
Unknown 10	ECM	K80C32T269a	631	AM901986	Uncultured basidiomycete	828	0.00	90

Table 3-3: Results of UNITE queries from sporocarps or root tips (ECM) and consent species identification based on comparison of GenBank and UNITE sequences.

Sample morphological species/ ECM morphotype	Source	Sample label	UNITE Accession #	UNITE Organism	UNITE score	e-value	Consent
Amanita muscaria	Sporocarp	K80S5	AB080983	*Amanita muscaria*	1187	0.00	*Amanita muscaria*
Amanita muscaria	ECM	K91C37T357a	AB080983	*Amanita muscaria*	1302	0.00	*Amanita muscaria*
Chalciporus piperatus	Sporocarp	K80S25	AF335457	*Chalciporus piperatus*	1308	0.00	*Chalciporus piperatus*
Hebeloma sp.	Sporocarp	K06S1	EF411103	Uncultured ECM (*Hebeloma* sp.)	1237	0.00	*Hebeloma sp.*
Hebeloma sp.	ECM	K06C5T156a	EF411103	Uncultured ECM (*Hebeloma* sp.)	1239	0.00	*Hebeloma sp.*
Inocybe sp. 1	Sporocarp	K80S33	UDB002392*	*Inocybe sindonia*	1352	0.00	*Inocybe sindonia*
Inocybe lacera	Sporocarp	K04S3	UDB000617*	*Inocybe lacera*	1279	0.00	*Inocybe lacera*
Inocybe sp. 2	Sporocarp	K98S25	DQ822810	*Inocybe* sp. KGP109	507	e-173	*Inocybe* sp.
Inocybe sp.	ECM	K91C37T359	DQ822810	*Inocybe* sp. KGP109	599	e-170	*Inocybe sp.*
Laccaria proxima	Sporocarp	K04S5	UDB001490	*Laccaria proxima*	1358	0.00	*Laccaria proxima*
Lactarius rufus	Sporocarp	K80S7	DQ097868	*Lactarius rufus*	1356	0.00	*Lactarius rufus*
Rhizopogon rubescens	Sporocarp	K04S6	AF158018	*Rhizopogon rubescens*	1134	0.00	*Rhizopogon rubescens*
Rhizopogon rubescens	ECM	K06C3T138b	AF158018	*Rhizopogon rubescens*	1095	0.00	*Rhizopogon rubescens*
Rhizopogon luteorubescens	ECM	K91C31T310b	AJ810038	*Rhizopogon luteorubescens*	1348	0.00	*Rhizopogon luteorubescens*
Rhizopogon sp. 1	Sporocarp	K98S35	AJ810042	*Rhizopogon pseudoroseolus*	1340	0.00	*Rhizopogon pseudoroseolus*
Rhizopogon pseudoroseolus	ECM	K98C31T213a	AJ810042	*Rhizopogon pseudoroseolus*	1340	0.00	*Rhizopogon pseudoroseolus*
Rhizopogon sp. 2	Sporocarp	K80S64	DQ068966	*Rhizopogon luteolus*	1380	0.00	*Rhizopogon luteolus*
Scleroderma bovista	Sporocarp	K80S9	UDB002179*	*Scleroderma bovista*	1083	0.00	*Scleroderma bovista*
Suillus sp.	Sporocarp	K98S8	AY880932	*Suillus granulatus*	1296	0.00	Uncultured ECM (*Suillaceae*

*UNITE specific sequences, not available through GenBank

Table 3-3 cont.: Results of UNITE queries from sporocarps or root tips (ECM) and consent species identification based on comparison of GenBank and UNITE sequences.

Sample morphological species/ ECM morphotype	Source	Sample label	UNITE Accession #	UNITE Organism	UNITE score	e-value	Consent
Thelephora terrestris	Sporocarp	K91S27	AY822747	uncultured ECM	1283	0.00	*Thelephora terrestris*
Thelephora terrestris	ECM	K98C34T230b	U83486	*Thelephora terrestris*	1295	0.00	*Thelephora terrestris*
Tricholoma sp.	Sporocarp	K91S9	UDB001502*	*Tricholoma pessundatum*	1376	0.00	*Tricholoma pessundatum*
Wilcoxina mikolae	ECM	K04C38T193a	DQ069000	*Wilcoxina mikolae*	1154	0.00	*Wilcoxina mikolae*
Pseudotomentella sp.	ECM	K98C35T239a	UDB001617*	*Pseudotomentella griseopergamacea*	1291	0.00	*Pseudotomentella sp.*
Pseudotomentella tristis	ECM	K91C38T363a	AJ889968	*Pseudotomentella tristis*	684	0.00	*Pseudotomentella tristis*
Tomentella sp.	ECM	K80C37T323b	EF619832	uncultured *Tomentella*	1053	0.00	*Tomentella sp.*
Tuber sp.	ECM	K06FC8T13a	AY748861	uncultured ECM *(Tuber)*	1328	0.00	*Tuber sp.*
unknown Basidiomycete	ECM	K04C35T201a	AB211143	uncultured mycorrhizal Basidiomycete	1328	0.00	unknown Basidiomycete
unknown *Pezizaceae*	ECM	K06FC10T18a	DQ974687	Uncultured ECM *(Pezizaceae)*	1179	0.00	unknown *Pezizaceae*
unknown 8	ECM	K80C33T282a	AY641466	uncultured ECM (Basidiomycota)	855	0.00	Unknown 8
unknown 9	ECM	K80C32T280a	DQ309132	uncultured fungus	1135	0.00	Unknown 9
unknown 10	ECM	K80C32T269a	AB089818	mycorrhizal Basidiomycete	799	0.00	Unknown 10

*UNITE specific sequences, not available through GenBank

ECM ROOT TIP IDENTIFICATION

Overall, 20 ECM root tip morphotypes were distinguished in the soil core assessments (Table 3-4 and Figure 3-1).

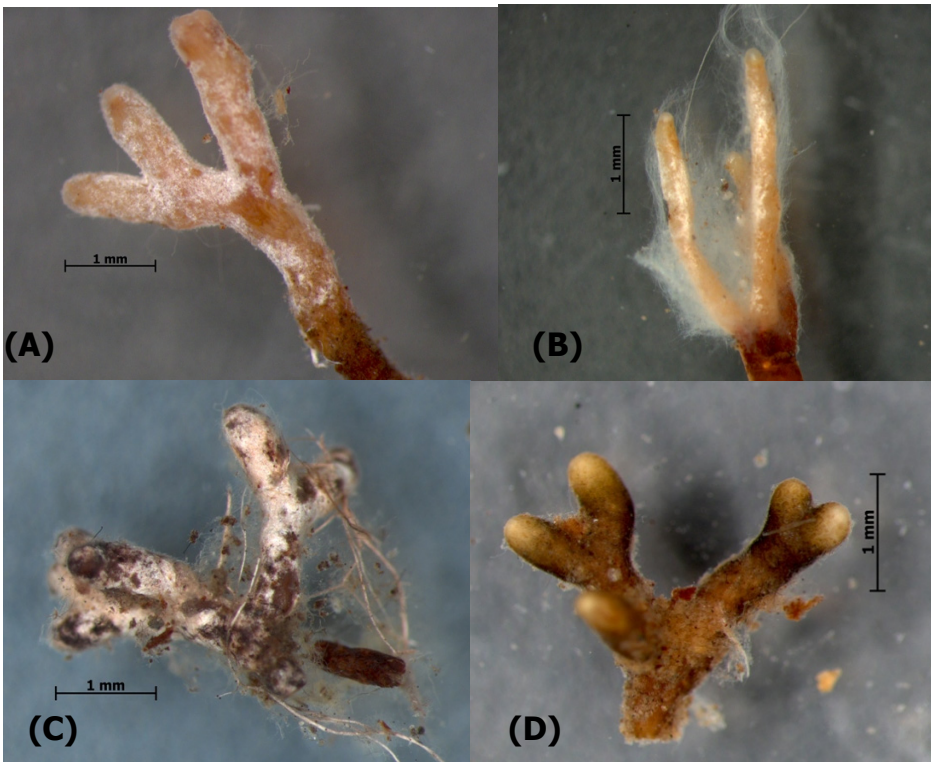

Figure 3-1: ECM morphotypes of *Amanita muscaria* (A), *Hebeloma* sp. (B), *Pseudotomentella tristis* (C) and *Tomentella* sp. (D).

During the analysis of the soil cores several ECM types occurred only sporadically and could not be placed into any of the established morphotype groups. These where not analysed further. These tips were combined in the group 'various unknown' as the main focus of the study was not to investigate rare species. The ITS region was amplified from the ECM root tips and RFLP patterns of each morphotype were compared (Table 3-1 represents *in silico* RFLP patterns based on aligned sequences, Appendix 5 Table A5-1 lists RFLP patterns based on 2% agarose gel pictures).

Table 3-4: ECM morphotypes observed in soil core assessments in 2005 and 2006. For detailed descriptions refer to Appendix 4.

ECM species/type	Description
Amanita muscaria	silver-white, tortuous, frequent irregularly branched
Cenococcum geophilum	black, straight, not branched, woolly texture with emananting black hyphal fans
Hebeloma sp.	frosty white, straight, irregular branched, cottony surface, net of white hyphae in flat angle
Inocybe sp.	velvet-white to milky yellow, dichotomous, straight, no rhizomorphs
Thelephora terrestris	milky white-skin colour, irregular, straight
Pezizales sp.	clear-white, dichotomous branched, short, straight no rhizomorphs
Pseudotomentella sp.	black with white patches, dichotomous, straight, short, net of dark hyphae in flat angle
Pseudotomentella tristis	frosty white with dark patches, dichotomous, straight, short, net of white hyphae in flat angle, rhizomorphs
Rhizopogon luteorubescens	frosty white, dichotomous, coralloid or irregular; white rhizomorphs
Rhizopogon pseudoroseolus	frosty white, dichotomous or short and coralloid; white thick rhizomorphs
Rhizopogon rubescens	frosty white, tortuous, dichotomous branching, white rhizomorphs
Tomentella sp.	brown to black-green with white apices, dichotomous, straight, velvety-flake structure, some hyphae at base
Tuber sp.	clear to honey brown with white apices, smooth, dichotomous, no rhizomorphs
Wilcoxina mikolae	milky white when young, brown with white apices with age, unbranched to irregular branched, no hyphae or rhizomorphs
unknown Basidiomycete	brown with frosty white patches, white apices, irregular branching, straight, cottony surface with hyphal fans
unknown 2	black, irregular, tortuous
unknown 8	velvety black to purple, smooth, dichotomous
unknown 9	red-brown, thin, irregular, tortuous, smooth
unknown 10	dirty white with dark patches, dichotomous, straight, short, net of white hyphae in flat angle, rhizomorphs
unknown 12	purple-iridescent, dichotomous, tortuous, smooth

As the first step of identification of the collected ECM root tips, the ECM RFLPs (based on 2% agarose gel pictures, Appendix 5, Table A5-1) were compared against each other to confirm the morphological grouping. With RFLP matching and sequencing, some morphotypes could be grouped together, as they represented different age stages of the ECM fungus colonising the root tips. Root tips colonised by *Amanita muscaria* and *Wilcoxina mikolae* (Figure 3-2) varied morphologically with age and young and old stages were initially separated during the morphotyping process.

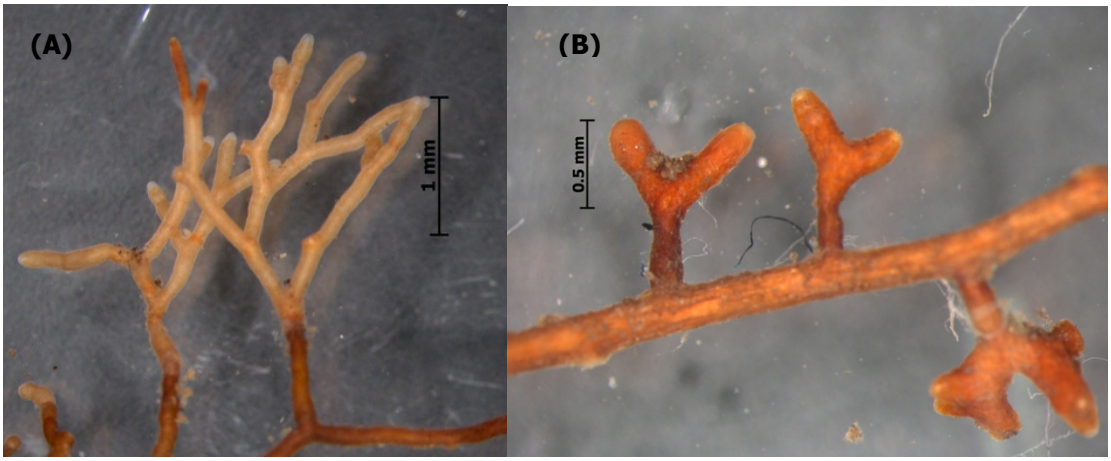

Figure 3-2: Young (A) and mature (B) stage of *Wilcoxina mikolae* colonising root tips of *Pinus radiata*.

Uncertainties within the morphotypes unknown 8, 10 and *Pseudotomentella tristis* could also be resolved with sequence information. These types looked similar to other morphotypes and were only tentatively placed in a separate group, which was confirmed with RFLP matching. Next, the RFLP patterns from morphotypes were compared to the RFLP patterns from sporocarp specimens (based on 2% agarose gel pictures, Appendix 5 Table A5-1 lists RFLP patterns). The ECM RFLP matched those for *Amanita muscaria* and *Hebeloma* sp. sporocarps and were positively identified. RFLP patterns from *Inocybe* sp., *Thelephora terrestris* and *Rhizopogon rubescens* ECM were only tentatively matched to the respective sporocarp RFLP patterns, as each of them was missing bands with *Alu*I. For *Inocybe* sp., bands of 180 bp and 409 bp were missing, for *T. terrestris* bands of 370 bp and 594 bp and *R. rubescens* the band of 650 bp length was missing. These species identifications were confirmed with direct sequencing and the *in silico* RFLP patterns. The restriction endonuclease cut sites were identical in the case of the *Inocybe* sp. ECM and sporocarp sequences and almost identical (1 bp difference in sequence length) in the case of *R. rubescens* and *T. terrestris* ECM and sporocarp sequence. The restriction endonuclease *Alu*I gave rise to many partial digests, explaining some of the differences in RFLP patterns and indicating that this enzyme was not

very robust for species discrimination. Samples of *Rhizopogon pseudoroseolus* from ECM root tips did not match the RFLP pattern of the respective sporocarp, as it was missing one band each with *Alu*I, *Hin*fI and *Mbo*I (missing band of 371, 156 and 163 bp, respectively), however, the other bands matched positively. This identity of *R. pseudoroseolus* was confirmed through direct sequencing of cloned material, as well as the *in silico* RFLP pattern.

A representative of each morphotype was cloned and sequenced as described in Chapter 2. BLAST was used to query the sequence data in the GenBank and UNITE databases (Table 3-2 and Table 3-3). The UNITE query confirmed the GenBank results with identical sequence results except for *Pseudotomentella* sp. (K98C35T239a) and *Inocybe* sp. (K91C37T359). For the latter, however, it did not change the result as in both queries the organism was the same. Species labels were assigned with confidence for *Amanita muscaria, Cenococcum geophilum, Thelephora terrestris, Rhizopogon rubescens, Rhizopogon luteorubescens, Rhizopogon pseudoroseolus, Pseudotomentella tristis* and *Wilcoxina mikolae*. Organism labels to genus level could be applied for *Tuber* sp., *Inocybe* sp. and *Tomentella* sp. Types unknown 8, 9 and 10 had low scores and results did not provide evidence of genus, family or order classification, furthermore sequence results varied greatly, consequently the morphotype labels were 'unknown'. Type unknown 2 did amplify successfully, however neither a RFLP pattern nor a successful ITS sequence was obtained in several attempts.

Sequence query results were identical for sequences generated from sporocarp and ECM root tip material for *Amanita muscaria, Hebeloma* sp., *Inocybe* sp., *Rhizopogon rubescens, Rhizopogon pseudoroseolus* and *Thelephora terrestris.* The similarity between the ECM and sporocarp sequences was as follows: *A. muscaria* 96% identical*, Hebeloma* sp. 97% identical, *Inocybe* sp. 99.7% identical (same species), *R. rubescens* 96% identical*, T. terrestris* 93% identical and *R. pseudoroseolus* was also very similar, although ambiguities in the sporocarp sequence made the exact percentage unclear. The *in silico* RFLP patterns of the ECM and sporocarp ITS of *Inocybe* sp. and *R. pseudoroseolus* were absolute identical, in the case of the *in silico* RFLP patterns of *A. muscaria, Hebeloma* sp., *R. rubescens* and *T. terrestris* differences of 1 -2 bp were observed (Table 3-1).

For the remainder of this thesis, the term 'ECM species' will be applied to morphotypes which were positively identified to species level through either RFLP matching to sporocarp or sequencing, the term 'ECM type' will be applied to morphotypes that were either identified to genus level or where no classification could be made.

ECM SPECIES IDENTITY CLARIFICATION

ECM taxa *Tricholoma pessundatum, Laccaria laccata* and *Hebeloma crustuliniforme* are reported ECM associates of *P. radiata* in New Zealand and were identified by Chu-Chu and Grace during their research on mycorrhizal fungi associated with *P. radiata* (Chu-Chou, 1979; Chu-Chou, 1980; Chu-Chou & Grace, 1988). These putative species were collected in the

sporocarp assessments during this study; however sequencing of the collected material did not confirm the suggested species names. To confirm the identity of these species, reference material from the Landcare Research Herbarium

(**http://nzfungi.landcareresearch.co.nz/html/mycology.asp**) was obtained and sequenced. Furthermore, species identities for collected *Inocybe* spp. and *Rhizopogon* spp. were clarified with molecular analysis. These are examined individually below.

"Tricholoma pessundatum"

Putative *Tricholoma pessundatum* specimens collected in site K80-26 and K91-15 (K80S34 and K91S9) were analysed; GenBank and UNITE database BLAST results are presented in Appendix 5. Both samples matched either *Tricholoma ustale* or *Tricholoma aurantium,* the highest maximum identity of sample K80S34 was below the cut-off of 97%. Only one *T. pessundatum* sequence is currently available in the GenBank database (AY2207305) and this GenBank sequence was not in the results of the BLAST search for samples K80S34 and K91S9. Both specimens had a positive match with a UNITE sequence from *Tricholoma pessundatum*, the next closest match for both is a UNITE sequence from *Tricholoma tridentinum*. These samples were compared to DNA extracted from a specimen collected in New Zealand and morphologically identified as *T. pessundatum* from the Landcare Research Herbarium. The sequence was BLAST searched in the GenBank and UNITE databases and the results of this sample were as for K80S34; *Tricholoma ustale* and *Tricholoma aurantium* were the closest matches. In New Zealand no specimen that is an absolute positive morphological identification of "*T. pessundatum*" has been collected (G. Ridley, personal communication, 30 April 2007) and the species group *T. pessundatum/tridentinum/stans* is currently not confidently clarified taxonomically. Since both morphological and molecular methods resulted in uncertain identity of this organism, *Tricholoma* sp. was only noted to the genus level in this thesis.

"Laccaria laccata"

Laccaria specimens collected in the first sporocarp assessment were initially morphologically identified as either *L. laccata* or *L. proxima*. The *Laccaria* species collected and published from M. Chu-Chou's work was identified and labelled by M. Chu-Chou as *Laccaria laccata* (Chu-Chou, 1979). The ITS region from specimens of "*L. laccata*" and "*L. proxima*" were fully sequenced and the sequences were BLAST searched in GenBank and UNITE (Table 3-2 to Table 3-35). For both samples, the GenBank match had a 99% maximum identity to *Laccaria proxima.* Consequently, the species label *Laccaria proxima* was applied with confidence for specimens collected in this study. A specimen from the Landcare PDD herbarium was obtained (PDD62700) and sequenced for comparison purposes, however sequencing was unsuccessful, likely due to degradation of DNA in the herbarium specimen.

"Hebeloma crustuliniforme"

Basidiocarps collected in the nursery in 2005 and 2006 were morphologically identified as *Hebeloma crustuliniforme.* BLAST searching of ITS sequence from K06S1 in GenBank and UNITE databases did not confirm this species identification (Appendix 5). In both database queries the ITS matched sequences from organisms only identified to genus level (*Hebeloma*), with a 99% maximum identity. For comparison purposes, herbarium specimens from the Landcare Research Herbarium were obtained and analysed (JAC8966 and JAC9848). ITS sequences from both samples were BLAST searched in the GenBank and UNITE databases (Appendix 5). In the case of JAC8966, the highest sequence match had only 88% identity and a low score in both, GenBank and UNITE (identical query results). Sample JAC9848 had a 99% maximum identity for the three highest ranked sequences which were all different species of the genus *Hebeloma*. In contrast, the UNITE result indicated the sequence for *Hebeloma populinum* was the closest match. Sequencing results of *Hebeloma* spp. collected during this study, compared with the samples obtained from Landcare Research herbarium specimens were unclear. Since a clear species identification was not possible, the organism label *Hebeloma* sp. was used for *Hebeloma* specimens collected during this research.

***Inocybe* spp.**

Several types of the genus *Inocybe* were collected during the sporocarp assessments in 2005 and 2006. *Inocybe* sp. 1 was distinct due to its white colour and size, but several smaller, brown coloured specimens were collected (Figure 3-3). The morphological identification of *Inocybe* spp. is very difficult and it was not possible to identify the collected *Inocybe* specimens to species level using morphology; however RFLP analysis and ITS sequencing of DNA from the sporocarps made it possible to distinguish the collected specimens and identify them to species level.

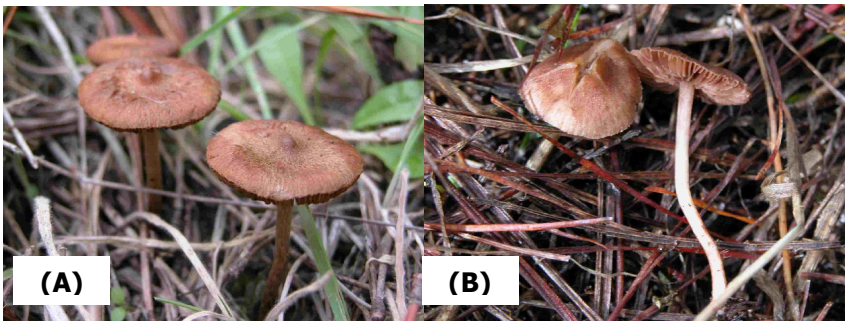

Figure 3-3: Sporocarps of *Inocybe lacera* (A) and *Inocybe* sp. (B) collected during this study.

The small, brown specimens collected represented two species. *Inocybe lacera* was positively identified to species level by ITS sequencing (Table 3-2 and Table 3-3). In the case of *Inocybe* sp. 2 it was not possible to find a positive species match, hence only the genus name was applied to these specimens. In the case of the white *Inocybe* sp. 1, the sequence match results for samples K80S29 and K80S33 did not match the same species between the GenBank and UNITE database (Appendix 5). With GenBank, the maximum identity for K80S29 was below the threshold value and the highest ranked results identified the organism to genus level only. The UNITE query resulted in a match with high scores for both samples with the UNITE sequence from *Inocybe sindonia*. Dried fruit bodies collected in the sporocarp assessments were sent to Dr Geoff Ridley for morphological identification. He confirmed the suggested species identity *Inocybe sindonia* (G. Ridley, personal communication, 21 March 2007). DNA was extracted from a Landcare Research Herbarium specimen presented under the synonym for this species, *Inocybe eutheles* (PDD750021). The sequence was BLAST searched in GenBank and UNITE (Appendix 5) and the results matched the earlier analysis; therefore the name *Inocybe sindonia* was used.

***Rhizopogon* spp.**

Hypogeous fruiting bodies of *Rhizopogon* spp. were collected in the nursery, site K04-2, K98-8 and K80-26. *Rhizopogon*-like morphotypes were observed in the nursery, and sites K98-8 and K91-15. Specimens collected in the nursery and K04-2 were identified as "*Rhizopogon rubescens*" based on morphology. ECM morphotypes collected from the nursery were positively matched with RFLP analysis to the fruiting bodies of *R. rubescens*. BLAST searching of ITS sequence data from sporocarp and ECM root tip material (K04S6 and K06C3T138b) in GenBank and UNITE resulted in positive matches to the species label "*R. rubescens*" as well as "*R. roseolus*". Considerable confusion exists in the literature over the correct name for this species and it has been suggested that the species names are synonymous (Martin *et al.*, 1998; Cairney & Chambers, 1999), based on Cairney and Chambers (1999) it was decided to use the species label "*R. rubescens*". Both, ECM root tips and a hypogeous fruiting body collected in site K98-8 were positively identified as "*R. pseudoroseolus*" by sequence analysis. Rhizopogon-type root tips were observed in site K91-15 and sequence analysis identified this type as "*R. luteorubescens*". The sporocarp collected in site K80-26 was positively identified as "*R. luteolus*" by both, GenBank and UNITE database searches. A parsimony analysis by Dr. Maria P. Martin, comparing sequence data generated in this study with unpublished material matched the sequence of a *R. verii* species collected in Tunisia under *Pinus pinaster* (M.P. Martin, personal communication, 28 July 2007). As the parsimony analysis utilized unpublished sequence data, it was decided to use the name *R. luteolus* for this specimen collected at K80-26.

3.2.2 ABOVEGROUND ECM DIVERSITY

The species richness, diversity indices, abundance and composition of the aboveground ECM communities associated with *Pinus radiata* were analysed and showed the following trends: (*i*) species richness and diversity increased from the nursery to the outplanting, richness was highest in site K98-8 whereas diversity was highest in K91-15; (*ii*) species composition changed from site to site; (*iii*) aboveground, each site was dominated by one species.

ABOVEGROUND ECM FUNGAL SPECIES RICHNESS

Over the two assessments in 2005 and 2006, a total of 18 ECM taxa were observed in all plots combined. The species that were identified are presented in Table 3-5. In 2005, 16 ECM taxa were identified and in 2006 this number increased to 18 ECM taxa, as both *Thelephora terrestris* and *Rhizopogon luteolus* were added.

Species richness (*S*, total number of species) between the sporocarp assessments in 2005 and 2006 was calculated using pooled data for each year (Figure 3-4). Species richness increased from three species in the nursery to ten species in site K98-8. Richness decreased to six species in site K91-15 and increased back to ten species in site K80-26. The Margalef's species richness index (*d*) of the 2006 assessment was calculated as the mean over the transects (Figure 3-5). In contrast to the richness, the Margalef's index declined from the nursery to site K04-2. The index was highest in site K91-15 (1.00) and decreased in site K80-26. Jackknife estimates of the aboveground species richness (Table 3-6) were identical to the observed richness in the nursery and slightly higher in all other sites (indicating robust enough sampling). Rarefaction curves for the estimated number of aboveground ECM species are presented in Appendix 6.

Table 3-5: Ectomycorrhizal taxa observed in sporocarp assessments 2005 and 2006 within each *P. radiata* stand investigated.

ECM taxa	Nursery		K04-2		K98-8		K91-15		K80-26	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Amanita muscaria (L.) Lam. (1783)					+	+	+	+	+	+
Chalciporus piperatus (Bull.) Bataille (1908)					+	+			+	+
Hebeloma sp.	+	+								
Inocybe lacera (Fr.) P. Kumm. (1871)			+	+	+	+	+	+	+	+
Inocybe sindonia (Fr.) P. Karst. (1879)							+	+	+	+
Inocybe sp.					+			+	+	+
Laccaria proxima (Boud.) Pat. (1887)			+	+	+	+	+		+	+
Lactarius rufus (*Scop.*) Fr. (1838)					+	+			+	+
Lycoperdon gunnii Berk. (1860)					+	+				
Lycoperdon sp.					+	+				
Rhizopogon luteolus Fr. (1817)										+
Rhizopogon rubescens (Tul. & C. Tul.) Tul. & C. Tul. (1844)	+	+	+	+						
Rhizopogon pseudoroseolus A. H. Sm. (1966)					+	+		+		
Scleroderma bovista Fr. (1829)				+	+					
Suillus sp.							+			
Thelephora terrestris Ehrh. (1787)						+		+		+
Tricholoma sp.							+		+	+
Wilcoxina mikolae (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf (1985)	+	+								

Nursery: Te Ngae Nursery; K04-2: outplanted in 2004; K98-8: outplanted in 1998; K91-15: outplanted in 1991; K80-26: outplanted in 1980; all plantation sites in Kaingaroa Forest.

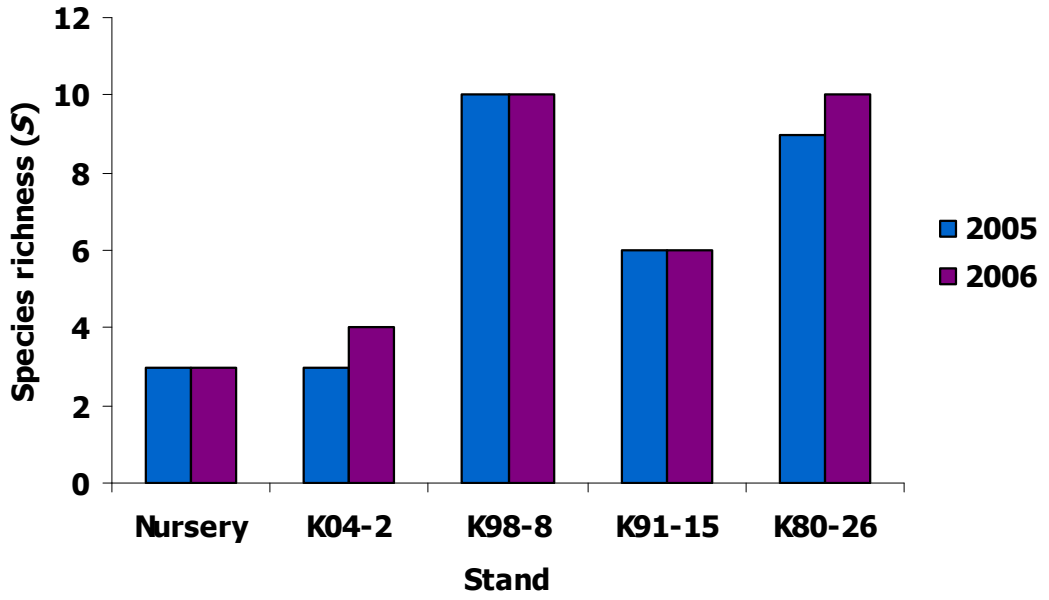

Figure 3-4: Species richness (*S*) at the study sites investigated during the 2005 and 2006 assessments. Values are pooled over the assessed transects (n=5 except for K91-15 2006: n=2).

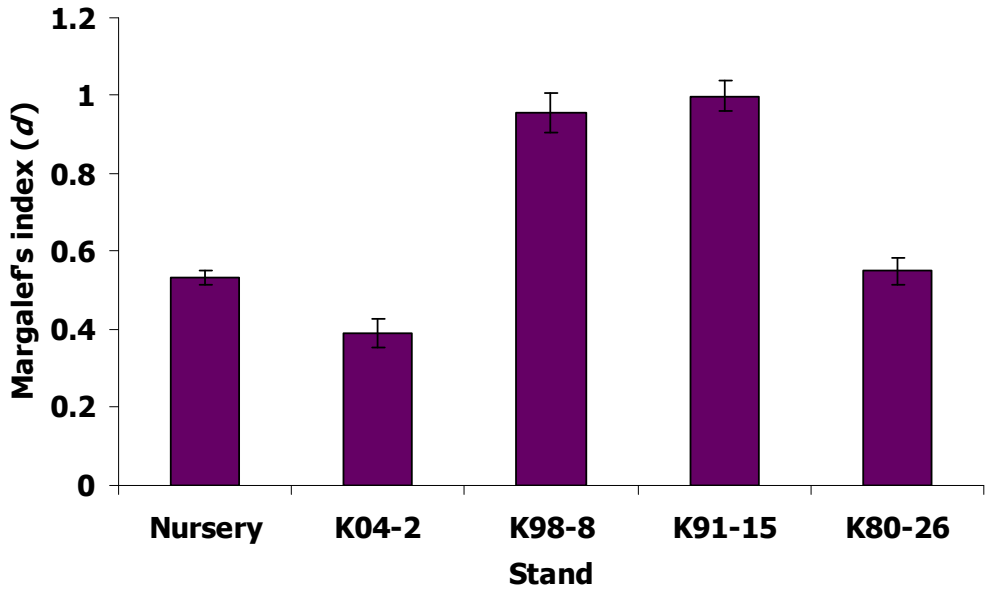

Figure 3-5: Mean Margalef's species richness index (*d*) for the study sites investigated during sporocarp assessment 2 in 2006. (n= 5 for nursery, K04-2, K98-8 and K80-26, n=2 for K91-15, ± standard error).

Table 3-6: Estimated species richness with Jackknife estimate (± standard error) for the study sites investigated during sporocarp assessment 2 in 2006.

	Observed richness	Jackknife estimate (± SE)
Nursery	3	3.00 (± 0.00)
K04-2	4	4.80 (± 0.80)
K98-8	10	10.80 (± 0.80)
K91-15	6	7.00 (± 0.00)
K80-26	10	12.40 (± 1.60)

ABOVEGROUND ECM ABUNDANCE

The absolute and relative abundance of ECM sporocarps counted at each site in assessment 2 in 2006 are presented in Appendix 7. The absolute and relative abundance of ECM sporocarps observed at all sample sites in the 2006 assessment is presented in Table 3-7. The most abundant species observed was *Lactarius rufus* (33%), which was almost exclusively found in site K80-26. *Laccaria proxima, Inocybe sindonia* and *Inocybe lacera* were the next most abundant ECM taxa with 22%, 18% and 14% abundance, respectively. *Inocybe sindonia* was found in two sites only, whereas *L. proxima* and *I. lacera* were distributed over all the sites.

Table 3-7: Absolute and relative abundance of total specimens collected between April and June 2006 in sporocarp assessment 2. Numbers are total of all visits and transects.

ECM Taxa	Absolute abundance	Relative abundance (%)
Lactarius rufus	1589	33.23
Laccaria proxima	1037	21.69
Inocybe sindonia	865	18.09
Inocybe lacera	648	13.55
Rhizopogon rubescens	151	3.16
Amanita muscaria	150	3.14
Thelephora terrestris	143	2.99
Inocybe sp.	99	2.07
Tricholoma sp.	33	0.69
Hebeloma sp.	27	0.56
Lycoperdon sp.	11	0.23
Scleroderma bovista	10	0.21
Chalciporus piperatus	6	0.13
Lycoperdon gunnii	5	0.10
Wilcoxina mikolae	5	0.10
Rhizopogon sp.	2	0.04
Rhizopogon luteolus	1	0.02
Total specimen counted	**4782**	

ABOVEGROUND ECM SPECIES DIVERSITY INDICES AND PIELOU'S EVENNESS

The Shannon and Simpson diversity indices and Pielou's evenness were calculated as described in Chapter 2 for sporocarp assessment 2. Indices could not be calculated for sporocarp assessment 1 as only presence/absence data was collected. The Shannon index ranges between 0 and 5 (high diversity), while the Simpson index ranges between 0 and 1. Index values presented (Table 3-8) are the mean of the five surveyed transects at each site (two for site K91-15).

Diversity was lowest in the nursery site (0.37 for Shannon, 0.26 for Simpson index) and highest in site K91-15 (1.14 and 0.58 for Shannon and Simpson indices, respectively). As with the diversity indices, Pielou's evenness increased from the nursery and was highest in site K91-15.

Table 3-8: Mean Pielou's evenness, Shannon and Simpson diversity index (± standard error).

	Pielou's evenness (±SE)	Shannon diversity (±SE)	Simpson diversity (±SE)
Nursery (n=5)	0.42 (±0.10)	0.37 (±0.12)	0.26 (±0.08)
K04-2 (n=5)	0.46 (±0.06)	0.46 (±0.06)	0.26 (±0.04)
K98-8 (n=5)	0.46 (±0.08)	0.83 (±0.12)	0.41 (±0.08)
K91-15 (n=2)	0.71 (±0.03)	1.14 (±0.04)	0.58 (±0.02)
K80-26 (n=5)	0.55 (±0.04)	1.05 (±0.10)	0.55 (±0.04)
Overall (n=22)	0.63	1.80	0.79

n = number of transects assessed

ABOVEGROUND ECM SPECIES RANK-ABUNDANCE CURVES

The rank-abundance curves from all sites were characterised by the dominance of one or two species in the site and in the case of the older plantation sites, many rare species (Figure 3-6). The nursery and site K04-2 had a low species richness and evenness, as indicated by the steep gradient of the curve. Species richness increased in site K98-8 but the evenness was still low. In site K91-15 species richness declined and the slope of the rank-abundance curve was less steep, indicating higher evenness. Overall evenness and richness increased from site K98-8 to K80-26.

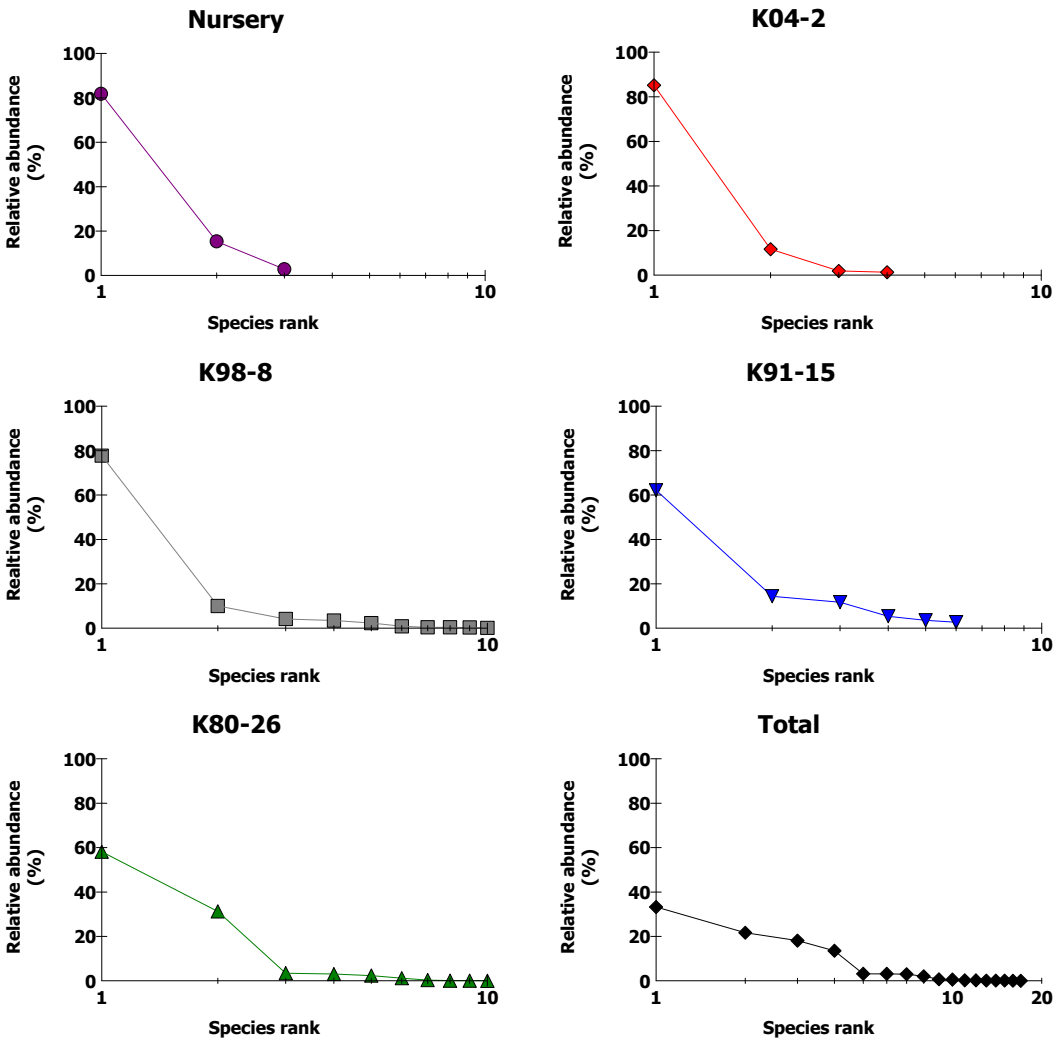

Figure 3-6: Rank-abundance curves for sites investigated and total of sporocarp assessment 2 in 2006.

SPOROCARP ABUNDANCE IN RELATION TO CLIMATE DATA

The relationship between sporocarp fruiting patterns and climatic conditions was not the main focus of this study, however climate data was obtained from the closest meteorological station to the study sites in Kaingaroa Forest and plotted against the monthly sporocarp abundance in the 2006 assessment (Appendix 8). Climate data details are presented and summarised in Appendix 8.

The overall trend was that sporocarp production was high in the month of March 2006 and declined progressively with falling temperatures in the following months. Exceptions were species *Thelephora terrestris* and *Hebeloma* sp., which were more abundant in the colder months, May and June, and *Lactarius rufus*, which was most abundant in May, following a fortnight of constant rain.

3.2.3 BELOWGROUND ECM DIVERSITY

To assess belowground diversity of ECM fungi associated with *P. radiata*, three surveys were conducted as described in Chapter 2. In all surveys, ECM type abundance was recorded; data from the soil cores collected at each site was not pooled and is presented as mean values for each site.

The species richness, diversity indices, abundance and composition of the belowground ECM communities associated with *Pinus radiata* in a plantation in New Zealand were analysed and showed the following trends: (*i*) species richness and diversity decreased from the nursery to the first years of outplanting and increased again thereafter and was highest in site K80-26; (*ii*) species composition was similar in the nursery and early years of outplanting but changed in the older plantation sites (K98-8, K91-15 and K80-26); (*iii*) the nursery and early outplanting sites were dominated by one, and two ECM types, respectively, whereas species frequency was more evenly distributed in the older plantation sites.

BELOWGROUND ECM SPECIES RICHNESS

In total, 19 distinct ECM types were observed (Table 3-9). Fourteen species/types were found in soil core assessment (SCA) 1 and 2. In SCA 3, five new ECM species/types were found as well as the 14 in SCA 1 and 2. The Margalef's index was 1.45, 1.57 and 1.90 for SCA 1, 2 and 3, respectively and 1.82 overall (Figure 3-8). Species richness (*S*; total number of species) and the respective Margalef's species richness index (*d*) were calculated for each soil core, the mean values from all three surveys are presented in Figure 3-7 and Figure 3-8.

Table 3-9: ECM types found in soil core assessments 1, 2 and 3 in 2005 and 2006 within each *P. radiata* age group investigated.

	Nursery		K05F-1	K04-2			K98-8			K91-15			K80-26		
ECM type	SCA 1	SCA 3	SCA 3	SCA 1	SCA 2	SCA 3	SCA 1	SCA 2	SCA 3	SCA 1	SCA 2	SCA 3	SCA 1	SCA 2	SCA 3
Amanita muscaria							+	+	+	+	+	+	+	+	+
unknown Basidiomycete						+	+	+	+	+	+	+	+		+
Cenococcum geophilum									+	+	+	+	+	+	+
Hebeloma sp.	+	+	+												
Inocybe sp.									+			+	+	+	+
Pseudotomentella sp.									+		+	+	+	+	+
Pseudotomentella tristis							+					+	+		+
Rhizopogon luteorubescens												+			
Rhizopogon pseudoroseolus								+	+						
Rhizopogon rubescens	+	+	+	+	+	+									
Thelephora terrestris									+		+				+
Tomentella sp.								+		+	+	+	+	+	+
Tuber sp.	+	+	+	+	+										
Wilcoxina mikolae	+	+	+	+	+	+									
unknown 2	+	+	+												
unknown 8										+					+
unknown 9									+					+	+
unknown 10															+
unknown 12					+			+	+		+	+		+	+
unknown various					+		+	+	+		+	+		+	+

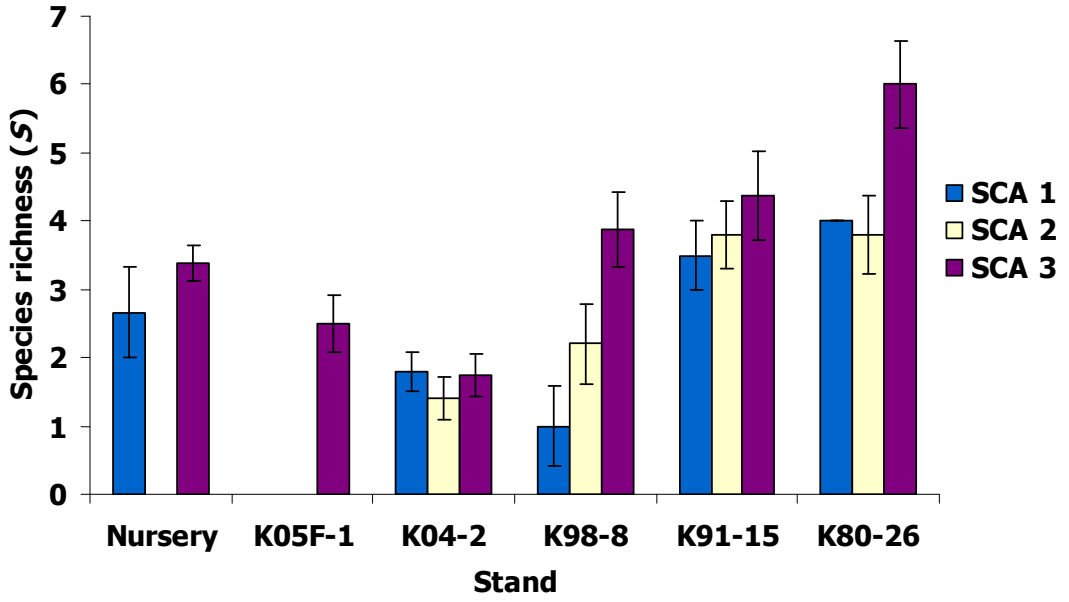

Figure 3-7: Mean species richness (*S*) of ECM taxa observed in soil core assessments (SCA) 1, 2 and 3 in 2005 and 2006 (± standard error, standard error = 0 for K80-26 in SCA1). K05F-1 was only assessed in SCA 3; the nursery was not assessed in SCA 2.

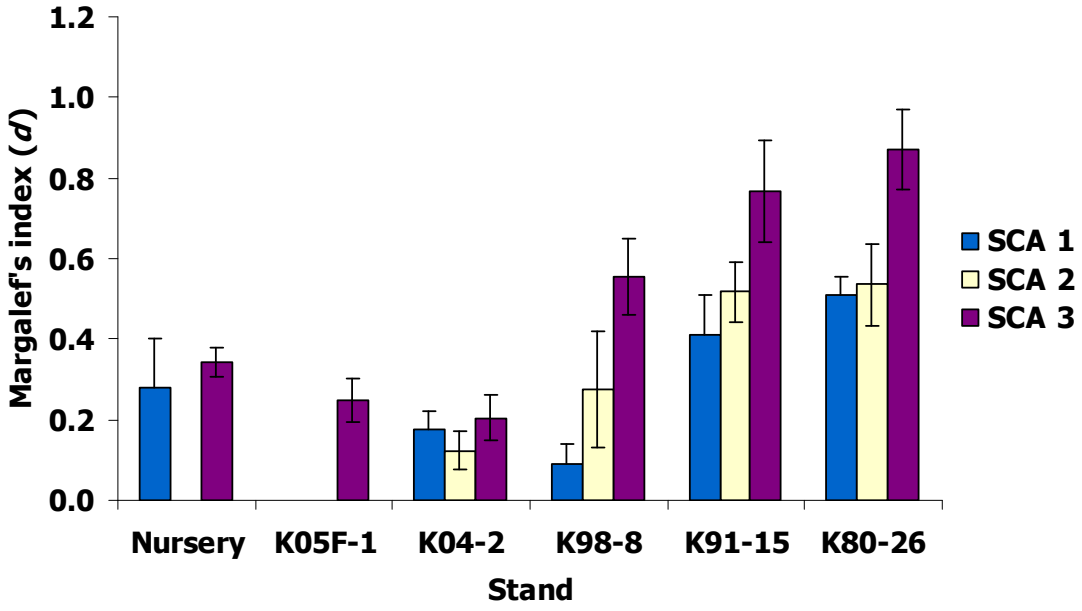

Figure 3-8: Mean Margalef's index (*d*) of ECM taxa observed in soil core assessments (SCA) 1, 2 and 3 in 2005 and 2006 (± standard error). K05F-1 was only assessed in SCA 3, the nursery was not assessed in SCA 2.

In general, species richness and Margalef's index initially declined in the outplanting sites, but increased in the later stages of the plantation. Site K98-8 at the June 2005 assessment had the lowest richness and Margalef's index values, while the maximum values were calculated for sites K80-26 in 2006. Overall, values for species richness and Margalef's index increased in all instances except for site K04-2 over the course of the three assessments from 2005 to 2006. Data from SCA 1 in 2005 is the least reliable data. Due to working out sampling and core processing difficulties not all collected cores were processed. Increased sampling and processing efficiency makes data from SCA 3 the most reliable data set. Table 3-10 shows the Jackknife estimates for species richness of all sites investigated over the three soil core assessments in 2005 and 2006. Jackknife values followed the trend of the observed richness, but were higher compared to the observed richness values. However, differences were minor in the third assessment, indicating robust sampling in this assessment.

Table 3-10: Estimated species richness with Jackknife estimate (±standard error) for the study sites investigated during soil core assessments (SCA) 1 and 2 in 2005 and soil core assessment 3 in 2006.

	SCA 1		SCA2		SCA 3	
	Observed richness	Jackknife estimate (± SE)	Observed richness	Jackknife estimate (± SE)	Observed richness	Jackknife estimate (± SE)
Nursery	5	7.00 (± 1.16)	-	- -	5	5.00 (± 0.00)
K05F-1	-	- -	-	- -	5	5.88 (± 0.88)
K04-2	3	3.00 (± 0.00)	4	6.75 (± 1.15)	3	3.00 (± 0.00)
K98-8	4	6.00 (± 0.00)	6	8.40 (± 1.60)	10	10.88 (± 0.88)
K91-15	5	11.00 (± 2.12)	8	9.60 (± 0.98)	10	10.88 (± 0.88)
K80-26	7	10.00 (± 0.00)	8	8.00 (± 0.00)	13	14.75 (± 1.15)

BELOWGROUND ECM ABUNDANCE

The absolute and relative abundance of ECM types at each soil core assessment and per site are presented in Appendix 7. Table 3-11 presents the absolute and relative abundance of ECM types as the sum of all three assessments and sites investigated in 2005 and 2006. Overall the most abundant type belowground was *R. rubescens* with 32%. The next most abundant ECM types were *W. mikolae*, *Tuber* sp., *A. muscaria* and *Hebeloma* sp., the abundance ranged from 16% to 7%. All remaining types had an overall abundance under 4%.

Table 3-11: Total absolute and relative abundance of ECM types as the sum of all three soil core assessments (SCA) and sites investigated in 2005 and 2006 (n=92 soil cores).

ECM Type	Overall Absolute abundance	Overall Relative abundance (%)
Rhizopogon rubescens	10727	31.58
Wilcoxina mikolae	5568	16.39
Tuber sp.	3958	11.65
Amanita muscaria	2909	8.56
Hebeloma sp.	2442	7.19
Cenococcum geophilum	1388	4.09
unknown Basidiomycete	1305	3.84
Rhizopogon pseudoroseolus	1004	2.96
Inocybe sp.	986	2.90
unknown various	885	2.61
unknown 9	739	2.18
Tomentella sp.	557	1.64
unknown 12	349	1.03
unknown 2	271	0.80
Pseudotomentella sp.	261	0.77
Pseudotomentella tristis	209	0.62
Thelephora terrestris	176	0.52
Rhizopogon luteorubescens	105	0.31
unknown 10	76	0.22
unknown 8	49	0.14
Total ECM root tips analysed	**33964**	

BELOWGROUND ECM SPECIES DIVERSITY INDICES AND PIELOU'S EVENNESS

The Shannon and Simpson diversity indices and Pielou's evenness were calculated as described in Chapter 2. Diversity indices are the mean values of the soil cores analysed for each site (Table 3-12).

Diversity was higher in the nursery sites than in the first years of outplanting (stands K05F-1 and K04-2). In site K98-8 the diversity increased in this stand to values which were found in the 15 yr old stand K91-15. The highest diversity value (1.31 for Shannon and 0.66 for Simpson diversity) in the study was found in the last assessment, SCA 3, of the oldest stand investigated, K80-26, which had also increased over the course of the assessments. The values for Pielou's evenness showed a pattern similar to the diversity indices; evenness decreased in the young outplanting sites K05F-1 and K04-2. Site K98-8 showed a broad range in the evenness values between the assessments and the value for the third assessment was the highest overall in the study. Evenness in K91-15 and K80-26 was slightly lower. Even though more ECM types were found in SCA 3 than in SCA 2 (19 compared with 14 in SCA 2),

the total diversity values and Pielou's evenness for each soil core assessment were lower in SCA 3.

Table 3-12: Mean Pielou's evenness, Shannon and Simpson diversity index (± standard error) for all sites investigated, overall Pielou's evenness, Shannon and Simpson diversity index for each site and all three soil core assessments (SCA 1-3).

		Pielou's evenness (±SE)	Shannon diversity (±SE)	Simpson diversity (±SE)
Nursery	SCA 1 (n=3)	0.88 (± 0.06)	0.79 (± 0.14)	0.52 (± 0.06)
	SCA 2	n/a	n/a	n/a
	SCA 3 (n=8)	0.72 (± 0.08)	0.88 (± 0.12)	0.50 (± 0.00)
K05F-1	SCA 1	n/a	n/a	n/a
	SCA 2	n/a	n/a	n/a
	SCA 3 (n=8)	0.48 (± 0.11)	0.41 (± 0.11)	0.25 (± 0.00)
K04-2	SCA 1 (n=10)	0.48 (± 0.12)	0.38 (± 0.10)	0.24 (± 0.07)
	SCA 2 (n=8)	0.38 (± 0.14)	0.30 (± 0.12)	0.20 (± 0.08)
	SCA 3 (n=8)	0.57 (± 0.15)	0.44 (± 0.12)	0.29 (± 0.08)
K98-8	SCA 1 (n=4)	0.29 (± 0.20)	0.20 (± 0.14)	0.13 (± 0.10)
	SCA 2 (n=5)	0.70 (± 0.23)	0.50 (± 0.28)	0.29 (± 0.16)
	SCA 3 (n=8)	0.81 (± 0.06)	0.98 (± 0.08)	0.56 (± 0.00)
K91-15	SCA 1 (n=2)	0.79 (± 0.08)	0.99 (± 0.22)	0.59 (± 0.08)
	SCA 2 (n=5)	0.72 (± 0.11)	0.88 (± 0.12)	0.50 (± 0.07)
	SCA 3 (n=8)	0.76 (± 0.08)	1.10 (± 0.18)	0.59 (± 0.00)
K80-26	SCA 1 (n=2)	0.68 (± 0.01)	0.95 (± 0.02)	0.50 (± 0.01)
	SCA 2 (n=5)	0.72 (± 0.13)	1.00 (± 0.25)	0.53 (± 0.13)
	SCA 3 (n=8)	0.75 (± 0.03)	1.31 (± 0.09)	0.66 (± 0.00)
SCA 1 overall (n=21)		0.74	1.94	0.80
SCA 2 overall (n=23)		0.84	2.23	0.86
SCA 3 overall (n=48)		0.70	2.10	0.81
Study overall (n=92)		0.75	2.24	0.84

n = number of soil cores assessed; n/a = not applicable

BELOWGROUND ECM SPECIES RANK-ABUNDANCE CURVES

The overall trend for rank abundance curves from the belowground ECM communities (SCA 2 and 3, assessment SCA 1 not representative) for the nursery and sites K98-8, K91-15 and K80-26 were relatively flat curves, representing the broken-stick type. Evenness increased and the species rank-abundance curves progressively became flatter. Sites K04-2 and K05F-1 have abundance curves of the geometric-type which is typical for sites characterised by disturbance. Figure 3-9 illustrates the curves from soil core assessment 3; rank abundance curves for soil core assessment 1 and 2 are presented in Appendix 9.

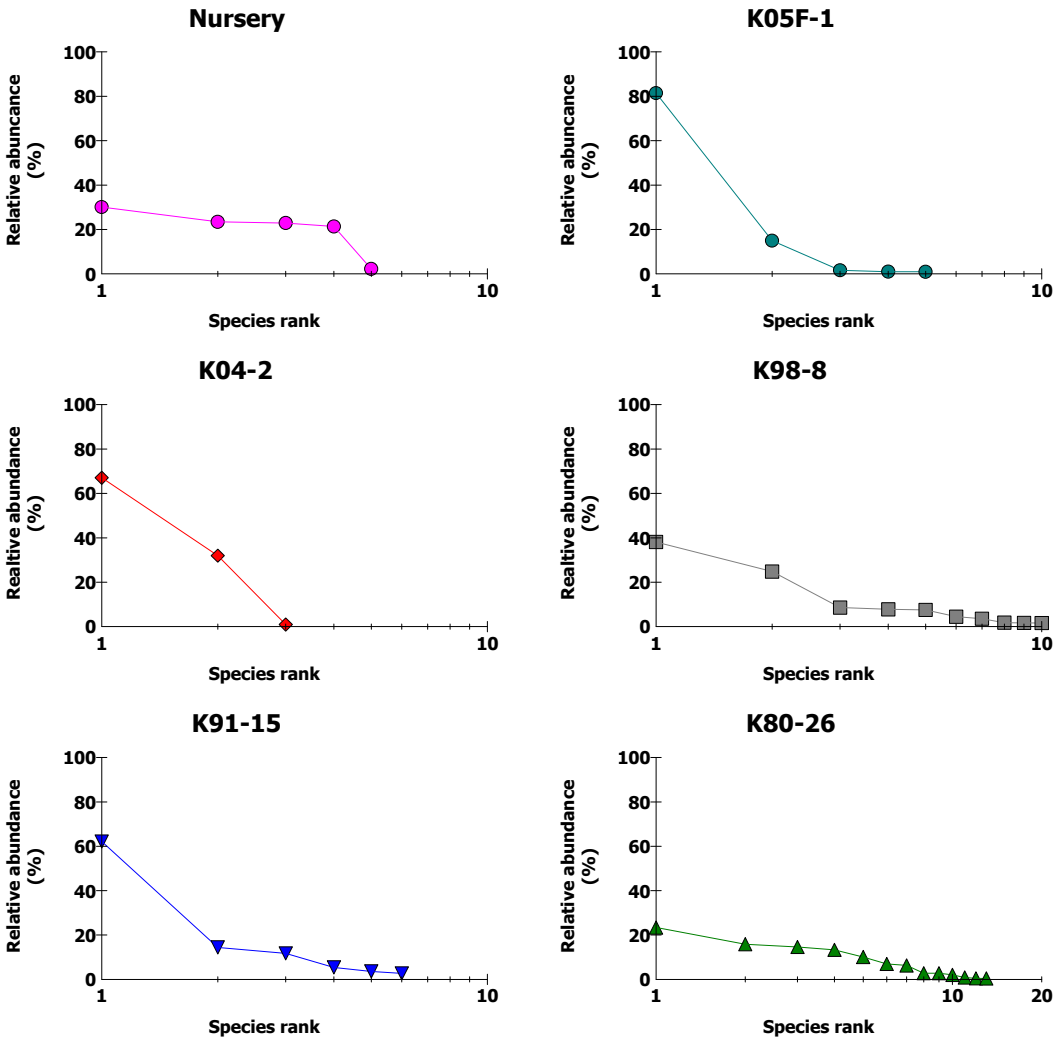

Figure 3-9: Rank abundance curves for sites investigated in soil core assessment 3, 2006.

SEASONALITY OF BELOWGROUND ECM DIVERSITY – COMPARISON OF SOIL CORE ASSESSMENTS 2 AND 3

To investigate the possible influence of seasonality on the diversity of ECM communities associated with *P. radiata,* soil core assessments 2 and 3 were compared. Cores for SCA 2 were collected in December 2005 (summer); cores for SCA 3 were collected from May – July 2006 (autumn). For comparison purposes and statistical analysis, sites K04-2, K98-8, K91-15 and K80-26 were used. Sites nursery and K05F-1 were not included as these were only sampled in SCA 3.

Comparing richness, diversity indices, abundance and composition of the summer and autumn assessments showed the following trends: (*i*) overall, more ECM species/types were found in the autumn; (*ii*) mean richness and diversity values for each investigated site did not differ significantly except for richness in site K80-26; (*iii*) species composition and dominant

species differed between the summer and autumn assessment. Only site K91-15 had the same dominant species in both assessments.

Seasonal ECM species richness

A Student's t-test was performed to compare the means of the richness and Margalef's index between the SCA 2 and SCA 3. Total species richness and Margalef's index (Table 3-13) were higher in SCA 3 compared with SCA 1. Richness and Margalef's index values of K04-2, K98-8 and K91-15 in SCA 3 were higher than in SCA 2, but differences were not significant. However, for K80-26 both species richness and Margalef's index were significantly higher (p=0.026 and 0.039, respectively) in SCA 3.

Table 3-13: Mean species richness and Margalef's index for sites K04-2, K98-8, K91-15 and K80-26 in soil core assessment (SCA) 2 and SCA 3 (± standard error) and respective p-value between the assessments. Total Species richness and Margalef's index for both SCA 2 and SCA 3 (SCA 2: n= 25 soil cores, SCA 3: n=34 soil cores).

		Species richness (±SE)	p-value	Margalef's index (±SE)	p-value
K04-2	SCA 2	1.40 (± 0.31)	0.436	0.12 (± 0.05)	0.544
	SCA 3	1.75 (± 0.31)		0.21 (± 0.06)	
K98-8	SCA 2	2.20 (± 0.58)	0.063	0.27 (± 0.15)	0.152
	SCA 3	3.88 (± 0.55)		0.56 (± 0.09)	
K91-15	SCA 2	3.80 (± 0.49)	0.496	0.52 (± 0.08)	0.117
	SCA 3	4.38 (± 0.65)		0.77 (± 0.13)	
K80-26	SCA 2	3.80 (± 0.58)	0.026	0.54 (± 0.10)	0.039
	SCA 3	6.00 (± 0.63)		0.87 (± 0.10)	
SCA 2 overall		14.00		1.57	
SCA 3 overall		17.00		1.82	

p-value calculated from Student's t-test

Seasonal ECM abundance

The absolute and relative abundance, irrespective of site, for soil core assessments 2 and 3 is illustrated in Figure 3-10.

Irrespective of sampling sites, more ECM species/types and ECM root tips were found and counted in the autumn assessment SCA 3 (17 types *versus* 13 in SCA 2). Species frequency differed between the two seasonal assessments; in SCA 2, *R. pseudoroseolus*, *A. muscaria* and *R. rubescens* were the most abundant species whereas ECM species *A. muscaria* and Type unknown Basidiomycete were the dominant types in SCA 3. The trend for the individual sampling sites was similar to the trend for the overall survey. More ECM species/types were found in the autumn assessment and species frequency and dominant species differed in all sites except for site K91-15 where *A. muscaria* was dominant in both assessments. The

percentage of shared species increased from 33% to 62% over the sampling sites (2 to 26 yrs) concurrent with the increase in ECM richness in these sites (from six species in K04-2 to thirteen in K80-26).

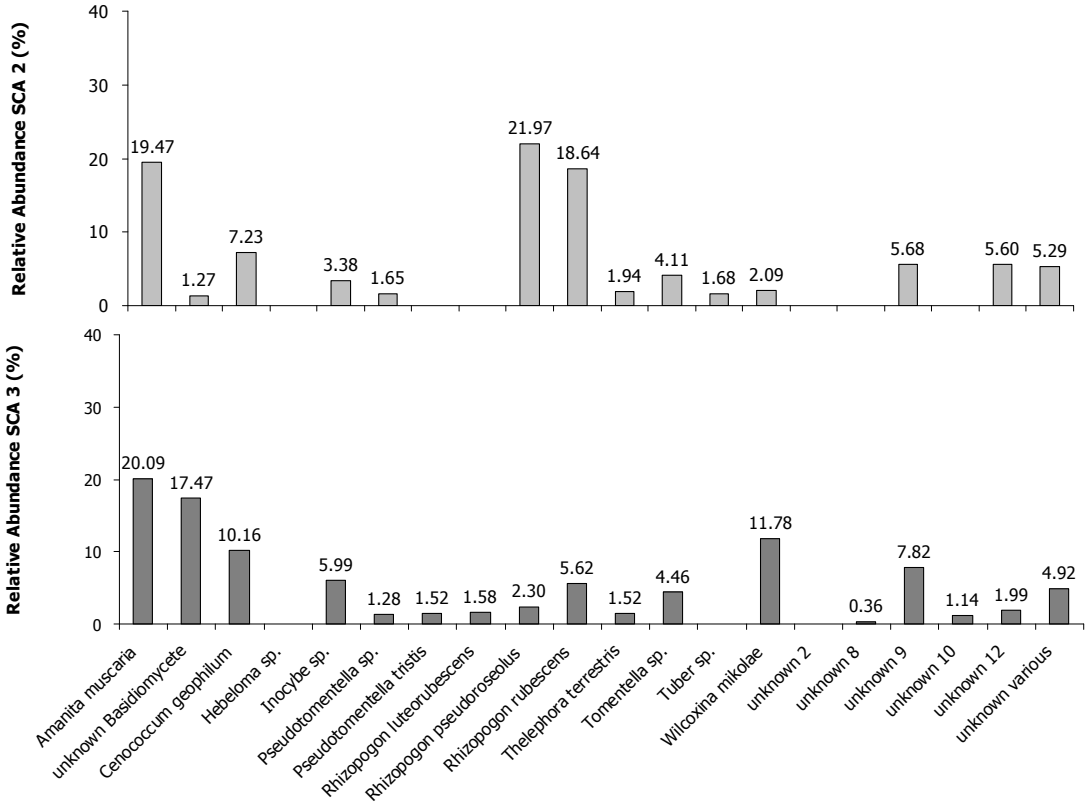

Figure 3-10: Relative abundance of ECM types found overall in K04-2, K98-8, K91-15 and K80-26. Top: soil core assessment (SCA) 2, bottom: SCA 3 (nursery and K05F-1 sites not included as only assessed in SCA 3). The corresponding absolute abundance values are given above each bar.

Seasonal ECM species diversity indices and Pielou's evenness

Total Pielou's evenness, Shannon diversity and Simpson diversity of SCA 2 and 3 are presented in Table 3-14. Neither evenness nor Shannon or Simpson diversity indices of each site were significantly different between the two seasonal assessments, as determined by t-test analysis.

Table 3-14: Mean Pielou's evenness, Shannon and Simpson diversity index (± standard error) for sites K04-2, K98-8, K91-15 and K80-26 investigated in soil core assessment (SCA) 2 and 3 and respective p-value between assessments. Total Pielou's evenness, Shannon and Simpson diversity indices for both SCA 2 and SCA 3 (SCA 2: n= 25 soil cores, SCA 3: n=34 soil cores).

		Pielou's evenness (±SE)	p-value	Shannon diversity (±SE)	p-value	Simpson diversity (±SE)	p-value
K04-2	SCA 2	0.38 (± 0.14)	0.305	0.30 (± 0.12)	0.437	0.20 (± 0.08)	0.366
	SCA 3	0.57 (± 0.15)		0.44 (± 0.12)		0.29 (± 0.08)	
K98-8	SCA 2	0.70 (± 0.23)	0.700	0.50 (± 0.28)	0.161	0.29 (± 0.16)	0.128
	SCA 3	0.81 (± 0.06)		0.98 (± 0.08)		0.56 (± 0.00)	
K91-15	SCA 2	0.72 (± 0.11)	0.726	0.88 (± 0.12)	0.328	0.50 (± 0.07)	0.402
	SCA 3	0.76 (± 0.08)		1.10 (± 0.18)		0.59 (± 0.00)	
K80-26	SCA 2	0.72 (± 0.13)	0.806	1.00 (± 0.25)	0.299	0.53 (± 0.13)	0.499
	SCA 3	0.75 (± 0.03)		1.31 (± 0.09)		0.66 (± 0.00)	
SCA 2 overall		0.84		2.23		0.86	
SCA 3 overall		0.85		2.41		0.89	

p-values derived from Student's t-test

3.2.4 ABOVEGROUND *VERSUS* BELOWGROUND ECM DIVERSITY

To assess the differences between the above- and belowground diversity of ECM associated with *P. radiata* the 2006 sporocarp (SA 2) and soil core (SCA 3) assessments were compared. Both datasets consisted of abundance data and were collected at the same time of year (June/July). The nursery and K04-2, K98-8, K91-15 and K80-26 were included in the comparison, site K05F-1 was excluded from the analysis as it was not surveyed in SA 2. A Student's t-test was performed to test the differences between the age classes.

Comparing the species richness, diversity indices, abundance and composition revealed the following patterns: (*i*) total species richness and diversity were similar above- and belowground; (*ii*) mean richness differed significantly in sites K04-2 and K98-8, however, the number of ECM species observed belowground was higher in all age classes; (*iii*) only a small percentage of species were found both, as fruiting bodies and colonising root tips; (*iv*) dominant species aboveground were not detected in the belowground ECM communities.

ABOVE- AND BELOWGROUND SPECIES RICHNESS

Total aboveground ECM species richness was 18 and the Margalef's index was 1.89, the total belowground ECM species richness was slightly higher (19) and the Margalef's index was similar (1.90). Values for each site are listed in Table 3-15, with Table 3-16 showing the ECM species/types found in SA 2 and SCA 3. Seven species were common between the above- and belowground ECM communities: *Amanita muscaria, Hebeloma* sp., *Inocybe* sp., *Rhizopogon pseudoroseolus*, *Rhizopogon rubescens*, *Thelephora terrestris* and *Wilcoxina mikolae*.

The mean richness and Margalef's index for SA 2 and SCA 3 were compared using Student's t-test. Species richness above- and belowground in the nursery was not significantly different. The Margalef's index for the sporocarp assessment was higher than the soil core assessment ($p=0.001$), reflecting the influence of abundance on this index. Sites K04-2 and K98-8 varied significantly in species richness and Margalef's index ($p=0.017$ and 0.02 for K04-2, respectively and $p=0.005$ and 0.004 for K98-8, respectively). In both sites more species were found in the aboveground sporocarp assessment. Species richness and Margalef's index in sites K91-15 and K80-26 were not significantly different above- and belowground.

Table 3-15: Mean species richness (*S*) and Margalef's index (*d*) for the nursery, K04-2, K98-8, K91-15 and K80-26 sites in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3 (± standard error) and respective p-value between the assessments, total Species richness and Margalef's index for both SA 2 and SCA 3.

		Species richness (±SE)	p-value	Margalef's index (±SE)	p-value
Nursery	SA (n=5)	2.60 (± 0.40)	0.150	0.53 (± 0.02)	0.001
	SCA (n=8)	3.38 (± 0.26)		0.34 (± 0.04)	
K04-2	SA (n=5)	2.80 (± 0.20)	0.017	0.39 (± 0.04)	0.02
	SCA (n=8)	1.75 (± 0.31)		0.21 (± 0.06)	
K98-8	SA (n=5)	6.20 (± 0.37)	0.005	0.96 (± 0.05)	0.004
	SCA (n=8)	3.88 (± 0.55)		0.56 (± 0.09)	
K91-15	SA (n=2)	5.00 (± 0.00)	0.370	1.00 (± 0.04)	0.111
	SCA (n=8)	4.38 (± 0.65)		0.77 (± 0.13)	
K80-26	SA (n=5)	6.80 (± 0.58)	0.370	0.55 (± 0.04)	0.599
	SCA (n=8)	6.00 (± 0.63)		0.87 (± 0.10)	
SA overall		18.00		1.89	
SCA overall		20.00		1.90	

n = number of transects/soil cores; p-value derived from Student's t-test

Table 3-16: Comparison of ECM taxa (sporocarp and ECM) collected in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3 associated with *P. radiata* in different aged stands in June/July 2006.

	Nursery		K04-2		K98-8		K91-15		K80-26	
ECM taxa/ECM type	**SA**	**SCA**	**SA**	**SCA**	**SA**	**SCA**	**SA**	**SCA**	**SA**	**SCA**
Amanita muscaria					+	+	+	+	+	+
Cenococcum geophilum						+		+		+
Chalciporus piperatus					+				+	
Hebeloma sp.	+	+								
Inocybe lacera			+		+		+		+	
Inocybe sindonia							+		+	
Inocybe sp.					+	+	+	+	+	+
Laccaria proxima			+		+		+		+	
Lactarius rufus					+				+	
Lycoperdon gunnii					+					
Lycoperdon sp.					+					
Pseudotomentella sp.						+		+		+
Pseudotomentella tristis						+		+		+
Rhizopogon luteolus									+	
Rhizopogon luteorubescens								+		
Rhizopogon pseudoroseolus					+	+	+			
Rhizopogon rubescens	+	+	+	+						
Scleroderma bovista			+		+					
Suillus sp.							+			
Thelephora terrestris						+		+	+	+
Tomentella sp.						+		+		+
Tricholoma sp.							+		+	
Tuber sp.		+		+						
Wilcoxina mikolae	+	+		+						
unknown Basidiomycete				+		+		+		+
unknown 2		+								
unknown 8								+		+
unknown 9						+				+
unknown 10										+
unknown 12				+		+		+		+
unknown various				+		+		+		+

ABOVE- AND BELOWGROUND ABUNDANCE

The absolute and relative abundance, irrespective of site, was compared and are shown in Figure 3-11. Slightly more ECM species/types were found belowground and fruiting aboveground (18 *versus* 19). Species frequency and dominant species differed between the above- and belowground assessments. *Lactarius rufus, Laccaria proxima, Inocybe sindonia* and *Inocybe lacera* were abundant aboveground, whereas in the soil core assessment *Rhizopogon rubescens*, *Tuber* sp., *Wilcoxina mikolae* and *Hebeloma* sp. were the most abundant species.

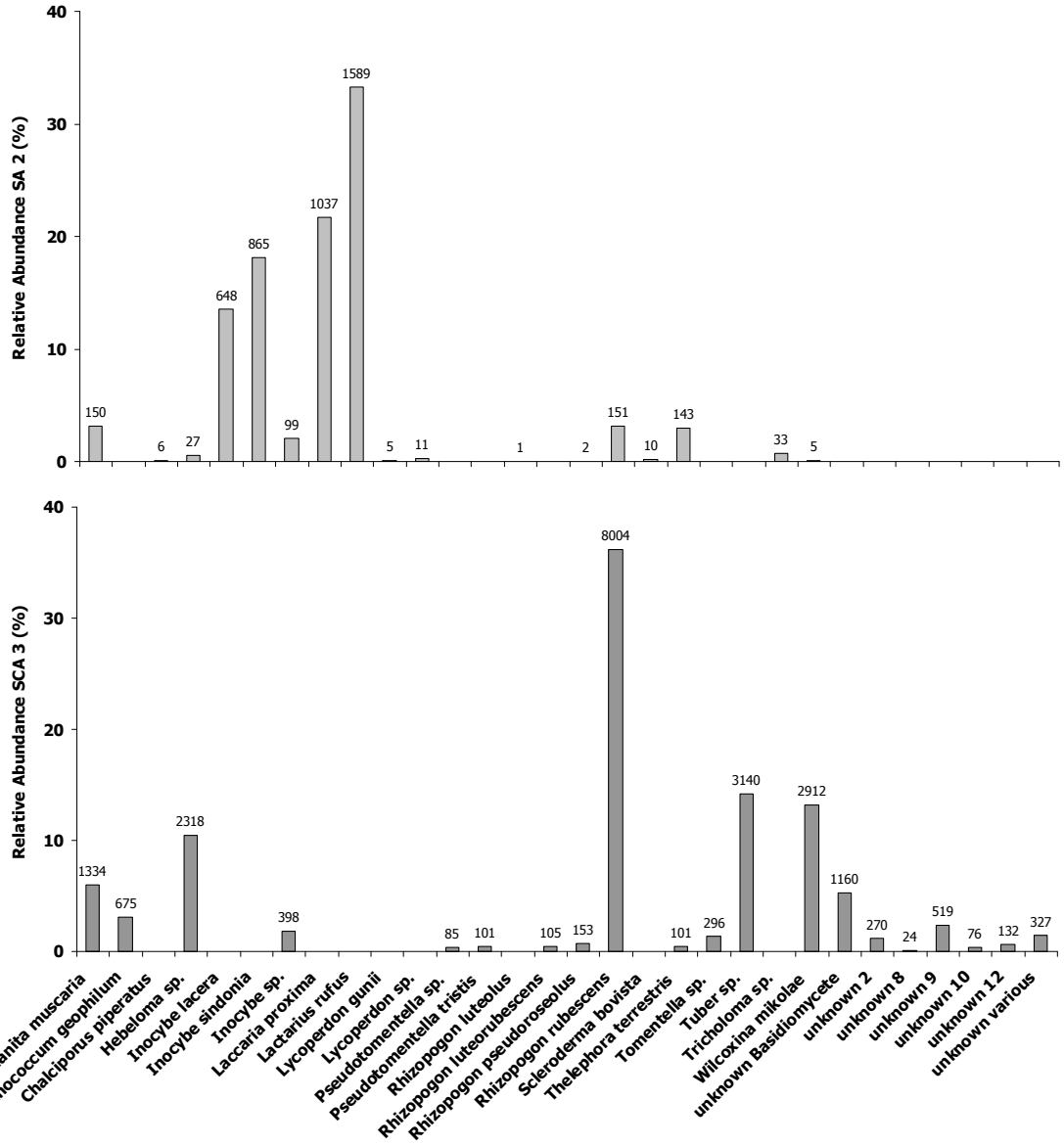

Figure 3-11: Relative abundance of ECM sporocarps and morphotypes found overall in the nursery, K04-2, K91-15, K98-8 and K80-26 sites in sporocarp assessment 2 (top) and in soil core assessment 3 (bottom). The corresponding absolute numbers are shown above each bar.

The absolute and relative abundance of ECM in the individual sites are presented in Table 3-17 to Table 3-19. Species richness increased from the nursery to the oldest plantation site (K80-26, 26 yrs) but the number of species was higher belowground than aboveground. With the exception of the nursery site, the general trend for the sites investigated was that more species were observed belowground and the species frequency and dominant species differed above- and belowground. The percentage of species that were observed both above- and belowground in the forest plantation sites ranged from 14% to 25%. In the nursery, three of the five species (60%) observed were present above- and belowground (*Hebeloma* sp., *Wilcoxina mikolae* and *Rhizopogon rubescens*) and the same species, *R. rubescens* was dominant both above- belowground.

Table 3-17: Absolute and relative abundance of ECM observed in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3 in the nursery and K04-2. Absolute numbers are total of all transects in the sporocarp assessment and total of soil cores processed at each assessment.

	Nursery				K04-2			
	SA (n=5)		SCA (n=8)		SA (n=5)		SCA (n=8)	
ECM Taxa/ ECM type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	-	-	-	-
Cenococcum geophilum	-	-	-	-	-	-	-	-
Chalciporus piperatus	-	-	-	-	-	-	-	-
Hebeloma sp.	27	15.34	2223	22.91	-	-	-	-
Inocybe lacera	-	-	-	-	453	85.15	-	-
Inocybe sindonia	-	-	-	-	-	-	-	-
Inocybe sp.	-	-	-	-	-	-	-	-
Laccaria proxima	-	-	-	-	62	11.65	-	-
Lactarius rufus	-	-	-	-	-	-	-	-
Lycoperdon gunnii	-	-	-	-	-	-	-	-
Lycoperdon sp.	-	-	-	-	-	-	-	-
Pseudotomentella sp.	-	-	-	-	-	-	-	-
Pseudotomentella tristis	-	-	-	-	-	-	-	-
Rhizopogon luteolus	-	-	-	-	-	-	-	-
Rhizopogon luteorubescens	-	-	-	-	-	-	-	-
Rhizopogon pseudoroseolus	-	-	-	-	-	-	-	-
Rhizopogon rubescens	144	81.82	2921	30.10	7	1.32	373	31.99
Scleroderma bovista	-	-	-	-	10	1.88	-	-
Thelephora terrestris	-	-	-	-	-	-	-	-
Tomentella sp.	-	-	-	-	-	-	-	-
Tuber sp.	-	-	2274	23.43	-	-	-	-
Tricholoma sp.	-	-	-	-	-	-	-	-
Wilcoxina mikolae	5	2.84	2073	21.36	-	-	782	67.07
unknown Basidiomycete	-	-	-	-	-	-	11	0.94
unknown 2	-	-	214	2.21	-	-	-	-
unknown 8	-	-	-	-	-	-	-	-
unknown 9	-	-	-	-	-	-	-	-
unknown 10	-	-	-	-	-	-	-	-
unknown 12	-	-	-	-	-	-	-	-
unknown various	-	-	-	-	-	-	-	-
Total Specimen/ ECM types counted	176		9705		532		1166	

n = number of transects/soil cores

Table 3-18: Absolute and relative abundance of ECM in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3 observed in K98-8 and K91-15. Absolute numbers are total of all transects in the sporocarp assessment and total of soil cores processed at each assessment.

	K98-8				K91-15			
	SA (n=5)		SCA (n=8)		SA (n=2)		SCA (n=8)	
ECM Taxa/ ECM type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative	Absolute	Relative (%)
Amanita muscaria	52	4.19	587	38.12	4	3.70	314	25.99
Cenococcum geophilum	-	-	55	3.57	-	-	257	21.27
Chalciporus piperatus	4	0.32	-	-	-	-	-	-
Hebeloma sp.	-	-	-	-	-	-	-	-
Inocybe lacera	43	3.46	-	-	69	63.89	-	-
Inocybe sindonia	-	-	-	-	13	12.04	-	-
Inocybe sp.	29	2.33	26	1.69	6	5.56	200	16.56
Laccaria proxima	966	77.78	-	-	-	-	-	-
Lactarius rufus	5	0.40	-	-	-	-	-	-
Lycoperdon gunnii	5	0.40	-	-	-	-	-	-
Lycoperdon sp.	11	0.89	-	-	-	-	-	-
Pseudotomentella sp.	-	-	28	1.82	-	-	1	0.08
Pseudotomentella tristis	-	-	-	-	-	-	88	7.28
Rhizopogon luteolus	-	-	-	-	-	-	-	-
Rhizopogon luteorubescens	-	-	-	-	-	-	105	8.69
Rhizopogon pseudoroseolus	2	0.16	69	4.48	-	-	84	6.95
Rhizopogon rubescens	-	-	-	-	-	-	-	-
Scleroderma bovista	-	-	-	-	-	-	-	-
Thelephora terrestris	125	10.06	25	1.62	16	14.81	-	-
Tomentella sp.	-	-	-	-	-	-	21	1.74
Tuber sp.	-	-	-	-	-	-	-	-
Tricholoma sp.	-	-	-	-	-	-	-	-
Wilcoxina mikolae	-	-	-	-	-	-	-	-
unknown Basidiomycete	-	-	382	24.81	-	-	129	10.68
unknown 2	-	-	-	-	-	-	-	-
unknown 8	-	-	-	-	-	-	-	-
unknown 9	-	-	120	7.79	-	-	-	-
unknown 10	-	-	-	-	-	-	-	-
unknown 12	-	-	116	7.53	-	-	4	0.33
unknown various	-	-	132	8.57	-	-	5	0.41
Total Specimen/ ECM types counted	1242		1540		108		1208	

n = number of transects/soil cores

Table 3-19: Absolute and relative abundance of ECM in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3 observed in K80-26. Absolute numbers are total of all transects in the sporocarp assessment and total of soil cores processed at each assessment.

	K80-26			
	SA (n=5)		SCA (n=8)	
ECM Taxa/ ECM type	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	94	3.45	433	15.88
Cenococcum geophilum	-	-	363	13.31
Chalciporus piperatus	2	0.07	-	-
Hebeloma sp.	-	-	-	-
Inocybe lacera	83	3.05	-	-
Inocybe sindonia	852	31.28	-	-
Inocybe sp.	64	2.35	172	6.31
Laccaria proxima	9	0.33	-	-
Lactarius rufus	1584	58.15	-	-
Lycoperdon gunnii	-	-	-	-
Lycoperdon sp.	-	-	-	-
Pseudotomentella sp.	-	-	56	2.05
Pseudotomentella tristis	-	-	13	0.48
Rhizopogon luteolus	1	0.04	-	-
Rhizopogon luteorubescens	-	-	-	-
Rhizopogon pseudoroseolus	-	-	-	-
Rhizopogon rubescens	-	-	-	-
Scleroderma bovista	-	-	-	-
Thelephora terrestris	2	0.07	76	2.79
Tomentella sp.	-	-	275	10.08
Tuber sp.	-	-	-	-
Tricholoma sp.	33	1.21	-	-
Wilcoxina mikolae	-	-	-	-
unknown Basidiomycete	-	-	638	23.40
unknown 2	-	-	-	-
unknown 8	-	-	24	0.88
unknown 9	-	-	399	14.63
unknown 10	-	-	76	2.79
unknown 12	-	-	12	0.44
unknown various	-	-	190	6.97
Total Specimen/ ECM types counted	2724		2727	

n = number of transects/soil cores

ABOVE- AND BELOWGROUND SPECIES DIVERSITY INDICES AND PIELOU'S EVENNESS

Pielou's evenness, Shannon and Simpson diversity are presented in Table 3-20, a Student's t-test was performed to compare the above- and belowground values. The total Pielou's evenness, Shannon and Simpson diversity values were higher in the belowground assessment (SCA 3). Diversity indices did not differ significantly between the sites except for the Shannon diversity in the nursery (p=0.014). For Pielou's evenness a significant difference was found in the nursery, K98-8 and K80-26.

Table 3-20: Mean Pielou's evenness, Shannon and Simpson diversity index (± standard error) for nursery, K04-2, K98-8, K91-15 and K80-26 sites investigated in sporocarp assessment (SA) 2 and soil core assessment (SCA) 3, total Pielou's evenness, Shannon and Simpson diversity index for each SA 2 and SCA 3.

		Pielou's evenness (±SE)	p-value	Shannon diversity (±SE)	p-value	Simpson diversity (±SE)	p-value
Nursery	SA (n=5)	0.42 (± 0.10)	0.047	0.37 (± 0.12)	0.014	0.26 (± 0.08)	0.062
	SCA (n=8)	0.72 (± 0.08)		0.88 (± 0.12)		0.50 (± 0.00)	
K04-2	SA (n=5)	0.46 (± 0.06)	0.535	0.46 (± 0.06)	0.830	0.26 (± 0.04)	0.724
	SCA (n=8)	0.57 (± 0.15)		0.44 (± 0.12)		0.29 (± 0.08)	
K98-8	SA (n=5)	0.46 (± 0.08)	0.007	0.83 (± 0.12)	0.350	0.41 (± 0.08)	0.079
	SCA (n=8)	0.81 (± 0.06)		0.98 (± 0.08)		0.56 (± 0.00)	
K91-15	SA (n=2)	0.71 (± 0.02)	0.523	1.14 (± 0.04)	0.841	0.58 (± 0.02)	0.915
	SCA (n=8)	0.76 (± 0.08)		1.10 (± 0.18)		0.59 (± 0.00)	
K80-26	SA (n=5)	0.55 (± 0.04)	0.002	1.05 (± 0.10)	0.075	0.55 (± 0.04)	0.159
	SCA (n=8)	0.75 (± 0.03)		1.31 (± 0.09)		0.66 (± 0.00)	
SA overall		0.63		1.80		0.79	
SCA overall		0.71		2.10		0.81	

n = number of transects/soil cores; p-value derived from Student's t-test

3.3 DISCUSSION

3.3.1 ECM FUNGI COMMONLY ASSOCIATED WITH *PINUS RADIATA* IN NEW ZEALAND

In the nursery, *Hebeloma* sp., *Rhizopogon rubescens* and the ascomycete *Wilcoxina mikolae* were found both as sporocarps and colonising root tips. *Tuber* sp. was present on the root tips but no fruiting bodies were found. Both *Hebeloma* sp. and *R. rubescens* are well known nursery fungi and the latter is likely to be the most effective, adaptable and beneficial associate with *P. radiata* in New Zealand and other exotic plantations in the nursery stage (Chu-Chou & Grace, 1985; Duñabeitia *et al.*, 2004). Chu-Chou & Grace (1985) found *R. rubescens* more beneficial in promoting growth of radiata pine seedlings in New Zealand than *Laccaria proxima* and *Hebeloma* sp. In addition, Duñabeitia *et al.* (2004) compared the species to *R. luteolus* and *Scleroderma citrinum* for their ability to tolerate adverse environmental conditions, such as water stress, change in pH and toxic metals and showed that *R. rubescens* is the most resistant species. The species was also the most beneficial to radiata pine seedlings and significantly increased shoot growth (height, diameter and dry weight) and dehydration tolerance of the seedlings. In this present study *R. rubescens* was the most abundant species in the nursery (82%), confirming data from previous nursery studies in New Zealand (Chu-Chou, 1979) and Spain (Duñabeitia *et al.*, 1996). The species is a successful species in the nursery and also the outplanting environment because of its resistance to disturbance (Karkouri *et al.*, 2002; Liu *et al.*, 2004) and its long rhizomorphs, which allow successful colonization of new roots in the nurseries and outplantings (Cline *et al.*, 2005) and fast establishment. In general *Rhizopogon* spp. are among the most common introduced fungi in exotic pine plantations (Dunstan *et al.*, 1998; Giachini *et al.*, 2004; Tedersoo *et al.*, 2007).

In both, the present and prior studies on ECM fungi associated with *P. radiata* in New Zealand (Chu-Chou, 1979), *Hebeloma* sp. was only found in the nursery stage. This is in contrast to reports from Western Australia where the taxon is widely distributed in exotic plantations and was associated with pines up to 60 years of age (Dunstan *et al.*, 1998). Sequencing results of *Hebeloma* sp. specimens collected during this study and Landcare Research PDD herbarium material showed that a clear species classification for this taxon was not possible. Aanen *et al.* (2000) and Boyle *et al.* (2006) found in their studies on phylogenetic relationships in the genus *Hebeloma* that the ITS sequences differed by only a few nucleotides. Both publications suggested that there has been a rapid recent speciation within the genus and that the variability of the ITS region was not divergent enough to reflect the morphological species differences. On the basis of these publications and the sequencing results of this study, the organism label *Hebeloma* sp. was used for *Hebeloma* specimens collected during this

research. This recent speciation of *Hebeloma* could imply that *Hebeloma* species associated with *Pinus* sp. found in Australia is different to that observed in New Zealand.

The species *W. mikolae* is a known ECM associate of conifers and a number of angiosperms internationally (Yu *et al.*, 2001), but to date has not been reported in the literature as being an ECM associate of *P. radiata*, in New Zealand or other countries. The ascomycete is an ectendomycorrhiza as the hyphae penetrate the epidermal cells of the host tree, however, the fungus also produces the characteristic ECM features of a Hartig net and mantle. The species is common in conifer nurseries and habitats which have experienced disturbance, such as clearcutting, burning and wildfires (Yu *et al.*, 2001). In such sites, the resistant chlamydospores of *Wilcoxina* spp. are the main source of inoculum when new seedlings emerge and the species is important for the re-vegetation of disturbed sites (Yu *et al.*, 2001).

Laccaria proxima was a dominant species in the aboveground ECM assessments of the 8 yr old site K98-8 (77%) and present in all other sites except the nursery. *Laccaria* is a cosmopolitan and common genus which is frequently associated with planted pines and eucalypts throughout the world (Cairney & Chambers, 1999). Some taxa such as *L. proxima*, *L. laccata* and *L. bicolour* are often found in disturbed sites and in association with young stands, but not mature forests. The high plasticity within the *Laccaria* genus makes it difficult to distinguish species morphologically, especially in the case of *L. laccata* and *L. proxima* where only minor differences in spore size, shape and ornamentation are discriminating features (Gardes *et al.*, 1990). Moreover, the opinion on defining values for spore sizes and spine length for each species varies amongst mycologists (NZFUNGI, 2007b), which makes morphological species identification difficult. In McNabb's (1972) monograph on the *Trichlomataceae* of New Zealand the species *L. laccata* was not reported to occur in New Zealand. Nevertheless, Chu-Chou (1979) labelled the *Laccaria* species collected in her studies as *L. laccata***.** To clarify the species identity, this identification was followed up in this study with DNA sequencing of *Laccaria* specimens collected from Kaingaroa Forest and based on the results the species label *Laccaria proxima* was applied. It is assumed that the taxa *L. laccata* is actually not present in New Zealand, based on inspections of Landcare Research Herbarium material by the leading *Laccaria* expert G. Mueller (G. Mueller, personal communication, 10 May 2006). The application of molecular methods in this study was able to resolve the confusion over *L. laccata* and *L. proxima* in New Zealand.

Thelephora terrestris sporocarps were only identified in the 2006 survey and although likely to have been present in 2005 were not counted due to inexperience in identifying sporocarps. This resupinate taxa is a frequent ECM symbiont of conifers (Cairney & Chambers, 1999) and generally common in the nursery, where it usually outcompetes ECM species that have been artificially inoculated onto the crop. In this study, *T. terrestris* was not found in the nursery but only in the older plantation sites, which is similar to reports from plantations in Western

Australia (Dunstan *et al.*, 1998). Chu-Chou & Grace (1990) found the species only in nurseries established on peaty soil and it was the main associate of containerised radiata pines seedlings where a peat and bark potting mix was used (Minchin, Jones & Ridgway unpublished, personal communication, 4 December 2007). This indicates that *T. terrestris* might only grow under these specific soil conditions in nurseries in New Zealand, which are different from the soil conditions in the nursery investigated in this study. The species was also found colonising root tips of trees in the 8, 15 and 26 yr old stands.

Amanita muscaria, found above- as well as belowground in the 8, 15 and 26 yr old stand is of special interest for New Zealand. This species is considered as a "regulated pest" by the Ministry of Agriculture and Forestry (MAF) in New Zealand and is of concern to the native ECM fungal "flora" as it has been found associated with native *Nothofagus* spp. in the Nelson Lakes National Park and other locations in the northern half of the South Island (Johnston *et al.*, 1998; NZFUNGI, 2007a). In 2007 the species was also reported fruiting under natives in the Tongariro National Park in the North Island (I. Dickie, personal communication, 14 April 2007). *Amanita muscaria* has also recently been observed in *Nothofagus cunninghamii* forest in Tasmania (Sawyer *et al.*, 2002; Orlovich & Cairney, 2004). As in New Zealand, this appearance is raising concerns that the fungus might replace the indigenous ECM fungi in these forests (Sawyer *et al.*, 2002), however, to date this has not been investigated in detail. Belowground this species had a high abundance, especially in the 8 and 15 yr old sites (23 – 38% in 8 yr old site, 26 – 52% in 15 yr old site). Similar values were found by Natarajan *et al.* (1992) in a 17 yr old *Pinus patula* plantation in India, where 45% of root tips were colonised by *A. muscaria.* In Chu-Chou & Grace's (1988) study on mycorrhizal fungi of radiata pine in the North and South Islands in New Zealand, *A. muscaria* was isolated from root tips from 10 and 17 yr old trees, however abundance was greater in the 17 yr old sites (<5% relative abundance in the 10 yr old site, approximately 10% relative abundance in 17 yr old site). As in the cited study, *Amanita muscaria* was not found in the younger sites in this present study, but it was more prevalent in the older plantation sites. This may be due to favourable growing conditions for this fungus in the investigated sites, or due to the invasive potential of *A. muscaria.* This species was more abundant overall and it was found noticeably more often in the 8 yr site compared to the study of Chu-Chou & Grace (1988), indicating that it might be outcompeting other species in these sites.

Cenococcum geophilum was also dominant belowground in the older sites. This species is one of the most frequently encountered ECM fungi in nature (LoBuglio *et al.*, 1996). This ubiquitous species, which lacks a known sexual reproductive state, has a wide host and habitat range and is extremely drought resistant, however it is not limited to dry sites. The species also grows and forms mycorrhizae over a wide pH range (2.4 - 7.5) and at high salt concentrations (Cairney & Chambers, 1999; Jany *et al.*, 2003; Menkis *et al.*, 2007). *Cenoccocum geophilum* is of interest as it could potentially be the only native ECM species

associated with *P. radiata* in New Zealand. The species has been found to be associated with *Nothofagus solandri* var. *cliffortoides* in New Zealand and the variant found here is suspected to be native (Mejstrik, 1972).

The genus *Inocybe*, a known ectomycorrhizal associate of conifers and hardwoods, was dominant in the aboveground assessment of the 15 yr old stand, but also present in the 8 and 26 yr old stands. The species found were *I. lacera, I. sindonia* and *Inocybe* sp. Belowground only *Inocybe* sp. was observed, its abundance increased from the 8 yr to the 26 yr old site. *Inocybe* spp. sporocarps are difficult to distinguish based on morphological traits (Arora, 1979). In this study, species were distinguished using RFLP patterns and DNA sequence data. ECM taxa *I. lacera* and *Inocybe* sp. are both small and brown coloured specimens, which were only distinguishable using molecular tools. In the case of *Inocybe sindonia*, it was only possible to positively identify this taxon to species level using the Nordic-baltic sequence databank UNITE. This species has, to date, not been reported as an associate of *P. radiata* in New Zealand. *Inocybe sindonia* is present in Europe and America and known from *Pinus* spp. plantations and other conifer forests. The species is also known under its synonym *I. eutheles*.

Specimens of the genus *Tricholoma* were collected in the aboveground assessments and morphologically identified as *T. pessundatum*, however the morphological identification of this species was not certain. Recently, the label *Tricholoma stans* or its synonym *T. testaceum* has also been applied to this species (G. Ridley, personal communication, 30 April 2007 and 7 November 2007). Sequencing searches from collected specimens and reference material from the Landcare Research PDD gave mixed results. When BLAST searched in GenBank, species matched either *T. ustale* or *T. aurantium*, but not a *T. pessundatum*. When BLAST searched with the UNITE database, specimens matched the species *T. pessundatum* or *T. tridentinum,* with much higher scores. As the scores associated with the UNITE matches were higher, it was assumed that these were better species matches that the sequences in GenBank. The species *T. pessundatum, T. tridentinum* and *T. stans*, all belonging to the section Genuina, are morphologically difficult to distinguish and closely related, as the sequencing results in this study showed. It is suggested that the species associated with *P. radiata* in New Zealand could potentially belong to the *T. tridentinum* complex (J. H. Clausen, personal communication, 23 April 2007). The collections *T. pessundatum* and *T. tridentinum* are likely to fit with the true *T. tridentinum,* which occurs in Europe in regions climatically similar to the *P. radiata* growing regions in New Zealand (J. H. Clausen, personal communication, 23 April 2007). However, as only a limited number of collections of the *T. tridentinum/pessundatum/stans* complex have been sequenced to date, a re-organisation of this group is possible. Since the species–group is currently not confidently clarified and neither morphological nor molecular methods are able to identify the *Tricholoma* specimens

collected for this research to a species level, the species label *Tricholoma* sp. was used in this thesis. The species was not found colonising root tips.

The species *Lactarius rufus* was dominant in the aboveground assessment of the oldest site (26 yrs), but never found colonising root tips. The species is a known ectomycorrhizal associate of pines (Cairney & Chambers, 1999), but has not been listed as an associate of *P. radiata* in New Zealand in the literature to date. In this study the species was often found growing on rotting wood; it is known that *L. rufus* can make use of additional saprotrophic sources for its carbon demand (Iwanski & Rudawska, 2007).

Rhizopogon pseudoroseolus was found above- and belowground in the 8 yr old site and had an exceptionally high abundance in the second assessment in the 8 yr old site. This can be explained by the morphology of the species, as its mycorrhizae are of tuberculate structure. In the assessments, each colonised root tip was counted individually, resulting in high counts for the large clump extracted in this specific assessment. It was also observed belowground in the 15 yr old stand. *Rhizopogon pseudoroseolus* is known to be an ECM associate of *Pinus contorta* (Molina & Trappe, 1994), however, it has not been reported as a mycorrhizal associate of radiata pine in New Zealand to date. The same applies to *R. luteorubescens*, which was only observed belowground in the 15 yr old stand. *Rhizopogon luteolus* was only found fruiting once in the oldest stand and this species is known to be an associate of *P. radiata* in New Zealand (Chu-Chou & Grace, 1985).

Of the rare ECM fungal types that were observed belowground, the tomentelloid species *Pseudotomentella* sp., *Pseudotomentella tristis* and *Tomentella* sp. are noteworthy. Resupinate thelephoroid fungi (tomentelloid fungi) have a world-wide distribution, but their ability to form ECM has only been realised recently. Kõljalg *et al.* (2000) investigated this group further and demonstrated the symbiotic nature of several *Tomentella* spp. species. Based on their studies, tomentelloid species seem to be common ECM symbionts in boreal and temperate forest. This group of closely related species is known to form resupinate sporocarps on decaying wood debris and it has been suggested that the preference of tomentelloid fungi for decaying plant materials might be related to their capability in utilizing organic sources of nutrients and decomposition of decayed conifer stumps and logs. No *Pseudotomentella* spp. has been collected and deposited in the main fungal herbarium in New Zealand (Landcare Research Herbarium, PDD) to date and no reports of this species in New Zealand are known to the author. Two *Tomentella* spp. have been collected under *P. radiata* in New Zealand and deposited in the PDD Herbarium (*T. castanea*, *T. pilosa*), another two *Tomentella* spp. are described from New Zealand (*T. fusca, T. punicea*) (Cunningham, 1957), however all are reported as saprotrophs. This present study is the first to find these resupinate species colonising root tips and identifying them as an ECM associate of *P. radiata* in New Zealand.

3.3.2 ABOVEGROUND ECM DIVERSITY

ABOVEGROUND SPECIES RICHNESS

In this study of ectomycorrhizal (ECM) sporocarp diversity over the course of two sampling periods in 2005 and 2006, a total of 18 ECM taxa were observed. It is not possible to compare these values of New Zealand *P. radiata* as an exotic host species to richness and diversity values of ECM fungal species associated with *P. radiata* in its natural range in California, USA as no studies have been carried out in its native range. As a further measure of species richness, Margalef's index was calculated for the 2006 assessment. Values for the index were low, reflecting the overall low species richness of the ECM community associated with *P. radiata* in a plantation in New Zealand.

This research presents an in depth look at the ECM species in Kaingaroa Forest using both morphological and molecular criteria. The only other significant research in New Zealand on ECM fungal diversity in *P. radiata* plantations was by Chu-Chou & Grace in the late 1970s – 90s (Chu-Chou & Grace 1979, 1980, 1983 a, 1983 b, 1984 a, 1984 b, 1987, 1988, 1990). This research was prior to the widespread use of molecular technologies for species identification. The methods used during those studies were sporocarp field observations and collection and isolation of fungal symbionts from sporocarps and mycorrhizae, which were compared to each other for species identification of mycorrhizae. Chu-Chou & Grace's studies covered a wide range of nurseries and plantations in both, the North and South Island of New Zealand and were undertaken over a period of 20 years, with only 17 taxa identified as ECM associates of *P. radiata* in plantations (Table 3-21). In this study, 18 ECM taxa were identified to be associated with *P. radiata*. Even though the present work covers a much smaller area and timeframe, the species diversity is similar, indicating that ECM diversity in *P. radiata* plantations in New Zealand is reasonably small.

Table 3-21: List of ECM taxa found associated as sporocarps with *P. radiata* in New Zealand identified in Chu-Chou and Chu-Chou & Grace publications and this study.

ECM taxa	Chu-Chou and Chu-Chou & Grace publications (year of publication)	Present in this study
Amanita muscaria	1979, 1983 a,1987, 1988	+
Chalciporus piperatus	1979	+
*Hebeloma sp.**	1979, 1983 a, 1985, 1987, 1988, 1990	+
Endogone flammicorona	1983 a, 1983 b, 1984,1988	-
Inocybe sp.	-	+
Inocybe sindonia	-	+
Inocybe lacera	1979, 1983 a, 1987, 1988,	+
*Laccaria proxima***	1979, 1983 a, 1985, 1987, 1988	+
Lactarius rufus	-	+
Lycoperdon sp.	-	+
Lycoperdon gunnii	-	+
Lycoperdon perlatum	1983 b	-
Rhizopogon pseudoroseolus	-	+
Rhizopogon rubescens	1979, 1983 a, 1983 b, 1985, 1987, 1988, 1990	+
Rhizopogon luteolus	1979, , 1983 a, 1983 b, 1985, 1988, 1990	+
Scleroderma aurantium	1979, 1983 a	-
Scleroderma bovista	1983 b, 1987	+
Scleroderma verrucosum	1979, 1983 a, 1983 b, 1987	-
Suillus luteolus	1979, 1983 a, 1987, 1988, 1990	-
Suillus granulatus	1979, 1988	-
Suillus sp.	-	+
Thelephora terrestris	1979, 1983 a, 1988, 1990	+
Tricholoma sp. ***	1983 a, 1988	+
Tuber sp.	1983 b, 1984, 1987, 1988,1990	-
Wilcoxina mikolae	-	+

* *Hebeloma* sp. listed as *Hebeloma crustuliniforme* in Chu-Chou & Grace's publications

** *Laccaria proxima* listed as *Laccaria laccata* in Chu-Chou & Grace's publications

*** *Tricholoma* sp. listed as *Tricholoma pessundatum* in Chu-Chou & Grace's publications

A number of species identified by Chu-Chou & Grace were not detected in the present study as sporocarps (*Endogone flammicorona, Tuber* sp., *Scleroderma aurantium*, *Scleroderma verrucosum, Suillus luteolus* and *Lycoperdon perlatum*). There are several reasons that may explain this discrepancy. Firstly, although *Endogone flammicorona* and *Tuber* sp. were found in Chu-Chou & Grace's work in nurseries and forests located in similar areas to our research sites, these species were not observed in this study. Chu-Chou & Grace specifically searched for the hypogeous fruiting bodies by raking and searching through the litter and upper layer

of soil (Chu-Chou & Grace, 1984). This intensive searching method was not applied in the present study, potentially explaining the lack of the two species in our sporocarp assessments. The dependency on certain soil conditions for fruiting is another potential explanation for the absence of some species. *Scleroderma* spp. for example are more common in forests established on farmland where higher levels of nitrogen, phosphorus and calcium and lower levels of magnesium and potassium are found (Chu-Chou & Grace, 1987).

Several species identified during the sporocarp assessments for this study were not noted in the research by Chu-Chou & Grace. *Lycoperdon gunnii* and an unidentified *Lycoperdon* sp. were collected in this study. The *Lycoperdon* spp. were included in the sporocarp assessment, as *L. perlatum* has been reported as an ECM fungus of *P. radiata* (Chu-Chou & Grace, 1983) and *Lycoperdon* spp. as an associate of *Eucalyptus* spp. in New Zealand (Chu-Chou & Grace, 1981). However, the mycorrhizal status of these other species is not clear and *Lycoperdon* spp. are often reported to be saprotrophs only (Martinez-Amores *et al.*, 1990). In the case of the genus *Inocybe, Inocybe sindonia* was identified using sequencing of the ITS region of the rDNA and sequence databank searches. Despite this technology the identity of a further *Inocybe* sp. remained unresolved. The ascomycete *Wilcoxina mikolae* was another new record as an associate of *P. radiata* in New Zealand. This species was initially detected during the belowground assessment in the present study and following this observation, ascocarps were observed fruiting in the nursery. It is not clear if this species is a recent addition to the suite of mycorrhizal species of radiata pine in New Zealand or if was not included in the studies of Chu-Chou & Grace since teleomorphs of this genus were only identified in the mid 1980s (Yu *et al.*, 2001). Another species that was not recorded by Chu-Chou & Grace is *Lactarius rufus*. This species is a known ectomycorrhizal associate of *Pinus* spp. but can also use decaying wood as an alternative carbon source, therefore, it could have been categorised as a saprotroph only and not been included in the sporocarp surveys. The species *Wilcoxina mikolae*, *Inocybe sindonia*, and *Lactarius rufus* have been found as ECM taxa associated with *Pinus* spp. in other countries, however, they have not been previously reported as associates of *P. radiata* in New Zealand. These species may have been overlooked in prior surveys or may be new associations in this exotic plantation system. In the case of *W. mikolae* and *I. sindonia* this research shows that with using molecular tools it is possible to identify more species and clarify species identities of cryptic taxa.

Compared to values for similar forest systems in the Northern Hemisphere species richness in Kaingaroa Forest is low. Numbers of epigeous fruit body taxa from a four year long ECM study in western North America in conifer forests (5 yr old plantation and 75 - 125 yr old mature forest) ranged from 70 – 241 species (Durall *et al.*, 2006), with abundance of fruit bodies varying widely between sampling years and being dependent on precipitation. A range of 42 – 124 ECM species were reported from a six year study of a 100 yr old spruce forest (*Picea* spp.) in Sweden (Dahlberg *et al.*, 1997) and Smith *et al.* (2002) recorded 263

macrofungal ECM species from a *Pseudotsuga menziesii* system in the North-western USA, where 30 - 35 yr, 45 - 50 yr and >400 yr old forests were investigated over four years.

In contrast with the Northern Hemisphere forests, species richness values from Southern Hemisphere plantations have been found to range from 11 to 55 (Garrido, 1986; Dunstan *et al.*, 1998; Giachini *et al.*, 2000). Sporocarp studies in Argentina on *Pinus ponderosa* in nurseries and plantations ranging from 12 – 47 yrs identified 18 ECM species associated with the exotic plantation tree (Barroetaveña *et al.*, 2005), which is the same number of species were found in this study. Species richness of *P. ponderosa* and *P. menziesii* was also compared between native western North American forests and plantations in Argentina by Barroetaveña *et al.* (2007)(Barroetaveña *et al.*, 2007). The comparison showed that species richness in plantations was much lower (18 taxa for *P. ponderosa* and 15 for *P. menziesii*) than in native forests (157 taxa in *P. ponderosa* and 514 for *P. menziesii*). The low species richness in these plantations in Patagonia can partially be explained by the fact that pine plantations there occur in first rotations on grasslands with typical vesicular-arbuscular mycorrhizal (VAM) flora lacking ECM fungi compatible with pines. This is in contrast to Kaingaroa sites which are currently in the third rotation of radiata pine. Another reason for the low richness might be the age and history of *P. ponderosa* plantations in Argentina, as these are young and the first plantation in this country was started in 1920, which is similar to the situation in New Zealand. A large number of first rotation forests in New Zealand in the late 1980s and 1990s were also planted on grasslands. No data on ECM associates for these first rotation sites is available (G. Ridley, personal communication, 7 November 2007). This study on ECM of a *P. radiata* plantation, and the results of Barroetaveña *et al.* (2007), show that ECM species richness of an introduced plantation species is lower than native systems. Thus, as radiata pine in New Zealand is an introduced species with a young history is likely that this is the main reason that ECM species richness is low.

Being a plantation forest means the investigated system is a monoculture, consisting of the same plant cohort in each stand, whereas in native forests, multiple ECM host species with a mixed age class structure are likely to be present. ECM species richness increases with the number of hosts present (Ishida *et al.*, 2007), hence, more ECM fungal species are likely to be found in native systems. Soil and microclimatic conditions are likely to vary from the native forests to the plantation and may not be suitable for some ECM species. For this reason some ECM species present in natural forests might not be able to grow in exotic plantations. *Pinus radiata* plantations in New Zealand have been planted using seedlings from nurseries with a low diversity of ECM. In contrast, in native forests seeds germinate in soil with a high diversity of ECM. Once a plantation is established, new ECM species may arrive and come into the system through new inoculum sources, such as neighbouring plantations of a different species (for example *Pseudotsuga menziesii* in New Zealand) or as new associations with native ECM species. The age of a plantation forest compared to native forests can contribute

to the difference in species richness. Species richness values of natives cited earlier are from systems that are at least 100 yrs of age (400 yrs in Smith *et al*. 2007), whereas exotic plantation trees in New Zealand never reach their full potential age. In the case of *P. radiata* in New Zealand, trees are harvested at an age of 25-30 yrs, therefore only a short period of the total potential life of the tree has been assessed. Also, certain forestry practices, such as clearcutting, reduce ECM species richness and fruit body production (Jones *et al.*, 2003; Heinonsalo, 2004; Durall *et al.*, 2006). This was also seen in this study. This drastic disturbance which reduces the carbon source for ECM fungi is not usually a factor in the native forests.

Finally, the low species richness found in this present study could be related to the duration of the study and the frequency of sampling. Sporocarp production and composition are known to be irregular and vary annually with changing weather patterns (Vogt *et al.*, 1992; Durall *et al.*, 2006). The dependency of sporocarp fruiting patterns on climatic conditions was not a main focus of this study, however, climate data was made available and plotted against the monthly sporocarp abundance in the assessment in 2006. A general pattern was obvious; sporocarp production was high in autumn and progressively declined with falling temperatures. However, not all species are triggered to fruit by the same exogenous factors. *Thelephora terrestris* and *Hebeloma* sp. for example were more abundant in the colder months of late autumn. In contrast, *Lactarius rufus* was prevalent in the month of May, following a fortnight of constant rain. The influence of moisture and temperature on the fruit body production of ECM fungi was also observed by Durall *et al.* (2006). Exogenous environmental factors trigger sexual reproduction of fungi, but these factors vary between species and some species produce sporocarps very irregularly (Gardes & Bruns, 1996; Kranabetter & Wylie, 1998). Due to this variability the characterization of an ECM community requires long-term site monitoring (Durall *et al.*, 2006). Sporocarp assessments in this two year long study were performed over four months of the year in two to three weekly intervals, and irregular or infrequent fruiting species may have been missed with this approach.

ABOVEGROUND SPECIES DIVERSITY AND EVENNESS

Overall, the low diversity indices in exotic plantation forests can be explained by the same factors as discussed for species richness.

Shannon and Simpson diversity indices were calculated and total values for the composite data from all sites were 1.80 and 0.79, respectively (ranging from 0.37 to 1.14 and 0.26 to 0.55, respectively). The maximum value possible for the Shannon index is around five and for the Simpson index one, indicating that the exotic *P. radiata* system at Kaingaroa Forest has a low diversity, especially in the early phases in the nursery and the young plantation forests. Similar low values were found in a study on species diversity of an exotic *Pinus taeda*

plantation in Brazil, where stands of one, nine and 18 yrs of age were assessed over a period of 12 months (Giachini *et al.*, 2004). The total Shannon value for the study was 0.84, which is less than observed in this study. The higher Shannon value for the Kaingaroa sites is probably due to the inclusion of the nursery stage and the later stage of the plantation (oldest stand was 27 yrs of age), which contributed to the increased total species richness and hence diversity.

In comparison to the diversity values in exotic plantations, the values in native systems were much higher. For example the ECM diversity indices for an old growth *Quercus ilex* forest (170 yrs) in the Mediterranean basin in Europe were 4.48 for the Shannon index and 0.95 for the Simpson index (Richard *et al.*, 2004), close to the potential maximum for each index.

Rank-abundance curves are a way of visualising species richness and evenness and therefore diversity in an ecosystem and are widely used in ecological studies (Magurran, 1988; Begon *et al.*, 1998). Rank-abundance curves in the ectomycorrhizal literature are commonly used in belowground studies (Visser, 1995; Bigg, 2000; Erland & Taylor, 2002), but to the knowledge of the author no study based on sporocarp assessments has used rank-abundance curves. Rank-abundance curves from sporocarp ECM communities from all investigated sites were characterised by the dominance of one or two species in the site and in the case of the older plantation sites, many rare species. Rank-abundance curves from the nursery and the 2 yr old site are of the geometric series type, which is typical of recently disturbed habitats or of habitats that experience harsh climatic conditions (Magurran, 1988). In this case the resource (root tip) is captured and held by the first species to arrive; fast colonising species such as *R. rubescens* are more successful in this habitat. If the species arriving first do not use all of the resources, a part of the resource left for other species, but the remaining resource becomes smaller and smaller. This habitat is highly competitive, where slow colonising species are at a competitive disadvantage to the species that are able to colonise readily. Evenness increases in the 8, 15 and 26 yr old sites and these curves are of the lognormal type. In this type it is assumed that part of the resource is under competition between the species holding the resource and the newcomer, contrary to the geometric series where the resource is held by the first coloniser (Visser, 1995).

3.3.3 BELOWGROUND ECM DIVERSITY

BELOWGROUND SPECIES RICHNESS

Over the course of the three ECM soil core assessments a total of 19 distinct ECM types (unknown various not included) were detected from root tips in this study. As it is the first time that a study on the belowground diversity of ectomycorrhizal fungi of *P. radiata* spanning from seedling to harvest has been carried out, numbers for belowground ECM species richness of *P. radiata* cannot be compared to other studies on the same host. To

date, research on belowground ECM of *P. radiata* has mainly concentrated on the nursery or outplanting stage in plantations only (Duñabeitia *et al.*, 2004; Ortega *et al.*, 2004; Liu *et al.*, 2004; Chen *et al.*, 2006). No publications on the ECM root tip communities associated with *P. radiata* in its native range, California, have been found.

Our findings on the belowground ECM species richness for this exotic monoculture plantation system are lower than has been reported for other host species. For example Gebhardt *et al.* (2007) found 61 ECM types in a *Quercus rubra* chronosequence study in East Germany and Goodmann and Trofymow (1998) reported 69 ECM types from a study on old growth and mature *Pseudotsuga menziesii* forests in Canada. Similar numbers are reported when *Pinus* spp. are the host, for example Yamada & Katsuya (2001) reported 54 ECM taxa from *P. densiflora* seedlings and mature trees (45 yrs) in Japan and richness was even higher in a *P. sylvestris* forest (1- 62 yrs) in Sweden where 135 ECM types were found (Jonsson *et al.*, 1999). In all of these studies the host is a native species and the system a natural forest and not an exotic monoculture plantation as in our study. In natural forests other similar natural forests can act as a source of inoculum and increase diversity, whereas in monoculture, especially exotic, plantations, the inoculum is more limited to what is present in the plantation itself. Also in both studies other multiple host species were present in these forests. It is known that multiple host species influence the ECM fungal richness, for example Ishida *et al.* (2007) investigated eight host species in a mixed conifer broadleaf forest and reported up to 209 ECM morphotypes. This is an additive effect as multiple-host ECM species are more likely to be present in the system.

As plantations in New Zealand are young exotic systems this will also contribute to the low species richness found in this study. Relative to other mycorrhizal forests in Canada or the USA, the plantation forests in New Zealand are young, and were established around 1870 with seedlings originating from England and Australia (Weston, 1957; Bannister, 1973). As pointed out by Ishida *et al.* (2007), the time elapsed after the establishment of the host species is an important host effect on the ECM community as certain species need longer to establish themselves in a system. When the exotic seedlings were imported to New Zealand it is highly likely that the mycorrhizal associates were also imported. Based on numbers from similar systems, however, it seems likely that *P. radiata* in New Zealand is not yet associated with its full suite of mycorrhizal species. Except for *Cenococcum geophilum*, no native fungi were found associated with *P. radiata* in this study. This has been reported from other studies of exotic *Pinus* spp. plantations, illustrating that *P. radiata* may not be able to associate with native fungi, resulting in plant disappearance when co-introduced ECM fungi were lacking (Tedersoo *et al.*, 2007). The lack of native fungal associates in our study could either be because native species are not compatible with the exotic plantation tree, or that native species have not yet infiltrated the system.

Another reason for the lower species richness could be the harvesting age of *P. radiata* trees in New Zealand, which is around 25 – 30 yrs of plantation age. Species richness values from this study are not of a mature but rather a maturing forest and hence lower than values from studies cited previously on mature forests of 40 yrs or older (Visser, 1995). As stated in Kranabetter *et al.* (2005), managed forests typically only have few older stands because of the relatively short intervals planned for commercial stand rotations. This change in the forest age-class distribution can in turn be the reason for the reduction in the richness of ECM communities, depending upon the extent of ECM species reliant on mature forest conditions.

In addition to species richness, the Margalef's index was calculated as this species richness index also incorporates the total number of individuals (root tips colonised by ECM fungi). In our study, the Margalef's index ranged from 0.09 to 0.87, which are low values. The overall index for the total of the soil core assessments was 1.82. Comparison values for the Margalef's index were only found in Mah *et al.* (2001). In that study (Mah *et al.* 2001) values for hybrid spruce seedlings ranged from 0.59-1.07, which was higher than the values for seedlings in our site. This again reflects the low species richness in the radiata pine system, and as discussed previously, values are likely to be lower than in other systems as the focus of this study is a young, introduced, monoculture plantation system.

BELOWGROUND SPECIES DIVERSITY AND EVENNESS

Compared to studies of mature forests the maximum diversity value for the *P. radiata* plantation system in New Zealand was very low. In this study, the maximum diversity was recorded in the 27 yr old site, Shannon diversity was 1.31 and 0.66 for Simpson, evenness was 0.75. In Yamada & Katsuya's (2006) study on a 45 yr old *Pinus densiflora* forest in Japan the Shannon diversity index ranged from 2.04 to 2.59. Gebhardt *et al.* (2007) found a Shannon diversity of 1.83 in a 21 yr old *Quercus rubra* forest, which increased to 2.8 in the 43 yr old forest, evenness values were 0.69 and 0.83, respectively. Only in Korkama *et al.*'s (2006) study of *Picea abies* clones were similar values to the present study found, the maximum Shannon diversity was 1.43 and evenness ranged from 0.75 to 0.79. In their study the experimental set up consisted exclusively of *P. abies*, similar to the monoculture situation in the present study whereas in the other cited studies other ECM hosts were present. Again, the influence of multiple ECM hosts could explain a higher diversity (Ishida *et al.*, 2007).

Although diversity in this study is on the lower side of the range of the potential diversity for ECM systems, the values for evenness were high and correspond to those found in the literature. In our study about five ECM types were highly ranked overall, the rest of the ECM types were rare. This high number of rare species is a common pattern described for almost all forest ecosystems. Characteristically, a belowground ECM community consists of a few common species, colonising about 50-70% of the fine roots, and a large number of rarer species (Gardes & Bruns, 1996; Gehring *et al.*, 1998).

Diversity values were lowest in the youngest plantation site investigated (K04-2, 2 yrs old). The drop in diversity compared to the previous age group, can be explained by the fact that this site was harvested by clearcutting in 2000. It is known that clearcutting results in a substantial reduction in ECM fungal diversity (Kranabetter & Wylie, 1998; Jones *et al.*, 2003; Palfner *et al.*, 2005; Luoma *et al.*, 2006). Compared to other studies on the effect of clearcutting on ECM diversity of outplanted seedlings (Hagerman *et al.*, 1999; Mah *et al.*, 2001), ECM diversity in *P. radiata* in New Zealand is low. In Kaingaroa Timberlands it is common practise to leave the site fallow for one year after clearcutting and to then replant it in the following year. It has been shown that after clearcutting the remaining stumps and dying roots are able to support living hyphae of ectomycorrhizal fungi for up to two years (Hagerman *et al.*, 1999; Luoma *et al.*, 2006), hence it is expected that enough viable inoculum was present in the outplanting site in Kaingaroa Forest. The low diversity observed in this study is therefore somewhat surprising but in turn does reflect the overall low ECM diversity and species richness associated with *P. radiata* in New Zealand.

The rank-abundance curves from the belowground ECM communities in the nursery and the older plantation sites (8, 15 and 26 yrs) had a similar pattern, the species curve was relatively flat and represented the broken-stick type. Evenness increased from the 8, 15 to 26 yr old site, hence species rank-abundance curves progressively became flatter as the resource became shared more or less evenly between the species. In the one and two year old sites (K04-2 and K05F-1) however, a geometric rank-abundance curve was found, the curve was very steep and the evenness low with the community being dominated by one species. This kind of rank distribution is typical for communities in disturbed habitats. This pattern of species rank-abundance curves in outplanted sites was also observed by Visser (1995) in a *Pinus banksia* stand. Older sites in their study (41-122 yrs) followed a log-normal distribution, which is the form of distribution that follows the broken-stick and is characteristic of mature systems (Magurran, 1988).

TEMPORAL PARTITIONING OF BELOWGROUND ECM COMMUNITIES

Seasonality is one of the factors that is suspected to influence species composition of the ECM colonising root tips. It is known to occur in soil bacteria communities, however there is little evidence for temporal partitioning within ECM fungal communities (Koide *et al.* 2007 b). In this present study, the belowground communities of ECM fungi of *P. radiata* from the summer and autumn assessments were compared.

Even though more ECM species/types were observed in the autumn assessment, the diversity indices did not vary significantly between the assessments. However, species composition and abundance of dominant species varied between the summer and autumn collections. Koide *et al.* (2007 b) investigated the frequencies and occurrences of hyphae and ECM root tips of a *Pinus resinosa* plantation (65 yr old) over a thirteen-month period and found similar

results. No clear evidence for temporal partitioning within ECM root tips was found, but two distinct patterns in relative frequencies of the species groups were obvious, with one group having a consistent frequency and the second group varying depending on time of the year. Buée *et al.* (2005) monitored the diversity in a beech forest (*Fagus silvatica*) over the course of a year and found that the species structure of the ECM community changed depending on the season, temperature and soil moisture. However both cited studies were not over a long enough timeframe to draw firm conclusions.

In the present study, the increase in total species richness can be partially explained with the increased quality of data for the third assessment, as the author's skill to distinguish morphotypes increased. Other reasons for the increase and the change in frequencies may be due to changes in soil conditions, such as temperature and moisture, as optimum growing conditions are species specific (Buée *et al.* 2005, Koide *et al.* 2007 b and references therein). Also a change in root growth and the presence of colonisable roots for ECM fungal species could be a determining factor (Koide *et al.* 2007 b and references therein), as SCA 3 was conducted after SCA 2, the abundance of roots available for colonisation likely increased over the summer, leading to the increase in ECM types that were observed belowground. As roots are produced under a variety of exogenous factors, different cohorts of roots may be colonized by different groups of fungi, depending on their tolerance to the conditions under which the roots become available. The precise seasonal effect on ECM species composition cannot be determined from this data, as only one season each was sampled. The data however does indicate that the season and the associated changes in soil conditions and host root growth could influence species richness, composition and frequency and therefore warrants further investigation.

3.3.4 ABOVE-VERSUS BELOWGROUND ECM DIVERSITY

The ECM communities in a *Pinus radiata* nursery and plantation forest in New Zealand was examined and the species composition above- and belowground was compared. It has been reported in the ECM literature that there is no or only little correlation between ECM species fruiting aboveground and colonising the hosts' root tips belowground (Gardes & Bruns, 1996; Peter *et al.*, 2001). No study to date has investigated the correlation between the above-belowground species composition in an exotic plantation in New Zealand.

ABOVE – AND BELOWGROUND SPECIES RICHNESS AND DIVERSITY

The overall species richness of the above- and belowground ECM communities were similar. Across all stand ages, 18 ECM taxa were detected during the aboveground assessment of ECM sporocarps, whereas 19 ECM types/species were found to colonise root tips of *P. radiata*. The mean species richness of the above- and belowground community for each stand age only varied significantly in the 2 yr and 8 yr old plantation sites where more species were found aboveground.

The literature describes conflicting results for above- *versus* belowground fungal diversity in plantations. A six year long study on the above- and belowground ECM communities in a 100 yr old *Picea abies* forest in Sweden by Dahlberg *et al*. (1997) found 48 ECM species as sporocarps and 22 ECM species belowground. On a per annum basis, 18-26 species were observed as sporocarps. ECM root tips were only sampled once within the study (compared with 26 sporocarp surveys) with the 22 ECM species identified representing species richness on a per annum basis. Consequently, species richness of the *P. abies* system on a per annum basis is, like in the present study, similar above- and belowground. Other studies however showed different results. Species richness was higher aboveground in studies comparing above- and belowground species diversity by Yamada *et al*. (2001) on a native *Pinus densiflora* forest (45 yrs old) and by Peter *et al*. (2001) on a *Picea abies* forest in Switzerland. In the first case 40 species were found above and 28 species belowground, in the latter study 128 and 79 ECM species, respectively. Other studies again report the opposite, namely higher species richness belowground. Gardes & Bruns (1996) reported ten species aboveground from a 40 yr old *Pinus muricata* system and twice as many species belowground. Gehring *et al.* (1998) investigated species richness in a mature *Pinus sylvestris* forest in the Northern USA and identified 22 ECM species as sporocarps and 51 ECM types, a study by Jonsson *et al*. (1999) on the same species, but in Sweden, reported 66 species above- and 135 belowground.

From this literature no clear trend is apparent. The variation in species richness above- and belowground between the cited studies could be attributed to several factors. The duration of the study, sampling designs and frequency of sampling are the most likely factors. As the study by Dahlberg *et al.* (1997) demonstrated, more species are likely to be found as sporocarps the longer the study lasts, because the probability of sampling during climatic conditions favourable for fruiting increases as the length of time for the study increases. Of the 48 species identified as sporocarps, only 21 species were found within a single assessment, illustrating the infrequency in fruiting patterns of some species. It is known that many species have irregular fruiting patterns, as they need specific exogenous environmental factors to trigger sexual reproduction, due to this irregularity long-term site monitoring is needed to fully characterise the aboveground fungal community (Vogt *et al.*, 1992; Durall *et al.*, 2006). Duration of sampling is not the only important factor, frequency of sampling is another one affecting species richness, as the fruit body formation in most fungal species is known to be limited to a few days or weeks during the year and to be an unpredictable phenomenon (Guidot *et al.*, 2003).

The Margalef's species richness index was significantly lower belowground in the 2 yr and 8 yr old plantation site, concurrent with the species richness. This is not surprising for the 2 yr old site, as this was the clearcut site. Seedlings in this site were in the process of establishing on site and especially in the first year, developing their root system that had been trimmed

prior to the outplanting. In this case, and due to the age of the seedling, carbon provision from the host was limited and some ECM species are likely to have used saprotrophic means of food acquisition instead of colonising the root tips. In the case of the 8 yr old plantation site, the saprotrophic ability of some species could be a possible explanation for the higher species richness aboveground. Also, *Lycoperdon* spp. were found aboveground in this site and the ECM status of these with *P. radiata* is unclear. Although the richness in the nursery was higher belowground, the Margalef's index was lower than aboveground. The Margalef's index takes richness and abundance into account; 176 specimens were counted in the sporocarp assessment, whereas 9705 ECM root tips were analysed in the soil core assessment, which explains the discrepancy between the richness and Margalef's index for the assessments in this site. The total Margalef's index did not differ significantly between the above- and belowground assessments.

Similar to the results of the species richness, the Shannon and Simpson diversity indices did not differ greatly but were slightly higher in the aboveground communities. Only in the nursery site was a significant difference found between the above- and belowground diversity indices, with diversity lower aboveground. As discussed above for the Margalef's index, the diversity indices are not only influenced by the species found, but also the respective abundance. Considerably more ECM root tips than sporocarps were analysed in the nursery, which would greatly influence the diversity value.

ABOVE- AND BELOWGROUND ABUNDANCE

There was little correlation in the total relative abundance between the above- and belowground ECM communities. Only seven species were found both, above- and belowground and furthermore the dominant species aboveground were not present in the belowground ECM communities. This striking lack of shared species between the above – and belowground communities was also observed by Peter *et al*. (2001) and Gehring *et al*. (1998), where only 22% and 14% of species, respectively, were observed in both environments. Durall *et al.* (1999) also reported only a weak correlation between mycorrhizae and sporocarps in stands less than 20 yrs old (*Pinus contorta* and *Tsuga heterophylla*), which is a similar maximum stand age to the present work (oldest stand 26 yrs old).

Species found belowground, but not producing sporocarps in this study were *Cenococcum geophilum, Pseudotomentella sp., Pseudotomentella tristis, Rhizopogon luteorubescens, Tomentella* sp*., Tuber* sp. and a group of unknown ECM types. There are several reasons for this finding. Species such as *Tuber* spp. or *Rhizopogon* spp. form hypogeous sporocarps, yet this study did not specifically search for these types of fruiting bodies. Also, for some species a sexual state is unknown, as it is the case for *Cenoccocum geophilum* (LoBuglio *et al*., 1996), hence they will not be found in aboveground assessments. Other species form inconspicuous sporocarps, which are easily overlooked and therefore potentially missed in

sporocarp surveys. For example, thelephoroid or corticoid Basidiomycota such as the *Tomentella* sp. and *Pseudotomentella* spp. collected in this study produce inconspicuous sporocarps only (Visser, 1995; Gardes & Bruns, 1996; Kårén *et al.*, 1997; Kõljalg *et al.*, 2000). ECM types unknown Basidiomycete, unknown 2, 8, 9 19 and 12 could not be matched to RFLPs from collected sporocarps or identified by direct sequencing of the ITS region. These species could either be native ECM fungi or new ECM fungi that are not yet present in the available databases. At this stage these species identities remain unknown, but it shows that additional species are found when assessing the belowground ECM communities with the aid of molecular methods.

Species found above- but not belowground were *Chalciporus piperatus, Laccaria proxima, Inocybe lacera, I. sindonia, Lactarius rufus, Lycoperdon gunnii, Lycoperdon* sp.*, Rhizopogon luteolus, Scleroderma bovista, Suillus* sp. and *Tricholoma* sp. Abundant fruiters, such as *L. rufus*, are likely to additionally access saprotrophic sources of carbohydrates, hence there is no need for them to colonise the root tips in abundance (Gardes & Bruns, 1996; Iwanski & Rudawska, 2007). In the present study *L. rufus* was frequently observed growing on decaying wood. The unresolved mycorrhizal status of *Lycoperdon* spp. with radiata pine, as mentioned earlier may also explain why it was not detected belowground. In the case of *L. proxima* the lack of mycorrhizae belowground was also observed by Chu-Chou & Grace (1987, 1988) in *Pseudotsuga menziesii* nurseries and *P. radiata* plantations in New Zealand. The species was found in large numbers in their study sites, but it was not isolated from mycorrhizae samples. However, this also may be due to a poor recovery rate during re-isolation from *L. proxima* mycorrhizae (Chu-Chou & Grace, 1987).

Other studies often rarely detected the species that are commonly found fruiting on the belowground roots (Gardes & Bruns, 1996; Kranabetter & Wylie, 1998), however in this study they were not found at all in the belowground assessment. This was also reported from Peter *et al.* (2001) in their study on the above- and belowground community structure of ECM fungi in *Picea abies*. Peter *et al.* (2001) attributed this to the sampling strategy of the belowground diversity, which could have also been an issue in the present study. ECM fungi are known to have a clumped and patchy distribution, horizontally as well as vertically (Kranabetter & Wylie, 1998; Gagne *et al.*, 2006). In this study, soil cores were taken randomly within the survey plot, but at a constant distance from the tree (60 cm). Species with a clumped or non-uniform distribution around the stem are likely to be missed in this sampling approach. A sampling scheme based on a gradient away from the host tree with more samples collected may enable more species to be detected. ECM species can also differ in their preference for the organic and mineral soil layers (Taylor & Bruns, 1999; Dickie *et al.*, 2002). In a study in a mixed coniferous forest on the vertical distribution of ECM species (Rosling *et al.*, 2003), 15 out of the 25 identified ECM taxa were exclusively found in the mineral soil, amongst these *Suillus luteus* and *Lactarius utilis*. Sampling depth in the present study was 40 cm, given the

potential rooting depth of pines (1.5 – 2 m, Rosling *et al.*, 2003); some species may only colonise roots found deeper in the soil and thus missed. In addition, seasonality of ECM species colonising root tips is another potential factor for the discrepancy, as soil cores were only sampled once in autumn and summer, although belowground seasonality has not been scientifically proven to date. The varying efficiency of nutrient acquisition by different mycorrhizal species could also explain missing the root tips of abundant fruiters. Some species may be very efficient at acquiring nutrients from its host and only need to colonize few root tips to develop a large biomass of fruit bodies (Gardes & Bruns, 1996).

ABOVE- AND BELOWGROUND SPECIES COMPOSITION

Species abundance was depended on stand age, with only the nursery site sharing the same abundant species above- and belowground. In the plantation sites in Kaingaroa Forest there was a great difference in species composition and the respective abundance between the above- and belowground communities.

Some ECM species are able to additionally access saprotrophic sources of carbohydrates and may fruit abundantly even though the belowground biomass is small (Gardes & Bruns, 1996). In the nursery the only carbohydrate source is the seedling itself, as the nursery is free of decaying logs or leaf and needle debris, which may explain that only ECM species present on the root tips are actually found fruiting. The only species not found during the sporocarp assessments was *Tuber* sp. This ascomycete produces hypogeous fruiting bodies, which were not specifically searched for during this study. In studies of Chu-Chou & Grace (1987) on hypogeous fungi in radiata pine plantations in New Zealand, a *Tuber* sp. (not identified to species level) was frequently found in nurseries as it was searched for by examining the seedling root systems and surrounding soil. Alternatively, soil and microclimatic conditions may not favour fruiting of this species in this specific nursery.

The 15 yr old site in this study was thinned in 2002 and it is likely that this affected the above- and belowground ECM species. Species richness was low in the sporocarp assessment (five species) with *Inocybe lacera* being the most abundant species. In comparison species richness belowground did not decline (10 ECM species/types) and the ECM community was dominated by *Amanita muscaria* and *Cenoccocum geophilum*. A study by Shaw *et al.* (2003) on the effects of thinning on ECM communities of *Pinus sylvestris* found that thinning does not affect the sporocarp production in the long term, but an initial response to thinning can be expected. This is in contrast to our observations, the site was thinned in 2002, and our first observation started in 2005, three years after the thinning. In the study by Shaw *et al.* (2003) all brush was removed from the experimental area, whereas in our study site the brush remained in the site. This made it more difficult to find sporocarps. The thinning also increased the space and light available in the site and favoured the growth of weeds like *Rubus fruticosus* and *Pteridium esculentum*. These factors may have changed the

microclimate and soil conditions within this site, which in turn influenced the fruiting abundance of the ECM species.

ABOVE- AND BELOWGROUND RANK ABUNDANCE CURVES

Rank-abundance curves for the investigated study sites differed for all sites between the above- and belowground assessments, except for site K04-2, the two year old site. Rank-abundance curves for the belowground ECM communities do not correlate to their aboveground counterparts, except for the two year old site. In the nursery and the older plantation sites (8, 15 and 26 yrs) the species curve for the belowground ECM was relatively flat and represented the broken-stick type. Evenness increased through the 8 to 15 to 26 yr old site, hence species rank-abundance curves progressively became flatter as the resource was shared more evenly between the species. The difference between the trends in the rank-abundance curves was due to the broken stick model being concerned with just one resource. Belowground the ECM fungi can only use the root tips to colonise and receive the carbon through the host plant whereas aboveground ECM fungi can utilise other means of carbon sources like plant debris, as discussed earlier. In the two year old site however a geometric rank-abundance curve was found in the belowground community, as seen aboveground, reflecting the fact that the seedlings are in a stressed and disturbed environment with seedlings subject to 'transplantation shock' and are planting in a habitat that has been disturbed by clearcutting.

3.3.5 METHODOLOGY LIMITATIONS

SPOROCARP ASSESSMENTS

As recorded in this and other studies (Vogt *et al.*, 1992; Dahlberg *et al.*, 1997; Durall *et al.*, 2006), sporocarp fruiting patterns vary between species and are irregular. The factors that trigger sporulation vary annually and are influenced by weather patterns. In order to assess sporocarp diversity, sites have to be monitored regularly over the long-term. In this PhD study, sporocarp fruiting patterns were assessed for two sampling seasons; however, a longer term study would provide a better indication of the factors regulating sporocarp production. Aboveground ECM studies are an integral part of ECM community and ecology studies, but there is little correlation between above- and belowground ECM communities. Therefore to accurately estimate ECM diversity of a system it is important to sample above and belowground. Even if time is a limiting factor, aboveground assessments should always be included in ECM studies.

SOIL CORE ASSESSMENTS

In the first soil core assessment 20 cores were collected at each site in one sampling effort. When processing the samples, it became apparent that the number of samples collected was too high, thus only a sub sample of the collected samples was actually processed (21 cores in

total). Furthermore, the extended storage of samples complicated identification and PCR amplification because the samples became degraded in storage. Over the course of the study the number of samples collected was reduced to eight soil cores per site, and one site was sampled at each sample collection time. After collection, soilcores were processed immediately and not stored for more than 10 days at 4°C. A recommendation missing in the present literature on ECM studies is that samples must be processed quickly, especially when dealing with DNA fingerprinting methods. Ideally samples should be processed as soon as possible as fresh material is preferred for DNA extraction. Samples should also not be stored for longer than one to two weeks, otherwise material can be infected by other soil fungi and degrade, which reduces the quality of DNA extracted.

Soil cores were taken at a constant distance of 60 cm from randomly selected trees. This approach was utilized in order to sample a large enough variety across the site. Yet this did not take the fungal niche partitioning along distance gradients into account; certain species are only found either proximal or distal to the tree (Bruns, 1995; Dickie & Reich, 2005). A future recommendation would be to take several samples from randomly selected trees at different distances from the tree.

MOLECULAR ANALYSIS

Both sporocarp and ECM colonising root tips were identified using a combined morphological and molecular approach. RFLP patterns were used to identify ECM species and morphotypes, based on comparisons with RFLP patterns generated from sporocarp samples. Whilst this is a fast approach to group unknown ECM species it has some disadvantages. RFLP pattern comparison to sporocarps left several ECM types unidentified. In this study, only two ECM types were matched positively to a species name, a further three were tentatively matched and ten ECM types could not be matched. This was partially due to the lack of RFLP reference material to compare the unknowns to as well as the fact that RFLP patterns showed variations and background signals. In the case of *A. muscaria, Inocybe* sp., *R. rubescens, R. pseudoroseolus* and *T. terrestris*, where both sporocarp and ECM RFLP patterns were available, additional fragments were found in the ECM RFLPs with the enzymes *Hin*fI and *Mbo*I. This might be because the ECM DNA was extracted from mixed material (root and fungus) which may have resulted in some cross contamination in the PCR amplification. Even though only single root tips were used for DNA extraction, colonisation of a root tip by multiple ECM or contamination from saprobic fungi cannot be excluded, which could also cause problems in the RFLP analysis. The enzyme *Alu*I was found to be unsatisfactory for the analysis as it gave rise to many partial digests and was not robust for species discrimination. This partial digest was surprising as the enzyme was fresh, however, an explanation could be that the batch was faulty. A more plausible explanation could be an inhibitory effect on the enzyme by the elution solution used during the PCR cleanup process (supplied with

GenElute™ PCR Clean-Up Kit, Sigma Aldrich, Missouri, USA) which is a different buffer and pH to that recommended for *AluI* activity. The supplied and recommended buffer was used for the digestion with *AluI* however this solution could be modified by compounds present in the PCR elution solution. Even though samples were processed as fast as possible and stored at 4°C, additional steps to reduce the risk of contamination and the resulting contamination of the DNA might be necessary. Martin (2007) suggested the application of ethanol in incremental steps to 100% ethanol may help to decrease solubility of DNA and slow degradation or storage in detergents.

Even though RFLP analysis did not resolve species identities of most unknown ECM types, it is a strong tool to group ECM types and, combined with cloning and direct sequencing, it works well for ECM community analysis. Methods such as T-RFLP (terminal restriction fragment length polymorphism) or LH-PCR (length heterogeneity PCR) are newer methods developed for mycorrhizal community analysis (Martin, 2007). These are rapid and more reliable techniques than RFLP alone; however they are more expensive and were not obtainable for this research.

In this study, the most effective method of species identification was direct sequencing of ITS PCR products. With the recent drop in the cost of sequence analysis this approach for species identification is becoming more feasible for community studies and in future studies, RFLP analysis would be omitted in favour of direct sequencing.

3.3.6 CONCLUSION

As demonstrated by this and the cited studies, it is necessary to investigate the above- and belowground species to assess overall ECM species diversity of an ecosystem. In this study species richness of the exotic *P. radiata* stand was found to be lower than has been reported for native coniferous forests in the northern hemisphere, yet similar to the richness observed in an exotic plantation in Argentina. Several factors, including the relatively young age of *P. radiata* plantations and the fact that the tree was imported into New Zealand with a limited suite of ectomycorrhizal associates are likely to be responsible for this. Combining the above- and belowground assessments, 28 ECM taxa were identified as ECM species associated with *P. radiata* in a North Island plantation in New Zealand. When the diversity values of the above- and belowground environments were compared, only seven species were common to both environments, furthermore the dominant species aboveground were not observed in the belowground ECM communities. In the aboveground study, five species were found associated with *P. radiata* that were previously not reported with this host in New Zealand (*Inocybe sindonia, Lactarius rufus, Lycoperdon gunii, Rhizopogon pseudoroseolus* and *Wilcoxina mikolae*). Belowground, the species *Pseudotomentella* sp., *P. tristis, R. luteorubescens, Tomentella* sp., *Wilcoxina mikolae* were found as new associates of *P. radiata* in New Zealand. Additionally, nine ECM types were found that could not be identified with

molecular analysis. Of the species that were observed, only *Cenococcum geophilum* has been reported as a potential native fungus.

Diversity is defined as the richness and evenness of a group of species in a specific environment (Begon *et al.*, 1998). Values such as species richness, frequency, species diversity indices and evenness numerically describe the diversity of a community in an ecosystem and were used to portray the within-site (α-) diversity of the stands that were investigated. In this study it became apparent that the driving factors for the α-diversity of the fungal symbionts are factors such as host specificity, host origin, source of inoculum, plantation forest age, temporal partitioning and disturbance. Species diversity of an ecosystem, however, is not only driven by the within-site factors but also by the larger scale factors, which describe the between-site (β-) diversity, such as stand age. As stand age may drive changes in species composition both above and belowground, the relationship between stand age and ECM species composition are investigated in Chapter 4.

4 ECM COMMUNITIES ASSOCIATED WITH DIFFERENT AGE CLASSES OF *PINUS RADIATA*

4.1 INTRODUCTION

Chapter 3 assessed the overall diversity of ECM communities associated with *Pinus radiata* in a plantation in New Zealand and small scale factors driving the α-diversity were discussed. A large range of factors determines which fungal species will associate with a given host. This chapter will assess and discuss host age, which is hypothesised to influence between-site diversity (β-diversity). It has been hypothesised that the composition of ECM communities is correlated with host age and that a succession of ECM species occurs over the lifetime of the host (Visser, 1995).

Succession is defined as the 'non-seasonal, directional and continuous pattern of colonisation, and extinction on a site by species populations' (Begon *et al.*, 1998). The concept of mycorrhizal succession originated from studies of mycorrhizal fruit body production observed during the growth of a mixed birch stand situated on agricultural soil in Scotland (Mason *et al.*, 1982, 1983). Based on these studies it has been postulated that the species richness of ECM fungi increases until canopy closure and then decreases as host physiology and forest floor organic matter changes (Dighton & Mason, 1984; Last *et al.*, 1987). More recent studies (Visser, 1995; Kranabetter *et al.*, 2005) do not support the theory that some species can only colonise the host tree roots early, while some others can colonize only in later growth stages of the trees (Heinonsalo, 2004).

Studies of mycorrhizal succession in New Zealand by Chu-Chou & Grace (Chu-Chou, 1979; Chu-Chou, 1980; Chu-Chou & Grace, 1988; Chu-Chou & Grace, 1990) on *Pinus radiata* plantations were conducted from the 1970s to mid 1990s. Their research included nurseries and plantation forests in the North and South Island and they observed successional patterns, primarily by sporocarp surveys. With the application of DNA fingerprinting methods for ECM species identification in succession studies of ECM fungi, it became evident that there is little correlation between the above- and belowground ECM communities (Visser, 1995). In regards to succession this raises the questions 'Have only sporocarp fruiting patterns been observed?' and 'Can successional patterns be observed on root tips?'.

Typically, the analysis of temporal changes of mycorrhizal root communities in natural ecosystems is difficult because the plants supporting the fungal symbionts are normally mixed in age and species composition. In this study, a monoculture plantation in which all trees of a

cohort were established synchronously and all cohorts occurred within a short distance of one another on the same soil type was used, thus eliminating confounding factors which could interfere with investigating the effect of the tree host age on the ECM community structure. The aim of this chapter was to assess ECM communities of different age groups of *Pinus radiata* plantations that were investigated in Chapter 3, both above- and belowground, to determine if there are discriminating species for each age class both above and below ground. The hypotheses investigated were (*i*) the ECM fungal species richness and diversity changes with stand age; (*ii*) the ECM fungal composition changes with stand age.

4.2 RESULTS

ECM communities of a *Pinus radiata* chronosequence were assessed in 2006 in Te Ngae nursery and Kaingaroa Forest. The stand ages investigated were nursery (K06), 2 yrs (K04-2-2), 8 yrs (K98-8-8), 15 yrs (K91-15) and 26 yrs (K80-26) after outplanting. The results of this chapter are based on sporocarp assessment (SA 2) and belowground assessment (SCA 3) and were performed as outlined in Chapter 2. For both, sporocarps and ECM fungi colonising root tips, species identification was accomplished with a combined morphological approach, as outlined in Chapter 3 and Appendices 4 and 5.

4.2.1 CHANGE IN SPECIES RICHNESS AND DIVERSITY OVER TIME

The species richness and diversity indices of the nursery, 1, 2, 8, 15 and 26 yr old stands were assessed above- and belowground in 2006 and the following trends were observed: (*i*) α-diversity: aboveground species richness increased as stand age increased, while belowground richness decreased from the nursery to the two year old stand and increased thereafter; (*ii*) β-diversity: aboveground the 8, 15 and 26 yr old stands were similar, while belowground, two different species compositions were observed: the nursery and 1 and 2 yr old stand had similar species composition, while the 8-, 15-, and 26 yr old stands had similar species composition; (*iii*) aboveground diversity increased from the nursery to the 15 year old stand, belowground diversity decreased from the nursery to the one year old stand and increased thereafter; (*iv*) species richness was higher aboveground, whereas species diversity was higher belowground.

SPECIES RICHNESS

Aboveground, richness increased with increasing age class (Table 4-1a). Richness almost doubled between site K04-2 (2.8) and K98-8-8 (6.2) and richness was significantly different between the sites ($F=22.95$, $p<0.0001$). The Tukey's test indicated that the nursery and K04-2 were significantly different from sites K98-8 and K80-26. In contrast to species richness, Margalef's index decreased from the nursery to site K04-2. The index increased to

more than double in site K91-15 and did not change much thereafter. The one-way ANOVA showed that there was a significant difference ($p<0.0001$) between sites in the Margalef's index, with the Tukey's test showing that the index in the nursery and K04-2 site were significantly lower compared with the K98-8, 91-15 and K80-26 sites.

Belowground, richness decreased form the nursery to the first two years of outplanting in Kaingaroa forest (K05F-1 and K04-2) (Table 4-1b). Richness increased progressively thereafter in the older plantation sites to the maximum value in site K80-26 (26 yrs), which was almost twice the richness observed in the nursery. The one-way ANOVA showed that the species richness was significantly different ($F=8.22$, $p<0.0001$) between sites. The Tukey's test indicated that K80-26 was significantly different from the nursery, K05F-1, K04-2 and K98-8 sites. Margalef's index showed the same trend as the richness values. The maximum value of 0.87 was observed in site K80-26. This value was approximately 2.5 times higher than the value calculated for the nursery. The Tukey's test indicated that the nursery, K05F-1 and K04-2 sites were significantly different ($p<0.0001$) from the K91-15 and K80-26 sites.

Table 4-1: Total and mean species richness and mean Margalef's index for the study sites investigated during sporocarp assessment 2 (Table a) and soil core assessment 3 (Table b) in 2006 (± standard deviation over the mean). F- and p-values derived from one-way ANOVA, means with the same letter are not significantly different to each other (Tukey's Test).

a)

Aboveground	**Species richness**			**Margalef's index**	
	Total	Mean (± SD)	Tukey's test	Mean (± SD)	Tukey's test
Nursery (n=5)	3	2.6 (± 0.89)	a	0.53 (± 0.04)	a
K04-2 (n=5)	4	2.8 (± 0.45)	a	0.39 (± 0.08)	a
K98-8 (n=5)	9	6.2 (± 0.84)	b	0.96 (± 0.12)	b
K91-15 (n=2)	6	5.0 (± 0.00)	a, b	1.00 (± 0.06)	b
K80-26 (n=5)	10	6.8 (± 1.30)	b	0.94 (± 0.21)	b
	F = 22.95 p < 0.0001		LSD = 0.89	F = 21.18 p < 0.0001	LSD = 0.13

n=number of transects sampled

b)

Belowground	**Species richness**			**Margalef's index**	
	Total	Mean (±SD)	Tukey's test	Mean (±SD)	Tukey's test
Nursery (n=8)	5	3.38 (± 0.74)	a, b	0.34 (± 0.10)	a
K05F-1 (n=8)	5	2.50 (± 1.20)	a, b	0.25 (± 0.15)	a
K04-2 (n=8)	3	2.00 (± 0.58)	a	0.23 (± 0.15)	a
K98-8 (n=8)	10	3.88 (± 1.55)	a	0.55 (± 0.27)	a, b
K91-15 (n=8)	10	4.38 (± 1.85)	b, c	0.77 (± 0.35)	b
K80-26 (n=8)	13	6.00 (± 1.77)	c	0.87 (± 0.28)	b
	F = 8.22 p < 0.0001		LSD = 1.39	F = 10.43 p < 0.0001	LSD = 0.24

n=number of soil cores sampled

SPECIES DIVERSITY INDICES

The β-diversity of the sites was measured with the Sorensen diversity index. The Sorensen similarity coefficient was calculated based on presence/absence data and the results are presented in Table 4-2. The index ranges from 0, if the sites are dissimilar and have no species in common to 1 in cases of complete similarity. In the aboveground study, the nursery site was not very similar to the K04-2 site and had no species in common with the K98-8, K91-15 and K80-26 sites. The next age class, the K04-2 site was not very similar to any of the other sites. The K98-8, K91-15 and K80-26 sites had a high similarity and most species were common to all sites. Belowground, the nursery and K05F-1 site were identical to each other and both shared 50% of species with the K04-2 site but were different to the K98-8, K91-15 and K80-26 sites. The K04-2 site was not similar to the K98-8, K91-15 and K80-26 sites and only shared a few species with these. The K98-8, K91-15 and K80-26 sites had, as in the aboveground assessment, a high similarity, with even more species being shared belowground.

Table 4-2: Matrix for Sorensen's index of all study sites investigated in sporocarp assessment 2 (Table a) and soil core survey 3 (Table b) in 2006.

a)

	Nursery	**K04-2**	**K98-8**	**K91-15**	**K80-26**
Nursery	1	0.29	0	0	0
K04-2	0.29	1	0.29	0.20	0.29
K98-8	0	0.29	1	0.63	0.70
K91-15	0	0.20	0.63	1	0.63
K80-26	0	0.29	0.70	0.63	1

b)

	Nursery	**K05F-1**	**K04-2**	**K98-8**	**K91-15**	**K80-26**
Nursery	1	1	0.50	0	0	0
K05F	1	1	0.50	0	0	0
K04	0.50	0.50	1	0.15	0.15	0.13
K98	0	0	0.15	1	0.70	0.78
K91	0	0	0.15	0.70	1	0.78
K80	0	0	0.13	0.78	0.78	1

The α-diversity of each site was measured with the Shannon and Simpson diversity index (Chapter 3) and these indices were compared here using a one-way ANOVA to assess differences between the stands. The indices are based on abundance data and listed in Table 4-3. The Shannon diversity index calculated for the aboveground assessment increased gradually from the nursery to the maximum value in site K91-15 (15 yrs), which was three times higher than in the nursery. The index was slightly lower in the oldest site investigated, K80-26. There was a significant difference ($F=9.09$, $p<0.0001$) between the young (0-8 yr) and older (8-26 yr) sites (Tukey's test). The Simpson index for the aboveground diversity did increase as well, however it remained the same in the nursery and the second year of outplanting (site K04-2). As with the Shannon diversity, the Simpson diversity values were highest in site K91-15 and slightly lower in the oldest site, K80-26. As with the Shannon index, the Simpson index was a significantly different ($F=5.45$, $p=0.006$) between the young (0-8 yr) and older (8-26 yr) sites (Tukey's test).

Diversity values were higher in the belowground assessment and the trends between the sites were different as well. Both the Shannon and Simpson diversity index decreased from the nursery to the first year of outplanting in site K05F-1 but increased thereafter. The highest value of both indices was calculated for site K80-26 (26 yrs), the oldest site that was assessed. The belowground nursery values were comparatively higher and almost as high as in site K91-15 (15 yrs). The diversity indices were significantly different between the sites (Table 4-3). The Shannon diversity ($F=8.3$, $p<0.0001$) of the one and two year old sites were significantly different to those of the 15 and 26 yr old sites. The Simpson diversity index however ($F=6.21$, $p<0.0001$) differed significantly between the 1 yr old and the 8, 15 and 26 yr old sites.

Table 4-3: Mean Shannon and Simpson diversity indices for all study sites investigated in sporocarp assessment 2 (Table a) and soil core assessment 3 (Table b) in 2006 (± standard deviation of the mean). F- and p-values derived from one-way ANOVA, means with the same letter are not significantly different to each other (Tukey's Test).

a)

Aboveground	**Shannon diversity index**		**Simpson diversity index**	
	Mean (± SD)	Tukey's test	Mean (± SD)	Tukey's test
Nursery (n=5)	0.37 (± 0.28)	a	0.26 (± 0.17)	a
K04-2 (n=5)	0.46 (± 0.14)	a	0.26 (± 0.09)	a
K98-8 (n=5)	0.83 (± 0.28)	a, b	0.41 (± 0.17)	a, b
K91-15 (n=2)	1.14 (± 0.06)	b	0.58 (± 0.03)	b
K80-26 (n=5)	1.05 (± 0.21)	b	0.55 (± 0.08)	b
	F = 9.09 p < 0.0001	LSD = 0.23	F = 5.45 p = 0.006	LSD = 0.13

n=number of transects sampled

b)

Belowground	**Shannon diversity index**		**Simpson diversity index**	
	Mean (± SD)	Tukey's test	Mean (± SD)	Tukey's test
Nursery (n=8)	0.88 (± 0.33)	a, b	0.50 (± 0.19)	a, b
K05F-1 (n=8)	0.41 (± 0.32)	a	0.25 (± 0.20)	a
K04-2 (n=8)	0.50 (± 0.31)	a	0.33 (± 0.21)	a, b
K98-8 (n=8)	0.98 (± 0.23)	b, c	0.56 (± 0.09)	b
K91-15 (n=8)	1.10 (± 0.51)	c	0.59 (± 0.23)	b
K80-26 (n=8)	1.31 (± 0.26)	c	0.66 (± 0.09)	b
	F = 8.30 p< 0.0001	LSD = 0.34	F = 6.21 p < 0.0001	LSD = 0.18

n=number of soil cores sampled

4.2.2 ECM COMMUNITY ANALYSIS AND DISCRIMINATING SPECIES

The above- and belowground relative abundance and species composition of the nursery, 1, 2, 8, 15 and 26 yrs old stands in Kaingaroa Forest were analysed. A hierarchical cluster and a MDS analysis as well as a similarity analysis were performed and discriminating species for each age group or clustering were determined. Trends were as follows: (*i*) one species dominated in each age group aboveground, whereas belowground the relative abundance was more evenly spread; (*ii*) the hierarchical clustering grouped ECM communities aboveground according to their respective stand age, whereas belowground two distinct groups were observed: nursery, 1 and 2 yr old stand and 8, 15 and 26 yr old stand; (*iii*) discriminating species were found for all groups as defined by hierarchical clustering above- and belowground. Based on these groups, species that were discriminating aboveground were found not to be of importance belowground and *vice versa*.

RELATIVE ABUNDANCE

The absolute and relative abundance of ECM sporocarps counted in assessment 2 in 2006 are presented in Table A7-1 Appendix 7. Tables A7-2 to A7-4 in Appendix 7 present the absolute and relative abundance of ECM types in the three soil core assessments from 2005 to 2006. The relative abundance of ECM species, with abundance greater than 2%, is illustrated for the aboveground (Figure 4-1a) and belowground communities (Figure 4-1b).

The overall trend in the aboveground ECM communities for each age group was that only one species dominated and had a high relative abundance in the respective site. This was *Rhizopogon rubescens* in the nursery, *Inocybe* sp. in K04-2 and K91-15, *Laccaria proxima* in K98-8 and *Lactarius rufus* in K80-26 (Figure 4.1). The relative abundance of all dominant species of the aboveground assessment were significantly different between the investigated age groups, with the exception of *Inocybe* sp. ($F=1.69$, $p=0.198$) (Table 4-4).

In contrast to the aboveground ECM communities, relative abundance belowground was distributed more evenly amongst several species in the investigated age groups, the nursery, K98-8, K91-15 and K80-26 and no discriminating species could be determined (Figure 4.1). However, in the early outplanting sites K05F-1 and K04-2, one species dominated the ECM community at each site. In K05F-1 it was *Wilcoxina mikolae,* in site K04-2 it was *R. rubescens*. Overall the relative abundances of *Hebeloma* sp., *R. rubescens, W. mikolae* and ECM type unknown Basidiomycete were significantly different between the investigated age groups, but the remainder of the species were not significantly different between sites (Table 4-4).

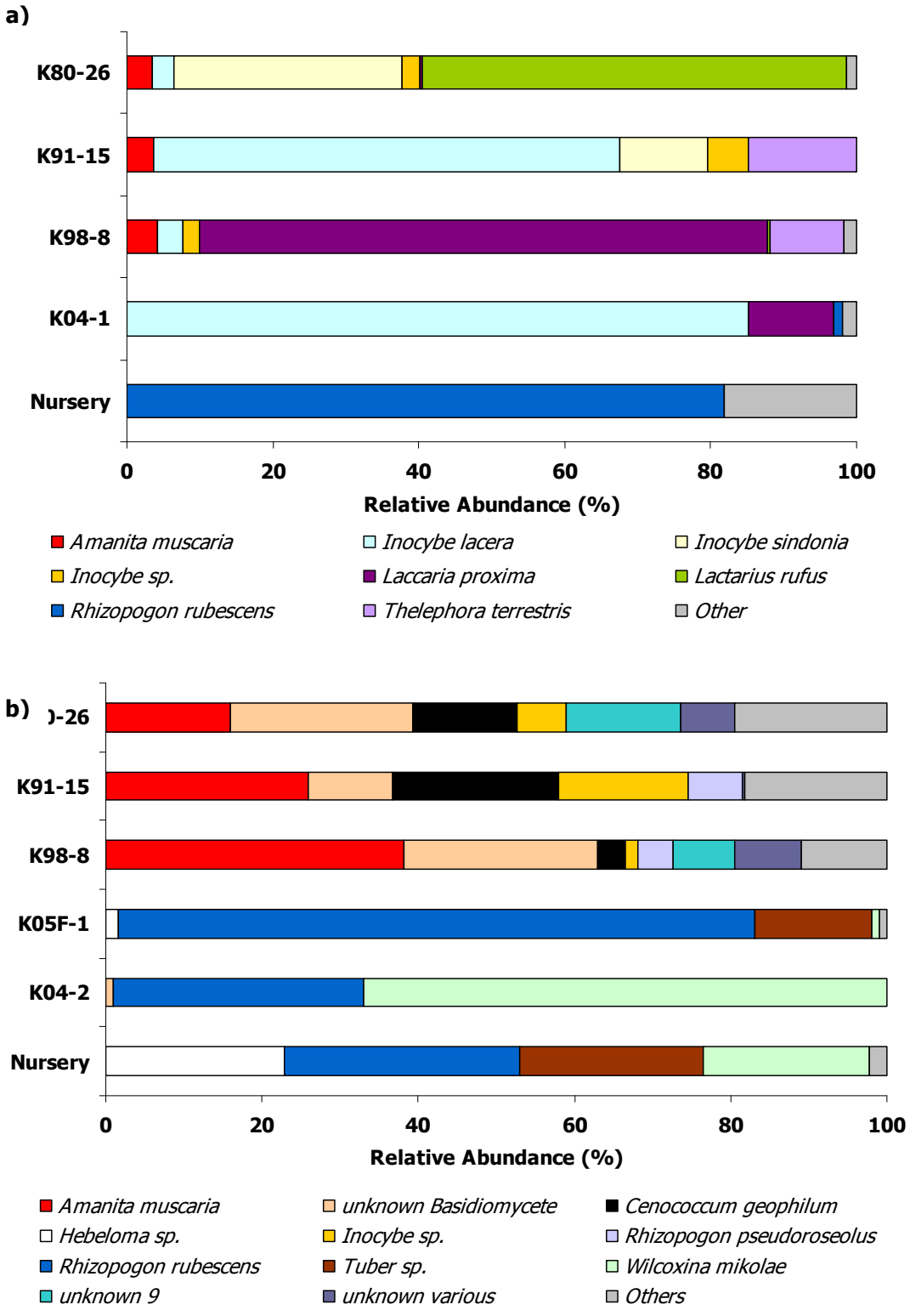

Figure 4-1: Relative abundance of the overall highest contributing above- and belowground ECM species (2% relative abundance cut off percentage) Figure a) sporocarp assessment 2 in 2006, sites assessed were nursery, K04-2, K98-8, K91-15 and K80-26. Figure b) soil core assessment 3 in 2006, sites assessed were nursery, K05F-1, K04-2, K98-8, K91-15 and K80-26.

Table 4-4: One-way ANOVA results of overall higher contributing ECM species between sites for each species in the aboveground (sporocarp assessment 2) and belowground (soil core assessment 3) communities.

Species	Sporocarp assessment 2		Soil core assessment 3	
Amanita muscaria	F=6.13	p=0.003	F=2.05	p=0.091
Cenococcum geophilum	n/a		F=1.09	P=0.379
Hebeloma sp.	n/a		F=8.04	p<0.0001
Inocybe sp.	F=1.69	p=0.198	F=2.37	p=0.056
Inocybe lacera	F=15.84	p<0.0001	n/a	
Inocybe sindonia	F=7.10	p=0.001	n/a	
Laccaria proxima	F=11.60	p<0.0001	n/a	
Lactarius rufus	F=10.80	P<0.0001	n/a	
Rhizopogon rubescens	F=9.53	p<0.0001	F=5.21	p=0.001
Rhizopogon pseudoroseolus	n/a		F=2.28	p=0.064
Thelephora terrestris	F=4.57	p=0.011	n/a	
Tuber sp.	n/a		F=2.14	p=0.079
Wilcoxina mikolae	n/a		F=9.20	p<0.0001
Unknown Basidiomycete	n/a		F=7.84	p<0.0001
Unknown 9	n/a		F=1.89	p=0.116
Unknown various	n/a		F=2.13	p=0.08

n/a=not applicable

CLUSTER ANALYSIS AND MDS ORDINATION

In order to study species assemblages in the above- and belowground ECM communities at the investigated study sites, hierarchical clustering and non-metric multidimensional scaling (MDS) was performed. The hypothesis was that each sampled age group had a unique ECM fungal species assemblage and species composition within those assemblages changed over time as host age increased.

The hierarchical clustering based on a Bray-Curtis similarity matrix of the aboveground ECM assessment in 2006 is illustrated in Figure 4-2. The clustering technique grouped ECM communities/transects according to their sample sites at the arbitrary similarity level of 47%. All stands exhibited stand-specific ECM fungal communities and no overlap in groups was detected and the similarity in the ECM communities decreased as tree age increased. The belowground cluster analysis of the assessment in 2006 is illustrated in Figure 4-3. In contrast to the aboveground analysis, the belowground ECM communities were not grouped relative to sample sites. Two distinct clusters were obvious which were grouped at the arbitrary similarity level of 2%. Cluster one consisted of ECM communities sampled from the

nursery, K05F-1 and K04-2, whereas cluster two was made up of the communities from K91-15, K98-8 and K80-26 sites. Between these two clusters no overlap in communities was observed. Within both clusters, no further distinction based on sample sites was possible because species composition of the sites overlapped and were therefore not site specific.

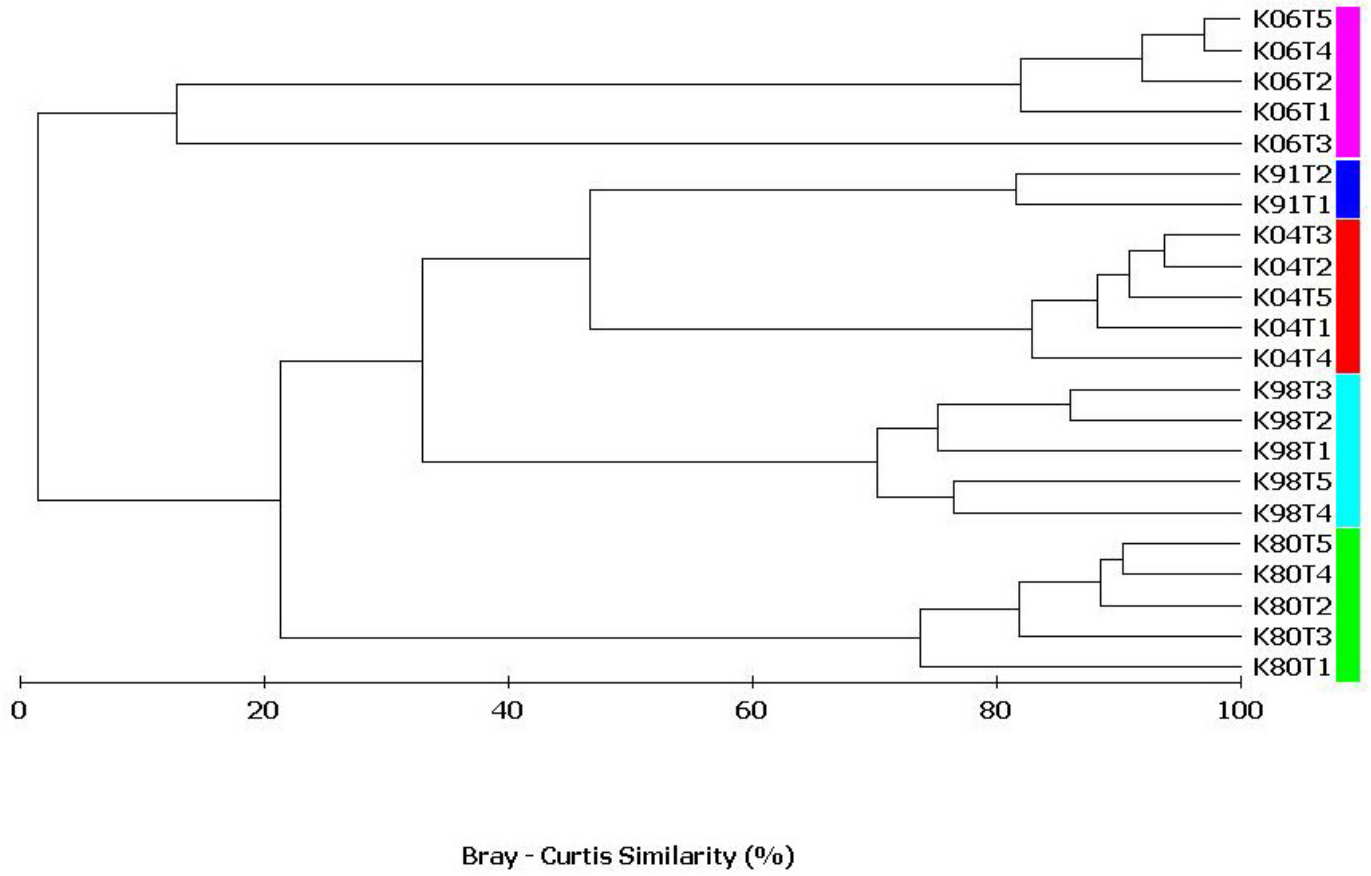

Figure 4-2: Dendrogram for hierarchical clustering (using group-average linking) of five (two for K91-15) replicate transects from each of sites K06 (nursery), K04-2, K91-15, K98-8 and K80-26 from sporocarp survey 2 in 2006, based on Bray-Curtis similarity. Colour coding: nursery – pink; K04-2 – red; K98-8 – turquoise; K91-15 – blue; K80-26 – green.

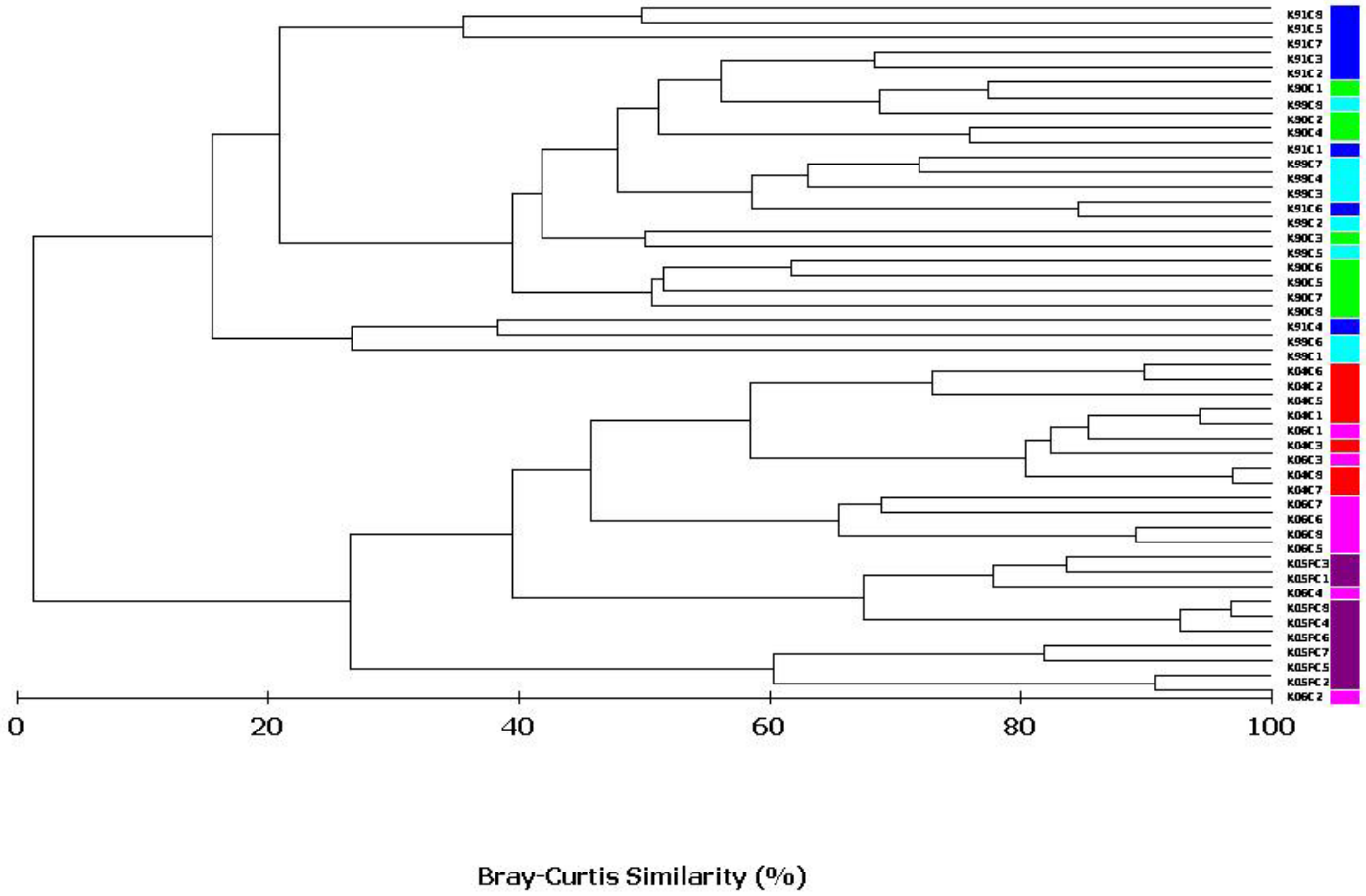

Figure 4-3: Dendrogram for hierarchical clustering (using group-average linking) of eight replicate soil cores from each of sites K06 (nursery), K05F-1, K04-2, K91-15, K98-8 and K80-26 from soil core survey 3 in 2006, based on Bray-Curtis similarity. Colour coding: nursery – pink; K05F-1 – purple; K04-2 – red; K98-8 – turquoise; K91-15 – blue; K80-26 – green.

The MDS ordination of the ECM communities from the above- and belowground assessment is illustrated in Figure 4-4. The MDS ordination for the aboveground has a stress level of 0.02, which is an excellent representation (Figure 4-4a)(Clarke & Warwick, 2001). As with the cluster analysis, the ECM communities of a sample site were grouped together. No overlap in ECM communities was found and the similarity between the communities decreased with stand age. The stress level for the belowground MDS ordination was 0.07, representing a good ordination (Figure 4-4b). Again, the trend for the belowground ECM communities was as seen in the cluster analysis, two distinct groups were apparent, group nursery/K05F-1/K04-2 and group K91-15/K98-8/K80-26. Within these groups, communities from the sampled stands overlapped.

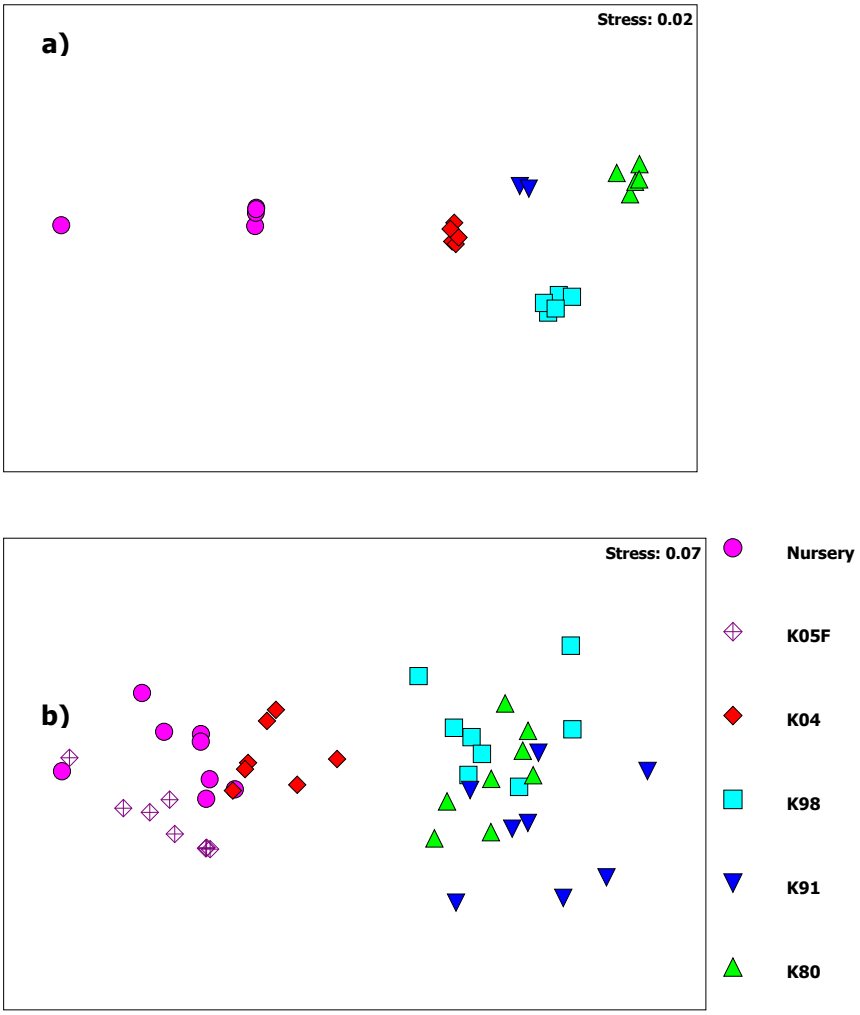

Figure 4-4: MDS ordination of the above- (a) and belowground ECM communities (b), data square root transformed and based on Bray-Curtis similarity. Figure a) ordination of five (two for K91-15) replicate transects from nursery, K04-2, K91-15, K98-8 and K80-26 sites from soil core survey 3 in 2006. Figure b) ordination of eight replicate soil cores from nursery, K05F-1, K04-2, K91-15, K98-8 and K80-26 sites from soil core survey 2 in 2006.

SIMILARITY ANALYSIS OF ECM COMMUNITIES AND DETERMINATION OF DISCRIMINATING SPECIES

The SIMPER routine within the PRIMER statistical package was used to analyse the similarity within sites and to determine which species were discriminating species within the above- and belowground ECM communities. This analysis indicates which species are responsible for the observed clustering patterns in the hierarchical clustering and MDS analysis. The ratio similarity/standard deviation (Sim/SD) identifies the discriminating species of a site. If the ratio is high, a species contributes much to the similarity within a group and does so consistently in inter-comparisons of all samples.

Table 4-5 presents the similarity analysis of the aboveground ECM communities based on sampling sites; these groups were confirmed through the hierarchical clustering and MDS analysis. Aboveground, each site, with the exception of K91-15, was found to have a different discriminating species. In the nursery, *Rhizopogon rubescens* had the highest average abundance, but *Wilcoxina mikolae* was the discriminating species for this site as it had the highest Sim/SD ratio and was found in all transects. Site K04-2 had the highest within site similarity (80%) and its discriminating species was *Inocybe lacera*. In site K98-8, *Laccaria proxima* had the highest overall average abundance and was the discriminating species. For site K91-15, no discriminating species could be determined with the SIMPER analysis, as only two transects were analysed; however, *Inocybe lacera* had the highest average abundance and similarity over the two transects. In the oldest site that was assessed, K80-26, *Amanita muscaria* was the discriminating species, *Lactarius rufus* had the highest abundance, but was not as evenly distributed over the site.

Table 4-5: Similarity analysis of the aboveground ECM communities from sporocarp assessment 2 in 2006. Sites sampled: nursery, K04-2, K91-15, K91-15 and K80-26, with five transects in each site (K91-15 – 2 transects). Listed are the higher contributing species only (90% contribution cut off percentage) with the discriminating species underlined.

	Average Abundance	Average Similarity	Sim/SD	Contribution (%)
Nursery (n=5) Average similarity: 59.64				
Rhizopogon rubescens	28.8	28.54	1.16	41.13
Wilcoxina mikolae	1.0	25.84	3.62	37.24
Hebeloma sp.	5.4	15.01	1.16	21.63
K04-2 (n=5) Average similarity: 79.8%				
Inocybe lacera	90.6	48.16	14.67	58.95
Laccaria proxima	12.40	28.57	8.81	34.97
K98-8 (n=5) Average similarity: 68.44%				
Laccaria proxima	193.2	27.13	10.62	38.00
Thelephora terrestris	25.0	14.60	3.27	20.44
Amanita muscaria	10.4	13.60	7.76	19.05
Inocybe lacera	8.6	11.69	6.51	16.37
K91-15 (n=2) Average similarity: 76.1				
Inocybe lacera	34.5	27.75	n/a	35.56
Thelephora terrestris	8.0	18.88	n/a	24.19
Inocybe sindonia	6.5	17.06	n/a	21.86
Amanita muscaria	2.0	14.35	n/a	18.38
K80-26 (n=5) Average similarity: 72.14%				
Lactarius rufus	316.8	23.90	8.05	30.68
Inocybe sindonia	170.4	18.74	5.71	24.06
Inocybe lacera	16.6	11.51	7.77	14.77
Amanita muscaria	18.8	11.40	14.16	14.63
Inocybe sp.	12.8	5.50	1.09	7.06

n = number of transects; Sim/SD = Similarity/Standard deviation; n/a = not applicable as only 2 transects analysed.

For the belowground ECM communities, the SIMPER analysis was based on clusters as defined through the hierarchical clustering and MDS analysis (Table 4-6), as well as a SIMPER analysis based on sample sites (Appendix 11). Cluster 1 included the nursery site, K04-2 and K05F-1 whereas cluster 2 included sites K98-8, K91-15 and K80-26. Compared to the aboveground analysis, within site similarity was low (30 - 34% similarity belowground versus 60 – 80% similarity aboveground). For cluster 1, *Rhizopogon rubescens* was the discriminating species with the highest average abundance and similarity over all 24 soil cores collected from the nursery, K05F-1 and K04-2. For cluster 2, which comprised the older

plantation sites sampled (K98-8, K91-15 and K80-26), two species were found to be the discriminating species, ECM type unknown Basidiomycete and *Amanita muscaria*.

Table 4-6: Similarity analysis of the belowground ECM communities from soil core assessment 3 in 2006, clusters based on hierarchical clustering and MDS analysis. Cluster 1: nursery/K04-2/K05F-1, cluster 2: K98-8/K91-15/K80-26, with 24 soil cores analysed for each cluster. Listed are higher contributing species only (90% contribution cut off percentage). The discriminating species are underlined.

	Average Abundance	Average Similarity	Sim/SD	Contribution (%)
Cluster 1 (Nursery/K05F-1/K04-2) Average similarity: 34.14%				
Rhizopogon rubescens	348.00	17.56	0.91	51.43
Wilcoxina mikolae	126.61	10.32	0.69	30.22
Tuber sp.	136.52	3.80	0.42	11.14
Cluster 2 (K98-8/K91-15/K80-26) Average similarity: 30.25%				
unknown Basidiomycete	47.88	10.92	1.05	36.09
Amanita muscaria	55.58	8.65	0.92	28.61
Inocybe sp.	16.58	3.10	0.46	10.24
unknown various	13.63	2.06	0.39	6.82
Pseudotomentella sp.	7.17	1.41	0.37	4.66
Cenococcum geophilum	28.13	1.19	0.30	3.94

Sim/SD = Similarity/Standard deviation

4.3 DISCUSSION

In order to investigate the hypothesis that ECM fungal communities are correlated with host age and that a succession of ECM species occurs as the stand ages, the above- and belowground ECM communities of a *Pinus radiata* plantation were investigated within different age classes (nursery, 1, 2, 8, 15 and 26 yrs old stands, 1 yr old not included in aboveground assessment). Since the study was conducted in a plantation, the oldest age group that could be investigated was limited by the harvesting age, which is approximately 25 - 30 yrs of age in New Zealand. Compared to other international studies on successional patterns of ECM fungi (Visser, 1995; Goodman & Trofymow, 1998; Kranabetter *et al.*, 2005; Palfner *et al.*, 2005; Fernández-Toirán *et al.*, 2006; Gebhardt *et al.*, 2007; Twieg *et al.*, 2007; Smith *et al.*, 2007) the scale of investigated age groups in the present study is narrower and the oldest age group that was investigated was much lower.

4.3.1 ABOVE- AND BELOWGROUND SPECIES RICHNESS AND DIVERSITY INDICES

Aboveground, species richness increased from the nursery to the oldest age group investigated (26 yrs), with a slight decrease in the 15 yr old stand. The largest increase was between the two and eight year old stands. Aboveground, diversity indices increased over the chronosequence to reach its maximum in the 15 yr old stand and decreased slightly to the oldest age group. This study confirms the observations by Chu-Chou & Grace (1988, 1990) on the ECM species richness of radiata pine plantations in New Zealand, where considerably more species were found in stands over five years old compared to the nursery. Our results are also in agreement with the early work on ECM fungal succession by Dighton & Mason (1984) and Mason *et al.* 1987 which was based on sporocarp observations. These authors found an increase in species richness and diversity until canopy closure, which occurred around 27 yrs of forest age, the same age as the oldest stand sampled here. A six year long sporocarp succession study by Fernández-Toirán *et al.* (2006) on a *Pinus pinaster* chronosequence (0-10, 11-20, 21-40, 41-60, 61-90, 90 yrs) in central Spain found a clear effect of stand age on cumulative species richness per plot. Other studies on sporocarp succession on *Pinus* spp., *Tsuga* spp. and *Pseudotsuga menziesii* (Visser, 1995; Smith *et al.*, 2002; Kranabetter *et al.*, 2005) covered forests of an older age (up to 400 yrs) and age groups were subdivided on a broader scale. These studies found an increase in species diversity over the investigated chronosequence, with the highest increase appearing between the young (<10 yrs) stands and stands older than 40 yrs. In contrast to the theory of Dighton and Mason (1984) a decline in species richness after canopy closure was not observed in these studies.

The 15 yr old site, K91-15, showed the effects of thinning on ECM sporocarp diversity and frequency. Species richness dropped to five ECM taxa and overall 108 observations of ECM specimens were made in the sporocarp abundance count in 2006, the lowest count of all the sites observed in 2006. Due to the unforeseen growth of the under storey, especially *Pteridium aquilinum* (bracken fern) and *Rubus fruticosus* (blackberry), and the associated difficulty in safely accessing the site, sporocarps were counted along two transect lines only, resulting in the low total count. Extrapolation of the data to five transects would still result in the second to lowest count, half as much as what was counted in site K04-2 in 2006. As discussed in Chapter 3, it is likely that the remains of the thinning rubbish in the site may be the cause of the drop in sporocarp richness in this stand. The species richness increased in the 26 yr old stand, however diversity indices decreased. This was due to a considerably higher production of sporocarps in this stand, which influenced the diversity values.

Unlike aboveground, the belowground species richness initially decreased from the nursery to the one and two year old stands. Richness increased thereafter over the rest of the chronosequence. Diversity indices showed the same trend, however indices decreased only

from the nursery to the one year old stand and increased again. As aboveground, the biggest increase was observed between the two and eight year old stands. None of the cited studies includes a nursery stage in their observations, as in the present study. The decrease in diversity from the nursery to outplanting is investigated in depth in Chapter 5. The reason for a further reduction in species richness from the one to the two year old stands is mainly due to the disappearance of the nursery species *Hebeloma* sp. and Type unknown 2, presumably because conditions at the outplanting site are unfavourable and these species are not able to survive in the outplanting site.

As mentioned earlier, the maximum stand age studied was limited by the short rotation for radiata pine in New Zealand; comparable studies of belowground ECM succession that are recorded in the literature are of much older forests (Visser, 1995; Goodman & Trofymow, 1998; Palfner *et al.*, 2005; Gebhardt *et al.*, 2007; Twieg *et al.*, 2007). Host species in these studies include *Pseudotsuga menziesii, Quercus rubra, Picea sitchensis* and *Pinus banksiana*. The trend observed in the present study is in accordance with results from the cited studies; the belowground species diversity increased from young stands to the mature forest. In the present study, ECM species richness had not yet reached the climax stage and diversity had not plateaued off, as was the case in the cited studies of mature forests.

In this study, the highest increase in both, above- and belowground diversity was found from the two to the eight year old stands, thereafter diversity values increased aboveground until age 15 and belowground until age 26, in the latter only by minor increments, but diversity had still not plateaued off. Other studies have not investigate age groups on such a fine age scale as in the present study, but species richness generally increased rapidly in young stands, matching the pattern of root development and increasingly higher root density (Kranabetter & Friesen, 2002). Depending on the age groups investigated, diversity in other studies had the greatest increase between 5 and 26 yrs (with *P. menziesii*, Twieg *et al.* 2007), 5 and 21 yrs (Gebhardt *et al.*, 2007) and between 6 and 41 yrs (Visser, 1995), after around 30+ yrs the diversity generally levelled out. It is not possible to compare the observed steep increase in diversity in the early years of the forest to other studies because none have been done on such a fine scale. Seedlings were planted on a clearcut site, this forestry practise is known to reduce ECM species richness as well as the fruit body production (Jones *et al.*, 2003; Heinonsalo, 2004). In the present study there was a great difference between the two and the eight year old stands that were assessed. In the 8 yr old stand, there was an established understorey, leading to changes in soil characteristics which may facilitate the establishment of different fungal species.

Our results confirm the general trend observed in ECM succession studies of an increase in diversity after outplanting, supporting the hypothesis that ECM fungal species richness and diversity increase with stand age. The ECM species diversity of the *P. radiata* plantation in the

present study plateaued off at the oldest stand investigated, as it has not reached the peak stage. *Pinus radiata* grows extraordinarily well in New Zealand, due to the climate in this country the plantation tree is able to grow without a dormancy stage and all year round. This continuous growth means the trees mature faster, it also suggests that the trees have a high root turnover, indicating the trees might be 'physiologically older' than other radiata pine growing in their native range with a dormancy stage. If ECM species diversity and succession are directly related to host age, the ECM succession of the investigated forest should have reached its maximum under these circumstances. However, although the tree might grow and mature relatively quickly, the forest floor/soil does not necessarily undergo the same rapid development and might not have the same characteristics such as nutrient status or moisture content of a typical climax forest at canopy closure, suggesting that the soil conditions might have more influence on ECM succession than the actual tree 'age'.

4.3.2 ABOVEGROUND ECM COMMUNITIES RELATED TO HOST AGE

Aboveground, a clear sequence of ECM species and increasing complexity related to increasing stand age was observed. The nursery and younger stands had low species richness, low fruit body density and were dominated by early seral fungi such as *Hebeloma* sp., *Rhizopogon rubescens, Laccaria proxima* and *Inocybe* spp. This data is in agreement with international studies on sporocarp succession (Visser, 1995; Kranabetter *et al.,* 2005; Fernández-Toirán *et al.*, 2006). These early stage or pioneer species are successful in the young stands and the nursery due to their primary dispersal mechanisms by spores rather than living mycelia, which requires the attachment to a mature host (Kranabetter & Friesen, 2002). *Rhizopogon rubescens* for example is likely to be dispersed by distribution of their spores via wind or animal dispersal such as pigs or possums who have been feeding on the hypogeous sporocarps. Pioneer species are of the r-selective type, which is characterised by a rapid dispersal, high productivity, tolerance a of wide range of soil conditions and relatively small basidiomata (Visser, 1995; Frankland, 1998). The discriminating species for the nursery was the ascomycete *Wilcoxina mikolae,* whilst in the youngest stand in the plantation, the two year old stand, *Inocybe lacera* was the clear discriminating species. *Hebeloma* sp., *R. rubescens* and *W. mikolae* were only found in the nursery and two year old stand and not thereafter. The nursery species do not survive outplanting, probably due to changes in soil conditions, carbon supply by the host and competitive pressure from ECM fungi dominating in older stands (Danielson & Visser, 1989; Erland & Taylor, 2002).

According to the original successional theory of Dighton & Mason (1984) and Last *et al.* (1984), the early stage fungi are being replaced by taxa termed late stage fungi. Species composition and dominant ECM species from the eight year old stand changed in the present study, however, species categorised as early stage fungi, did persist next to species which

were only found in the older stands. For example, *Inocybe* sp. and *Laccaria proxima* were still present in the older stands. *Thelephora terrestris* has been categorised as an early stage fungus (Visser, 1995) however in the present study it was first observed in the eight year old stand. Obviously some species that are successful in colonising seedlings and young trees are persistent and competitive enough to survive in the more competitive environment of the older stages. Also these species must be able to tolerate a wider range of growing conditions to enable them to grow in the early stage of a forest as well in the older stage, where soil conditions have changed considerably (Simard *et al.*, 2002). This pattern of ECM succession being an accumulation of species adding richness to the ECM community, with only few species being completely replaced, has also been found in other ECM successional studies (Visser, 1995; Goodman & Trofymow, 1998), contrasting with the theory of early and late stage fungi.

Species categorised as late stage fungi, such as *Amanita muscaria* or *Lactarius rufus,* are K-selected species, characterised by being host-selective, slower growing and more dependent on energy from their hosts with larger, more persistent basidomata. The driving force behind succession patterns was thought to be the accumulation of nutrients rather than exhaustion of the food supply (Dighton & Mason, 1984). Fungi of the later stage in a succession need a larger supply of carbohydrates, particularly glucose, hence the need to be attached to a mature host (Frankland, 1992; Bigg, 2000). Species found exclusively in the later stage of a forest predominantly disperse by living mycelia, which requires the attachment to a mature host. Furthermore, these species are only found in the later stage of a forest, as they only persist when the fine root density is high enough to ensure a constant supply of new roots in close proximity to the fungus (Fleming *et al.*, 1984). Rather than colonisation being a function of root age, it is more likely that the persistence of these fungi required the higher rooting density found on older parts of the root system (Kranabetter & Friesen, 2002). Species exclusively found in the later stage of a forest are slower to enter the system, however once established they are highly competitive (Kranabetter & Friesen, 2002). Specific soil conditions, such as pH, nutrient status, temperature and/or moisture are required for successful colonisation and establishment by some species, thus limiting their dispersal in either the young or old stands of a forest. There is strong evidence that changes in the soil environment are just as important for successional patterns as colonisation capacity (Jones *et al.*, 2003; Kranabetter *et al.*, 2005).

The discriminating species for the 8 year old stand was *Laccaria proxima*, which dominated this stand. This age class is probably a transitional stage between young and old stands, as it was dominated by pioneer species, but late stage fungi such as *A. muscaria*, were also present. No discriminating species could be determined for the 15 yr old stand, due to the limited sampling that was possible in this thinned site. The most abundant species however was *Inocybe lacera*, a species found in young as well as in old stands. The 15 yr old stand is

somewhat of an exception in the chronosequence due to the impact of thinning on the stand, which decreased diversity, as discussed in Chapter 3. In the oldest stand investigated, *Amanita muscaria* was the discriminating species, followed by *Lactarius rufus*, both species are typical for older forests.

The second hypothesis, that ECM fungal species communities change with the age of the host, was supported for the aboveground ECM species associated with *P. radiata* plantations in New Zealand. However, it is unlikely that the successional pattern observed was only a function of host age, it is more likely to be a combination of fungal dispersal and colonisation potential and soil conditions as well as the age of the host. Climatic conditions also have a strong effect on sporocarp production and climatic conditions can potentially obscure a successional pattern observed in any given year (Fernández-Toirán *et al.*, 2006).

4.3.3 BELOWGROUND ECM COMMUNITIES RELATED TO HOST AGE

No change in ECM composition directly related to the age of the host was observed in this belowground assessment of the *P. radiata* chronosequence, but two distinct groups of ECM species were found – a young group and a plantation forest group. This trend is in contrast to what has been found in other international studies on mycorrhizal succession (Visser, 1995; Kranabetter *et al.*, 2005; Gebhardt *et al.*, 2007; Twieg *et al.*, 2007). In these studies a change in fungal communities associated with stand age was observed. Yet these studies investigated forests on a different age scale, generally the second oldest age class in these studies was equivalent to our oldest class or even older.

The young ECM communities consisted of species found in the nursery and the one and two year old stand. *Rhizopogon rubescens* and *Wilcoxina mikolae* were present and dominant in all three sites, the latter species being the most consistent over all three sites and soil cores sampled and hence the discriminating species for this group. The nursery and the one year old stand had the same ECM composition, *Hebeloma* sp., *Tuber* sp. and Type unknown 2 were also present. All species in this young group are considered to be pioneer species. Members of the Ascomycetes (*W. mikolae, Tuber* sp.) are known to be dominant in nurseries and clearcuts (Menkis *et al.*, 2005; Tedersoo *et al.*, 2006). Gebhardt *et al.* (2006) found *Tuber* sp. and another Ascomycete type prevailing in their youngest stand (5 yrs) in a *Quercus rubra* chronosequence. Danielson (1991) also found that Ascomycetes often dominate early stages of mycorrhizal succession. These species are r-selected types and can readily colonise seedlings in the nursery and outplanting from spores, but due to their low competitiveness tend to decrease in population after the outplanting phase (Iwanski *et al.*, 2006). *Hebeloma* sp. and *R. rubescens* are also known pioneer symbionts in the early stage of the forest; the latter suggested as being the most beneficial fungus for *P. radiata* seedlings (Duñabeitia *et al.*, 2004). *Rhizopogon rubescens* is able to tolerate a wider spectrum of soil

conditions and disturbance, hence its survival until the second year after outplanting. *Hebeloma* sp. however was only present in the first year after outplanting and disappeared thereafter, likely as it was not well suited to the changing soil conditions. Both species are rhizomorph forming and therefore long distance exploration types (Agerer, 2001), these features would have facilitated inoculation of host roots in the nursery and outplanting. The one and two year old stands were dominated by one species only (*R. rubescens* and *W. mikolae,* respectively) and the respective rank-abundance curves (Chapter 3) were of the geometric type, which is typical of disturbed sites (Magurran, 1988); disturbance in the present study was the outplanting of seedlings on a clearcut site. The stands from the young group were characterised by the same suite of fungal species being present, but also showed the effects of disturbance and outplanting.

The plantation forest group was made up of ECM species observed in the 8, 15 and 26 yr old stands. None of the species found in the young group was present in any of the soil cores collected from these older stands. The ECM composition of the plantation forest group was very similar (Sorensen's index between the stands was 0.78 and 0.70); only rare species were slightly different between the stands. In all three stands the relative abundance was evenly spread and rank-abundance curves for the stands (Chapter 3) were of the broken stick type, indicating the presence of a few dominant species and numerous rare species. This species rank-abundance type is typical for maturing stands (Magurran, 1988; Visser, 1995; Simard *et al.*, 2002). The next sequential type would be the log normal type, which was found in the study by Visser (1995) in *Pinus banksiana* stands older than 40 yrs, showing that these stands had reached the maturity and canopy closure stage. This comparison implies that even the oldest stand investigated in this study had not reached the maturity level but was still developing. Hence the ECM fungal similarity in these plantation forest stands is not surprising as the conditions found from the 8 to the 26 yr old stand favour the same type of ECM species.

The discriminating species for the plantation forest group were Type unknown Basidiomycete and *Amanita muscaria*. According to the early successional theories *A. muscaria* is a late stage fungus and only able to associate with mature hosts (Frankland, 1998), however in this study it was found to be associated with 8 yr old trees and was present in all subsequent stands. These observations conflict with the late stage theory and indicate that more factors than purely host age determine the presence or absence of an ECM species. *Cenococcum geophilum* was another abundant species in all stands. This species is ubiquitous and found associated with a wide range of host species, over a wide habitat range and is resistant to changes in pH and other soil conditions changes (Cairney & Chambers, 1999; Jany *et al.*, 2003; Menkis *et al.*, 2007). Less abundant species in the *P. radiata* stands that were assessed were members of the *Thelephoraceae*, *Rhizopogon* spp., *Inocybe* sp. and a group of unknown ECM types. As observed in the aboveground ECM communities earlier, species such

as *Thelephora terrestris* and *Inocybe* sp. were previously categorised as early stage fungi (Visser, 1995). None of these species were found associated with the seedlings in the nursery or the early outplanting years, and the presence of these species in all of the plantation stands does contradict the early – late stage theory based on the influence of the age of the host. It is more likely that these species are found in these sites because of their colonisation strategies and due to the soil conditions after the initial years after the outplanting. As covered in the aboveground discussion, colonisation strategies are another major factor determining the successful colonisation of root tips and explaining why a different suite of ECM fungi is found in the plantation forest. In the early stage of a forest or in the nursery, the dominant species colonise by spores or propagules and these species are successful in this environment because competition is low. Whereas species found in the later stage of a forest are generally better competitors but take longer to establish in an environment as their main way of colonising is by living mycelium, which requires the attachment to a living host. A further requirement for the survival of these species is a high enough root density for a constant supply of new root tips (Kranabetter & Friesen, 2002). In this situation with a higher root turnover the competitive pressure increases and species from the late stages, which are known to be good competitors, are more successful in colonising and persisting.

The soil conditions from the 8 to the 26 yr old stand were suitable for the suite of species found in the plantation forest group and obviously did not change too much to displace some species. However, a change in conditions from the first two years to the later stage in the forest is possibly another reason for the difference in ECM species in the two different groups found belowground. Jones *et al*. (2003) hypothesised that changing environmental conditions are a major factor in the change in ECM community structure. The clearcut site had changed from a disturbed site with no vegetation growth to a forest with a developed root system, an understorey and a developed organic layer. These factors will influence the soil conditions and other edaphic factors, and over the four years of development the soil density and pH of the site would have changed, as well as the nutrient status and water availability. All these factors in turn influence the growth potential of ECM fungal species and coupled with a higher root turnover and space available for colonisation, the result is increased competitive pressure between fungal species because the conditions within the plantation have significantly changed.

According to the hypothesis of Dighton & Mason (1984), the succession of fungi can be explained by changes in carbohydrate supply from the host tree and by an increase in the litter and humus layer in the forest. The change in the supply of carbohydrates might be caused by decrease of net photosynthesis, tree vitality or altered distribution of photosynthates over root and shoot; furthermore, the ECM might be affected by an increased internal recycling of nutrients as the trees age (Termorshuizen, 1991). Changes in nitrogen availability as the forest stand ages are caused by a depletion of nitrogen in the mineral soil

and an accumulation of nitrogen in the organic layer, accompanied by a redistribution of fine roots (and ECM) to the organic layer (Goodman & Trofymow, 1998). These explanations for succession are more likely to apply to forests older than the stands investigated in the present study. The driving forces behind the changes in ECM species communities in this *P. radiata* plantation are likely due to changes in edaphic conditions, colonisation strategies and competitive pressure in-between species.

For the belowground ECM communities, our second hypothesis that ECM species communities change with the age of the host, has to be rejected. The ECM communities clearly changed after the second year of the radiata pine seedlings being in the plantation; however the composition of the fungal symbionts did not change as the host matured. In this specific *P. radiata* monoculture plantation environment it does not seem appropriate to apply the mycorrhizal succession concept of early stage, late stage and multistage fungi, but to categorize species into nursery species, which survive the first few of years of outplanting and plantation forest species, which are present throughout the majority of the plantation forest lifetime. The clear change in ECM communities in the *P. radiata* plantation forest investigated in this study occurs between the two and eight yr old stands. It would be interesting to see at what point the ECM colonizing plantation forest trees become the dominant species in those six years that were not investigated and if this changeover in composition happens gradually or suddenly.

4.3.4 COMPARISON OF ABOVE- AND BELOWGROUND SUCCESSIONAL PATTERNS

As seen in this study and discussed in Chapter 3, the similarity between above-and belowground ECM community composition is low (Dahlberg & Stenström, 1991; Gardes & Bruns, 1996). Also apparent in this study was that the successional patterns observed in the aboveground ECM communities differed from the belowground successional patterns. Aboveground ECM communities changed with the host age and stands investigated and were dominated by one main species. The ECM species in the nursery differed from the plantation forests and within these early stage fungi were accompanied by species categorised as late stage fungi, but not replaced by the latter. Belowground ECM communities changed only between the two and eight year old stands, but not thereafter with the development of the host.

This difference in successional patterns is striking, but is possibly explained by exogenous factors that influence sporocarp fruiting patterns. Fernández-Toirán *et al.* (2006) found a clear effect of stand age on cumulative species richness per plot in a *Pinus pinaster* chronosequence study in Spain. But no effect was found between yearly species richness per plot and stand age, which is thought to be an effect of the presence of many species of fungi that infrequently produce sporocarps and strong climatic driven inter-annual variability. The

variability in fruiting patterns has been discussed earlier in Chapter 3 and it was pointed out that, in order to obtain a complete picture of ECM sporocarp diversity, forests have to be investigated over a long period. This study however covered only two sampling seasons and for this specific successional part of the research only the data of 2006 was used. The data presented here is a snapshot in time, and does not present the full picture of the sporocarp diversity and succession. Also, as covered in the previous chapter, several species either form inconspicuous sporocarps or do not fruit at all, hence the sporocarp assessment alone do not represent all ECM fungal species which are of importance for a host species and ecosystem.

Due to the limited time scale of this study, it was not possible to repeat the sampling in identical tree age classes at other sites or to repeat the sampling over several years. Therefore the results presented here shall be interpreted as a snapshot in time and space of the ectomycorrhizal communities associated with *Pinus radiata* in a plantation in New Zealand. However, the successional trends observed, aboveground ECM communities change with the age of the host whereas the belowground ECM communities only change between the very young stage and plantation forests. This suggests that the driving force behind successional patterns is not solely the age of the host. Aboveground succession can be obscured by fruiting patterns which are triggered by exogenous factors and overall the mechanism driving succession is likely a combination of host age, edaphic factors and the fungal colonisation and competitive success. As one of the clearest successional trends was the replacement of nursery species by forest species, Chapter 5 assesses the question of 'When do ECM species changes occur?' in more detail and follows up the fate of the nursery species and the incursion of non-nursery species in the first year of the outplanting in Kaingaroa Forest.

5 CHANGES IN ECM DIVERSITY FROM NURSERY TO OUTPLANTING

5.1 INTRODUCTION

Chapter 4 investigated the change in above- and belowground ECM fungal community composition in different age classes of *P. radiata*. While a clear change in species composition aboveground occurred between each stand age that was investigated, the species composition belowground was not observed to change after age eight. These results lead to the question of the survival of species from the nursery following outplanting and when species from the nursery are replaced by forest ECM fungi. The establishment and performance of outplanted seedlings is dependent on ECM fungi (Stenström & Ek, 1990; Duñabeitia *et al.*, 2004; Menkis *et al.*, 2007) as they facilitate the establishment through enhanced nutrient and water uptake and protection against pathogens. In situations where seedlings are outplanted on clearcut land, the soil is depauperate of ECM inoculum as the host tree is missing and the site has been highly disturbed through the previous logging process (Jones *et al.*, 2003). In this situation the mycorrhization of the seedlings at outplanting is beneficial for the tree establishment. Hence information about the presence and persistence of nursery ECM in the early stages of the outplanting is of importance for forestry practises such as seedling treatment and inoculation programs. It is essential that the species present in the nursery survive and facilitate the establishment of seedlings, as a lack of ECM is likely to lead to a failure of the trees in the plantation (Gilmour, 1958; Hall & Perley, 2006). Prior research on the change in ECM communities of inoculated and naturally colonising ECM species on *Pinus* spp. seedlings (Danielson & Visser, 1989; Dahlberg & Stenström, 1991; Menkis *et al.*, 2007) was in contrast to that observed in the present study. The prior studies showed that forest ECM entered the ECM communities soon after the outplanting and displaced the nursery ECM. These findings increase the need to answer the question of how long nursery ECM persisted on *P. radiata* seedlings in a New Zealand plantation with a specific focus on the first years of outplanting.

The objective of this chapter was to determine when the ECM fungi colonising roots of *P. radiata* in the nursery were displaced by other species. It was also of interest to determine if the ECM diversity and richness of the *P. radiata* seedlings decrease after the initial outplanting process and how it changed over the first year of being in the plantation. A further point of interest was to know how long the naturally colonising ECM species from the nursery persist and if they facilitate the establishment of the seedlings in the clearcut sites or if this role was taken over by forest ECM species. Also, it was relevant to know when the first forest ECM species occur and at what abundance. Therefore the species richness, diversity,

abundance and composition of the belowground ECM communities associated with *P. radiata* seedlings in the nursery and first year of outplanting were analysed, as well as the ECM communities of a two and eight year old plantation site.

5.2 RESULTS

Seedlings were outplanted in Kaingaroa Forest in June 2006 and ECM diversity was assessed monthly from August 2006 to February 2007, followed by a final assessment in June 2007 as outlined in Chapter 2. Furthermore ECM species diversity of sites planted in 1998 and in 2004 was assessed in 2005 and 2006. The ECM species diversity of *P. radiata* nursery seedlings from Te Ngae Nursery was assessed in May 2006 with eight seedlings collected and analysed as described in Chapter 2. Seedlings raised in this nursery were outplanted in site K06F-OS in Kaingaroa Forest in June 2006, three seedlings were randomly sampled from a 100 m x 100 m plot within the site and analysed monthly from this site from August 2006 to February 2007, with a final assessment in June 2007. To investigate the ECM species composition of recently established seedlings, three more sites (K04-2, K05F-1 and K98-8, as in Chapter 3) were investigated in June and December 2005 and June 2006 (K05F-1 only in June 2006). Site K04-2 in Kaingaroa Forest was planted in 2004 with seedlings which were raised at Te Ngae Nursery (Chapter 2) and represented the 1, 1 ½ and 2yr old stage of the host tree in the plantation at the time of sampling. Site K98-8 was planted in 1998 (Chapter 2) and represented the 7, 7 ½ and 8yr old stage of *P. radiata* at respective sampling time. Site K05F-1 was planted in 2005 and represented the 1 yr old stage.

ECM ROOT TIP IDENTIFICATION

ECM root tip identification was performed, based on a combined morphological and molecular approach, as outlined in Chapter 2. Over the course of this study, ten ECM species/types were identified (Table 5-1). Of these, seven have been identified previously and are described in Chapter 3 and Appendix 4. Three additional species were found during the monthly assessment of outplanted seedlings, ECM type *Pezizales* sp., unknown 13 and unknown 14. For a detailed description of these species, RFLP patterns, and/or sequence results refer to Appendix 4.

Table 5-1: ECM species/types found in the soil core assessment in May 2006 in the nursery and in the monthly assessments of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007.

	Nursery	Plantation site K06F-OS							
ECM species/type	**May**	**August**	**September**	**October**	**November**	**December**	**January**	**February**	**June**
Amanita muscaria									+
Hebeloma sp.	+		+	+	+	+	+	+	+
Pezizales sp.					+	+		+	+
Rhizopogon rubescens	+	+	+	+	+	+	+	+	+
Tuber sp.	+			+	+	+	+	+	+
Wilcoxina mikolae	+		+						+
unknown Basidiomycete									+
unknown 2	+	+	+						
unknown 13									+
unknown 14									+

5.2.1 MONTHLY SEEDLING ASSESSMENT

The species richness, diversity, abundance and composition of the belowground ECM communities associated with *P. radiata* seedlings in the nursery and first year of outplanting were analysed and showed the following trends: (*i*) richness and diversity decreased from the nursery to outplanting and increased within a year to a level higher than found in the nursery; (*ii*) apart from one, all nursery species were present belowground one year after outplanting, with *Rhizopogon rubescens, Hebeloma* sp. and *Tuber* sp. dominating; (*iii*) ECM species composition changed at the end of the first year of outplanting; (*iv*) the first non-nursery species were observed six months after outplanting.

SPECIES RICHNESS

Mean species richness and Margalef's index for the nursery assessment in May 2006 and the monthly assessment of the outplanted seedlings from August 2006 to June 2007 are shown in Figure 5-1 and Figure 5-2, respectively. In the nursery, mean richness was 3.38 and Margalef's index 0.3. After the outplanting on a clearcut site in Kaingaroa Forest the richness and the index decreased significantly ($p=0.008$ and $p=0.023$, respectively) to 0.67 and 0.1, respectively, in the August assessment. Mean species richness increased in September and remained the same in October, thereafter it increased again to 3.00 in January and February. In the last assessment in June 2007 richness was the highest, being 4.67. The Margalef's index showed a slightly different picture. In September and October 2006 the index remained low (0.09 and 0.06, respectively). By November the value had increased to a value similar to that recorded in the nursery. Between November and February it fluctuated and reached its maximum value of 0.61 in June 2007. Both richness and Margalef's index were significantly different between the nursery and the monthly assessments in K06F-OS ($F=3.57$, $p=0.008$ and $F=2.86$, $p=0.023$, respectively). The Tukey's test showed that richness and Margalef's index in June 2007 was significantly higher compared to August, September and October 2006. The remaining monthly assessments did not differ between each other.

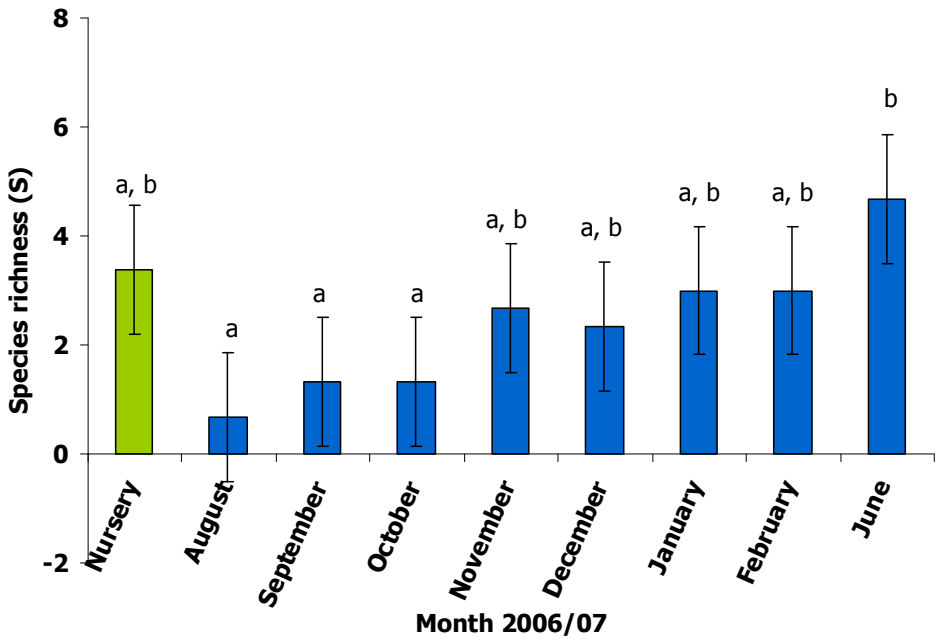

Figure 5-1: Mean species richness of ECM species/taxa observed in the soil core in the follow up assessments of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007 (± pooled standard deviation). The same letter above the bar indicates that monthly richness is not significantly different as calculated by Tukey's test.

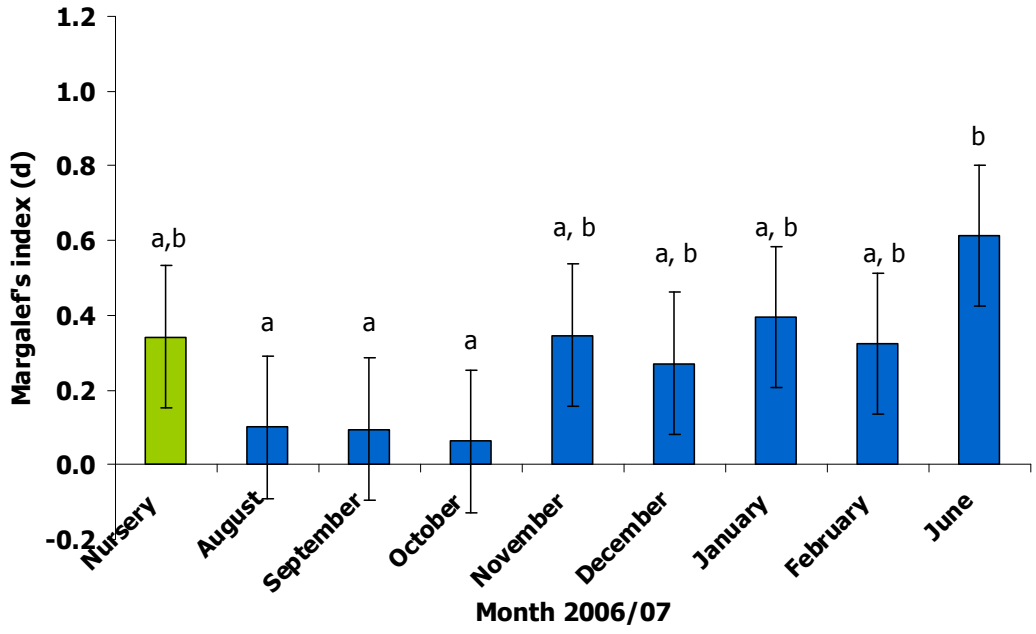

Figure 5-2: Mean Margalef's index of ECM species/taxa observed in the soil core assessments in the follow up assessments of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007 (± pooled standard deviation). The same letter above the bar indicates that monthly Margalef's index is not significantly different as calculated by Tukey's test.

ECM ABUNDANCE IN THE NURSERY AND OUTPLANTING SITE K06F-OS

The mean number of ECM root tips analysed during the assessments is shown in Figure 5-3. In May 2005, eight seedlings were assessed in the nursery and a total of 9705 ECM root tips were processed. Due to the trimming of seedlings before being outplanted, a total of only 28 ECM root tips were observed in the first assessment in August in Kaingaroa Forest, and 42 tips were counted in September. In the October assessment, there was abundant root growth and the root tip number processed rose to 370. ECM root tip numbers remained similar in the following month of November and increased to 546 in December 2006. In February 2007, the number doubled to more than to 1378. A total of 1038 ECM root tips were observed in the final assessment in June 2007.

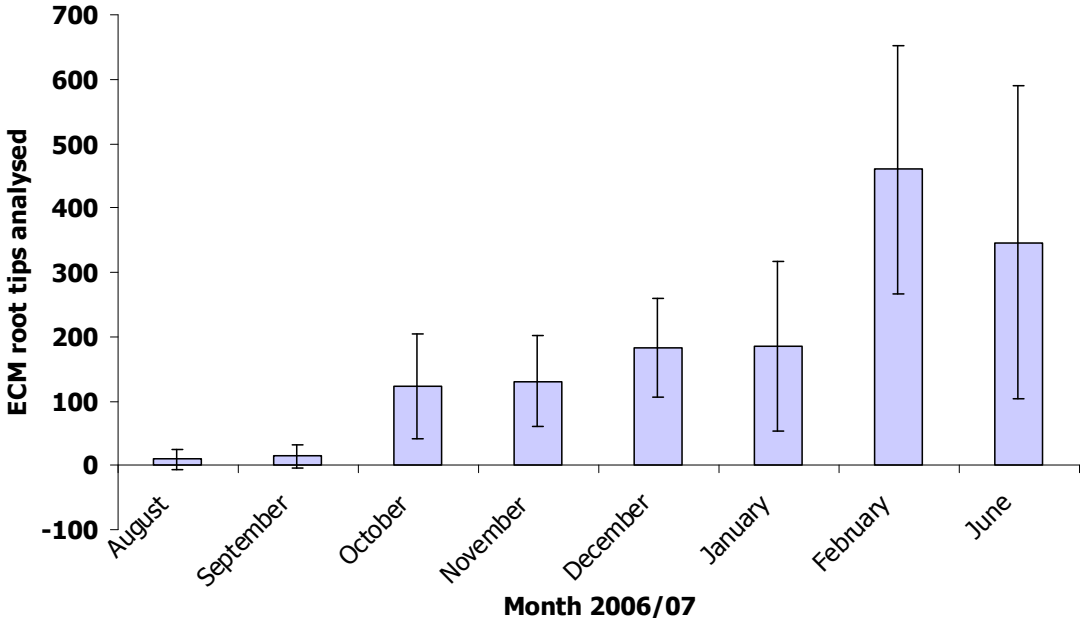

Figure 5-3: Mean number of ECM root tips per seedling analysed in the assessments of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007 (± standard deviation of mean).

The relative abundance of ECM species/types collected in the assessment of outplanted *P. radiata* seedlings in Kaingaroa Forest is shown in Figure 5-4, for detailed values on absolute and relative abundance of the nursery assessment refer to Appendix 7 (Tables A7-5 and A7-6).

In the nursery, the fungal ECM species observed were: *Rhizopogon rubescens*, *Tuber* sp., *Wilcoxina mikolae* and *Hebeloma* sp.; all with a similar abundance (21 – 30%). Seedlings from this nursery were outplanted in Kaingaroa Forest in June 2006 (site K06F-OS) and

assessed for the first time in August 2007. Before being outplanted, seedlings were trimmed, hence the root system was rudimentary in August and only two species were present, *R. rubescens* and Type unknown 2. In September, richness increased to four species, with *W. mikolae* being the dominant species (57%). Both *R. rubescens* and Type unknown 2 were present and collectively comprised 35% of the ECM type/species. *Hebeloma* sp. was of minor abundance. Species composition in October had changed compared to the previous months and only three species were present, *R. rubescens, Hebeloma* sp. and *Tuber* sp. with *R. rubescens* present on almost half of the root tips that were analysed. In November, *Pezizales* sp., a fourth ECM species, was observed. This species was only of minor abundance (7%) but was the first non-nursery ECM species found during this study. In contrast to the previous month, the most abundant species was *Tuber* sp., followed by *R. rubescens* and *Hebeloma* sp. In the December assessment, although species composition remained the same, the relative abundance had altered with *R. rubescens* accounting for 76% of the root tips analysed, compared to 28% in the previous month. *Tuber* sp., *Hebeloma* sp. and *Pezizales* sp. had similar abundance ranging from 8 – 8.5%. *Rhizopogon rubescens* remained the most abundant in January and *Pezizales* sp. was not found in this assessment. In February 2007 the suite of ECM species colonising the root tips remained the same. *Rhizopogon rubescens* continued to be the dominant species (73%) and *Pezizales* sp. was present again in this assessment with an abundance of 2%. The final assessment was made in June 2007, one year after outplanting in Kaingaroa Forest. Species abundance increased from four to nine species, with four new species colonising the root tips, *Amanita muscaria*, Type unknown Basidiomycete, Type unknown 13 and Type unknown 14. The dominant species was still *R. rubescens* with 34% abundance and two other nursery species, *Hebeloma* sp. and *Wilcoxina mikolae*, had high relative abundance (14% and 11%, respectively).

Overall, the nursery species *R. rubescens*, *Hebeloma* sp. and *Tuber* sp. were present with relatively high abundance in nearly all assessments, with *R. rubescens* being the most abundant ECM species on *P. radiata* in the months of August, October and December to June.

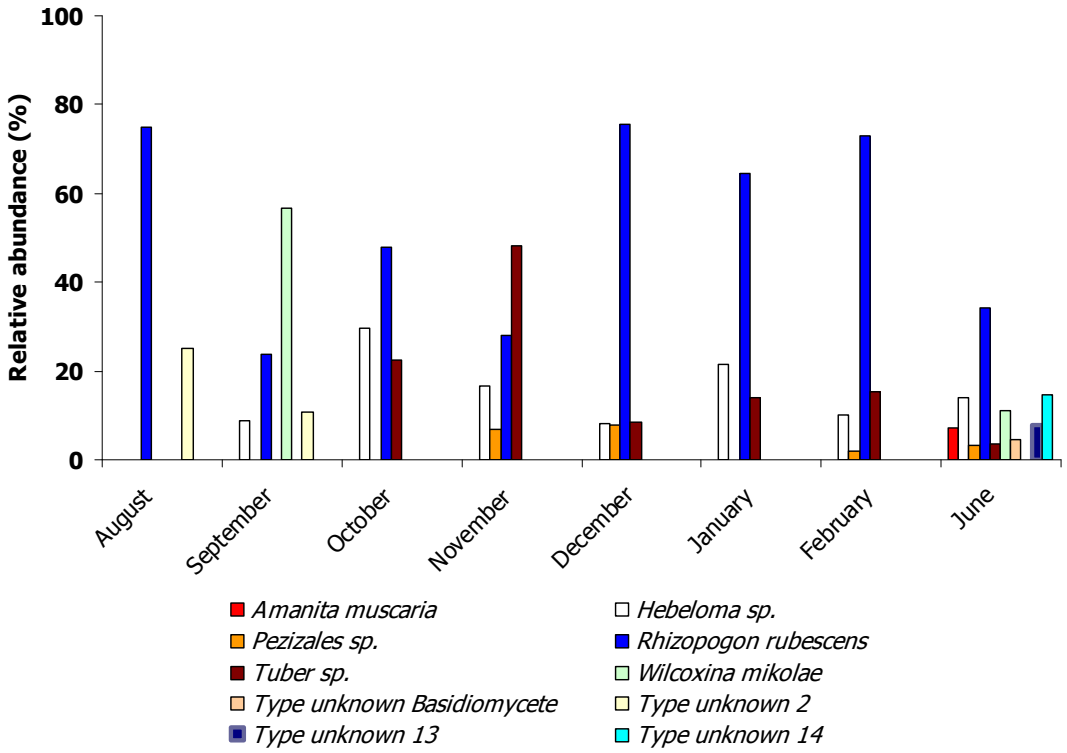

Figure 5-4: Relative abundance of ECM species/types collected in the follow up assessment of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007.

Figure 5-5 illustrates the change in the presence of nursery to non-nursery species over the course of the assessments. Nursery species were categorised as ECM species/types that were found in the original assessment of the seedlot source in Te Ngae nursery for the Kaingaroa plantation in 2006. These species were *Hebeloma* sp.*, R. rubescens, Tuber* sp.*, W. mikolae* and Type unknown 2. Nursery species dominated until the last assessment in June 2007, the first occurrence of a non-nursery species was in November 2006 (*Pezizales* sp.). This ECM species was the sole non-nursery species until the final assessment in June 2007, where four more non-nursery species were identified. In this last assessment the nursery species *W. mikolae*, which was abundant in the nursery but not present from the October to the February assessment, was collected again.

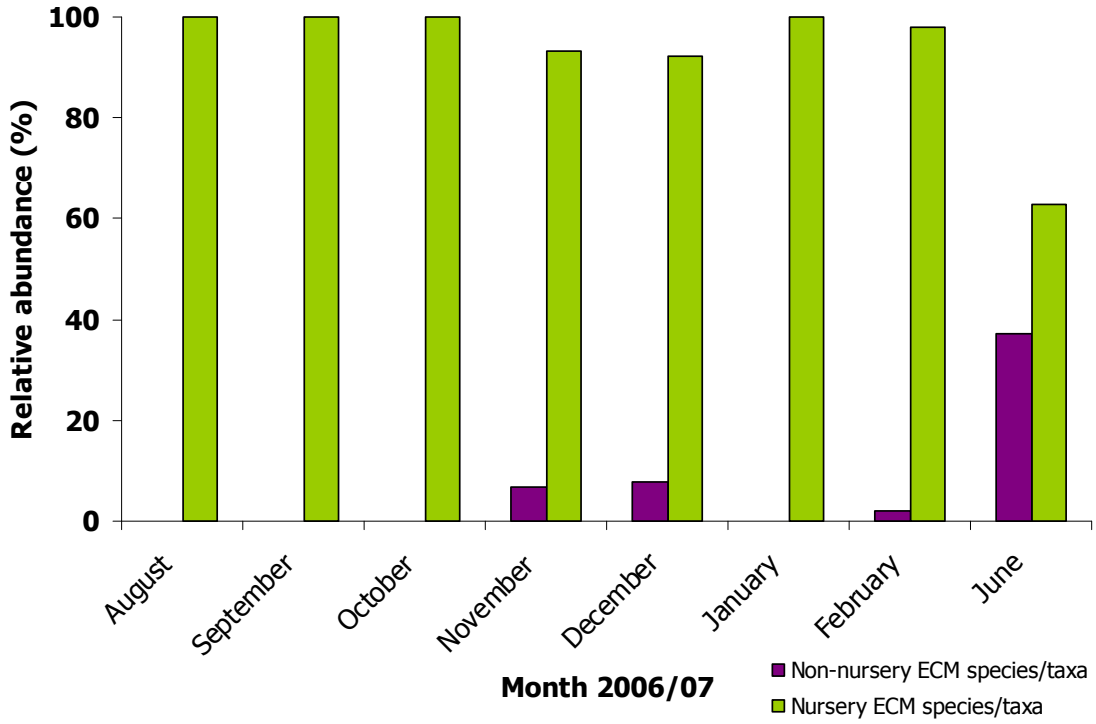

Figure 5-5: Relative abundance of nursery and non-nursery species collected in the follow up assessment of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007.

VISUAL ASSESSMENT OF SEEDLING HEALTH AND GROWTH

Due to root trimming before outplanting, seedling roots were short and lacked new feeder roots at the first assessment in August 2006 (Figure 5-6). The foliage of the plant had yellow shoot tips, which was probably due to winter frosts and outplanting shock. This was also apparent in September (Figure 5-7), however root tip growth had resumed and new feeder roots were present in September. Although root growth increased in October, seedling foliage still showed yellow shoot tips and no new growth (Figure 5-8). Over the next eight months, the root systems continued to grow well. By the November assessment, terminal buds were visible on the foliage and increased growth of new roots observed (Figure 5-9). This was also the case in December, seedlings did not increase visibly in height but more new terminal buds were present (Figure 5-10 A). In the January (Figure 5-10 B) and February assessments seedlings had increased in height and were greener. By the final assessment in June 2007, seedlings had grown substantially and had a well developed root system.

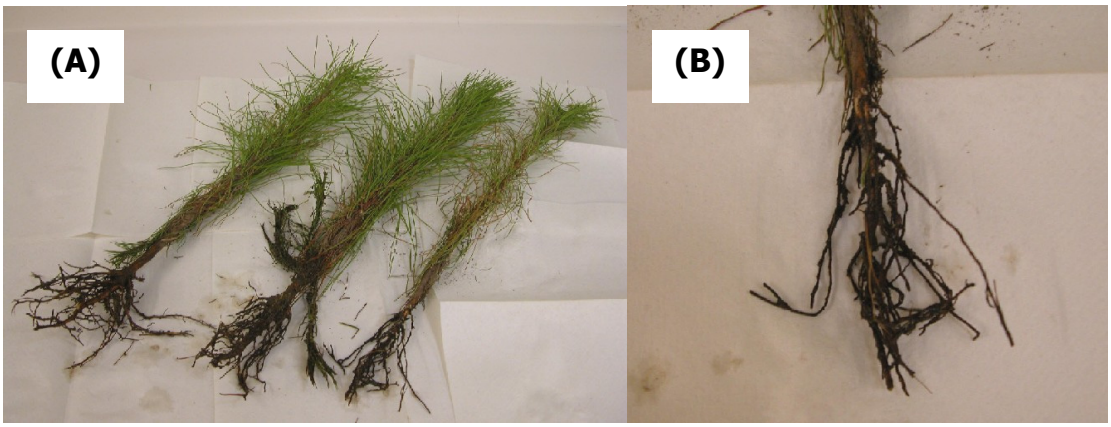

Figure 5-6: *Pinus radiata* seedlings collected in August 2006 assessment of K06F-OS (A), close up of the seedling root system (B).

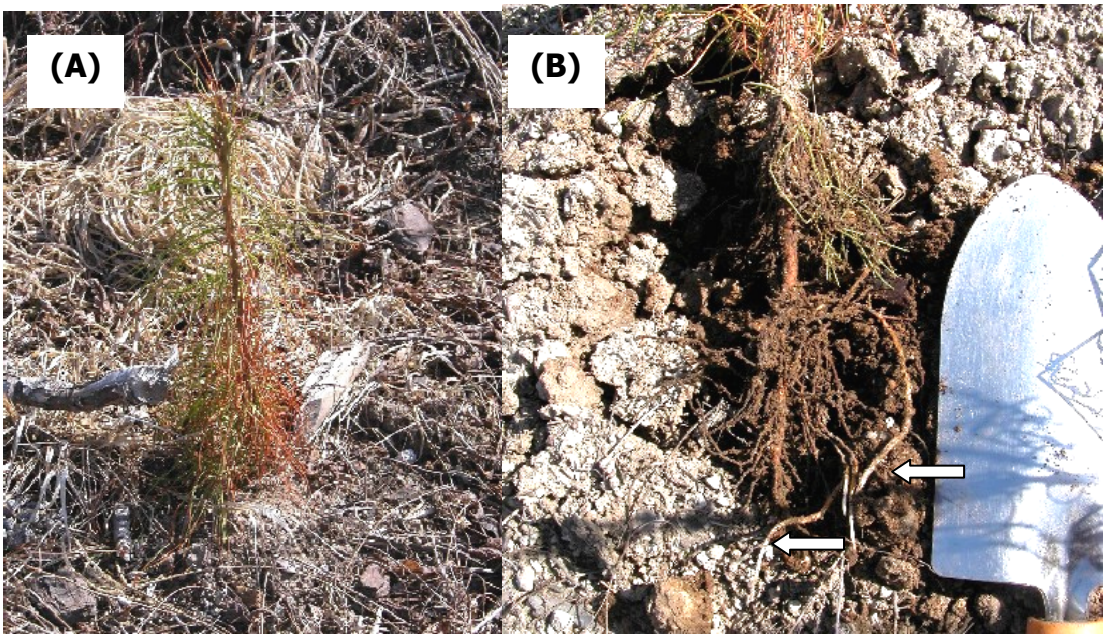

Figure 5-7: *Pinus radiata* seedling collected in September 2006 (A) showing symptoms of transplanting shock and yellow needle tips. Close up of the root system shows new root tips growth (B; arrow).

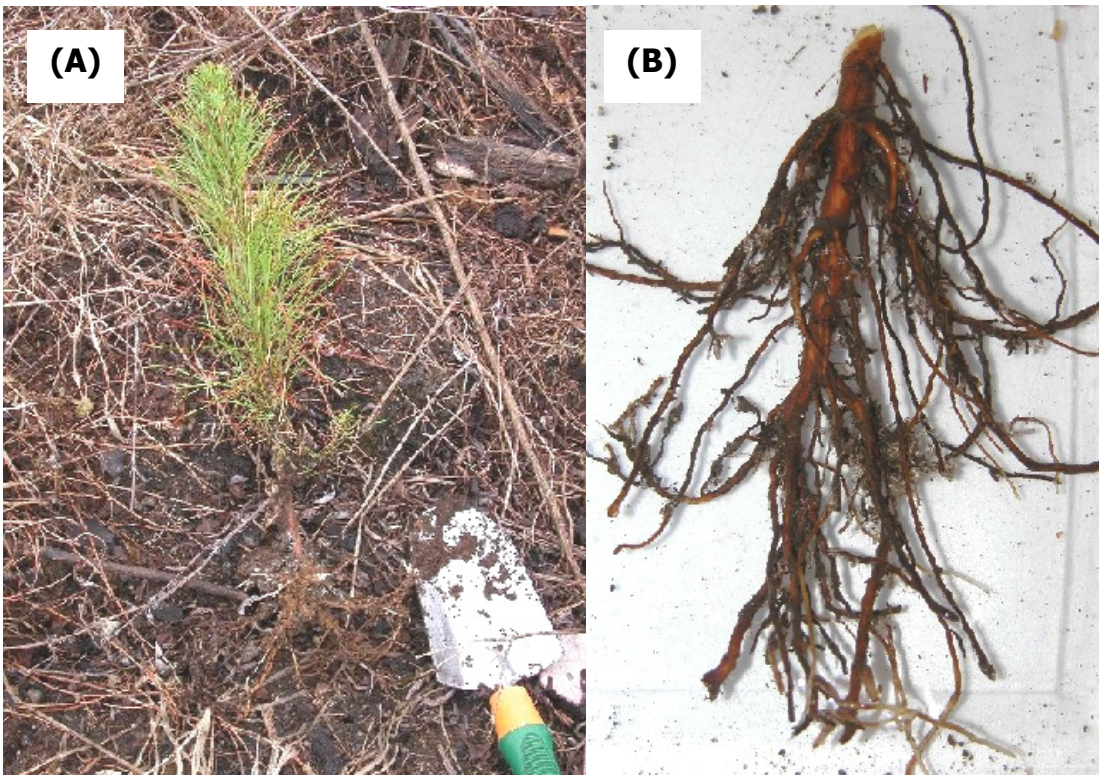

Figure 5-8: *Pinus radiata* seedling collected in October 2006 (A), close up of the root system of a seedling collected in October 2006 (B).

Figure 5-9: *Pinus radiata* seedling collected in November 2006, showing the increased growth of the root system.

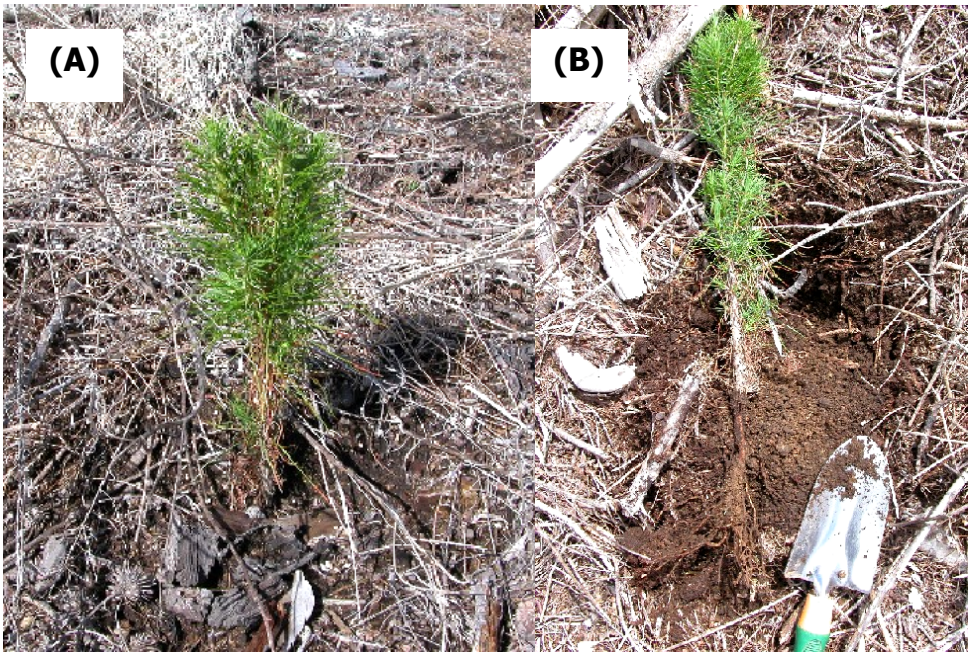

Figure 5-10: *Pinus radiata* seedling collected in December 2006 (A) and January 2007 (B).

SPECIES DIVERSITY INDICES AND PIELOU'S EVENNESS

The diversity measures, Pielou's evenness and the Shannon and Simpson diversity indices, were calculated as outlined in Chapter 2. Values are presented in Table 5-2 for the mean of seedlings analysed per assessment.

Initially, diversity indices and evenness were comparatively high in the nursery assessment in May 2006. Evenness increased in the first assessment in the outplanting site K06F-OS, whereas both the Shannon and Simpson diversity indices declined. For the first three months, diversity remained low, as calculated by the Shannon (maximum of 0.23) and Simpson (maximum of 0.39) diversity indices. Evenness was high in September, decreased in October and recovered again in November. Between November to January, the diversity values fluctuated slightly. In the February assessment, both diversity and evenness decreased. By June however, the values had recovered and both, the Shannon and Simpson diversity reached their maximum values in the June outplanting assessment with 1.21 and 0.60, respectively. Evenness was also high in the final assessment (0.82). Neither evenness, Shannon or Simpson diversity indices were significantly different between the nursery and the K06F-OS monthly assessments (evenness $F=1.38$, $p=0.255$; Shannon diversity $F=2.45$, $p=0.055$; Simpson diversity $F=2.00$, $p=0.93$).

Table 5-2: Mean Pielou's evenness, Shannon and Simpson diversity index (± standard error) for the nursery assessment in May 2006 (eight seedlings) and in the follow up assessments of the outplanted seedlings in Kaingaroa Forest plantation site K06F-OS from August 2006 to June 2007 (three seedlings per assessment).

	Month	Pielou's evenness (±SE)	Shannon diversity (±SE)	Simpson diversity (±SE)
Nursery	May	0.72 (± 0.08)	0.88 (± 0.12)	0.50 (± 0.00)
Plantation site K06F-OS	August	0.81 (± 0.00)	0.19 (± 0.19)	0.39 (± 0.00)
	September	0.91 (± 0.00)	0.21 (± 0.21)	0.15 (± 0.15)
	October	0.33 (± 0.33)	0.23 (± 0.23)	0.16 (± 0.16)
	November	0.84 (± 0.12)	0.79 (± 0.12)	0.52 (± 0.06)
	December	0.88 (± 0.09)	0.64 (± 0.33)	0.39 (± 0.20)
	January	0.75 (± 0.08)	0.79 (± 0.19)	0.46 (± 0.10)
	February	0.48 (± 0.15)	0.55 (± 0.27)	0.30 (± 0.17)
	June	0.82 (± 0.08)	1.21 (± 0.39)	0.60 (± 0.13)

5.2.2 ECM ROOT TIP ASSESSMENTS OF SEEDLINGS UP TO 8 YEARS AFTER OUTPLANTING

Species richness, abundance and composition of the belowground ECM communities of recently established seedlings (1 – 8 yr old plantations) were analysed and the following trends were observed: (*i*) species richness increased about threefold from the 1st to the 8th year after outplanting; (*ii*) species composition changed considerably between the 2nd and 7th year following outplanting; (*iii*) no nursery species was observed in the 7th year after outplanting.

The relative abundance of ECM in sites K04-2 (1 – 2 yrs of outplanting) and K98-8 (7 – 8 yrs of outplanting) for all three assessments in 2005 and 2006 was analysed. Table 5-3 presents the absolute and relative abundance of ECM species/types analysed on ECM root tips of *P. radiata* in site K04-2 and K98-8 at soil core assessments (SCA) 1, 2 and 3. In the first assessment of site K04-2 in June 2005 (1 yr), three species were identified on *P. radiata* root tips, with *R. rubescens* being the most abundant species (44%). In the December 2005 assessment (1 ½ yrs), richness increased to 5 species/types, *R. rubescens* still being the most abundant taxa (75% abundance). Additionally, new non-nursery species/types were detected in this assessment, ECM type unknown 12 and Type unknown various. In the June 2006 assessment (2 yrs), species richness decreased to three species/types. The nursery species still present were *R. rubescens* and *W. mikolae*, however dominance had shifted to *W. mikolae*, which had an abundance of 67%, whereas *R. rubescens* was 32%. ECM Type unknown Basidiomycete was first detected in K04-2 in 2006 with 1% abundance.

Four ECM species/types were found in the first assessment of site K98-8 in June 2005 (7 yrs), when Type unknown various made up 60% of the abundance. In the second assessment of

the site in December 2005 (7 ½ yrs), species richness increased to six. The abundance of Type unknown various dropped to 5% and the most dominant type in this assessment was *R. pseudoroseolus* with 69% abundance. In this assessment *Tomentella* sp. and Type unknown 12 were identified for the first time at this site. In the third assessment of site K98-8 in June 2006 (8 yrs), species richness was ten, with *Amanita muscaria* (38%) and Type unknown Basidiomycete (25%) dominating. In this assessment the ECM species *Cenococcum geophilum, Inocybe* sp., *Pseudotomentella* sp., *Thelephora terrestris* and ECM Type unknown 9 were identified for the first time. Overall, no nursery species were found in any of the three assessments of site K98-8.

Next, ECM species identified from all soil core assessments during this study in 2005 and 2006 as a chronosequence from the nursery to the early years of outplanting (nursery, K05F-1, K04-2 and K98-8) were analysed (Table 5-4). For absolute and relative numbers refer to Chapter 3 (Table 3-10) with Table 5-3 presenting species/types present at each site. Species richness was five in the nursery and K05F-1, which is the first year of the *P. radiata* seedlings being in the plantation. In both sites the same species were present: *Hebeloma* sp., *R. rubescens*, *Tuber* sp., *W. mikolae* and Type unknown 2. *Hebeloma* sp. and Type unknown 2 were not collected again after this stage. New in K04-2, however, was Type unknown Basidiomycete, unknown 12 and a group of unknown ECM types, resulting in species/type richness of six in this site. In K98-8 (which represented the 8th year of outplanting in 2006) the overall species richness was 12 and out of these species, nine species were observed for the first time: *Amanita muscaria, C. geophilum, Inocybe* sp*., Pseudotomentella sp., Pseudotomentella tristis, R. pseudoroseolus, T. terrestris, Tomentella* sp. and Type unknown 9. None of the nursery ECM species/types were still present.

Table 5-3: Absolute and relative abundance of ECM species/types observed in soil core assessments (SCA) 1, 2 and 3 in 2005 and 2006 in site K04-2 (outplanted in 2004, 2yrs old in 2006) and K98-8 (outplanted in 1998, 8yrs old in 2006).

	K04-2						K98-8					
	SCA 1 June 2005 (n=10)		**SCA 2 December 2005 (n=8)**		**SCA 3 June 2006 (n=8)**		**SCA 1 June 2005 (n=4)**		**SCA 2 December 2005 (n=5)**		**SCA 3 June 2006 (n=8)**	
ECM species/type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	-	-	157	26.52	284	22.94	587	38.12
unknown Basidiomycete	-	-	-	-	11.00	0.94	63	10.64	10	0.81	382	24.81
Cenococcum geophilum	-	-	-	-	-	-	-	-	-	-	55	3.57
Inocybe sp.	-	-	-	-	-	-	-	-	-	-	26	1.69
Pseudotomentella sp.	-	-	-	-	-	-	-	-	-	-	28	1.82
Pseudotomentella tristis	-	-	-	-	-	-	19	3.21	-	-	-	-
Rhizopogon pseudoroseolus	-	-	-	-	-	-	-	-	851	68.74	69	4.48
Rhizopogon rubescens	933	44.13	722	74.97	373	31.99	-	-	-	-	-	-
Thelephora terrestris	-	-	-	-	-	-	-	-	-	-	25	1.62
Tomentella sp.	-	-	-	-	-	-	-	-	19	1.53	-	-
Tuber sp.	605	28.62	65	6.75	-	-	-	-	-	-	-	-
Wilcoxina mikolae	576	27.25	81	8.41	782	67.07	-	-	-	-	-	-
unknown 9	-	-	-	-	-	-	-	-	-	-	120	7.79
unknown 12	-	-	19	1.97	-	-	-	-	18	1.45	116	7.53
unknown various	-	-	76	7.89	-	-	353	59.63	56	4.52	132	8.57
Total ECM root tips analysed	2114		963		1166		592		1238		1540	
Average number of ECM root tips/seedling	211		120		146		149		248		193	

n=number of soil cores processed

Table 5-4: The overall ECM species/types found in all soil core assessments in 2005 and 2006 in the nursery site and the plantation sites K05F-1, K04-2 and K98-8 in Kaingaroa Forest.

ECM species/type	Nursery	K05F-1	K04-2	K98-8
Amanita muscaria				+
unknown Basidiomycete			+	+
Cenococcum geophilum				+
Hebeloma sp.	+	+		
Inocybe sp.				+
Pseudotomentella sp.				+
Pseudotomentella tristis				+
Rhizopogon pseudoroseolus				+
Rhizopogon rubescens	+	+	+	
Thelephora terrestris				+
Tomentella sp.				+
Tuber sp.	+	+	+	
Wilcoxina mikolae	+	+	+	
unknown 2	+	+		
unknown 9				+
unknown 12			+	+
unknown various			+	+

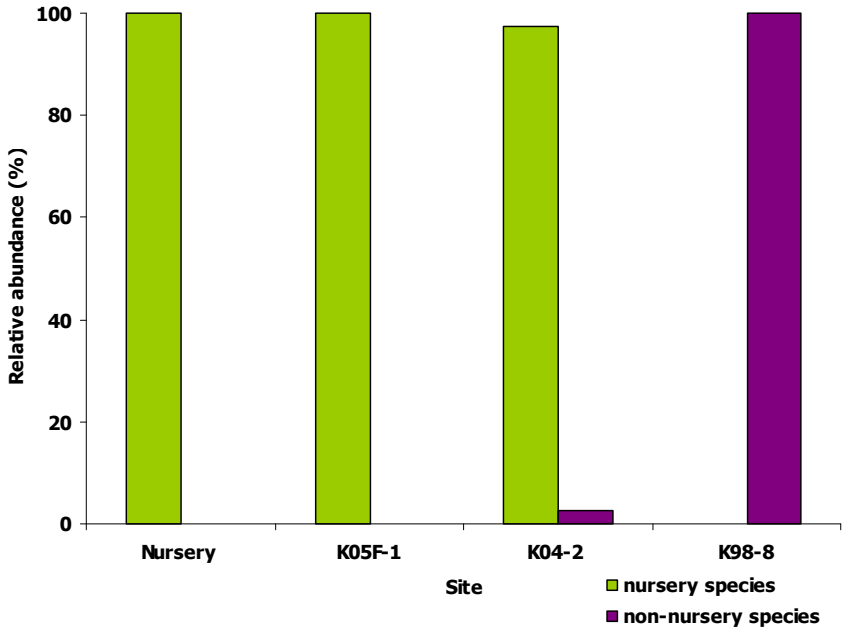

Figure 5-11: Relative abundance of nursery and non-nursery species collected in soil core assessment 3 in May – June 2006 in the nursery site and the plantation sites K05F-1, K04-2 and K98-8 (outplanted in 2005, 2004 and 1998, respectively).

Figure 5-11 illustrates the change from nursery to non-nursery species over the chronosequence of the nursery site, K05F-1, K04-2 and K98-8. As previously described, nursery species were categorised as ECM species/types that were found in the nursery

assessment in Te Ngae nursery. The change from nursery to non-nursery species occurred between the 1-2 yr and 7-8 yr old site.

5.3 DISCUSSION

This is, to the knowledge of the author, the first study where the composition of naturally colonising ECM fungal communities of seedlings at regular intervals from the nursery through to the first year of outplanting in the plantation has been assessed. Information about the presence and persistence of nursery fungi in the early stages of the outplanting is of importance for forestry and inoculation programs. It is essential that species present in the nursery survive and facilitate the establishment of seedlings, as a lack of persistent species can result in a failure in the establishment in the plantation (Gilmour, 1958; Hall & Perley, 2006). The majority of studies have investigated seedlings inoculated with specific ECM in the nursery (Danielson & Visser, 1989; Menkis *et al.*, 2007), where the follow up was done after the first year of outplanting. A study by Dahlberg & Stenström (1991) however, investigated ECM communities on outplanted *Pinus sylvestris* nursery seedlings in Sweden over two years, with the seedlings assessed at two, four and 15 months after outplanting. The present study investigated the survival of naturally colonised nursery ECM during first year following planting on a more frequent scale, with eight assessments over a 12 month period.

Species richness and diversity of ECM fungi of *Pinus radiata* seedlings initially decreased from the nursery during the first few months of outplanting on a clearcut site. The initial decrease in ECM colonisation can be mainly attributed to the "transplant shock", which occurs when seedlings are transferred from the nursery environment to the clearcut plantation site. One of the main causes of transplant shock is water stress, which results from poor root proliferation and insufficient root-soil contact. This problem can be pronounced in the case of bareroot seedlings, where root-soil contact is highly disrupted through the loss of fine roots at lifting (Davis & Jacobs, 2005). It can be assumed that during this time the plant is under stress and that it expends considerable resources in growing new roots. The second reason for the sudden decrease in species richness and diversity is due to trimming of the seedlings. In New Zealand it is common practice to trim the roots of the seedlings before outplanting in order to ensure a good establishment in the soil. Bent or twisted roots would provide poor anchorage to the seedling and result in toppling at a later stage (MacLaren, 1993). This trimming not only physically removes nursery ECM fungi and their hyphae, but it also reduces the surface available for re-colonisation by ECM fungi until the re-growth of the seedling root system. Species richness recovered once the seedlings had re-grown their root system and seedlings were able to resume growth and produce new terminal buds after six months.

The Margalef's index, which is an index incorporating richness as well as the total number of individuals observed (Magurran, 1988), increased over the year of the study, but did not replicate the trends between months seen with species richness only. For example the mean

species richness increased (but not significantly) from 0.67 in August to 1.33 in September, but the Margalef's index for both months was similar (0.06 and 0.09, respectively). These values reflect the influence of the total number of individuals assessed in the respective month, as more ECM root tips were analysed in September. The values for the Margalef's index increased less gradually when compared to the species richness values alone, the major increases occurred after five months and between 9 and 12 months after outplanting, when new root tip growth was prominent.

By six months after outplanting, richness and diversity had recovered and then increased over the remaining six months. The nursery species *Rhizopogon rubescens, Hebeloma* sp., *Tuber* sp. and *Wilcoxina mikolae* survived the first year of outplanting and dominated until the third year, but by six years after planting they were replaced. The persistence of nursery species following outplanting has been shown to be highly variable, ranging from three to ten years. A study by Danielson and Visser (1989) on the host response to inoculation and behaviour of introduced and indigenous ECM fungi of *Pinus banksiana* in Canada, found that the nine inoculated nursery fungi were replaced over three years. In their study, indigenous fungi increased from 4% in the first year to 33% in the second and finally to 72% in the third year after outplanting. A similar picture occurred in a study on the dynamic changes in nursery and indigenous mycorrhizal of *Pinus sylvestris* seedlings planted in clearcuts and forests in Sweden (Dahlberg & Stenström, 1991), in this case seedlings were not intentionally inoculated in the nursery but became naturally colonised by nursery ECM species. The first indigenous species occurred after two months with a frequency of 9% and this increased to 28%, 15 months after outplanting. Additionally, Menkis *et al.* (2007) found a low persistence of inoculated ECM species on *Pinus sylvestris* and *Picea abies* seedlings when outplanted on abandoned farmland in Sweden. Other studies have reported a high persistence of nursery mycorrhizal symbionts in outplanting, as was also found in the present study. For example the pre-inoculated *Laccaria bicolour* survived for three to ten years in *Pseudotsuga menziesii* plantations in France (Selosse *et al.*, 1998). In Mediterranean pine plantations, the inoculated *Suillus collinitus* remained in the sites for four years (Karkouri *et al.*, 2006). These reports highlight the large amount of variability in the survival of nursery ECM fungi (Menkis *et al.*, 2007). The variability in survival of nursery ECM fungi following planting is likely to be due to a range of interacting factors including the specific characteristics of individual ECM species, their interaction with the host and the effect of the new soil environment. ECM fungal species can vary in their affinity to their host. Some species are more optimal for a host than others (Harley & Smith, 1983) and, as discussed in Chapter 4, a succession of fungal species occurs over the lifetime of a host. A change of carbon allocation by the host plant over the course of time is a crucial element for ECM species composition. Fungi that appear in the later stages of succession need a larger supply of carbohydrates (particularly glucose), hence the need to be attached to a mature host (Frankland, 1992; Bigg, 2000). Carbon demand values by ECM are

reported to range from 10 – 50% of net primary production in field studies (Simard *et al.* 2002 and references therein). A fluctuation in carbon supply from the host can change fungal species composition if the ECM species is sensitive to carbon availability. The fungal species also have individual needs and requirements in their soil environment, which is another determining factor for the survival of nursery species and replacement by other ECM. Soil conditions, such as pH, temperature and moisture, vary between the nursery and the plantation and change during the development of the plantation site. Iwanski & Rudawska (2007) demonstrated that microhabitat affects the colonization patterns of ECM fungi and consequently the composition of the ECM community of the regenerating seedlings.

In this study, *Rhizopogon rubescens* was the dominant fungal species in most assessments, followed by *Tuber* sp. during the first year of outplanting. This confirms our results from Chapter 4, where *R. rubescens* was found to be the discriminating species for the 'young stands' which included the nursery and the first two years of outplanting. As discussed in Chapter 3, the ubiquitous species *Rhizopogon rubescens* is a successful and beneficial coloniser in nurseries and plantations, since it is relatively resistant to disturbance and able to tolerate adverse environmental conditions (Karkouri *et al.*, 2002; Duñabeitia *et al.*, 2004) with *Rhizopogon* spp. in general being among the most common introduced fungi in exotic pine plantations (Dunstan *et al.*, 1998; Giachini *et al.*, 2004; Tedersoo *et al.*, 2007). *Rhizopogon rubescens* is a long-distance exploration type (Agerer *et al.*, 1996) and with extensive rhizomorphs, the species is able to successfully compete for new roots in an environment such as clearcut sites, where the degree of competition from other species is low. These characteristics of *R. rubescens* enable it to be a successful species in the nursery as well as in the early stages of the outplanting and to be prevailing in the harsh environment of the clearcut site. In this study it was the dominant species in the nursery and in most months after outplanting. Similar results were reported from studies in both New Zealand (Chu-Chou, 1979) and Spain (Duñabeitia *et al.*, 1996). Duñabeita *et al.* (2004) demonstrated that *R. rubescens* is a very beneficial species for radiata pine in its early growing stage, as it not only enhances growth due to better nutrient acquisition but is also effective in mycorrhization and able to adapt and tolerate adverse environmental conditions.

Members of the order *Pezizales* (Ascomycetes), in particular *Wilcoxina* spp. and *Tuber* spp., are reported to be dominant species in nurseries and clearcut situations (Menkis *et al.*, 2005; Tedersoo *et al.*, 2006). In contrast to *R. rubescens, Tuber* spp. do not have the features of long-distance exploration types their success in colonisation in the nursery and early phases of the outplanting is more likely due to their propagules. These thick walled chlamydospores are very resistant and able to persist over time (Baar *et al.*, 1999; Tedersoo *et al.*, 2006). Hence they are readily available to infect the roots of the seedlings in the nursery and outplanting environment where only a few other species are present and when the degree of competition is low. Further increasing the survival and enhancing the dispersal mechanism of

Tuber spp. is the fact that some species are able to produce conidia and chlamydospores in a vegetative state (anamorph), enabling the species to persist for extended periods in ECM communities in the absence of a sexual state (Tedersoo *et al.*, 2006). Nonetheless, due to their low competitiveness *Tuber* spp. do not withstand the competitive pressure once new species enter the community and their abundance decreases following the initial years of outplanting (Iwanski *et al.*, 2006), as observed in this present study. In addition, studies on *Pinus* spp. and *Populus* spp. seedlings in the nursery and following outplanting showed that *Tuber* spp. have a growth enhancing effect on the host species (Cairney & Chambers, 1999).

In year two, *R. rubescens* and *Tuber* sp. were still present and dominant, but the third nursery species which was dominant in the first year, *Hebeloma* sp., was not observed. *Hebeloma* spp. are another group of ECM fungi that are successful pioneer species that occur in young plantations and disturbed habitats (Iwanski *et al.*, 2006). Similar to *R. rubescens, Hebeloma* sp. is a long-distance exploration type that has rhizomorphs and an extensive hyphal net which enables it to colonise root tips quickly and efficiently in the disturbed habitat of nurseries and outplanting sites. *Hebeloma* sp. (formerly identified as *H. crustuliniforme*, see Chapter 3) was observed for the first time in New Zealand belowground in a plantation forest. Up until this detailed follow-up study on outplanted *P. radiata* seedlings the fungus was only found fruiting and colonising root tips in nurseries, but was never observed in the plantation forests (Chapter 3 and 4 in present study; Chu-Chou, 1979). Studies which used *Hebeloma crustuliniforme* for inoculation purposes on *Pinus* spp. (Danielson & Visser, 1989; Menkis *et al.*, 2007), reported good survival of the species in the first year after outplanting, but, as observed in this present study, this species was absent after the second year. The reason behind the disappearance of the species is unknown, however it is suspected that changes in the host physiology or soil conditions may be the cause (Danielson & Visser, 1989). With the establishment and growth of the host, the carbon flow changes towards the symbiont, which may disadvantage *Hebeloma* sp. and cause its disappearance (Danielson & Visser, 1989). As discussed earlier, fungal species are sensitive to change the surrounding soil conditions and have growing optima for factors such as pH, temperature and chemical composition (Brundrett, 1991; Erland & Taylor, 2002). With the development of the host and the outplanting site, these factors change over time and are likely to be outside the growing optimum needed by *Hebeloma* sp. Studies in exotic *Pinus* spp. plantations in Australia observed *Hebeloma crustuliniforme* in plantations of up to 60 years of age (Dunstan *et al.*, 1998), which is in contrast to this study and the cited studies, indicating that *Hebeloma* sp./*H. crustuliniforme* is not well suited to the conditions in the investigated plantation sites. As discussed in Chapter 3, species in the genus *Hebeloma* exhibit a genetic plasticity and it has been suggested that recent speciation has taken place in the genus *Hebeloma* (Aanen *et al.*, 2000; Boyle *et al.*, 2006). This could possibly mean that the *H. crustuliniforme* species

found in mature plantation forests in Australia may be a different species which is better adapted to plantation forest conditions.

Wilcoxina mikolae was abundant in the nursery and early outplanting, but not during the remainder of the first year of outplanting. The species, however, was present again one year after outplanting as well as in the second year. The reason for the interim disappearance of this species is not clear; a change in carbon supply by the host during the first year might have caused the loss of this species. Seasonality could be another potential explanation, as outlined earlier, soil conditions influence the survival of a fungal species and may have been unfavourable for *W. mikolae* from November to June.

The nursery species were clearly dominant in the first years of outplanting. These species were already associated with seedlings from the nursery and had colonised the available surface, providing their 'own' inoculum for the outplanting site in Kaingaroa Forest. It seems the observed nursery species are better adapted to successfully colonise disturbed habitats such as the clearcut sites either through fast growing rhizomorphs or persistent propagules acting as inoculum. This initial dominance of nursery ECM indicate their important role in seedling establishment (Dahlberg & Stenström, 1991). Seedlings need ECM fungi that are able to persist in the first few years of outplanting to provide the stressed seedling with the required nutrients and to assist with water uptake, especially if the root surface area is reduced due to trimming before planting. Non-nursery species will eventually enter the system and take over, however the invasion by these species can be slow as the new inoculum may have to enter from surrounding plantation forests via wind or animal dispersal. Fungal species found in the later stages of tree development may also have a higher carbon demand than the young seedling can provide and would be unable to associate with the seedlings in the early phase of the plantation. Without persistent ECM fungi from the nursery there is the potential that outplanted seedlings have a period without being associated with ECM fungi, which in turn can cause malnutrition and lead to a failure in establishment of the tree. In this study, of the four surviving nursery species, *Hebeloma* sp. was the least competitive one, whereas *R. rubescens* was the most persistent species following outplanting.

The first non-nursery ECM species, *Pezizales* sp., appeared six months after the outplanting of the radiata pine seedlings in Kaingaroa Forest and was of minor abundance. No further non-nursery species were observed until one year after outplanting. *Amanita muscaria* and ECM types unknown Basidiomycete, unknown 13 and 14 were the next ECM to colonise the roots of the outplanted seedlings. The abundance of non-nursery species was lower than that of the nursery species at this point in time.

As discussed earlier, the perseverance of nursery fungi in the outplanting is both fungal and host species specific. In several studies on inoculated and naturally colonising ECM species on *Pinus* spp. seedlings (Danielson & Visser, 1989; Dahlberg & Stenström, 1991; Menkis *et al.*,

2007), indigenous species entered the ECM communities soon after the outplanting and replaced the nursery species. Indeed, Dahlberg and Stenström (1991) found these to occur two months after outplanting. These results are in contrast to our findings, where the non-nursery species only played a minor role in the first two years after outplanting and the sites are dominated by nursery species. The main source of new ECM fungal inoculum for the outplanted seedlings is surviving mycelium, pre-senescent mycorrhizae, and spores or sclerotia at the site that seedlings are planted on or spores from surrounding plantation forests. One year old seedlings in this study were planted on clearcut sites which had been felled two years prior and remained fallow for a year. Clearcutting is known to reduce the fungal ECM species richness and diversity due to the removal of the carbon source and cause severe changes in the soil conditions, as covered in Chapter 3 (Heinonsalo *et al.*, 2001; Simard *et al.*, 2002) and also by removing the living hyphae which would act as a means of fungal dispersal. However, it is known that the remaining stumps and dying roots in clearcut sites support living hyphae of ECM fungi for up to three years (Hagerman *et al.*, 1999). As the sites in Kaingaroa Forest were fallow for only one year, viable inoculum should have been available for colonisation of the seedling roots by non-nursery species. Furthermore, mature forests were close to the outplanting site and provide another source of non-nursery fungi for colonisation through wind or animal dispersal. It seems that the comparatively slow intrusion of non-nursery species into the outplanting sites in our study is not due to a lack of inoculum, but probably to edaphic factors such as pH, soil moisture or temperature which appeared to be unfavourable for non-nursery species for the early years of the stand. Jones *et al.* (2003) found that many forest fungi cannot survive in clearcut sites, even if they were already present prior to harvest. The authors hypothesised that the changing environmental conditions and the ability of the seedlings to support the different mycorrhizal types are the driving factors behind the change in community structure and not the availability of ECM fungal inoculum. The dependency on mature hosts may be another reason for the slow establishment of non-nursery species. As mentioned, many species present in the later stages in the forest disperse via living mycelia and require attachment to a mature host (Jones *et al.*, 2003; Kranabetter, 2005). Studies on the influence of retention trees on ECM colonization in clearcut sites (Cline *et al.*, 2005; Kranabetter, 2005; Luoma *et al.*, 2006) demonstrated that the establishment of forest fungi is enhanced by the presence of retention trees and without them some ECM species might take decades to disperse from nearby intact forests into younger stands.

Amanita muscaria was found in the assessment of *P. radiata* seedlings one year after outplanting. This is the first time in New Zealand that this species has been reported at such an early stage. In the present study (Chapter 3) and studies of Chu-Chou & Grace (1988), *A. muscaria* was never observed fruiting or occurring on root tips in stands younger than seven years. Additionally, overseas studies have not reported the species to be present on trees of

such a young age (Garrido, 1986; Dunstan *et al.*, 1998). This species, as discussed in Chapter 3, is of interest due to its invasiveness, and since it has been found associated with native *Nothofagus* spp. (Johnston *et al.*, 1998) it is actally of concern for New Zealand and considered as a "regulated pest" by the Ministry of Agriculture and Forestry (MAF). *Amanita muscaria* is of the medium distance exploration type, characterised by forming rhizomorphs (Agerer, 2001), which enable the species to explore more diverse areas and colonise new roots. These features may be one of the reasons why *A. muscaria* is such a successful coloniser and able to invade new roots and younger systems, but it must also be a highly competitive species which is able to outcompete other ECM to establish itself successfully in a new environment. A further reason thought to be important for its invasive potential is that *A. muscaria* is a 'multi stage fungus' which is able to associate with older as well as a younger hosts and is less sensitive to the amount of carbon supplied by the host. It would be interesting if *A. muscaria* is also capable of associating with radiata pine seedlings in the nursery stage; however this has not been investigated. If this was the case then the effect on ECM fungal species composition currently found associated with the seedlings in the nursery stage and any displacement of species, especially species beneficial to host growth such as *R. rubescens* would need to be determined. The presence of *Amanita muscaria* in nurseries could also increase the spread of the species into forests surrounding the nursery or even forests further away, as spores of the fungus are able to attach to the footwear of nursery workers (preliminary study in Australia, T. Lebel, personal communication, 24 August 2006). This anthropogenic spore dispersal could be of concern since a nursery is more highly frequented by workers than plantation forest sites.

After seven years in the plantation, it was observed that nursery ECM species had been completely displaced by non-nursery species. The change in species composition is driven by changes in the host, the colonisation and competitive potential of species and the surrounding soil environment. Over the course of time, the host tree has established itself in the plantation and is developing its root system. Because of the growth of new roots the rates at which fungi establish new contacts with the root system increases, which in turn can lead to extinctions of less competitive species (Erland & Taylor, 2002). During the flush of root growth a large number of non-colonised root tips are produced, which is likely to contribute to an enhanced diversity (Bruns, 1995), as observed in this study. As discussed earlier, the carbon allocation by the host changes with increasing age, and as ECM species vary in respect to their carbon needs, different ECM species will be able to associate with the host over the lifetime of the host. The increase in carbon supply and the surface available for colonisation leads to an increase in the competitive pressure between the fungal species, and the nursery species will be replaced by more competitive species (Bruns, 1995).

The clearcut site changes from bare land to a forest with an understorey, an organic layer and a well developed root system. This in turn changes the soil and microclimatic conditions

of the site, such as pH, nutrient status, water availability, soil density and light availability. As covered previously, fungi do have specific growth optima for these factors and are sensitive to the change of their surrounding environment (Brundrett, 1991; Erland & Taylor, 2002), which determines their colonisation potential and survival and is a major driving factor in the change from nursery to non-nursery species.

The history of *Pseudotsuga menziesii* plantations in New Zealand is an example of the importance of ECM colonisation of seedlings from the nursery to the outplanting. The tree species failed in its establishment in the early years because of a lack of mycorrhization of the seedlings (Gilmour, 1958). Since the introduction of persistent and beneficial nursery species, in particular *Rhizopogon parksii,* the tree is able to establish in the plantation (Hall & Perley, 2006). In this present study on the survival of nursery ECM species of *P. radiata* seedlings in the plantation, the species colonising the seedlings in the nursery were dominant until the third year after outplanting and non-nursery species were only of minor frequency until then. The environmental conditions did not favour non-nursery species until about seven years after outplanting. If seedlings were not equipped with ECM fungi at the time of outplanting, it is likely that seedlings would be without beneficial ECM symbionts until the soil conditions change to become conducive for the non-nursery species. This could lead to a prolonged period without the necessary symbionts and hence a deficit in nutrient and water uptake and no protection against pathogens, which in turn may lead to failure in establishment of the plantation tree. It has been shown that *R. rubescens* is the best suited and beneficial symbiont for *P. radiata* in the nursery (Chu-Chou & Grace, 1985; Duñabeitia *et al*., 2004) and as seen in this study, the species is also persistent and prominent in the early outplanting years. *Hebeloma* sp. was dominant in the nursery, but has been shown to be less beneficial for *P. radiata* growth and did not survive in the outplanting for more than one year. These results indicate not only that ECM fungal colonisation of seedlings in the nursery and during early outplanting is important, but that the beneficial species persist and survive the early years of the outplanting and facilitate a successful establishment of the tree in the plantation.

6 CONCLUDING DISCUSSION AND FUTURE WORK

The above- and belowground ECM communities of the exotic plantation species *Pinus radiata* were the subject of this study. The overall and stand specific diversity and species composition was investigated and the correlation between the species found fruiting aboveground and the species found colonising the root tips belowground was analysed. Successional patterns for both, above- and belowground ECM were examined and the survival of the nursery species in the first year of outplanting on a clearcut site was assessed.

The need for ECM colonisation for tree growth and health is a well recognised fact (Smith & Read, 1997). Chu-Chou & Grace's research on ECM fungi associated with New Zealand's exotic plantation species was extensive; however, it was restricted to mainly aboveground diversity because the methods necessary to identify belowground ECM species were limited at the time of their research. Even though forestry is New Zealand's third largest export earner, mycorrhizal associates of *P. radiata* have not been a research focus since the 1990s. One of the aims of this study was to resume this research topic and expand on historic data of Chu-Chou & Grace using improved methods. With the means of molecular fingerprinting (Restriction Fragment Length Polymorphism, RFLP in this research) and direct sequencing it is possible to identify species more precisely, in particular ECM fungi colonising root tips. Since it is the belowground ECM that are the important factors influencing the nutrient uptake and protection of the host plant it is necessary to focus the research directly on the belowground ECM. The knowledge of the ECM associated with *P. radiata* in the nursery stage and in the plantation forests will be useful for sustainable forestry practises, pathogen protection and inoculation programs in order to improve growth of healthy seedlings in the nurseries and their establishment in the plantation forests.

The present study investigated the diversity of the above- and belowground ECM communities and their correlation to each other. It was found that ECM diversity in this exotic monoculture system of *P. radiata* was low both above- and belowground. The driving forces behind the α -diversity (within site) are multifaceted and dictate which species are present in an ecosystem. The host itself is one of the crucial factors and its influence on diversity can be broken down into several aspects. For one, there is host specificity which pre-determines the initial plant-fungus association and sometimes this can be an absolute factor due to genetic and physiological incompatibilities (Malajczuk *et al.*, 1982). Ishida *et al.* (2007) showed that species diversity increases with the number of hosts present in a system, which is likely to be one of the main reasons for the low diversity found in this present study. *Pinus radiata* in New Zealand is planted as a monoculture system, stands consist of a cohort of single age trees and no other tree species is present in the stands. This leads to the next reason behind

the low diversity, the fact that the host is exotic and a relatively recent introduction into New Zealand. *Pinus radiata* is native to California/USA, but was introduced to New Zealand *via* England and Australia and mainly through seed importation (Shepherd, 1990). Also, with regards to exotic plantation trees, the time elapsed since establishment of the host can also possibly influence ECM fungal diversity. Exotic species may establish initially with a minimal number of ECM symbionts, whatever inoculum came with the host but ECM fungal richness can increase over time as new associations with native ECM fungi occur. In this study no native fungi were identified on radiata pine root tips or found as sporocarps in the plantations and no reports of native ECM fungi in exotic plantations in New Zealand are known with the possible exception of *Cenococcum geophilum*. This ubiquitous species, which lacks a sexual reproduction stage and is only found colonising root tips, is potentially a native species.

This was the first study of belowground ECM communities associated with *P. radiata* plantations utilizing molecular methods for species identification. The understanding of the diversity of ECM fungi associated with *P. radiata* is increasing, however gaps still exist. In this study, six ECM species were unidentifiable using DNA sequence database searches which raises the question regarding what species are 'hiding' in the assemblages of unknown ECM types found in during this research. Potentially, these could be native ECM species, but to date this is unknown as sequence analysis data from native ECM species is currently limited. Due to this lack of information on ECM species associated with both native and exotic hosts, it is uncertain if native ECM species have become associated with *P. radiata* in New Zealand to date. However, species exchange between the systems has happened; the exotic ECM species *Amanita muscaria* has been found to associate with native *Nothofagus* spp. (Johnston *et al.*, 1998; Orlovich & Cairney, 2004). To fill the gaps in this area of research, more work on both, native and exotic ECM is required. Native ECM fungi need to be analysed with molecular methods and sequenced to build the reference database, especially the ECM communities of *Nothofagus* spp. would be of interest. Since a known ECM associate of *P. radiata* is able to colonise this native tree it would be interesting if this ECM fungal exchange could happen *vice versa*. If so, it would be interesting to determine if native ECM species are beneficial to the growth of *P. radiata* and maybe even being superior in their effects than the current exotic associates. In addition their potential to protect against pathogenic fungi would be of interest to the forestry sector.

Seasonality, or temporal partitioning, is another aspect influencing diversity that was considered in this study. The results indicated that the time of year influences which species may be present in a system due to exogenous factors such as soil temperature and moisture as well as the hosts root turnover. Knowledge of the effects of temporal partitioning on ECM diversity however is lacking and a field for future research. This information would assist in finding the best surveying and sampling period in order to sample ECM root tips during the

time of year where they are actively colonising root tips and clearly expressing distinguishing features (mantle growth and colour) required for morphological grouping.

Differences in ECM diversity in relation to both, soil depth (vertical variation) and distance to the tree (horizontal variation), was not investigated in this study; however, Dickie *et al.* (2002) and Rosling *et al.* (2003) showed that ECM diversity changes with soil depth and species have a varying vertical distribution. Likewise, Huda *et al.* (2006) found that that ECM infection in roots of *Dipterocarpus turbinatus* in a plantation in Bangladesh varied considerably with distance from the host; however the exact ECM species were not determined in their study. Different species may be found at different distances from the tree base, hence a sampling designed which is based on a horizontal gradient may result in a higher ECM species diversity compared to sampling from only one distance per tree. For future work a horizontal gradient sampling design would be applied in at least an initial survey to investigate if this approach would increase ECM species diversity. Since the ECM symbiosis is an association between a plant and a fungus where the fungal partner plays a connecting role between the plant and the soil, the edaphic factors take on a crucial role in determining the diversity and performance of ectomycorrhizal communities (Brundrett, 1991). Consequently another valuable area for future study would be the influence of different soil conditions on the ECM diversity of a given host. This information would be particularly valuable in the forestry sector to aid the selection of future plantation or nursery sites. Disturbance is yet another important factor in diversity, as it opens up areas for new colonization. Fire disturbance and clearcutting are well-studied factors and are known to alter ECM fungal diversity (Jones *et al.*, 2003; Heinonsalo, 2004). In the case of clearcutting, diversity is generally reduced due to the drastic removal of the carbon source and changes in the soil conditions (Heinonsalo, 2004), as observed within this study. Within a plantation, the rotation history can also play a factor in ECM diversity. If the plantation species remains the same over all rotations, a change in ECM fungal diversity is unlikely. However, if host species are rotated, species diversity is likely to be affected as the previous host species may introduce ECM fungi that have a broad host range and their spores can inoculate the following tree host and increase the suite of ECM species of the following tree host. In the case of the low diversity found in New Zealand, this could be of potential interest, as a host species change, for example *Pseudotsuga menziesii* following *P. radiata,* may increase ECM diversity. High ECM diversity is a desirable feature of an ecosystem. Species poor communities are likely to perform their ecological functions less efficiently than a species rich community because they will be less resistant to environmental fluctuations and pathogens (Iwanski & Rudawska, 2007). ECM diversity is essential for tree growth and ecosystem health, in particular in stressful environments such as plantations (Perry *et al.*, 1989; Hagerman *et al.*, 1999; Simard *et al.*, 2002).

With the use of RFLP fingerprinting and direct sequencing for species identification, four previously unreported ECM species associated with *P. radiata* in New Zealand were detected in the belowground ECM assessment. Given that only a limited area and stand ages in one forest in the North Island were investigated, diversity of ECM on radiata pine may be higher than expected and warrants further investigation. Nurseries and plantations with different soil conditions, different site characteristics (i.e. hilly site) and locations (i.e. South Island) should be investigated. Also, the influence of site history is of interest – how does the ECM diversity and community differ from a first to a third rotation forest?

The ECM richness and diversity values from the above- and belowground assessments were similar, but no correlations were found in the overall and stand specific ECM species composition, confirming results of a lack of correlation between these communities from other studies (Gehring *et al.*, 1998; Hagerman *et al.*, 1999; Peter *et al.*, 2001). Reasons behind this discrepancy can primarily be found in the fruiting patterns of species. Fruiting is irregular and triggered by exogenous factors which not only vary between species but also between years. Also, some species do not fruit at all or produce inconspicuous fruiting bodies which may be easily overlooked. This study confirmed that sporocarp surveys have to be conducted over a long period of time to compile a complete picture of the sporocarp diversity and to get reliable results. It also confirmed that in order to assess the ECM fungal diversity of the system, both the above- and belowground communities must be considered. However to determine which ECM fungal species is the most important and beneficial to its host tree, the focus has to be on the ECM actually colonising the root tips, as this is where the nutrient exchange takes place.

The influence of age host on the β-diversity (between-site) of ECM communities both above- and belowground was assessed. Compared to other ECM successional studies, the forest investigated was younger, due to the rotation age in New Zealand, and the stand ages were on a finer scale. A clear succession in ECM communities related to host age was found aboveground and characteristic species were identified for each age group. As discussed previously, sporocarp fruiting patterns are under the influence of external factors such as temperature and rainfall and results on successional patterns are only reliable if these fruiting patterns are observed over a long period. In this research however only two fruiting periods were assessed. The results from the belowground assessment are more robust, as colonisation patterns are more consistent. Unlike that seen aboveground, belowground only two cohorts of ECM communities were detected and the change in ECM composition occurred between the second and seventh year of outplanting. This latter finding is novel as other successional studies have not investigated stand ages on a comparable detailed scale (Visser, 1995; Gebhardt *et al.*, 2007). As our data cannot be compared to other studies this result raises several questions. The first is 'Is the time of our observed change in ECM species composition from predominantly nursery species to predominantly forest species the norm in

forests?' or 'Does it occurs more quickly in a *P. radiata* plantation?'. Since our study was conducted in a monoculture plantation situation, this raises the next question: 'Are successional mechanisms different in an exotic monoculture plantation compared to a native forest?'. It would be of great interest to investigate a *P. radiata* forest in New Zealand which is substantially older than the investigated oldest stand (26 yrs) to see if and how the ECM communities of these older trees change and if similar patterns to other studies were seen (Visser, 1995; Palfner *et al.*, 2005; Gebhardt *et al.*, 2007; Twieg *et al.* 2007).

As observed in this study, ECM diversity of *P. radiata* in New Zealand is depauperate compared to other systems and may be lacking potential ECM symbionts which in turn could influence successional patterns. With respect to this plantation study, it would also be interesting to determine if the observed change in ECM communities happen gradually or suddenly during the six years that were not investigated. This question is currently being followed up by the author in a research project at Scion, Rotorua. The one year old (K06F-OS) and two year old (K04-2) site from this PhD study are being investigated to determine when ECM species change from nursery to forest ECM fungi occurs.

This research on succession in ECM communities in *P. radiata* in New Zealand confirmed the recent opinion in mycorrhizal ecology that succession is a function of ECM colonisation strategies and soil condition changes rather than root age (Simard *et al.*, 2002; Jones *et al.*, 2003). The influences of changes in carbohydrate supply, an increase in the litter and humus layer and nitrogen availability on ECM succession seem to be of more importance in older forests than were investigated here (Visser, 1995; Simard *et al.*, 2002). Our research only spanned the younger stage of a forest, as at 27 yrs the host trees had not reached the climax stage. This brings up the questions: 'What would happen in the ECM communities of *P. radiata* in New Zealand if the host tree was not harvested, but left to develop into an old growth forest?'. 'Would ECM succession follow the trends observed in other (native) forests that have been investigated?' (Visser, 1995; Gebhardt *et al.*, 2007) and 'What would be the driving forces of succession in these stages?'. Furthermore it became apparent that aboveground succession can be masked by fruiting patterns which are triggered by environmental factors. Sporocarp studies require a much longer period of investigation to unravel true successional patterns rather than fruiting patterns caused by exogenous factors.

The fate of nursery species in the first year of outplanting in a clearcut site and the entry of non-nursery ECM species was investigated in this research. Again, this study investigated the change of the ECM species on a more detailed scale compared with other studies (Danielson & Visser, 1989; Menkis *et al.*, 2007) and found that the ECM species from the nursery persisted for at least two years after outplanting of the seedlings. The acquisition of forest ECM species was slower than has been reported in other systems (Dahlberg & Stenström, 1991; Menkis *et al.*, 2007). This result highlights the need and importance of persistent

species from the nursery to the outplanting. In the case of *P. radiata,* the symbiont *Rhizopogon rubescens* was found to be the most beneficial species for growth and performance of *P. radiata* (Chu-Chou & Grace, 1990; Duñabeitia *et al.*, 2004) and as seen in this study, it is the most persistent species in the outplanting. These persistent species are of great importance if seedlings are planted in clearcut sites, as the fungal inoculum is generally low (Heinonsalo, 2004). The nursery species are needed to promote the establishment of the seedlings until the forest ECM species colonise the young trees. Forest species were observed colonising the seedlings one year after planting, but the change in dominant species was not until seven years after planting, which is longer than reported in other systems (Dahlberg & Stenström, 1991; Menkis *et al.*, 2007). It has been shown that the ECM diversity of seedlings growing close to established ectomycorrhizal colonized vegetation is higher and declines with increasing distance (Cline *et al.*, 2005; Dickie & Reich, 2005). In several countries this mechanism is now being implemented into sustainable forestry practises through retention trees that are left in clearcut sites to facilitate inoculation of newly outplanted seedlings, amongst other roles, in these highly disturbed sites (Cline *et al.*, 2005; Luoma *et al.*, 2006). This forestry practise has not been implemented in New Zealand, but it would be of interest to learn whether retention trees would have a beneficial effect on ECM diversity of outplanted seedlings in New Zealand. Since ECM diversity in *P. radiata* plantation forests is low, especially in the first years of outplanting, it would be desirable to increase ECM diversity in order to facilitate good establishment and increase the resistance of the trees in these highly disturbed sites. Furthermore the impact of nursery practises such as fungicide, herbicide and nutrient application, as well as outplanting practises root trimming and washing of seedling roots before outplanting on the ECM colonisation on nursery seedlings and their respective survival in the outplanting are a field of future research. Knowledge about the impact of these factors can improve the seedling treatments and lead to more sustainable forestry and nursery practises, utilising the ECM species to increase the health and growth of seedlings and facilitate their establishment instead of the external application of supplements.

Another novel finding in this outplanting study was the observation of *Amanita muscaria* on root tips of *P. radiata* seedlings one year after planting. This species has never been observed so early in an exotic plantation; seven year old plantations were previously the youngest reported (present study; Chu-Chou & Grace 1988). *Amanita muscaria* appears to be highly invasive, which raises two questions. 'Would *A. muscaria* also be able to associate with radiata pine seedlings in the nursery?' and, if so, 'Would it be as beneficial for growth and performance as *R. rubescens*?'. The invasiveness of the species is also of interest in New Zealand since it has been found to be associated with native *Nothofagus* spp. in various locations in the North and South Island. These observations could indicate that *A. muscaria* is able to change ECM communities of native host trees and displace native ECM fungi.

Current research (Duñabeitia *et al.*, 2004) suggests that the most abundant nursery species found associated with *P. radiata* in this research, *Rhizopogon rubescens*, is the most beneficial species to the host tree in the early stages of the seedling. However, it would be interesting to investigate if a group of species, each with different functional attributes would be more beneficial to seedling growth and vigour compared with a single, that has the desired effect or are species combinations needed to get the best out of the mycorrhizal symbiosis. This as already discussed would provide resilience to the system being able to withstand and respond to biotic and abiotic change. In addition, the ECM function of pathogen protection is yet another area of future research, especially for sustainable forestry practices. It would be desirable to know which species in the nursery and plantation forests are the most effective at protecting against root pathogens such as *Phytophtora* spp. to reduce infection of the plantation trees.

The research outlined in this thesis provides a snapshot of the ectomycorrhizal fungal community of *P. radiata* in a plantation situation in New Zealand. Overall, the ECM fungal diversity was low compared to what has been recorded in natural and long standing forest ecosystems. As the plantations of New Zealand are relatively young forests that were established with exotic *P. radiata,* it is not surprising that the ECM diversity is low. As the *P. radiata* plantations of New Zealand age, it will be interesting to assess fungal ECM species richness and diversity, to determine if recruitment of the native ECM flora has occurred.

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APPENDIX 1 PURE TISSUE CULTURE MEDIA

HAGEM MEDIA (HG)

(Modess 1941: Zur Kentniss der Mykorrhizabildner von Kiefer und Fichte. Symbolae Botanica Upsaliensis 5: 1 –147).

1 litre:

KH_2PO_4	0.5 g
$MgSO_4$ $7H_2O$	0.5 g
NH_4CL	0.5 g
$FeCl_3$ (1% sol.)	0.5 ml
Agar (Danisco Cultor, Dingley, Australia)	15 g
Glucose (BDH Chemicals Ltd, Poole, England)	5.0 g
Malt extract (Danisco Cultor)	5.0 g
Yeast (BDH Chemicals Ltd)	1.0 g
Biotin (BDH Chemicals Ltd) (1µg/ml)	4.0 ml
Distilled water	1000 ml

Bring pH to 4.6 using 0.1M HCl after autoclaving for 20 min.

BENLATE/MALT EXTRACT AGAR

Malt extract (Danisco Cultor)	20 g
Agar (Danisco Cultor)	20 g
Benlate	0.02 g
Distilled water	1000 ml

Autoclave for 20 min, let cool down to 60°C. Add 0.1 g streptomycin sulfate or 2.5 ml of 40 mg/ml streptomycin stock/litre.

1.5% MALT EXTRACT AGAR

Malt extract (Danisco Cultor)	15 g
Agar (Danisco Cultor)	15 g
Distilled water	1000 ml

Autoclave for 20 min.

MODIFIED MELIN NORKRANS MEDIUM (MMN)

(Marx, D. H. (1969). *Phytopathology* 59 153-163.)

STOCKS

$CaCl_2$ $2H_2O$ (0.05g/l agar)

5 g in 100 ml distilled water; add 1 ml /litre agar

KH_2PO_4 (0.5g/l agar)

12.5 g in 200 ml distilled water; add 8 ml /litre agar

NaCl (0.025g/l agar)

2.5 g in 100ml distilled water; add 1 ml /litre agar

FeNaEDTA (2% solution)

2g in 100ml distilled water; add 1.2ml /litre agar

$(NH_4)_2HPO_4$ (0.25g/l agar)

25.0g in 100ml distilled water; add 1 ml /litre agar

Thiamine HCl (1% solution)

1 g in 100 ml distilled water; add 1 ml /litre agar

$MgSO_4$ $7H_2O$ (0.15g/l agar)

15 g in 100 ml distilled water; add 1 ml /litre agar

$CaCl_2$ $2H_2O$	0.05 g (1 ml stock)
NaCl	0.025 g (1 ml stock)
$(NH_4)_2HPO_4$	0.25 g (1 ml stock)
$MgSO_4$ $7H_2O$	0.15 g (1 ml stock)
KH_2PO_4	0.5 g (8 ml stock)
FeNaEDTA (2% solution)	1.2 ml
Thiamine HCl (1% solution)	1.0 ml
Glucose (BDH Chemicals Ltd)	2.5 g
Malt extract (Danisco Cultor)	10 g
Agar (Danisco Cultor)	15 g
Distilled water	1000 ml

Bring pH to 4.7 using 0.1M HCl after autoclaving for 20 min.

APPENDIX 2 IDENTIFYING LITERATURE

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APPENDIX 3 MOLECULAR PROTOCOLS

DNA EXTRACTION PROTOCOLS

DNA EXTRACTION FASTDNA® KIT

Q BIOgene Inc., Valencia, CA, USA

1. Place ceramic bead in Lysing Matrix A tube, add sample to the tube.
2. *For Fungal Tissue*: Add 1.0 ml CLS-Y solution (provided), place second ceramic bead to the tube.

   For Plant Tissue (ECM): Add 800 µl CSL-VF solution (provided) and 200 µl PPS solution (provided)
3. Homogenize in the FastPrep® Instrument for 20 sec at a speed setting of 4.00 three times, place on ice for 2 min between homogenizing steps.
4. Centrifuge at 14,000 x g for 15 min to pellet debris.
5. Transfer supernatant (about 650 µl) to a 2.0 ml microcentrifuge tube; add equal volume of binding matrix. Mix.

   Note: shake binding matrix well before and during use
6. Incubate for 5 min at room temperature.
7. Centrifuge at 14,000 x g for 1 min to pellet binding matrix. Discard supernatant.
8. Add 500 µl SEWS-M solution (provided). Gently resuspend the pellet with pipette tip.
9. Centrifuge at 14,000 x g for 1 min and discard supernatant.
10. Centrifuge at 14,000 x g for 10 sec and remove rest of residual liquid with a medium pipette.
11. Elute DNA by gently resuspending binding matrix in 100 µl of DES solution (provided). Incubate for 5 min at room temperature.
12. Centrifuge at 14,000 x g for 1 min. Transfer eluted DNA (about 100 µl) to a clean microcentrifuge tube.

CTAB EXTRACTION PROTOCOL

Gardes & Bruns (1993), modified

1. Add 300 µl 2x CTAB buffer to 1.5 ml centrifuge tube containing fresh or dried tissue.

2. Freeze (-20°C freezer for 5 min) and thaw (65°C water bath for 3 min) three times; crush tissue after final thaw with a micropestle.
3. Incubate at 65°C for 30 min.
4. Add 1 volume of chloroform, vortex.
5. Centrifuge for 15 min at 14,000 x g
6. Remove the aqueous phase and transfer to a new microcentrifuge tube.
7. Precipitate DNA with an equal volume of cold isopropanol (stored at -20°C) for 10 min.
8. Centrifuge at 14,000 x g for 10 min.
9. Discard supernatant, wash pellet with 600 µl 70% ice-cold ethanol solution.
10. Spin at 14,000 x g for 5 to 10 min.
11. Discard supernatant and invert tubes on a clean lint free tissue, dry in laminar flow.
12. Resuspend in 50 µl pure, distilled H_2O

CTAB Buffer:
2 % CTAB 2 g

1.4 M NaCl 28 ml of 5 M NaCl

100 mM Tris-HCl pH 8.0 10 ml of 1 M

20 mM EDTA 4 ml of 0.5 M

1 % PVP-40 1 gr

Make up to 200ml with distilled H_20, pH 8 and autoclave.

Add 0.2% β-mercaptoethanol, 20 µl per 10 ml solution

MODIFIED CTAB & PHENOL EXTRACTION METHOD (SAMBROOK ET AL. 1987)

1. Add 500 µl 2x CTAB to material in microcentrifuge tube, heat at 65°C for 10 min, freeze at -20°C for 10 min; repeat 1 – 2 times.
2. Macerate with sterile micropestle.
3. Incubate at 65°C for 10 min.
4. Centrifuge at 14,000 x g for 10 min.
5. Remove aqueous phase and transfer into new 1.5 ml microcentrifuge tube.

Next steps carried out in a fume hood:

6. Add equal volume (400 – 450 µl) phenol (Sigma-Aldrich, St. Louis, Missouri, USA), mix gently.
7. Centrifuge at 14,000 x g for 10 min.
8. Pipette top layer into new 1.5 ml microcentrifuge tube (do not disturb layers).
9. Add equal volume (400 -450 µl) chloroform : isoamylalcohol (24:1), mix gently.
10. Centrifuge at 14,000 x g for 10 min.
11. Remove aqueous phase and transfer into new 1.5 ml microcentrifuge tube.
12. Again, add equal volume (400 -450 µl) chloroform : isoamylalcohol (24:1), mix gently.
13. Centrifuge at 14,000 x g for 10 min.
14. Remove aqueous phase and transfer into new 1.5 ml microcentrifuge tube.
15. Add 1/10 3M natrium acetate and 2x Volume 95% Ethanol
16. Store at -20°C for 1hr.
17. Centrifuge at 4°C at 14,000 x g for 15 min.
18. Pour of supernatant.
19. Wash with 600 µl of ice cold 70% Ethanol.
20. Centrifuge at 4°C at 14,000 x g for 15 min.
21. Pour of supernatant, let pellet dry inverted on a lint free tissue in laminar flow.
22. Resuspend DNA in 100 µl TE.

DNEASY® PLANT MINI KIT

Qiagen Inc., Hilden, Germany

1. Grind fungal tissue under liquid nitrogen to a fine powder using mortar and pestle. Transfer the tissue powder to a 1.5 ml microcentrifuge tube.
2. Add 400 µl of Buffer AP1 and 4 µl of RNase A stock solution (20 µg/ml) to the fungal tissue. Vortex vigorously.
3. Incubate the mixture for 10 min at 65°C. Mix 2-3 times during incubation by inverting tube.
4. Add 130 µl of Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.
5. Centrifuge the lysate for 5 min at 14,000 x g.

6. Apply the lysate to the QIAshredder Mini Spin Column placed in a 2 ml collection tube and centrifuge for 2 min at 14,000 x g.
7. Place flow through into new tube without disturbing cell-debris pellet
8. Add 1.5 volumes of Buffer AP3/E to the cleared lysate and mix by pipetting.
9. Apply 650 µl of the mixture from above (incl. any precipitate) to the DNeasy® Mini Spin Colum sitting in a 2 ml collection tube. Centrifuge for 1 min at 10,000 x g and discard flow-through.
10. Repeat step 9 with remaining sample. Discard flow-through and collection tube.
11. Place DNeasy® Mini Spin Column in a new 2 ml collection tube, add 500 µl Buffer AW to the DNeasy® Mini Spin Column and centrifuge for 1 min at 10,000 x g. Discard flow-through and reuse the collection tube in step 12.
12. Add 500 µl Buffer AW to the DNeasy Mini Spin Column and centrifuge for 2 min at 14,000 x g to dry the membrane.
13. Transfer the DNeasy Mini Spin Column to a 1.5 ml microcentrifuge tube and pipette 100 µl of Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at 10,000 x g to elute.

    Repeat this step.

REDEXTRACT-N-AMP™ PLANT PCR KIT

Sigma, St. Louis, Missouri, USA

Extraction from ECM root tip material

1. Place ECM root tip into collection tube; break tip into 2 pieces (with pipette)
2. Add 50 µl of Extraction Solution. Vortex briefly. Make sure the root tip is covered by the extraction solution and soaked up.
3. Incubate at 95°C for 10 min.
4. Add 50 µl of Dilution Solution. Vortex.
5. Store the diluted ECM extract at 2-8°C.
6. For PCR – use a 1:10 dilution to avoid solidifying of the extract.

Extraction from Sporocarp material

1. Place small piece of clean sporocarp tissue into collection tube.
2. Add 100 µl of Extraction Solution. Vortex briefly. Make sure the root tip is covered by the extraction solution and soaked up.

3. Incubate at 95°C for 10 min.
4. Add 100 µl of Dilution Solution. Vortex.
5. Store the diluted sporocarp extract at 2-8°C.
6. For PCR – use a 1:10 dilution to avoid solidifying of the extract

PCR PRODUCT PURIFICATION

QIAQUICK® PCR PURIFICATION KIT

Qiagen Inc., Hilden, Germany

All centrifuge steps are at 14,000 x g

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. Place a QIAquick® spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick® column and centrifuge for 30-60 sec.
4. Discard flow-through, place the QIAquick® column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the QIAquick® column and centrifuge for 30-60sec.
6. Discard flow through and place the QIAquick® column back in the same tube. Centrifuge the column for an additional 1 min.
7. Place QIAquick® column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 50 µl Buffer EB to the centre of the QIAquick® membrane and centrifuge the column for 1 min. Alternatively, to increase DNA concentration, add 30 µl elution buffer to the centre of the QIAquick® membrane, let the column stand for 1 min, and then centrifuge.

GENELUTE PCR CLEAN-UP KIT

Sigma, St. Louis, Missouri, USA

1. Put GenElute Miniprep Binding Column into a collection tube. Add 500 µl of the Column Preparation Solution. Centrifuge at 10,000 x g for 1 min. Discard eluate.
2. Add 5 volumes (79 µl) of Binding Solution to 1 volume of the PCR reaction and mix, transfer solution into the binding column. Centrifuge at maximum speed for 1 min. Discard the eluate.
3. Apply 500 µl of diluted Wash Solution to the column and centrifuge at maximum speed for 1 min. Discad the eluate.

4. Centrifuge the column at maximum speed for 2 min, without any additional wash solution. Discard residual eluate and collection tube.
5. Transfer the column to a fresh 2 ml collection tube. Add 50 µl of Elution Solution. Centrifuge at 14,000 x g for 1 min. Store at -20°C.

CLONING PROTOCOL

pGEM®-T Easy Vector Cloning Protocol for Promega pGEM®-T and pGEM®-T Easy Vector System

Promega Corporation, Madison, USA

Cells used: XL-1 Blue MRF'KAN electroporation competent cells (grown from PCR-Script Amp Electroporation-Competent Cells Cloning Kit, Stratagene, La Jolla, CA, USA)

The following Protocol is based on the Promega pGEM®-T and pGEM®-T Easy Vector System, the transformation step has been altered to electroporation instead of heat/shock.

All recipes for solutions and reagents needed are listed at the end of the protocol.

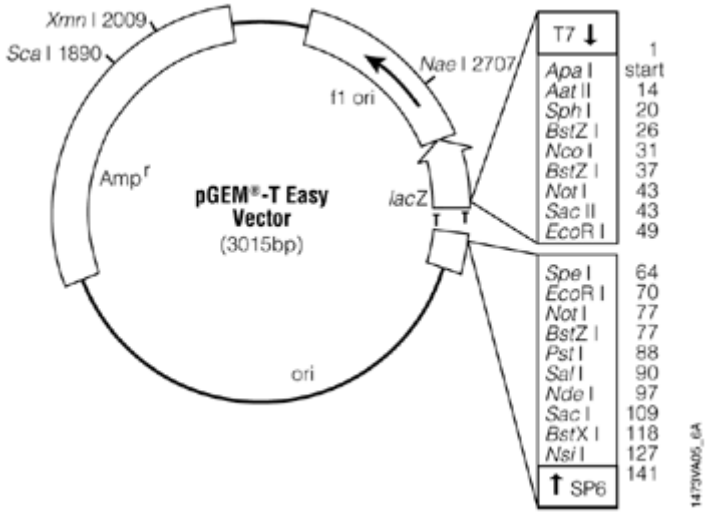

Figure A3-1: pGEM®-T Easy Vector circle map and sequence reference points (from www.promega.com)

LIGATION

- Briefly centrifuge the pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tubes.
- Vortex the 2X Rapid Ligation Buffer vigorously before each use.

- Set up ligation reactions in the following order:

Stock	1x (µl)
2X Rapid Ligation Buffer, T4 DNA Ligase	5.0
pGEM®-T Easy Vector (50 ng)	1.0
PCR Product	2.5
T4 DNA Ligase (3 Weiss units/µl)	1.0
H_2O	0.5
Total	10

- Mix the reactions by pipetting.
- Incubate the reactions 1 hr at room temperature.
- Alternatively, if a maximum number of transformants is required, incubate the reactions overnight at 4°C

TRANSFORMATION

The following steps have to be carried through on ice:

- Thaw XL-1 Blue MRF'KAN electroporation competent cells on ice (aliquoted to 40 µl in 1.5 ml tubes)
- Add 2 µl ligation reaction. Mix gently.
- Transfer to chilled BioRad 0.1 cm gap electroporation cuvette (Bio-Rad Laboratories, Hercules CA, USA). Tap to remove all bubbles, make sure bacteria are evenly distributed across the gap.
- Put tube in electroporator (Bio-Rad Laboratories GenePulser). Make sure that contacts are dry.
- Zap: voltage at 1700 V for field strength of 17 KV/cm, resistance at 200 Ω capacitor at 25 µF.
- Pulse once.
- Quickly remove cuvette and add 960 µl sterile SOC. Transfer to 1.5 ml tube.
- Put in shaker at 37°C, 230 r.p.m. for 1 hr (Eppendorf Thermomixer 5423, Hamburg, Germany).
- While incubating, add 100 µl SOC, X-gal and IPTG each to LB+Amp plates (recipes below).

- Put 100, 200 and 300 µl transformed cells on plate and spread.
- Incubate inverted at 37°C overnight.
- White colonies are successful transformants.

GROWING TRANSFORMANTS

- Put 6 µl Ampicillin (10 mg/ml) and 3 ml 2-YT in 15 ml tube.
- Pick a single white colony with sterile toothpick, swish in pre-prepared PCR reaction and then put in media.
- Shaker 200 rpm at 37°C overnight (innova™ 4080 Incubator Shaker, New Brunswick Scientific)
- Check for positive transformants by colony PCR:

Colony PCR Cocktail

Stocks	1 x (µl)
H_2O	8.11
10 x reaction buffer (Roche Applied Science, Penzberg, Germany)	2.001
dNTPs (0.2 mM each of dATP, dGTP, dCTP, dTTP (Roche Applied Science))	1.5
ITS-1F (1 µM)	0.15
ITS-4 (1 µM)	0.15
Taq (1.5 U, Roche Applied Science)	0.09
DNA template	n/a
Total Mix	12

PCR program: Initial step of 94°C for 5min, followed by 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 45 s, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 2 min, 13 cycles of 94°C for 35 s, 53°C for 55 s and 72°C for 3 min, followed by a final extension step of 72°C for 7 min on an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany).

STORING TRANSFORMANTS (OPTIONAL)

Mix 300 µl sterile glycerol with 700 µl bacteria and place at –20°C

PLASMID PURIFICATION

Harvesting:

- Place 1.5 ml of transformants in 1.5 ml microcentrifuge tube.
- Centrifuge at 14,000 x g for 5 min.
- Discard supernatant.
- Add rest of transformants into the microcentrifuge tube.
- Centrifuge at 14,000 x g for 5 min.

Purification – Lysis by alkali (modification of Birnboim and Doly 1979 and Ish-Horowicz and Burke 1981, in Sambrook *et al.* 1987):

- Resuspend the bacterial pellet in 100 µl of ice-cold Solution I by vigorous vortexing.
- Vortex two tubes simultaneously with their bases touching to ensure that the bacterial pellet is completely dispersed in Solution I (Store Solution I at 4°C).
- Add 200 µl of Solution II.
- Mix the contents by inverting the tubes rapidly five times. Make sure that the entire surface of the tube comes in contact with Solution II. Do not vortex.
- Store the tubes on ice.
- Add 150 µl of ice-cold Solution III.
- Vortex the tubes inverted for 10 sec. Store the tube on ice for 3-5 min.
- Centrifuge at 14,000 x g for 5 min at 4°C. Transfer the supernatant to a fresh tube.
- Precipitate the DNA with 2 volumes of 95% ethanol (900 µl) at room temperature. Mix by vortexing. Allow the mixture to stand for 2 min at room temperature.
- Centrifuge at 14,000 x g for 5 min at 4°C.
- Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a lint free paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.
- Rinse the pellet of DNA with 1 ml of 70% ethanol at 4°C. Remove the supernatant as described in step above. Allow the pellet to dry in the air for 10 min.
- Re-dissolve the nucleic acids in 50µl of TE containing DNAse free RNase (20 µg/ml) Vortex briefly. Store the DNA at -20°C.

- Check insert size with restriction digestion *EcoR*I:

Stocks	1 x (µl)
H_2O	6.15
Supplied buffer	1.25
Plasmid DNA	5.00
EcoR I (1 U)	0.1
Total Mix	12.5

Incubate at 37°C for 1 hr. Run on a 2% agarose gel (Invitrogen), at 80 Volt for 60 min. Stain for 10 min using ethidium bromide (12.5 µl of 1% EtBr in 500 ml distilled H_2O) and visualise under UV light (300 nm). Capture gel picture and measure band size (Bio-Rad Quantity One® Image Acquisition & Analysis Software (Bio-Rad Laboratories, Hercules CA, USA)). Determine product size by comparison to maker (Track-It™ 1 kb plus DNA ladder (Invitrogen)), Vector size ~3.5 kb.

REAGENTS FOR TRANSFORMATION

All filter sterilization of reagents is done with the Millex Syringe driven filter unit (Millipore Corporation, Bedrofd, USA).

Luria Bertani agar amended with ampicillin (LB-Amp)(1 litre)

10 g Bacto-tryptone (Becton, Dickson and Co., New Jersey, USA)

5 g Bacto-yeast extract (Becton, Dickson and Co.)

5 g NaCl

15 g Agar (Danisco Cultor, Dingley, Australia)

Adjust pH to 7.5 with 0.1 M HCl; autoclave for 20 min and let cool down to 55°C, then add 2.5 ml of 10 mg / ml Ampicillin (Sigma-Aldrich, filter sterilized). Pour plates (9 cm diameter, Biolab Ltd, Auckland, NZ) and store upside down.

SOC (100 ml):

2.0 g	Bacto-tryptone
0.5 g	Bacto-yeast extract
1 ml	1 M NaCl
0.25 ml	1 M KCl
1 ml	2 M $MgCl_2$ stock, filter-sterilized
1 ml	2 M glucose, filter-sterilized

Add Bacto-tryptone, Bacto-yeast, NaCl and KCl to 97ml distilled water and stir to dissolve. Autoclave for 20 min and let cool to room temperature before adding 2 M $MgCl_2$ stock and 2 M glucose, each to a final concentration of 20 mM. Make up to 100 ml with sterile distilled water and adjust to final pH 7.0 with 0.1 M HCl.

Stock solutions:

1 M NaCl	2.9 g dissolved in 50 ml distilled H_2O
1 M KCl	3.75 g dissolved in 50 ml distilled H_2O
2 M Glucose	3.60 g dissolved in 10 ml distilled H_2O

2-YT: liquid media, reconstitute as required.

X-gal: 100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Dissolve in 2 ml dimethylformamide and aliquot into 500 µl volumes in 1.5 ml tubes. Store at –20°C and dilute to a 2% solution for spreading on the plates.

IPTG (Isopropyl β-D-1-thiogalactopyranoside): 0.1 M (10x stock). Put 0.12 g in 15 ml tube and add 4.88 ml sterile distilled water. Filter sterilize and store at –20°C. Dilute to a 10% solution for spreading on the plates.

To prepare plates for transformed bacteria use LB-Amp plates and pipette 100µl of SOC onto them, add 100 µl of 2% X-gal and 100 µl of 10% IPTG and spread. Make up 30 min prior to plating.

REAGENTS FOR PLASMID DNA PURIFICATION

Solution I
50 mM Glucose

25 mM Tris HCl (pH 8)

10 mM EDTA (pH 8)

Autoclave for 20 min and store at 4°C.

Solution II
0.2 M NaOH (freshly diluted from a 10 N stock)

1% SDS

Do not autoclave, filter sterilize.

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

TE-Buffer
10 mM Tris, adjust to pH 8 using 0.1 M HCl

1 mM EDTA

PRIMERS USED IN THIS STUDY

ITS1F	5' CTTGGTCATTTAGAGGAAGTAA3'
ITS4	5' TCCTCCGCTTATTGATATGC3'
ITS4B	5' CAGGAGACTTGTACACGGTCCAG 3'
M13Forward	5' CCCAGTCACGACGTTGTAAAACG3'
M13Reverse	5' AGCGGATAACAATTTCACACAGG3'

APPENDIX 4 ECM SPECIES AND TYPE DESCRIPTIONS

The position in the classification hierarchy for all species is based on Ainsworth and Bisby's Dictionary of Fungi, 9th Edition (2001), morphological descriptions of species are based on NZ Fungi database (http://nzfungi.landcareresearch.co.nz/html/mycology.asp) and literature used for species identification as applicable from Appendix 2. Photographs, unless otherwise stated, were taken by the author and are from the sporocarp and soil core assessments in 2005 and 2006.

The restriction enzyme *Alu*I gave rise to many partial digests, marked in italics and red in the RFLP tables for each species. Only bands greater than 170 bp in size were generally seen on the agarose gels.

AMANITA MUSCARIA (L.) LAM. (1783)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Pluteaceae; Genus Amanita

Figure A4-1: *Amanita muscaria* sporocarp (left) and colonising *P. radiata* root tip (right).

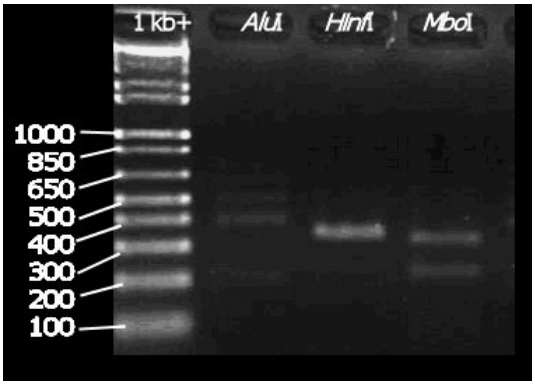

Figure A4-2: RFLP banding pattern for *Amanita muscaria* sporocarp (K80S5) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-1: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Amanita muscaria*.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S5 [a]	711	*is*	19, 105, 197, 390	8, 347, 356	60, 97, 224, 330
			g	201, 387, *484*	334	208, 310
ECM	K91C37T357	711	*is*	19, 104, 198, 391	8, 348, 356	59, 98, 225, 330
	K80C31T259		*g*	225, 416, *512*	362	237, 342

a- This sequences appeared to be from mixed PCR products. To gain a full sequence for *in silico* RFLP analysis the sequence from the ITS1F and ITS4 primer was aligned with the GenBank result and consensus bases assigned.

Main morphological sporocarp characteristics:

Detailed description in: Ridley, G.S. (1991). The New Zealand species of *Amanita* (Fungi: Agaricales). *Australian Systematic Botany* 4(2): 325-354

Main ECM colonising root tips characteristics:

Silver-white, tortuous, frequent irregularly branched.

CENOCOCCUM GEOPHILUM FR. (1829)

Position in classification hierarchy: Phylum Ascomycota; Class Ascomycetes; Order Dothideomycetes; Genus Cenococcum

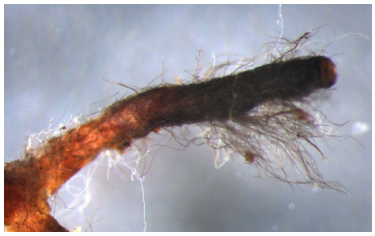

Figure A4-3: *Cenococcum geophilum* colonising *P. radiata* root tip.

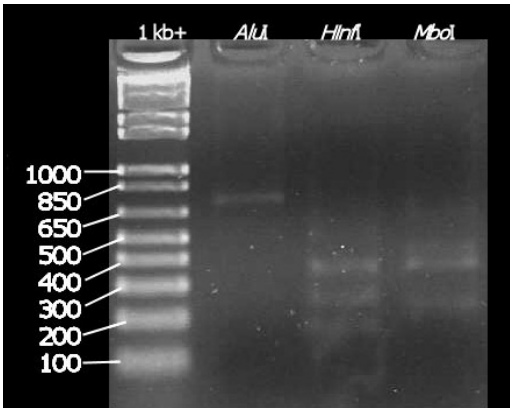

Figure A4-4: RFLP banding pattern for *Cenococcum geophilum* ECM (K91C31T307) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-2: RFLP gel (*g*) banding patterns for *Cenococcum geophilum*. No *in silico* cut sites available.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K91C31T307b	n/a	*g*	*752*	197, 268, 354	239, 366

Main ECM colonising root tips characteristics:
Black, straight, not branched, woolly texture with emananting black hyphal fans.

CHALCIPORUS PIPERATUS (BULL.) BATAILLE (1908)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Boletaceae; Genus Chalciporus

Figure A4-5: Sporocarp of *Chalciporus piperatus.*

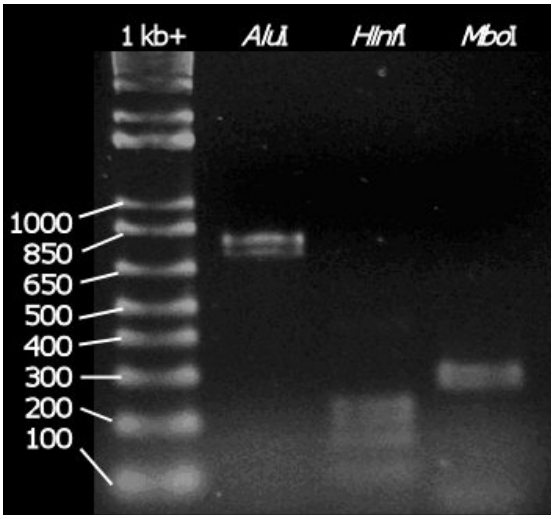

Figure A4-6: RFLP banding pattern for *Chalciporus piperatus* ECM (K80S25) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-3: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Chalciporus piperatus*.

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S25	786	*is*	59, 727	8, 62, 111, 165, 205, 235	10, 53, 60, 76, 280, 307
			g	744, *809*	120, 178, 236	295, 314

Main morphological sporocarp characteristics:

Detailed description in: McNabb, R.F.R. (1968). The Boletaceae of New Zealand. *New Zealand Journal of Botany* 6(2): 137-176

HEBELOMA SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Bolbitiaceae; Genus Hebeloma

Figure A4-7: *Hebeloma* sp. sporocarp (left) and colonising *P. radiata* root tip (right).

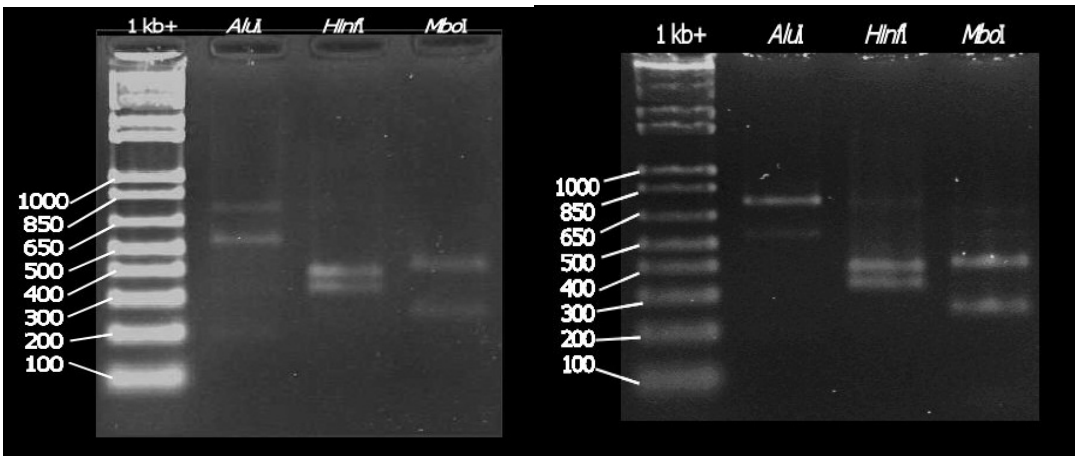

Figure A4-8: RFLP banding pattern for *Hebeloma* sp. sporocarp (K06S4, left) and ECM (K06C5T156a, right) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-4: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Hebeloma* sp.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K06S1	731	*is*	191, 211, 329	8, 289, 362	30, 260, 369
			g	*547, 746*	337, 395	266, 425
ECM	K06C5T156	733	*is*	190, 213, 330	8, 331, 394	60, 262, 411
			g	*553, 753*	344, 407	273, 435

Main morphological sporocarp characteristics:
Cap white, 3-6cm, convex then expanded with low umbo, margin inrolled when young; cuticle smooth and slightly viscid when moist. Gills sinuate, whitish, stem short, stout, white with powdery apex. Spores cigar-brown. Strong smell of radish.

Main ECM colonising root tips characteristics:
Frosty white, straight, irregular branched, cottony surface, net of white hyphae in flat angle.

INOCYBE LACERA (FR.) P. KUMM. (1871)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Cortinariaceae; Genus Inocybe

Figure A4-9: *Inocybe lacera* sporocarps.

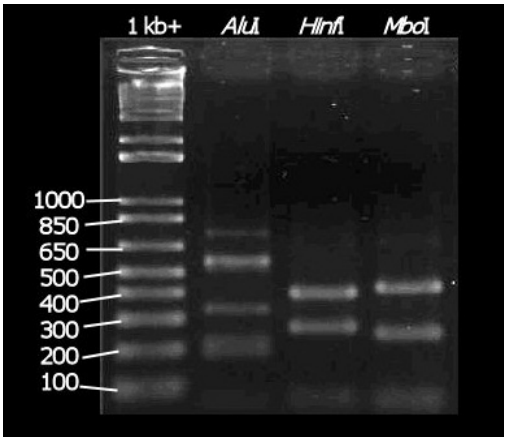

Figure A4-10: RFLP banding pattern for *Inocybe lacera* sporocarp (K04S3) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-5: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Inocybe lacera*.

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K04S3	722	*is*	182, 214, 326	8, 74, 258, 382	20, 60, 230, 412
			g	214, 334, *555, 734*	272, 393	253, 419

Main morphological sporocarp characteristics:

Detailed description in: Kuo, M. (2005, February). *Inocybe lacera*. Retrieved from the MushroomExpert.Com Web site: http://www.mushroomexpert.com/inocybe_lacera.html

INOCYBE SINDONIA (FR.) P. KARST. (1879)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Cortinariaceae; Genus Inocybe

Figure A4-11: *Inocybe sindonia* sporocarps.

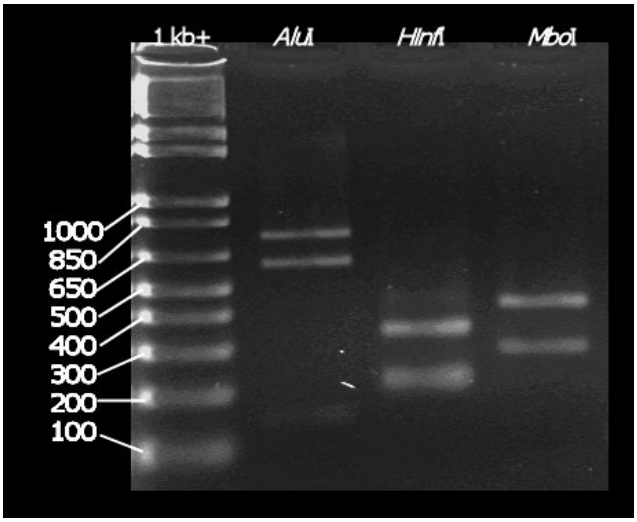

Figure A4-12: RFLP banding pattern for *Inocybe sindonia* sporocarp (K80S56) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-6: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Inocybe sindonia.*

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S33	731	*is*	144, 587	5, 8, 187, 209, 322	60, 269, 402
			g	*617, 775*	208, 336	321, 463

Main morphological sporocarp characteristics:

Detailed description in: Karsten, P.A., 1879, Bidrag till Kännedom of Finlands Natur Folk 32: 465

INOCYBE SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Cortinariaceae; Genus Inocybe

Figure A4-13: *Inocybe* sp. sporocarp (left) and colonising *P. radiata* root tip (right).

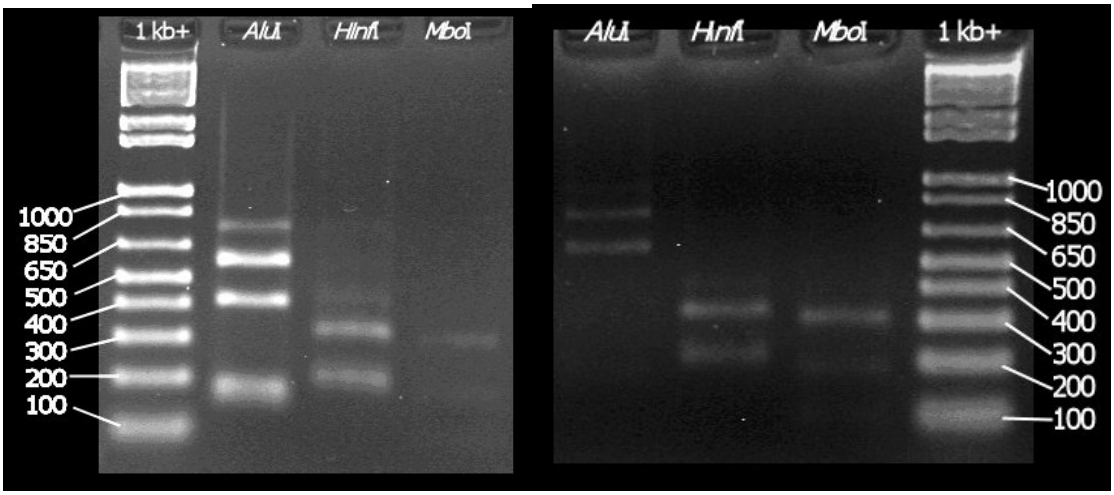

Figure A4-14: RFLP banding pattern for *Inocybe* sp. sporocarp (K98S25) and ECM (K91C35T344) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-7: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Inocybe* sp.

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K98S25	745	*is*	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
			g	180, 409, *574, 757*	200, 319, 414	185, 291
ECM	K91C37T359	745	*is*	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
	K91C37T361		*g*	*584, 743*	215, 327, 452	190, 302

Main ECM colonising root tips characteristics:
Velvet-white to milky yellow, dichotomous, straight, no rhizomorphs.

LACCARIA PROXIMA (BOUD.) PAT. (1887)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Hydnangiaceae; Genus Laccaria

Figure A4-15: *Laccaria proxima* sporocarps.

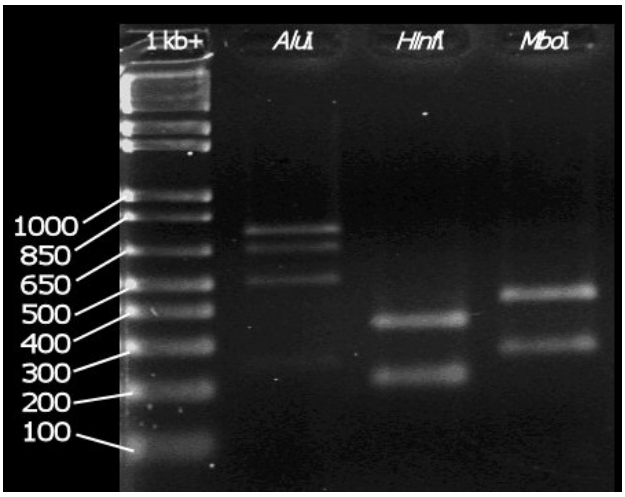

Figure A4-16: RFLP banding pattern for *Laccaria proxima* sporocarp (K04S11) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-8: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Laccaria* sp.*:*

Source	Label	Uncut (bp)		*AluI* fragment length (bp)	*HinfI* fragment length (bp)	*MboI* fragment length (bp)
Sporocarp	K04S5	730	*is*	15, 94, 96, 128, 397	8, 191, 202, 329	60, 261, 409
			g	*245, 505, 644, 764*	209, 352	277, 439

Main morphological sporocarp characteristics:

Detailed description in: McNabb, R.F.R. (1972). The Tricholomataceae of New Zealand. 1. *Laccaria* Berk. & Br. *New Zealand Journal of Botany* 10(3): 461-484.

LACTARIUS RUFUS (SCOP.) FR. (1838)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Russulales; Family Russulaceae; Genus Lactarius

Figure A4-17: *Lactarius rufus* sporocarps.

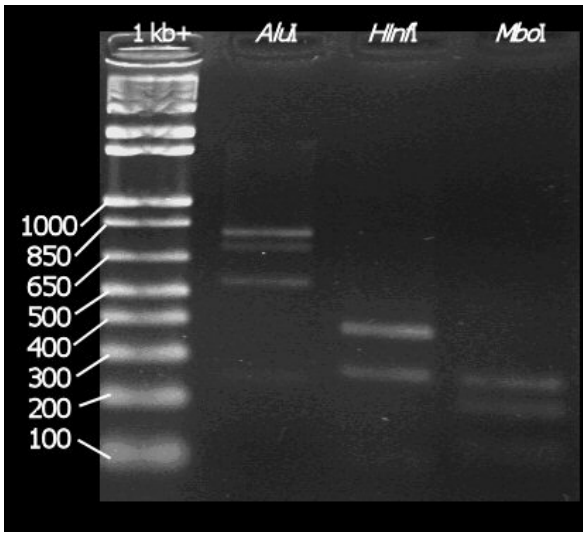

Figure A4-18: RFLP banding pattern for *Lactarius rufus* sporocarp (K80S60) on 2% agarose gel with AluI, HinfI and MboI restriction enzymes.

Table A4-9: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Lactarius rufus*.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S7	684	*is*	79, 162, 522	8, 108, 264, 383	60, 106, 134, 206, 257
			g	522, *683, 758*	268, 379	206, 256

Main morphological sporocarp characteristics:
Dark red-brown cap, 4-8 cm, convex then soon expanded and often depressed. Gills adnate-decurrent, red. Stem hollow, colour as cam. Milking when wounded.

LYCOPERDON SPP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Lycoperdaceae; Genus Lycoperdon

Figure A4-19: *Lycoperdon* sp. sporocarp.

Main morphological sporocarp characteristics:
Small puffballs, characterised by a cellular sterile base, flesh white when young, powdery and brown when mature, opening through a well-defined pore.

No RFLP pattern applicable.

PSEUDOTOMENTELLA SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Thelephorales; Family Thelephoraceae; Genus Pseudotomentella

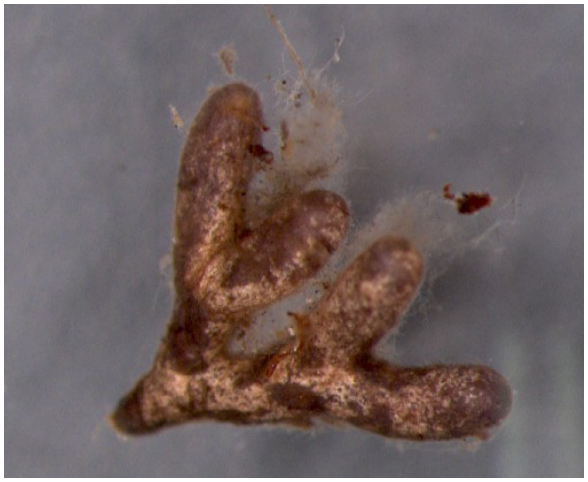

Figure A4-20: *Pseudotomentella* sp. colonising *P. radiata* root tips.

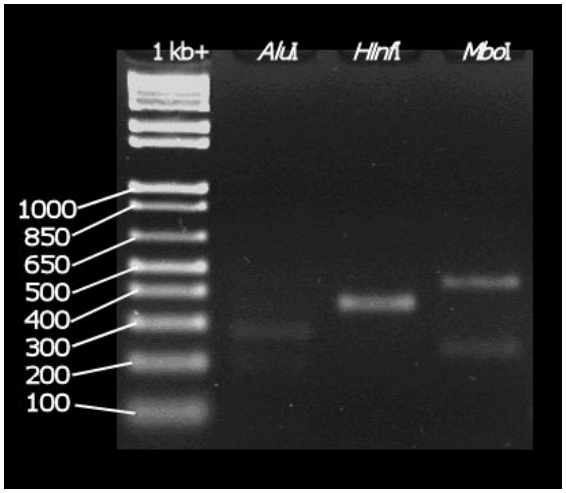

Figure A4-21: RFLP banding pattern for *Pseudotomentella* sp. ECM (K98C35T239) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-10: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Pseudotomentella* sp.

ECM species/type	Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Pseudotomentella sp.	ECM	K98C35T239	721	*is*	16, 68, 75, 96, 193, 273	8, 349, 364	60, 232, 429
				g	212, 278	359	239, 439

Main ECM colonising root tips characteristics:

Black with white patches, dichotomous, straight, short, net of dark hyphae in flat angle.

PSEUDOTOMENTELLA TRISTIS (P. KARST.) M.J. LARSEN (1971)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Thelephorales; Family Thelephoraceae; Genus Pseudotomentella

Figure A4-22: *Pseudotomentella tristis* colonising *P. radiata* root tips.

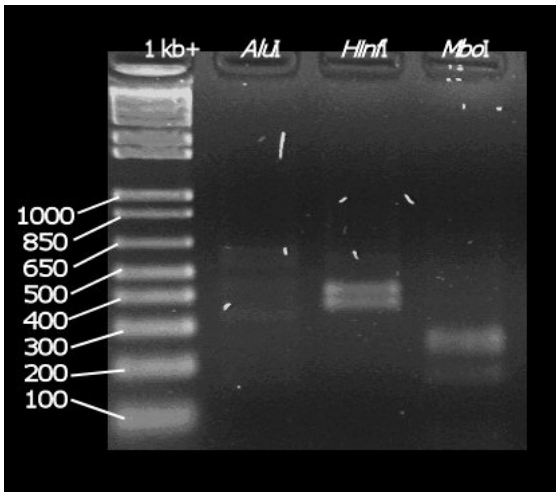

Figure A4-23: RFLP banding pattern for *Pseudotomentella* tristis ECM (K91C38T363) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-11: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Lactarius rufus:*

ECM species/type	Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Pseudotomentella tristis	ECM	K91C38T363	789	*is*	19, 32, 43, 64, 69, 96, 149, 316 336	8, 366, 414	60, 64, 170, 234, 260
				g	*417, 533, 595*	364, 410	178, 253

Main ECM colonising root tips characteristics:
Frosty white with dark patches, dichotomous, straight, short, net of white hyphae in flat angle, rhizomorphs

RHIZOPOGON LUTEOLUS FR. (1817)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Rhizopogonaceae; Genus Rhizopogon

Figure A4-24: *Rhizopogon luteolus* hypogeous sporocarp htttp//www.bioimages.org.uk/html/P6/P60126.php.

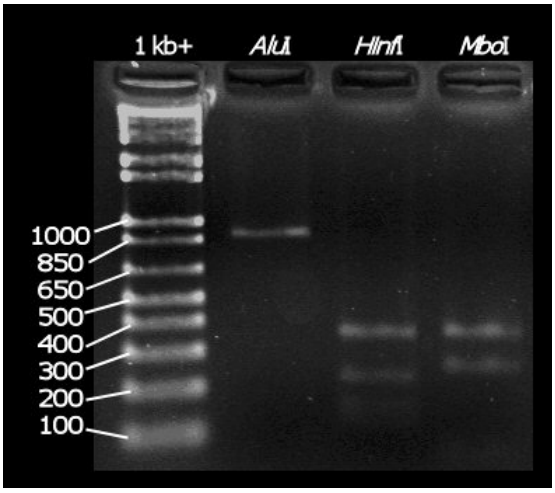

Figure A4-25: RFLP banding pattern for *Rhizopogon luteolus* sporocarp (K80S64) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-12: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Rhizopogon luteolus:*

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S64	889	*is*	67, 221, 601	7, 8, 128, 159, 228, 359	60, 62, 74, 78, 254, 361
			g	243, 639, *309, 701*	192, 258, *406*	295, *410*

Main morphological sporocarp characteristics:

Detailed description in: Beaton, G.W.; Pegler, D.N.; Young, T.W.K. (1985). Gasteroid Basidiomycota of Victoria State, Australia: 5–7. *Kew Bulletin* 40(3): 573–598.

RHIZOPOGON LUTEORUBESCENS A.H. SM. (1966)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Rhizopogonaceae; Genus Rhizopogon

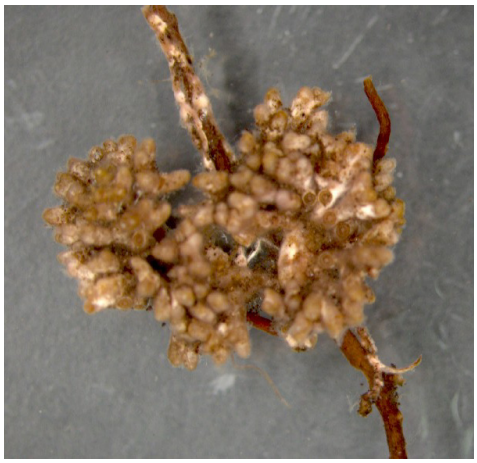

Figure A4-26: *Rhizopogon luteorubescens* colonising *P. radiata* roots.

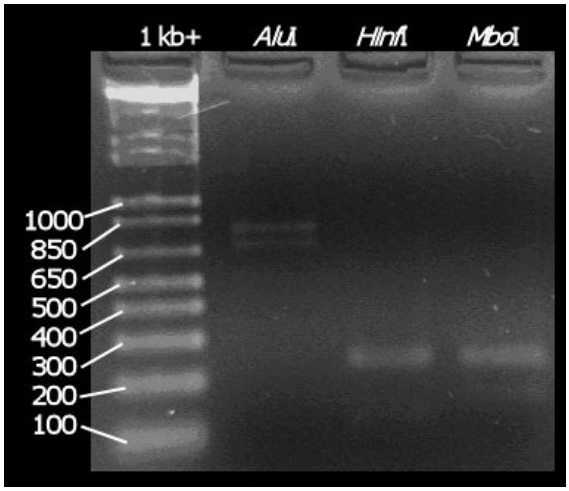

Figure A4-27: RFLP banding pattern for *Rhizopogon luteorubescens* ECM (K91S31T310) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-13: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Rhizopogon luteorubescens*:

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K98C31T310	762	*is*	94, 668	11, 23, 112, 137, 236, 243	60, 62, 161, 234, 245
			g	*768*	240	247

Main ECM colonising root tips characteristics:
Frosty white, dichotomous, coralloid or irregular; white rhizomorphs.

RHIZOPOGON PSEUDOROSEOLUS A.H. SM. (1966)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Rhizopogonaceae; Genus Rhizopogon

Figure A4-28: *Rhizopogon pseudoroseolus* hypogeous sporocarp (left) and colonising *P. radiata* root tips.

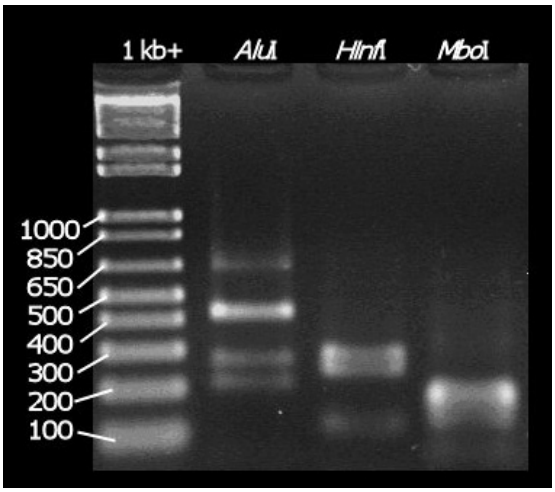

Figure A4-29: RFLP banding pattern for *Rhizopogon pseudoroseolus* sporocarp (K98S35) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-14: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Rhizopogon pseudoroseolus.*

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K98S35	765	*is*	94, 277, 394	8, 23, 112, 144, 235, 243	60, 62, 160, 238, 245
			g	284, 371, *490, 671, 752*	156, 241	163, 235
ECM	K98C31T213	765	*is*	94, 277, 394	8, 23, 112, 144, 235, 243	60, 62, 160, 238, 245
			g	280, *494, 685, 774*	265	238

Main ECM colonising root tips characteristics:
Frosty white, dichotomous or short and coralloid; white thick rhizomorphs.

RHIZOPOGON RUBESCENS (TUL. & C. TUL.) TUL. & C. TUL. (1844)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Rhizopogonaceae; Genus Rhizopogon

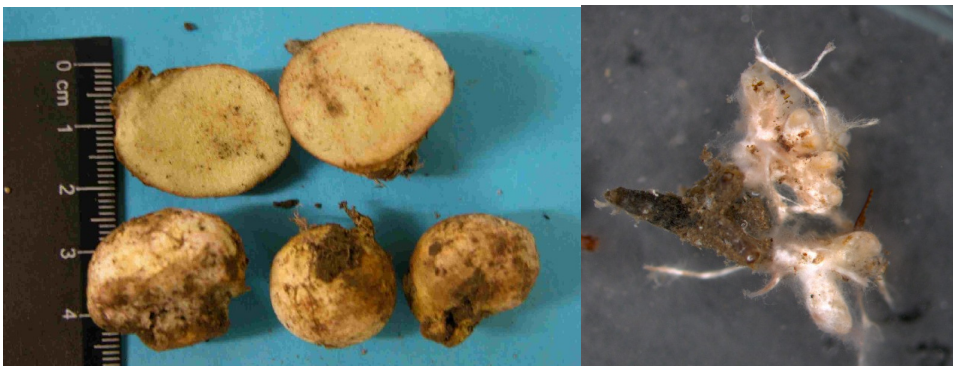

Figure A4-30: *Rhizopogon rubescens* hypogeous sporocarp (left) and colonising *P. radiata* root tips (right).

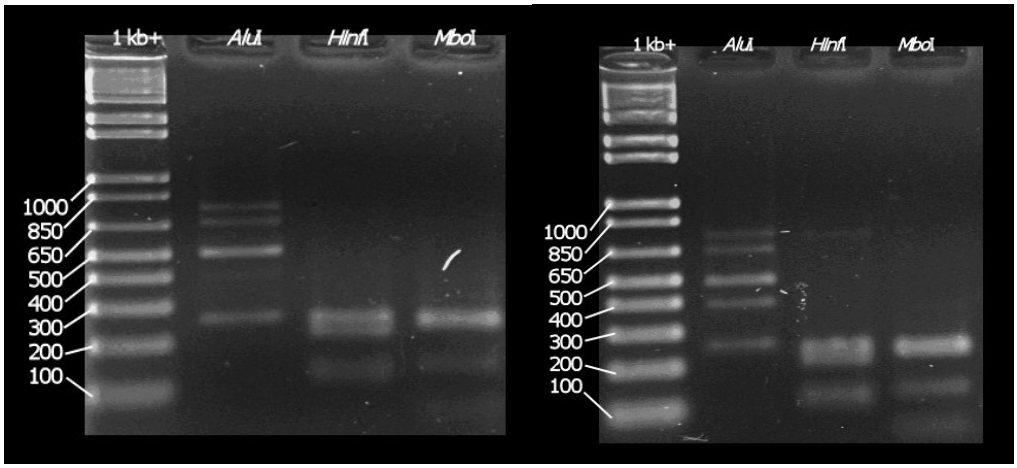

Figure A4-31: RFLP banding pattern for *Rhizopogon rubescens* sporocarp (K06S2) and ECM (K06C3T141) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-15: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Rhizopogon rubescens*.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K04S6	737	*is*	94, 251, 392	8, 11, 23, 113, 129, 210, 243	60, 62, 135, 234, 246
			g	257, *499, 650, 758*	215, 239	171, 274
ECM	K06C3T138	738	*is*	94, 252, 392	8, 11, 23, 113, 129, 211, 243	60, 62, 136, 234, 246
			g	271, *500, 759*	215, 242	124, 227

Main morphological sporocarp characteristics:

Detailed description in: Detailed description in: Beaton, G.W.; Pegler, D.N.; Young, T.W.K. (1985). Gasteroid Basidiomycota of Victoria State, Australia: 5–7. *Kew Bulletin* 40(3): 573–598.

Main ECM colonising root tips characteristics:

Frosty white, tortuous, dichotomous branching, white rhizomorphs.

SCLERODERMA BOVISTA FR. (1829)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Sclerodermataceae; Genus Scleroderma

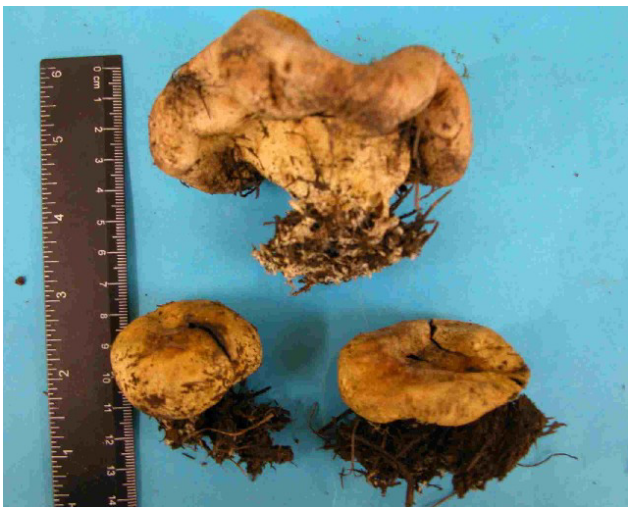

Figure A4-32: *Scleroderma bovista* sporocarp.

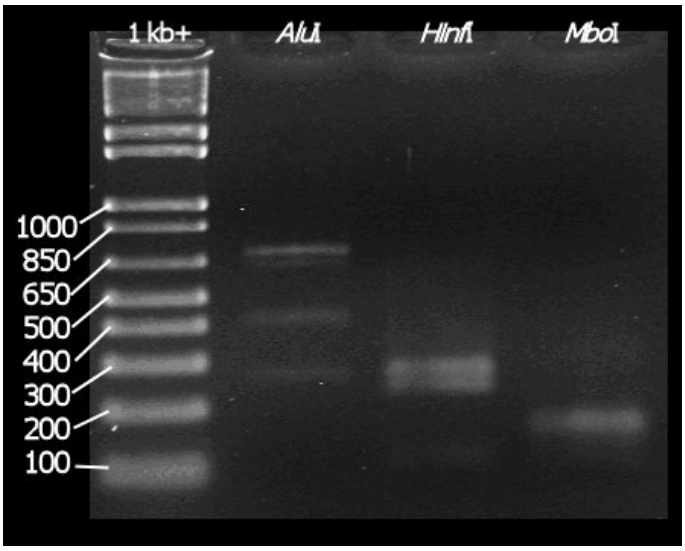

Figure 33: RFLP banding pattern for *Scleroderma bovista* sporocarp (K04S14) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-16: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Scleroderma bovista.*

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K80S9 [a]	699	*is*	18, 59, 212, 410	8, 37, 116, 252, 286	12, 60, 124, 152, 173, 178
			g	218, 431*, 273, 651*	120, 250, 273	182

a- This sequences appeared to be from mixed PCR products. To gain a full sequence for *in silico* RFLP analysis the sequence from the ITS1F and ITS4 primer was aligned with the GenBank result and consensus bases assigned.

Main morphological sporocarp characteristics:

Detailed description at: Kuo, M. (2006, February). *Scleroderma bovista.* Retrieved from the *MushroomExpert.Com* Web site: http://www.mushroomexpert.com/scleroderma_bovista.html

SUILLUS SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Boletales; Family Suillaceae; Genus Suillus

Figure A4-34: *Suillus granulatus* sporocarps. From: http://nzfungi.landcareresearch.co.nz/html/data.asp?TID=&ID=&NAMEPKey=11079.

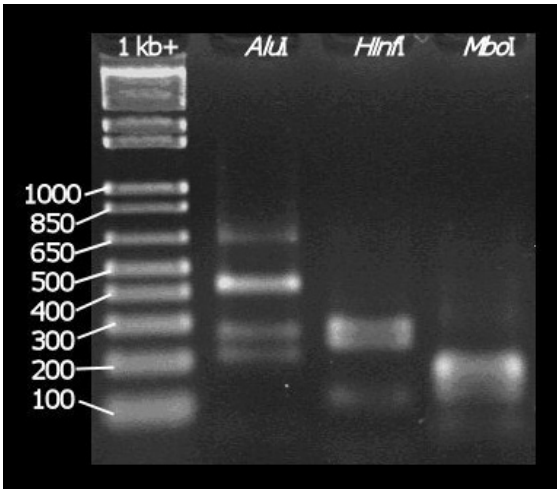

Figure A4-35: RFLP banding pattern for *Suillus* sp. sporocarp (K91S8) on 2% agarose gel with AluI, HinfI and MboI restriction enzymes.

Table A4-17: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Suillus* sp.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K98S8	723	*is*	109, 614	8, 11, 23, 37, 74, 92, 124, 129, 225	60, 62, 69, 141, 157, 234
			g	610, *738*	124, 230	147, 230

Main morphological sporocarp characteristics:
Detailed description in: McNabb, R.F.R. (1968). The Boletaceae of New Zealand. *New Zealand Journal of Botany* 6(2): 137-176

THELEPHORA TERRESTRIS EHRH. (1787)

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Thelephorales; Family Thelephoraceae; Genus Thelephora

Figure A4-36: *Thelephora terrestris* sporocarp (left) and colonising *P. radiata* root tips (right).

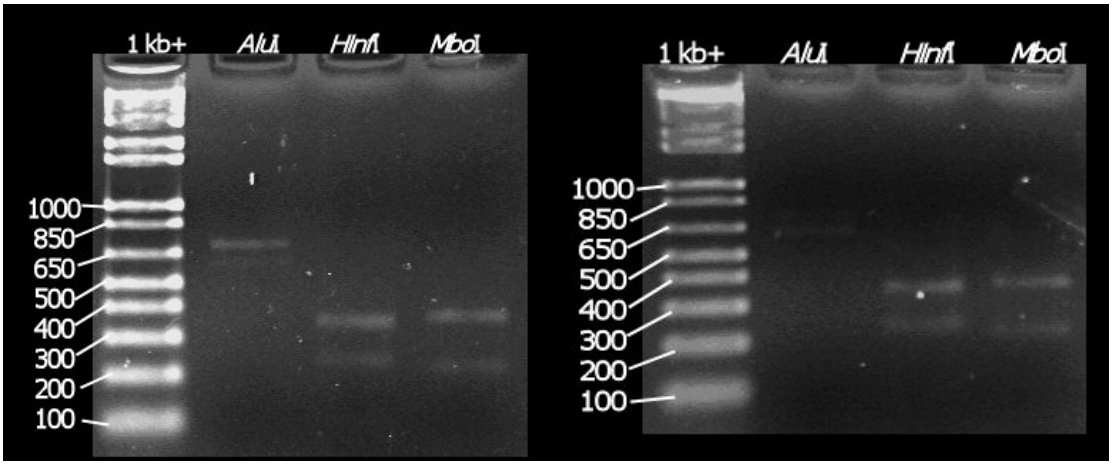

Figure A4-37: RFLP banding pattern for *Thelephora terrestris* sporocarp (K80S62) and ECM (K80C32T273) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-18: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Thelephora terrestris*.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Sporocarp	K91S27	701	*is*	30, 70, 601	8, 100, 241, 352	56, 60, 220, 365
			g	594, *370, 692*	238, 351	219, 367
ECM	K98C34T230	700	*is*	30, 69, 601	8, 100, 240, 352	55, 60, 220, 365
			g	*724*	226, 335	208, 347

Main morphological sporocarp characteristics:
Deep brown, fringed, fan-like bodies on soil and leaf litter. No gills or pores, spores being produced on the smooth to wrinkled surface.

Main ECM colonising root tips characteristics:
Milky white-skin colour, irregular, straight.

TOMENTELLA SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Thelephorales ; Family Thelephoraceae; Genus Tomentella

Figure A4-38: *Tomentella* sp. colonising *P. radiata* root tips

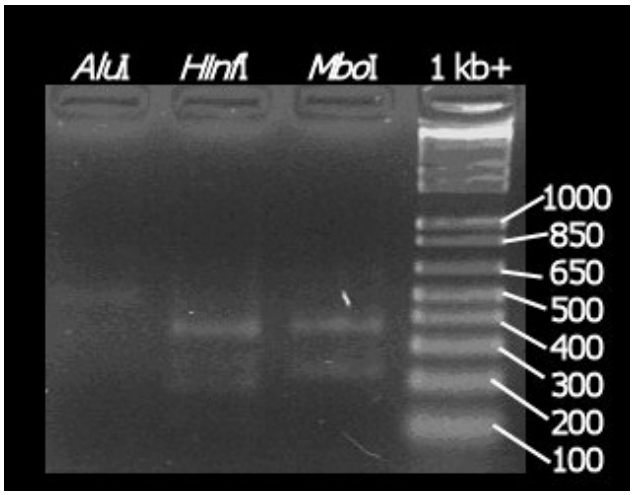

Figure A4-39: RFLP banding pattern for *Tomentella* sp. ECM (K80C37T324) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-19: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Tomentella* sp.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K80C37T323	700	*is*	30, 54, 67, 91, 458	8, 144, 177, 177, 194	55, 60, 363
			g	478	202, *351*	375, *242*

Main ECM colonising root tips characteristics:
Brown to black-green with white apices, dichotomous, straight, velvety-flake structure, some hyphae at base.

TRICHOLOMA SP.

Position in classification hierarchy: Phylum Basidiomycota; Class Basidiomycetes; Order Agaricales; Family Tricholomataceae; Genus Tricholoma

Figure A4-40: *Tricholoma* sp. sporocarp.

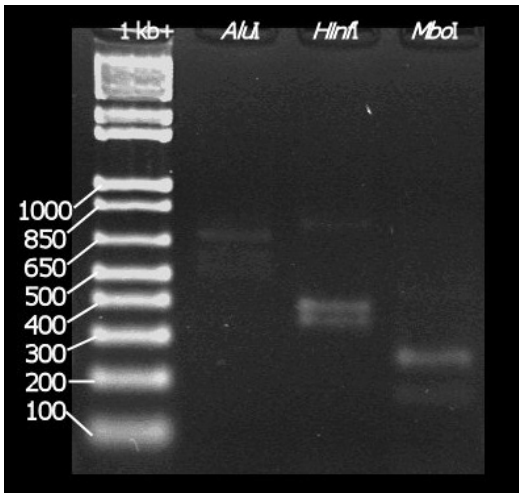

Figure A4-41: RFLP banding pattern for *Tricholoma* sp. (K91S9) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-20: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Tricholoma* sp.

Source	Label	Product Length (bp)	*Alu*I Fragment length (bp)	*Hinf*I Fragment length (bp)	*Mbo*I Fragment length (bp)
Sporocarp	K91S9	640	28, 40, 69, 503	8, 247, 385	52, 152, 175, 261

Main morphological sporocarp characteristics:
See Stevenson, G. (1964). The Agaricales of New Zealand: V. *Kew Bulletin* 19(1): 1-59.

TUBER SP.

Position in classification hierarchy: Phylum Ascomycota; Class Ascomycetes; Order Pezizales; Family Tuberaceae; Genus Tuber

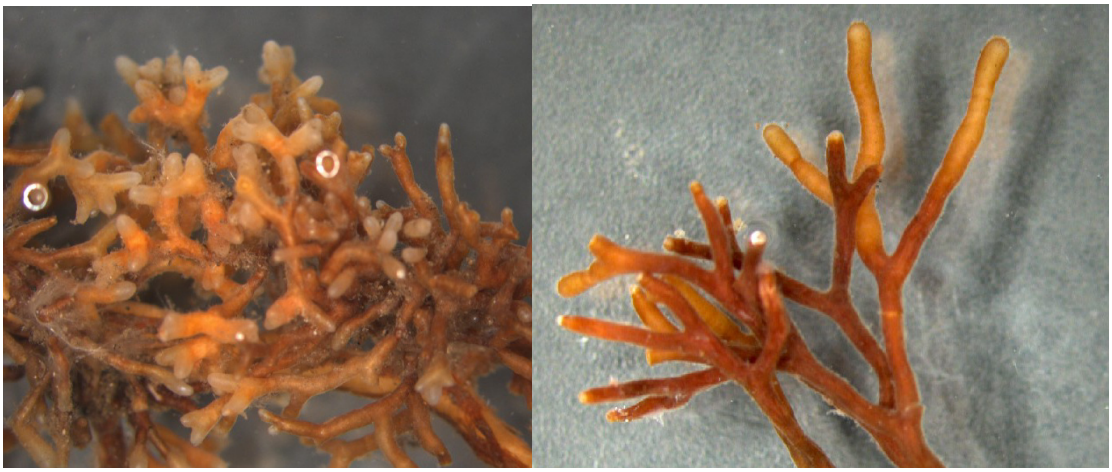

Figure A4-42: *Tuber* sp. colonising *P. radiata* root tips.

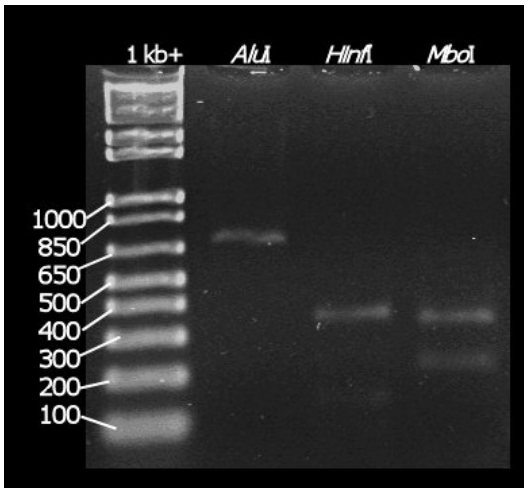

Figure A4-43: RFLP banding pattern for *Tuber* sp. ECM (K06FC8T13) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-21: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Tuber* sp.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K06FC8T13	688	*is*	no cut site	8, 70, 101, 147, 362	49, 60, 230, 349
			g	*715*	364	220, 331

Main ECM colonising root tips characteristics:
Clear to honey brown with white apices, smooth, dichotomous, no rhizomorphs.

WILCOXINA MIKOLAE (CHIN S. YANG & H.E. WILCOX) CHIN S. YANG & KORF (1985)

Position in classification hierarchy: Phylum Ascomycota; Class Ascomycetes; Order Pezizales; Family Pyronemataceae; Genus Wilcoxina

Figure A4-44: *Wilcoxina mikolae* fruiting bodies and colonising *P. radiata* root tips.

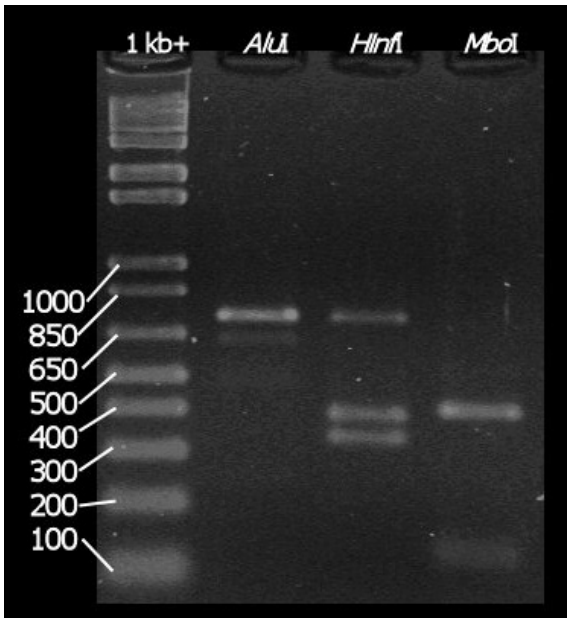

Figure A4-45: RFLP banding pattern for *Wilcoxina mikolae* ECM (K04C38T193) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-22: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Wilcoxina mikolae*.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K04C38T193	636	*is*	no cut site	8, 161, 183, 284	49, 60, 97, 212, 218
			g	*644, 745*	340, 395, *745*	410

Main ECM colonising root tips characteristics:

Milky white when young, brown with white apices with age, unbranched to irregular branched, no hyphae or rhizomorphs.

TYPE *PEZIZACEAE* SP.

Position in classification hierarchy: Phylum Ascomycota; Class Ascomycetes; Order Pezizales; Family Pezizaceae

Figure A4-46: Type *Pezizaceae* colonising *P. radiata* root tips.

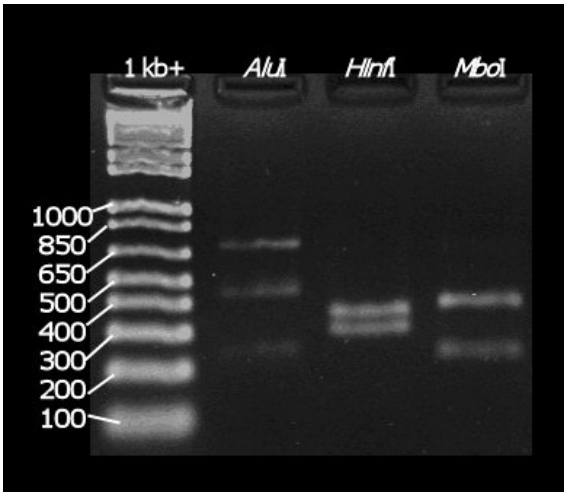

Figure A4-47: RFLP banding pattern for Type *Pezizaceae* ECM (K06FC8T13) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-23: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for *Pezizaceae* ECM.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K06FC10T18	681	*is*	206, 232, 243	8, 9, 304, 360	60, 237, 384
			g	256, *442, 699*	312, 370	252, 409

Main morphological sporocarp characteristics:

Clear-white, dichotomous branched, short, straight no rhizomorphs.

TYPE UNKNOWN BASIDIOMYCETE

Position in classification hierarchy: n/a

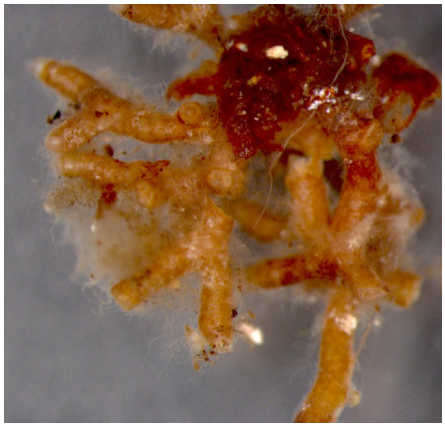

Figure A4-48: Type unknown Basidiomycete colonising *P. radiata* root tips.

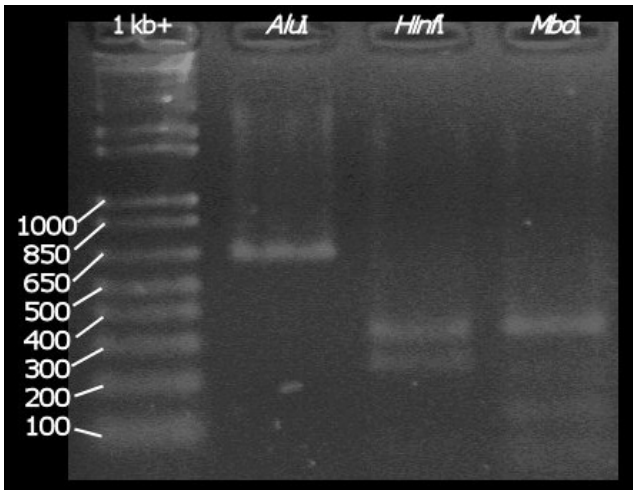

Figure A4-49: RFLP banding pattern for Type unknown Basidiomycete ECM (K06FC8T13) on 2% agarose gel with *Alu*I, *Hinf*I and *Mbo*I restriction enzymes.

Table A4-24: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for Type unknown Basidiomycete.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K04C35T201	722	*is*	10, 15, 94, 95, 128, 380	8, 328, 386	60, 121, 133, 408
			g	*625*	174, 275	197

Main ECM colonising root tips characteristics:

Brown with frosty white patches, white apices, irregular branching, straight, cottony surface with hyphal fans.

TYPE UNKNOWN 2

Position in classification hierarchy: n/a

Figure A4- 50: Type unknown 2 colonising *P. radiata* root tips.

Main ECM colonising root tips characteristics:
Black, irregular, tortuous.

TYPE UNKNOWN 8

Position in classification hierarchy: n/a

Figure A4-51: Type unknown 8 colonising *P. radiata* root tips

Table A4-25: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for Type unknown 8.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K80C33T282	769	*is*	183, 586	93, 142, 171, 363	60, 188, 231, 290
			g	400	356	330, 476

Main ECM colonising root tips characteristics:
Velvety black to purple, smooth, dichotomous

TYPE UNKNOWN 9

Position in classification hierarchy: n/a

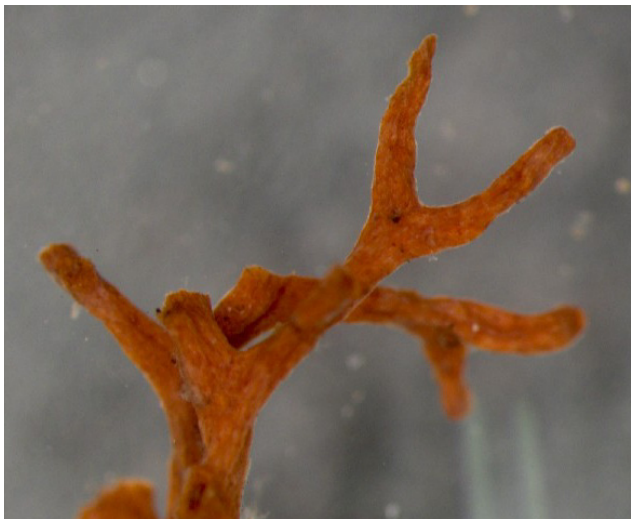

Figure A4-52: Type unknown 9 colonising *P. radiata* root tips.

Table A4-26: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for Type unknown 9.

Source	Label	uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K80C32T280	636	*is*	no cut site	8, 66, 241, 321	49, , 58, 60, 130, 339
			g	501	276, 387	144, 226, 274

Main ECM colonising root tips characteristics:
Red-brown, thin, irregular, tortuous, smooth

TYPE UNKNOWN 10

Position in classification hierarchy: n/a

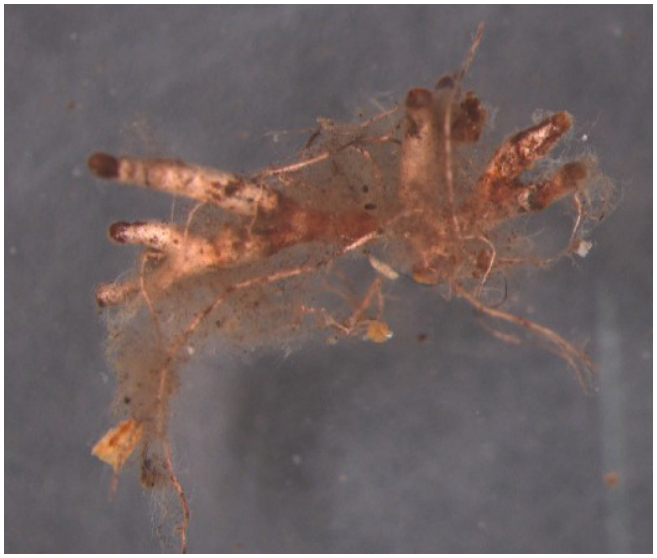

Figure A4-53: Type unknown 10 colonising *P. radiata* root tips.

Table A4-27: RFLP *in silico* (*is*) cut sites and gel (*g*) banding patterns for Type unknown 10.

Source	Label	Uncut (bp)		*Alu*I fragment length (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
ECM	K80C32T269	632	*is*	183, 449	66, 248, 318	49, 50, 60, 185, 288
			g	No band	388	255

Main ECM colonising root tips characteristics:

Dirty white with dark patches, dichotomous, straight, short, net of white hyphae in flat angle, rhizomorphs.

TYPE UNKNOWN 12

Position in classification hierarchy: n/a

Main ECM colonising root tips characteristics:

Purple-iridescent, dichotomous, tortuous, smooth.

TYPE UNKNOWN 13

Position in classification hierarchy: n/a

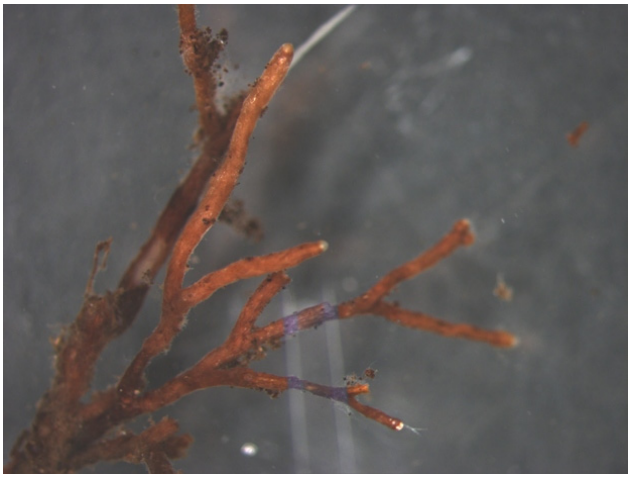

Figure A4-54: Type unknown 13 colonising *P. radiata* root tips.

Main ECM colonising root tips characteristics:
brown, dichotomous, white apex, smooth.

TYPE UNKNOWN 14

Position in classification hierarchy: n/a

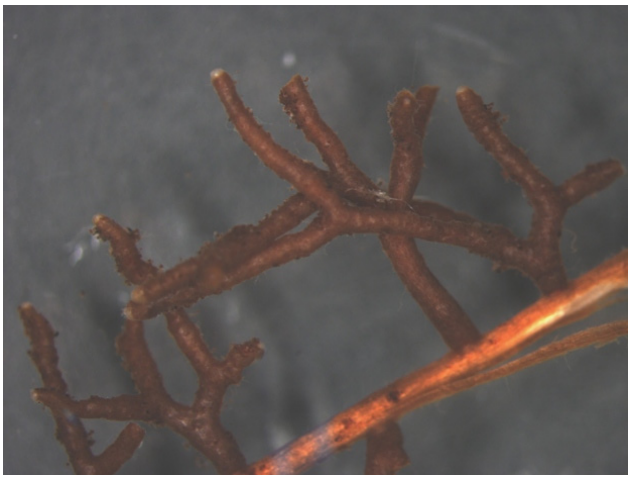

Figure A4-55: Type unknown 14 colonising *P. radiata* root tips.

Main ECM colonising root tips characteristics:
Dark brown, dichotomous, smooth.

APPENDIX 5 SUPPLEMENTARY DATA ECM SPECIES IDENTIFICATION

RFLP PATTERNS

Table A5-1: Banding sizes of internal transcribed spacer (ITS) – restriction fragment length polymorphism (RFLP) generated by restriction digest with enzymes *Alu*I, *Hinf*I and *Mbo*I of ectomycorrhizal fungi from root tip (ECM) or sporocarps collected in sporocarp and soil core assessments in 2005 and 2006. Based on 2% agarose gels, banding sizes acquired with Bio-Rad Quantity One® Image Acquisition & Analysis Software (Bio-Rad Laboratories, Hercules CA, USA). Uncut size of ITS region based on sequence data.

ECM species/type	Source	Label	Uncut (bp)	RFLP banding size (bp) *Alu*I [a]					*Hinf*I			*Mbo*I	
Amanita muscaria [b]	Sporocarp	K80S5	711	201	387	*484*			334			208	310
Amanita muscaria	ECM	K80C31T259	711	225	416	*512*			362			237	342
Cenococcum geophilum	ECM	K91C31T307	n/a	*752*					197	268	354	239	366
Chalciporus piperatus	Sporocarp	K80S25	786	744	*809*				120	178	236	295	314
Hebeloma sp.	Sporocarp	K06S1	732	*547*	*746*				337	395		266	425
Hebeloma sp.	ECM	K06C5T156a	733	*553*	*753*				344	407		273	435
Inocybe sp.	Sporocarp	K98S25	745	180	409	*574*	*757*		200	319	414	185	291
Inocybe sp.	ECM	K91C37T361	745	*584*	*743*				215	327	452	190	302
Inocybe lacera	Sporocarp	K04S3	722	214	334	*555*	*734*		272	393		253	419
Inocybe sindonia	Sporocarp	K80S33	731	*617*	*775*				208	336		321	463
Laccaria proxima	Sporocarp	K04S5	730	*245*	*505*	*644*	*764*		209	352		277	439
Lactarius rufus	Sporocarp	K80S7	763	522	*683*	*758*			268	379		206	256
Rhizopogon rubescens	Sporocarp	K04S6	737	257	*499*	*650*	*758*		215	239		171	274
Rhizopogon rubescens	ECM	K06C3T138	738	271	*500*	*759*			215	242		124	227
Rhizopogon luteolus	Sporocarp	K80S64	889	243	*309*	639	*701*		192	258	*406*	295	*410*
Rhizopogon luteorubescens	ECM	K91C31T310	762	*768*					240			247	
Rhizopogon pseudoroseolus	Sporocarp	K98S35	765	284	371	*490*	*671*	*752*	156	241		163	235
Rhizopogon pseudoroseolus	ECM	K98C31T213	765	280	*494*	*685*	*774*		256			238	
Scleroderma bovista [b]	Sporocarp	K80S9	699	218	*273*	431	*651*		120	250	273	182	
Suillus sp.	Sporocarp	K91S8	723	610	*738*				124	230		147	230
Thelephora terrestris	Sporocarp	K91S27	701	*370*	594	*692*			238	351		219	367
Thelephora terrestris	ECM	K98C34T230	700	*724*					226	335		208	347
Tricholoma sp.	Sporocarp	K91S9	737	508	*674*				352	391		222	299
Pseudotomentella sp.	ECM	K98C35T239	721	212	278				359			239	439
Pseudotomentella tristis	ECM	K91C38T363	788	336	*417*	*533*	*595*		364	410		178	253
Tomentella sp	ECM	K80C37T324	700	478					202	351		242	375
Tuber sp.	ECM	K06FC8T13	688	*715*					364			220	331
Wilcoxina mikolae	ECM	K04C38T193	636	*644*	*745*				340	395	745	410	
unknown 1 (Pezizaceae)	ECM	K06FC10T18	681	256	*442*	*699*			312	370		252	409
unknown Basidiomycete	ECM	K04C35T201	722	625					174	275		197	

Primer pairs ITS1F and ITS 4 were used for PCR amplification of the ITS region

a – *Alu*I gave rise to many partial digests and was not the most robust for morphotype discrimination; uncut fragments in red and italics.

b – These sequences appeared to be from mixed PCR products. To gain a full sequence for *in silico* RFLP analysis the sequence from the ITS1F and ITS4 primer was aligned with the GenBank result and consensus bases assigned.

ECM SPECIES IDENTITY CLARIFICATION

Supplementary tables for ECM species clarification, Chapter 3, section 3.2.1 Sporocarp and ECM root tip identification – "ECM species identity clarification"

"*TRICHOLOMA PESSUNDATUM*"

Table A5-2: GenBank query for samples K80S34, K91S0 from the present study and Landcare Research herbarium specimen PDD58858. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (bp)	GenBank Accession #	Organism	GenBank score	e-value	Maximum Identity
K80S34	717	AF458435	*Tricholoma ustale*	1180	0.00	96.00
		DQ367919	*Tricholoma aurantium*	1171	0.00	96.00
		AB036894	*Tricholoma ustale*	1164	0.00	96.00
K91S9	704	DQ367919	*Tricholoma aurantium*	123	0.00	97.00
		AF458436	*Tricholoma ustale*	1221	0.00	96.00
		AF458437	*Tricholoma ustale*	1219	0.00	96.00
PDD58858	733	AF458435	*Tricholoma ustale*	1195	0.00	96.00
		DQ367919	*Tricholoma aurantium*	1186	0.00	96.00
		AB036894	*Tricholoma ustale*	1180	0.00	96.00

Table A5-3: UNITE query for *Tricholoma* specimens K80S34, K91S9 from the present study and Landcare Research herbarium specimen PDD58858. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (Bp)	UNITE Accession #	Organism	UNITE score	e-value
K80S34	717	UDB001502*	*Tricholoma pessundatum*	1376	0.00
		UDB001442*	*Tricholoma tridentinum*	1322	0.00
		AF458435	*Tricholoma ustale*	1215	0.00
K91S9	704	UDB001502*	*Tricholoma pessundatum*	1229	0.00
		UDB001442*	*Tricholoma tridentinum*	1189	0.00
		UDB000805*	*Tricholoma pessundatum*	1112	0.00
PDD58858	733	UDB001502*	*Tricholoma pessundatum*	1352	0.00
		UDB001442*	*Tricholoma tridentinum*	1308	0.00
		AF458435	*Tricholoma ustale*	1223	0.00

* UNITE specific sequences, locked by R. Kjõller and not free to download (August 2007)

"LACCARIA LACCATA"

Table A5-3: GenBank query for samples K04S5 and K98S32 from the present study. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (bp)	GenBank Accession #	Organism	GenBank score	e-value
K04S5	730	DQ068958	*Laccaria proxima*	1240	0.00
		AY750156	*Laccaria proxima*	1227	0.00
		DQ367906	*Laccaria bicolor*	1196	0.00
K98S32	705	DQ068958	*Laccaria proxima*	1211	0.00
		AY750156	*Laccaria proxima*	1198	0.00
		DQ367906	*Laccaria bicolor*	1189	0.00

Table A5-4: UNITE query for samples K04S5 and K98S32 from the present study. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (bp)	UNITE Accession #	UNITE Organism	UNITE score	e-value
K04S5	730	DQ068958	*Laccaria proxima*	1355	0.00
		AY750156	*Laccaria proxima*	1330	0.00
		DQ367906	*Laccaria bicolor*	1304	0.00
K98S32	705	DQ068958	*Laccaria proxima*	1330	0.00
		AY750156	*Laccaria proxima*	1304	0.00
		DQ367906	*Laccaria bicolor*	1255	0.00

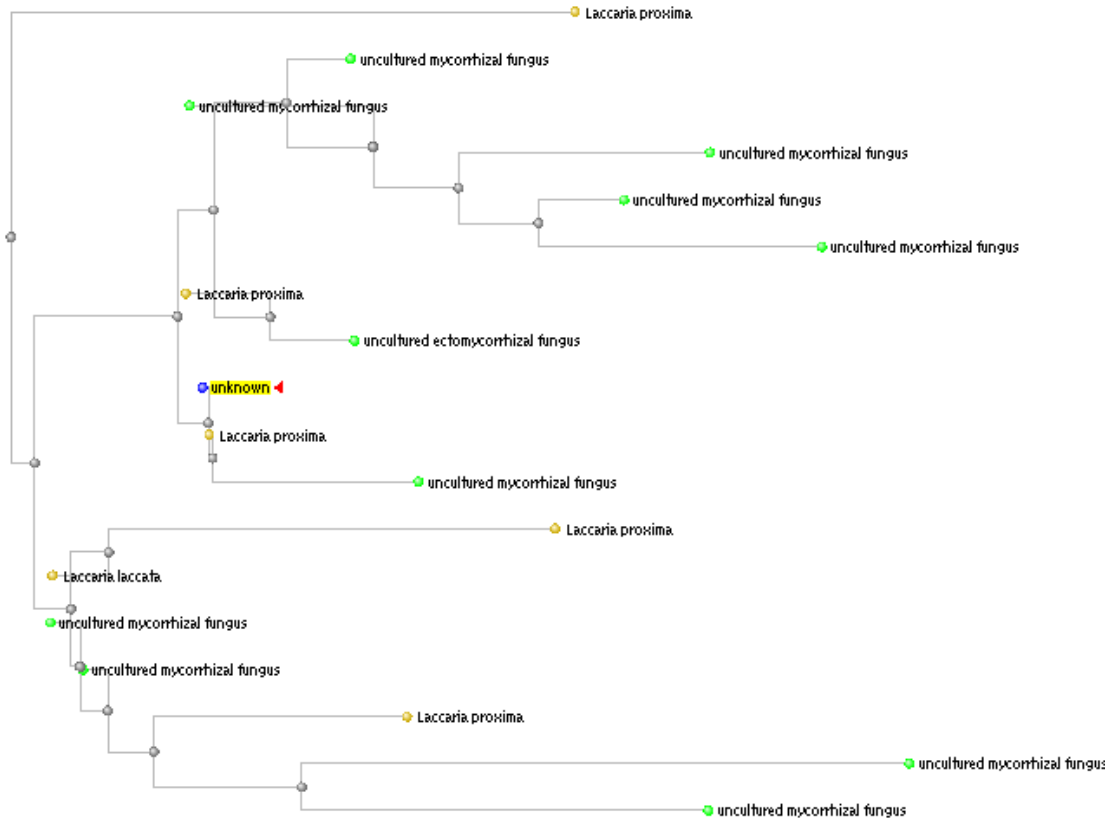

Figure A5-1: Partial distance tree of results from GenBank query for K04S5 labelled as 'unknown' (Neighbour joining method, maximum sequence difference = 0.75).

"*HEBELOMA CRUSTULINIFORME*"

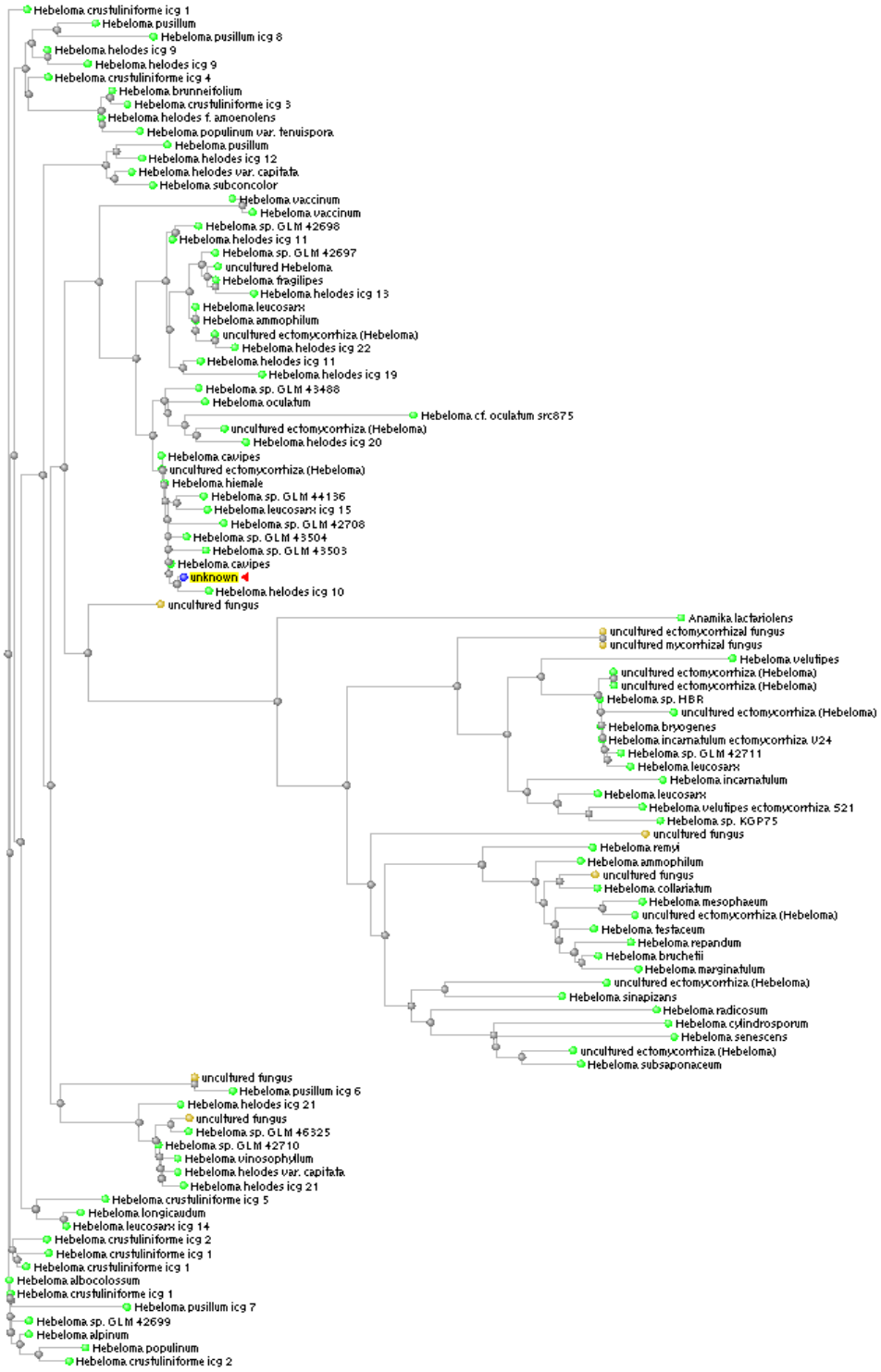

Figure A5-2: Distance tree of results from GenBank query for K06S1 labelled as 'unknown' (Neighbour joining method, maximum sequence difference = 0.75).

Table A5-5: GenBank query for sample K06S1 from the present study and Landcare Research herbarium specimen JAC8966 and JAC9848. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (Bp)	GenBank Accession #	Organism	GenBank score	e-value
K06S1	731	EF411103	Uncultured ECM	1205	0.00
		AY320389	*Hebeloma* sp. GLM 43504	1197	0.00
		AY320391	*Hebeloma* sp. GLM 44136	1192	0.00
JAC8966	701	AY818351	*Hebeloma velutipes*	897	0.00
		AF430291	*Hebeloma incarnatulum*	857	0.00
		EF093151	*Hebeloma* sp. HBR	856	0.00
JAC9848	657	AY308583	*Hebeloma albocolossum*	1147	0.00
		AY311518	*Hebeloma longicaugum*	1144	0.00
		AY320383	*Hebeloma* sp. GLM 42699	1142	0.00

Table A5-6: UNITE query for sample K06S1 from the present study and Landcare Research herbarium specimen JAC8966 and JAC9848. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (Bp)	UNITE Accession #	Organism	UNITE score	e-value
K06S1	731	EF411103	Uncultured ECM	1237	0.00
		AY320389	*Hebeloma* sp. GLM 43504	1229	0.00
		AY320391	*Hebeloma* sp. GLM 44136	1229	0.00
JAC8966	701	UDB002445*	*Hebeloma velutipes*	753	0.00
		AY818351	*Hebeloma velutipes*	737	0.00
		EF093151	*Hebeloma* sp.	716	0.00
JAC9848	657	UDB000697*	*Hebeloma populinum*	1235	0.00
		AY308583	*Hebeloma albocolossum*	1215	0.00
		AY320383	*Hebeloma* sp. GLM 42699	1207	0.00

*UNITE specific sequences, not available through GenBank

INOCYBE SPP.

Table A5-7: GenBank query for samples K80S29, K80S33 from the present study and Landcare herbarium specimen PDD750021. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (Bp)	GenBank Accession #	Organism	GenBank score	e-value
K80S29	674	AY751556	Cf. *Inocybe* sp. EC258 B207	958	0.00
		AY310829	Uncultured ectomycorrhizal fungus TAM205	922	0.00
		DQ974742	*Inocybe* sp. src527	751	0.00
K80S33	731	AY751556	Cf. *Inocybe* sp. EC258 B207	987	0.00
		AY310829	Uncultured ectomycorrhizal fungus TAM205	928	0.00
		AY825515	Uncultured ectomycorrhiza (*Inocybe*)	809	0.00
PDD750021	701	AY751556	Cf. *Inocybe* sp. EC258 B207	989	0.00
		DQ822816	*Inocybe* sp. KGP59	957	0.00
		EF417819	Uncultured ectomycorrhiza (*Inocybe*)	942	0.00

Table A5-8: UNITE query for samples K80S29, K80S33 from the present study and Landcare herbarium specimen PDD750021. The three highest ranked scores are listed for each sample.

Sample label	Sequence length (Bp)	UNITE Accession #	Organism	UNITE score	e-value
K80S29	674	UDB002392*	*Inocybe sindonia*	1225	0.00
		UDB001757*	*Inocybe sindonia*	1154	0.00
		AY751556	Cf. Inocybe sp. EC258 B207	858	0.00
K80S33	731	UDB002392*	*Inocybe sindonia*	1352	0.00
		UDB001582*	*Inocybe sp.*	1315	0.00
		UDB001757*	*Inocybe sindonia*	1259	0.00
PDD750021	701	UDB002392*	*Inocybe sindonia*	1289	0.00
		UDB001582*	*Inocybe sp.*	1271	0.00
		UDB001757*	*Inocybe sindonia*	1204	0.00

*UNITE specific sequences, not available through GenBank

APPENDIX 6 SPECIES RICHNESS RAREFACTION CURVES

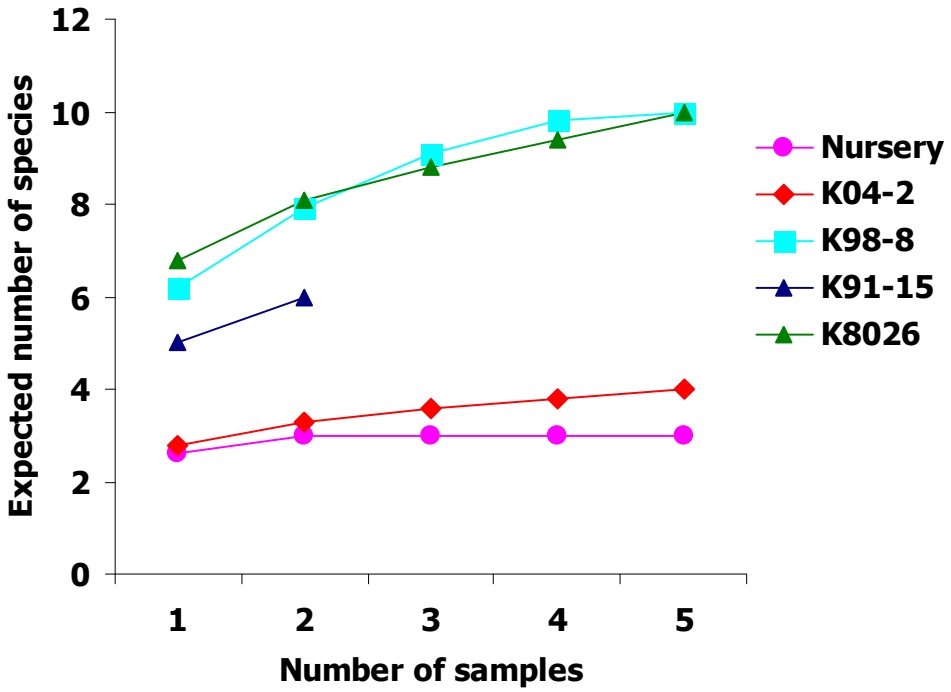

Figure A6-1: Rarefaction curves for expected number of aboveground ECM species at the study sites investigated during the 2006 sporocarp assessment (SA2).

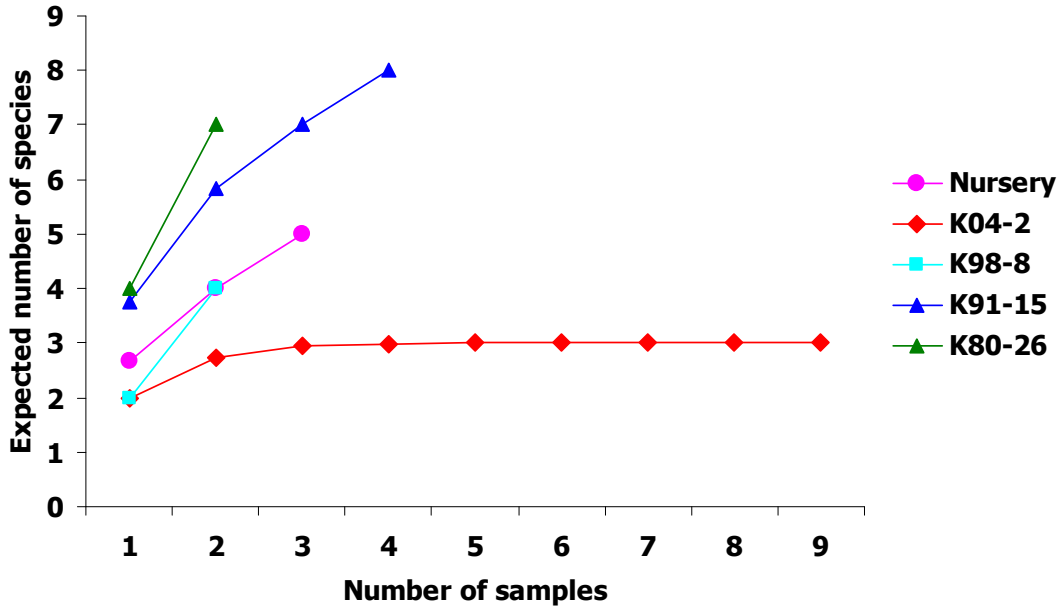

Figure A6-2: Rarefaction curves for expected number of belowground ECM species at the study sites investigated during soil core assessment 1 (SCA1).

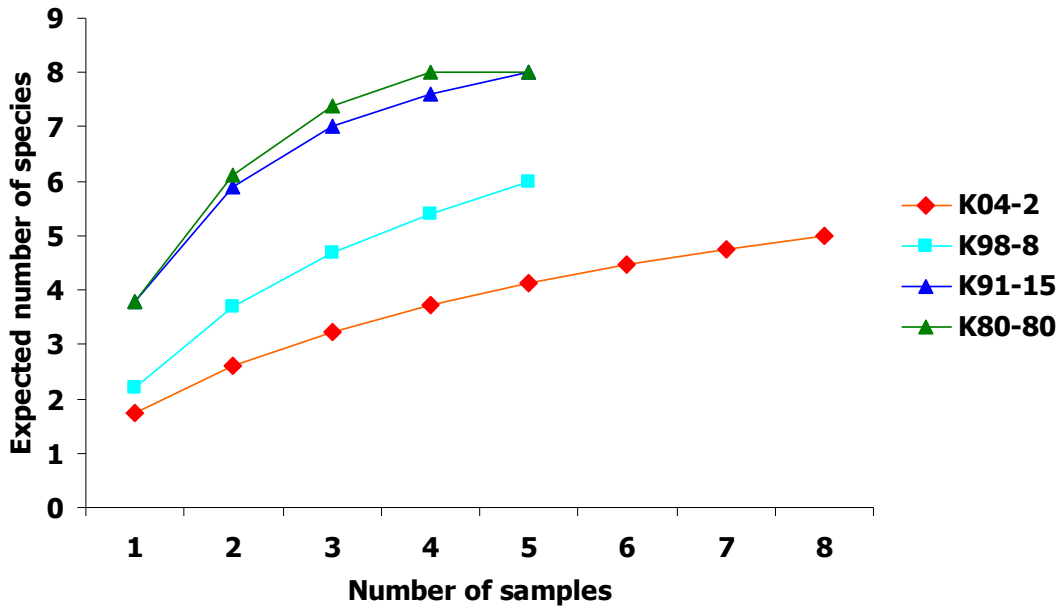

Figure A6-3: Rarefaction curves for expected number of belowground ECM species at the study sites investigated during soil core assessment 2 (SCA2).

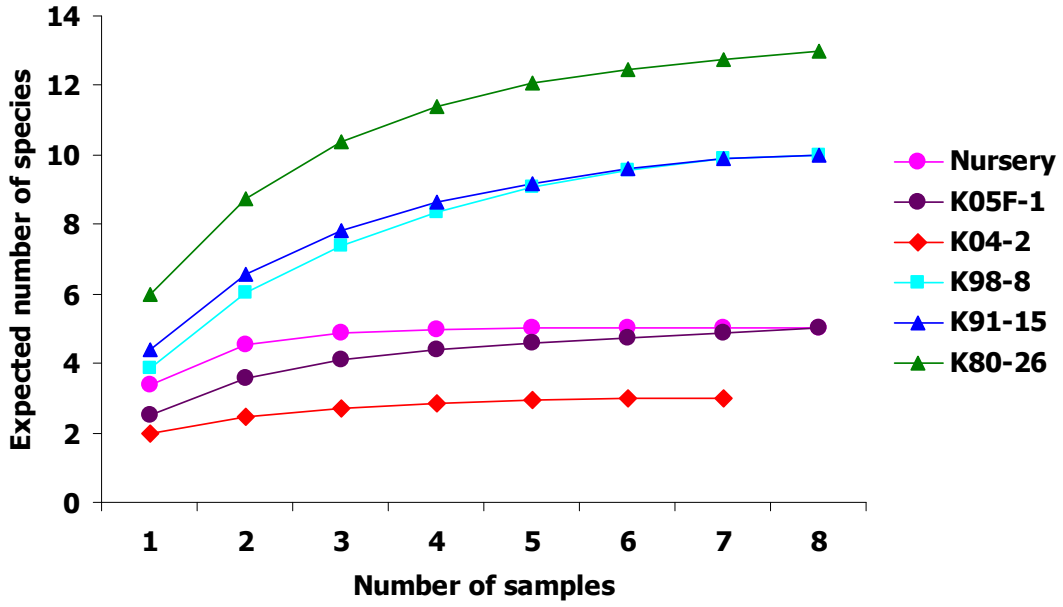

Figure A6-4: Rarefaction curves for expected number of belowground ECM species at the study sites investigated during soil core assessment 3 (SCA3).

APPENDIX 7 ABSOLUTE AND RELATIVE ABUNDANCE OF ABOVE- AND BELOWGROUND ECM

The absolute and relative abundance of ECM sporocarps counted in assessment 2 in 2006 are presented in Table A7-1. In the nursery, *Rhizopogon rubescens* was the most common of the three species (82%). In K04-2, four taxa were observed, *Inocybe lacera* accounted for 85% of the recorded specimens. The K98-8 site was dominated by *Laccaria proxima* (78%); however, ten taxa were observed overall. In the K91-15 site, *Inocybe lacera* was the dominant species (64%) of the five species recorded. With 58%, *Lactarius rufus* was the most abundant species in K80-26, followed by *Inocybe sindonia* (31%), but eight other species were also recorded in this site.

Tables A7-2 to A7-4 present the absolute and relative abundance of ECM types in the three soil core assessments from 2005 to 2006. In the first assessment of the nursery in June 2005 (SCA 1), *Wilcoxina mikolae* dominated (60%) over the other four species present (*Hebeloma* sp*., R. rubescens, Tuber* sp. and unknown 2). In the third assessment in 2006 (SCA 3) abundance was more evenly distributed between *R. rubescens* (30%), *Hebeloma* sp., *Tuber* sp. (23% each) and *W. mikolae* (21%). The K05F-1 site was planted with seedlings that were assessed in the nursery in 2005 and was hence only assessed in 2006. The same five species observed in the nursery were present in this site, but the distribution of abundance changed considerably. In this first year of outplanting *R. rubescens* composed 81% of the sample. The K04-2 site was established in 2004 with seedlings from Te Ngae nursery. Three ECM species were identified, *R. rubescens* was dominant (44%, SCA 1). The same three species were found in SCA 2 with *R. rubescens* being the most abundant species (75%). Additionally, ECM type unknown 12 and unknown various were observed in this assessment. Species numbers, composition and abundance changed in K04-2 in the third assessment in 2006. Three ECM species/types were identified including a low abundance of a new ECM type, unknown Basidiomycete.

Species composition and abundance changed in the older plantation sites. Across all three assessments in the K98-8 site, the number of ECM species/types detected, increased. In the first assessment of K98-8 (7 yrs at time of assessment) four ECM species/types were found. None of these were ECM species observed in the nursery, K05F-1 or K04-2 sites, except for ECM type unknown Basidiomycete. The group 'unknown various' composed 60%, reflecting the initial problems with identification of ECM morphotypes. In the second assessment of K98-8, six ECM species/types were identified. *Amanita muscaria* and unknown Basidiomycete were present in both assessments, but ECM species *Rhizopogon pseudoroseolus*, first

observed in SCA 2, was the most abundant type (69%). In the third assessment of K98-8 (8 yrs at time of assessment), ten ECM species/types were identified, *Amanita muscaria* (38%) and unknown Basidiomycete (25%) being the most abundant species. In the K91-15 site more ECM species/types were identified as the assessments progressed from SCA 1 to SCA 3. *Amanita muscaria*, *Cenococcum geophilum, Tomentella* sp. and Type unknown Basidiomycete were found in all three assessments in this site. *Amanita muscaria* and *C. geophilum* were the most abundant ECM species in all three cases, the relative abundance of *Tomentella* sp. decreased from SCA 1 to SCA 3, whereas the relative abundance of Type unknown Basidiomycete increased. Species richness increased from five to eleven over the three assessments. The highest diversity of ECM species/types were found in the oldest stand investigated, K80-26. Richness increased from seven to thirteen over the three assessments. Again, *A. muscaria, C. geophilum* and *Tomentella* sp. were present in all three surveys, as well as *Inocybe* sp. and *Pseudotomentella tristis.* In the SCA 1, *Inocybe* sp. was the most abundant species (50%), however the species composition changed in the second assessment. Species were more evenly distributed, Type unknown 9 was the most abundant ECM type (25%). All the species/types from SCA 2 were recorded again in SCA 3 with an additional five species/types being detected.

Table A7-1: Absolute and relative abundance of specimen collected in each study site during April – June 2006 in sporocarp assessment 2. Absolute numbers are the total of all visits and transects. Relative abundance was calculated as follows: (number sporocarps for each ECM taxa) / (total number of sporocarps sampled per study site) x100.

	Nursery (n=5)		**K04-2** (n=5)		**K98-8** (n=5)		**K91-15** (n=2)		**K80-26** (n=5)	
ECM Taxa	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	52	4.19	4	3.70	94	3.45
Chalciporus piperatus	-	-	-	-	4	0.32	-	-	2	0.07
Hebeloma sp.	27	15.34	-	-	-	-	-	-	-	-
Inocybe lacera	-	-	453	85.15	43	3.46	69	63.89	83	3.05
Inocybe sindonia	-	-	-	-	-	-	13	12.04	852	31.28
Inocybe sp.	-	-	-	-	29	2.33	6	5.56	64	2.35
Laccaria proxima	-	-	62	11.65	966	77.78	-	-	9	0.33
Lactarius rufus	-	-	-	-	5	0.40	-	-	1584	58.15
Lycoperdon gunii	-	-	-	-	5	0.40	-	-	-	-
Lycoperdon sp.	-	-	-	-	11	0.89	-	-	-	-
Rhizopogon rubescens	144	81.82	7	1.32	-	-	-	-	-	-
Rhizopogon sp.(pseudoroseolus)	-	-	-	-	2	0.16	-	-	-	-
Rhizopogon luteolus	-	-	-	-	-	-	-	-	1	0.04
Suillus sp.	-	-	-	-	-	-	-	-	-	-
Scleroderma bovista	-	-	10	1.88	-	-	-	-	-	-
Thelephora terrestris	-	-	-	-	125	10.06	16	14.81	2	0.07
Tricholoma sp.	-	-	-	-	-	-	-	-	33	1.21
Wilcoxina mikolae	5	2.84	-	-	-	-	-	-	-	-
Total Specimen counted	**176**		**532**		**1242**		**108**		**2724**	

n = number of transects assessed

Table A7-2: Absolute and relative abundance of ECM types collected in the Nursery, K05F and K04 for the three soil core assessments (SCA) 1, 2 and 3 in 2005/06. Absolute numbers are total of all soil cores processed at each assessment.

	Nursery				K05F-1		K04-2					
	SCA 1 (n=3)		SCA 3 (n=8)		SCA 3 (n=8)		SCA 1 (n=10)		SCA 2 (n=8)		SCA 3 (n=8)	
ECM Type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	-	-	-	-	-	-	-	-
unknown Basidiomycete	-	-	-	-	-	-	-	-	-	-	11	0.94
Cenococcum geophilum	-	-	-	-	-	-	-	-	-	-	-	-
Hebeloma sp.	124	3.71	2223	22.91	95	1.64	-	-	-	-	-	-
Inocybe sp.	-	-	-	-	-	-	-	-	-	-	-	-
Pseudotomentella sp.	-	-	-	-	-	-	-	-	-	-	-	-
Pseudotomentella tristis	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopogon luteorubescens	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopogon pseudoroseolus	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopogon rubescens	1068	31.98	2921	30.10	4710	81.43	933	44.13	722	74.97	373	31.99
Thelephora terrestris	-	-	-	-	-	-	-	-	-	-	-	-
Tomentella sp.	-	-	-	-	-	-	-	-	-	-	-	-
Tuber sp.	148	4.43	2274	23.43	866	14.97	605	28.62	65	6.75	-	-
Wilcoxina mikolae	1999	59.85	2073	21.36	57	0.99	576	27.25	81	8.41	782	67.07
unknown 2	1	0.03	214	2.21	56	0.97	-	-	-	-	-	-
unknown 8	-	-	-	-	-	-	-	-	-	-	-	-
unknown 9	-	-	-	-	-	-	-	-	-	-	-	-
unknown 10	-	-	-	-	-	-	-	-	-	-	-	-
unknown 12	-	-	-	-	-	-	-	-	19	1.97	-	-
unknown various	-	-	-	-	-	-	-	-	76	7.89	-	-
Total ECM root tips analysed	3340		9705		5784		2114		963		1166	
Average number of ECM root tips/soil core	1113		1213		723		211		120		146	

n = number of soil cores assessed

Table A7-3: Absolute and relative abundance of ECM types collected in K98 and K91 for the three soil core assessments (SCA) 1, 2 and 3 in 2005/06. Absolute numbers are total of all soil cores processed at each assessment.

	K98-8						K91-15					
	SCA 1 (n=4)		SCA 2 (n=5)		SCA 3 (n=8)		SCA 1 (n=2)		SCA 2 (n=5)		SCA 3 (n=8)	
ECM Type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	157	26.52	284	22.94	587	38.12	512	50.79	408	52.37	314	25.99
unknown Basidiomycete	63	10.64	10	0.81	382	24.81	13	1.29	39	5.01	129	10.68
Cenococcum geophilum	-	-	-	-	55	3.57	397	39.38	184	23.62	257	21.27
Hebeloma sp.	-	-	-	-	-	-	-	-	-	-	-	-
Inocybe sp.	-	-	-	-	26	1.69	-	-	-	-	200	16.56
Pseudotomentella sp.	-	-	-	-	28	1.82	-	-	16	2.05	1	0.08
Pseudotomentella tristis	19	3.21	-	-	-	-	-	-	-	-	88	7.28
Rhizopogon luteorubescens	-	-	-	-	-	-	-	-	-	-	105	8.69
Rhizopogon pseudoroseolus	-	-	851	68.74	69	4.48	-	-	-	-	84	6.95
Rhizopogon rubescens	-	-	-	-	-	-	-	-	-	-	-	-
Thelephora terrestris	-	-	-	-	25	1.62	-	-	75	9.63	-	-
Tomentella sp.	-	-	19	1.53	-	-	61	6.05	36	4.62	21	1.74
Tuber sp.	-	-	-	-	-	-	-	-	-	-	-	-
Wilcoxina mikolae	-	-	-	-	-	-	-	-	-	-	-	-
unknown 2	-	-	-	-	-	-	-	-	-	-	-	-
unknown 8	-	-	-	-	-	-	25	2.48	-	-	-	-
unknown 9	-	-	-	-	120	7.79	-	-	-	-	-	-
unknown 10	-	-	-	-	-	-	-	-	-	-	-	-
unknown 12	-	-	18	1.45	116	7.53	-	-	18	2.31	4	0.33
unknown various	353	59.63	56	4.52	132	8.57	-	-	3	0.39	5	0.41
Total ECM root tips analysed	592		1238		1540		1008		779		1208	
Average number of ECM root tips/soil core	149		248		193		504		156		151	

n = number of soil cores assessed

Table A7-4: Absolute and relative abundance of ECM types collected in K80 for the three soil core assessments (SCA) 1, 2 and 3 in 2005/06. Absolute numbers are total of all soil cores processed at each assessment.

	K80-26					
	SCA 1 (n=2)		SCA 2 (n=5)		SCA 3 (n=8)	
ECM Type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	152	16.76	62	6.94	433	15.88
unknown Basidiomycete	20	2.21	-	-	638	23.40
Cenococcum geophilum	36	3.97	96	10.75	363	13.31
Hebeloma sp.	-	-	-	-	-	-
Inocybe sp.	457	50.39	131	14.67	172	6.31
Pseudotomentella sp.	112	12.35	48	5.38	56	2.05
Pseudotomentella tristis	89	9.81	-	-	13	0.48
Rhizopogon luteorubescens	-	-	-	-	-	-
Rhizopogon pseudoroseolus	-	-	-	-	-	-
Rhizopogon rubescens	-	-	-	-	-	-
Thelephora terrestris	-	-	-	-	76	2.79
Tomentella sp.	41	4.52	104	11.65	275	10.08
Tuber sp.	-	-	-	-	-	-
Wilcoxina mikolae	-	-	-	-	-	-
unknown 2	-	-	-	-	-	-
unknown 8	-	-	-	-	24	0.88
unknown 9	-	-	220	24.64	399	14.63
unknown 10	-	-	-	-	76	2.79
unknown 12	-	-	162	18.14	12	0.44
unknown various	-	-	70	7.84	190	6.97
Total ECM root tips analysed	907		893		2727	
Average number of ECM root tips/soil core	454		447		341	

n = number of soil cores assessed

Table A7-5: Absolute and relative abundance of ECM types collected from May (nursery) to November 2006 in K06F during the outplanting assessment. Absolute numbers are total of all soil cores processed at each assessment.

	May (n=8)		August (n=3)		September (n=3)		October (n=3)		November (n=3)	
ECM species/type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	-	-	-	-	-	-
Hebeloma sp.	2223	22.91	-	-	4	8.70	110	29.73	65	16.62
Pezizales sp.	-	-	-	-	-	-	-	-	27	6.91
Rhizopogon rubescens	2921	30.1	21	75.00	11	23.91	177	47.84	110	28.13
Tuber sp.	2274	23.43	-	-	-	-	83	22.43	189	48.34
Wilcoxina mikolae	2073	21.36	-	-	26	56.52	-	-	-	-
unknown Basidiomycete	-	-	-	-			-	-	-	-
unknown 2	214	2.21	7	25.00	5	10.87	-	-	-	-
unknown 13	-	-	-	-	-	-	-	-	-	-
unknown 14	-	-	-	-	-	-	-	-	-	-
Total ECM root tips analysed	9705		28		46		370		391	
Average number of ECM root tips/seedling	1213		9		15		123		130	

n = number of soil cores assessed

Table A7-6: Absolute and relative abundance of ECM types collected from December 2006 to June 2007 in K06F during the outplanting assessment. Absolute numbers are total of all soil cores processed at each assessment.

	December (n=3)		January (n=3)		February (n=3)		June (n=3)	
ECM species/type	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)
Amanita muscaria	-	-	-	-	-	-	73	7.03
Hebeloma sp.	44	8.06	115	21.66	138	10.01	146	14.07
Pezizales sp.	43	7.88	-	-	26	1.89	35	3.37
Rhizopogon rubescens	413	75.64	342	64.41	1004	72.86	355	34.20
Tuber sp.	46	8.42	74	13.94	210	15.24	36	3.47
Wilcoxina mikolae	-	-	-	-	-	-	115	11.08
unknown Basidiomycete	-	-	-	-	-	-	49	4.72
unknown 2	-	-	-	-	-	-	-	-
unknown 13	-	-	-	-	-	-	76	7.32
unknown 14	-	-	-	-	-	-	153	14.74
Total ECM root tips analysed	546		531		1378		1038	
Average number of ECM root tips/seedling	182		177		459		346	

n = number of soil cores assessed

APPENDIX 8 SUPPLEMENTARY DATA SPOROCARP ABUNDANCE PER SITE VISIT AND RELATION TO CLIMATE DATA

CLIMATE DATA

During the sporocarp assessment 1 in 2005 (April – June) the average temperature ranged from 6°C to 11°C with lowest temperatures in June. Total rainfall ranged from 25mm to 163mm and was lowest in April and highest in May 2005. Temperatures during the second sporocarp assessment in 2006 (March – June) were similar to the previous year and ranged from 5°C to 13°C and again, were lowest in June. Total rainfall was lower than in 2005 ranged from 22mm to 99mm, April being the wettest month in the assessment 2006 with 99mm compared to 25mm in 2005. May 2006 had less rainfall than in 2005 (44mm and 163mm, respectively), June 2006 as well had less rain with 44mm/month than compared to the previous year (74mm).

Table A8-1: Minimum, maximum and average Temperature (°C), average relative Humidity (%) and total rainfall per month (mm) of the Kaingaroa Forest station "Goudie Road". Data kindly provided by Kaingaroa Timberlands.

		Minimum Temperature (°C)	Maximum Temperature (°C)	Average Temperature (°C)	Average relative Humidity (%)	Total Rainfall (mm)
2005	January	6.7	28.3	15.8	78.2	33
	February	7.8	29.2	17.4	79.8	74
	March	5.0	23.4	14.8	81.6	125
	April	0.1	21.4	10.9	81.5	25
	May	-0.2	17.5	9.9	90.0	163
	June	-1.7	8.9	6.4	85.5	74
	July	-3.3	13.5	6.7	90.0	109
	August	-1.7	16.4	7.1	85.7	74
	September	-2.8	18.3	10.3	80.8	44
	October	3.1	20.6	10.8	85.5	153
	November	1.4	23.0	12.0	78.4	26
	December	7.7	24.2	15.5	81.6	148
2006	January	5.4	29.6	16.2	79.9	101
	February	4.1	25.2	15.9	80.4	134
	March	1.1	23.4	13.0	80.8	22
	April	3.9	20.0	12.4	88.2	99
	May	-0.2	17.4	9.0	88.6	44
	June	-1.5	15.1	5.2	85.8	42
	July	0.1	12.1	7.0	88.3	34

SUPPLEMENTARY FIGURES

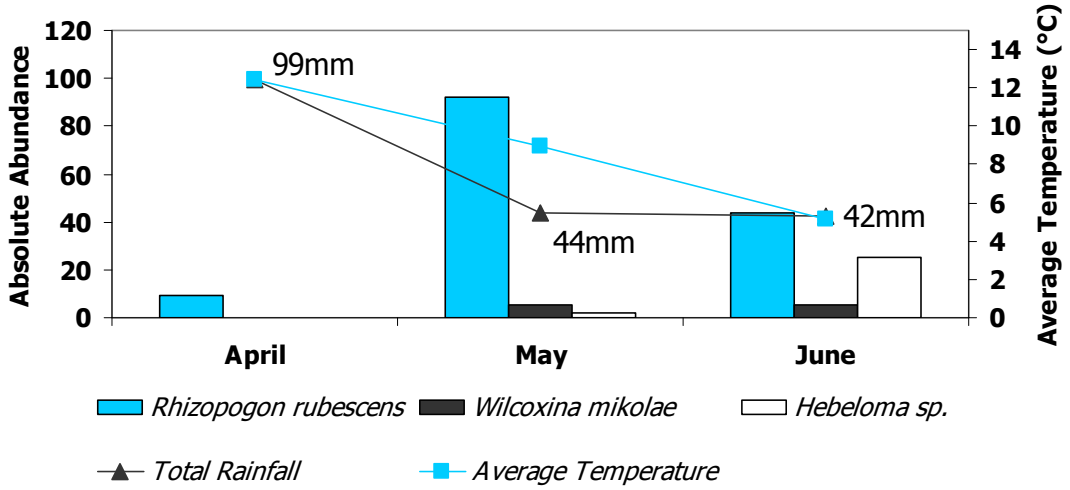

Figure A8-1: Total monthly absolute abundance of the Nursery site in sporocarp assessment 2 (left axis), average monthly temperature (°C, right axis) and total monthly rainfall (mm, values in graph).

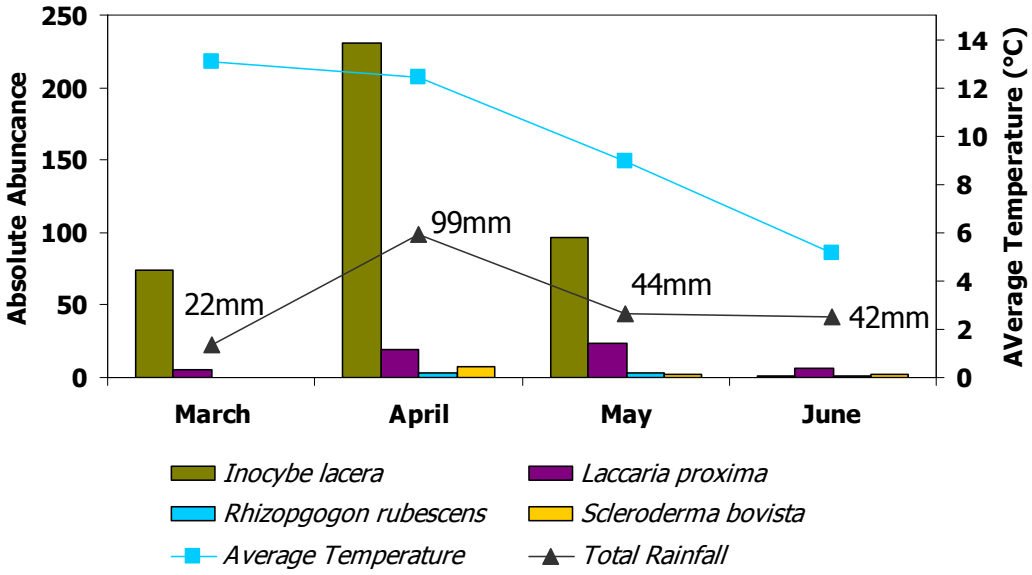

Figure A8-2: Total monthly absolute abundance of K04-2 site in sporocarp assessment 2 (left axis), average monthly temperature (°C, right axis) and total monthly rainfall (mm, values in graph).

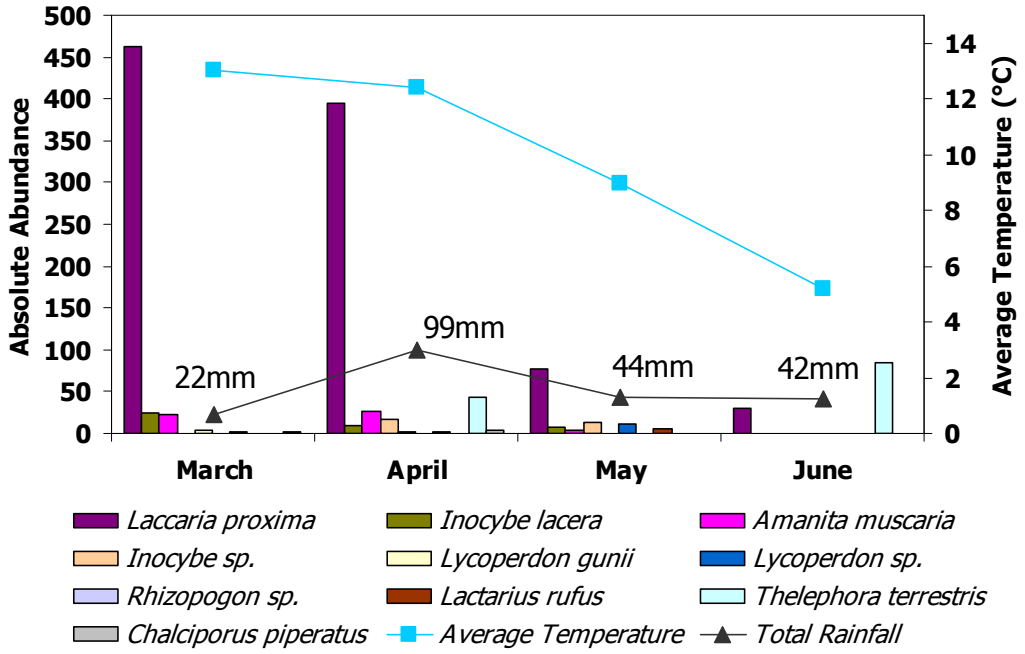

Figure A8-3: Total monthly absolute abundance of K98-8 site in sporocarp assessment 2 (left axis), average monthly temperature (°C, right axis) and total monthly rainfall (mm, values in graph).

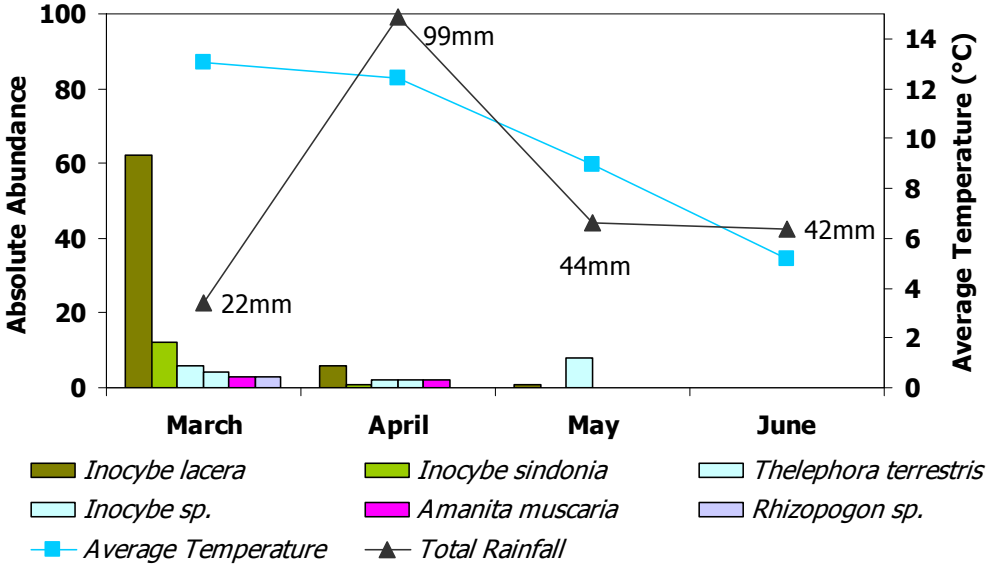

Figure A8-4: Total monthly absolute abundance of K91-15 site in sporocarp assessment 2 (left axis), average monthly temperature (°C, right axis) and total monthly rainfall (mm, values in graph).

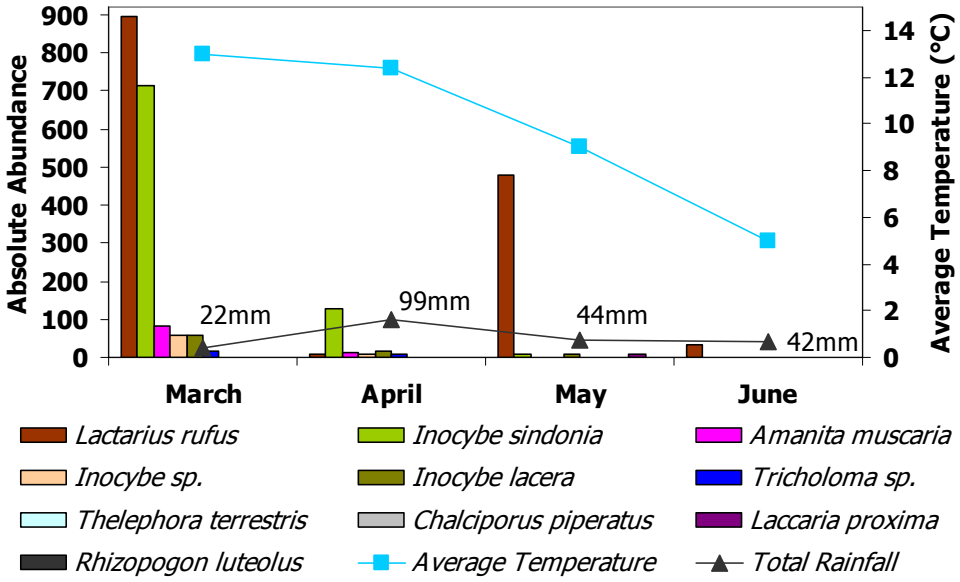

Figure A8-5: Total monthly absolute abundance of K80-26 site in sporocarp assessment 2 (left axis), average monthly temperature (°C, right axis) and total monthly rainfall (mm, values in graph).

APPENDIX 9 SUPPLEMENTARY DATA BELOWGROUND ECM SPECIES RANK-ABUNDANCE CURVES

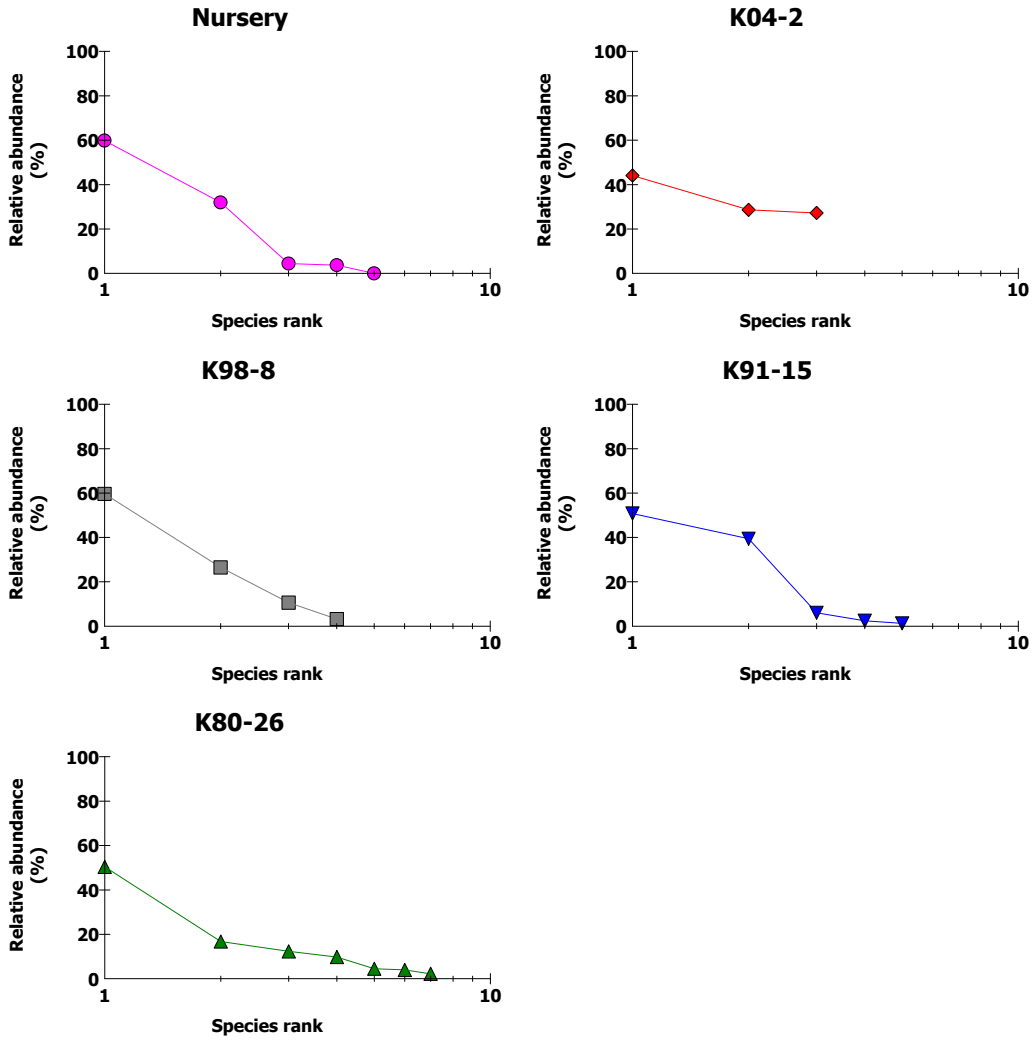

Figure A9-1: Rank abundance curves for sites investigated in soil core assessment 1, 2005.

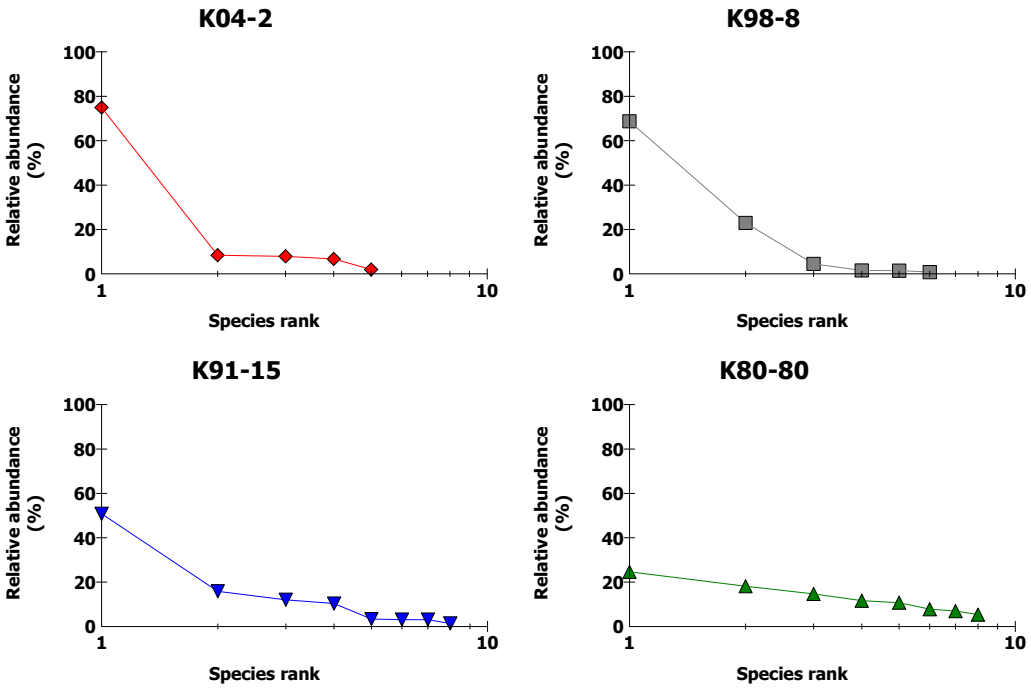

Figure A9-2: Rank abundance curves for sites investigated in soil core assessment 2, 2005.

APPENDIX 10 SUPPLEMENTARY DATA ANOVA ANALYSIS

Table A10-1: One-way Analysis of Variance (ANOVA) of the mean species richness, Margalef's index, Shannon and Simpson diversity index of the sporocarp assessment 2 in 2006, with five different host age groups (nursery, 2, 8, 15 and 26 yrs) as treatments.

Analysis	Source of variation	d.f	ss.	m.s.	F value	p value
Species richness	treatment	4	73.49	18.37	22.97	<0.0001
	residual	17	13.60	0.80	-	-
Margalef's index	treatment	4	1.36	0.34	21.18	<0.0001
	residual	16	0.26	0.02	-	-
Shannon diversity	treatment	4	1.89	0.47	9.09	<0.0001
	residual	17	0.89	0.05	-	-
Simpson diversity	treatment	4	0.36	0.09	5.45	0.006
	residual	16	0.26	0.02	-	-

d.f = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

Table A10-2: One-way Analysis of Variance (ANOVA) of the mean species richness, Margalef's index, Shannon and Simpson diversity index of the soil core assessment 3 in 2006, with five different host age groups (nursery, 2, 8, 15 and 26 yrs) as treatments.

Analysis	Source of variation	d.f	ss.	m.s.	F value	p value
Species richness	treatment	5	78.78	15.76	8.22	<0.0001
	residual	23	31.88	1.39	-	-
Margalef's index	treatment	5	2.91	0.58	10.43	<0.0001
	residual	41	2.29	0.06	-	-
Shannon diversity	treatment	5	4.77	0.95	8.30	<0.0001
	residual	41	4.71	0.12	-	-
Simpson diversity	treatment	5	0.99	0.19	6.21	<0.0001
	residual	41	1.3	0.03	-	-

d.f = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

Table A10-3: One-way Analysis of Variance (ANOVA) of the mean species richness, Margalef's index, Shannon and Simpson diversity index of the outplanting survey in 2006/07.

Analysis	Source of variation	d.f	ss.	m.s.	F value	p value
Species richness	treatment	8	39.63	4.95	3.57	0.008
	residual	23	31.88	1.39	-	-
Margalef's index	treatment	8	0.76	0.09	2.86	0.2
	residual	23	0.76	0.03	-	-
Shannon diversity	treatment	8	3.25	0.41	2.45	0.04
	residual	23	3.8	0.17	-	-
Simpson diversity	treatment	8	0.85	0.11	2.00	0.093
	residual	23	1.23	0.05	-	-

d.f = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

APPENDIX 11 SUPPLEMENTARY DATA SIMPER ANALYSIS

In the nursery, *Wilcoxina mikolae* was the discriminating species. *Rhizopogon rubescens* had a higher average abundance in the nursery but was not as evenly distributed over the cores that were analysed. Site K05F was characterised by *Rhizopogon rubescens*. In site K04, *Wilcoxina mikolae* was the discriminating species belowground. *Amanita muscaria* and ECM type unknown Basidiomycete were the characterising species for site K98. Site K91 was discriminated belowground from the other sampling sites by *Inocybe* sp. In K80, the discriminating species belowground was ECM type unknown Basidiomycete.

Table A8-1: Similarity analysis of the belowground ECM communities from soil core assessment 3 in 2006. Sites sampled: nursery, K05F, K04, K91, K91 and K80, eight soil cores in each site. Listed are higher contributing species only (90% contribution cut off percentage). Discriminating species are underlined.

	Average Abundance	Average Similarity	Sim/SD	Contribution (%)
Nursery (n=8) Average similarity: 50.19%				
Wilcoxina mikolae	259.13	18.83	1.60	37.51
Rhizopogon rubescens	365.13	13.60	0.93	27.09
Hebeloma sp.	277.88	10.52	0.68	20.96
Tuber sp.	284.25	6.11	0.65	12.17
K05F (n=8) Average similarity: 46.27%				
Rhizopogon rubescens	588.75	34.70	1.25	75.00
Tuber sp.	108.25	9.51	0.66	20.56
K04 (n=8) Average similarity: 54.1%				
Wilcoxina mikolae	111.71	41.93	2.69	77.5
Rhizopogon rubescens	53.29	11.24	0.75	20.78
K98 (n=8) Average similarity: 33.11%				
unknown Basidiomycete	47.75	12.70	0.91	38.35
Amanita muscaria	73.38	12.23	0.96	36.92
various unknown	16.5	4.33	0.50	13.09
K91 (n=8) Average similarity: 29.96%				
Inocybe sp.	25.00	11.93	1.02	39.84
Amanita muscaria	39.25	5.18	0.67	17.3
unknown Basidiomycete	16.13	4.15	0.68	13.84
Pseudotomentella sp.	11.00	3.47	0.57	11.6
Pseudotomentella tristis	13.13	3.46	0.49	11.54
K80 (n=8) Average similarity: 40.98%				
unknown Basidiomycete	79.75	19.43	4.61	47.42
Amanita muscaria	54.13	8.02	1.31	19.58
unknown 9	49.88	4.09	0.72	9.98
Tomentella sp.	34.38	2.05	0.56	4.99
unknown various	23.75	1.84	0.42	4.50
Cenococcum geophilum	45.38	1.57	0.33	3.82

n=soil cores; Sim/SD = Similarity/Standard deviation