Eucalypt decline and ectomycorrhizal fungal community ecology of *Eucalyptus delegatensis* forest, Tasmania, Australia.

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Submitted in fulfilment of the requirements for the

Degree of Doctor of Philosophy

University of Tasmania

October 2011

Photograph: North-western Tasmanian *E. delegatensis* forest in morning smoke haze. Photographer: Bob Hamilton, Forestry Tasmania.

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Abstract

Ectomycorrhizal fungi underpin critical ecosystem processes which affect tree health. Eucalypt decline is widespread throughout Australia, and its cause has been attributed to a variety of factors, including forest management. In Tasmania, dieback in *Eucalyptus delegatensis* R.T. Baker has been linked to altered fire regimes and associated changes in mycorrhizal communities. This thesis presents a study that explores ectomycorrhizal species richness and community composition in relation to eucalypt health, understorey vegetation and soil chemistry in the context of fire history. The results further our understanding of ectomycorrhizal ecology and elucidate factors important to the maintenance of a healthy forest ecosystem.

Study sites were established in *E. delegatensis* forest with either sclerophyll understorey (six plots) or rainforest understorey (six plots). Eight of the plots, located in north-east Tasmania, had been established for a study of fire ecology and had known fire histories ranging from 42 years since the last fire to long unburnt (>120 years). Four plots located in north-western Tasmania were long unburnt but had been disturbed by logging 22-25 years previously. Ectomycorrhizal fungal sporocarps, root tips and soil samples were collected during a three-year period from all 12 plots. Samples from soil, root tips and sporocarps gave rise to different but complementary information about ectomycorrhizal communities. Fungal operational taxonomic units were identified through DNA sequencing and phylogenetic analysis. At each site, understorey vegetation was characterised, soil and eucalypt foliage chemistry was analysed, and eucalypt crown condition was assessed. Primary crown dieback was identified as the most effective method for the measurement of eucalypt health. Multivariate statistical analyses were used to explore the relationships among ectomycorrhizal communities, eucalypt health, vegetation and abiotic variables.

E. delegatensis forest with rainforest understorey was more than likely to be affected by severe eucalypt decline, had higher concentrations of soil inorganic nitrogen (nitrate and ammonium) and eucalypt foliar nitrogen, and had lower concentrations of soil and eucalypt foliar phosphorus, than forest with sclerophyll understorey. As forest declined in health the ecosystem moved from being nitrogen limiting to phosphorus limiting due to reduced phosphorus availability and plant uptake, potentially due to altered mycorrhizal activity. Ectomycorrhizal communities differed between moderately and severely declining forest and were correlated to crown health and altered soil chemistry associated with the two

levels of decline. The Cortinariaceae had high species richness in healthiest sites while the Russulaceae and Thelephoraceae were rich in forest affected by severe decline. In north-western and north-eastern Tasmania unique and distinctly different ectomycorrhizal fungal communities were found to occur in *E. delegatensis* forest with rainforest understorey versus those with sclerophyll understorey.

Irrespective of understorey type and health status, the Cortinariaceae were highly diverse, and were the most species-rich family within the ectomycorrhizal community of *E. delegatensis* forest. The Cortinariaceae also was the most abundant family in the root tip community. The Helotiales, Russulaceae and Thelephoraceae also were important components. The importance of the Cortinariaceae in *E. delegatensis* forest is similar to other Australian eucalypt forests but distinctly different from northern hemisphere forests, which tend to be dominated by the Russulaceae, Thelephoraceae and Corticeaceae.

Distance-based multiple linear regression models using only significant predictor variables based on soil and foliage nutrient concentrations and crown health were able to explain 52% of the variation in fungal community composition, and 44% of the variation in ectomycorrhizal community proportional composition at the family level. Soil pH, total soil nitrate, soil organic carbon and soil phosphorus were significant in predicting ectomycorrhizal species composition and proportional composition in the final models. A multiple linear regression model showed that available soil nitrate and phosphorus were significant in predicting ectomycorrhizal community richness. High richness was associated with low available soil nitrate or phosphorus.

Northern hemisphere studies which show that changes in soil chemistry, especially mineral nitrogen, can strongly influence mycorrhizal species richness, species composition and community structure corroborate the likely influence of soil nitrogen on the ectomycorrhizal communities of *E. delegatensis* forest. This is the first study to find a strong correlation between ectomycorrhizal fungal communities and the status of eucalypt forest health. The results support the currently proposed model that, in the absence of fire, premature decline of temperate Australian eucalypt forests is closely linked to changes in soil chemistry, understorey vegetation and mycorrhizal communities.

Abbreviations

ANGIS	Australian National Genome Information Service
ANOVA	Analysis of Variance
asl	Above Sea Level
bp	Base-pair (nucleic acid)
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CAP	Canonical Analysis of Principal Coordinates
CCorA	Canonical Correlations Analysis
DBH	Diameter at Breast Height
dNTP	Deoxynucleotide triphosphate
ECM	Ectomycorrhizal
EDTA	Ethylene di-amino tetra-acetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal Transcribed Spacer
LB	Luria-Bertani
LSU	Large Subunit
MDS	Multi-Dimensional Scaling
OTU	Operational Taxonomic Unit
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
rDNA	DNA coding for ribosomal RNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SE	Standard Error
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SOC	Super Optimum Catabolite
SSU	Small Subunit
TE	Tris-EDTA
T-RFLP	Terminal Restriction Fragment Length Polymorphism
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Publications and communications

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Awards

- Recipient of Bushfire CRC top-up scholarship 2006 (AUS $18 000).
- Recipient of the Holsworth Wildlife Research Endowment 2006, 2007, 2008 (AUS $15 000).
- Inaugural recipient of the Ecological Society of Australia Jill Landsberg Trust applied ecology award, 2007 (AUS $6 000).
- Recipient of the Institute of Foresters Maxwell Jacobs Trust Award, 2007 (AUS $2 500).
- Recipient of Forestry CRC top-up 2009 (AUS $9 000).

Acknowledgements

First and foremost I wish to thank my supervisors: Associate Professor Caroline Mohammed, Dr Morag Glen, Dr Neil Davidson, Dr Dugald Close and Dr Tim Wardlaw, for their support and advice over the years.

I am grateful to the many people that provided field and technical assistance for this project, especially David Page, Chris Ware, Dr Genevieve Gates, Professor David Ratkowsky, Luci Agustini, Malcolm Hall, the volunteers who attended the fungal foray, Tim Watson, Dr Robert Archibald and Dr Perry Swanborough. I also wish to thank Dr Christine Stone and Dr Anthony Francis for their input into the project and Professor David Ratkowsky and Dr Greg Lee for providing statistical advice.

I am indebted to all of the Forestry Tasmania staff that assisted with the project including the fire staff and foliage shooters, especially Tony Blanks for all his help organising the burn treatment and Bob Knox for overseeing the burnt treatment.

Thanks to all the friendly and helpful members of the Bruns Lab, at the University of California Berkeley, especially Professor Tom Bruns and Tim Szaro, for making my stay so enjoyable and productive.

A big thank-you to my loved ones: Mum, Dad, Laurence, Steve, and all my friends who have encouraged and supported me throughout my studies. A special thanks to my Grandparents who fully supported my studies and assisted financially enabling me to visit UC Berkeley.

This work was funded by a number of agencies: The Bushfire CRC, the Holsworth Wildlife Trust, the Jill Landsberg Trust, the Maxwell Jacobs Trust, Forestry Tasmania, the CRC for Forestry, the School of Plant Science University of Tasmania, and the Tasmanian Institute of Agricultural Research (School of Agricultural Science) University of Tasmania.

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1.1.1 Forest decline

Decline has been observed in many different forests across the globe (Mueller-Dombois 1988; Ciesla & Donaubauer 1994; Wardle, Walker *et al.* 2004) affecting European (Innes 1992b; Innes 1992a) and North American (Chevone & Linzon 1988) coniferous and broadleaf forests dominated by species such as *Picea* A. Dietr.(Pastor, Gardner et al. 1987; Estivalet, Perrin et al. 1990), *Quercus* L. (Thomas, Blank *et al.* 2002), *Fagus* L. (Stribley & Ashmore 2002) and *Acer* L. (Horsley, Long *et al.* 2000). Southern hemisphere forests are affected, including those dominated by *Nothofagus* Blume (Wardle & Allen 1983) and *Eucalyptus* L'Her (Withers & Ashton 1977; Old, Kile *et al.* 1980; Landsberg 1985; Heatwole & Lowman 1986; Jurskis 2005b).

The term decline refers to the chronic or progressive deterioration in health of an individual tree or group of trees resulting in premature death (Manion 1991; Innes 1992b). The interaction of biotic and abiotic factors producing a cycle of stress-response events that results in gradual decline in health and mortality differentiates decline from other forest diseases (Manion 1991; Percy 2002). Symptoms of decline include an alteration or reduction in growth including tree girth, the presence of cankers, foliar discolouration and crown dieback (Schulze 1989; Innes 1992b). Dieback differs from decline in that loss of vigour in the crown follows a single stress event after which recovery can occur, whereas decline is a progressive deterioration in health (Manion 1991). Dieback involves crown thinning due to foliage loss, failure of normal new growth to replace foliage and death of branches beginning at the growing tips and spreading to the primary branches (Ellis 1964; Innes 1992b) .

Forest decline has a complex aetiology, and causes differ between different forests. The decline of northern hemisphere forests has been attributed to increasing air pollution and associated soil acidification due to anthropogenic causes (Johnson & Siccama 1983; Pitelka

& Raynal 1989; Schulze 1989; Führer 1990; Innes 1992a; Innes 1993a; Stribley & Ashmore 2002; Thomas, Blank *et al.* 2002). Altered soil chemistry has been identified as playing an important role in forest decline. Nitrogen deposition and saturation have been identified as causal agents of northern hemisphere temperate forest decline (Pastor, Gardner *et al.* 1987; Aber 1992; Aber, McDowell *et al.* 1998; Magill, Aber *et al.* 2000; Fenn, Poth *et al.* 2006; Wallace, Lovett *et al.* 2007) and more recently have been identified as a possible cause of eucalypt decline in the southern hemisphere forests (Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). Other general factors associated with forest decline are climatic stress, vegetation dynamics, foliage chemistry and defoliation, pathogens and microbial community alterations (Estivalet, Perrin *et al.* 1990; Führer 1990; Horsley, Long *et al.* 2000; Stribley & Ashmore 2002; Thomas, Blank *et al.* 2002; Close, Davidson *et al.* 2009).

1.1.2 Eucalypt dieback

Tree dieback has been observed in several species throughout Australia (Withers & Ashton 1977; Lunt 1998; Grigg, Close *et al.* 2009; Scott, Burgess *et al.* 2009) but is widespread amongst the genus *Eucalyptus* (Ellis 1964; Bird, Kile *et al.* 1974; Podger, Kile *et al.* 1980; Heatwole & Lowman 1986; Wardlaw 1989; Lowman & Heatwole 1992; Granger, Kasel *et al.* 1994; Davidson, Close *et al.* 2007). Eucalypt dieback has been categorised into a number of different types depending on species affected, geographic location, and possible causes. Types of dieback include *Eucalyptus marginata* Sm. dieback (Podger 1968; Grigg, Close *et al.* 2009) and *E. gomphocephala* DC. dieback (Fox & Curry 1980; McCaw & Sneeuwjagt 2002; Archibald, Bowen *et al.* 2005) in Western Australia. Regrowth dieback affects *E. regnans* forest (Podger, Kile *et al.* 1980; Wardlaw 1989) and high-altitude dieback affects *E. delegatensis* and *E. coccifera* Hook.f. forest in Tasmania (Ellis 1964; Ellis, Mount *et al.* 1980; Harvest, Davidson *et al.* 2008). Other forms of eucalypt dieback include bell miner dieback, which affects *E. saligna* Sm. in NSW (Stone 1996; Florence 2005; Stone, Kathuria *et al.* 2008), rural and farmland tree dieback (Landsberg 1985; Landsberg, Morse *et al.* 1990; Close & Davidson 2004; Davidson, Close *et al.* 2007), lime-induced chlorosis dieback (Mundulla Yellows) (see Parsons & Uren 2007 for review; Grigg, Close *et al.* 2009).

Crown condition, the state of health of the upper part of the tree containing the leaves and branches, is often used as a basis for the measurement of tree and forest decline (Manion 1991; Innes 1993b; Zarnoch, Bechtold *et al.* 2004). General symptoms of decline in crown condition are observed despite the multiple causes of eucalypt decline. These symptoms

include altered distribution and arrangement of branches and foliage in the canopy, changes in foliage density, the proportion of dead twigs and branches, and the amount of epicormic growth (growth from dormant buds below the bark) (Grimes 1978; Innes 1990; Stone 1999; Zarnoch, Bechtold *et al.* 2004). A number of methods have been developed and applied to assess eucalypt decline (Grimes 1978; Fox & Curry 1980; Kile, Turnbull *et al.* 1981; Wardlaw 1989; Stone, Wardlaw *et al.* 2003a; Jurskis, Selby *et al.* 2005; Stone & Haywood 2006); a standardised, rigorous, and efficient field-based method has not been identified and adopted.

1.1.3 Causes of eucalypt decline

Eucalypt decline does not have one single cause but rather is the result of complex biotic and abiotic interactions and alterations within an ecosystem (Podger, Kile *et al.* 1980; Heatwole & Lowman 1986; Stone 1999; Jurskis & Turner 2002; Florence 2005; Harvest, Davidson *et al.* 2008; Close, Davidson *et al.* 2009).

Altered fire regimes have been implicated in eucalypt forest decline (Ellis, Mount *et al.* 1980; McCaw & Sneeuwjagt 2002; Florence 2005; Jurskis 2005a; Close, Davidson *et al.* 2009). Fire plays an important role in nutrient cycling within Australian eucalypt forests. Fire combusts organic material and releases nutrients released from the litter, soil, and understorey (Raison 1980; Grove, O'Connell *et al.* 1986; Attiwill, Polgase *et al.* 1996; Grogan, Bruns *et al.* 2000). The role of fire in the Australian landscape has been debated thoroughly (Bowman 1998; Jurskis 2000) and there is agreement that Australian vegetation evolved in a fire prone landscape and is well adapted to fire (Gill, Grove *et al.* 1981).

Australian Aboriginal peoples often manipulated burning patterns to create a more open landscape (Jones 1969; Bowman 1998). Many Australian landscapes have seen an alteration in fire regime since European settlement (Bowman 1998; Jurskis 2000). In the absence of fire, ecological processes and abiotic variables such as nutrient cycles, and competition for water, the soil microclimate and microbial communities are altered (Granger, Kasel *et al.* 1994; Jurskis & Turner 2002; Jurskis 2005b; Close, Davidson *et al.* 2009).

One aspect of the forest ecosystem, which may be particularly sensitive to altered fire regimes, is the nitrogen cycle. Changes in the pattern of nitrogen cycling have also been identified as contributing to eucalypt decline (Granger, Kasel *et al.* 1994; Jurskis & Turner 2002; Turner, Lambert *et al.* 2008). Long-term accumulation of nitrogen, which can occur in

the absence of fire, and can lead to biochemical imbalances in the soil and foliage together with root morphological changes, is suggested as a causal process (Jurskis & Turner 2002; Jurskis 2005b; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). Invasion of a shrubby understorey affecting both soil and vegetation dynamics also contributed to the declines (Jurskis 2005a; Jurskis and Turner 2002; Close, Davidson *et al.* 2009), and water stress may exacerbate the decline (Jurskis and Turner 2002; Close, Davidson *et al.* 2009). Secondary repercussions including altered microbial communities, increased herbivory, pests, and disease, may also occur (Lowman & Heatwole 1992; Stone 1999; Jurskis & Turner 2002; Jurskis 2005a; Harvest, Davidson *et al.* 2008; Close, Davidson *et al.* 2009; Scott, Burgess *et al.* 2009).

1.1.4 *Eucalyptus delegatensis* forest in Tasmania

Tasmania is an island state of Australia, lying between the latitudes of 40° and 43°40’ S and is separated from mainland Australia by Bass Strait. Tasmania covers an area of 6.82 million ha, of which approximately 3 million ha are forested.

E. delegatensis forest covers an estimated area of 600 000 ha of Tasmania, of which over half is managed for wood production (Ellis & Lockett 1991). This forest type is described as wet temperate forest dominated by tall eucalypt dominants with open canopies (National Forestry Inventory 2002, Figure 1.1). This community is mainly located on the Central Plateau, the North East highlands and Western Tiers between an altitude of 600 m and the tree line at 1100 m on steep mountain slopes and cool mountain valleys (Ellis & Lockett 1991; National Forestry Inventory 2002). *E. delegatensis* forest is commonly found on deep basalt, dolerite, and granite soils and is dependent on high and regular rainfall (Ellis 1985; National Forestry Inventory 2002). The forest is dominated by *E. delegatensis* solely, or in association with *E. amygdalina*, Labill. *E. dalrympleana* Maiden., *E. pauciflora* Sieber ex Spreng. and *E. coccifera* (Ellis 1992).

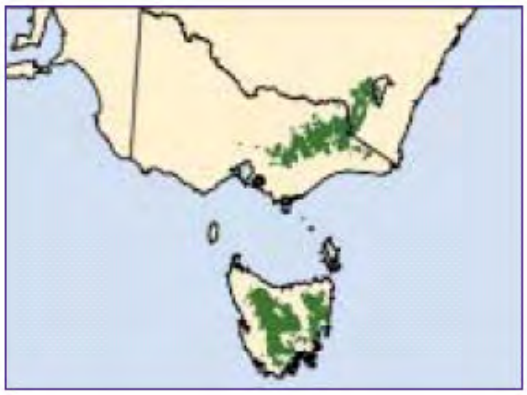

Figure 1.1 The distribution of *E. delegatensis* forest in Australia (National Forestry Inventory 2002). Green indicates forested areas dominated by *E. delegatensis*.

Fire is an important factor shaping eucalypt communities of Tasmania (Jackson 1968; Ellis 1985; Fensham & Kirkpatrick 1992). *E. delegatensis* forest relies on intense fires for regeneration, which results in even-aged stands in forests with a closed wet sclerophyll or rainforest understorey, or when fire is less intense, well defined multiple aged classes of eucalypts occur in forest with an open shrubby understorey (National Forestry Inventory 2002). A model proposed by Jackson (1968) describes the interactions between fire frequency and vegetation community composition and structure of Tasmanian eucalypt forests. High frequencies of fire (15-25 years) result in open communities, and as fire interval is decreased there is an increase in wet-scrub and rainforest plants, with a mixed eucalypt and rainforest community resulting from fire interval of 25-200 years. Eucalypts are lost from the system when fire intervals exceed the lifespan of eucalypts (approximately 350 - 500 years) (Jackson 1968). Ellis (1985) built on Jackson's (1968) model and proposed a model for secondary succession in *E. delegatensis* forest. The model incorporates the complex relationship between fire frequency, soil and vegetation. The absence of fire from this eucalypt forest produces a rainforest climax community, which can regenerate through gap-phase replacement. Forests dominated by *E. delegatensis* over a ground layer of grass or ferns with a variable understorey of shrubs are part of this successional sequence to rainforest. These forests occur when fire intervals are between 30-150 years and the speed of succession depends on altitude (more rapid succession takes place at lower altitudes). When the fire interval is shorter, rainforest species are eliminated allowing the establishment of eucalypts over a grassy understorey.

Prior to European settlement, large areas of *E. delegatensis* forest in Tasmania were utilised by Aborigines as hunting grounds, which involved regular patch-burning to aid hunting and

travelling (Ellis & Lockett 1991; Ellis & Pennington 1992). At the time of European settlement these forests were open, containing mixed-age eucalypts with old and mature trees and ground cover of either grass or scrub (Ellis & Lockett 1991). After European settlement the early settlers maintained regular burning over much of the highlands that were used for grazing (Ellis 1995). Remote areas, and areas of high rainfall experienced a decrease in burning or have remained unburnt for long periods (Ellis & Lockett 1991). Areas that were burnt experienced fires that were less frequent and more intense than those lit by the Aborigines (Ellis 1992).

E. delegatensis is economically important to the timber industry providing a high-quality wood resource (National Forestry Inventory 2002). Much of the area containing *E. delegatensis* forest has been utilised for sawn timber since the second World War, and after the 1970's forestry became a major activity (Ellis 1995). Most of the *E. delegatensis* forest in Tasmania has had some partial harvesting or timber removal since European settlement (Rob Stuart and Bob Hamilton, Forestry Tasmania, pers. comm.). Timber harvesting has mostly been carried out by clear-felling or partial-harvest (where some trees are left in the forest) followed by slash burning (National Forestry Inventory 2002; Neyland & Cunningham 2004). These techniques have resulted in poor *E. delegatensis* forest regeneration on the coldest sites (high plains and valley bottoms) and alternative strategies such as uneven-management techniques, high intensity burning following clear felling, are now employed to ensure sustainable management (Neyland & Cunningham 2004).

1.1.5 *E. delegatensis* decline in Tasmania

E. delegatensis decline, occurs above 800 m in forest where mean annual rainfall is greater than 1200 mm (Ellis 1964). This type of decline was first observed in the 1960's in the north-east of Tasmania (Ellis 1964). Decline affects trees of all age classes and canopy positions and severity increases with altitude (Ellis 1964). Premature decline of *E. delegatensis* has been observed in areas unburnt for 50 years at the highest elevations and 70 years at elevations of 800 m (Ellis 1964). The senescence of eucalypt trees often less than 200 years old is extremely premature considering their natural lifespan of 350-500 years (Ellis 1985). Visible decline begins with thinning of the foliage and then proceeds to death of small branches with some epicormic growth from the main stem and branches (Ellis, Mount *et al.* 1980). The leaves of affected *E. delegatensis* are abscised whilst still

green and turgid, and death of the tree occurs within a few years of appearance of the symptoms (Ellis, Mount *et al.* 1980).

1.1.6 Causes of *E. delegatensis* decline

The progression of *E. delegatensis* decline has been consistently correlated to the development of a rainforest understorey where fire has been excluded for long periods (Ellis 1964; Ellis, Mount *et al.* 1980; Ellis & Pennington 1992; Harvest, Davidson *et al.* 2008). Ellis, Mount *et al.* (1980) conducted a study to test the effect of understorey removal and burning on *E. delegatensis* decline. When the rainforest understorey was removed, no change in eucalypt health was observed, but when the understorey was burnt, the eucalypts exhibited an increase in health (Ellis, Mount *et al.* 1980). The amelioration of decline symptoms by fire suggests that fire alters the ecosystem in a way that allows the eucalypts to recover.

Changes in soil chemistry have been identified as an important factor in the decline of many forests and may occur as a result of changing vegetation dynamics in the absence of fire. One of the most recent theories explaining eucalypt decline holds that the accumulation and saturation of mineral nitrogen in the soil in the absence of fire leads to a decline in health (Turner & Lambert 2005; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). Immobilisation of nutrients in biomass and changes in availability of nutrients, especially nitrogen, through the process of mineralisation and nitrification, are important, and can lead to changes in nutrient uptake (Turner & Lambert 2005), as well as changes in N:P stoichiometry. Where tree uptake of nutrients is greater than available nutrients the soil nutrient pools decrease, but where nutrient inputs are greater than uptake, nutrients can accumulate and availability of nutrients can be altered (Close, Davidson *et al.* 2009). The results of Harvest, Davidson *et al.* (2008) give support to the role of altered soil chemistry in *E. delegatensis* decline. Soils collected from under declining eucalypt forest had higher concentrations of total pools of nitrogen, phosphorus and organic carbon than soils collected from under healthy eucalypt forest (Harvest, Davidson *et al.* 2008). Other differences between forest with declining *E. delegatensis* trees and forest containing healthy trees include the development of a litter layer in which humus decomposition is compromised (Ellis 1964), the accumulation of allelochemicals at cool, moist sites (Bowman & Kirkpatrick 1986), and reduced soil temperatures (Ellis 1971) in forest exhibiting decline.

Another factor that may be contributing to *E. delegatensis* forest decline is altered microbial, including mycorrhizal fungal, communities. Ectomycorrhizal associations of healthy and declining *E. delegatensis* forest were explored by Ellis and Pennington (1992) using seedling pot trials. Eucalypt seedlings grown in soil collected from declining forest had sparse root systems and formed few mycorrhizal associations, whereas seedlings grown in soil collected from healthy forest formed frequent ectomycorrhizal associations on vigorous root systems (Ellis & Pennington 1992). Furthermore, when soil from healthy forest was added to soil from declining forest, eucalypt seedlings showed a marked increase in growth compared to seedlings grown in only unhealthy soil (Ellis & Pennington 1992). It is possible that altered vegetation and soil conditions present in declining *E. delegatensis* forests result in a corresponding succession in the soil community from one that supports eucalypt forest to one that is antagonistic (Ellis & Pennington 1992).

Germination bioassays conducted by Harvest, Davidson *et al.* (2008) using soil filtrates support this theory. Filtrates contained either bacteria and fungi, or only bacteria. Soil filtrates from declining *E. delegatensis* forest and rainforest with dead eucalypts induced a significant dysplastic germination response in linseed compared to soil filtrates from healthy eucalypt forest. There was a greater dysplastic response from filtrates containing only bacteria, suggesting that an antagonistic bacterial component rather than a fungal component was the cause (Harvest, Davidson *et al.* 2008). Although soil filtrates from declining *E. delegatensis* forest affected the germination response of linseed, the filtrates from declining *E. coccifera* forest did not cause any dysplastic germination response (Harvest, Davidson *et al.* 2008). The differing germination responses to the different soils suggest that the bacterial component causing the dysplastic response in linseed is not common to all sites with overstorey eucalypt decline (Harvest, Davidson *et al.* 2008).

1.2 Ectomycorrhizal function and ecology

1.2.1 Mycorrhizal associations

Mycorrhizal associations are symbiotic relationships between a plant and fungus whereby both are benefitted. The fungus is provided with carbohydrates from the plant and the plant benefits through increased access to nutrients and water via the fungus (Smith & Read 1997). More than 90% of all plants form mycorrhizal associations (Aerts 2002). There are many different types of mycorrhizal associations including:

- Ectomycorrhizas that occur between woody plants and a variety of fungi (Brundrett & Cairney 2002). Fungal hyphae grow within the plant root but do not penetrate the internal part of the root cells (Imhof 2009).
- Vesicular-arbuscular (VA) associations, which are formed by Glomeromycota (Schussler, Schwarzott *et al.* 2001)that penetrate root epidermal cells and form nutrient exchange structures inter-cellularly (Imhof 2009).
- Ericoid mycorrhizas, which occur on plants from the Ericaceae and mycorrhizal structures appear as coils within root epidermal cells (Read 1996).
- Ectendomycorrhizas, which are a combination of ecto- and endo- mycorrhizal associations (Yu, Egger *et al.* 2001) in that they anatomically predominate inter-cellular space, but based on fungal taxa involved are a variant of ectomycorrhizas (Imhof 2009).
- orchid mycorrhizas (Rasmussen 2002; Imhof 2009).

1.2.2 Ectomycorrhizal associations

Many forest trees around the world form ectomycorrhizal (ECM) associations with a fungal partner (Newman & Reddell 1987; Harley 1989), including the tree genus *Eucalyptus* (Myrtaceae) (Bougher 1995). Eucalypts form ectomycorrhizas with a variety of Basidiomycetes, Ascomycetes and some Mucoromycetes (Molina, Massicotte *et al.* 1992; Taylor & Alexander 2005). Individual eucalypts may associate with many species of fungi (Brand 1992; Molina, Massicotte *et al.* 1992) each with physiological attributes operative under different environmental conditions (Molina, Massicotte *et al.* 1992; Bougher & Tommerup 1996).

Ectomycorrhizal structures are morphologically distinct with physiological changes in both plant and fungus and these are the sites of nutrient exchange between the plant and fungus (Smith & Read 1997; Taylor & Alexander 2005). The fungus grows over short lateral roots forming a sheath from which hyphae grow inter-cellularly into the outer cortex of the root producing a ‘Hartig Net’. The fungus does not normally penetrate the endodermis or the stele. Ectomycorrhizas lack root hairs and the outer cortical and epidermal cells are usually elongated.

1.2.3 Ectomycorrhizal function

Ectomycorrhizal fungi release and transfer nutrients through the ecosystem and are critical for nutrient cycling and ecosystem function (Marschner & Dell 1994; Tommerup & Bougher 1999; Lilleskov & Bruns 2001; Alvarez, Huygens *et al.* 2009). Mycorrhizal fungi increase the absorbing area of plant roots and can explore a greater volume of soil than plant roots alone (Harley & Smith 1983; Bolan 1991). ECM fungi also increase plant nutrient uptake by releasing enzymes into the soil that dissolve tightly bound nutrients such as phosphorous, iron and zinc (Bolan 1991; Baxter & Dighton 2001; Alvarez, Gieseke *et al.* 2006; Bucher 2007; Alvarez, Huygens *et al.* 2009; Nygren & Rosling 2009; Tatry, Kassis *et al.* 2009).

Some ECM fungi are involved with decomposition and actively degrade organic materials through the production of enzymes such as protease, phosphatases and lignase (Courty, Buée *et al.* 2010). Also, the degradation of soil organic matter can be a significant source of carbon for ECM fungi (Bougher & Tommerup 1996; Read & Perez-Moreno 2003; Courty, Buée *et al.* 2010). There is evidence to show that some ECM fungi may also access nutrients through the process of mineral weathering by the dissolution of soil minerals (Courty, Buée *et al.* 2010).

Nitrogen availability is limiting to plant growth in many soils (Aerts 2002) so the ability of an ECM species to uptake and transfer various nitrogen sources to its host is important (Bruns 1995; Courty, Buée *et al.* 2010). ECM fungi differ in their ability to utilise and transfer various forms of nitrogen including ammonium, nitrate or organic nitrogen, such as proteins (Abuzinadah & Read 1986; Grove, O'Connell *et al.* 1986; Abuzinadah & Read 1989; Lilleskov, Hobbie *et al.* 2002; Sawyer, Chambers *et al.* 2003; Guidot, Verner *et al.* 2005; Müller, Avolio *et al.* 2007; Corrêa, Strasser *et al.* 2008). In Australia, ECM associations are particularly important for phosphorus acquisition, which is often limiting (Bougher 1995; Tommerup & Bougher 1999). ECM may be particularly important for plant nutrient uptake under stressful conditions, such as drought (Alvarez, Huygens *et al.* 2009).

Ectomycorrhizal functional diversity arises when species exhibit differential abilities to utilise resources, transfer nutrients or demand carbon from hosts (Koide, Courty *et al.* 2007; Courty, Buée *et al.* 2010), for example inter-specific variability to produce phosphomonoesterase, which is used to degrade organic P (Nygren & Rosling 2009). These differences in function lead to the possibility of functional complementarily within forest ecosystems, which may be important for ecosystem resilience (Courty, Buée *et al.* 2010).

Mycorrhizal fungi influence plant community structure, composition and productivity (Allen, Allen *et al.* 1995; Aerts 2002; Kernaghan 2005; van der Heijden, Bardgett *et al.* 2008; van der Putten, Bardgett *et al.* 2009). In general, plants that are involved in mutualistic associations profit greatly and often exhibit increased establishment, growth and survival (Burgess, Malajczuk *et al.* 1993; Smith & Read 1997; Tommerup & Bougher 1999; Brundrett & Cairney 2002; Swaty, Deckert *et al.* 2004; Alvarez, Huygens *et al.* 2009; van der Heijden & Horton 2009). The mycelium of mycorrhizal fungi link plants together forming a network through which resources, such as carbon and water, can be distributed through the ecosystem, generally along source-sink gradients (Finlay & Read 1986; Kennedy, Izzo *et al.* 2003; Booth 2004; Leake, Johnson *et al.* 2004; Simard & Durall 2004; Simard 2009; van der Heijden & Horton 2009; Courty, Buée *et al.* 2010). These networks may influence plant community diversity and structure through mediating resource transfer (Simard & Durall 2004; van der Heijden & Horton 2009; Courty, Buée *et al.* 2010). Common mycorrhizal networks are also known to help with plant regeneration following disturbance, aiding host recruitment and survival (Simard 2009; van der Heijden & Horton 2009). Furthermore, the presence of below-ground symbionts may be important for the migration of plant communities because of climate change, and survival of both the plant and mycorrhizal communities may be dependent on joint responses to change (Perry, Borchers *et al.* 1990; Courty, Buée *et al.* 2010). The mycorrhizal community may also influence the succession of plant communities under changing climatic conditions (Perry, Borchers *et al.* 1990).

Mycorrhizal fungi are involved with a variety of other activities including soil stabilisation, water uptake and storage, heavy metal tolerance and herbivore and pathogen resistance (Perrin 1990; Gehring & Whitham 1994; Cairney & Chambers 1999; Tommerup & Bougher 1999; Swaty, Deckert *et al.* 2004; Müller, Avolio *et al.* 2007; Plamboeck, Dawson *et al.* 2007; Tang, Zhang *et al.* 2008; Tripathi, Kamal *et al.* 2008; Claridge, Trappe *et al.* 2009; Luo, Li *et al.* 2009). Additionally, the sporocarps of ECM fungi are an important food source of mycophagous mammals (Claridge 2002). Like all fungi, ECM fungi store carbon in biomass, which has important implications for carbon cycling and climate change mitigation (Treseder & Allen 2000; Rooney, Killham *et al.* 2009).

Recently ECM research has begun to focus on functional diversity including metabolic processes using enzymatic tests, stable isotopes and fungal genomics (Courty, Pritsch *et al.* 2005; Courty, Pouysegur *et al.* 2006; Koide, Courty *et al.* 2007; Martin & Nehls 2009; Mayor, Schuur *et al.* 2009; Courty, Buée *et al.* 2010). ECM ecology and function, such as tissue-scale

carbon and nitrogen pathways and flux of photosynthates to mycorrhizal roots, can now be studied in the field through the advancement in techniques like Ion Mass Spectrometry and $^{13}CO_2$ pulse-labelling (Courty, Buée *et al.* 2010). The first full DNA sequence of an ECM species was a huge advancement in the area of fungal genomics (Martin & Selosse 2008) and the identification of fungal genes that are involved with specific functions, such as nutrient transport, will be valuable in increasing our understanding of ECM function (Martin, Kohler *et al.* 2007; Martin & Nehls 2009).

1.2.4 Ectomycorrhizal community ecology

Ectomycorrhizal communities are the result of complex relationships between numerous biotic and abiotic variables. Competition for resources, such as space and nutrients, by fungi resulting in niche partitioning is a large determinant of ECM community composition and diversity (Bruns 1995; Dickie 2007; Peay, Kennedy *et al.* 2008b). Ecological processes such as dispersal and inoculum potential also influence ECM community composition and structure (Newton 1992; Cairney & Chambers 1999; Peay, Bruns *et al.* 2007). These factors result in patchy distributions of ECM fungi, which vary in both the vertical and horizontal planes (Dickie, Xu *et al.* 2002; Tedersoo, Koljalg *et al.* 2003) and influence community structure so that in general few species dominate ECM communities while the majority of species are rare (Dahlberg 2001; Peter, Ayer *et al.* 2001a; Taylor 2002; Kennedy & Hill 2010).

Fungal preference for plant hosts also influences ECM community composition (Molina, Massicotte *et al.* 1992; Dickie 2007; Tedersoo, Jairus *et al.* 2008) as some species are host-specific while others, such as *Laccaria laccata* (Scop. : Fr.) Cooke, are generalists (Bruns, Bidartondo *et al.* 2002; Taylor 2008). Genetic diversity within a host plant species may also influence the diversity of associated ECM fungi (Taylor 2008). The number of host plants present in the community may play a role in ECM community diversity within ecosystems (Dickie 2007). Carbon allocation from host plants to mycorrhizal fungi has been shown to alter the abundance of mycorrhizas, ECM reproduction and diversity of mycorrhizal fungi (Hobbie 2006; Peter, Ayer *et al.* 2008; Andrew & Lilleskov 2009; Druebert, Lang *et al.* 2009; Yarwood, Myrold *et al.* 2009). The connectedness and interactions between the above- and below-ground communities mean that changes in the productivity of either community is likely to affect the other (van der Putten, Bardgett *et al.* 2009). Plant carbon allocation to mycorrhizal fungal partners may increase under higher CO_2 contributing to global warming

that may also result in increased mycorrhizal respiration, potentially altering mycorrhizal communities (Staddon 1998; Courty, Buée *et al.* 2010). Other climate change effects on mycorrhizal fungi may also be linked to plant host responses (Staddon, Heinemeyer *et al.* 2002).

ECM communities are further determined by climatic and edaphic factors (Lilleskov & Bruns 2001; Anderson & Cairney 2007; Craine, Elmore *et al.* 2009). ECM communities are known to vary greatly with soil chemistry (Bougher, Grove *et al.* 1990; Tommerup & Bougher 1999; Avis, McLaughlin *et al.* 2003). Changes in soil conditions such as increased soil nitrogen modifies ECM community richness, composition and structure (Wallenda & Kottke 1998; Lilleskov, Fahey *et al.* 2001; Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003; Treseder 2004; Parrent, Morris *et al.* 2006; Parrent & Vilgalys 2007; Kranabetter, Friesen *et al.* 2009). Likewise, soil pH, organic carbon, and soil phosphorus are also known to affect ECM communities (Treseder 2004; Dickie 2009; Twieg, Durall *et al.* 2009). Soil moisture and water availability affects sporocarp production, mycorrhizal root tip colonisation and abundance, and ECM community composition (Launonen, Ashton *et al.* 2004; Swaty, Deckert *et al.* 2004; Lilleskov, Bruns *et al.* 2009). Elevated CO_2, altered precipitation and increased temperatures that are predicted to occur with climate change may affect ECM fungi directly by impacting on growth and function, or indirectly through effects on the host plant (Staddon, Heinemeyer *et al.* 2002; Treseder 2004; Parrent & Vilgalys 2007; Cudlin, Kieliszewska-Rokicka *et al.* 2008; Garcia, Ovasapyan *et al.* 2008; Taylor 2008; Courty, Buée *et al.* 2010; Vargas, Baldocchi *et al.* 2010). Atmospheric and soil pollution may also affect ECM richness and root tip abundances (Jansen 1991; Kielszewska-Rokicka, Rudawska *et al.* 1997).

Changes in ECM community structure and diversity occur with forest development (Blasius & Oberwinkler 1989; Jansen 1991; Frankland 1992; Twieg, Durall *et al.* 2007), and early-stage and late-stage fungi have been identified (Mason, Wilson *et al.* 1993; Bruns 1995). Succession within the ECM community following disturbances such as fire or forestry has been identified (Visser 1995; Stendell, Horton *et al.* 1999; Chen & Cairney 2002; Hart, DeLuca *et al.* 2005; Cairney & Bastias 2007; Dickie 2009). Fungi present at different successional stages exhibit differing characteristics in dispersal, competition, resource utilisation and resource requirements (Bruns 1995).

ECM fungi interact with many other organisms within the ecosystem including the soil bacterial community, which can promote ECM associations (Garbaye 1994; Frey-Klett, Garbaye *et al.* 2007). Fungivores may aid with dispersal of spores, while pathogens play a role in ECM community ecology through negative effects on fungal and host growth and survival, and host herbivores may cause alterations in carbon allocation from host to ECM fungi, reducing root colonisation (Gehring & Whitham 1994; Gehring & Whitham 2002; Taylor & Alexander 2005).

1.2.5 Australian ectomycorrhizal fungi

Between 7000 and 25000 species of ECM fungi are thought to occur worldwide (Read & Perez-Moreno 2003; Taylor & Alexander 2005; Rinaldi, Comandini *et al.* 2008). Australia has a high diversity of ECM species with an estimated 6500 ECM species of which many are endemic (Bougher 1995; Neale Bougher pers. comm. 2009). In 1999, 700 species of fungi have been described as ectomycorrhizal with eucalypts (Tommerup & Bougher 1999). This figure has changed little since that date because of lack of taxonomic expertise in Australia (see section 1.3.3).

Research focusing on ECM diversity and function in Australia is sparse in comparison to other parts of the world. Early research includes recording Australian ECM plants and the structure and type of mycorrhizas of these plants (Chilvers & Pryor 1965; Warcup 1980; Brundrett & Abbott 1991) as well as documenting ECM morphotypes (Chilvers 1967; Ashton 1976; Malajczuk & Hingston 1981). Other studies have investigated the formation of ectomycorrhizas following inoculation experiments (Malajczuk, Molina *et al.* 1982; Malajczuk, Dell *et al.* 1987), and the function of ectomycorrhizas under controlled laboratory conditions (Bougher, Grove *et al.* 1990; Burgess, Malajczuk *et al.* 1993; Dell, Malajczuk *et al.* 1994; Chen, Brundrett *et al.* 2000; Duponnois, Duponnois *et al.* 2003; Launonen, Ashton *et al.* 2004).

The effects of disturbances such as fire (Malajczuk & Hingston 1981; Warcup 1990; Launonen, Ashton *et al.* 1999; Glen 2001; Chen & Cairney 2002; McMullan-Fisher, May *et al.* 2002; Bastias, Huang *et al.* 2006), mining (Gardner & Malajczuk 1988; Reddell, Gordon *et al.* 1999; Glen, Bougher *et al.* 2008), forestry (Lu, Malajczuk *et al.* 1999) and environmental variables such as litter (Reddell & Malajczuk 1984) on mycorrhizal function or diversity in Australian environments, have also been explored. Other work has focused on the taxonomy of Australian fungi, and more recently on molecular identification and genetic

variation of ECM species (Chambers, Sawyer *et al.* 1999; Sawyer, Chambers *et al.* 1999; Glen, Tommerup *et al.* 2001a; Glen, Tommerup *et al.* 2001b).

1.2.6 Tasmanian ectomycorrhizal fungi

There are very few studies dedicated to ECM fungi in Tasmania. Ashton (1976) was one of the first studies to publish information on the ectomycorrhizas of *E. regnans* F. Muell. More recently Warcup (1991) studied eucalypt mycorrhizas in relation to the regeneration of native forestry coups and Tedersoo, Jarius *et al.* (2008) studied host specificity in *E. regnans* forest. Other research on ectomycorrhizal fungi in Tasmania has focused on diversity and seasonality of hypogeous sporocarps and mycophagy (Johnson 1994). There are a number of more general publications on the fungi of Tasmania including macrofungal community diversity and ecology (Packham, May *et al.* 2002; Ratkowsky & Gates 2005), a comparison of macrofungi in silviculture forests (Gates, Ratkowsky *et al.* 2005) and a study of the macrofungi associated with coarse woody debris over a fire chronosequence (Gates 2009).

1.3 Sampling and identifying ectomycorrhizal species and communities

1.3.1 Challenges of sampling ectomycorrhizal fungi

There are a number of challenges to sampling ECM fungi. These can be viewed in terms of separate issues; the high diversity of ECM communities, the high temporal and spatial variability of ECM structures, and the different information that each of the ECM substrates provide.

ECM fungal communities of temperate and boreal ecosystems are highly diverse (Taylor 2002; Taylor & Alexander 2005; Tedersoo 2007; Taylor 2008) and ECM communities tend to be composed of many rare species and few common species (Dahlberg 2001; Peter, Ayer *et al.* 2001a; Taylor 2002). This high diversity and associated community structure has meant that ECM communities are very difficult to completely characterise.

ECM species can be difficult to sample as fungal sporocarps. Sporocarps can be easily missed as these reproductive structures are ephemeral, appearing at irregular, unpredictable intervals in response to various climatic stimuli and some species do not, or rarely reproduce sexually (Bougher & Tommerup 1996; Tommerup & Bougher 1999; O'Brien, Parrent *et al.* 2005). Also, many fungal species are inconspicuous or hypogeous and can be overlooked (Peter, Ayer *et al.* 2001a; O'Brien, Parrent *et al.* 2005). ECM fungi are

also highly spatially variable having patchy distributions and as such ECM structures such as hypogeous sporocarps and mycorrhizal root tips may be missed during sampling (Dickie, Xu *et al.* 2002; Taylor 2002; Taylor 2008). This is also true for collecting soil samples (Anderson & Cairney 2004). Due to the variability of ECM communities, sampling needs to be intense with numerous samples collected from frequent visits and over different seasons and long time periods. Even with long-term sampling, measurements of species richness and diversity may be inaccurate. For example, a study of fungal sporocarp richness and abundance conducted over 21 years in a stable mixed forest recorded new species every year, and the number of species would likely have continued to increase if sampling had continued (Straatsma, Ayer *et al.* 2001).

ECM species and communities can be sampled via sporocarp surveys, mycorrhizal root tip sampling, or through collection of soil. As discussed above, sporocarp sampling provides challenges in that they can only be collected as certain times of years following specific climatic conditions. Also, the presence of species sporocarps does not necessarily indicate the presence of ectomycorrhizas, and inversely, the absence of sporocarps does not indicate lack of ectomycorrhizal activity with the ecosystem. Root tip sampling provides a means of sampling active ectomycorrhizas, and provides information on below-ground ECM community composition and structure. Root tips sampling is time consuming as many root tips need to be collected and processed, and many can missed because of their small size. Soil can also be used to study ECM communities and is advantageous over other substrates as it is quick to collect, and process, and does not require technical expertise required for root tip collection and processing. One of the main disadvantages of studying soil is that is does not provide any information on ECM function, as do mycorrhizal root tips (which are actively involved in symbioses), or sporocarps (which are sexually reproducing), as DNA from dead mycelium or spores may be amplified along with active mycelium (Anderson & Cairney 2004).

The high diversity of ECM communities, along with the reproductive strategies of different ECM species, and the differential sampling techniques used to asses above and below-ground ECM communities, means little congruence between the above- and below-ground ECM communities is seen, and in general, a combination of sampling strategies provides the most comprehensive information about ECM communities (Gardes & Bruns 1996; Dahlberg, Jonsson *et al.* 1997; Peter, Ayer *et al.* 2001a; Taylor 2002; Porter, Skillman *et al.* 2008).

1.3.2 Techniques for studying ectomycorrhizal communities

Modern techniques for studying ECM communities are based on molecular identification or profiling. Advances in molecular methods have facilitated the accurate identification and analysis of fungal communities, and in combination with morphological techniques, provide a powerful means of studying ECM communities. Common molecular techniques that are used to identify ECM fungi and study ECM communities are summarised in Table 1.1 (see Anderson & Cairney 2004 for review).

Molecular methods for ECM identification and community profiling are based on ribosomal DNA (rDNA). The Internal Transcribed Spacer (ITS) is a non-coding region of the rDNA that separates the small-subunit (SSU) and the large-subunit (LSU) and has been widely applied to the study of fungi, especially ECM fungi. The ITS region is highly conserved within species but exhibits enough variation between species allowing taxonomic resolution often to the species level (Gardes, White *et al.* 1991; Kåren, Hogberg *et al.* 1997; Taylor & Bruns 1999; Anderson & Cairney 2004; Nilsson, Ryberg *et al.* 2009; Ryberg, Kristiansson *et al.* 2009).

DNA can be extracted and amplified using fungal specific primers from any fungal structure such as sporocarps, mycorrhizal root tips, or mycelium. Two universal primers ITS1 and ITS4 were developed by White, Bruns *et al.* (1990) to amplify the ITS spacer of the rDNA using Polymerase Chain Reaction (PCR). These primers were first applied the study of mycorrhiza by Gardes, White *et al.* (1991). The primers ITS1-F and ITS4B were developed by Gardes and Bruns (1993) for specific amplification of fungal and Basidiomycete DNA. Also the reverse primer, ITS4A was designed for increased specificity for Ascomycete DNA (Larena, Salazar *et al.* 1999). These primers have been subsequently used extensively for fungal community studies (Erland, Henrion *et al.* 1994; Kåren, Hogberg *et al.* 1997; Farmer & Sylvia 1998; Chambers, Sawyer *et al.* 1999; Jonsson, Dahlberg *et al.* 1999; Viaud, Pasquier *et al.* 2000; Burke, Martin *et al.* 2005).

Many molecular techniques result in the identification of operational taxonomic units (OTUs), which are groups of fungi that have similar molecular profiles or sequences. OTUs are often used as a proxy for a single species, but differ from a species unit, as their morphological or sexual compatibilities are generally not considered. Both the molecular and morphological variation of an organism needs to be considered to determine the appropriate limits to each OTU.

PCR-RFLP and T-RFLP are techniques that are useful for assessing community shifts and tracking presence and absence in samples (Horton & Bruns 2001; O'Brien, Parrent *et al.* 2005). The advantages of using these techniques are that each sample, either root tip or sporocarp, can be profiled, and profiles can then be grouped on similarity, removing the need for taxonomic identification of each individual sample. These techniques can be time consuming as most research has indicated that two restriction enzymes are needed to adequately differentiate fungal taxa, but in some cases, three or more may be required (Kåren, Hogberg *et al.* 1997; Glen, Tommerup *et al.* 2001b). Also, these techniques are limited in their ability to provide taxonomic affiliation or enumerate species richness of communities unless a large reference database is available, or they are combined with DNA sequencing (Horton & Bruns 2001; O'Brien, Parrent *et al.* 2005). Using PCR-RFLP or T-RFLP, in combination with DNA sequencing, does allow grouping of like profiles, which is beneficial as it minimises the number of sequences (and therefore minimises time invested and expense) that need to be obtained for taxonomic identification.

T-RFLP is advantageous over PCR-RFLP as it allows automated sizing with great precision of restriction fragments and a high output of data, as well as multiple species identification from a single sample but also has many disadvantages including the formation of pseudo-restriction fragments (Egert & Freidrich 2003; see Avis, Dickie *et al.* 2006; and Dickie & FitzJohn 2007 for review).

Cloning of PCR amplicons to isolate individual DNA fragments from environmental samples has been used for assessing soil fungal diversity (Viaud, Pasquier *et al.* 2000; Chen & Cairney 2002; O'Brien, Parrent *et al.* 2005; Porter, Skillman *et al.* 2008). This method allows mixed samples, such as soil, or multiple mycorrhizal root tips to be processed together. The main limitation to this method is determining the number of clones for PCR-RFLP and sequencing so that common and rare sequences can be determined and diversity is fully sampled (Anderson & Cairney 2004). In highly diverse communities a large number of clones may need to be screened. For example O'Brien, Parrent *et al.* (2005) sampled 100 soil cores and sequenced all 863 clones that were recovered from these cores resulting in 412 fungi, with no saturation in the species-effort curve.

DNA sequencing consists of a series of biochemical reactions, fluorescently labels the nucleotides, and their order is determined by a DNA sequencer. Sequences from different samples can be aligned and checked for similarity, enabling groups of like sequences to be

determined (e.g. species or operational taxonomic units) or searched for matches to known DNA sequences within a database.

Next-generation DNA sequencers (454- or Pyro-sequencing) that allow high quality sequence output based on the "sequencing by synthesis" principle are likely to supersede current instruments in the near future and will allow large-scale sequencing of complete fungal communities (Mardis 2008; Buee, Reich *et al.* 2009; Nilsson, Ryberg *et al.* 2009; Öpik, Metsis *et al.* 2009).Like all methodologies, 454-sequencing poses challenges in that the limited read lengths may be problematic for identification (lengths of individual reads of DNA sequence are in the neighbourhood of 300-500 nucleotides, shorter than the 800-1000 obtainable with chain termination methods). The composition depicted through this new style of sequencing and traditional sequencing may not be comparable (Nilsson, Ryberg *et al.* 2009). An alternative technique to sequencing, which also has the capacity for high throughput, is microarray analysis (Courty, Buée *et al.* 2010). In the past this technique has been used for genome-wide transcription profiling but is now being applied to the identification of ectomycorrhizal communities (Reich, Kohler *et al.* 2009).

For this research, amplification of the ITS region by PCR of root tips and sporocarps using the fungal specific primers ITS1 and ITS4 was utilised in combination with DNA sequencing. PCR, soil cloning, PCR-RFLP and DNA sequencing were all used in combination for identification of environmental samples (bulk soil).

Table 1.1 Summary of common molecular techniques used for the identification and characterisation of ectomycorrhizal species and communities.

Molecular technique	**Description of process**
DNA sequencing	DNA extractions followed by PCR, then the order of nucleotides is determined by a DNA sequencer (Gardes, White *et al.* 1991; Stendell, Horton *et al.* 1999; Tedersoo, Jairus *et al.* 2008). The key principle of most methods of DNA sequencing (e.g. Sanger sequencing) is chain termination with dideoxynucleotide triphosphates (ddNTPs). 454- or Pyro-sequencing with next generation sequencers differs from Sanger sequencing, in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides (Buee, Reich *et al.* 2009; Nilsson, Ryberg *et al.* 2009; Jumpponen, Jones *et al.* 2010; Tedersoo, Nilsson *et al.* 2010).
Restriction fragment length polymorphism (RFLP)	DNA amplification, followed by restriction digestion with endonucleases that cleave the DNA at certain restriction sites. Digested DNA, containing restriction fragments is then electrophoresed on high resolution agarose or acrylamide gel to determine RFLP patterns and restriction fragment sizes. The RFLP profiles from unknown material are then matched to profiles obtained from identified sporocarps or cultures (Erland, Henrion *et al.* 1994; Kåren, Hogberg *et al.* 1997; Glen, Tommerup *et al.* 2001a).
Terminal restriction fragment length polymorphism (T-RFLP)	Similar to PCR-RFLP but uses a fluorescent label unique to each PCR primer for detection of the terminal fragments of restriction-digested PCR products. These terminal restriction fragments contain the labelled primer and extend to the restriction site for the enzyme used (Liu, Marsh *et al.* 1997; Edel-Hermann, Dreumont *et al.* 2004; Burke, Martin *et al.* 2005).

Molecular technique	Description of process
Soil cloning	DNA is extracted from an environmental sample, amplified and then cloned using bacterial phage cells. These cells are then cultured after which DNA is extracted, and amplified. RFLP or sequencing can then be used to identify species or community patterns (Viaud, Pasquier *et al.* 2000; Chen & Cairney 2002; O'Brien, Parrent *et al.* 2005; Porter, Skillman *et al.* 2008).

1.3.3 Challenges of identifying ectomycorrhizal species

Robust identification of fungal species is required in any study about the fungal community but there are a number of challenges to achieving this aim.

Fungal culture is one of the earliest methods employed for the study of ECM fungi and involves isolation of a single fungal species, from sporocarps or environmental samples. There are a number of limitations associated with culture based identification but the most important characterisation of ECM communities is that this technique is biased toward species that are able to be isolated and grown as cultures (Anderson & Cairney 2004; O'Brien, Parrent *et al.* 2005). Many symbiotic fungi have specialised growth requirements and have proved difficult to grow well in culture (Courty, Buée *et al.* 2010). The ECM genus *Cortinarius* Pers. (Gray) is one group that has proven difficult to isolate and maintain in culture (Brundrett, Bougher *et al.* 1996) and has high diversity in Australian eucalypt forests (Bougher & Malajczuk 1986; Malajczuk, Dell *et al.* 1987; Bougher 1995).

The observation of morphological characteristics of fungal sporocarps is a common technique used for the identification of fungi, including ECM species. Historically, morphological identifications of sporocarps were difficult as they were based on northern hemisphere taxonomic classification, which does not fit many Australian species (Bougher 1995). In Australia, fungal identification difficulty is exacerbated by taxonomic constraints, as many species that are examined are either new to science or have not yet been formally described (Glen, Tommerup *et al.* 2001b). Some sporocarps, for example those of the genus *Cortinarius*, are very difficult to identify to species using morphology as many species share similar morphological characteristics (Glen, Tommerup *et al.* 2001b). A further limitation to

morphological identification of sporocarps is lack of mycological expertise in Australia, resulting from limited mycological teaching and funding for mycological studies.

Ectomycorrhizal root tip morphological classification (morphotyping) is used to group like fungi giving a proxy for species that are forming ectomycorrhizas (Agerer 1991; Agerer 2001) but does not usually allow taxonomic identification to species (Egger 1992; Burke, Martin *et al.* 2005). Gradations in morphological characteristics cause difficulties in distinguishing between two morphotypes of similar appearance (Horton & Bruns 2001; Burke, Martin *et al.* 2005). This can cause over estimations of species richness. A study conducted by Peter *et al.* (2001a) revealed that morphotyping has a lower resolution of ECM community composition than either sporocarp inventories or molecular analyses. Furthermore this method requires high labour and skill input.

There are also a number of issues in relation to identifying ECM fungi through molecular techniques. Despite the assumption that the ITS region is conserved within species, this region has been shown to exhibit polymorphism with some ECM species yielding identical profiles while other species yield multiple profiles (Gardes, White *et al.* 1991; Kåren, Hogberg *et al.* 1997; Chambers, Sawyer *et al.* 1999; Glen, Tommerup *et al.* 2001b; Horton 2002). Kåren, Hogberg *et al.* (1997) found seven out of 44 species exhibited polymorphism of the ITS region and Glen *et al.* (2001) identified intra-specific variation in the ITS region in 12 of the 42 (29%) Australian fungal species analysed. More recently, genomic regions, such as the SSU, LSU, meta-genomics, multi-gene studies, or even complete genomic sequencing, are now applied to the study of mycorrhizal fungi and these techniques highlight where the future of fungal molecular research may lie (Binder & Hibbett 2006; Fierer, Breitbart *et al.* 2007; Tedersoo, Jairus *et al.* 2008; Courty, Buée *et al.* 2010). Despite the disadvantages of using the ITS region, it is generally agreed that a more accurate and extensive estimate of species richness is provided from the ITS region than other genomic regions, and its use provides a standardised approach to identification (Lord, Kaplan *et al.* 2002; Nilsson, Ryberg *et al.* 2009).

There has been discussion over potential bias of primers to amplify a specific DNA template in mixed DNA sample (Anderson & Cairney 2004), but in a study of primer bias conducted by Anderson , Campbell *et al.* (2003), the ITS primer pair was found to equally amplify the Ascomycota, Basidiomycota, Chytridiomycota and Mucoromycotina, suggesting that primer bias is less significant than originally thought. Alternative fungal specific primers

have been developed (Martin & Rygiewicz 2005; Tedersoo, Jairus *et al.* 2008; Vancov & Keen 2009), but no primers exist that differentiate ECM fungi from fungi with other ecological roles (genes identified as essential for mycorrhizal function need to be identified in order to develop mycorrhizal, or ECM, primers, and this discovery of functional mycorrhizal genes is unlikely).

Despite DNA sequencing providing the highest possible level of taxonomic resolution, and therefore the most complete representation of community diversity, there are a number of drawbacks, which impede the identification of ECM species via sequencing. DNA sequencing is often expensive and thus may limit the scale of sampling. Many species of ECM fungi, especially from Australia, have not been sequenced and do not match identified database sequences, limiting their identification to the genus, family or even higher taxonomic levels (Horton & Bruns 2001; Ryberg, Kristiansson *et al.* 2009). Also, public database sequences may not have been identified correctly to begin with, and matches to incorrectly identified sequences can occur (Nilsson, Ryberg *et al.* 2006). Another problem that can be encountered is the presence of chimeric DNA sequences generated from environmental DNA samples, which can often be overlooked, and can influence the level of diversity detected in a sample (Anderson & Cairney 2004).

1.4 Ectomycorrhizal fungi and forest health

Ectomycorrhizal fungi are considered to be keystone species in many ecosystems because of their functional importance (Mills, Soule *et al.* 1993; Wolters, Silver *et al.* 2000; Leake 2001; Leake, Johnson *et al.* 2004; Fitter, Gilligan *et al.* 2005; Kernaghan 2005; van der Heijden, Bardgett *et al.* 2008; Antoninka, Wolf *et al.* 2009; van der Heijden & Horton 2009). ECM fungi have the potential to influence the health of their hosts, vegetation dynamics, and other biota and therefore can affect the health of the forest ecosystem. Considering that ECM fungi provide many ecosystem services, an understanding of the links among ECM communities, their functions and the effect of altered ECM communities on forest health and other ecosystem services, are vital. Although ECM fungi are known to significantly increase the growth and survival of their hosts, relatively few studies have investigated the link between the mycorrhizal community and forest health *in situ*.

In the northern hemisphere declining forests, ECM communities and mycorrhizal abundance have been shown to be altered in comparison to healthy forests (Estivalet, Perrin *et al.* 1990; Perrin & Estivalet 1990; Arnolds 1991; Fellner & Pešková 1995; Vinceti,

Paoletti *et al.* 1998; Coughlan, Dalpe *et al.* 2000; Blom, Vannini *et al.* 2009). Arnolds (1991) noted that the occurrence of ECM fungal sporocarps declined following visible forest deterioration due to nitrogen deposition from atmospheric pollution. Fellner & Pešková (1995) reported a similar observation that ECM sporocarp production, along with active mycorrhizal roots, declined following visible damage to forests caused by air pollution, acidification, or fertilisation. In Norway Spruce (*Picea abies*) forest, declining trees were found to have fewer mycorrhizal roots than healthy trees (Estivalet, Perrin *et al.* 1990; Perrin & Estivalet 1990) and declining forests had a higher frequency of mycorrhizal morphotypes but with less complex ramification and a marked decrease in diversity with depth compared to healthy forest (Vinceti, Paoletti *et al.* 1998). In another study of Norway Spruce, ECM species richness, both on root tips and as sporocarps, was reduced in declining forest compared to less severely affect forests (Peter, Ayer *et al.* 2008).

Congruent with Harvest, Davidson *et al.*'s (2008) findings in *E. delegatensis* forest, Estivalet, Perrin *et al.* (1990) conclude that decline of Norway Spruce in the Vosges Forest region of France, was associated with a depressive soil factor. This factor was identified as microbial, and may involve an alteration of rhizospheric microorganisms, which act to suppress mycorrhizal development of the trees. The pattern of reduced mycorrhizal richness with decline was also seen in Douglas Fir forest in the Netherlands (Jansen 1991) where tree vitality was inversely related to ECM richness and root tip abundance. Furthermore altered ECM community composition has been detected in declining sweet chestnut (*Castanea sativa* Mill.) forest in Italy, compared to healthy forest (Blom, Vannini *et al.* 2009).

In the southern hemisphere, both the intensity of ectomycorrhiza formation and the diversity of ectomycorrhizal fungi on *E. delegatensis* were found to differ between declining and healthy forests (Ellis & Pennington 1992). Also, soil microbial communities differed between healthy and declining *E. delegatensis* forest (Harvest, Davidson *et al.* 2008).

The importance of ECM fungi for ecosystem function and their response to different management strategies and disturbances (Lagana, Salerni *et al.* 2002; McMullan-Fisher, May *et al.* 2002; Packham, May *et al.* 2002; Wolfaardt & van der Merwe 2002) have led to the suggestion that ECM fungi be used as bioindicators of forest health (Fellner 1990; Folke & Knudsen 1994; Fellner & Peskova 1995; Old, Coops *et al.* 1999; Tommerup & Bougher 1999; Wolfaardt & van der Merwe 2002). By measuring shifts in ECM communities, a better understanding of the ecological relationships between ECM fungi and forest decline will be

gained, allowing the identification of options to manage forest decline and improve forest health.

1.5 Aims

This thesis aims to explore the ECM fungal community of *E. delegatensis* forest in relation to eucalypt forest decline. To achieve this aim a number of factors needed to be considered. Firstly, crown health assessment methodology was reviewed and assessed to determine the most effective way to measure the health of eucalypts in the field (Chapter 2). This was a vital step in linking eucalypt health and decline to the ECM community. Secondly, to further develop methodology for the study of ECM fungi, the influence of sampling strategy on the detection of soil fungi was investigated and is presented in Chapter 3. ECM community richness and structure of *E. delegatensis* forest was described and characterised using molecular identification tools from native forest in northern Tasmania (Chapter 4). The relationship of ECM fungi and forest health is considered in Chapter 5. In this chapter, the ecosystem properties of eucalypt health, understorey vegetation, soil nutrients and foliage nutrients are investigated in relation to ECM species richness, and community composition and structure. Finally, Chapter 6 synthesises the information contained in the body of the thesis and highlights the important outcomes in terms of our understanding of eucalypt decline, the role of ECM fungi in eucalypt forests, and implications for the management of these forests.

To summarise, the aims of this thesis are:

- to compare methods for assessing eucalypt crown health and identify the most effective methods for use in the field;
- to develop a sampling strategy for soil fungi within Australian forests and assess the applicability of soil analyses for the detection of ECM fungi;
- to characterise the ECM community of *E. delegatensis* forest in Tasmania using molecular techniques;
- to explore the relationship between the ECM fungal community and *E. delegatensis* health using a number of ecosystem properties; and
- to establish base-line data for a larger study investigating the use of fire for the management of eucalypt forest health.

2.0 An accurate, precise and efficient method for assessing eucalypt crown condition

2.1 Introduction

Crown condition is frequently used as an indicator of tree decline and dieback (Manion 1991) and is often incorporated into monitoring for forest health. Crown condition is the state of the upper part of the tree containing the branches and foliage.

A number of field methods have been developed to assess crown condition and include measurement of live crown ratio, crown class, crown light exposure, crown diameter, crown density, crown volume, crown dieback and foliage transparency (Grimes 1978; Innes 1990; Zarnoch, Bechtold *et al.* 2004). Crown condition assessment methods, such as those of Zarnoch, Bechtold *et al.* (2004), were developed in the northern hemisphere for conifers or deciduous broadleaf species, and are not entirely transferrable to eucalypt species because of differing growth patterns and the different responses to stress in eucalypts (Stone 1999). Crown condition assessment methods that have been developed particularly for eucalypts, are largely based on categorisation of crown size, shape and density, branching, and the extent of epicormic growth (Grimes 1978; Fox & Curry 1980; Podger, Kile *et al.* 1980; Kile, Turnbull *et al.* 1981; Wardlaw 1989; Stone, Wardlaw *et al.* 2003a; Jurskis, Selby *et al.* 2005). These different crown attributes are altered with the onset of dieback and as the tree declines. The reduction of primary growth, the amount and persistence of replacement epicormic growth, the death and loss of shoot tips, and finally an overall alteration of crown structure are all important indicators of eucalypt dieback.

The approach to assessing eucalypt crown condition varies across methods (see Table 2.1 for a summary). For example Grimes (1978) uses weighted scales so that some parameters contribute differently to the overall crown condition score, whereas the Stone, Wardlaw *et al.* (2003a) method modifies Grimes (1978) by using the same scale for each parameter. Fox and Curry (1980) and Jurskis, Selby *et al.* (2005) are based on ordinal scales, whereas Wardlaw (1989) is based on a continuous scale. Some parameters are indices based on proportional data (Kile, Turnbull *et al.* 1981; Wardlaw 1989; Stone & Haywood 2006).

Methods also differ in the number of parameters used to assess crown condition. The Fox and Curry (1980) method includes all crown parameters within a single pictorial scale. The Jurskis, Selby *et al.* (2005) method also uses a single score that encompasses many crown parameters but unlike the Fox and Curry (1980) method, descriptions of each score are provided. Podger, Kile *et al.* (1980) also use a single score but assess only one parameter described as the proportion of primary branch dieback. The Grimes (1978), Kile, Turnbull *et al.* (1981), Stone, Wardlaw *et al.* (2003a) and Wardlaw (1989) methods use a number of crown parameters that are scored separately (referred to as single-parameter methods), and can then be added to give an overall score for crown condition (referred to as additive parameters) . The Grimes (1978) and Stone, Wardlaw *et al.* (2003a) methods describe each condition level (score) for each of the parameters, whereas the Podger, Kile *et al.* (1980), Kile, Turnbull *et al.* (1981) and Wardlaw (1989) methods use a continuous scale.

For widespread utility, methods for assessment of crown condition need to be capable of assessing different species of eucalypts (which may have different canopy structure or foliage colour) and different types of dieback (Stone and Haywood 2006) from a range of geographic regions (Innes 1993). Furthermore, methods need to be practical and efficient so that data can be collected quickly from many trees over large areas with limited resources. Assessments also need to be repeatable (Stone & Haywood 2006) and comparable among different studies and over time (Innes 1993b). Although many methods have been applied to the assessment of eucalypt dieback, their effectiveness has not been evaluated and a standard field method that is rigorous and easily applied to the assessment of crown condition is currently lacking.

The aims of this study were to develop a crown condition method that could be used to indicate eucalypt health, which could then quantitatively be related to other biotic and abiotic variables, such as ectomycorrhizal community compositions and soil nutrient concentrations. The method therefore needed to be accurate, precise and efficient for the assessment of eucalypt crown condition in the field. In order to address this objective five performance criteria were developed and considered: 1 capacity to assess dieback; 2 observer bias; 3 repeatability; 4 capacity to assess different species and; 5 efficiency to score.

The development of a uniform widely applicable method that meets these criteria will simplify comparisons of crown condition between trees, allow assessment of a large

number of trees and allow tree health to be directly correlated to other component of the forest ecosystem.

Table 2.1 A comparison of eucalypt crown condition assessment methods detailing the attributes that are observed in each method. + denotes the attribute is assessed in the method, — denotes that the attribute is not included in the method.

Crown condition attribute	**Fox & Curry (1980)**	**Grimes (1978)**	**Jurskis, Selby *et al.* (2005)**	**Kile, Turnbull *et al.* (1981)**	**Podger, Kile *et al.* (1980)**	**Stone, Wardlaw *et al.* (2003a)**	**Wardlaw (1989)**
primary crown dieback	—	+	+	+	+	+	+
crown position	—	+	—	—	—	—	—
foliage density	—	+	—	—	—	+	—
dead branches	—	+	+	—	—	+	—
dead top	—	—	—	—	—	—	+
crown shrinkage	—	—	—	+	—	—	+
foliage damage	—	—	—	—	—	+	—
epicormic growth	—	+	+	+	—	+	+
overall crown condition	+	+	+	—	—	+	—
data type	ordinal	categorical	ordinal	continuous, proportional	continuous, proportional	categorical	continuous, proportional
scale	0 - 6	varies	0 - 5	0 - 100	0 - 100	0 - 5	0 - 100

2.2 Methodology

2.2.1 Study areas

The first study area was located in Mersey State Forest, 81 km west southwest of Launceston, Tasmania (41.58 °S, 146.17 °E). Soils were dark brown ferrasols derived from Tertiary Basalt (Grant, Laffan *et al.* 1994). The site was partially harvested in the 1980's. The second study area was located in Ringarooma State Forest 60 km east northeast of Launceston (41.35 °S, 147.67 °E and 41.37 °S, 147.61 °E). The second study area was on brown dermasols derived Devonian granodiorite (Grant, Laffan *et al.* 1994) and had a mix of

fire histories with some areas long unburnt (since 1880), and some burnt during the 1960's. Both areas were dominated by alpine ash (*E. delegatensis*) open forest with either a rainforest understorey dominated by myrtle (*Nothofagus cunninghamii* (Hook.) Oerst.), or a dry sclerophyll understorey. Both locations were at an altitude of 800 – 900 m asl with a gentle slope of 0 – 5°. Mean annual rainfall for the two study areas is in the range of 1320 mm (Ben Nevis) to 1564 mm (Lorinna) and mean annual temperature is 9.1°C (Ben Nevis) and 9.3°C at Lorinna (ESOCLIM module of ANUCLIM 5.2 Houlder, Hutchinson *et al.* 2000).

The third study area was situated on a valley flat approximately 20 km north of Fingal (41.64 °S, 147.97 °E) in north-eastern Tasmania. Sites consisted of black peppermint (*E. amygdalina*) forest with a dry sclerophyll native understorey or remnant fragments of manna gum (*E. viminalis* Labill.) – white mountain gum (*E. dalrympleana*) forest with open grassy understorey. The *E. viminalis* – *dalrympleana* population at this site is an intergrade population with characteristics intermediate between these two closely related species. The average annual rainfall at Fingal (Station No. 92012 1882 – 2008) is 608 mm and the mean annual temperature is 10.2°C (Australian Bureau of Meteorology 2009). The underlying rock type was 'Mathinna Beds' (derived from Silurian-Devonian siltstone and sandstone), which weathers to a sandy soil of low inherent fertility (Laffan, Grant *et al.* 1998). All sites were flat or very gently sloping (0 - 5°).

The fourth study area was established approximately 130 km south of Perth, Western Australia in the Yalgorup National Park (32.83 °S, 115.39 °E), which had not been burnt since at least 1972, and the adjacent State forest (32.91 °S, 115. 72 °E), which was burnt every 5 -15 years for the past six decades. Sites were dominated by tuart (*Eucalyptus gomphocephala*) woodland with West Australian peppermint (*Agonis flexuosa (*Willd.) Sweet.) midstorey and an open to dense dry sclerophyll understorey. Soils were sandy grey-brown with limestone generally within 1 m depth. Mean annual rainfall is 738 mm and a mean annual temperature of 17.3°C at Bunbury (Station No. 09965 1995-2008) (Bureau of Meteorology www.bom.gov.au 2009).

Sites one and two from this study were also used for additional research on the relationships between eucalypt decline and the ECM community presented in this thesis (see Chapters 3, 4 and 5). Site one includes Sites E and S used in Chapter 3 and plots 1 to 8 used in Chapters 4 and 5, and site two includes plots 9 to 12 that were used in Chapters 4 and 5. Site four formed part of a broader Australia wide study investigating the ecological

relationships of eucalypt decline in the absence of fire, to which the research in this thesis also contributed. The third site was part of a study investigating agricultural land management and eucalypt dieback (Close, Davidson *et al.* 2008). The four sites were selected as they contained eucalypt trees that ranged in age and in condition from healthy to being severely affected by dieback.

2.2.2 Crown condition assessments

The four methods selected for comparison were Fox and Curry (1980), Jurskis, Selby *et al.* (2005), Stone, Wardlaw *et al.* (2003a) and Wardlaw (1989). These methods were selected as they represented two different approaches to measuring crown condition (combined- and single/additive-parameter methods), they use a variety of different attributes that are known to change with dieback, and have been specifically applied to a range of different eucalypt species from different locations across Australia.

The Jurskis, Selby *et al.* (2005) method, and the Fox and Curry (1980) method encompass a range of attributes within a single score (referred to as combined-parameters). The Fox and Curry (1980) method relies on a pictorial representation of eucalypt crowns in various conditions (F), while Jurskis, Selby *et al.* (2005) describe the condition of a variety of attributes associated with each crown condition score (J). The other two methods measure crown attributes independently of one another (single-parameters), which can then be summed to give an overall score for crown condition (additive-parameters). The Wardlaw (1989) method uses four crown attributes as parameters; primary crown dieback (PCD), the proportion of epicormic growth (E), crown contraction defined as the current extent of the crown as a proportion of the original crown (CC), and the length of the dead top (DT) (Table 2.2). The Stone, Wardlaw *et al.* (2003a) method also uses five attributes, but only four are used here. These attributes are; canopy size and shape (SS), crown density (density of foliage) (FD), the number and distribution of dead branches in the crown (DB), and the proportion and distribution of epicormic growth (EG) (Table 2.2). Full details of each of the methods are provided in Appendix 1. Codes and definitions for each of the parameters analysed are summarised in Table 2.2.

Crown condition was assessed by observers that were familiar with eucalypt crown structure and foliage. These observers had some training in eucalypt crown assessment but were not extensively experienced. A total of 516 randomly selected trees were assessed across the four study areas (Table 2.3).

Each tree was assessed from the ground, where there was a clear view of the tree canopy. *E. delegatensis* trees were assessed by all four methods described above while *E. amygdalina*, *E. viminalis-dalrympleana* and *E. gomphocephala* were assessed by only the Fox and Curry (1980), Jurskis, Selby *et al.* (2005), and Stone, Wardlaw *et al.* (2003a) methods. The Wardlaw (1989) method was designed specifically for the ash group of eucalypts and testing across different groups would have been useful, but was not applied because of resource constraints. In the case of *E. gomphocephala* only three parameters from the Stone, Wardlaw *et al.* (2003a) method were used for analysis (FD, DB and EG) due to different definitions applied by observers for the SS parameter. The crown condition scores of *E. gomphocephala* for these three parameters still provided useful data for a comparison of methods and were included in all of the statistical analyses except the Spearman's correlations.

Crown condition assessments were repeated one year later by the same observer for 47 of the *E. delegatensis* trees using the Fox and Curry (1980), Jurskis, Selby *et al.* (2005) and Stone, Wardlaw *et al.* (2003a) methods to determine repeatability between years. During this second survey, 15 trees were assessed by two independent observers to determine observer bias. The number of trees assessed for observer bias and repeatability represent the maximum number of trees that could be found and assessed within the short survey period allocated for this second survey.

Table 2.2 Codes, definitions, parameter type and score range for each crown condition parameter assessed.

Code	Method	Parameter type	Definition	Score range
F	Fox and Curry (1980)	combined	overall dieback using pictorial guide	0 - 6
J	Jurskis, Selby *et al.* (2005)	combined	overall dieback using pictorial guide and descriptions	0 - 5
SS	Stone, Wardlaw *et al.* (2003a)	single	overall canopy size and shape - descriptive	0 - 5
FD	Stone, Wardlaw *et al.* (2003a)	single	crown density (density of foliage) - descriptive	0 - 5
DB	Stone, Wardlaw *et al.* (2003a)	single	dead branches - descriptive	0 - 5
EG	Stone, Wardlaw *et al.* (2003a)	single	epicormic growth - descriptive	0 - 5
T1	Stone, Wardlaw *et al.* (2003a)	additive	addition of SS, FD, DB and EG	0 - 20
T3	Stone, Wardlaw *et al.* (2003a)	additive	addition of SS, DB and EG	0 - 15
PCD	Wardlaw (1989)	single	proportion of the primary branches that have died back	0 - 100
E	Wardlaw (1989)	single	proportion of the current tree crown that is of epicormic origin	0 - 100
CC	Wardlaw (1989)	single	current crown as a proportion of the estimated original crown	0 - 100
DT	Wardlaw (1989)	single	Metres of dead top for the tree standardised by dividing by the maximum meters of dead top recorded from any tree from the same site	unlimited
T2	Wardlaw (1989)	additive	addition of PCD, E, CC and DT	unlimited
T4	Wardlaw (1989)	additive	addition of PCD, E and CC	0 - 300

Table 2.3 The number and species of eucalypt trees assessed for crown condition. The species, observer and number of trees assessed at each site are shown.

Study site	Location	Species	Observer	Number of trees assessed
One	Mersey State Forest, Tasmania	*E. delegatensis*	One	35
Two	Ringarooma State Forest, Tasmania	*E. delegatensis*	One	75
Three	Fingal Valley, Tasmania	*E. amygdalina*	Two	146
Three	Fingal Valley, Tasmania	*E. viminalis-dalrympleana*	Two	60
Four	Yalgorup, Western Australia	*E. gomphocephala*	Three	200
			Total	**516**

2.2.3 Statistical analyses

Scores for the Jurskis, Selby *et al.* (2005) parameter and the Wardlaw (1989) parameters PCD, E and DT were converted (by subtracting from the maximum score) so that high scores represented the healthiest state. Scores for all of the parameters, including the additive parameters, were standardised by dividing by the maximum score possible so that each parameter was directly comparable. These standardised parameters were used for all subsequent analyses. Mean per site for each species and standard errors were calculated for each of the parameters from the Fox and Curry (1980), Jurskis, Selby *et al.* (2005) and Stone, Wardlaw *et al.* (2003) methods for all of the trees assessed.

Pair-wise correlations of the crown condition parameters using Spearman's correlation analysis were determined using GenStat v7.1 (VSN International Ltd 2003). Correlations were first calculated for the *E. delegatensis* data and then for all of the available data, with the omission of the Western Australian data. The Western Australian data were omitted from the correlation analysis as SS was not scored. Correlation coefficients were used to indicate which of the parameters were measuring the same or similar canopy parameters (i.e. had a positive strong correlation) and which were assessing unique parameters. Data were also analysed by principal components analysis (PCA) to determine which of the

parameters were contributing to the variation in the *E. delegatensis* data set, and all species data set (including the Western Australian data).

The correlation analysis and PCA indicated that two parameters, DT and FD, were only weakly positively correlated to the other parameters, and contributed less to the PCA component than the other parameters. Due to this outcome, the additive parameters, which can be derived from the Stone, Wardlaw *et al.* (2003a) and Wardlaw (1989) methods (T1 and T2 respectively), were recalculated with either DT or FD removed from the totals (to give the parameters T3 and T4 respectively).

Repeat assessments of individual trees over two years by the same observer, and during the same year but by different observers, were used to determine repeatability and observer bias. Bias and repeatability for each tree assessed were determined by calculating the difference between the first score for each individual tree and each individual parameter (year one or observer one) and the second score (year two or observer two). The mean and standard errors of these differences for each parameter were calculated. For many types of dieback, where there are not repeat episodes of dieback, symptoms stabilise within the first few years of onset (Wardlaw 1990)and as such the perceived stability of crown health symptoms over the successive years in which the experiments were conducted are likely to have measured differences in repeatability of assessments rather than actual changes in crown condition.

2.2.4 Assessment of crown condition parameters against performance criteria

All of the parameters compared in this study were judged against five performance criteria;

1. capacity to measure dieback (correlation of parameters and the variability of these correlations (standard deviation), and capacity to indicate the presence of dieback symptoms in the crown);
2. observer bias (the difference in scores between observers and the variability in these differences (standard error);
3. repeatability (the difference in scores between years, and the variability of these differences (standard error));
4. capacity to assess different species (species bias (the rank order of health of each species by each parameter) and;

5. efficiency to score (time to score parameter). Each parameter was assessed as performing in one of three categories: high, moderate, or low.

Categories were based on the ranges for each criterion that arose from the study. For example a parameter performed well in its capacity to measure dieback if the mean correlation to all other parameters was > 0.86, and did not perform well if its mean correlation was < 0.8. The parameter that consistently performed well against the five criteria was determined to be the best parameter for standard adoption for the assessment of eucalypt health.

2.3 Results

2.3.1 Capacity to measure dieback

All sites contained trees that had a complete range of crown conditions, as indicated by the maximum and minimum crown condition scores for each of the parameters (Table 2. 4). Crown condition scores for each of the species at each of the sites varied with the different parameters but within a site, crown condition scores for each parameter had low variation (SE ≤ 0.05) (Table 2.4).

The Spearman's correlation analyses of the *E. delegatensis* data and the complete data set showed strong positive correlations among all parameters, with the exception of FD and DT (Table 2.5). The parameters that showed the highest correlations were EG and E for the *E. delegatensis* data (Rho=0.95), and DB and F (Rho=0.91) for all of the data (Table 2.5).

The mean correlation coefficients for each of the parameters are depicted in Figure 2.1. There were similar trends in the correlations between the parameters for both the *E. delegatensis* data and the data set including all species. The additive-parameters derived from the Stone, Wardlaw *et al.* (2003a) showed the highest correlations. T3 had the highest correlation coefficient mean with the lowest standard error (Rho = 0.943, SE=0.011) for the *E. delegatensis* data and T1 had the highest coefficient mean and lowest standard error (Rho= 0.945, SE=0.014) for the data on all species. The additive-parameter from the Wardlaw (1989) method, T4, had the second highest mean with the lowest standard error of the *E. delegatensis* data (Rho = 0.942, SE=0.011), and T3 had the second highest mean from the all species data (Rho = 0.926, SE=0.018).Of the single-parameters both SS (from the all species data) and PCD (from the *E. delegatensis* data) had similarly high correlations

comparable to the additive-parameters (SS Rho=0.909 and SE=0.021, PCD Rho = 0.932 and SE=0.012). Of the single-parameters CC (for the *E. delegatensis* data) and EG (all data) showed the lowest mean correlation coefficients as well as the highest standard error (CC Rho = 0.855 and SE=0.019, EG Rho = 0.871 and SE=0.029).

The PCA analyses performed on the *E. delegatensis* data, and all species data, showed that the majority of the variance (84% for *E. delegatensis* and 88% for all species data) in the data set could be explained by a single component (Figure 2.2). In the *E. delegatensis* PCA all of the parameters contributed to Component 1 in the same direction with weights ranging from 0.18 to 0.275. The parameters FD and DT contributed less to Component 1 than the other parameters. T4 had the largest weighting of the additive parameters (0.275) and PCD had the largest weighting of the single-parameters (0.27). The PCA analysis of all species data supported the *E. delegatensis* PCA. The parameter weights for Component 1 had a smaller range than the *E. delegatensis* PCA (0.31 to 0.37 in the same direction) (Figure 2.2). Of the single-parameters, SS had the greatest weighting of 0.36, and T1 was the most strongly associated variable with Component 1 (weight was 0.37).

Table 2.4 Mean, standard error, maximum, and minimum crown condition scores for each eucalypt species at each site. Shaded rows indicate that the parameter was not measured for that species and the particular site. Min. = minimum crown condition score recorded, Max. = maximum crown condition score recorded.

	Site	Statistic	Site 1	Site 2	Site 3	Site 3	Site 4
	Species		*E. delegatensis*	*E. delegatensis*	*E. amygdalina*	*E. viminalis-dalrympleana*	*E. gomphocephala*
	n		35	75	146	60	200
Crown condition parameter	F	mean	0.70	0.60	0.62	0.76	0.67
		SE	0.03	0.02	0.02	0.03	0.02
		max.	1.0	1.0	1.0	1.0	1.0
		min.	0.4	0.3	0.2	0.0	0.2
	SS	mean	0.71	0.53	0.59	0.76	
		SE	0.04	0.03	0.02	0.03	
		max.	1.0	0.9	1.0	1.0	
		min.	0.2	0.2	0.0	0.0	
	FD	mean	0.67	0.59	0.62	0.71	0.55
		SE	0.03	0.02	0.02	0.02	0.02
		max.	1.0	0.9	1.0	1.0	0.9
		min.	0.4	0.2	0.0	0.0	0.1

	Site	Statistic	Site 1	Site 2	Site 3	Site 3	Site 4
	Species		*E. delegatensis*	*E. delegatensis*	*E. amygdalina*	*E. viminalis-dalrympleana*	*E. gomphocephala*
	DB	mean	0.54	0.46	0.52	0.64	0.59
		SE	0.03	0.02	0.02	0.03	0.02
		max.	0.8	0.8	1.0	0.9	1.0
		min.	0.2	0.2	0.0	0.2	0.2
	EG	mean	0.69	0.56	0.59	0.89	0.66
		SE	0.02	0.03	0.03	0.02	0.02
		max.	1.0	1.0	1.0	1.0	1.0
		min.	0.2	0.2	0.0	0.2	0.3
	J	mean	0.61	0.48	0.49	0.68	0.58
		SE	0.04	0.03	0.02	0.03	0.02
	J	max.	0.9	0.8	1.0	1.0	1.0
		min.	0.2	0.1	0.0	0.2	0.1
	PCD	mean	0.67	0.47			
		SE	0.05	0.04			
		max.	1.0	1.0			
		min.	0.1	0.0			

	Site	Statistic	Site 1	Site 2	Site 3	Site 3	Site 4
	Species		*E. delegatensis*	*E. delegatensis*	*E. amygdalina*	*E. viminalis-dalrympleana*	*E. gomphocephala*
	E	mean	0.70	0.40			
		SE	0.05	0.04			
		max.	1.0	1.0			
		min.	0.1	0.0			
	CC	mean	0.71	0.56			
		SE	0.04	0.03			
		max.	1.0	1.0			
		min.	0.1	0.1			
	DT	mean	0.92	0.87			
		SE	0.03	0.03			
		max.	1.0	1.0			
		min.	0.3	0.2			

Table 2.5 Spearman's Rho correlations for each of the crown condition parameters. Correlations for the *E. delegatensis* data set (*E. delegatensis* only) and the data set containing all species (all species) are shown separately. The highest non-identity correlation within a row (excluding additive parameters) for each parameter is highlighted. Additive parameters are indicated by superscript of A.

Data set	Parameter	PCD	E	CC	DT	T2 [A]	T4 [A]	SS	FD	DB	EG	T1 [A]	T3 [A]	J	F
E. delegatensis	PCD	1.00	0.93	0.86	0.61	0.97	0.97	**0.93**	0.57	0.91	0.89	0.95	0.97	0.92	0.89
E. delegatensis	E	0.93	1.00	0.81	0.65	0.96	0.96	0.85	0.45	0.89	**0.95**	0.90	0.94	0.88	0.84
E. delegatensis	CC	**0.86**	0.81	1.00	0.52	0.92	0.93	0.84	0.57	0.78	0.79	0.85	0.85	0.80	0.82
E. delegatensis	DT	0.61	**0.65**	0.52	1.00	0.69	0.63	0.54	0.31	**0.65**	0.58	0.58	0.60	0.60	0.52
E. delegatensis	T2 [A]	0.97	0.96	0.92	0.69	1.00	0.99	0.91	0.55	0.90	0.92	0.94	0.96	0.91	0.88
E. delegatensis	T4 [A]	0.97	0.96	0.93	0.63	0.99	1.00	0.92	0.56	0.90	0.92	0.95	0.97	0.91	0.89
E. delegatensis	SS	**0.93**	0.85	0.84	0.54	0.91	0.92	1.00	0.65	0.87	0.83	0.96	0.96	0.91	0.89
all species	SS	-	-	-	-	-	-	1.00	0.73	**0.90**	0.83	0.95	0.91	0.87	**0.90**
E. delegatensis	FD	0.57	0.45	0.57	0.31	0.55	0.56	**0.65**	1.00	0.54	0.46	0.72	0.59	0.57	0.68
all species	FD	-	-	-	-	-	-	0.73	1.00	0.71	0.63	0.82	0.85	**0.75**	0.71
E. delegatensis	DB	**0.91**	0.89	0.78	0.65	0.90	0.90	0.87	0.54	1.00	0.83	0.92	0.93	0.88	0.84
all species	DB	-	-	-	-	-	-	0.90	0.71	1.00	0.81	0.94	0.89	0.86	**0.91**
E. delegatensis	EG	0.89	**0.95**	0.79	0.58	0.92	0.92	0.83	0.46	0.83	1.00	0.90	0.94	0.85	0.81
all species	EG	-	-	-	-	-	-	**0.83**	0.63	0.81	1.00	0.91	0.93	0.80	0.82
E. delegatensis	T1 [A]	0.95	0.90	0.85	0.58	0.94	0.95	**0.96**	0.72	0.92	0.90	1.00	0.98	0.92	0.92
all species	T1 [A]	-	-	-	-	-	-	**0.95**	0.82	0.94	0.91	1.00	0.99	0.90	0.92
E. delegatensis	T3 [A]	**0.97**	0.94	0.85	0.60	0.96	0.97	0.96	0.59	0.93	0.94	0.98	1.00	0.93	0.90
all species	T3 [A]	-	-	-	-	-	-	0.91	0.85	0.89	**0.93**	0.99	1.00	0.88	0.89
E. delegatensis	J	**0.92**	0.88	0.80	0.60	0.91	0.91	0.91	0.57	0.88	0.85	0.92	0.93	1.00	0.90
all species	J	-	-	-	-	-	-	**0.87**	0.75	0.86	0.80	0.90	0.88	1.00	0.84
E. delegatensis	F	0.89	0.84	0.82	0.52	0.88	0.89	0.89	0.68	0.84	0.81	0.92	0.90	**0.90**	1.00
all species	F	-	-	-	-	-	-	0.90	0.71	**0.91**	0.82	0.92	0.89	0.84	1.00

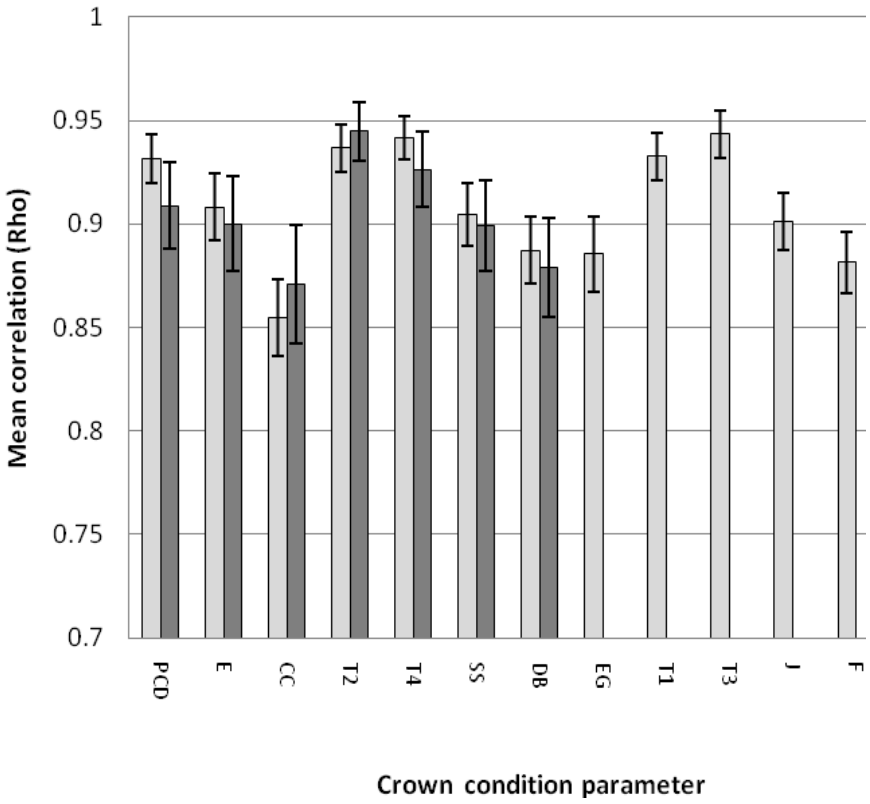

Figure 2.1 Spearman's correlation coefficient means for each crown condition parameter (i.e. the correlation of each parameter with all other parameters (FD and DT omitted) was averaged for each individual parameter). Error bars show standard error. Pale grey represents *E. delegatensis* data and dark grey represents all data. Parameters that were highly correlated with all other parameters (i. e. high mean correlation) and had a low standard error accurately and precisely assessed crown condition.

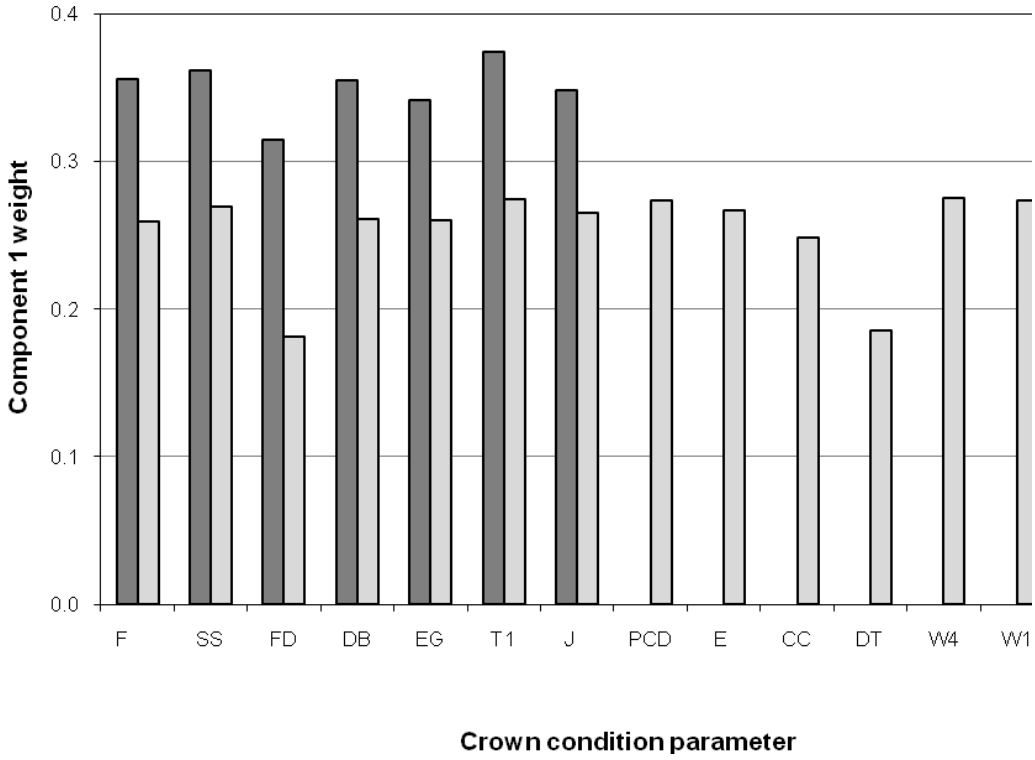

Figure 2.2 PCA component 1 weights for each of the parameters analysed by the two PCA analyses. Analysis one used the Fox and Curry (1980), Stone *et al.* (2003a) and Jurskis *et al.* (2005) parameters for all eucalypt species data (represented by dark grey). Analysis two used the Jurskis *et al.* (2005), Fox and Curry (1980), Stone *et al.* (2003a) and Wardlaw (1989) parameters on the *E. delegatensis* data (represented by pale grey). Component weights did not differ greatly among each parameter for the *E. delegatensis* data, with FD contributing the least. For the second analysis, again there is little variation among the majority of the parameters with FD and DT contributing less to PCA component 1.

2.3.3 Observer bias and repeatability

The differences in crown condition scores by different observers and by the same observer over two years were used to assess observer bias and repeatability. A small difference in crown condition scores among different observers and by the same observer at different times indicated low observer bias and high repeatability. The mean differences in scores from observers within the same year, and by the same observer between years were very small (Figure 2.3). These ranged from 0.01 to 0.05 points difference. Standard errors were

at least the same or larger than the differences, indicating high variability in observer bias and repeatability. The parameters SS, T1 and F showed the least amount of observer bias (1% difference), but T1 had the smallest standard error. EG showed the highest observer bias (5% difference in mean scores among observers and a large standard error). For the repeatability assessments, SS, T3 and T1, performed the best with equal differences in scores between years, but T1 had the smallest standard error. Both DB and EG showed poor repeatability sharing the largest difference in scores between years, but EG had the larger standard error.

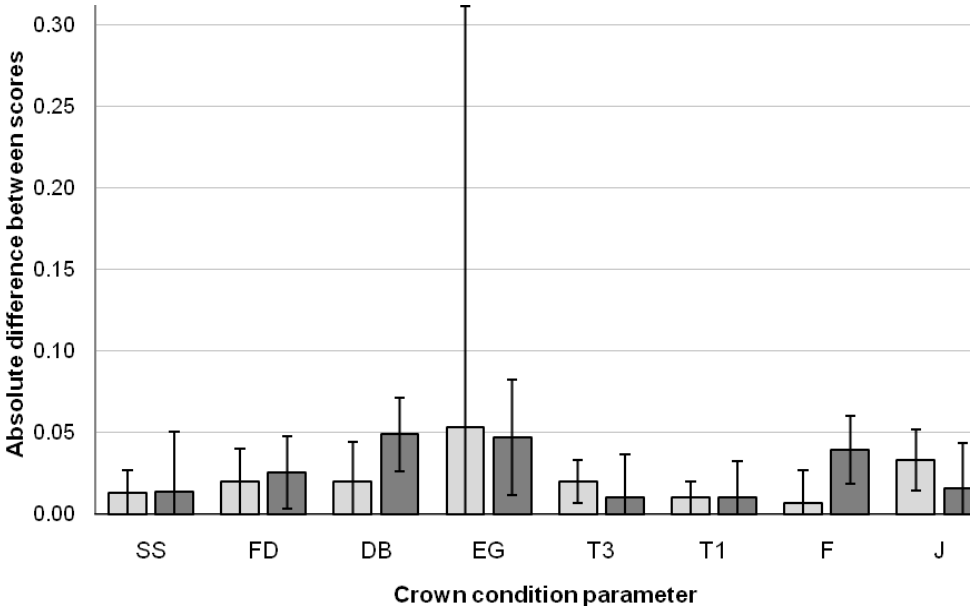

Figure 2.3 Differences in *E. delegatensis* scores for each parameter, by different observers and over different years. Error bars show standard error. Pale grey represents the mean difference in crown scores for all trees assessed within the same year but by two different observers (performance criterion 2 observer bias). Dark grey represents the mean difference in crown condition scores for all trees assessed by the same observer over two successive years (performance criterion 3 repeatability). EG showed the largest observer bias, and both DB and EG showed low repeatability. T1 was the most repeatable parameter and also had the lowest observer bias.

2.3.4 Species comparison

The rank order of the crown condition scores for the four eucalypt species changed depending on the parameter used (Figure 2. 4). For example, the Fox and Curry (1980) method scored *E. gomphocephala* as the second healthiest species, then *E amygdalina* and the least healthy was *E. delegatensis*. Whereas using the Jurskis *et al.* (2005) method,

E. delegatensis was the second most healthy, *E. gomphocephala* the third and *E. amygdalina* was the least healthy species (Figure 2.4). Due to different observers assessing each of the species, the differences in crown condition of the species may equally be because of observer differences, rather than species differences. Given the large error bars associated with each of the parameters for each species, no significant differences between the species or parameters are evident (Figure 2.4) indicating that there are no species/parameters effects. In this case the parameters are equally suitable to apply to any of the species (i. e. one parameter is not more/less suitable for a particular species). Alternatively, real differences may not have been detected among scores.

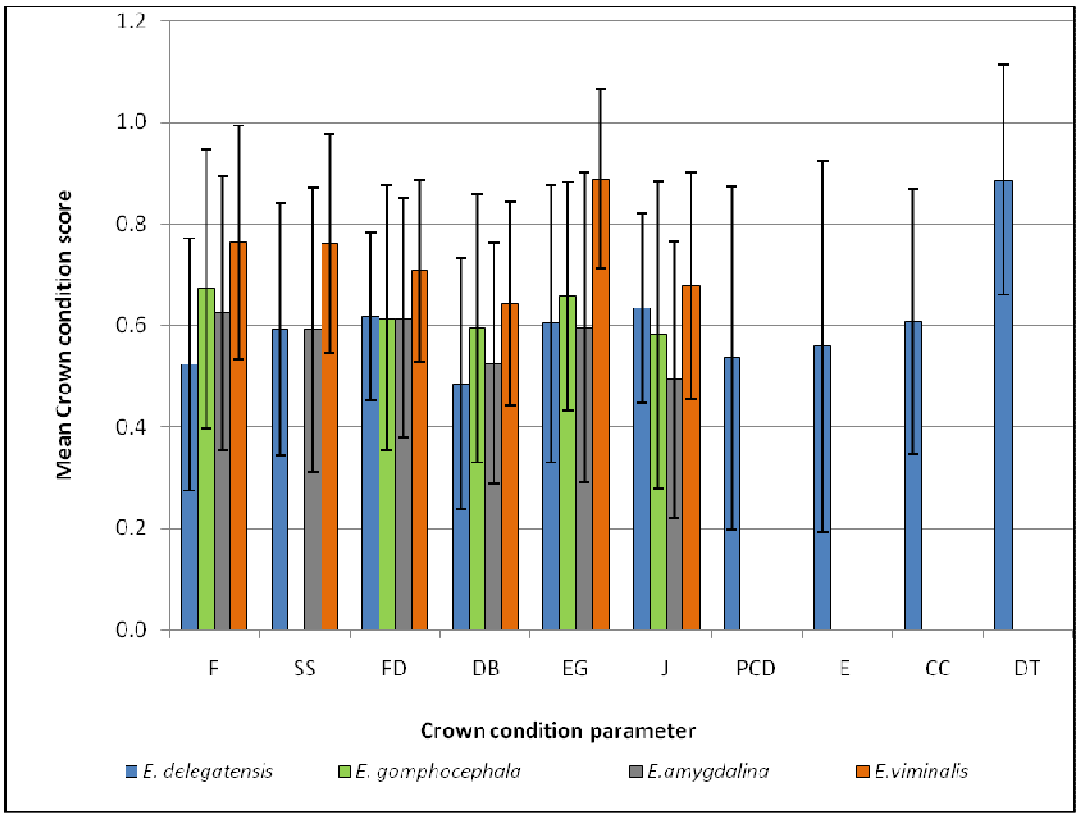

Figure 2.4 Mean crown condition score for each of the parameters used to assess the four eucalypt species. Error bars show standard deviation. Large standard errors indicate that none of the parameters differ significantly among species.

2.3.5 Efficiency to score

Each of the four methods compared showed varying efficiency to score because of their level of technical complexity, which dictated the time required to score each of the parameters. Assessments of efficiency to score are shown in Table 2.6. The Fox and Curry (1980) method was the simplest and easiest to use being based on uncomplicated pictures and was the quickest method to score (< 2 minutes per tree). The Jurskis, Selby *et al.* (2005) was a combined-parameter method that utilised clear descriptions of each crown condition score. This method was slightly more time consuming to score, but was relatively quick overall (2-5 minutes per tree). The two additive-parameters methods were the most technical demanding and least efficient. Each of the single-parameters for these additive methods was well defined and thus moderately efficient to score (2-5 minutes per parameter per tree). When determining the efficiency of the two parameters overall, i.e. the derived additive parameter, these methods were both inefficient in comparison to the combined-parameter methods (between 5 and 15 minutes per tree).

2.3.6 Overall method utility

The performance of each parameter was judged against the five criteria used to assess the accuracy, precision and efficiency of the parameters. The explicit range of each of the high, moderate and low categories for performance were determined by examining the numerical range of each parameter. The performance of each parameter against the criteria is summarised in Table 2.6. When considering the first and most important performance criterion, capacity to assess dieback, T1 performed the best. The parameters SS, T3, T2, T4, PCD and J were found to measure highly correlated crown attributes that are associated with dieback and so also have a relatively high capacity to assess dieback. Two parameters, FD and DT did not correlate well to other parameters and had high variance. These parameters measure unique attributes that are probably not as consistently related to dieback as those that show high correlations. FD and DT can therefore be considered to have a low capacity to assess dieback. Of the parameters that had a moderate to high capacity to assess dieback, the Stone *et al.* (2003a) parameters all met the criteria of low observer bias and high repeatability whereas the J parameter did not. Observer bias and repeatability were not assessed for the Wardlaw (1989) parameters. Overall the additive parameter T1 met the highest number of performance criteria as only one criterion, efficiency to score, did not fall into the best-performance category. The parameter SS

performed the second best meeting five out of eight performance criteria, with two criteria, species bias and efficiency to score, falling into the second best-performance category. FD performed the worst of all parameters meeting only one of the eight performance criteria, low species bias. Appendix 2 depicts photographs of a selection of *E. delegatensis* trees with the scores for T1, SS, FD and PCD shown. Highest values indicate the best health.

Table 2.6 The ranking of parameters used to assess eucalypt crown condition according to five criteria; 1. Capacity to assess dieback (correlation [high ≥ 0.86, moderate 0.86-0.80, low < 0.8] and correlation variance [high > 0.13, moderate 0.12-0.13, low < 0.12]); 2. Observer bias (difference in scores between observers [low = 0.01, moderate = 0.02, high > 0.02] and variance [low = 0.01-0.02, moderate = 0.021-0.04, high > 0.04] in these differences); 3. Repeatability (difference in scores between years [high = 0.01, moderate = 0.02, low > 0.02] and variance of these differences [low = 0.01-0.02, moderate = 0.021-0.04, high > 0.04]); 4. Species bias (the difference in rank order of species health compared to mean score of all parameters [low=no change in rank, moderate = 1 species ranked differently, high ≥ 2 species ranked differently]), and; 5. Efficiency to score (time to score [high < 2 minutes, moderate 2-5 minutes, low > 5 minutes]). Values in bold indicate the parameter fell within the best category. n/a denotes that the parameter was not assessed against that particular criterion.

Parameter	**Parameter type**	**Capacity to assess dieback**		**Observer bias**		**Repeatability**		**Species bias**	**Efficiency to score**	**Number of criteria in best category**
SS	single	**high**	mod	**low**	**low**	**high**	**low**	mod	mod	5
FD	single	low	high	mod	mod	low	mod	**low**	mod	1
DB	single	**high**	mod	**low**	mod	low	mod	**low**	mod	3
EG	single	mod	high	high	high	low	**low**	**low**	mod	2
T3	additive	**high**	mod	mod	**low**	**high**	mod	n/a	low	3
T1	additive	**high**	**low**	**low**	**low**	**high**	**low**	n/a	low	6
PCD	single	**high**	mod	n/a	n/a	n/a	n/a	n/a	mod	1
E	single	**high**	high	n/a	n/a	n/a	n/a	n/a	mod	1

Parameter	Parameter type	Capacity to assess dieback		Observer bias		Repeatability		Species bias	Efficiency to score	Number of criteria in best category
CC	single	mod	mod	n/a	n/a	n/a	n/a	n/a	mod	0
DT	single	low	high	n/a	n/a	n/a	n/a	n/a	mod	0
T4	additive	**high**	mod	n/a	n/a	n/a	n/a	n/a	low	1
T2	additive	**high**	mod	n/a	n/a	n/a	n/a	n/a	low	1
F	combined	mod	mod	**low**	mod	low	**low**	**low**	**high**	4
J	combined	**high**	mod	high	mod	mod	mod	mod	mod	1

2.4 Discussion

2.4.1 Defining crown condition parameters

There are many definitions of parameters that can be assessed in the estimation of eucalypt tree decline. Primary branch dieback is used to express crown condition in many studies (Podger, Kile *et al.* 1980; Kile, Turnbull *et al.* 1981; Wardlaw 1989) although there are slight differences in definition. Podger, Kile *et al.* (1980), Kile, Turnbull *et al.* (1981) and Wardlaw (1989) all describe primary crown dieback as the proportion of primary branches in the actively extending part of the crown that have died-back from the branch terminal (PCD). Stone, Wardlaw *et al.* (2003a) described dieback as the present extent of living foliage compared to the estimated amount that would have been present in the original fully extended crown (SS). This definition given by Stone, Wardlaw *et al.* (2003a) is more similar to the crown shrinkage parameter (CC) defined by Wardlaw (1989) than Wardlaw's (1989) definition of dieback (PCD). The different definitions of dieback have created difficulties surrounding the selection of crown characteristics for the measurement of crown condition and dieback.

2.4.2 Strengths and limitation of the crown condition parameters

The results of this study indicate that there is significant inter-dependence among the various crown condition parameters. For example if a large proportion of branches have died-back it is likely that the tree will have a severely contracted crown, foliage will be sparse and there will be significant epicormic growth. Highly correlated parameters are therefore likely to indicate the presence of a range of symptoms associated with dieback. Also, there was a consistently high correlation between SS (crown size and shape) and PCD indicating that they are measuring a similar crown condition parameter. This is important when considering the various definitions of crown dieback as although they have distinct definitions, quantitative comparisons indicate that these parameters measure the same attribute. The single- parameters that estimated primary crown dieback (SS and PCD) also had the highest correlation to other parameters and varied the least in their correlation to other parameters. Consistent with this, Kile, Turnbull *et al.* (1981) found that the percentage of primary crown dieback, the percentage of existing crown composed of epicormics and the existing crown volume compared with initial crown volume, were highly correlated. As a result Kile, Turnbull *et al.* (1981) assessed only the degree of primary crown dieback for their study of dieback severity. Due to the high correlation among the

different parameters, the assessment of a single parameter is almost as accurate and precise as assessing numerous parameters to determine crown condition.

A number of parameters had important limitations apart from the significant inter-dependence among the various crown condition parameters. Of the parameters assessed, two parameters were weakly correlated relative to the other parameters. These parameters, foliar density (FD) and metres of dead top (DT), appear to be measuring crown parameters that are not well correlated to overall dieback, and thus may be less suitable to assessing crown condition. Eucalypt trees can have a high severity of dieback but have a large proportion of the crown comprising dense epicormic foliage. Crown foliage density also fluctuates seasonally. Growth and leaf senescence peak in summer for temperate eucalypt species (Pook 1984; Pook 1985) making it difficult to monitor crown condition if assessments are not performed at the same time of year. Foliage density is therefore not always a useful crown characteristic for the assessment of dieback as it may be misinterpreted and may not actually represent the health of the tree. In many cases of dieback in *E. delegatensis*, and other eucalypt species but not all, a stag is present at the top of the crown. The length of dead top (DT) can sometimes be indicative of the severity of the dieback, although this is not the case when the dead top has decayed and dropped or been thrown by wind. In these cases trees can be severely affected by dieback, but do not score for length of dead top. The parameter, metres of dead top, is most useful when monitoring the long-term progression of dieback from early onset which would see the development of a dead top over time as the crown contracts. Foliage density and metres of dead top may not be consistently useful to measure crown condition as they depend on the stage of dieback and the species being observed.

Low observer bias and high repeatability was found in most of the parameters compared in this study. The difference in crown condition scores for all parameters was ≤ 5%, but the standard errors were variable. This may have been an artefact of the sample size used and with an increase in sample size both the difference in crown condition scores and standard errors would be predicted to decrease. Despite this, the range of standard errors of the differences for most parameters was less than those reported by Solberg and Strand (1999). Two parameters, crown size and shape (SS), and the combination of all the Stone, Wardlaw *et al.* (2003a) parameters (T1), were the most repeatable over time. T1 had one of the lowest standard deviations in crown condition scores between observers and years indicating that errors are not cumulative as scores are added. The proportion of epicormic

foliage (EG) shared the lowest repeatability with the amount of dead branches (DB) which also had the highest observer bias. Consistent with the results from our study, Grimes (1978) reported that bias was greatest for the crown epicormic score, followed by dead branches in a comparison of 27 observers for each of the crown parameters.

There are a number of reasons why the proportion of epicormic foliage and dead branches were not repeatable. Untrained observers may have difficulty in differentiating between primary and epicormic growth, which is especially important and challenging in mid-range values where there is a mix of primary and epicormic foliage. Solberg and Strand (1999) found that the largest differences in estimates of crown density between observers occurred for intermediate crown densities, possibly because like epicormic growth, intermediate levels of parameters are more difficult to score. It is also difficult to ascertain whether epicormic foliage represents continuing decline or recovery from stress such as growth suppression, fire or drought (Ellis, Mount *et al.* 1980; Podger, Kile *et al.* 1980; Stone 1999). This could only be determined through repeated measures. Furthermore, dead branches may be difficult to observe as they can be hidden or obvious from only one point around the tree. These two issues, identification and observation, are likely to be the main reasons causing higher bias in the epicormic and dead branches parameters, and thus causing these parameters to be less suitable for measurements of crown condition in the field.

All of the parameters that were compared allowed a comparison of health across different species. The scores of the crown condition parameters did not differ significantly with the species of eucalypt assessed. This is important as a species/parameter effect would restrict the comparison of crown condition to within a single species from the same location, or same vegetation type such as woodland with an open understorey or closed understorey, and forest with closed understorey, or closed midstorey. This finding indicates that the parameter that best meets the performance criteria will be suitable for application across different eucalypt species, of different ages, from different locations and forest types.

Each method investigated in this study had strengths and weaknesses due the varying performance of the distinct parameters used in the four different crown condition methods. The combined-parameter methods for assessment of crown condition (i. e. Fox & Curry 1980; Jurskis, Selby *et al.* 2005) were simple and easy to use and thus were more efficient than the single-parameter methods. However, these combined-parameter

methods tended to generate relatively variable data and so were less suited to application across species. In contrast, single-parameter methods were more difficult to score as they were more technically demanding, and so were less efficient to score. The methods that utilised a variety of single-parameters to get an overall score (an additive-parameter) (i. e. Wardlaw 1989; Stone, Wardlaw *et al.* 2003a) gave a most rigorous estimate of crown condition. This is because of their high correlation to other parameters, high stability, low variation (lower sensitivity to bias) and they were sensitive enough to detect true changes. Thus, the additive-parameter methods have the ability to provide detailed descriptions on the condition of various crown characteristics that can be related to specific stages of decline, which provide a more complete view that is ecologically applicable.

2.4.3 Identification of an accurate, precise and efficient crown condition method

Both of the methods that produce an additive-parameter performed well against the criteria. This is partly due to the inevitable result that the addition of parameters results in successively greater proportions of the variance explained in any regression model. Although the Wardlaw (1989) method was not assessed for observer bias or repeatability, these biases are likely to be similarly low to those found for Stone *et al.* (2003a) given the high correlations between the two methods. The advantage of using the Stone, Wardlaw *et al.* (2003a) method (*cf*. Wardlaw 1989) is the classification of each parameter with descriptions of different levels. Being descriptive, the Stone, Wardlaw *et al.* (2003a) values are easier to rate by an inexperienced observer than a scalar (continuous) method (i.e. a measure based on actual values that form part of a linear continuum between a minimum and maximum value) such as the Wardlaw (1989) method. However, a disadvantage of the Stone, Wardlaw *et al.* (2003a) method is that the change from one value to the next does not necessarily reflect a linear change in condition. This becomes a particular issue when attempting to relate condition of the tree with some tree response (e. g. growth). Despite these drawbacks, the additive-parameter T1 that uses four different crown parameters (Stone, Wardlaw *et al.* 2003a) met the highest number of criteria and so provides the best overall tool for monitoring crown condition.

2.4.4 Minimising bias in eucalypt crown condition assessments

A number of factors can influence the assessment of crown condition including observer experience (Smith, Pinkard *et al.* 2005) and bias, weather and the appearance and view of the tree (Innes 1988). The influence of these factors can be reduced by providing observer

training, providing reference material, and assessing under optimal weather conditions i.e. when there is good light and good contrast of the crown with the sky (Innes 1988; Solberg & Strand 1999). Reference material such as photographs of healthy crowns from each of the eucalypt species being assessed provides a good base-line to judge the extent of dieback in the crown. One of the main challenges in applying field-based assessments was finding a position where was a clear and full view of the crown. This is especially difficult when there is a thick understorey/midstorey that restricts overhead views. This problem can be minimised by first observing the tree from various positions and then using this knowledge when assessing the canopy from the clearest position.

2.5 Conclusion

The identification and development of a method that is rapid to score, accurate and precise allows crown condition to be related to other ecosystem variables such as soil nutrients and species assemblages. This is particularly important for the study of eucalypt decline, which is a huge problem throughout Australia affecting many different species of *Eucalyptus*, and is often caused by complex ecological interactions. To determine the influence of different abiotic and biotic variables on tree health, a quantitative method of determining tree health is required. This method needs to represent tree health, be accurate, precise, and efficient to score in the field so that time consuming and costly physiological measurements of tree health can be avoided.

The capacity of a parameter to measure dieback is the main selection criterion. This capacity was established through correlation with other parameters (so that it encompasses the condition of different attributes associated with dieback), the repeatability of the parameter to measure dieback across observers and time, and the sensitivity of the parameters (that detect change but are not susceptible to bias). Overall the primary crown dieback parameter (the proportion of primary branches that have died back, either as a percentage or as scale, i.e. SS or PCD), performed well and was the key parameter for the accurate and precise assessment of crown condition (other parameters within the additive parameters did not perform well against the criteria). Primary crown dieback can likely be differentiated across a wide range of eucalypt species, sites and locations and can indicate change in condition with time.

Thus primary crown dieback meets the criteria for an accurate, precise and effective method for assessment of crown condition and is the most suitable method for the assessment of crown condition in mature eucalypts.

3.0 The effect of spatial scale and fungal material on sampling and detecting *E. delegatensis* forest soil fungi

3.1 Introduction

With advances in molecular techniques, the study of fungal communities using environmental samples, such as soil, has become widespread (Viaud, Pasquier *et al.* 2000; Chen & Cairney 2002; Dickie, Xu *et al.* 2002; Landeweert, Leeflang *et al.* 2003; Anderson & Cairney 2004; Cairney 2005; Landeweert, Leeflang *et al.* 2005; O'Brien, Parrent *et al.* 2005; Avis, Dickie *et al.* 2006; Fierer, Breitbart *et al.* 2007; Berthrong, Schadt *et al.* 2009; Reich, Kohler *et al.* 2009; van der Linde, Alexander *et al.* 2009; Vancov & Keen 2009). Bulk soil contains fungal mycelium including extraradical ectomycorrhizal (ECM) mycelium (Anderson & Cairney 2004). Fungal spores and other material, such as broken roots tips including ECM root tips, and sclerotia are also found in soil (Dickie, Xu *et al.* 2002; Anderson & Cairney 2004; Landeweert, Leeflang *et al.* 2005). Thus, DNA extracted from soil provides a means of studying ECM fungi in the environment, and ectomycorrhizal fungal taxa from the Agaricales, Russulales, Boletales and Cantharellales have all been sampled from soil (Porter, Skillman *et al.* 2008). Even so, the extent to which soil can replace fungal sporocarp or ECM root tip sampling, to study ECM fungal communities is still largely unknown (Chen & Cairney 2002; Landeweert, Leeflang *et al.* 2003; Porter, Skillman *et al.* 2008).

The advantage of using bulk soil for the study of ectomycorrhizal fungi, over using separated root tips, or sporocarps, is the ease of obtaining and processing soil. Root tips, especially those of eucalypts, are time-consuming and difficult to separate from the surrounding sample, as they are often very fine and may be contained within a sticky soil/mycelium substrate. Similarly, sampling sporocarps in the Australian landscape often means conducting frequent surveys in difficult terrain (steep and/or thick with vegetation) to accommodate the unpredictable production of sporocarps. Additionally, identifying sporocarps requires a high level of expertise, skill and time, especially when sporocarps belong to taxa that are difficult to distinguish morphologically, such as the Cortinariaceae, which is widespread and has high diversity in Australian eucalypt forests (Bougher &

Malajczuk 1986; Malajczuk, Dell *et al.* 1987; Bougher 1995). Samples also need to be processed immediately as sporocarps degrade rapidly after collection. Furthermore, sporocarps provide little information of below-ground community structure. Despite these constraints, sporocarps, as either fresh or herbarium specimens allow species identification through morphological characteristics. These identified samples can then be sequenced or profiled to provide the reference information required to identify unknown fungal sequences and fungal profiles through similarity comparisons (Anderson & Cairney 2004). Without these sequences or profiles from identified herbarium specimens, many fungal operational taxonomic units (OTUs) detected from soil would remain as unknown species.

The extent of correspondence among ECM fungal communities sampled from sporocarps, root tips, or soil has been studied on a number of occasions (Gardes & Bruns 1996; Dahlberg, Jonsson *et al.* 1997; Peter, Ayer *et al.* 2001a; Landeweert, Leeflang *et al.* 2005; Porter, Skillman *et al.* 2008). Only 22% of ECM species recorded from sporocarps surveys were also recorded as ECM root tips in a Norway Spruce forest (Peter, Ayer *et al.* 2001a) and approximately 10% of OTUs that were sampled as sporocarps in a Canadian hemlock forest were also sampled from soil (Porter, Skillman *et al.* 2008). Other studies have indicated that there is some overlap between ECM species found in bulk soil and as separated ECM root tips. Landeweert, Leeflang *et al.* (2003) found 10 of the 25 OTUs detected in soil samples had over 98% sequence similarity to OTUs from root tips. In a similar study, Landeweert, Leeflang *et al.* (2005) found that of the 14 fungal taxa detected in soil, 11 were found on root tips and seven were identified as ECM taxa. The different views of the community that are obtained from different samples may be because of different sampling strategies employed for studies using sporocarps, ECM root tips or soil and/or inadequate sampling of sporocarps, root tips or soil resulting in many species present within the community remaining undetected (Horton & Bruns 2001; Taylor 2002; Peay, Kennedy *et al.* 2008b). Many ECM species, such as those from the Thelephoraceae and Sebacinaceae, do not produce large, easily detected sporocarps and are unlikely to be sampled from sporocarps. Both ECM mycelia and ectomycorrhizal root tips give a better understanding of fungal function within an ecosystem than sporocarps, as mycelium is the main body of the fungus where nutrient transfer takes place (Cairney 2005; Anderson & Cairney 2007) and ectomycorrhizal root tips are the site of transfer between fungus and host (Harley & Smith 1983; Courty, Buée *et al.* 2010). Although the use of soil has the potential to advance our understanding of ECM communities and function, this technique is still reliant on having a large database of readily available sequences and/or profiles from

described and identified fungal species to which unknown species can be compared to assist in identification.

Fungi are known to have spatially patchy, aggregated distributions varying in both the horizontal and vertical planes (Dickie, Xu *et al.* 2002; Taylor 2002; Peay, Kennedy *et al.* 2008b; Taylor 2008). The spatial distribution of soil microbes including ECM fungi varies with resource availability, vegetation community competition and structure, positioning of individual trees and population processes, such as dispersal, reproduction and competition (Bruns 1995; Dickie, Xu *et al.* 2002; Ettema & Wardle 2002; Dickie 2007; Peay, Bruns *et al.* 2007; Peay, Kennedy *et al.* 2008b; Taylor 2008). In forests, patch size of soil microbes is thought to be one to several meters (Saetre & Baath 2000) and ECM fungi can form genets up to 10m width (Courty, Buée *et al.* 2010). ECM abundance and community composition have been shown to change over very small scales, and extramatrical mycelium may be spatially distant from ectomycorrhizal root tips (Tedersoo, Koljalg *et al.* 2003; Izzo, Agbowo *et al.* 2005; Genney, Anderson *et al.* 2006). Izzo, Agbowo *et al.*(2005) found that ECM community composition turnover between years was frequent at scales of <20cm. This trend is also supported by the work of Lilleskov, Bruns *et al.* (2004) who found ECM community similarities were only correlated at less than 2 to 3 meters. At larger spatial scales (i.e. forest stand), ECM community composition is more stable with dominant species recorded across multiple years (Izzo, Agbowo *et al.* 2005; Koide, Shumway *et al.* 2007). This temporal stability and spatial variability is highlighted by the result of Izzo, Agbowo *et al.* (2005) who showed that mixed-conifer forest plots were more similar in ECM composition across years than they were to one another within the same year. The question of how best to sample such aggregated and variable populations is central to our understanding of fungal ecology.

The spatial distribution of fungi in forest soil can give insight into how to best detect fungal species and assemblages. For example, at what spatial scale should soil fungi be sampled to understand species richness within a temperate forest? An understanding of the information that is obtained by sampling fungi at different spatial scales can aid in answering such questions. Currently, there is limited research assessing the benefits of different plot shapes and sizes to sample fungi leading to a lack of rigour in experimental design; there are no estimates for minimum sampling areas required to sample the ECM community on root tips nor is there an understanding of the optimum spatial distribution of samples (Taylor 2002). This is also true for studying fungi directly from environmental

samples. This lack of standardisation limits comparisons among studies based on different plot sizes as ecological patterns and processes are scale dependant (Vogt, Bloomfield *et al.* 1992; Taylor 2002; Zak & Willig 2004).

The increase in species richness with increasing area, known as the species-area relationship, is one of the earliest ecological models (Whittaker 1977). This relationship has been reported for many organisms but is only beginning to be investigated for fungi. Recent research indicates that species richness would indeed increase with increasing spatial scale as species turnover within communities occurs on a small scale (i.e. centimetre scale) (Tedersoo, Koljalg *et al.* 2003; Lilleskov, Bruns *et al.* 2004; Izzo, Agbowo *et al.* 2005). A couple of studies have also directly investigated the species-area relationship for fungi. One study found a strong relationship between ECM species richness and sample area which was similar to those reported for macro-organisms (Peay, Bruns *et al.* 2007). A study of fungal sporocarps within a temperate eucalypt rainforest in Tasmania found a steep positive relationship between fungal species richness and increasing area (measured by an increasing number of sub-plots) for both wood decay fungi and for ECM fungi (Gates 2009). In contrast to the above results, a study focusing on soil Ascomycota found a weak species-area relationship (Green, Holmes *et al.* 2004).

Within Australia, only a few studies have utilised soil as a means to study fungi (Chen & Cairney 2002; Anderson & Cairney 2004; Bastias, Huang *et al.* 2006; Bastias, Anderson *et al.* 2007; Bennett, Kasel *et al.* 2009; Vancov & Keen 2009) and there is little information on the effectiveness of sampling soil to study Australian fungi, especially ECM communities, nor has there been any attempt to determine an optimal sampling strategy or species-area relationship for Australian soil fungi. This study aimed to assess the applicability of using soil as a substrate for the study of ECM communities. This study further aimed to explore the effects of sample distribution and area on measures of species richness and composition by testing the hypotheses that species richness increases with increasing spatial distribution of samples, and with increasing sample-area. An efficient sampling strategy that would provide a comparable view of the soil fungal community from different plots and regions based on these findings is recommended.

3.2 Methods

3.2.1 Study sites

Three sites were established along Ben Ridge Road, near Mt Maurice in the highlands of northeast Tasmania 60 km east northeast of Launceston (Figure 3.1). Sites were on brown dermasols derived from Devonian granodiorite (Grant, Laffan *et al.* 1994) between 850 m and 900 m asl. Mean annual rainfall is 1320 mm and mean annual temperature is 9.1 °C at Ben Nevis (ESOCLIM module of ANUCLIM 5.2 Houlder, Hutchinson *et al.* 2000).

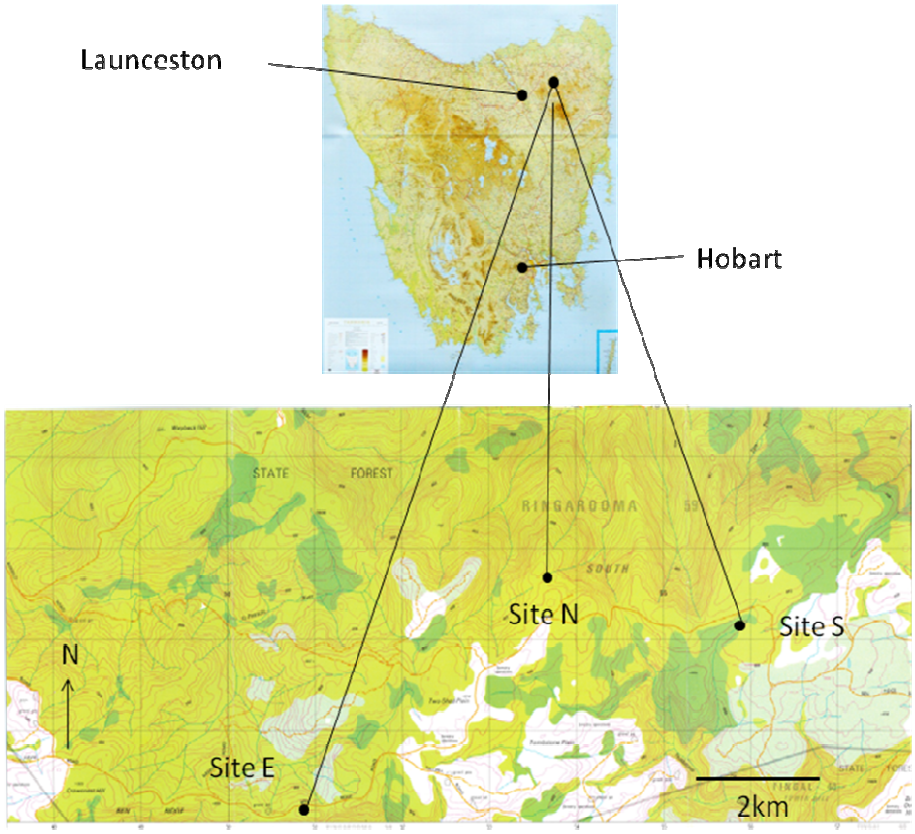

Figure 3.1 Locations of the three study sites used in the soil fungal study. The map of Tasmania indicates location of the site and the two major cities, Hobart and Launceston. Below the map of Tasmania is the topographic map (Maurice 1:25 000) showing the location of Site S, Site N and Site E.

The characteristics of the three study sites used for the soil fungal study are summarised in Table 3.1

Site E is located in the same place as plots 5 and 6 that are described in Chapter 4 and used for sampling in Chapters 4 and 5. Site S is located in the same place as plots 1 and 2 that are described in Chapter 4 and used for sampling in both Chapter 4 and 5.

Table 3.1 Summary of soil fungal study site characteristics.

Characteristic	**Site E**	**Site S**	**Site N**
Situation	41.37 °S, 147.61 °E Broad ridge-top	41.35 °S, 147.67 °E Low ridge between wetlands and creeks	40.35 °S 147.66 °E Low rise amidst wetlands
Drainage	Well-drained	Moderately well-drained	Poorly drained
Aspect	South-easterly	n/a	South-easterly
Dominant sp.	*E. delegatensis* to 50 m, 35% cover	*E. delegatensis to* 55 m, 25% cover	*E. delegatensis* to 50 m
Mid-storey	*Acacia dealbata* Link.to 15 m	*T. lanceolata* (Poir.) A.C.Sm., *Leptospermum lanigerum* (Sol. Ex Aiton.) Sm., *Pittosporum bicolor* Kook., *A. dealbata* to 24 m, *Dicksonia antarctica* Labill. to 15 m	*T. lanceolata*, *L. lanigerum* and *Nothofagus cunninghamii* to 22 m
Under-storey	*Pultenaea juniperina* Labill., *Tasmannia lanceolata*, *Persoonia gunnii* Hook.f., and *Coprosma nitida Hook.f.* to 4 m.	absent	absent
Ground cover	*Histiopteris* sp. (Agardh) J. Smith, *Hypolepis* sp. Bernh., *Polystichum proliferum* (R.Br.) C.Presl., *Pteridium esculentum* (G.Forst.) Cockayne., *Poa* sp. L.	Mostly litter, sparse *Gahnia grandis* (Labill.) S.T.Blake. *Hypolepis* sp. and *P. proliferum*.	*G. grandis* and *P. proliferum*.
Management history	Grazing lease until 1935 (Ellis, Mount *et al.* 1980). Dedicated as State forest c. 1948-1960. No record of forestry.	Unallocated crown land dedicated as State forest c. 1948-1960. No record of forestry.	Unallocated crown land dedicated as State forest c. 1948-1960. No record of forestry.
Fire history	1967, 1963, frequently prior to 1935 (Ellis, Mount *et al.* 1980).	1964, ~1880 (Ellis, Mount *et al.* 1980).	1964, ~1880 (Ellis, Mount *et al.* 1980).

3.2.2 Soil core collection and processing

From each of the study sites, five soil cores were collected from a 50 m x 50 m quadrat, five from a nested 7 m x 7 m quadrat and five from a nested 1 m x 1 m quadrat (Figure 3.2). The

southwest corner of each quadrat was determined by a randomly generated co-ordinate representing the easting and northing co-ordinates from the south-west corner of the site. A third random number indicated the angle of the western side of the plot in relation to north (i.e. 34 indicates 34°).

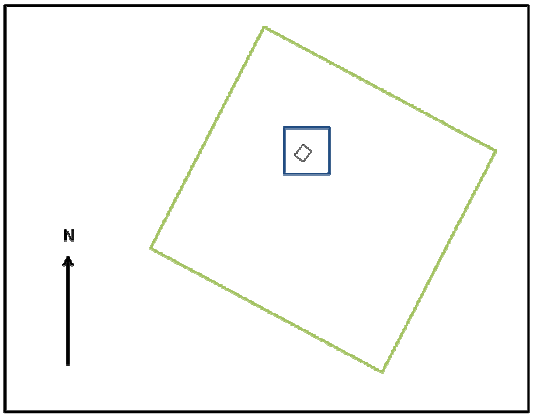

Figure 3.2 The arrangement of the nested quadrats at each of the three sites. The thick black indicates the site boundary. The green square indicates the largest randomly located plot (50 m x 50 m), with the 7 m x 7m plot nested within the 50 m x 50 m quadrat, and the 1 m x 1 m quadrat (thin grey square) nested within both the 7 m x 7m and 50 m x 50 m quadrats.

Soil cores were collected from all sites on one occasion. Soil core collection was not replicated over time because of time constraints. A randomisation strategy for the collection of soil cores was employed to ensure that there was no bias in sampling. Soil core collection was determined by generating random numbers that were used as the easting and northing co-ordinates (from the southwest corner of each quadrat). Soil cores were collected within the canopy drip line of nearest *E. delegatensis* tree to the random co-ordinate. Due to the random sampling strategy, cores could be collected very near to one another within each quadrat or very far apart. The number of trees in each quadrat from under which soil cores were sampled is summarised in Table 3.2. Litter was scraped away from the sample location and soil cores were cut from the ground using a sharp knife and the whole of the 5 cm x 5 cm x 10 cm deep core was removed and placed into a plastic zip-lock bag. Soil cores were kept at 4 °C until processed within one week of collection.

Each soil core was placed separately into a series of graded sieves (500 µm, 1000 µm, and 2360 µm) and shaken thoroughly to remove large material such as stones and roots (Landeweert, Leeflang *et al.* 2003). The finest soil particles were used to fill a 5 mL plastic screw-capped vial. Samples were frozen at -80 °C until analysed.

Table 3.2 The number of eucalypt trees from under which soil cores were taken in each quadrat.

Site	Quadrat (m)	No. eucalypts sampled
E	1x1	1
E	7x7	4
E	50x50	5
S	1x1	2
S	7x7	4
S	50x50	5
N	1x1	1
N	7x7	3
N	50x50	5

3.2.3 Root tip and sporocarp collection and identification

ECM root tips and sporocarps were collected from Site E and Site S according to the methods outlined in Chapter 4. Corresponding sites used throughout this thesis are described in section 3.2.1. ECM root tips and sporocarps were identified using DNA amplification followed by sequencing. A detailed account of these methods is outlined in Chapter 4.

3.2.4 Methodology to identify soil fungi

The soil fungi present in the soil collected from the three sites was identified through the extraction, amplification and cloning of DNA directly from the soil. Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP) was used to group the cloned DNA based on the similarity of their profiles. Representative samples from each group were then sequenced and identified. The methods used to identify the fungal species present in the soil cores are summarised in Figure 3.3.

3.2.5 DNA extraction

DNA was extracted from a sub-sample of 0.25 g taken from each soil core using a MoBio PowerSoil DNA Isolation Kit (Promega, Madison, USA) according to the manufacturer's instructions (see Appendix 3 for full protocol). DNA samples were stored at -80 °C until further analysis.

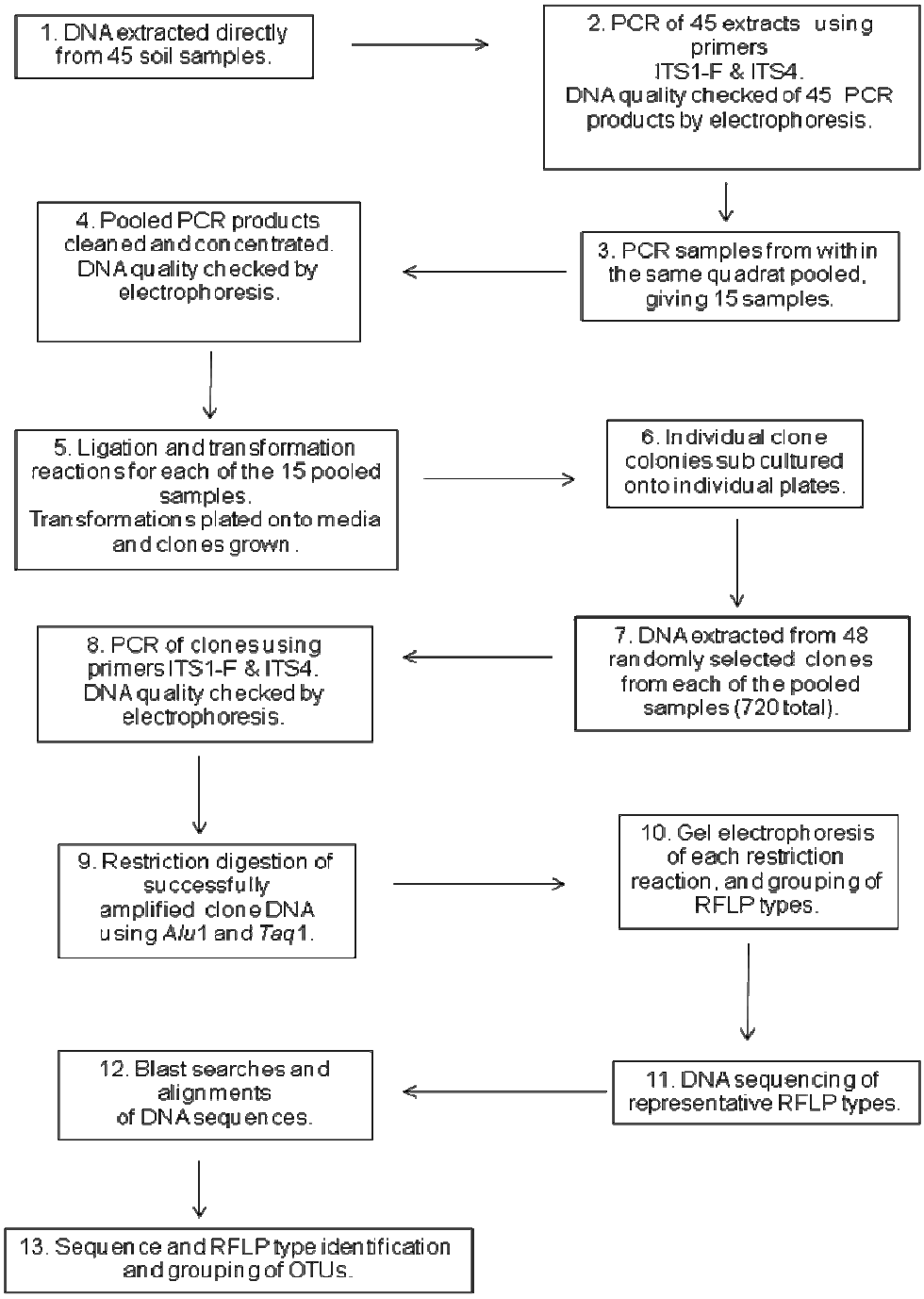

Figure 3.3 Flowchart showing the steps involved in identifying the soil fungi present in the quadrats from each of the three sites.

3.2.6 Polymerase Chain Reaction (PCR)

The internal transcribed spacer regions (ITS) of the rDNA were amplified by PCR using the primers, ITS1-F (Geneworks, Adelaide, Australia) (Gardes & Bruns 1993) and ITS4 (Geneworks, Adelaide, Australia) in the reverse direction (White, Bruns *et al.* 1990). These primers have been used widely for the study of fungal communities and are known to successfully amplify DNA from a range of fungal species including Ascomycota, Basidiomycota and Mucoromycotina (Anderson, Campbell *et al.* 2003). Alternative primers were not utilised because of time constraints. PCR protocol is shown in Appendix 4.

A 5 µL aliquot of each of the 45 soil PCR products was loaded into separate wells of a 1 % agarose gel (Fisher Biotec, Wembley, Australia) with 0.4 % (w/v) Bromophenol Blue loading buffer (Appendix 7). One lane of each gel was loaded with DNA size marker, lambda DNA cut with Eco RI and Hind III (Fisher Biotech, Wembley, Australia) and electrophoresed at 8 Vcm^{-1} for 30 minutes after which the gel was stained in 1 µg/mL ethidium bromide (MoBio Laboratories, Carlsbad, California) solution for 20 minutes and illuminated under a Vilber Lourmat UV transilluminator (Cedex, France) to check PCR product quality.

3.2.7 PCR product pooling, clean-up and concentration

The remaining 40 µL of each PCR product from soil samples in the same quadrat (e.g. the five PCR products from Site E quadrat 1 m x 1 m) were pooled together into a clean 1.5 mL tube. PCR products were pooled at this stage to minimise the number of soil samples for cloning, because of time and funding constraints. Samples were not pooled prior to this as DNA from each of the soil cores may not have been successfully amplified and so pooled samples may not have contained DNA from each of the soil cores. These pooled PCR products were cleaned and concentrated using the MoBio UltraClean PCR Clean-up DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions (see Appendix 5 for full protocol).

A 2 µL aliquot of the clean, concentrated PCR product was loaded on a 1 % agarose gel (Fisher Biotech, Wembley, Australia) and electrophoresed for 30 minutes at 8 Vcm^{-1}. The gel was then stained in 1 µg/mL ethidium bromide (MoBio Laboratories, Carlsbad, California) for 20 minutes and observed under a Vilber Lourmat UV transilluminator (Cedex, France). DNA concentration was approximated by visual comparison of band intensity with the lambda DNA size marker (Fisher Biotec, Subiaco, Western Australia).

3.2.8 Ligations, transformation and clone growth

Ligations and transformations were performed using a MoBio pGEM-T Vector Kit (Promega, Madison, USA) according to the manufacturer's instructions (see Appendix 6 for full protocol). Ligation reactions were performed immediately after PCR cleanup and concentration and were stored at 4°C until transformation.

Two aliquots (100 µL each) of transformation reaction were spread onto prepared petri-dishes containing Luria-Bertani (LB) medium (see Appendix 7 for recipe) with 100 µg/mL ampicillin (Sigma-Aldrich, Sydney Australia), previously spread with 100 µL 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega, Madison, USA) and 20 µL 50 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Promega, Madison, USA) using a sterilised L-shaped glass rod. Plates were incubated at 37 °C overnight then stored at 4 °C. From each plate individual white colonies were sub-cultured onto another LB/ampicillin/IPTG/X-gal plate. Sub-cultures were incubated at 37 °C overnight.

3.2.9 DNA extraction from clones and PCR-RFLP of cloned DNA

DNA was extracted from 48 randomly selected white colonies from each transformation reaction by lightly scraping the colony with an autoclaved pipette tip, and then placing the pipette tip into a single well of a 96 well PCR plate containing 200 µL of TE buffer (see Appendix 7 for recipe). Pipette tips were left in wells until the plate was full after which they were removed and the plate capped. The plate was vortexed briefly to break up cells and centrifuged for 1 minute so that all the cellular debris was contained in the bottom of the wells. The supernatant was used as a PCR template to amplify insert DNA in 50 µL reactions using the primers ITS1-F/ITS4 (Geneworks, Adelaide, Australia) (see Appendix 4 for PCR protocol).

A 5 µL aliquot of each PCR product was electrophoresed on a 1 % agarose gel (Fisher Biotech, Wembley, Australia) as above, after which the gel was stained in 1 µg/mL ethidium bromide (MoBio Laboratories, Carlsbad, California) solution for 20 minutes and illuminated under UV light (Vilber Lourmat transilluminator, Cedex, France) to check PCR product concentration.

3.2.10 Restriction fragment length polymorphism (RFLP)

All colony PCR products were digested using two restriction enzymes, *Alu*1 and *Taq*1 (Promega, Madison, USA) in two separate reactions. Digests were performed in 10 µL reaction volumes consisting of 3.8 µL water for injection (Baxter, Toongabbie, Australia), 1 µL of the appropriate 10X reaction buffer (Promega, Madison, USA) 0.1 µL BSA (Promega, Madison, USA), 0.1 µL enzyme (either 100 units/µL of *Alu1* or *Taq1*, Promega, Madison, USA) with 5 µL of PCR product and digested for 2 – 4 hours. Digests with *Alu1* were incubated at 37 °C while digests with *Taq*1 were incubated at 65 °C. Samples were stored at 4 °C until electrophoresis.

PCR-RFLP patterns were determined by running 10 µL of each sample with 0.4 % (w/v) Bromophenol Blue loading buffer (Appendix 7) on 3 % Hi-resolution agarose (Fisher Biotech, Wembley, Australia) at 6.5 Vcm^{-1} for 100 - 120 minutes. The molecular marker pUC19 digested with *Hpa*II (Fisher Biotech, Wembley, Australia) was used to estimate fragment lengths and differentiate PCR-RFLP profiles. Gels were stained in 1 µg/mL ethidium bromide (MoBio Laboratories, Carlsbad, California) for a minimum of 20 minutes after which they were observed under UV transilluminator (Cedex, France) and photographed with a Vilber Lourmat camera (Cedex, France). One or two representative clones of each PCR-RFLP type from each transformation reaction were selected for sequencing.

3.2.11 DNA sequencing, sequence editing, alignments and identification

PCR products of selected samples were purified and sequenced in one direction by Macrogen Laboratories, Korea (www.macrogen.com), unless sequence quality was poor in which case samples were also sequenced in the reverse direction.

Sequence chromatograms were examined for quality and edited when necessary using the software DNAStar SeqMan II v 5.05. Sequences were compared with sequences from public databases (GenBank, EMBL, DDBJ); by using the nucleotide–nucleotide (blastn) BLAST search option, available through the ANGIS online database (www.angis.org.au). Sequences were grouped according to accession number of the highest match and groups were aligned using ClustalW (Thompson *et al.* 1994). Alignments were used to determine operational taxonomic units (OTUs), which were initially defined as having ≥ 98% sequence match over the whole of the ITS region (ITS1, 5.8S and ITS2). OTUs were assigned to a

nearest taxonomic rank according to the level of match to database sequences. For example a sequence similarity ≥ 98% was taken to indicate probable con-specificity and sequence similarity of 95 - 98% similarity to indicate another species within a genus, unless more than one genus had comparable levels of sequence similarity, in which case it was assigned to a family or higher taxonomic level. Some samples had their highest match to environmental sequences and some samples had no close match to any database sequence. In these cases samples remained unidentified. Samples also remained unidentified if only a small part of the ITS region matched a sequence.

Taxonomic nomenclature followed Crous, Gams *et al.* (2004) (www.mycobank.org) apart from the Exidiaceae, the genus *Thaxterogaster* Singer. and Zygomycota. *Thaxterogaster* is a taxonomic synonym of *Cortinarius* (Peintner, Moser *et al.* 2002) and thus any OTUs with high sequence similarity to *Thaxterogaster* spp. were named *Cortinarius*. Taxonomy of the Zygomycota followed Hibbett, Binder *et al.* (2007) and were included under Mucoromycotina. The Exidiaceae followed current convention and were included as Sebacinaceae. OTUs were used as a proxy for species. All OTUs recorded within this chapter and following chapters were allocated a unique OTU name. OTUs were assumed to represent mycorrhizal species if they had high sequence similarity to known ectomycorrhizal taxa listed in Rinaldi, Comandini *et al.*(2008), or to sequences that were stated to be of mycorrhizal origin.

3.2.12 Species richness estimates, species-area and species-effort curves

Species richness was defined as the total number of OTUs observed within a given plot. Species-effort curves were constructed by graphing mean cumulative fungal OTU richness of the three sites against the number of pooled soil samples. The cumulative number of OTUs per five soil cores was calculated by adding the number of newly recorded OTUs from the 50 m x 50 m quadrats, then the 7 m x7 m quadrats and finally the 1x1 m quadrats. To assess the species-area relationship, plot size (m^2) and mean species richness were log transformed to improve homoscedasticity and increase linear regression fit (Peay, Bruns *et al.* 2007). Species-area curves were graphed by plotting $\log_{(10)}$ scale for plot size (m^2) against 1) $\log_{(10)}$ mean total fungal OTU richness of each quadrat size and 2) $\log_{(10)}$ mean mycorrhizal OTU richness. Simple linear regression models were fitted to the species-area and species-effort curves in Microsoft Excel.

3.2.13 Statistical analysis

Two-way ANOVA's to test for differences in total species richness and mycorrhizal species richness among different plot sizes and the three sites, was performed using GenStat v7.1 (VSN International Ltd 2003).

Canonical analyses of principal coordinates (CAP)(Anderson & Willis 2003) using the statistical program PRIMER v6 with PERMANOVA + (Anderson, Gorley *et al.* 2008) were used to assess differences in fungal community composition among the three different plot sizes, and for the three different sample sites, for all fungal OTUs recorded. CAP analyses were performed on Bray-Curtis similarity matrices, using 9999 unrestricted permutations (Anderson, Gorley *et al.* 2008).

The Bray-Curtis similarity measure calculates the similarity between every pair of samples, in this case between each pair of quadrats, using the following formula:

$$Similarity^{\text{Bray-Curtis}} = \frac{2A}{2A + B + C}$$

Where A is the number of species present in both samples, B is the number of species present in the first sample but absent from the second sample, and C is the number of species present in the second sample but absent from the first sample. This similarity measure ignores shared absences (i.e. 0), which is important as shared absences do not necessarily mean the samples are similar. Similarity is shown as a value between zero and one, where one represents perfect similarity.

CAP analysis is a discriminant analysis that uses a constrained ordination technique to display multivariate points by reference to an *a priori* hypothesis (Anderson & Willis 2003). The advantage of using CAP is that it can be used on any similarity matrix and can be used to reveal patterns that may be masked by unconstrained ordination analyses such as multi-dimensional scaling (Anderson & Willis 2003). CAP analysis is a useful tool for analysing similarities and differences of macrofungal species assemblages (Ratkowsky 2007) and has been used to analyse soil fungal communities from native eucalypt forest (Bastias, Huang *et al.* 2006; Bastias, Anderson *et al.* 2007). The first step of CAP analysis involves a Principal Coordinates analysis (PCoA) on the resemblance matrix (in this case Bray-Curtis). CAP then determines an appropriate set of axes (*m* axes) that maximises the group allocation success

(i.e. predicting group membership) whilst avoiding model over-parameterisation. CAP analysis finds axes through the multivariate data cloud that best discriminate among *a priori* groups. The hypotheses that species richness increases with increasing sample distribution and sample area were tested. CAP can also be analysed using permutations to give a p-value, which indicates if there are significant differences among the groups. CAP thus discriminates among groups by maximising group differences by finding axes that are best at separating the groups. Another feature of CAP is cross validation, which is achieved by the leave-one-out procedure that estimates misclassification of group allocation (the proportion of points, i.e. quadrats or plots that were mis-classified). This is achieved by removing one sample at a time and checking the ability of the model to correctly classify that sample into the appropriate group. Thus cross validation indicates how distinct the groups are and how well the model discriminated between the groups (Anderson, Gorley *et al.* 2008).

3.3 Results

3.3.1 PCR, cloning, PCR-RFLP, sequencing and fungal identification

DNA from all soil samples was successfully amplified. Overall there was a high success rate in amplifying the cloned DNA and with restriction digestion reactions (Table 3.3). The majority of the clones produced clear PCR-RFLP profiles (Appendix 8) although some clones did not digest with both of the *Alu1* and *Taq1* enzymes used (Table 3.3 and Appendix 8).

Of the 240 clones that were sequenced, 89 clones had <95% sequence similarity to sequences in public databases (Appendices 9 and 10). Fifty-four clones had ≤200 base pairs (bp) of sequence that matched public database sequences and 13 had a similarity match of ≤100 bp to publicly available sequences (Table 3.3). DNA sequence analysis resulted in 100 OTUs (Appendices 9 and 10). An additional three OTUs had distinct PCR-RFLP types but were not identified because of poor quality sequences. Two OTUs were identified to species level, 42 to genus, 10 to family, 9 to order, 19 to phyla and 21 remained unidentified because of poor matches to public database sequences or the sequences were similar to unidentified isolates or environmental samples in public databases (Appendices 9 and 10).

Nine sequence OTUs (9% of OTUs) had multiple PCR-RFLP profiles because of variation in restriction enzyme site locations; *Cortinarius* sp. 64 had four PCR-RFLP patterns,

Mucoromycete sp. 2 had three PCR-RFLP patterns Basidiomycete sp. 19, *Russula* sp. 9, *Mortierella* sp. 2, *Mortierella* sp. 4, *Mortierella* sp. 5, Saccharomycetales sp. 1 and *Zelleromyces* sp. 1 each had two PCR-RFLP patterns. In contrast, *Cryptococcus* sp. 3 and *Cryptococcus* sp. 5 had identical PCR-RFLP patterns. PCR-RFLP types were not compared across soil samples so the total number of PCR-RFLP types was not obtained.

The number of RFLP types and sequence OTUs differed in the majority of samples (Table 3.3). This is because some samples did not produce a clear RFLP pattern with both endonucleases (i.e. negative RFLP result), and thus not being counted as a unique RFLP type. In some cases, sequences of these clones were found to represent a unique OTU, and were therefore considered as a separate OTU. Furthermore, some samples that had similar sequences (and thus belonged to the same OTU) had multiple PCR-RFLP profiles, and some of the PCR-RFLP profiles were identical for different sequence OTUs. The number of PCR-RFLP types and the number OTUs established after DNA sequencing differed for all samples except two (Table 3.3). Sequence OTU richness was used for all following analyses.

Table 3.3 Success of PCR-RFLP and DNA sequence identification for fungi cloned from the pooled soil samples from the nine quadrats.

Site	**Quadrat**	**No. clones selected for PCR-RFLP**	**No. PCR-RFLP types**	**No. sequence OTUs**	**No. clones with only *Alu*1 digest**	**No. clones with only *Taq*1 digest**	**No. clones with Blast match <100bp**	**No. clones with Blast match ≤200bp**
E	1x1	47	13	13	2	0	1	3
E	7x7	47	22	20	6	0	2	11
E	50x50	36	9	14	11	1	6	8
S	1x1	48	11	10	1	0	0	1
S	7x7	28	10	13	4	1	1	2
S	50x50	48	31	31	2	0	2	10
N	1x1	38	12	9	0	2	1	6
N	7x7	37	11	13	0	5	0	8
N	50x50	38	7	8	4	2	0	5

3.3.2 Species richness, functional groups and community composition

A total of 100 DNA sequence OTUs were recorded from all three sites (Appendices 10, 11 and 12). Of these, 30 OTUs were determined to be mycorrhizal (Appendix 9). Site S had the highest fungal OTU richness (47 OTUs) and mycorrhizal richness (16 OTUs), and Site N had the lowest richness (29 fungal OTUs and 6 mycorrhizal OTUs). When considering individual quadrats, S50 had the greatest total fungal richness (31 OTUs) and mycorrhizal richness (6 OTUs), quadrat N50 had the lowest fungal richness (8 OTUs) and quadrat N7 had the lowest mycorrhizal richness (0 OTUs). Due to violation of assumptions traditional ANOVA could not be employed to test for statistical differences.

The majority of OTUs that were identified to order belonged to the Agaricales (16 OTUs), with the Russulales (10 OTUs), Mortierellales (7 OTUs), and Helotiales (6 OTUs) the next most dominant orders (Figure 3.4). This pattern in community dominance was reflected at Sites N and S which were dominated by the Agaricales whereas Site E was co-dominated by the Mortierellales and Russulales.

Of the mycorrhizal OTUs sampled (26 OTUs), OTUs belonging to the order Agaricales and Russulales each made up ~38%, Sebacinales 12%, Cantharellales ~8% and Trechisporales ~4% (*Trechispora* P. Karst. were considered as ECM fungi (Erland & Taylor 1999). Many common mycorrhizal genera were sampled including *Cortinarius*, *Russula* Pers., *Laccaria* Berk. & Broome., *Lactarius* Pers., and *Sebacina* Tul. & C.Tul. (Appendix 9). These data is comparable to the OTUs recorded in Chapter 4, where the Agaricales were the most species-rich order, and the Russulales the second most species rich order (Appendices 9 and 12).

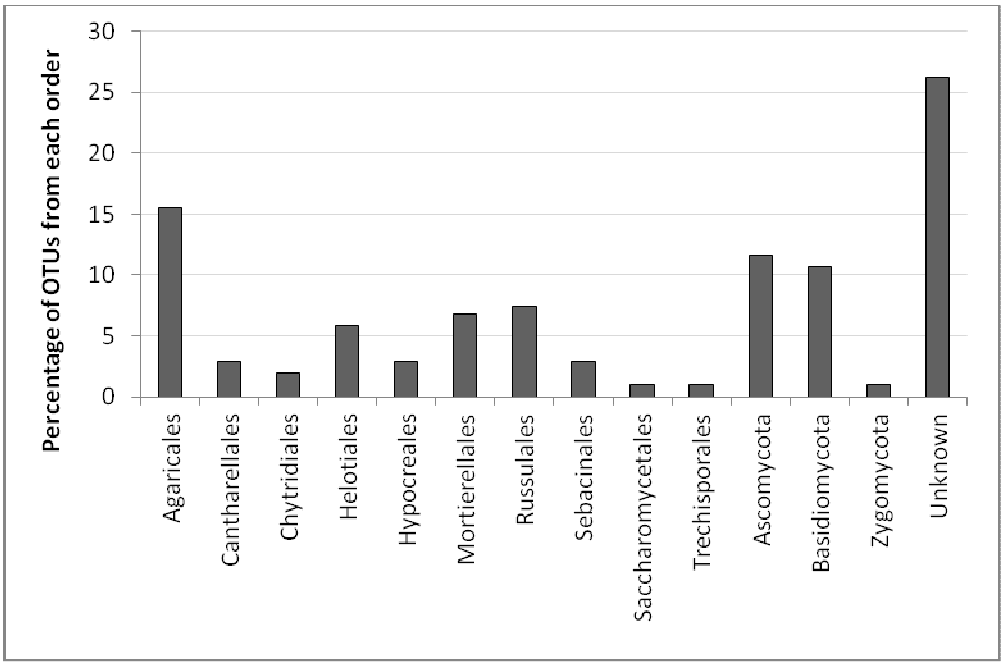

Figure 3.4 The number of fungal OTUs belonging to each order, or unable to be assigned to order, as a percentage of total OTU richness. OTUs of an unknown order made up the highest percentage of OTUs. Of those identified to order, the Agaricales was the most OTU rich.

No OTUs were sampled from every quadrat across all three sites and few OTUs were sampled from more than one quadrat from within each site (four from Site E and Site S, and one from Site N). Only two OTUs (*Cryptococcus* sp. 3 and *Leohumicola* sp. 1) were sampled from all three quadrats from a single site (Site S). Fourteen OTUs were sampled at more than one site, but only two (*Mortierella* sp. 4 and *Mortierella* sp. 5) were sampled at all three sites.

A canonical analysis of principal coordinates (CAP) was used to test the hypothesis that fungal community composition of each study site was different. Bray-Curtis similarities measures of community composition between sites are shown in Appendix 11. Fungal community composition among the three sites was not significantly different (with quadrats used as replicates) (Figure 3.5). Replicates from each site do not cluster to form groups based on site and there is no separation of quadrats from each site along either the CAP1 or CAP2 axes. The overall dissimilarity in fungal community composition among the sites is evident by the lack of clustering (Figure 3.5). The large misclassification error indicates the model is not strong and groups are not distinctive (Figure 3.5). The large p value indicates there are no significant differences among the communities from the different sites (Figure 3.5). Cross validation results indicated that although the fungal

composition of Site S is distinct, the compositions of the other two sites are not (Figure 3.5). The lack of difference in fungal community composition among sites is likely because of large dissimilarities in composition among replicates from each site, and the small number of OTUs that were recorded from multiple sites. This analysis was repeated using only OTUs that were sampled in more than one of the quadrats (i.e. singletons omitted leaving 17 OTUs), with no significant difference detected (misclassification was 56% and p = 0.13).

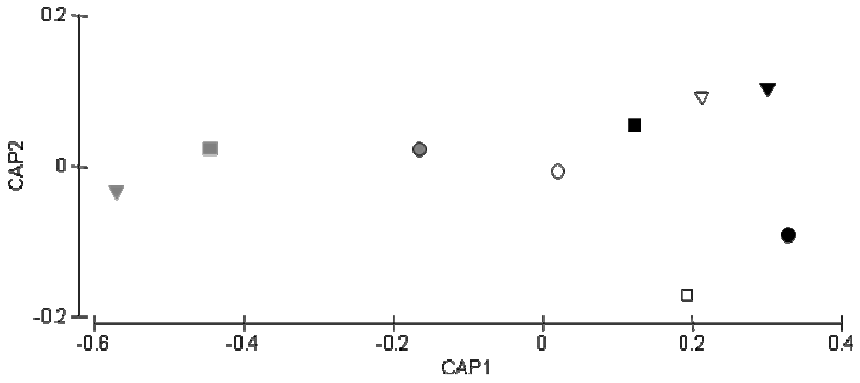

Figure 3.5 CAP analysis testing difference in fungal composition among the three sample sites. Misclassification error was 78% and p =0.32 for 9999 permutations. Individual markers represent the nine different quadrats, squares represent 1 m x 1 m, triangles are 7 m x 7 m quadrats, and circles are 50 m x 50 m quadrats. Black solid markers are Site N, grey solid markers are Site S, and open markers are Site E.

Taxa of unknown functional groups made up the greatest percentage of OTUs and were the predominant functional group at four quadrats (Figure 3.6). This is expected because of the large number of unidentified taxa, which therefore have unknown function. N1 had the same percentage of non-mycorrhizal OTUs as unknown OTUs, N50 had equal percentages of mycorrhizal and unknown OTUs, and S1 and S7 had equal percentages of mycorrhizal and non-mycorrhizal OTUs (Figure 3.6). Quadrat N7 has the greatest proportion of taxa of unknown function and the lowest percentage of mycorrhizal taxa and non-mycorrhizal taxa compared to all other quadrats. Quadrat E1 has the highest percentage of mycorrhizal taxa, S1 had the lowest percentage of unknown taxa, and N1 had the highest percentage of non-mycorrhizal taxa.

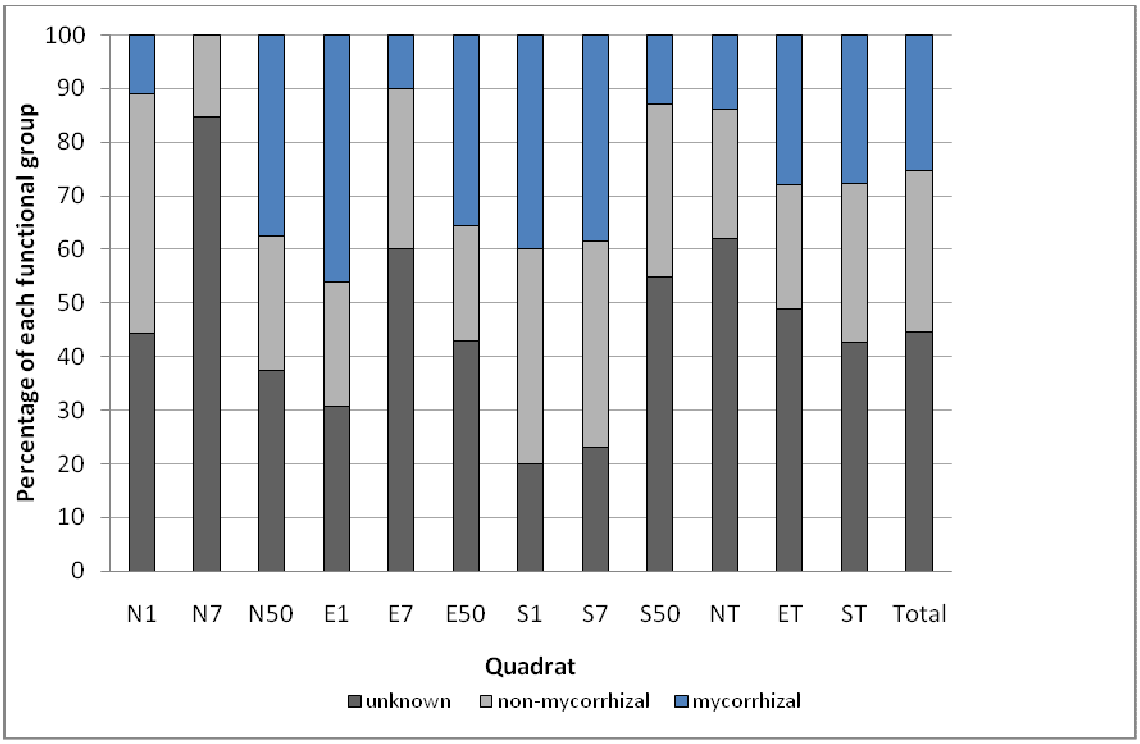

Figure 3.6 Percentage of mycorrhizal, non-mycorrhizal and OTUs of unknown function from each quadrat sampled (N1, N7, N50, E1, E7, E50, S1, S7, S50, letter indicates site and number indicates quadrat size), from each site (NT, ET, ST, letter indicates site) and the total combined samples (Total). Blue represents mycorrhizal OTUs, pale grey represents non-mycorrhizal OTUs and dark grey represent OTUs of unknown function.

3.3.3 Comparison of different types of sampling strategies to detect mycorrhizal taxa

A comparison of different sampling strategies to detect mycorrhizal taxa from *E. delegatensis* forest can be made by comparing the taxa detected from soil in this study, to the taxa detected by sampling mycorrhizal root tips and sporocarps from Chapter 4. Site E and Site S from this study were also used in Chapter 4 (Site E is in the same location as plots 5 and 6 and Site S is in the same location as plots 1 and 2) and thus a comparison can be made in terms of sample location. Sample effort is not equivalent as 20 soil cores for root tip sampling were collected from both Site E and Site S, where-as in this study, 15 soil cores were collected from each of the sites. Sporocarp surveys consisted of 29 person-hours per site over eight survey times (14.5 hours per plot).

Nine mycorrhizal OTUs detected in this soil study (from 45 soil cores) were also collected from *E. delegatensis* forest as root tips or sporocarps, which are reported in Chapter 4. These OTUS consisted of one Ascomycota OTU and the remainder were all Basidiomycota.

These OTUs were from one unknown and three known families; Clavulinaceae (1), Hydnangiaceae (2), Russulaceae (5), unknown (1). Five mycorrhizal OTUs detected in soils from Site E were also collected from the same site as sporocarps (Clavulinaceae sp. 1, *Laccaria* sp. 1, *Laccaria* sp. 5, *Russula* sp. 5, *Russula* sp. 8) and two OTU as root tips (*Russula* sp. 2 and *Laccaria* sp. 1). From Site S, *Laccaria* sp. 1 was sampled as both sporocarps and root tips, *Russula* sp. 9 was sampled as a sporocarp and *Zelleromyces* sp. 1 was sampled as root tips.

Some mycorrhizal taxonomic orders that were sampled from root tips from *E. delegatensis* forest in Chapter 4 were not sampled from either sporocarps, or soil in this study. These were the Boletales, Gomphales, Hysterangiales, Thelephorales and Pezizales. Only one order, the Corticiales was sampled solely as sporocarps and only one order, the Trechisporales, was sampled solely from soil. Three orders, the Agaricales, Cantharellales and Russulales were sampled by all three strategies (Table 3.4). Comparing the orders of mycorrhizal taxa that were recorded overall from root tip and sporocarp sampling from Chapter 4, and from soil in this chapter, soil recorded the lowest number of mycorrhizal fungus taxonomic orders, sporocarps recorded nine orders and root tip sampling recorded the greatest number of mycorrhizal orders (Table 3.4).

When comparing mycorrhizal OTU richness sampled from soil (in this study) or from root tips (from Chapter 4), difference between the two sampling strategies emerge. Overall, the number of mycorrhizal OTUs sampled per soil core for the same area was the same for both root tip and soil sampling (Table 3.5). When comparing sites directly, root tip sampling recorded a higher mycorrhizal OTU richness per soil core (Table 3.5).

Table 3.4 Comparison of ectomycorrhizal fungal orders sampled from *E. delegatensis* forest from soil, root tips and sporocarps. + indicates order was detected, - indicates the order was not detected.

Order	Soil	Root tips	Sporocarps
Agaricales	+	+	+
Boletales	-	+	+
Cantharellales	+	+	+
Corticiales	-	-	+
Elaphomycetales	-	+	+
Gomphales	-	+	-
Helotiales	+	+	+
Hysterangiales	-	-	+
Pezizales	-	+	+
Russulales	+	+	+
Sebacinales	+	+	-
Thelephorales	-	+	-
Trechisporales	+	-	-
Total	**6**	**10**	**9**

Table 3.5 A comparison of mycorrhizal OTU richness from *E. delegatensis* forest as sampled by soil (this chapter) or by root tips (using data from Chapter 4).

Site	Sample type	No. OTUs	No. Cores	Area sampled (ha)	Average per core (per 0.25 ha)
Overall	Soil	26	135	0.75	0.1
	Root tip	120	99	3	0.1
Site E	Soil	12	45	0.25	0.3
	Root tip	25	20	0.5	0.6
Site S	Soil	13	45	0.25	0.3
	Root tip	23	20	0.5	0.6

3.3.4 Species-area curves

The number of total fungal OTUs and mycorrhizal OTUs increased with the number of soil cores (volume of soil) sampled from each quadrat, and this trend was significant. Due to pooling of DNA from cores collected from the same quadrat, the final sample from each quadrat consisted of five soil cores and thus sampling effort increases in increments of five. The equation to describe the linear regression model for total fungal OTU richness = 2.3 (no. of soil cores) + 7.1, R^2=0.98 and p= <0.01, and for mycorrhizal OTU richness = 0.6 (no. soil cores) + 1.0, R^2 = 0.99 and p = <0.01.

For total fungal OTU richness and mycorrhizal richness, tripling the number of soil cores per plot from five to fifteen results in approximately double the number of fungal OTUs detected (Figure 3.7). The number of mycorrhizal OTUs increased at a slightly higher rate than the number of fungal OTUs so that tripling the sample effort resulted in 2.5 times the number of mycorrhizal OTUs detected (Figure 3.7).

The slope of the species-area curve is dependent on the order that quadrats are added, as the number of newly recorded OTUs varied for each quadrat size. To minimise this bias, OTUs from the larger quadrats were added to the cumulative richness before smaller quadrats.

Neither the mycorrhizal nor the total fungi species-area curves reached an asymptote indicating that ten soil cores for each site were not sufficient to completely sample the soil fungal or mycorrhizal communities present at any of the sites.

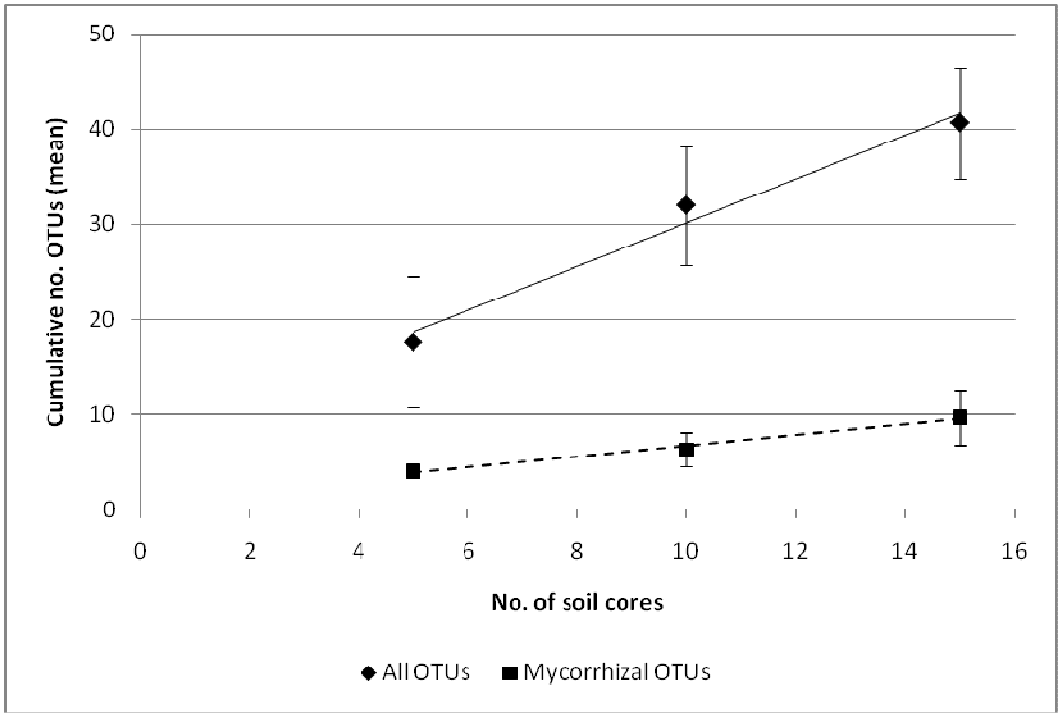

Figure 3.7 Species-area curves for the mean number of fungal OTUs and mycorrhizal OTUs sampled per five pooled soil cores, showing standard error as error bars. The first five pooled cores were sampled from the 50 m x 50 m quadrats, the second lot of soil cores were sampled from the 7 m x 7 m quadrats and the third lot of cores were sampled from the 1 m x 1 m quadrats.

3.3.5 Spatial distribution relationships

The mean number of fungal OTUs increased with quadrat size and variance also increased with quadrat size (Figure 3.8). Mean mycorrhizal OTU richness did not consistently increase with increasing quadrat size as the medium sized quadrat (7 m x 7 m) had the lowest mycorrhizal OTU richness, though some of the unknown OTUs may have been mycorrhizal (Figure 3.8). Due to this high variance and unequal variances of the groups, traditional ANOVA could not be used.

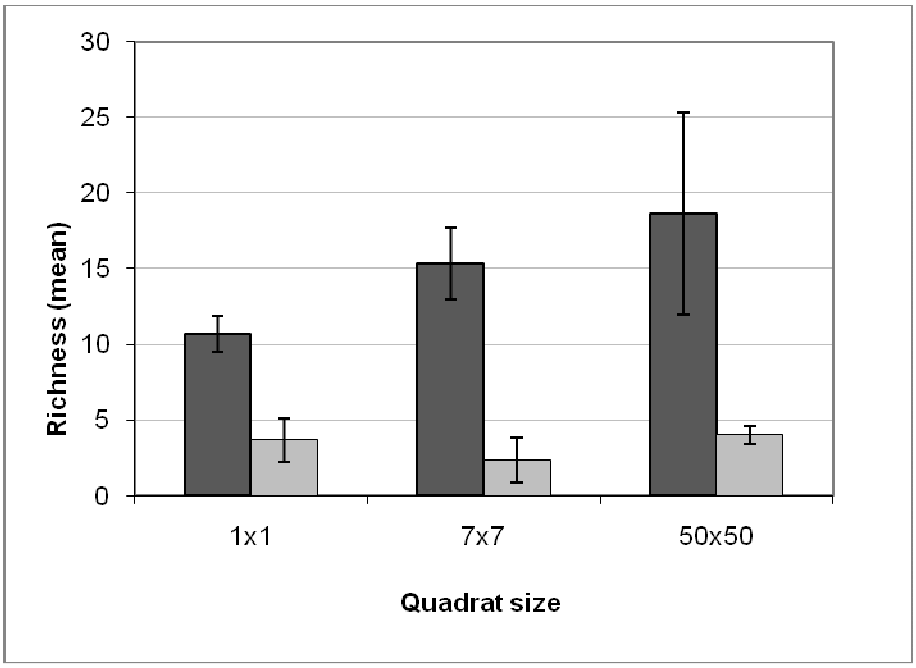

Figure 3.8 Mean total fungal OTU richness, and mycorrhizal OTU richness, for each of the quadrat sizes, showing standard error. Dark grey is total fungal OTU richness and light grey is mycorrhizal OTU richness. Mean richness increases with quadrat area for total fungal OTUs, but is inconsistent for mycorrhizal OTUs.

Bray-Curtis similarity measures among soil fungal community compositions (excluding singletons) of different quadrat sizes are shown in Appendix 11. A CAP analysis found no significant difference in species composition among the different quadrat sizes (Figure 3.9). Quadrats show some clustering on the basis of quadrat size to form three groups, which separate along the CAP1 axis, but groups do not separate along the CAP2 axis. The high misclassification error indicates that the model is not a good fit and does not explain the difference in fungal community composition among the three plots sizes well. The significance test and cross-validation results indicate that the fungal communities of the quadrat sizes are not distinct from one another. This analysis was repeated using only OTUs that were sampled in more than one of the quadrats (17 OTUs), with no significant difference detected for quadrat size (misclassification error was 56% and p =0.34) (figure not shown).

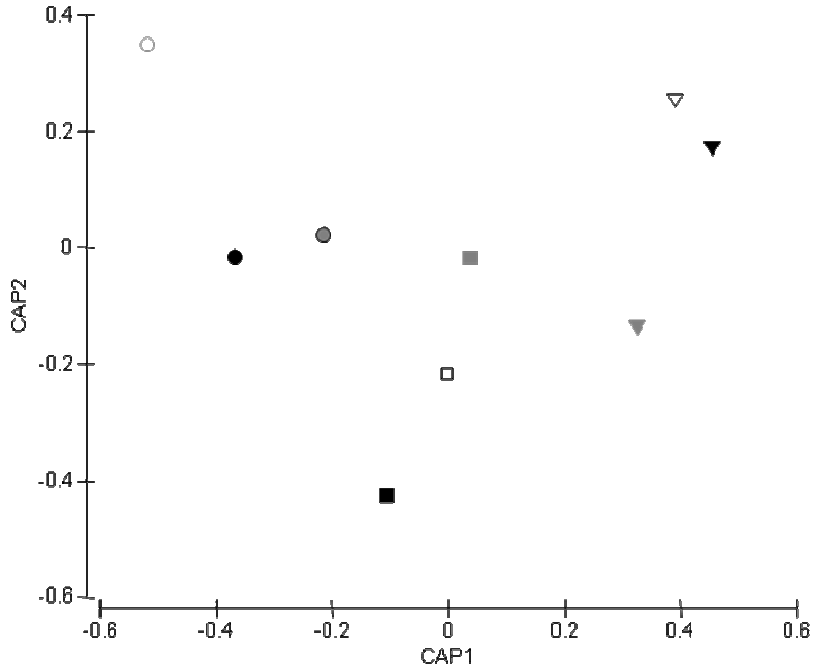

Figure 3.9 CAP analysis testing if there were differences in the fungal communities from each of the quadrat sizes. Misclassification error was 67%, p =0.25 for 9999 permutations. Individual markers represent the nine different quadrats, squares represent 1 m x1 m, triangles are 7 m x 7 m quadrats, and circles are 50 m x 50 m quadrats. Black solid markers are Site N, grey solid markers are Site S, and open markers are Site E.

3.4 Discussion

3.4.1 The fungal community of *E. delegatensis* forest detected by soil sampling

A total of 103 OTUs of soil fungi were recorded across ten orders and sixteen families. Like most fungal community studies conducted worldwide there was a large number of rare OTUs (Dahlberg 2001; Taylor 2002) and only few taxa were recorded from more than one quadrat or study site. Due to the large number of singletons and relatively few common taxa among the replicate quadrats from each site, the soil fungal assemblages did not significantly differ among the three *E. delegatensis* sites. The lack of statistical difference among the three different sites may be because of the three sites supporting similar fungal communities or it may be because of inadequate sampling as the majority of fungal OTUs

were only recorded in one of the sites, and thus the three sites were similar in that most species were unique to a single quadrat.

With such a large percentage of unknown OTUs it is difficult to determine the fungal community structure of this forest type, but representatives of many common saprotrophic and mycorrhizal fungal taxa were sampled. It is interesting that one functional group did not dominate the soil fungi community, with the percentage of mycorrhizal, non-mycorrhizal and unknown taxa, from the pooled samples not significantly different from one another. This in contrast to the northern hemisphere where most soil clones are identified as from ECM fungi. Even in Australia, soil has been found to be dominated by ECM fungi. Chen and Cairney (2002) found that mycorrhizal taxa dominated soil cores collected from eucalypt sclerophyll forest, except for one post-fire site where other soil fungi dominated. Dominance of a particular functional group in particular locations is expected as spatial separation of fungi with different resource use capabilities is known to occur (Dickie, Xu *et al.* 2002; Rosling, Landeweert *et al.* 2003; Lindahl, Ihrmark *et al.* 2007). Lindahl, Ihrmark *et al.* (2007) found that saprotrophic fungi were primarily found in the upper part of the litter on the surface of the forest floor while ECM fungi were found in the underlying decomposing litter and humus. Although spatial separation of functional groups within the 10 cm soil profiles collected in this study may exist, different soil horizons were not separated and examined as they were in the Lindahl, Ihrmark *et al.* (2007) study so functional separation within the cores could not be determined.

Soil fungal richness varies greatly across different forests and locations. In this study, the total taxonomic richness of three *E. delegatensis* forest sites was 103 OTUs from a total area of 1.25 ha and 45 soil cores. Another study of soil fungal diversity of eucalypt forest across three sclerophyll sites in Australia sampled 120 OTUs from 30 soil cores (Chen & Cairney 2002). Porter, Skillman *et al.* (2008) found 66 Agaricomycotina OTUs from 10 soils cores from a Hemlock forest. Lower observed richness was recorded in Scots Pine forest by Landeweert, Leeflang *et al.* (2005) who detected 14 OTUs from six soil cores from a single location, but per core, this species richness is similar to the species richness that was observed in this study.

The variation seen in soil fungal richness between studies may be because of true differences in richness of the different forest types sampled but also may be because of different methodologies employed. For example Chen and Cairney (2002) sampled 30 soil

cores and 0.5 g of soil, and Landeweert, Leeflang *et al.* (2005) sampled six cores and 0.5 g of soil, whereas this study sampled 45 soil cores and used 0.25 g of soil from each sample. Chen and Cairney (2002) employed four restriction enzymes whereas this study used only two, and they were different from the ones used by Chen and Cairney (2002). Furthermore, up to 48 clone colonies were selected from each pooled sample (5 soil cores from each quadrat size/location) in this study, with a minimum of 23 clones analysed, whereas Chen and Cairney (2002) selected 16 clones from each sample and samples were not pooled, so that more than double the number of clones were analysed by Chen and Cairney (2002) that were analysed in this study.

3.4.2 Species distribution and species-area relationships for soil fungi

The linear regression model between log mean fungal richness and log quadrat size showed no statistically significant relationship. The slope of the model was shallow suggesting that a large increase in quadrat size would be required to increase sampled fungal and mycorrhizal richness by even a small amount. For example to double the number of fungal OTUs sampled from five soil cores, plot size was increased 2500 times from $1m^2$ to $2500m^2$.

Other fungal studies have also detected a species-area relationship for fungi. A strong species-area relationship was found by Peay, Bruns *et al.* (2007) for ECM fungi sampled via root tips and sporocarps from different island sizes (R^2=0.74) and had a slope of 0.2. For red-listed fungal species throughout Finland the species-area slope ranged from 0.18 to 0.45 depending on forest locality (Tikkanen, Punttila *et al.* 2009). The slope of the species-area relationship found by Peay, Bruns *et al.* (2007)and Tikkanen, Punttila *et al.* (2009) was much higher that the slope of the species-area graph observed for all soil fungi in this study (0.05, Figure 3.7) and for mycorrhizal species-area (-0.05, Figure 3.7). The species-area slope for both the total fungal OTUs and mycorrhizal OTUs falls within those reported for microbial studies, which tend to have shallow, almost flat species-area curves: generally <0.1 (Green & Bohannan 2006).The relatively low slopes of microbial species-area curves may be because of insufficient sampling of rare taxa (Woodcock, Curtis *et al.* 2006).

In this study, many OTUs were not identified and were thus not assigned to a functional group. This may have resulted in many mycorrhizal OTUs not being included in the species-area curves calculated for mycorrhizal taxa, which would affect the species-area relationship for this group of fungi. Differences in fungal species-area relationships that are reported may also be because of different substrates and structures sampled (for example

mycelial abundance and distribution is highly temporally dynamic), different fungal groups studied, different taxonomic criteria and sampling efficiency.

The relationship between fungal OTU richness and spatial distribution was not significant suggesting that species richness is not necessarily higher for larger spatial areas that are sampled. The relatively small number of replicates (only three and no temporal replication), the high variance in OTU richness per quadrat area causing the relationship to be insignificant, and lack of asymptote in the species-area curve, makes it difficult to determine if the lack of a species-area relationship would be consistent across all studies and locations. Pooling the soil samples also restricted exploration of the species-area relationship as species richness estimates were unable to be obtained for each quadrat as effectively there was only one sample from each quadrat. The high variance in species richness observed for each plot area may be because of the patchiness of fungal taxa, the number of trees sampled around, or site specific differences, such as disturbance history, understorey vegetation, and tree age.

The species-area curves calculated for total fungal OTU richness and mycorrhizal richness (Figure 4.6) indicate that sampling was insufficient to effectively sample the fungi present in this forest soil (richness was still increasing with sampling effort at 15 soil cores), and may have resulted in inaccurate estimates of species diversity and composition (Taylor 2002; Anderson & Cairney 2007). Increasing the number of samples within a given area (increasing soil volume), rather than increasing the spatial spread from which the samples are taken, has a greater impact on the number of fungal OTUs that are recorded from *E. delegatensis* forest soil. For example doubling the number of fungal OTUs detected would require the spatial spread to be increased by a factor of 2500, or sample area to be tripled. It has been stressed by Anderson and Cairney (2007) who point out that sampling intensity and scale must both be considered when interpreting fungal community analyses and that there is a trade-off between the two, and Woodcock, Curtis *et al.* (2006) state that microbial community patterns will be strongly influenced by the sample size employed. Future studies should increase the number of soil cores (increase the sample area) collected so that the sampled species richness is closer to the true species richness. It is rare that an asymptote in fungal species-effort curves are reached (i.e. species richness ceases to increase with increasing sampling effort) (Taylor 2002). Peay, Bruns *et al.* (2007) found that sampling was adequate to capture the majority of ECM species richness of

different island sizes, which was evident through the flattening of species-area curves for each of their sampled islands, but their study differed substantially from the research here.

Although no statistical difference was detected in species composition among the different sized quadrats. This is possibly due to the high frequency of rare OTUs with only a few OTUs being represented in more than one replicate quadrat. The results suggest that soil fungal species have very small spatial patterns so that at different spatial scales species richness is similar, reflecting the small domains of most fungi with a small number of widespread fungi. Differences in community composition would be expected because of high variability and spatial turnover of fungal communities, leading to many rare and infrequently encountered taxa (Dahlberg 2001; Taylor 2002; Peay, Bruns *et al.* 2007). Thus any sized quadrat is likely to reveal a large number of OTUs that will not be sampled in other quadrats i.e. small quadrats sample just as many unique OTUs as large quadrats. Therefore sampling different sized quadrats does reveal differences in species composition, but this is masked by similarities in the percentage of singletons and unknowns.

More sampling is required to establish if there is an overlap of OTUs between the replicate quadrats and among the sites. Without further sampling, it is difficult to state if the three sites truly differ from one another in terms of soil fungal community composition. The slope of species-area curve was still increasing and therefore considerably more sampling is required to be able to describe the soil mycobiota. Also the increase in variance with increasing quadrat size suggests fine-scale structuring of the soil fungal communities.

The observed difference in species composition can be explained by the majority of fungi species present in only one quadrat indicating that at site level, the distribution of soil fungi is heterogeneous and dynamic with turnover of taxa in the forest occurring over small scales. Many ECM species are known to be patchily distributed (Tedersoo, Koljalg *et al.* 2003; Lilleskov, Bruns *et al.* 2004; Pickles, Genney *et al.* 2010). Lilleskov *et al.* (2004) found that dominant ECM fungi showed patchiness on a scale less than 3 m and recommend taking soil samples for forest stand root tip analyses every 3 m. Also, fungal mycelium has been shown to extend up to 3.3 m away from the sporocarp (van der Linde, Alexander *et al.* 2009). Tedersoo, Koljalg *et al.* (2003) found that the abundance of ECM fungi and root tips was highly variable on a 5 cm scale (Tedersoo, Koljalg *et al.* 2003) but patch size of individual ECM species and the location of these patches vary from year to year (Pickles, Genney *et al.* 2010). Considering that many of fungi were not ECM, the spatial scale of

these species is not surprising as many non-ECM species occupy small areas, and may reflect genets. It is difficult to know if this is the case for *E. delegatensis* forest as soil cores were pooled within each quadrat but because the observed species composition differed between the 1 m x 1 m quadrats and the 7 m x 7 m quadrats, it would appear that samples should be taken at small regular intervals (perhaps every 1 m), in order to detect the majority of fungal species present in *E. delegatensis* forest soil.

3.4.3 A comparison of sampling different fungal materials to study ectomycorrhizal fungi

Soil can be considered a useful tool to study mycorrhizal communities in this eucalypt forest. Almost one third of the OTUs recorded from the three study locations were mycorrhizal, and came from a variety of commonly found orders, families and genera. Most of the commonly encountered ectomycorrhizal orders from eucalypt forest were sampled from soil in this study (Appendix 9). Although mycorrhizal taxa were sampled from soil, there were differences in taxonomic orders sampled from soil compared to root tips, or sporocarps sampled from *E. delegatensis* forest in Chapter 4.

There is very little overlap at the OTU level between mycorrhizal taxa detected in soil compared to taxa identified from root tips or sporocarps in *E. delegatensis* forest. Also mycorrhizal OTU richness differed depending on the sampling strategy employed. Although the data from Chapter 4 is not directly comparable to this chapter because of differences in sampling strategies, it does provide some insight into the benefits of sampling different fungal structures. OTU richness was higher when sampled by root tips than when sampled from soil in this chapter. Only eight OTUs that were sampled from soil in this chapter were also sampled from root tips or sporocarps in Chapter 4. Northern hemisphere studies have found similar results with little overlap between taxa sampled in soil and either root tips or sporocarps indicating that different substrates give complementary views of the mycorrhizal community and total fungal community (Taylor & Bruns 1999). Landeweert, Leeflang *et al.* (2005) compared fungal taxa found on root tips to those detected in DNA extracted from soil and found that 11 of the 14 taxa detected in soil, were found on root tips, with 7 of these known ECM taxa. A similar result was obtained by Landeweert, Leeflang *et al.* (2003) where, of 25 OTUs detected from soil, 10 of these were also found on root tips from the same site, with 7 of these identified as known ECM taxa. Porter, Skillman *et al.* (2008) studied the overlap of Agaricomycotina OTUs (all functional groups) sampled

from soil and sporocarps and found only 13 OTUs were sampled from both soil and fruit bodies, out of 66 OTUs sampled from soil, and 132 OTUs sampled from sporocarps. Furthermore, the distribution of sporocarps has very little relationship to the below ground distribution of root tips and mycelial abundance (Genney, Anderson *et al.* 2006; van der Linde, Alexander *et al.* 2009) and so different strategies do not necessarily reveal similar information on spatial relationships.

The difference among soil, root tip and sporocarp diversity may be because many mycorrhizal taxa do not reproduce sexually and for those that do produce sporocarps, fruiting may be unpredictable (Bougher & Tommerup 1996; Tommerup & Bougher 1999; O'Brien, Parrent *et al.* 2005). Also root tip and sporocarp sampling focused only on detecting ECM OTUs whereas soil detected any mycorrhizal OTUs (i.e. species that form any type of mycorrhizal associations not just ectomycorrhizas). Furthermore the different strategies compared employ different techniques for sampling and different sampling effort, which may confound the comparisons. It is possible that different substrates give complementary views of the community because of insufficient sampling effort resulting in incomplete characterisation of the fungal community. Species detection is known to increase with sampling effort (Taylor 2002) and so if sampling effort was increased, fungal communities sampled from soil, sporocarps, or root tips, may be found to share more similarities.

The best substrate to detect ECM fungi will therefore depend on the aims of the study. If the aim is to get a quick idea of what ECM taxa may be present in a community then soil would be suitable as soil efficient to collect and process and relatively easy to analyse but gives lower mycorrhizal richness for the same sampling area and spatial distribution than sampling root tips. If detailed information on the ECM community composition is required, all three strategies, soil, root tips and sporocarp sampling would be needed to characterise the community as each strategy gives complementary information. If estimates of ECM species richness are required then root tip sampling would be best as it is less dependent on time of sampling as sporocarp surveys and gives a greater estimate of richness than soil. If the aim is to identify which species are forming ectomycorrhizas and functioning in nutrient transfer within the ecosystem then again, root tips should be sampled as neither soil or sporocarps can provide this information. On the other hand, if an understanding of the spatial distribution of fungi is of interest, then sampling soil is suitable as the

distribution of sporocarps and root tips does not indicate the extent of fungal genets within the ecosystem.

3.4.4 Critical examination of the use of soil for the study of fungal communities

Despite the proven usefulness of soil as a means to sample mycorrhizal fungi there are drawbacks. These include amplification of dormant spores and inactive DNA from dead mycelium or other fungal structures, which reduces community interpretation to presence of species without revealing functionality (Bridge & Spooner 2001; Avis, Dickie *et al.* 2006; Anderson & Cairney 2007).

Both sporocarp and root tip surveys require a significant amount of processing time. Finding quick, less laborious methods to study fungal communities is desirable and using soil DNA is a potential way of achieving this. For root tip sampling, removing and examining morphotypes (which requires expertise), and subsequent DNA extraction are the most time consuming steps, whereas removing a soil sample from a core and DNA extraction from soil using a kit are quick. The most time consuming steps for soil cloning are growing clone colonies and performing and analysing RFLP digests. Monetary constraints also restrict the application analysing DNA extracted directly from soil as a tool to study fungal communities as cloning can be very costly. With greater resources (time and money) every clone arising from each sample could potentially be analysed allowing more complete characterisation of the fungal community. Also, many samples are required to sufficiently sample the soil fungal community, and so in this way, soil is no more efficient than sampling root tips or sporocarps, which also require large sampling efforts. The efficacy of bulk soil sampling also comes from pooling, which could also be performed on root tip samples and has been used in other studies such as Landeweert, Leeflang *et al.* (2005). Also pooling sporocarps and amplifying using Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE) could also be feasible. Pooling would reduce processing time and costs when any fungal sample is employed. Although analysis of soil DNA is relatively efficient and does not rely on expert knowledge of mycorrhizal root tips, it cannot be used as a replacement for root tip studies. As mentioned above, the soil community does sample mycorrhizal taxa, but these tend to be different taxa, which complement information obtained from other structures. Similar conclusions were made by Porter, Skillman *et al.* (2008) who found that soil was complementary to sporocarp surveys.

A number of methodological biases, such as DNA extraction techniques, may have also influenced the results from this study (see Anderson & Cairney 2004 for review). This study extracted the DNA from a 0.25 g soil sub-sample which was taken from a well mixed larger sample. The size of the soil sample has been shown to influence perceived community structure with small samples detecting minor populations, which mask dominant populations (Ranjard, Lejon *et al.* 2003). For fungi, soil samples < 1 g showed changes in community structure with sample size (Ranjard, Lejon *et al.* 2003). Ranjard, Lejon *et al.* (2003) suggest large soil samples should be used for robust global descriptions of the soil community while many small samples should be used for a more complete inventory. Another bias is the number of clone colonies selected for PCR-RFLP, with highly diverse communities requiring the analysis of a large number of clones (Anderson & Cairney 2004). O'Brien, Parrent *et al.* (2005) found that fungal OTU richness increased with the number of clones sequenced and that even when a large number of clones are sequenced, species-effort curves may not reach saturation.

Another drawback when using restriction enzyme based profiling techniques for mycorrhizal community studies is that many mycorrhizal taxa have multiple RFLP profiles, and a number of taxa have been reported to have the same RFLP profiles (Kåren, Hogberg *et al.* 1997; Glen, Tommerup *et al.* 2001b; Bougoure & Cairney 2005). This can result in either over or under estimation of richness but RFLP is generally thought to underestimate true species richness (Avis, Dickie *et al.* 2006). In this study, PCR-RFLP profiles both overestimated richness (in three pooled samples) and underestimated richness (five pooled samples), when compared to estimates obtained from sequencing. Only a small number of OTUs had multiple PCR-RFLP profiles in this study and three of these belonged to two ECM families, the Cortinariaceae and Russulaceae. Mis-estimation of the richness from the RFLP profiles of these taxa may skew ECM community studies, especially in Australian eucalypt forest where the Cortinariaceae and Russulaceae make up a large proportion of the fungal biota and tend to dominate these ECM communities(Bougher & Malajczuk 1986; Malajczuk, Dell *et al.* 1987; Bougher 1995; Chambers, Sawyer *et al.* 1999). One way of minimising mis-estimation of species richness because of ITS polymorphism revealed by RFLP profiles is to increase the number of restriction enzymes that are used, which increases the chances of getting unique profiles for each OTU and thus improving confidence that the species richness is accurate. Being selective with the restriction enzymes used for RFLP analysis may also help to give more accurate measures of species richness and evenness (Alvarado &

Manjon 2009). Alternatively, DNA sequencing also allows a more accurate estimate of species richness as each sample is identified independently of one another.

Another aspect of this study, which influenced the effectiveness of detecting soil fungi, was the pooling of soil samples within each quadrat, which is especially important when coupled with the small amount of soil sub-sampled for DNA extraction. It is possible that the pooling of the PCR products within each quadrat reduced the number of OTUs detected, as more clones would have been screened if samples had not been pooled. If samples had not been pooled, there would have been replicate soil samples from each quadrat allowing the calculation of species richness estimates. These estimates are often used as observed species richness is thought to less accurately represent true species richness than estimated richness (Colwell & Coddington 1994). Also, species-area and species-effort curves would have had a finer resolution as sampling effort could be calculated in increments of one soil core, rather than five, and spatial heterogeneity of fungal species could have been established at a finer scale (i.e. within each quadrat as well as between each quadrat size). The application of new sequencing technologies are likely to supersede the need for cloning to separate DNA templates from different fungal species (Jumpponen, Jones *et al.* 2010), thus reducing the workload. While Jumpponen, Jones *et al.* (2010) used 454 sequencing to analyse DNA extracted and amplified from root tips, this technique could also be used with DNA extracted directly from soil.

Also, the nested plot design may have confounded results as quadrats were not independent of one another so that species richness of the smaller quadrats affected the species richness of the larger quadrats (Stohlgren 2007). Furthermore quadrats may have been located in species-poor areas of the site, which may confound results if species are patchily distributed (Stohlgren 2007). Results can also be biased by the starting location of the quadrats, but in this case, the smaller quadrats were randomly located within the successively larger quadrats in an attempt to minimise this bias (Stohlgren 2007).

A repeat of this study using a greater number of replicate sites, which would increase the number of replicates of each quadrat size, as well as using more soil for DNA extraction, using single DNA extracts instead of pooled extracts (and compare to pooled DNA results) and analysing more clones or utilising more advanced sequencing technologies would improve the detection of soil fungi and further our understanding of soil fungi and their area/habitat relationships.

The large proportion of taxa (approximately one quarter in this study) that are not identified to phyla or higher taxonomic levels is a further disadvantage of relying on environmental samples for fungal community studies. This problem has been encountered in a number of other studies (Landeweert, Leeflang *et al.* 2003; Landeweert, Leeflang *et al.* 2005; Bastias, Huang *et al.* 2006; Ryberg, Kristiansson *et al.* 2009). Bastias, Huang *et al.* (2006) describe two types of unknown fungi, which were encountered in this study; those that match uncultured or unidentified sequences, and those that do not match any known sequences and may represent new species. The large proportion of unknown OTUs in this study further highlights the limited understanding of soil fungi within these forest ecosystems. Unknown taxa are not a problem when studying shifts in community patterns over time, but when focusing on one part of the fungal community, for example the mycorrhizal community, interpretation is limited as many of the unknown OTUs may belong to the functional group of interest but would not be included in analyses and thus information is lost from the study.

3.4.6 Summary of the use of soil for the study of mycorrhizal communities

The appropriateness of different sampling strategies to study mycorrhizal communities depends on a number of factors. Firstly, sampling strategy will depend on the scientific question that is posed (see section 3.4.2 of this chapter), but available expertise, person-hours, and cost must be taken into account. Table 3.6 summarises each sampling strategy in terms of these different requirements and resources and gives examples of studies where each sampling strategy is appropriate. In some cases, it may be appropriate to use multiple sampling strategies such as when a more complete characterisation of the community is required, or when species need to be identified to determine functional attributes (i.e. unknown root tip samples need to be identified and matched to identified sporocarp samples through profiling techniques or DNA sequencing). Techniques such as microarray analysis, which can provide genetic information on the presence of different functional genes may also be appropriate following identification of samples (Berthrong, Schadt *et al.* 2009; Reich, Kohler *et al.* 2009). Other recent technologies such as 454-DNA sequencing may also provide a means for complete characterisation of soil mycorrhizal communities as many more samples can be analysed and sequenced efficiently (Buee, Reich *et al.* 2009; Öpik, Metsis *et al.* 2009), although this technique is currently very expensive.

Table 3.6 A comparison of three different sampling strategies for the study of mycorrhizal communities of *E. delegatensis* forest.

	Soil	**Root tips**	**Sporocarps**
Sampling time	any time, but best when hyphae and tree active	any time although best when tree is actively growing	only when species are fruiting
Expertise required	moderate	high	high for morphological ID, moderate for molecular
Cost	high	low for morphological grouping, high for molecular ID	low for morphological ID, high for molecular ID
Sample collection man power/time	low	low	high
Sample processing man power/time	low	high	high
Number of mycorrhizal orders sampled	10	11	8
Identification method	molecular - profiling, sequencing or both	Morphological, molecular-profiling, sequencing or both	morphological or molecular- profiling, sequencing or both
Information obtained	sub-sample community richness and composition, spatial distribution of mycelia	community richness and composition, influence of environment/disturbance on community and species, species function	taxonomy, reproduction, community, richness and composition
Example studies (of when the strategy is appropriate)	Spatial distribution of mycelia (van der Linde, Alexander *et al.* 2009). Comparison of community patterns (Chen & Cairney 2002).	Community composition changes with environmental variables (function) (Lilleskov, Fahey *et al.* 2002) and (Twieg, Durall *et al.* 2009).	New species description (Gates, Horton *et al.* 2009). Community characterisation using root tips and sporocarps (Gardes & Bruns 1996).

3.5 Conclusions

The extraction, amplification and cloning of DNA from *E. delegatensis* forest soils detected a variety of mycorrhizal taxa from a range of common mycorrhizal genera, families and orders. Soil samples are best used in combination with additional substrates to fully understand mycorrhizal community ecology as the different sampling strategies compared in this study (soil, root tips and sporocarps) provide complementary information about the mycorrhizal community of *E. delegatensis* forest.

Analysis of DNA extracted from soil suffers from bias in regards to soil sample size and number of clones screened, but is quick and requires generic technical skills, rather than specialist skills. This approach would be best employed for studies of ECM fungal community patterns rather than characterisation of an ECM community. This is because OTU identification, and functional group allocation, is limited because of the presence of many new species in soil and a limited understanding of soil fungal taxonomy.

Increasing the number of samples within a site (increasing the sample area), rather than increasing the spatial area over which the samples are collected, is likely to be a more efficient way of getting a more accurate estimate of species richness. This is because detected species richness increases more sharply with increases in sample area compared to increases in spatial distribution. Also, increasing the spatial distribution of the samples (sampling from a larger quadrat) may have crossed over a soil-fungal boundary confounding alpha and beta diversity. With only a limited number of replicates and minimal statistical analysis used in this study, there are no conclusive results and these hypotheses would require further testing.

Due to the high spatial heterogeneity of soil fungi, and large numbers of rare fungal species, sampling soil fungi from a larger area compared to a small area detects a different soil fungal community composition (the presence of different OTUs within each of the quadrat sizes). Few samples from a large area inadequately characterise the community. Hence comparisons of community composition are difficult because of lack of statistical support. Detected differences may arise because of stochastic reasons rather than true differences. If the aim is to compare assemblages among sites it may be most efficient to have a design that samples from several small sub-plots within a plot, leading to better detection of community similarity within a plot, and more distinct differences to sub-plots within other plots.

4.0 Ectomycorrhizal fungal community diversity of *E. delegatensis* forest

4.1 Introduction

Ectomycorrhizal (ECM) fungi form symbiotic relationships with many woody plants including trees of the genus *Eucalyptus*, which dominate much of Australia's forests and woodlands and are an important wood resource. Other members of the Myrtaceae such as *Leptospermum* Forst. are also known to be ECM, as are many other Australian woody plants including *Acacia* Mill.*, Casuarina* L., *Pomaderris* Labill. and *Nothofagus* (Chilvers & Pryor 1965; Ashton 1976; Warcup 1980).

Australian ECM fungi have an estimated diversity of 6500 species (Bougher 1995; Neale Bougher pers. comm. 2009) of the 7000 - 25000 species of ECM fungi that are thought to occur globally (Read & Perez-Moreno 2003; Taylor & Alexander 2005; Rinaldi, Comandini *et al.* 2008). A significant portion of Australia's ECM diversity is made up of hypogeous ECM fungi, which in 2001 were considered numbering up to 2450 species (Bougher & Lebel 2001).

Australia has a large proportion of endemic ECM fungi, including over 22 genera and three families that are unique to the continent (Castellano & Bougher 1994; Bougher 1995; Bougher & Lebel 2001). Australia also has many ECM taxonomic groups that are cosmopolitan, such as *Clavulina* J. Schröt., *Cortinarius*, *Laccaria*, *Lactarius*, *Russula* and *Tomentella* Pers. ex Pat. (Dickie & Moyersoen 2008). Some of these globally distributed genera, such as *Laccaria*, *Dermocybe* (Fr.) Wünsche and *Cortinarius,* contain Australian endemic species (May & Simpson 1997). The high level of endemism of Australian ECM fungi may be due to Australia's long evolutionary isolation, which not only resulted in evolutionary divergence and speciation, but has restricted the distribution of species with high host specificity to the range of their endemic hosts (Bougher, Fuhrer *et al.* 1994; Bougher 1995; Halling 2001). The high endemism and diversity of

4.0 Ectomycorrhizal diversity

Australian ECM fungi make a considerable contribution not only to Australia's biodiversity, but also global biodiversity.

ECM fungi perform a number of essential ecosystems functions such as soil stabilisation, water uptake and storage, heavy metal tolerance, herbivore and pathogen resistance, food for mycophagous mammals and influence plant community diversity (Perrin 1990; Gehring & Whitham 1994; Cairney & Chambers 1999; Tommerup & Bougher 1999; Claridge 2002; Simard & Durall 2004; Swaty, Deckert *et al.* 2004; Müller, Avolio *et al.* 2007; Plamboeck, Dawson *et al.* 2007; Tang, Zhang *et al.* 2008; Tripathi, Kamal *et al.* 2008; Claridge, Trappe *et al.* 2009; Luo, Li *et al.* 2009; van der Heijden & Horton 2009; van der Putten, Bardgett *et al.* 2009; Courty, Buée *et al.* 2010). Important among these functions is the uptake and transfer of nutrients through the ecosystem. ECM associations are particularly significant for phosphorus and nitrogen plant nutrition in impoverished soils, which are typical of much of Australia.

The significance of ECM fungal biodiversity, and the involvement of ECM fungi in ecosystem processes, which are imperative to the healthy functioning of forest ecosystems, makes this group of organisms particularly important. There is still much to be known about what processes shape ECM communities and what determines the diversity of these communities. Fungal host preference (Tedersoo, Jairus *et al.* 2008), and theories such as ecological niche and succession, along with ecological interactions, such as competition, and stochastic processes are thought to play a role in determining ECM diversity (Frankland 1992; Bruns 1995; Dickie 2007; Peay, Kennedy *et al.* 2008b). Compared to countries of the northern hemisphere, the number of ECM field studies conducted in Australia is proportionally low. However a number of studies have characterised the diversity of ECM communities of various Australian forest types (Malajczuk & Hingston 1981; Reddell, Gordon *et al.* 1999; Adams, Reddell *et al.* 2006; Midgley, Saleeba *et al.* 2007). Disturbances, such as fire (Malajczuk & Hingston 1981; Warcup 1990; Launonen, Ashton *et al.* 1999; Chen & Cairney 2002; Bastias, Huang *et al.* 2006; Robinson, Mellican *et al.* 2008), mining (Reddell, Gordon *et al.* 1999; Glen, Bougher *et al.* 2008) and forestry (Warcup 1991), have been shown to strongly influence the diversity of Australian ECM communities.

Most Australian ECM field studies have focused solely on either sporocarp or root tip surveys. It is now widely accepted that sampling the ECM community by either sporocarps or root tips

reveals different elements of the ECM community (Gardes & Bruns 1996; Dahlberg, Jonsson *et al.* 1997; Peter, Ayer *et al.* 2001a). For example, Gardes and Bruns (1996) found that some species of ECM fungi were sampled as both sporocarps and root tips, while others, such as *Suillus pungens* Thiers & A.H.Sm., were rarely sampled as root tips, and some species such as *Tomentella* and other Thelephoroid fungi, were only sampled as root tips. Below-ground abundance of ECM fungi as established by root tip analysis showed that at least half of the species did not produce conspicuous epigeous sporocarps (Dahlberg, Jonsson *et al.* 1997). Glen, Bougher *et al.* (2008) found little overlap between ECM fungal species collected as sporocarps and those identified on root tips from *Eucalyptus marginata* forest in Western Australia. The complementary data provided by collection of sporocarps and root tips provides a more comprehensive understanding of ECM community structure.

Many Australian ECM fungi are difficult to identify by morphology and do not fit taxonomic classifications based on the morphology of northern hemisphere fungi (Bougher 1995). Also, many ECM fungi share very similar sporocarp and root tip morphological characteristics making them difficult to differentiate. These challenges, along with the shortage of expert Australian mycologists, and limited teaching of mycology in Australia, have impeded studies of Australian ECM fungi. DNA analysis provides a means by which fungi can be identified independently of morphology and life-stage. DNA analysis is often less laborious than other techniques and is now widely employed for the study of ECM fungi (Horton & Bruns 2001; Peter, Ayer *et al.* 2001a; Glen, Bougher *et al.* 2008; Tedersoo, Jairus *et al.* 2008; Dickie 2009). Like morphological identification techniques, there are both advantages and disadvantages of utilising DNA analyses (see Chapter 1). The integration of molecular and morphological characterisation provides an efficient and powerful approach to discriminating species and determining relationships. There has been a marked increase in the study of native ECM communities in Australia since the development of molecular tools (see Glen, Tommerup *et al.* 2001b; Bastias, Huang *et al.* 2006; Bastias, Anderson *et al.* 2007; Midgley, Saleeba *et al.* 2007; Glen, Bougher *et al.* 2008; Tedersoo, Jairus *et al.* 2008).

Like most organisms, ECM fungi are threatened by habitat degradation and a changing climate (Tommerup & Bougher 1999; Staddon, Heinemeyer *et al.* 2002). The maintenance or loss of diversity of ECM species and their essential functions can have far reaching impacts beyond those on their immediate ecosystem. The involvement of ECM fungi in carbon storage and

release may have implications for understanding and mitigating climate change (Treseder & Allen 2000; Talbot, Allison *et al.* 2008; Paterson, Midwood *et al.* 2009), and ECM fungi may also aid the survival and adaption of their hosts under a changed climate (Perry, Borchers *et al.* 1990; Lynch & St. Clair 2004). An understanding of ECM fungal diversity, the determinants of diversity and the impact of disturbance on these communities will aid in their conservation and management, ultimately improving the management and conservation of native forests.

The above body of research and ideas leads to a number of questions:

- How diverse are the ECM communities of *E. delegatensis* forest?
- How does the richness of *E. delegatensis* ECM communities compare to other eucalypt forests and temperate forests in the northern hemisphere?
- Are the common ECM taxonomic groups found in *E. delegatensis* forest?
- What is the most species-rich taxonomic group of ECM in *E. delegatensis* forest?
- Are there common and rare ECM species?
- Does the ECM diversity differ between of *E. delegatensis* forest
    - differing in vascular plant species richness, or richness of host species?
    - that was frequently burnt and long unburnt?
    - sampled by sporocarp or root tips?
    - sampled at different times of the year? and
- Does DNA sequence analysis enable ECM diversity to be adequately measured?

The aim of this chapter is to address the above questions by assessing ECM and plant species' diversity in Tasmanian *Eucalyptus delegatensis* forest with differing disturbance histories. Sporocarp and root tip ECM community composition and structure were investigated through morphological characterisation and PCR (Polymerase Chain Reaction) amplification and sequencing of the rDNA ITS (ribosomal DNA Internal Transcribed Spacer) region.

4.2 Methods

4.2.1 Study sites

Twelve 0.25 ha plots with buffers were established in *E. delegatensis* forest in northern Tasmania. Plots were established as pairs that were adjacent (within 40 – 100 m of one another) within the same vegetation type and exposed to the same management history. Plots contained eucalypts ranging in health from severely declining to healthy (Appendices 2 and 14). In Australia, N in forest and cultivated soil are > 0.2%, whereas rangelands tend to be < 0.1%. Phosphorus content of most Australian soils is generally low (0.02%) but varies according to parent material. Basalt areas will have higher levels of soil P (so north-west Tasmanian sites have naturally higher soil P, > 0.05%, whereas the northeast will have lower soil P < 0.02%).

A schematic diagram of the experimental design is shown in Figure 4.1. Site and plot locations are shown in (Appendix 15).

Eight plots were established at two sites along Ben Ridge Road, near Mt Maurice in the highlands of north-east Tasmania, 60 km east northeast of Launceston (41.35 °S, 147.67 °E and 41.37 °S, 147.61 °E). Sites were between 850 m and 900 m asl, mean annual rainfall is 1320 mm and mean annual temperature is 9.1 °C (ESOCLIM module of ANUCLIM 5.2 Houlder, Hutchinson *et al.* 2000). Soils were brown dermasols derived from Devonian granodiorite (Grant, Laffan *et al.* 1994). Organic matter content of surface soil is normally high and medium in subsoil. Total phosphorus is high throughout the soil profile and nitrogen is medium in the surface soil but low in subsoil. Productivity is generally high but drops at high altitudes and the potential for nutrient depletion is moderate (Grant, Laffan *et al.* 1994).

The area in which the plots are situated was dedicated as State Forest (for the purpose of commercial wood production) in parcels from 1948 to 1960 and prior to this was unallocated crown land. There is no record of timber harvesting in the plots although it is likely that some partial harvesting has occurred in the adjacent areas since European settlement. The eight plots were selected on the basis that they had been used in earlier research of eucalypt dieback (Ellis, Mount *et al.* 1980) and had a range of understorey types, levels of eucalypt crown decline, and a known recent fire history.

Site 1, containing plots 1 to 4 (41.35 °S, 147.67 °E), was dominated by *E. delegatensis* with a rainforest understorey and fern ground cover. These plots were situated on a moderately well drained low ridge with wetland on two sides and gentle slopes to creeks on the other sides. Plots 1 and 2 were last burnt by a uniform high intensity fire in 1964 as part of the study conducted by Ellis, Mount *et al.* (1980) and prior to that by a low intensity fire c. 1880. Plots 3 and 4 were last burnt c. 1884, probably by a low intensity fire (Ellis *et al.* 1980). Plots 1 and 2 both contained more than 10 live eucalypts but plots 3 and 4 contained only six and nine live eucalypts, respectively.

Six km east of plots 1 to 4, along Ben Ridge road, plots 5 to 8 (41.37 °S, 147.61 °E) were established on a broad, well drained ridge-top with a gentle slope of south east aspect. This region had been part of a grazing lease until 1935 and been burnt relatively frequently prior to this date (Ellis, Mount *et al.* 1980). Plots 5 and 6 were burnt in 1963 and then again in 1967 by low intensity fires for a study conducted by Ellis, Mount *et al.* (1980). This site was part of an informal reserve dedicated c. 2005 under a Tasmanian Community Forest Agreement. The plots were dominated by *E. delegatensis* with a patchy mid-storey of *Acacia* and dry sclerophyll understorey with small grass patches. Plots 5 – 7 all contained greater than 10 individual eucalypt trees.

Site 2, containing four plots (9-12), was located one hundred and thirty km south-west of site one in northern Tasmania. Plots 9 to 12 were established along Gad's Hill Road in Mersey State Forest, 81 km west southwest of Launceston, Tasmania (41.58 °S, 146.17 °E). The region had been grazed by cattle since the 1940's and was dedicated as State Forest at an unknown time. Plots were at an altitude of 800 – 900 m asl with a gentle slope of 0 – 5°. Estimates of 1564 mm for mean annual rainfall and 9.3° C for mean annual temperature were based on long-term records from a nearby weather station at Lorinna (ESOCLIM module of ANUCLIM 5.2 Houlder, Hutchinson *et al.* 2000). Soils were dark brown ferasols derived from Tertiary Basalt (Grant, Laffan *et al.* 1994). These soils are generally high in surface organic matter, high in phosphorus throughout, and have moderate levels of nitrogen in surface soil and low levels in subsoil (Grant, Laffan *et al.* 1994). Soils are highly productive and resistant to nutrient depletion (Grant, Laffan *et al.* 1994).

Plots 9-12 were partially harvested in the early 1980's. These plots were selected on the basis of similarity to the north-east plots in terms of vegetation composition and structure. Plots 9 and 10 were dominated by alpine *E. delegatensis* with a rainforest mid-storey dominated by *N. cunninghamii* and sparse understorey of mixed rainforest and sclerophyll plants, with ferns. Plots 11 and 12 were co-dominated by *E. delegatensis* and *E. dalrympleana* and had a thick dry sclerophyll understorey with a ground layer of ferns. Plots 9, 11 and 12 all had greater than 10 eucalypt trees within the specified area, but plot 10 only had five individual eucalypt trees.

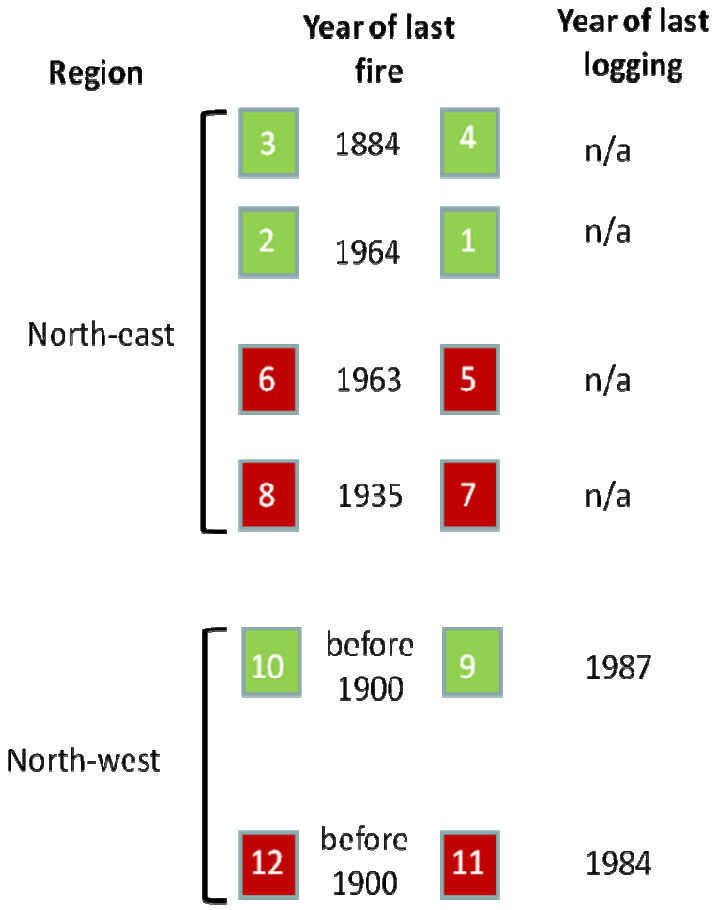

Figure 4.1 Schematic diagram showing the experimental design of the study. A total of twelve plots were established. Half of the plots had a rainforest understorey (green) and half had a sclerophyll understorey (red). Eight of the plots were located in north-eastern Tasmania and four of the plots were located in north-western Tasmania. Year of last fire and logging even for each of the paired control and treatment plots is shown in the centre of the diagram. n/a shows the plots have no record of being logged in recent history (last 100 years). Plot numbers are shown in the centre of the coloured squares.

4.2.2 Vegetation surveys

Vegetation surveys were conducted in November 2007 by scoring vascular plant species cover within four randomly located 5 m x 5 m quadrats within each of the study plots. Plant species cover was assessed using the Domin scale for all species present in the quadrat. Domin categories were converted to means for analysis. Domin cover categories (means in brackets) were: <1 (0.5), 1 - 4 (2.5), 5 - 9 (7), 10 - 24 (17), 25 - 32 (28.5), 33 - 49 (41), 50 - 74 (62), 75 - 94 (84.5), 95 -100 (97.5). Plant species richness for a plot was defined as the total number of plant species from all four quadrats surveyed within the plot. Plant species recognised as ECM hosts followed those of the Australian National Botanic Gardens records (http://mycorrhizas.info/ozplants.html).

4.2.3 Root tip sampling

Ten soil cores (5 cm x 5 cm x 10 cm deep) were collected from each of the 12 plots. Two soil cores from each plot were collected in July 2007, five in November 2007 and three in September/October 2008. Soil cores were collected throughout the year to ensure that root tips were sampled over different stages of the growing season. More intensive sampling took place in spring as ECM root tips are potentially more abundant following the resumption of root and shoot growth (Ashton 1975; Ashton 1976; Malajczuk & Hingston 1981). The unequal number of cores collected during the three sample times also reflects the resources available to process the soil cores at each sample time. Each soil core was collected from within the canopy drip zone of the *E. delegatensis* tree (greater than 90 cm DBH) nearest to a randomly generated co-ordinate. Occasionally this sampling technique resulted in more than one soil core collected from an individual tree, especially when plots contained five or fewer trees. Soil cores were stored at 4 °C until processed within four weeks of collection (Glen, Bougher *et al.* 2008). The tree from which each soil core was taken was labelled with a unique tree tag. Sampling from a tree did not affect any future sampling i.e. it was possible to collect multiple samples from around the same tree.

The soil from a single core was shaken through a series of graded sieves (500 µm, 1000 µm, and 2360 µm). Fine roots were cleaned and examined under a Zeiss semi 2000-C dissecting microscope with Zeiss KL500 electronic light. Each core was searched for 30 minutes for

mycorrhizal roots. All mycorrhizal root tips found within each soil core within the search time were grouped into morphotypes based on appearance such as colour, size, branching, mantle surface, emanating hyphae and rhizomorphs (Agerer 1986; Agerer 1991). Dark tips lacking turgidity were assumed dead. A sample (1-5 single root tips or a pyramidal, monopodial branching cluster of 3-5 root tips) of each morphotype was photographed using a Canon Powershot S40 digital camera and briefly described before being frozen at -80 °C prior to DNA analyses.

4.2.4 Sporocarp sampling

Sporocarp surveys were conducted on ten occasions in March, May, June, November and December of 2007; and February, March, May and June of 2008. Surveys were concentrated throughout autumn when conditions of higher rainfall and lower temperatures were established following summer, conditions often associated with sporocarp production (Johnson 1994; Bell & Adams 2004). A total of 15.5 person-hours were spent surveying each plot, split evenly between surveying for epigeous and hypogeous sporocarps. Survey times were not equal for each survey with less time spent surveying outside of the autumn/winter months (i.e. November and February) because of the lower abundance of sporocarps, and more intensive sampling during 2007 than 2008 as more resources were available for surveying and processing (10 person-hours per plot during 2007 and 5.5 person-hours per plots for 2008). Most surveys were conducted by two surveyors although the May 2007 survey team consisted of four volunteer surveyors per plot.

Epigeous surveys were conducted by systematically walking-through the plots and searching 5 m wide strips for sporocarps for the allocated time. All observed epigeous mycorrhizal sporocarps, as well as sporocarps of unknown functional status, were collected and stored in labelled paper bags until processed.

Surveys of hypogeous fungi were concentrated within the drip zone of *E. delegatensis* trees as distance from mature trees has a strong effect on hypogeous sporocarp density in Tasmanian forests (Johnson 1994). Approximately five to ten minutes was spent searching each tree, resulting in six to twelve trees searched every survey depending on the length of the survey. For example, for a 30 minute survey six trees were searched for 5 minutes each and for a two

hour survey, twelve trees were searched for 10 minutes each. Hand cultivators were used to remove the top litter layer and to dig in the upper 5 cm of the soil of the search zone surrounding each tree. All observed hypogeous sporocarps were collected and stored in labelled paper bags until processed. After searches were finished, the litter layer was returned to the search zone to minimise disturbance from surveying.

All collections were processed in the laboratory within three days of collection. Macroscopic (cap, stipe, gills) and other distinctive characteristics were noted for each collection, and where possible were used to identify the specimen to the level of genus using descriptions and taxonomic keys provided by Largent and Thiers (1977), Stuntz, Largent *et al.* (1977), Grgurinovic (1997), Bougher and Syme (1998), and Young (2000). Collections were photographed using a Canon Powershot S40 digital camera and given a unique record number. Small sections of the sporocarp cap and gills (or gleba) were cut from each collection for DNA extraction and stored in 1.5 ml tubes at -80 °C until processed. Collections were dried and frozen as a reference collection, and were deposited in the Tasmanian Herbarium.

4.2.5 DNA extraction

DNA was extracted from all root tips and sporocarps of ECM or unknown species by a silica-binding method (Glen, Tommerup *et al.* 2002 see Appendix 16 for full protocol). DNA was eluted in 25 µL TE buffer and aliquots diluted to 1:40, 1:20 or 1:10 in TE buffer (Sambrook, Fritsch *et al.* 1989 see Appendix 7 for recipe) then stored at -80 °C.

4.2.6 DNA amplification by PCR

PCR reactions are described in Appendix 17. Standard cycling parameters for the fungal specific primer combination ITS1-F and ITS4 were used (White *et al.* 1990; Gardes & Bruns 1993) (Appendix 17). PCR product (5 µL) was electrophoresed on a 1 % agarose gel (Fisher Biotec, Wembley, Australia) for 30 min at 8 Vcm^{-1}, stained in 1 µg/mL ethidium bromide (MoBio Laboratories, Carlsbad, California, USA), illuminated under UV light and photographed with a digital video camera (Vilber Lourmat, Cedex, France). When DNA amplification was not successful, alternative DNA dilutions were used as the PCR template until DNA amplification was optimised. PCR reactions that were sent to Macrogen Pty Ltd for sequencing were performed using 10 µL of diluted DNA in 50 µL reactions.

4.2.7 DNA sequencing

The majority of sequencing was performed by the University of California Berkeley DNA sequencing facility (USA) (http://mcb.berkeley.edu/barker/dnaseq/index.html) or Macrogen Pty Ltd (Korea) (www.macrogen.com). The remaining sequences were obtained according to the procedure outlined below. Sequencing was performed in a single direction, unless quality was poor, in which case the reverse direction was also sequenced.

PCR products were cleaned using ExoSAP IT (USB Corp, Cleveland, OH, USA). Cleanup reactions contained 3.5 µL of PCR product, 0.5 µL of ExoSAP and 1 µL of deionised water. Samples were vortexed and briefly centrifuged at 16 100 g briefly then incubated at 37 °C for 45 min, followed by 80 °C for 15 min in an Applied Biosystems 2720 Thermal Cycler.

Sequence reactions were carried out using an Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit. To each sample, 1 µL of Big Dye v3.1 sequence mix, 1 µL of Big Dye sequence Buffer (5x), 1 µL of 50 µM ITS1-F (Integrated DNA Technologies, USA) and 1 µL of sterile deionised water was added to 1 µL of clean PCR product in a 96 well PCR plate (Fisher Scientific, Santa Clara, USA). Reactions were heated for 1 minute at 96 °C, then 25 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C, and 4 minutes at 60 °C in an Applied Biosystems 2720 Thermal Cycler. Sequence reactions were precipitated by adding 1.25 µL 125 mM EDTA (Appendix 7) and 15 µL 100 % ethanol to each sample. Samples were briefly vortexed and incubated at room temperature for 15 minutes then centrifuged at 2254 g for 35 minutes. Plates were inverted and centrifuged at 700 g for 1 minute after which 15 µL 70 % ethanol was added to each well. Plates were centrifuged at 2254 g for 10 minutes then inverted and centrifuged at 700 g for 1 minute. Pellets were dried in the dark at room temperature then resuspended in 15 µL of A.C.E.™ formamide (Amresco, Solon, USA) and centrifuged briefly. Samples were heated at 65 °C for 2 minutes then 95 °C for 2 minutes then placed on ice for 1 minute before a brief centrifugation at 1000 g. Single pass sequencing was performed using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

4.2.8 Sequence analysis and fungal identification

Chromatograms were viewed and sequences edited using DNAStar SeqMan II v 5.05. For samples that had been sequenced in both directions but sequences were short or of variable quality, forward and reverse sequences were combined when both sequences had a good quality section that overlapped.

Grouping of fungal OTUs (Operational Taxonomic Units) and identification was based primarily on DNA sequence data, with some consideration given to consistency with macroscopic morphological characters. Searches of public DNA databases (GenBank, EMBL, DDBJ, UNITE), were carried out with the nucleotide–nucleotide (blastn) BLAST searches (Altschul, Madden *et al.* 1997; Kõljalg, Larsson *et al.* 2005), available through the ANGIS BioManager online database (www.angis.org.au). Sequences were grouped according to BLAST search results and aligned using Clustal W (Thompson *et al.* 1994). OTUs were initially defined as having ≥ 98% sequence similarity over the whole of the ITS region, then refined by phylogenetic analysis of related sequences. Phylogenetic trees were constructed using DNAML of the Phylip package (Felsenstein 1989) with default settings. Trees were rooted by outgroup. Phylogenetic trees were also constructed for groups of OTUs (four or more) that matched closely related taxa e.g. all sequences that were identified as belonging to the Hydnangiaceae from sporocarp collections, root tips as well as sequences from public databases were included in the phylogenetic analysis. In general, monophyletic clades that encompassed less than two percent variation in sequence similarity were assigned to a single OTU. Where possible, OTU groups were further refined by considering macroscopic similarity of sporocarps. In the few cases where monophyletic clades included over 2% sequence variation, or where a clade that could have been considered a single OTU on the basis of sequence data alone was split into two or more OTUs, the decision was based upon macroscopic morphological characters. In some cases where molecular identification failed, some sporocarp samples were allocated to an OTU based solely on their morphological similarity.

OTUs were assigned to a nearest taxonomic rank according to the level of sequence similarity to database sequences from known species. For example a sequence similarity ≥ 98% was taken to indicate probable con-specificity if sequence similarity to any other species was less than 97%. Likewise, sequence similarity of 95 - 98% was interpreted as likely to be another

species within that genus, unless more than one genus had comparable levels of sequence similarity, in which case it was assigned to a family or higher taxonomic level. Some OTUs had their highest match to environmental sequences and some samples had only low similarity to any database sequence. In these cases samples remained unidentified. Samples also remained unidentified if only a short fragment of the ITS region matched a sequence from a known fungal species.

Taxonomic nomenclature followed Crous, Gams *et al.* (2004) (www.mycobank.org) apart from the family Exidiaceae, the genus *Thaxterogaster* and Zygomycota. *Thaxterogaster* is a taxonomic synonym of *Cortinarius* (Peintner, Moser *et al.* 2002) and thus any OTUs with high sequence similarity to *Thaxterogaster* spp. were named *Cortinarius*. Taxonomy for the Zygomycota followed Hibbett, Binder *et al.* (2007) and were included under Mucoromycotina. The Exidiaceae followed current convention within the scientific community and were included under Sebacinaceae. OTUs were used as a proxy for species. All OTUs recorded within this chapter and following chapters were allocated a unique OTU name. OTUs were assumed to represent mycorrhizal species if they had high sequence similarity to known ECM taxa listed in Rinaldi, Comandini *et al.*(2008), or to sequences that were stated to be of mycorrhizal origin. DNA sequences were submitted to GenBank (accession numbers JF960600-JF960854).

4.2.9 Data analysis

Species richness was defined as the total number of OTUs observed within a given plot. If the same species was recorded from both sporocarp and root tip samples only a single data entry was made. Species richness estimates were calculated in EstimateS v8.0 (Colwell 2006) based on 500 randomisations of sample order without replacement.

S_{obs} (Mao Tau) interpolation estimator (Colwell, Mao *et al.* 2004) was used to simulate a randomised species accumulation curve for the observed data when the plots were selected in random order. The Mau Tau estimator uses the actual number of observed species from all plots as the total number of estimated species.

The non-parametric Jacknife estimator (Jacknife 1) of species richness was also calculated. The Jacknife estimator differs from Mao Tau in that Jacknife estimates the total number of species

as a function of the number of plots including both observed and unobserved species. The equation for the Jacknife estimator is given by:

$$J_n(S) = S_0 + \frac{n-1}{n}\sum_{i=1}^{n} r_i ,$$

where S_0 is the observed species count over all plots, r_i is the number of species that are found only in plot *i*, and *n* is the number of plots. The Jacknife estimator thereby provides a theoretical estimate of the total number of species present in the combined plots. This type of non-parametric estimator is advantageous as it uses presence and absence rather than abundance, and is known to reduce bias of richness estimations, although it tends to underestimate richness if there is a large number of rare species (Burnham & Overton 1978; Burnham & Overton 1979; Heltshe & Forrester 1983; Smith & van Belle 1984).

Estimated species richness (for sporocarp data and root tip data) was graphed against plot area and sample effort (the number of person-hours per survey for sporocarps and the number of soil cores for root tip data). ECM fungal species richness of each plot was also graphed against time since disturbance and plant species richness to determine any correlation with these variables.

A one-way analysis of variance (ANOVA) was performed in GenStat v7.1 (VSN International Ltd 2003) to test for the difference in ECM richness between the north-east and north-west plots, and between the two understorey types. Two-way analysis was not used because of the unbalanced samples sizes. Where percentages were used in the analysis, the data were angular transformed prior to inclusion in the analysis.

4.3 Results

4.3.1 Fire treatment

The experiment had been designed to include application of a fire treatment to half of the plots (i. e. a split plot design), which would have allowed a direct comparison between plots within the same site and understorey type that had been recently burnt, or burnt some time ago

(from 45 to 125 years). Fire treatment was only applied to the north-west rainforest and sclerophyll treatment plots in January 2009. The late timing of this treatment within the study timescale did not allow the collection of post-burn data within the scope of this study.

4.3.2 Plant species composition and richness

Plots 1 and 2 were dominated by a mixed age stand of *E. delegatensis* up to a height of 55 m with *T. lanceolata*, *L. lanigerum*, *Pittosporum bicolor* and *A. dealbata* forming a mid-storey to 24 m. *Dicksonia antarctica* was present in the mid-storey to a height of 15 m. Ground vegetation was sparse but where present consisted of *Gahnia grandis*, *Hypolepis* sp. and *Polystichum proliferum*. The majority of the ground was covered in litter. The mean Domin cover estimate for eucalypts was 25%.

Plots 3 and 4 were dominated by *E. delegatensis* with sparse cover up to 60 m. A mid-storey of *N. cunninghamii* was present to a height of 15 m, with scattered *Acacia melanoxylon* R.Br. trees. Ground cover was mostly absent but small patches of *G. grandis*, *P. proliferum* and *Histiopteris* sp. These plots were last burnt c. 1884 by a low intensity fire. The mean Domin cover estimate for eucalypts was less than 10%, and for *N. cunninghamii* was 25%.

Plots 5 to 8 were dominated by *E. delegatensis* of mixed ages ranging in height from 2 m to 50 m. A patchy mid-storey of *A. dealbata* to a height 15 m was present and a dry sclerophyll understorey consisting of *P. juniperina*, *T. lanceolata*, *Persoonia gunnii*, and *Coprosma nitida* to a height of 4 m was also present. Ground cover consisted of the ferns *Histiopteris* sp., *Hypolepis* sp., *P. proliferum* and *P. esculentum* along with small grass patches of *Poa* sp. The mean Domin cover estimate for eucalypts was 35%.

Plots 9 and 10 were dominated by *E. delegatensis* to a height of 60 m with a mid-storey of myrtle (*N. cunninghamii*) up to 30 m, *Leptospermum lanigerum* to 15 m and *Nematolepis squamea* (Labill.) Paul G. Wilson to a height of 15 m. Understorey was mostly absent with few *Leptecophylla juniperina* C.M.Weiller. shrubs and isolated *Dicksonia antarctica* ferns. Sparse ground cover mainly consisted of the fern *P. proliferum* and the sedge *G. grandis*. The mean Domin cover estimate for eucalypts was 20%, while that for *N. cunninghamii* was 30% and cover for tall shrubs (*L. lanigerum* and *N. squamea*) was 18%.

Plots 11 and 12 were co-dominated by *E. delegatensis* and *E. dalrympleana* reaching a height of 60 m. A thick dry sclerophyll understorey up to 5 m was dominated by *Monotoca glauca (*Labill) Druce., *P. juniperina* and *T. lanceolata*. Ground cover consisted of *Dianella tasmanica* Hook.f., *Blechnum wattsii* Tindale., and *P. esculentum*. The mean Domin cover estimate for eucalypts was 30%.

Complete plant species lists for each plot indicating abundance are provided in Appendix 18 (vegetation abundance).

Plant species richness ranged from ten to 33 species (Figure 4.2). Plot 6 had the greatest plant species richness, while plot 10 had the lowest plant species richness (Figure 4.2). In total across all plots, eight known ECM host species were recorded. These were *A. dealbata*, *A. melanoxylon*, *E. delegatensis*, *E. dalrympleana*, *L. lanigerum*, *N. cunninghamii*, *P. juniperina*, and *Melaleuca squarrosa* (Donn.) Sm. Plot 4 had the highest number of ECM host species and plots five and 10 had the lowest number of host plant species (Figure 4.2). There was no relationship between plant species richness and the number of ECM hosts found on each plot (Figure 4.2). There was no significant difference in plant species richness between the north-east and north-west plots (20.5 (N=8) v. 17 (N=4), $F_{1,10} = 0.1$, $p = 0.34$), but there was significantly higher richness in sclerophyll plots as opposed to rainforest plots (23.3 (N=6) v. 12.3 (N=6), $F_{1,10} = 11.38$, $p = 0.007$).

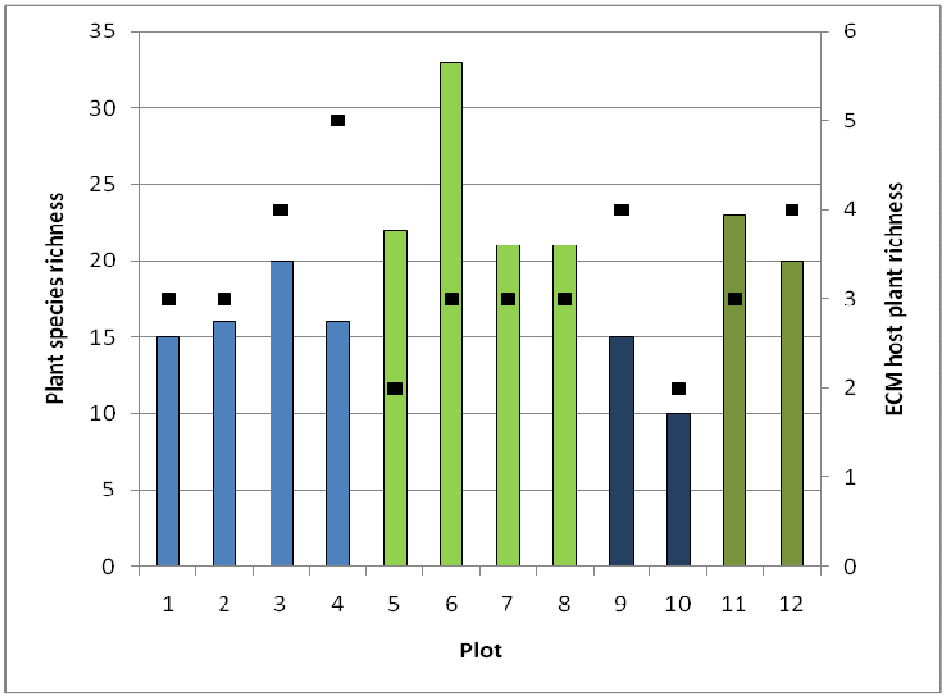

Figure 4.2 Plant species richness of each plot (total of species identified in four replicate quadrats assessed at each plot). Plots with a rainforest understorey are blue and plots with a sclerophyll understorey are green. North-east plots are light coloured (plots 1-8), and plots located in the north-west plot are dark coloured (plots 9-12). Paired plots are next to one another i.e. Plots 1 and 2, Plots 3 and 4, etc. Black squares show the number of ectomycorrhizal host species present in each plot (plotted on second axis).

4.3.3 Ectomycorrhizal fungal samples

Of the 120 soil cores collected from the 12 plots (10 per plot), sixteen soil cores had no mycorrhizal root tips, with the majority of these (10 soil cores) originating from plots seven and eight so that both plots had five cores with no mycorrhizas (Appendix 19). From the remaining 104 soil cores, a total of 493 root tip samples were taken (Appendices 19 and 20). Photographs of a selection of ECM root tips collected from *E. delegatensis* forest are shown in Appendix 21.

The eight sporocarp surveys of each of the plots resulted in 852 collections of fungi, of which 720 collections were considered, after morphological examination, to be ECM OTUs or unknown OTUs, and 129 collections were considered not to be ECM OTUs (Appendix 22). One hundred and thirteen collections were of hypogeous sporocarps and the remainder were

epigeous sporocarps (Appendix 22). A selection of ECM sporocarp photographs are shown in Appendix 23.

4.3.4 DNA extraction, PCR and sequencing

DNA amplification failed from all root tip samples from two soil cores and root tips from another five cores failed to produce legible DNA sequences because of poor quality sequence results, so that only 97 cores gave useful data (Appendix 19). DNA was extracted from 492 mycorrhizal root tip samples (Appendices 19 and 20), but ~18% of these samples failed to amplify using primers ITS1-F and ITS4 after three attempts, ~ 8% were amplified and sequenced but had poor quality sequences that were not able to be used for Blast searches, ~4% had sequences that matched non-mycorrhizal species and ~71% were allocated to ECM OTUs.

DNA was extracted from 672 of the sporocarp collections that were considered to be ECM OTUs or unknown species (DNA samples were not taken from 44 of these collections as originally these collections were to be identified morphologically). Of those, ~5% failed to amplify after three attempts, and ~4% produced sequences of poor quality that could not be used for identification. Six percent of the collection was identified as non-mycorrhizal through sequencing, ~54% (461 collections) of the collections were allocated to an ECM OTU based on DNA sequences and ~10% were identified to genus or family (Appendix 22). Only 7 collections (for which DNA samples were not taken) were identified to an ECM genus or family by morphological characteristics.

4.3.5 OTU discrimination and taxonomic identification

OTUs were grouped according to 98% sequence similarity of the ITS region and OTUs were determined by phylogenetic analyses. Maximum likelihood phylogenetic trees were constructed for the following ECM families; Amanitaceae, Boletaceae, Clavulinaceae, Cortinariaceae, Hydnangiaceae, Russulaceae, Thelephoraceae, Tricholomataceae and Sebacinaceae (Appendices 24 and 13).

Some 120 ECM OTUs were detected from root tips, and 178 as sporocarps, with 39 of these found as both root tips and sporocarps, giving a total of 259 ECM OTUs (Appendices 13 and 25).

This gives an OTU discovery rate (OTU: sample) 0.25 for root samples and 0.26 for sporocarps. Of the 259 ECM OTUs, 162 OTUs were identified to species or genus, 46 to family, 19 to order, 21 to phyla and 10 remained unidentified (Appendix 12). Over half of the OTUs (164 OTUs) represented a single sample (one root tip sample or one sporocarp collection).

4.3.6 Combined ectomycorrhizal species richness

A total of 259 ECM OTUs from 27 genera, 20 families and 12 orders were sampled from the identified OTUs (using the combined data from all 12 plots) (Appendix 12). Observed OTU richness of individual plots ranged from 62 in plot 9, to 22 in plot 7 (Figure 4.3) with a mean of 41 OTUs per plot and standard deviation of 12. With the exception of paired plots 1 and 2 which both recorded 32 OTUs, paired plots differed in species richness, in some cases by up to 30% (Figure 4.3). The presence and absence of OTUs within each plot is shown in Appendix 26.

Jacknife 1 estimated a total ECM species richness of 409 OTUs for 3 ha of *E. delegatensis* forest (Figure 4.4). This gives an estimated 19 new OTUs recorded for every 0.25 ha of forest sampled according to this sampling regime. These accuracy of these estimates may be compromised as sites from both regions (north-east and north-west Tasmania 200 km apart) were included.

Ectomycorrhizal OTU richness significantly differed between north-east and north-west forest plots. A one-way ANOVA found the north-west plots had a significantly higher ECM richness than the north-east plots (54 v. 34, $F_{1,10} = 13.42$, $p = 0.004$). There was no significant difference in total observed OTU richness per plot between the rainforest and sclerophyll plots (43 v. 38, $F_{1,10} = 0.39$, $p = 0.54$).

A correlation between ECM richness of plots and time since the plot had been disturbed (either by fire or by timber harvesting activities) was detected. This relationship was not linear, with observed ECM species richness greatest at around 20 years post-disturbance, then decreasing with increasing time until at about 80 years after which richness starts to rise again (Figure 4.5). This polynomial regression had a good fit with time since disturbance explaining ~ 76% of the variation in ECM richness between plots. Ectomycorrhizal OTU richness showed only a weak insignificant correlation with ECM host plant species richness (Figure 4.6). A linear regression of ECM richness and total plant species richness was slightly negative and again insignificant ($y = -0.55x + 51.26$, $R^2 = 0.06$, $p=0.45$, figure not shown).

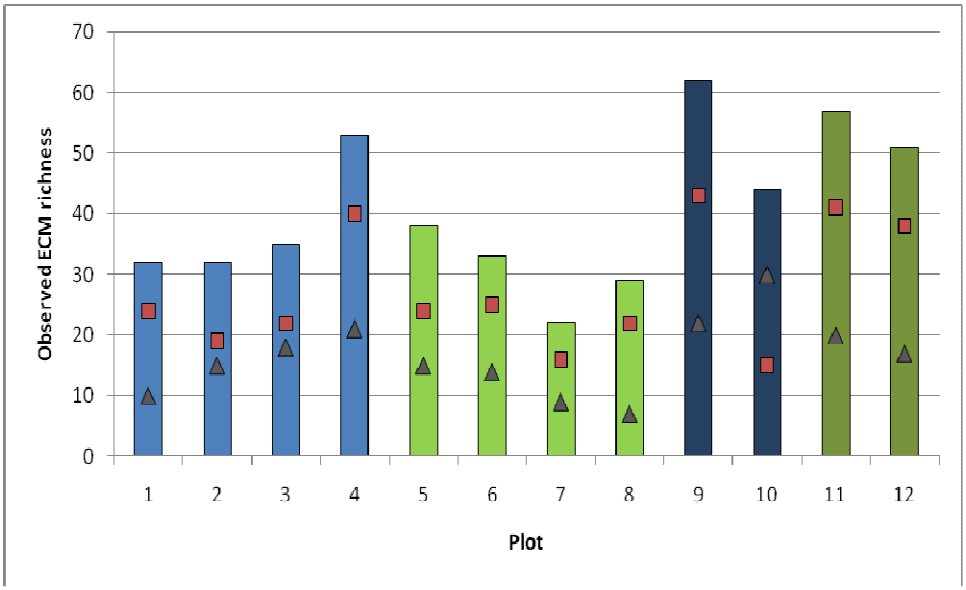

Figure 4.3 Observed ectomycorrhizal OTU richness for each plot using the combined sporocarp and root tip data (bars). Plots with a rainforest understorey are blue and plots with a sclerophyll understorey are green. North-east plots are light coloured (plots 1-8), and plots located in the north-west are dark coloured (plots 9-12). Paired plots are next to one another i.e. Plots 1 and 2, Plots 3 and 4, etc. Red squares indicate sporocarp richness and grey triangles indicate root tip richness.

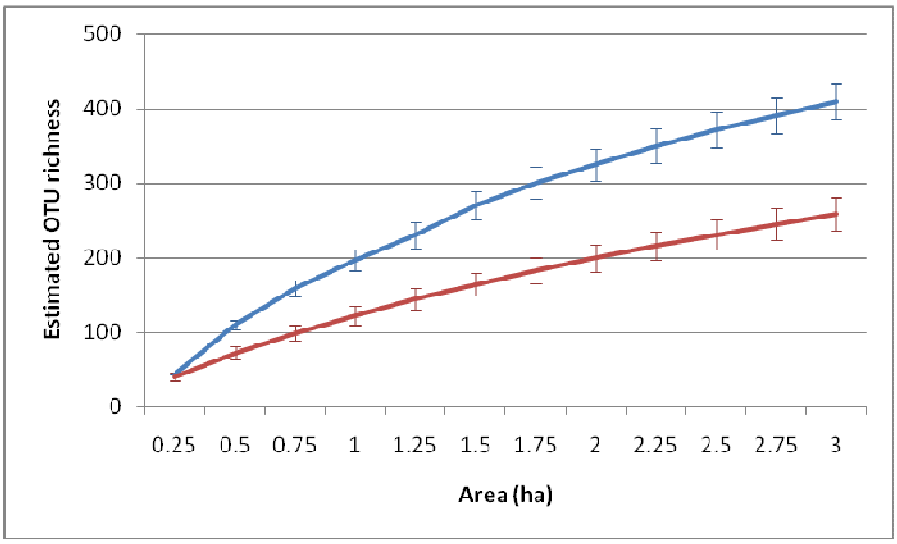

Figure 4.4 Species-area curve for combined sporocarp and root tip data showing Mao Tau (red) with 95% confidence level shown as error bars and Jacknife 1 (blue) species richness estimates showing standard deviation as error bars. Richness estimates show the number of species that are estimated to occur within a certain area of *E. delegatensis* forest.

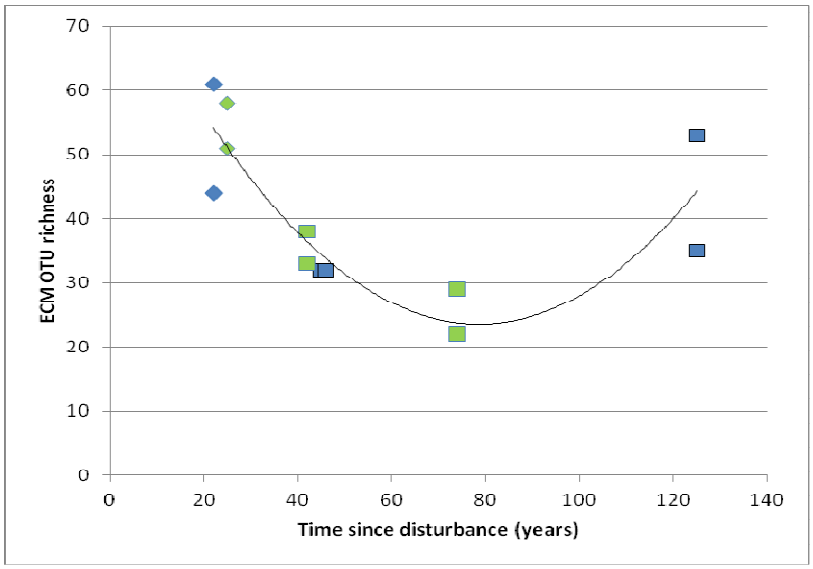

Figure 4.5 Relationship between observed ectomycorrhizal OTU richness of plots and time since disturbance (years). North east plots are shown as square and north west plots are shown as diamonds. Rainforest plots are blue and sclerophyll plots are green. The trend is shown by a second order polynomial, which has a good fit ($R^2 = 0.76$). The relationship is described by the equation $y = 0.01x^2 - 1.51x + 82.86$, p= 0.02.

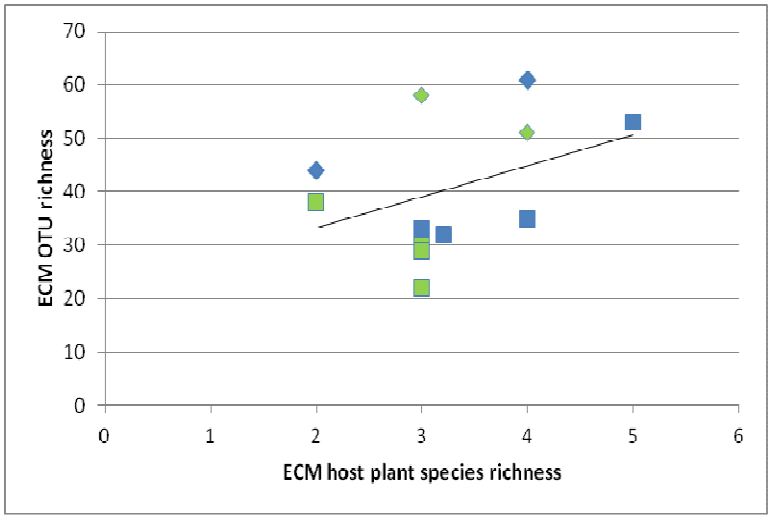

Figure 4.6 Relationship between observed ectomycorrhizal OTU richness of plots and plant species richness. North east plots are shown as square and north west plots are shown as diamonds. Rainforest plots are blue and sclerophyll plots are green. The linear regression model shows a weak positive relationship between the two variables (R^2=0.18). The relationship can be detrived by the equation y = 6.06*x + 20.97, p = 0.21.

4.3.7 Ectomycorrhizal sporocarp species richness

One hundred and seventy eight OTUs were sampled as sporocarps. The highest sporocarp OTU richness was in plot 9 (43 OTUs) and lowest was in the adjacent plot 10 (15 OTUs) (Figure 4.3).

Sporocarp species richness increased with the sample area, with approximately 13 new sporocarp OTUs recorded for every 0.25 ha (Figure 4.7). Jacknife 1 estimated that 279 sporocarp OTUs would be present in 3 ha of forest, which is 101 OTUs more than the actual recorded sporocarp richness of 178 OTUs. Again, this assumes that sporocarp species richness and area relationship is similar between regions.

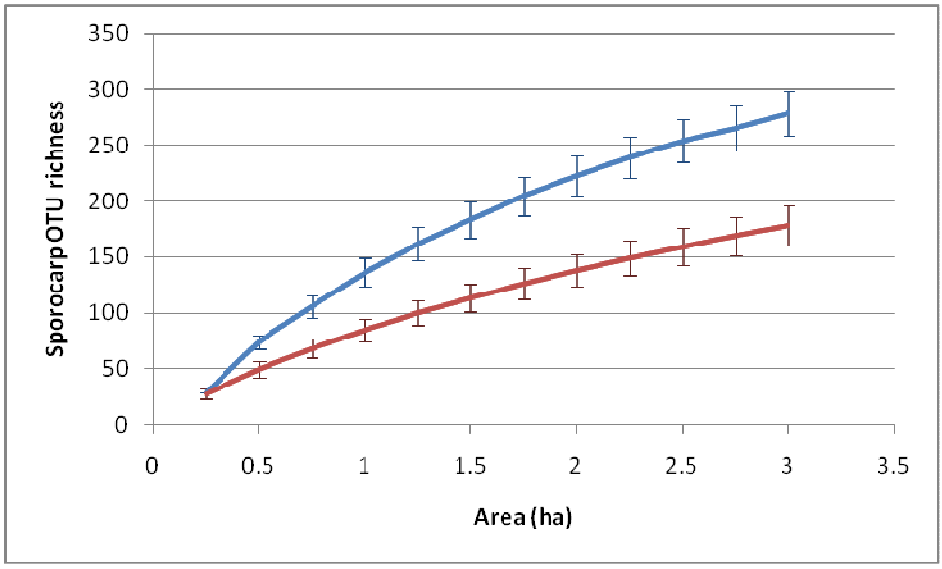

Figure 4.7 Species area curve for sporocarp ectomycorrhizal OTU richness data showing S_{obs} (Mao Tau) (red) with 95% confidence intervals shown as error bars and Jacknife 1 estimates (blue) with standard deviation shown as error bars. The number of OTUs sampled as sporocarps continues to rise with an increase in area.

Sporocarp OTU richness per plot did not significantly differ between understorey-type or between region (understorey - rainforest v. sclerophyll 27 vs. 28, $F_{1,10} = 0.007$, $p = 0.94$, and region north-east v. north-west , 24 vs. 34, $F_{1,10} = 2.86$, $p = 0.12$).

Of the sporocarp OTUs, ~ 15% (26 OTUs) formed hypogeous sporocarps. Hypogeous sporocarp OTU richness was highest in Plot 5, and zero in Plot 10 (Figure 4.8). Hypogeous sporocarp OTUs

comprised ~ 25 – 40% of the OTUs from the north-eastern sclerophyll plots (plots 5 – 8), and between 12 – 18% for the north-western sclerophyll plots (plots 11 and 12) (Figure 4.8). For the rainforest plots, hypogeous sporocarp OTUs made up 0 – 7% of sporocarp richness in the north-west (plots 9 and 10), ~ 13 – 20% for three of the north-east plots (plots 2 – 4) but only 8% for the fourth plot (plot 1) (Figure 4.8). Using the angular transformed data the percentage of hypogeous sporocarps within each plot was significantly greater in the sclerophyll plots than the rainforest plots for alpha = 0.05 (26% v. 12%, $F_{1,10}$=6.03, p = 0.04). There was also a significant difference between the north-east and the north-west plots, with the north-east plots having a greater percentage of hypogeous sporocarps in each plot (24% v. 9%, $F_{1,10}$=10.47, p = 0.01).

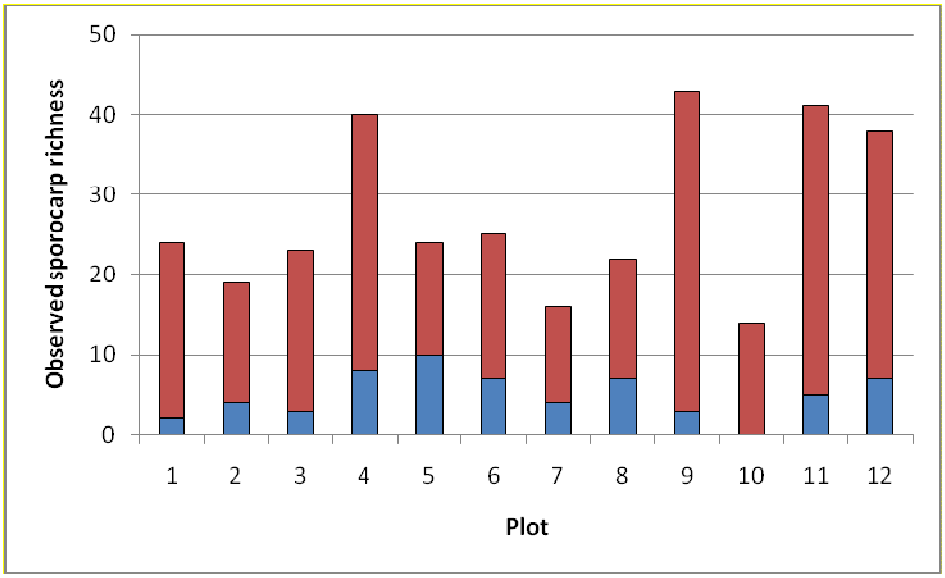

Figure 4.8 Observed epigeous or hypogeous sporocarp species richness. Plots 1-4 are in the north-east rainforest plots, plots 5-8 are north-east sclerophyll plots, plots 9-10 are north-west rainforest plots, and plots 11-12 are north-west sclerophyll plots. Epigeous species richness is shown in red and hypogeous species richness is shown in blue.

The species effort curve indicates that for every survey between two and six new sporocarp OTUs were recorded (Figure 4.9). The number of new species recorded was still rising at the end of the sampling period indicating that more sampling was required to completely characterise the sporocarp community (Figure 4.9).

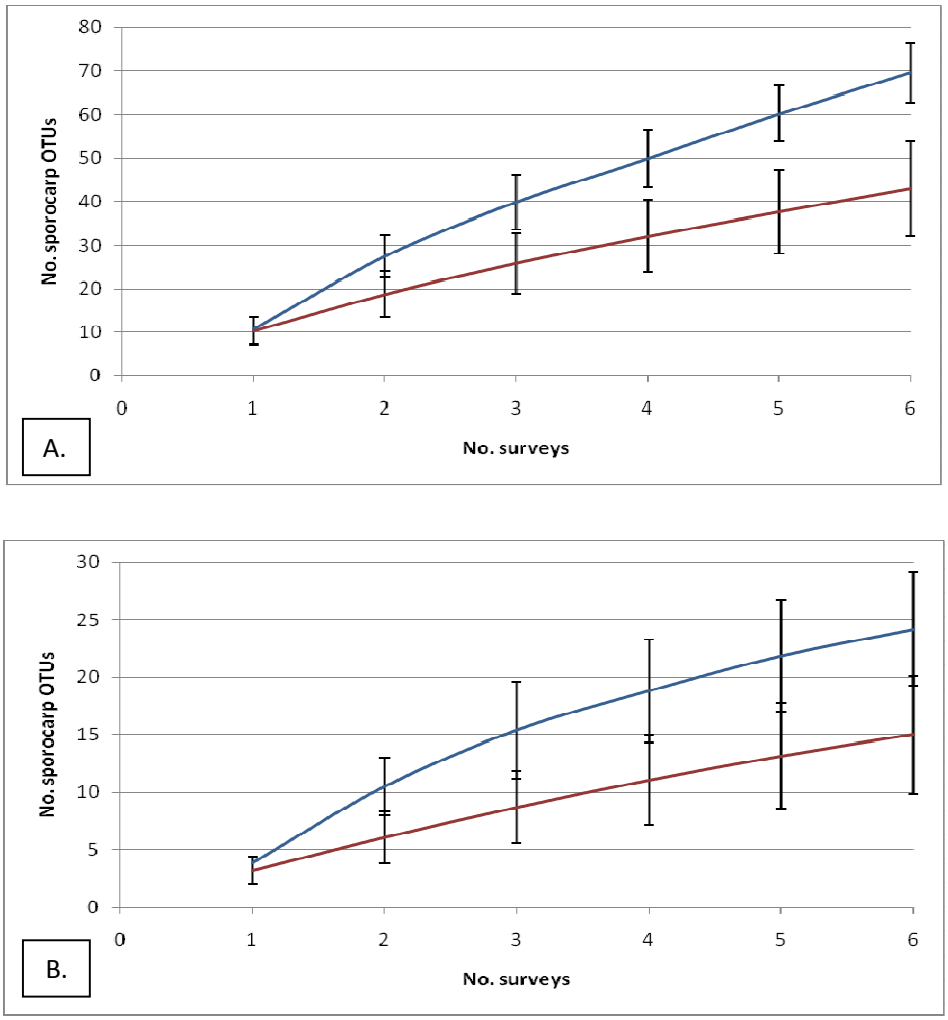

Figure 4.9 Species-effort curve for ectomycorrhizal sporocarp data showing S_{obs} (Mao Tau) (red) with 95% confidence intervals shown as error bars and Jacknife 1 estimates (blue) with standard deviation shown as error bars. A. Species effort curve for plot 9 that had the highest sporocarp OTU richness. The slope of the Mao Tau is 6.5 and the slope of the Jacknife 1 estimate is 11.5. B. Species effort curve for plot 10 that had the lowest sporocarp species richness.

The number of sporocarps recorded per survey hour (sporocarp richness per plot/number of survey hours per plot (15.5)) ranged from 1 (plot 7) to 3 (plots 9 and 11), giving an average of 2 sporocarp OTUs per survey hour. The average number of OTUs collected per survey hour was greater during 2007 than 2008 (Table 4.1).

There was little overlap of sporocarp OTUs recorded during 2007 and 2008 (Figure 4.10). One hundred and ten sporocarp OTUs were collected in 2007 that were not found in 2008 (55%

were from the Cortinariaceae) and 29 OTUs were only recorded in 2008 (55% were from the Cortinariaceae) (Figure 4.10a). Many more Cortinariaceae OTUs were recorded during 2007 than 2008 (Figure 4.10b).

Sporocarp richness varied temporally with the highest richness per survey hour occurring in late autumn, when temperatures begin to drop, and following an increase in rainfall (Figure 4.11). Lowest sporocarp richness was recorded in early summer as temperatures were rising and rainfall was dropping (Figure 4.11).

Table 4.1 Summary of ectomycorrhizal sporocarp OTUs found during the two years of surveying.

Sporocarp OTUs	**2007**	**2008**	**Total**
Observed richness	148	67	178
Mean richness per survey hour (total OTU/total hours)	1.2	1.0	1.0

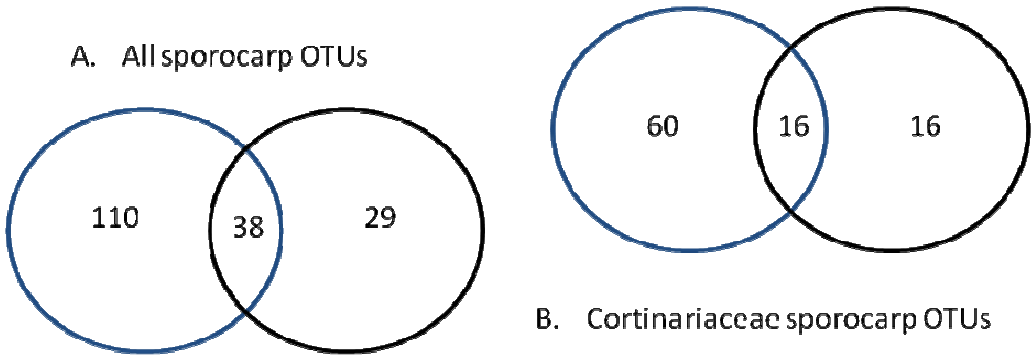

Figure 4.10 Venn diagrams showing the number of ectomycorrhizal sporocarp OTUs recorded from each year, and during both years. (A) All OTUs, (B) Cortinariaceae OTUs. Blue circles represent 2007 and black circles represent 2008.

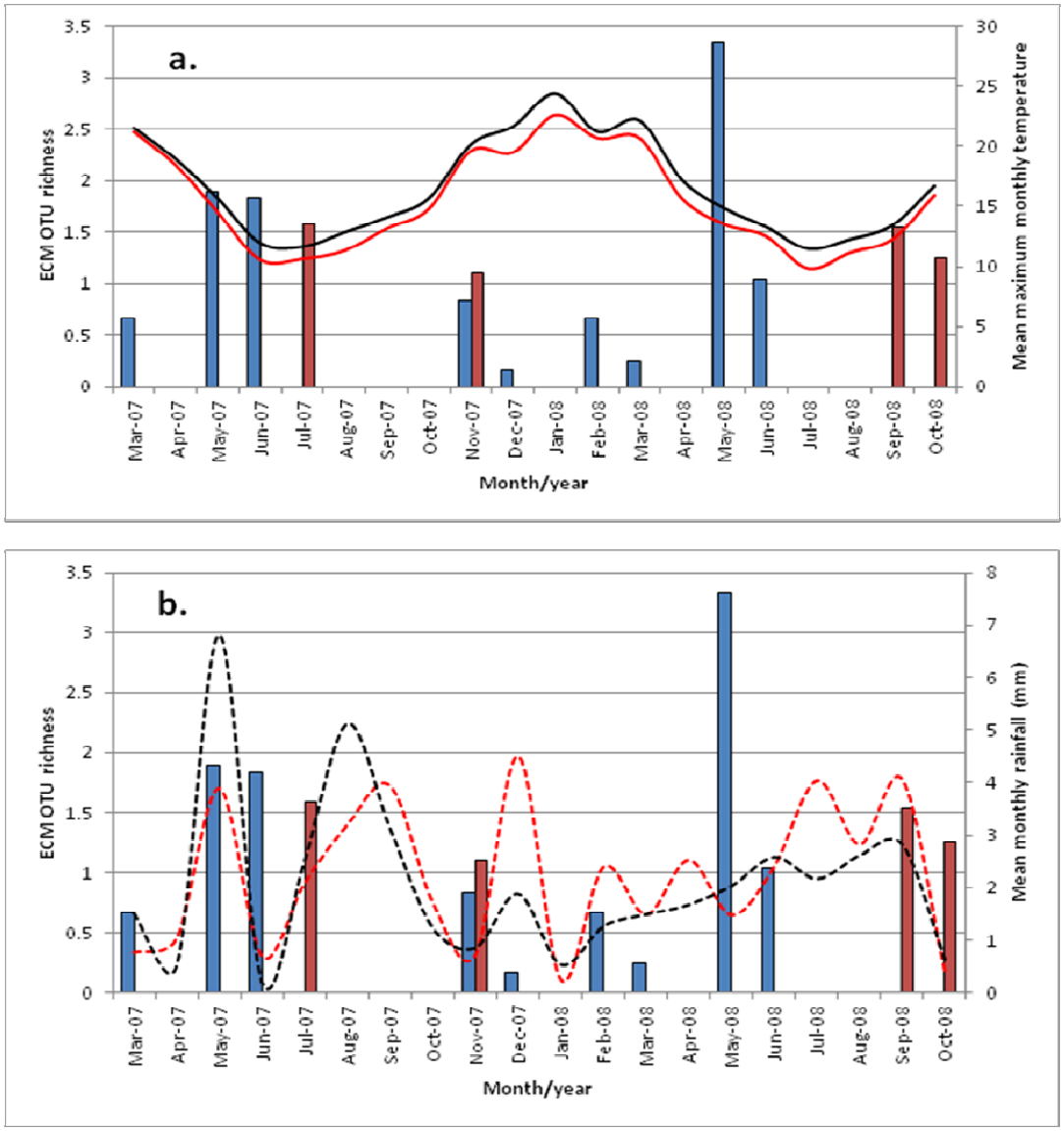

Figure 4.11 Sporocarp and root tip ectomycorrhizal richness at different sample times throughout the study period in relation to climatic data. Sporocarp richness per sample hour is shown as blue bars and root tip richness per soil core is shown as red bars. Climatic data are taken from two nearby weather stations. Sheffield School Farm in the north-west (station number 091291, 2007-2008, 294m) is shown in red, and Scottsdale (West Minestone Road) in the north-east (station number 091219, 2007-2008, 198m) is shown as black. A. Average maximum monthly temperatures are shown on the right hand side y-axis. B. Average monthly rainfall is shown on the right hand side y-axis.

4.3.8 Ectomycorrhizal root tip species richness

One hundred and twenty OTUs were detected as ECM root tips (Appendix 12). Highest root tip species richness was sampled in plot 10 (30 OTUs) and lowest richness was observed in plot 8 (7 OTUs) (Figure 4.3).

Ectomycorrhizal root tip OTU richness was estimated at 202 from Jacknife 1. Root tip OTU richness increased with area sampled as seen in Figure 4.12. Approximately nine new ECM OTUs were recorded for each 0.25 ha area sampled by 10 soil cores. The species-area curve does not reach an asymptote indicating that more ECM OTUs would continue to be recorded if the sample area was increased (Figure 4.12). Between one and three new OTUs were recorded for each soil core sampled. The species-effort curve does not begin to plateau indicating that there was insufficient sampling to characterise the below-ground ECM community (Figure 4.13).

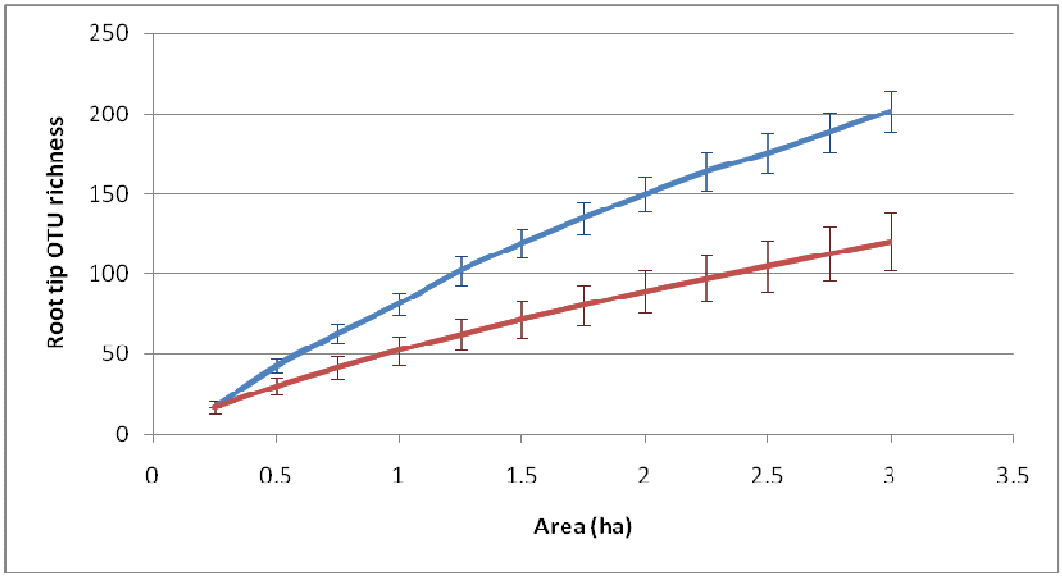

Figure 4.12 Species area curve for ectomycorrhizal root tip OTU richness data showing S_{obs} (Mao Tau) (red) with 95% confidence intervals shown as error bars and Jacknife 1 estimates (blue) with standard deviation shown as error bars.

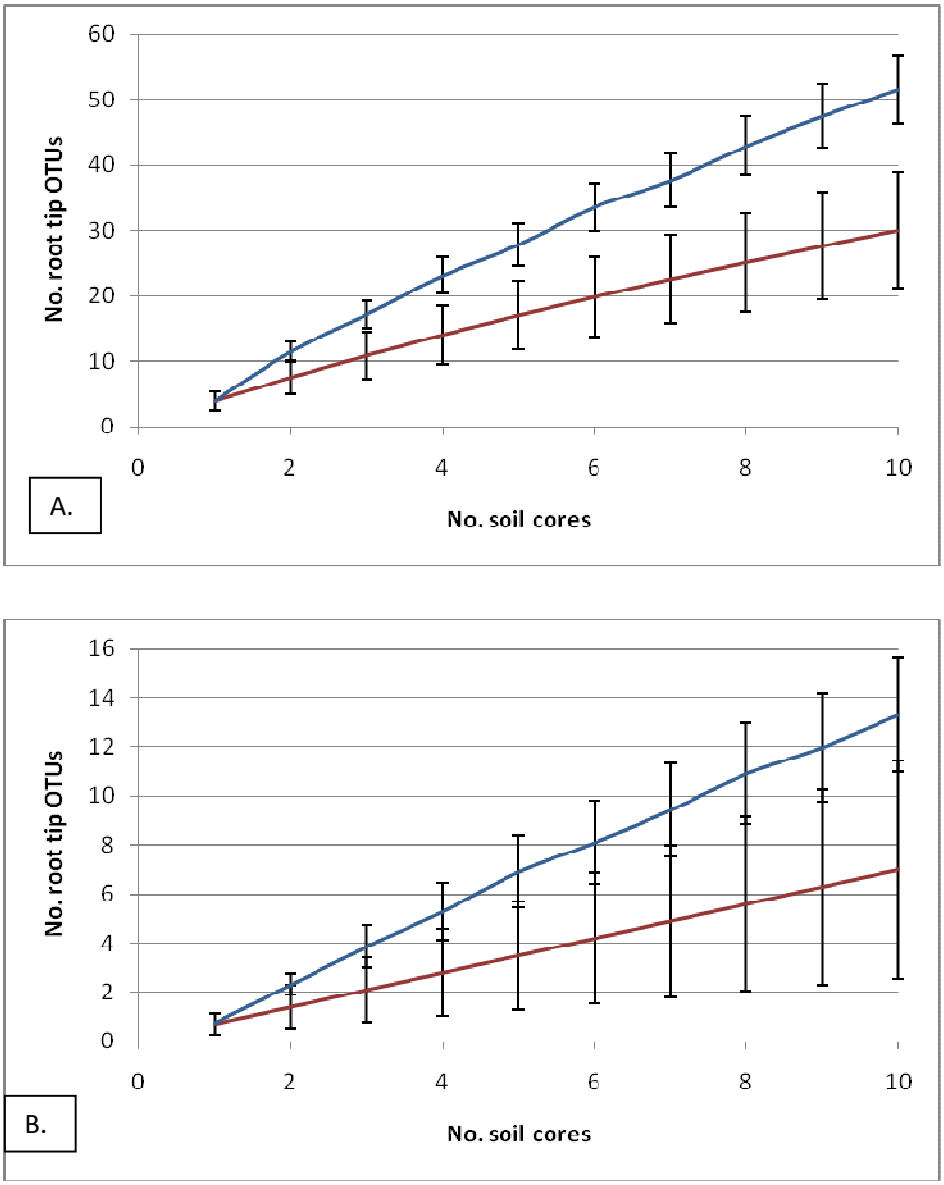

Figure 4.13 Species-effort curves for ectomycorrhizal root tip data showing S_{obs} (Mao Tau) (red) with 95% confidence interval shown as red error bars, and Jacknife 1 richness estimates (blue) with standard deviation shown as error bars. A. Species effort curve for plot 10 that had the highest root tip sppecies richness. The slope of the Mao Tau is 2.8 and the slope of the Jacknife 1 estimate is 5.2. B. Species effort curve for plot 8 that had the lowest root tip species richness.

Root tip OTU richness did not significantly differ between understorey-type (rainforest vs. sclerophyll 19 vs. 14, $F_{1,10} = 2.75$, $p = 0.13$) but was significantly higher in the north-west compared to the north-east (23 v. 14, $F_{1,10} = 8.28$, $p = 0.02$).

Eighty percent of root tip OTUs were detected at only one time of the year. Sixteen percent of root tip OTUs were only sampled in July 2007, 35 % were only sampled in November 2007, 29 % were only sampled in September/October 2008. The number of root tip OTUs that were sampled at the three different sample times are shown in Figure 4.14. Comparing the number of OTUs found per soil core for each sample time, July 2007 had the highest richness per core of 1.7 (for 24 cores), November 2007 had the lowest richness of 1.1 OTUs per soil core (for 60 cores) and September/October 2008 had a mean richness per core of 1.5 (for 36 cores). These values are so similar that there is no significant difference between the numbers of OTUs sampled at different times of the year (Figures 4.11 and Figure 4.14).

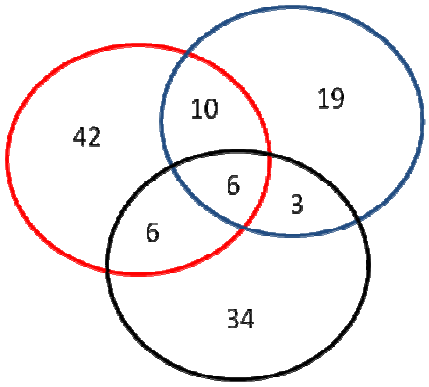

Figure 4.14 Venn diagram representing the number of ectomycorrhizal OTUs recorded from root tips at the three sample times, November 2007 (red), July 2008 (blue) and September/October 2008 (black). The number of soil cores collected in November 2007 was 60, in September/October 2008 was 36, and in July 2008 was 24.

4.3.9 Ectomycorrhizal community structure and dominance

Collections (root tips and sporocarps) included most families of ECM fungi that are known to occur in Australia (Appendix 12). Ascomycota OTUs made up ~ 10% of the OTUs and included members of the Elaphomycetales, Helotiales, and Pezizales. Basidiomycota taxa included

species in the Amanitaceae, Amylostereaceae, Bolbitiaceae, Boletaceae, Clavariaceae, Clavulinaceae, Corticiaceae, Cortinariaceae, Entolomataceae, Sebacinaceae, Gomphaceae, Hydnaceae, Hydnangiaceae, Inocybaceae, Russulaceae, Thelephoraceae and Tricholomataceae (Figure 4.15).

Overall (using the combined sporocarp and root tip OTUs from all plots) the Cortinariaceae had the highest species richness of all the families sampled (105 OTUs, Appendix 12 and Figure 4.15) and the Russulaceae was the next most diverse family (25 OTUs) (Figure 4.15). OTUs belonging to the Cortinariaceae made up the majority of the ECM community not only using the combined data but also within the root tip and sporocarp communities (Figure 4.15).

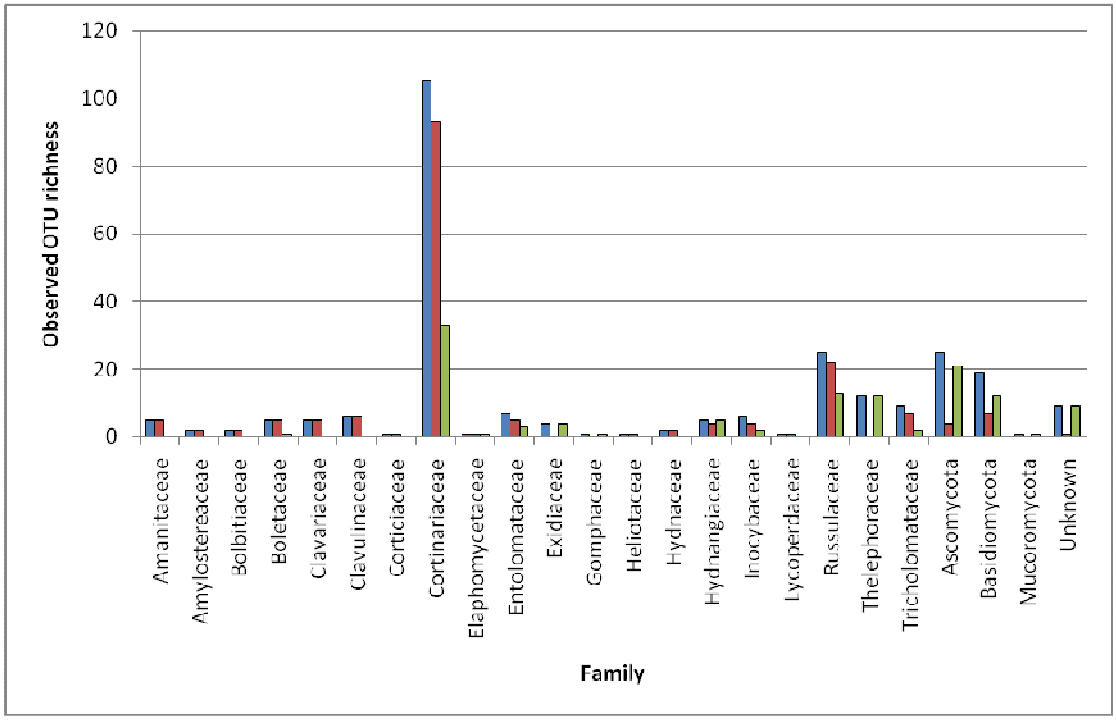

Figure 4.15 Number of observed ectomycorrhizal OTUs from each family and unknown OTUs, using the combined data from sporocarps and root tips (blue), only the sporocarp data (red), and only the root tip data (green), for all plots combined.

When looking at the combined community structure at a plot level, the Cortinariaceae were the most species- rich family in nine of the 12 plots (Figure 4.16). The Russulaceae were the most species-rich family in three north-eastern plots, (plots 2, 5 and 6). The Cortinariaceae was also the most species-rich family in the root tip community in seven of the plots (plots 2 – 4,

and 9 – 12). In the other plots, species of the Russulaceae made up a higher percentage of the root tip community. For the sporocarp communities, the Cortinariaceae was the richest family in all plots except plots 2, 5 and 6, where the Russulaceae had the greatest number of OTUs. Plots differed in the diversity of ECM families present and the richness of the non-dominant families (Figure 4.16).

Overall, the north-east sclerophyll plots are characterised by a large proportion of species from the Cortinariaceae and Russulaceae while the Thelephoraceae is almost absent (Figure 4.16). The north-east rainforest plots had a high species richness of the Cortinariaceae with a significant contribution by the Russulaceae. The Amanitaceae, Boletaceae and Thelephoraceae had a more conspicuous presence in these plots, and there was a high diversity of ECM families (Figure 4.16). All four north-west plots had a very high Cortinariaceae species richness and had a diversity of families present. The north-west rainforest plots had only a small proportion of Russulaceae with the Thelephoraceae making a slightly larger contribution to the community than the Russulaceae (Figure 4.16). The north-west sclerophyll plots had more Russulaceae and less Thelephoraceae-taxa than the rainforest plots (Figure 4.16).

A total of 27 OTUs were found as hypogeous sporocarps (fruiting form of OTUs was not inferred from DNA sequence analyses, and OTUS were only recorded as hypogeous when a sporocarp was sampled) (Figure 4.17). The Russulaceae showed the highest richness of hypogeous OTUs, with 12 of these identified to genus (one *Arcangeliella* sp., two Russulaceae spp., four *Russula* spp. and three *Zelleromyces* spp., and two of unknown genera). The next richest family was the Cortinariaceae with five OTUs identified to genus (three *Cortinarius* spp, one *Dermocybe* sp. and one *Descomyces* spp.). Only one hypogeous OTU, *Elaphomyces* sp. 1, was from the Ascomycota. Four OTUs were not identified to family with one belonging to the Pezizales, two to the Hysterangiales, and two to the Basidiomycota.

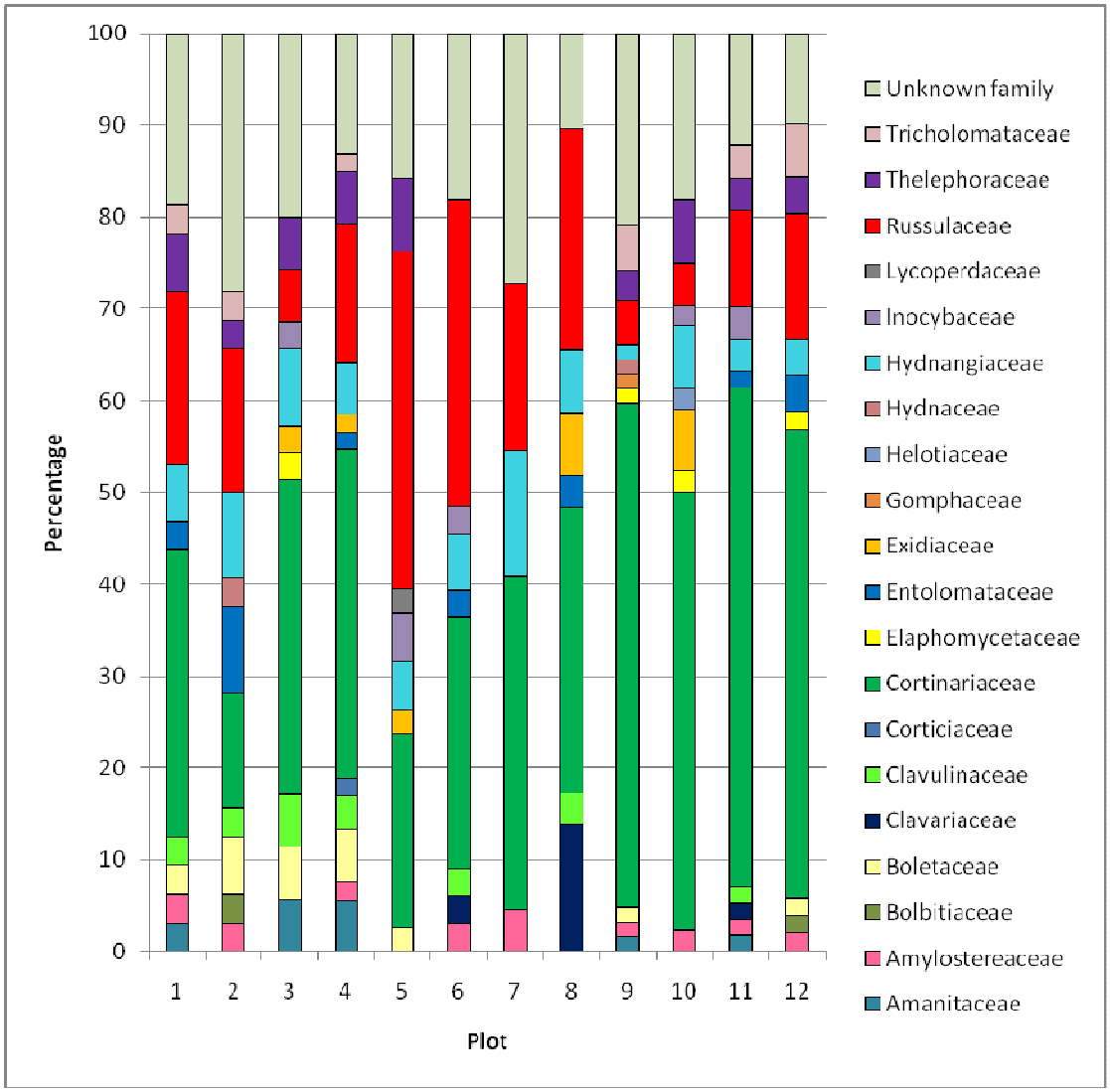

Figure 4.16 Ectomycorrhizal community compositions at the family level within each plot. The number of OTUs from each family within each plot is presented as a percentage of total ECM species richness from each plot. Plots 1 - 4 are north-east rainforest plots, Plots 5 - 8 are north-east sclerophyll plots, Plots 9 - 10 are north-west rainforest plots and Plots 11 - 12 are north-west sclerophyll plots. Paired plots are next to one another i.e. Plots 1 and 2, Plots 3 and 4 etc.

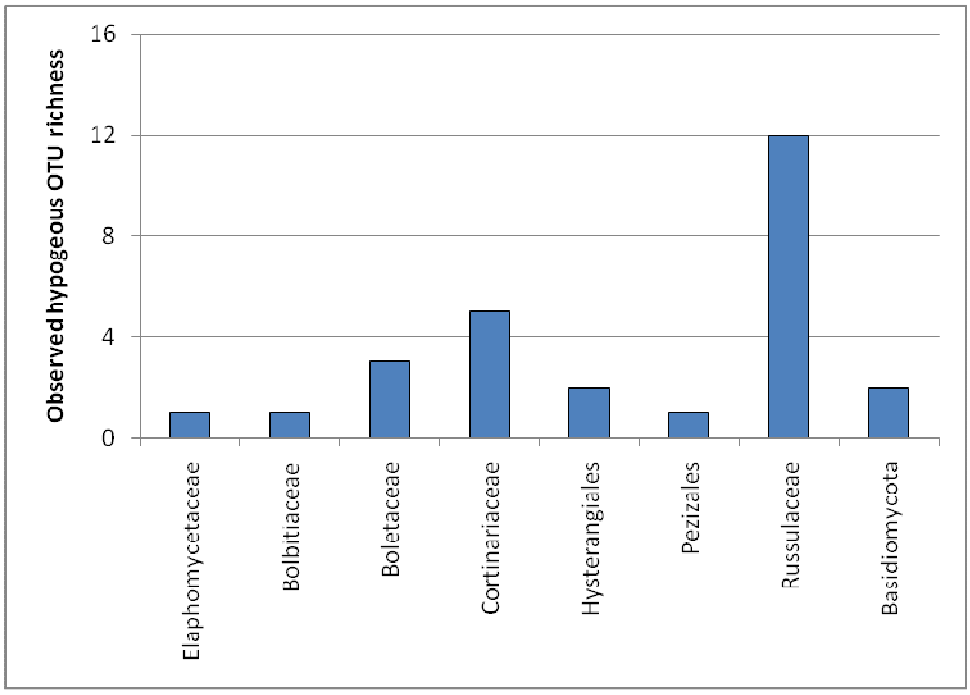

Figure 4.17 The number of ectomycorrhizal hypogeous sporocarp OTUs from each taxonomic group.

Although abundance was not measured in this study, the percentage of samples that belong to different taxonomic groups provides a rudimentary estimate of abundance for that taxon. The Cortinariaceae made up the majority of both sporocarp and root tip samples, and the Hydnangiaceae and Russulaceae made up a very similar percentage of all root tip and sporocarp samples (Figure 4.18).

The most frequently sampled OTUs on root tips were *Laccaria* sp. 1, *Russula persanguinea,* Fungal sp. 3, *Lactarius* sp. 1 and *Descolea recedens*, which together made up ~ 25% of all samples. For sporocarps, eight OTUs made up ~ 20% of all samples; *Lactarius* sp. 1, *Laccaria* sp. 5, *Cortinarius* aff. *rotundisporus*, *Cortinarius* aff. *tasmacamphoratus*, *Dermocybe* aff. *globuliformis*, *Laccaria* sp. 1, *Russula persanguinea* Cleland and *Zelleromyces* sp. 1 (Figure 4.19).

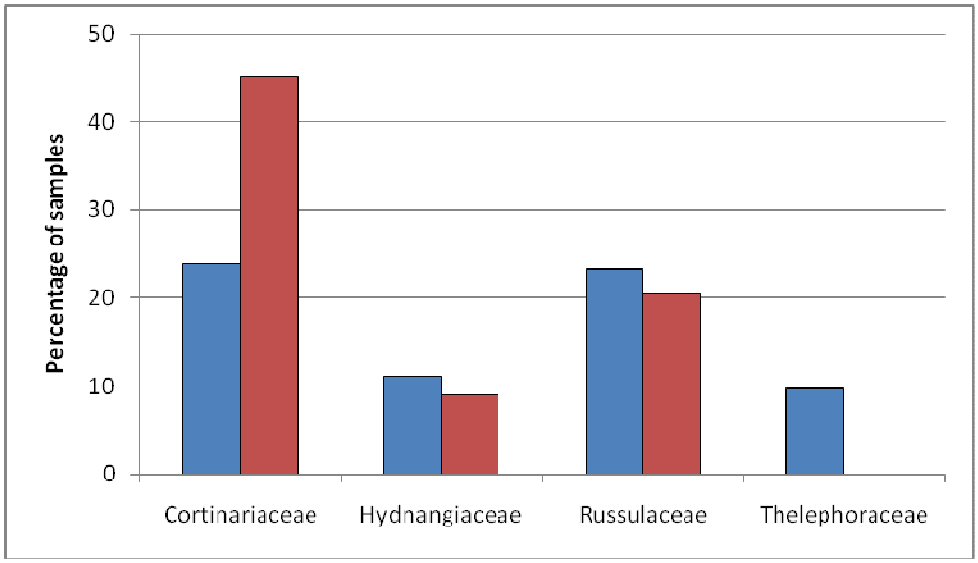

Figure 4.18 The percentage of root tip (blue) and sporocarp (red) samples from the four most frequently represented families.

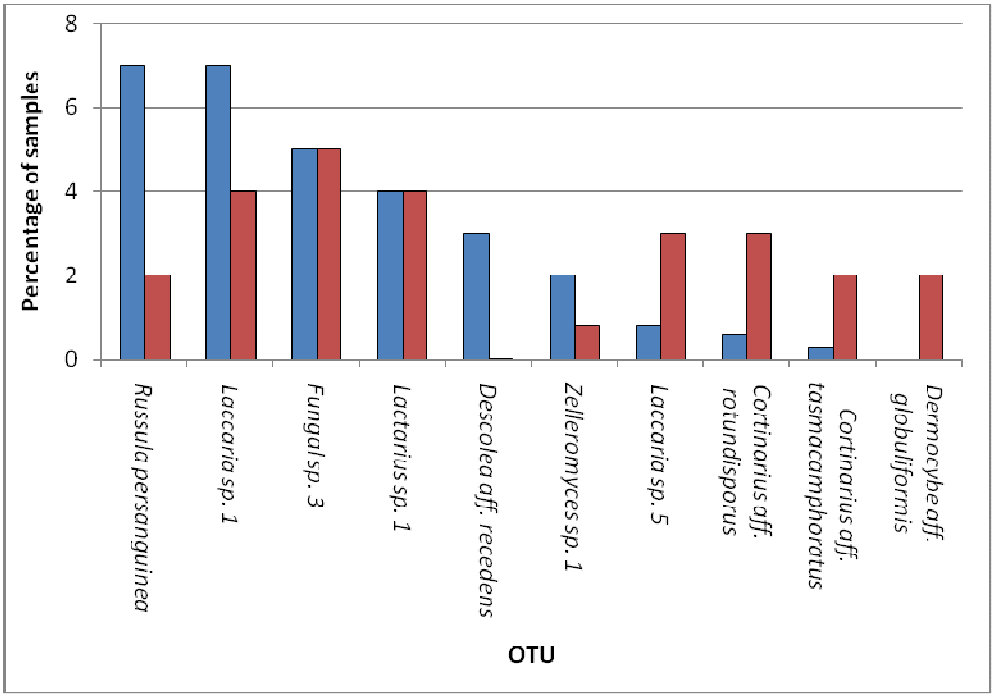

Figure 4.19 The percentage of samples of root tip (blue) and sporocarp (red) for the most abundant OTUs.

4.3.10 Ectomycorrhizal sporocarp and root tip community differences

Only 15% of observed OTUs were sampled as both sporocarps and root tips (Appendix 12). These OTUs were primarily from the Cortinariaceae (23 OTUs), Russulaceae (10 OTUs) and Hydnangiaceae (3 OTUs). OTUs that were observed as both sporocarps and root tips were not necessarily sampled as both from within the same plot. For example *Dermocybe* sp. 1 was sampled as a sporocarp in plot 6 and then detected in plot 10 as an ECM root tip.

The root tip community was more diverse at the level of order when compared to the sporocarp community (10 orders from root tips and 9 orders from sporocarps) whereas the sporocarp community was more diverse at the level of family (17 vs.12 families) and genus (23 genera from sporocarps and 15 from root tips), though this may be affected by the lower number of root tip samples, 493 vs. 672 sporocarp collections (Appendices 12, 20 and 22).

A larger proportion of Ascomycota OTUs were sampled from root tips than as sporocarps and a larger proportion of OTUs were unable to be identified to the level of family from root tips than from sporocarps (Figure 4.18). A notable ECM group that was not detected on root tips was the Amanitaceae. The Cortinariaceae made up a smaller proportion of root tips than sporocarp OTUs. Families that were not found as sporocarps but were detected on root tips included the Thelephoraceae and Sebacinaceae. The families of OTUs recorded from root tips and from sporocarps are compared in Figure 4.15.

4.3.11 Common, widespread and rare species

The majority of OTUs were only recorded in one plot, and many were only collected on one occasion. Seventy-four percent of root tip OTUs and 62% of sporocarp OTUs were found in only one plot (63% overall).

Descolea recedens, Laccaria sp. 1, and *Russula persanguinea* were the most widespread root tip OTUs and *Laccaria* sp. 1 was the most widespread sporocarp OTU (Table 4.2). These were all among the 10 most frequently sampled species (Figure 4.18). Overall (using combined data) the most widespread OTUs were *Laccaria* sp. 1 (found in all 12 plots), *Russula persanguinea*, *Lactarius* sp. 1 (both sampled from 10 plots) and *Descolea recedens (Cooke & Masse) Singer* (detected in 9 plots) (Table 4.2).

Table 4.2 Summary of OTUs that were widespread and sampled on multiple occasions. The maximum number of plots an OTU could be sampled from was 12. The maximum number of surveys was six for sporocarps, and three for root tips.

	Root tips		Sporocarp		Combined data
OTU	**no. plots**	**surveys**	**no. plots**	**surveys**	**no. plots**
Artomyces sp. 1	0	0	8	3	8
Cortinarius aff. *rotundisporus*	2	2	7	2	7
Descolea recedens	9	3	1	1	9
Laccaria sp. 1	9	2	9	6	12
Laccaria sp. 5	4	1	7	4	10
Lactarius sp. 1	6	2	10	6	10
Russula persanguinea	9	3	8	3	10

4.4 Discussion

4.4.1 Ectomycorrhizal species richness of Australian eucalypt forests

Studies of ECM forest communities are difficult to compare. This is not only due to inherent differences in forest floristic composition and structure, soils, topography, disturbance history, forest age and climate, but is also because of differences in experimental design such as sampling methods, sampling effort and the area of forest sampled. Despite this difficulty there are some similarities between ECM communities within Australian eucalypt forests (Glen 2001; Glen, Bougher *et al.* 2008; Tedersoo, Jairus *et al.* 2008; Gates 2009; Newbound 2009).

OTU richness as determined by root tip sampling within 3 ha of eucalypt forest ranges from 154 OTUs (from 140 soil cores) in jarrah forest to 120 OTUs in both *E. delegatensis* forest (120 soil cores) and 123 OTUs in southern Tasmanian *E. regnans* forest (45 soil cores) (Tedersoo, Jairus *et al.* 2008). Although species richness from root tips appears similar to other forest types the number of ECM fungi that are actively involved in symbioses in *E. delegatensis* forest is relatively greater in comparison to *E. regnans* forest (if differences in the sampling effort are considered, soil cores to sample *E. regnans* forest totalled 2 x volume sampled in

E. delegatensis forest). The species effort curves for *E. delegatensis* and *E. regnans* forest (Tedersoo, Jairus *et al.* 2008) clearly show that *E. delegatensis* root tip richness was estimated to be similar to *E. regnans* forest for the same sample effort, although volume of soil cores differed. For *E. delegatensis* forest Jacknife 1 estimated an average of 39 OTUs per 10 soil cores giving an estimate of 156 OTUs for 40 cores and 212 OTUs for Jacknife 1 (average was 53 OTUs per 10 cores). For *E. regnans* root tip species richness Jacknife 2 estimated 210 OTUs for 40 soil cores. These differences may be due to other sampling differences such as time of year, and times sampled, but may also be because forest age, the number of host species and past disturbances differ. The *E. regnans* forest sampled formed part of a national park and had not been disturbed for at least 100 years. Also, the rainforest/sclerophyll forest /grassland boundaries are much more dynamic for *E. delegatensis* forest than for *E. regnans* forest, which may contribute to differences in ECM species richness between forest types (Ellis 1985; Fensham & Kirkpatrick 1992).

Australian studies that have surveyed only sporocarps from eucalypt forest have also shown a high ECM species richness (Johnson 1994; Lu, Malajczuk *et al.* 1999; Claridge, Barry *et al.* 2000; Bougher & Lebel 2001; Gates, Ratkowsky *et al.* 2005; Ratkowsky & Gates 2005; Gates 2009; Newbound 2009). For example, Gates (2009) observed 330 ECM species as sporocarps in southern Tasmanian *E. obliqua* L'Her. forest and Johnson (1994) recorded 23 species of hypogeous sporocarps from *E. tenuiramis* Miq. forest in south-eastern Tasmania (27 were found in this study).

Only one other study of Australian eucalypt forest has sampled both ECM root tips and sporocarps. Total species richness observed in *E. delegatensis* forest was lower than for the same area of Western Australian jarrah forest surveyed in that study (Glen, Bougher *et al.* 2008).

The 120 root tip OTUs sampled in this study represents 17% of the total known fungi (700 species) that form ectomycorrhizas with *Eucalyptus* (Tommerup & Bougher 1999), but it is likely that some OTUs recorded in this study are undescribed species. When considering the number of ECM OTUs that are thought to occur in Australia (6500 species, Bougher 1995; Neale Bougher pers. comm. 2009), the 259 OTUs recorded in *E. delegatensis* forest represent 4% of the total estimated Australian ECM species, which is large for such a small study area.

Also the trajectories of the species-area and species-effort curves and the Jacknife 1 estimations of species richness, taking into account the high number of singletons, indicate that many more ECM OTUs are likely to be present that were not sampled. These OTUs may have been missed because of insufficient sampling (i.e. not enough time spent surveying, and not enough soil cores collected to ensure that spatially and temporally variable OTUs were recorded). Furthermore, the low overlap in OTUs between the sample years suggests that sampling over long periods is necessary in order to capture the diversity of the communities. It is also possible that some ECM roots were missed from the soil cores that were collected, and that some of the more inconspicuous sporocarps were missed during surveying.

The ECM fungal diversity of these Australian eucalypt forests is similar to other southern hemisphere forests such as New Zealand *Nothofagus* forest (Dickie 2009 yielded 140 OTUs (from root tip T-RFLP profiles). In contrast, many northern hemisphere coniferous forests appear to have lower fungal diversity (Visser 1995; Dahlberg, Jonsson *et al.* 1997; Stendell, Horton *et al.* 1999; Horton & Bruns 2001 see Table 2; Peay, Bruns *et al.* 2007; Twieg, Durall *et al.* 2007; Twieg, Durall *et al.* 2009), as do some Australian eucalypt forests (McMullan-Fisher, May *et al.* 2002; Packham, May *et al.* 2002; Newbound 2009).

The differences in species richness between *E. delegatensis* and other Australian eucalypt and northern hemisphere forests could be due to a number of reasons such as host species, edaphic and climatic factors, sampling effort and area, topography and disturbance history. For example Newbound's (2009) study focused on urban remnants, which are likely to have greater disturbance than other forests, and the study by McMullan-Fisher, May *et al.* (2002) relied on only sporocarp samples.

4.4.2 Differences in species richness of ectomycorrhizal communities determined by sampling of sporocarps or root tips

Root tip ECM diversity is generally thought to be higher than sporocarp diversity (Dahlberg 2001). ECM root tip observed richness was not greater than sporocarp richness in this study, although this is based on only ten soil cores from each plot. Only one plot had greater root tip richness than sporocarp richness. It is interesting that this plot also had the lowest plant species richness. Also there was no variation in the age of the dominant trees suggesting that

disturbance of this plot was minimal, or has been absent for a long time. The high root tip diversity may be related to succession and forest maturity. Lack of competition from regenerating, or understorey plant species, may allow the dominant ECM plant species, in this case *E. delegatensis* and *N. cunninghamii*, to produce a greater abundance of fine roots, allowing a greater diversity of ECM fungi to colonise root tips. This would mean that ECM root tips would be prevalent throughout the root system, and not only within the canopy drip line. If ECM root tips were generally found only within the canopy drip line, then ECM root tip richness would decline with tree age/maturity as the perimeter: area ratio of the canopy drip line decreased. It is possible that the sampling constraint to collect cores from within the drip line has contributed to the reduced root tip richness compared to sporocarp richness. Mineral nutrients within this zone will likely have already been exploited by mature trees and this area is therefore less likely to contain fine roots where mycorrhizas form.

Lower ECM root tip richness does not necessarily mean there will be fewer root tip ECM OTUs present, just that sampling may not have recorded all OTUs. The high spatial variability of mycorrhizal root tips that results in some cores containing no root tips, and the high number of cores required to sample the below-ground community sufficiently, highlight the difficulties in attempting to characterise the root tip community. It is well established that the distribution of mycorrhizal species is clustered and so shifting the location of many soil cores just a few centimetres may give a completely different view of species diversity (Stendell, Horton *et al.* 1999; Horton & Bruns 2001). Furthermore, the importance of sampling a large number of cores is seen in the case where soil cores may contain multiple species, while generally a single core tends to be dominated by only one or two species (Dahlberg, Jonsson *et al.* 1997; Stendell, Horton *et al.* 1999; Horton & Bruns 2001). Also, single cores often sample the same genet and so are not independent samples.

In a review of a number of ECM community studies, Dahlberg (2001) estimated that species forming macroscopic sporocarps make up only 10-30% of mycorrhizal OTUs. Peter, Ayer *et al.* (2001a) found that 22% of ECM detected in sporocarp surveys were also found as ECM root tips. If that is the case, and the 178 species sampled as sporocarps from this study make up 30% of the species that form mycorrhizas in this forest, we would expect a total estimation of 590 ECM taxa on root tips. This estimate is far greater than any computed Jacknife 1 estimates for root tip species richness (which was 202) and if true would represent a highly diverse ECM

community, which might be expected considering the range of vegetation types and the geographic range sampled. While providing additional information about taxa that were not sampled as sporocarps, sampling soil to study ECM fungi (Chapter 3) falls short of what is needed to provide reliable estimates of actual species richness.

The overlap between species that form mycorrhizas and species that form sporocarps varies among plots. Although methodological bias, such as different levels of sampling effort for root tips and sporocarps, may play a part in this, the extent of this overlap may also be because of the health of individual species. Furthermore, some fungal species fruit frequently and abundantly, while others fruit rarely. Environmental factors such as temperature and humidity may play a role in determining fruiting of species. The extent of overlap may also be determined by competition for space and resources by ECM species. Some of these reasons are discussed thoroughly below.

4.4.3 Determinants of ectomycorrhizal species richness

Differences in ECM species richness among plots in this study may partly be caused by differences in vegetation composition among the plots. The presence or absence of various ECM plant species such as *Nothofagus cunninghamii* may play a role in shaping the diversity of the ECM communities. ECM species diversity may also be related to plant host densities, or the number of living trees, as well as tree age and their health status. A degree of host specificity has been reported for a number of fungal species (Bougher, Grove *et al.* 1990; Molina, Massicotte *et al.* 1992; Ishida, Nara *et al.* 2007; Taylor 2008; Tedersoo, Jairus *et al.* 2008). In Tasmania, many dominant species of ECM fungi were found to have preference for host plant, including ECM species that had a preference for *E. regnans* (Tedersoo, Jairus *et al.* 2008). A linear pattern between the number of host species and estimated ECM richness, and the number of living trees and observed species richness, has been found in other forest communities (Dickie 2007; Gates 2009). The relationships between plant species richness and host species richness were explored in this study but no strong correlation between ECM species richness and either host species richness or plant species richness was observed. This may be because of the low overall diversity of ECM host plant species. Strong positive relationships between overstorey tree diversity and mycorrhizal diversity have been recorded (Kernaghan, Widden *et al.* 2003)and both plant productivity and plant diversity are known to

be positively correlated to mycorrhizal diversity, although this may be dependent on a number of factors (Baxter & Dighton 2001; Jonsson 2001; Kernaghan 2005; van der Heijden, Bardgett *et al.* 2008). The relationship between ECM communities and plant communities is explored fully in Chapter 5.

Succession in fungal assemblages is known to occur following disturbances, such as fire and timber harvesting (Visser 1995; Jones, Durall *et al.* 2003; Twieg, Durall *et al.* 2007; Dickie 2009) and has also been proposed to occur with forest-stand age (Dighton & Mason 1985; Dighton, Poskitt *et al.* 1986). Species associated with particular successional stages have been proposed (Bruns 1995). Early successional fungi tend to have a ruderal strategy (r-selected), where-as late successional fungi are stress tolerant or combative (S-selected or C-selected) (Bruns 1995). Late successional fungi are thought to require both host carbohydrates and nitrogen, than early successional fungi (Bruns 1995). Species such as those of *Laccaria* and *Inocybe* (Fr.) Fr. reportedly occur in the early stages of succession, *Cortinarius* and *Tricholoma* (Fr.) Staude at intermediate stages, while species of *Amanita* Pers., *Leccinum* Gray and *Russula* are primarily late-stage succession species (Dighton and Mason 1985), although this view of early- and late-stage fungi is often regarded as an over simplification (e.g. Twieg, Durall *et al.* 2007). This study did not find any evidence of succession occurring with forest age at the genus level. Species that are traditionally thought of as being early-successional, such as *Laccaria*, were found in all plots, and species that are regarded as late-successional fungi, such as *Russula* and members of the Boletaceae (most closely matching *Leccinum*), were recorded in plots that were disturbed c. 40 years ago. *Amanita*, traditionally a late-successional species, was found in both long undisturbed plots and the most recently disturbed plots.

Fungal succession within ECM communities may be important in determining ECM richness, with changes in forest resources, such as host species and soil nutrients, influencing the number of species that can be supported. Dighton and Mason (1985) proposed that ECM richness decreased in late successional forests and this relationship has been observed by Gates (2009) in Tasmanian *E. obliqua* forest. Other Australian studies found ECM richness to increase with time since rehabilitation following mining up to 16 years of age (Gardner & Malajczuk 1988; Glen, Bougher *et al.* 2008). Interestingly in this study of *E. delegatensis* forest, the number of ECM species, and especially the number of Cortinariaceae species, was highest in plots that had the most recent disturbance (the north-west plots were partially harvested c.

25 years ago). Similarly, Gates (2009) found that the largest number of ECM species, especially *Cortinarius*, was shared between the youngest *E. obliqua* forest plots, but not the mature plots. Although no linear relationship was found between ECM species richness and stand-age or time since disturbance, a polynomial correlation between ECM richness and time since disturbance was found in this study. From 20 to 80 years post disturbance there is a decline ECM richness, which is then followed by increasing in ECM richness with time. The polynomial regression between ECM richness and time since disturbance indicates that factors other than time since disturbance influence ECM richness. It is possible that initial increases are due to disturbance which shifted the forest to sclerophyll understorey where *E. delegatensis* is the main host, but as the community ages the ECM diversity declines until there is another shift in the community to rainforest, resulting in an increase in host diversity (albeit small), which may then increase ECM diversity. The curvilinear relationship may also be an artefact of the pair plot design (both of the final plots causing a polynomial relationship rather than a linear relationship were paired replicates). The number of individual hosts may also be important. At the point of eucalypt decline there may be reduction in ECM host density which is replaced by a rapidly developing *Nothofagus* stand. Other Myrtaceous plants may support similar mycorrhizas to *E. delegatensis* than those of *Nothofagus* which may imply that the establishment of *Nothofagus* leads to the establishment of ECM fungi that are incompatible with eucalypts, which could contribute to the decline of eucalypt forest. The reasons for changes in ECM richness following disturbances may also relate to availability of forest resources during succession, and other ecological interactions such as competition. Also, the plots in the north-east and north-west experienced different disturbances (fire on north-east and forestry in north-west) which may not produce entirely comparable affects considering inherent differences in soil fertility. Furthermore this pattern may also reflect regional forest types differences in OTU richness independent of disturbance history.

One of the most widely accepted explanations for ECM diversity is niche differentiation. Niche theory argues that by occupying distinct ecological niches within a site, multiple fungal species are able to co-occur (Bruns 1995; Dickie 2007). For example higher plant diversity is likely to create heterogeneous litter inputs, which may create opportunities for niche differentiation by ECM fungi (Reddell & Malajczuk 1984; Wardle 2006). It is probable that ECM fungi within *E. delegatensis* forest have different ecological roles differing in nutrient uptake and transfer

abilities, therefore partitioning forest resources (Bruns 1995; Peay, Kennedy *et al.* 2008a). Agerer (2001) suggests that root tip morphology, and other morphological characters of a species, can give insight into these functional roles. For example extensive hyphae and rhizomorphs are common in *Cortinarius* suggesting they are involved with exploration of soil nutrients and medium distance nutrient or water transfer. Although there is no evidence that high species richness indicates functional diversity, the high taxonomic diversity of ECM fungi, seen in this study, may reveal functional heterogeneity (Dickie & Moyersoen 2008). Forests that have greater ECM diversity are thought to have greater stability and resilience to ecosystem disturbance (Bougher 1995) and in the case of *E. delegatensis* forest, may be better placed to cope with and recover from decline.

Other than changes over long time frames, temporal partitioning throughout the year is likely to play a role in enabling the occurrence of diverse ECM communities. This study found no difference in ECM richness of root tips throughout the year, but did not examine the turn-over of species within the community throughout the year. Other studies have shown that ECM root tip diversity responds to host growth (Ashton 1976; Malajczuk & Hingston 1981), but this was not obvious here, perhaps because not enough samples were taken from different months/seasons. Sporocarp richness did differ throughout the year, being higher in the late autumnal and early winter months. In the northern hemisphere, species groups with different temporal patterns have been identified in *Pinus* plantations (Koide, Shumway *et al.* 2007). The proposed causes for temporal partitioning of ECM species include interactions among species, species' physiological tolerances, temporal environmental variability, temporal patterns of root production, and variation in fungal genet lifespan (Johnson 1994; Koide, Shumway *et al.* 2007).

Alternative explanations for the high ECM species richness seen in *E. delegatensis* forest include non-equilibrium theory where stochastic colonisation events, compensatory networks of ECM fungus species competitive abilities, and abiotic limitations may act to determine ECM community richness. Dispersal strategies adopted by different ECM species are also likely to play an important role in determining ECM richness and composition within these forests (Peay, Bidartondo *et al.* 2010).

A further discussion on the differences and similarities among the plots, and some of the factors that shape the ECM community, is provided in Chapter 5. In Chapter 5, vegetation

competition, soil nutrients, foliage nutrients and tree health are considered in the context of their influence on the composition and structure of the ECM community.

4.4.4 Diversity of Australian ectomycorrhizal fungi

The main ECM taxonomic groups known to occur in Australia (Brundrett 2008) were sampled in this study. The majority of OTUs recorded in this study from sporocarps and root tips belonged to the Cortinariaceae, Russulaceae, and Thelephoraceae. Twenty-five percent of root tip samples were from the Cortinariaceae, 23% from the Russulaceae, 11% from the Hydnangiaceae and 9% from the Thelephoraceae. The *Cortinarius*, *Russula-Lactarius*, and *Laccaria* lineages were also the most species rich and abundant lineages in other eucalypt and *Nothofagus* dominated Australian forests (Tedersoo, Jairus *et al.* 2008; Tedersoo, Gates *et al.* 2009). Members of the Sebacinaceae, namely *Sebacina*, were also recorded, and *Sebacina* spp. have been shown to be associates of Australian *E. marginata* (Glen, Tommerup *et al.* 2002), and *E. regnans* forests (Tedersoo, Jairus *et al.* 2008).

The few ECM taxonomic groups that were not sampled in this study include the Basidiomycota groups Hygrophoraceae and the Atheliales (Brundrett 2008). While every effort was made to ensure that only distinctively ECM roots were sampled (i.e. using morphological characteristics such as the presence of a Hartig Net), it is possible that some of the OTUs that were not identified to genus or species are not true ECM associates. Many species have been erroneously reported as ectomycorrhizal when they may actually be secondary root colonists that associate with ECM symbionts (Tedersoo, Pärtel *et al.* 2009).

Globally distributed genera such as *Cortinarius*, *Clavulina*, *Lactarius*, *Laccaria*, *Russula* and *Tomentella* were well represented in this forest and contributed significantly to overall diversity. Similar results were found by Tedersoo, Jairus *et al.* (2008) and Dickie and Moyersoen (2008) comment on the unremarkable nature of the fungal diversity of *E. regnans* forest when considering the biogeography of ECM fungi. They consider widespread genera as having broad-scale generic ecological niches (Dickie & Moyersoen 2008).

When considering ECM diversity at the species level, it is likely that many of the species from these globally distributed genera are endemic to Australia. May and Simpson (1997) collated information of the degree of endemism in common genera , which showed that of the eight

species of *Laccaria* present in Australia, five were endemic, 11 of the 14 *Dermocybe* species present in Australia are endemic and seven of the eight *Cortinarius* subgen. *Myxacium* are endemic. The low DNA sequence similarities of many OTUs to sequences in public databases, which are biased toward northern hemisphere species, indicate that these may represent novel, endemic species.

The majority of hypogeous taxa found in Australia are also endemic (Castellano & Bougher 1994; Bougher & Lebel 2001). Hypogeous OTUs made up a significant proportion (17%) of the diversity within *E. delegatensis* forest and represented a variety of ECM families. This result highlights the diversity of Australian hypogeous fungi, which play an important ecological role in Australian eucalypt forests by providing food for mycophagous animals, which disperse their spores (Claridge 2002). Four hypogeous OTUs had low DNA sequence similarity to public database sequences and may represent new species, genera, or even families. Interestingly, hypogeous OTUs made up a larger proportion of ECM diversity in four of the north-east plots than other plots. The north-east plots were much warmer and drier than the north-west plots (Figure 4.11), conditions that are conducive to hypogeous fruiting. Other factors that influence hypogeous fruiting are topographic position, soil fertility, time since last fire, vegetation and micro-habitat structures (Claridge, Barry *et al.* 2000).

The sporocarp samples and DNA sequences obtained from this study provide the resources to study and formally describe OTUs that are likely to represent novel species. Representative sporocarp collections will be deposited into the Tasmanian herbarium, and those species that have multiple collections will also be deposited into the Melbourne Botanic Gardens herbarium. DNA sequences will be submitted to a DNA sequence database to be made publicly available.

4.4.5 Differences in ectomycorrhizal diversity between sporocarp and root tip sampling

Sampling of sporocarps and root tips provides complementary information on the ECM community (Gardes & Bruns 1996; Dahlberg, Jonsson *et al.* 1997; Peter, Ayer *et al.* 2001a). The most notable difference between taxa detected on root tips and those collected as sporocarps are the absence of the Thelephoraceae and the Sebacinaceae from the sporocarp data set.

These differences may partly be because some ECM fungi do not reproduce sexually or do not produce macroscopic sporocarps e.g. *Cenococcum* Moug. & Fr. and *Sebacina* spp. Those that do reproduce sexually may produce sporocarps rarely, or produce sporocarps at times that were not sampled. The different sporocarp OTUs recorded between the two years sampled in this study provide evidence for temporal variability and the cryptic nature of many fungi. Other sporocarps may be inconspicuous and therefore collected less frequently e.g. Thelephoraceae, Corticiaceae, and species that produce hypogeous sporocarps. Differences in sample sizes and sampling effort for sporocarps and root tips may also contribute to the differences in the data provided by the different sampling methods. For example, above ground sporocarps were obvious and easier to observe than root tips or hypogeous sporocarps. Also, more of the OTUs that were recorded from above ground sporocarps matched sequences available on public databases.

A greater number of Ascomycota OTUs and unknown taxa were sampled as root tips than sporocarps. It is likely that some of the unknown OTUs from this study belong to the Ascomycota and possibly the Helotiales. Some root tip samples (9 OTUs) had highest sequence similarity to sequences derived from ericoid mycorrhizas in NCBI BLAST searches (Appendix 12). A number of OTUs were found to be from the Helotiales. Many members of the Helotiales have been recognised as putatively ectomycorrhizal and are phylogenetically related to root endophytes and ericoid mycorrhizal species (Tedersoo, Pärtel *et al.* 2009), though recent work indicates that some members of this lineage of Ascomycota are ectomycorrhizal-associated fungi rather than true ECM symbionts (Tedersoo, Pärtel *et al.* 2009). Although the Helotiales lineage has been reported as forming ECM and arbutoid mycorrhiza in the northern hemisphere (Rosling, Landeweert *et al.* 2003; Tedersoo, Koljalg *et al.* 2003; Tedersoo 2007), Tedersoo, Jairus *et al.* (2008) regard this group as rare in the northern hemisphere (except for *Meliniomyces bicolor* Hambl. & Sigler) and their presence in Australian forests may be a unique characteristic.

Amanita was not detected on root tips at all although it is a prominent ECM genus in Australia (Castellano & Bougher 1994). This is possibly because of vertical niche differentiation, with *Amanita* spp. residing lower in the soil profile (Dickie, Xu *et al.* 2002). Soil cores in this study were only 10 cm deep so species preferentially occupying soil beneath 10 cm may have been

missed. Alternatively, *Amanita* spp. may simply have been missed because of insufficient sampling.

4.4.6 Rare ectomycorrhizal species

Many ECM community studies, and other fungal studies, have found that the majority of species encountered are rare and very few species have a high or widespread abundance (Dahlberg 2001). This study is no exception to the rule with the majority (63%) of ECM taxa recorded only once. Tedersoo, Jairus *et al.* (2008) found that 55% of root tip taxa were only recorded once and Glen (2001) found 70% of ECM taxa were recorded in only one soil core. In a study of macro-fungal sporocarps 37% of species were singletons (Gates 2009). Greater sampling intensity may lower the percentage of singletons recorded as it would increase the probability that a species is detected, sampled greater than once, or sampled in greater than one core (Taylor 2002). It is possible that the high number of rare species is a sampling effect but likely that this pattern of rarity is a true reflection of species abundance. Neutral models of community ecology may explain this community structure and the occurrence of rarity (Hubbell 2001). This theory assumes that species within the same trophic level are essentially equivalent in their potential success, and abundances of species are determined stochastically within the confines of a fixed sized community or a determined number of individuals. Dispersal limitation may play a key role in determining the abundance of any species within the community (Peay, Bidartondo *et al.* 2010).

4.4.7 Common ectomycorrhizal species

Few taxa were recorded frequently but by examining taxa that were recorded in more than one plot, and at multiple times of the year, some taxa appear to be relatively common in *E. delegatensis* forest. Common OTUs included *Russula persanguinea*, *Lactarius* sp. 1, *Descolea recedens* and *Laccaria* sp. 1. It is likely that some of the OTUs recorded in *E. delegatensis* forest are the same species that were recorded by Tedersoo, Jairus *et al.* (2008) in *E. regnans* forest, and that these species are widespread throughout eucalypt forest in Tasmania. Gates (2009) also found *Lactarius eucalypti* O.K. Mill & R.N. Hilton and *Laccaria* spp. to be common and present as sporocarps in mature *E. obliqua* forest throughout the year in southern Tasmania

and *Descolea recedens*, *Russula persanguinea* and *Cortinarius* aff. *rotundisporus* were among the mostly frequently recorded species within this forest.

Species such as *Russula persanguinea* and *Lactarius eucalypti* are also common in other forest types on mainland Australia, and are most likely to be true generalists. Australian fungal species generally have a less restricted distribution than plant species and for eucalypt ECM fungi this may be because of their greater tolerance for functioning under a wider range of conditions (Bougher 1995). Thus, many ECM species that occur in Tasmania may also be present on mainland Australia.

It is interesting to note that despite the high diversity and abundance of the Cortinariaceae, many of these OTUs were infrequently encountered. Only two species, *Descolea recedens* and *Cortinarius* aff. *rotundisporus,* were recorded in more than half of the plots. *Descolea* is thought to have originated as an associate of Gondwanan plants and its distribution in the southern hemisphere is related to the disjunct distribution of *Nothofagus* (Bougher 1995) includling Tasmania where *Nothofagus* is widespread in temperate rainforest and mixed-forests.

4.4.8 The diversity and dominance of the Cortinariaceae in ectomycorrhizal communities

The Cortinariaceae were the most diverse family, and dominated the overall community composition, as determined both from sporocarps and root tips (46 % of all OTUs) with the genus *Cortinarius* having the highest number of species (33 % of all OTUs). Furthermore, almost a quarter (24 %) of all root tip samples were identified as belonging to the Cortinariaceae. Tedersoo, Jairus *et al.* (2008) found a similar result with *Cortinarius* being the most species-rich lineage detected on root tips in a southern Tasmanian *E. regnans* forest (making up 19% of all OTUs recorded). Macrofungal studies have also shown the Cortinariaceae to have high diversity in Tasmanian eucalypt forests (Gasparini 2004; Ratkowsky & Gates 2005; Gasparini 2007; Gasparini & Soop 2008; Gates 2009).

Cortinarius has a nearly global distribution with greater than 2000 described species having multiple lineages (Peintner, Moncalvo *et al.* 2004). Approximately 95 species of described *Cortinarius* are known to occur in Australia (www.rbg.vic.gov.au/dbpages/fungi/cat). Considering 94 *Cortinarius* OTUs were recorded in this study, many of which did not have high sequence similarity to any of the described and identified fungi with ITS sequences in public

DNA databases, it is highly likely that many of these OTUs represent undescribed species. Gates (2009) found that many of the fungal species recorded in Tasmanian *E. obliqua* forest were also previously undiscovered and undescribed and the majority of these new species belonged to the genus *Cortinarius*.

There have not been any studies on the importance of *Cortinarius* as ECM symbionts in southern hemisphere forests (Chambers, Sawyer *et al.* 1999) although Malajczuk *et al.* (1987) state that *Hysterangium* Vittad. and *Cortinarius* are a dominant component of the macro-fungal community of Western Australian eucalypt forests and their hyphae occupy 10% of the surface area. In Tasmanian *E. regnans* forest *Cortinarius* comprised 10.9% of colonised root tips (Tedersoo, Jairus *et al.* 2008) and in a Victorian *Nothofagus* forest 15% of root tips were colonised by *Cortinarius* (Tedersoo 2007). The Cortinariaceae are also among the most frequently recorded family in northern hemisphere temperate forests. The Cortinariaceae occupied 13% of the sampled root tips in a 65 year old stand of Jack Pine forest (Visser 1995), 14% of ECM roots within a Norway Spruce forest (Kåren, Hogberg *et al.* 1997) but < 5% of North American pine forests (Gardes & Bruns 1996; Taylor 2002).

Although the Cortinariaceae are frequently recorded in temperate forests, they are not necessarily the most abundant family. Ectomycorrhizal roots in mature northern hemisphere forests tends to be dominated by the Thelephoraceae and Corticiaceae rather than the Cortinariaceae (Stendell, Horton *et al.* 1999; Dahlberg 2001). Dahlberg (2001) reviewed the mycorrhiza literature in relation to abundance of ECM taxa and found that members of the Cortinariaceae were the most abundant in only 3 of 49 studies (two coniferous boreal forests and one tropical Indonesian forest) whereas the Russulales were most abundant in 14 studies in coniferous forests, and the Thelephoraceae most abundant in 17 studies (one broadleaf deciduous forest and the remainder were coniferous forests). In an *E. regnans* forest a single *Laccaria* species and *Lactarius eucalypti* were the most abundant colonisers with 44.4% and 42.2% root colonisation respectively, whereas *Cortinarius* spp. colonised 10.9% of roots (Tedersoo, Jairus *et al.* 2008).

The high species diversity, community dominance as determined by sporocarp and root tip sampling and widespread occurrence of members of the Cortinariaceae throughout Tasmania suggest that this family plays an important role in the ECM community of Tasmanian eucalypt

forest (Tedersoo, Jairus *et al.* 2008; Gates 2009), and probably other Australian eucalypt forests. Additional research on the ecology of the Cortinariaceae in Australian eucalypt forests is required to establish their exact roles and importance.

4.4.9 Challenges to identifying Australian ectomycorrhizal fungi

Many OTUs from this study had only low DNA sequence similarity to sequences in public databases, though the most similar sequence was often from a northern hemisphere species. The major limitation to this study, and other molecular studies, is the lack of southern hemisphere reference sequences available for comparison (Ryberg, Kristiansson *et al.* 2009). Even though there are more northern hemisphere sequences available, the majority of fungal species globally are still yet to be sequenced (Ryberg, Kristiansson *et al.* 2009). It is likely that many of the taxa from this study have been recorded for the first time, and of those that are not novel, many would not have been formally described or sequenced previously. The potentially high incidence of novel species is not surprising considering the high rate of newly discovered species in other Australian studies. Few sequences of Australian ECM taxa are publicly available and this study makes an important step in obtaining sequence data for Australian ECM taxa. The numerous sequences obtained from *Cortinarius* and other members of the Cortinariaceae in this study provides genetic information for a number of southern hemisphere members that may assist in resolving these complex phylogenies (Peintner, Moncalvo *et al.* 2004). Additional sequencing of known Australian species and herbarium collections are required before DNA sequences can provide confident identification of ECM species in Australia, as many sequences available in public databases are from inadequately identified and catalogued specimens (Ryberg, Kristiansson *et al.* 2009) and are from the northern hemisphere.

Determining an appropriate sequence divergence cut-off for operational taxonomic units that corresponds to traditionally recognised species is one of the main challenges to molecular studies (O'Brien, Parrent *et al.* 2005; Hughes, Petersen *et al.* 2009). No percentage sequence similarity can determine con-specific taxa across all taxonomic groups because the range of intra-specific variation differs among species (Hughes, Petersen *et al.* 2009). Determining the similarity cut-off is particularly challenging when species do not conform to traditional notions of species units, such as many members of the Cortinariaceae, which have cryptic morphology,

high inter-specific similarity (Glen, Tommerup *et al.* 2001b), high intra-specific polymorphism (Kåren, Hogberg *et al.* 1997; Chambers, Sawyer *et al.* 1999), and many appear to be part of species complexes. Further work using combined morphological and molecular characteristics, especially on difficult species, such as members of *Cortinarius,* is required to confirm intra- and inter-specific morphological and molecular variation and determine species delineations.

4.5 Conclusions

This study is one of the first field-based studies of ECM eucalypt forest communities in Australia. The ECM community of Tasmanian *E. delegatensis* forest is diverse, showing an extraordinary richness within the Cortinariaceae family, which dominated the community. Other important ECM lineages were the Russulaceae and Thelephoraceae. Hypogeous taxa made up a significant proportion of ECM diversity.

It is likely that novel taxa have been recorded from this study. Few taxa were widespread or frequently recorded. OTUs that were widespread and frequently recorded as both sporocarps and root tips are likely to be generalists. This includes *Russula persanguinea*, which had the highest percentage of root tips samples, *Lactarius* sp. 1, *Laccaria* sp. 1 and Fungal sp. 3.

This study is also one of few studies that have explored Australian ECM communities by sampling both sporocarps and ECM root tips. Differences between the data obtained by sporocarp collection and root tip sampling highlights the importance of sampling from different structures to get a comprehensive understanding of the ECM community.

The diversity of *E. delegatensis* forest ECM communities is likely to be influenced by a number of factors including the vegetation community, past disturbances, climate and soil. These influences on the ECM community are explored in more detail in Chapter 5.

The ECM community of *E. delegatensis* forest shared many similarities with other eucalypt communities within Australia e. g. Tasmanian *E. regnans* and *E. obliqua* forests, and *E. marginata* forest in Western Australia. These eucalypt forests are unique and distinct from northern hemisphere forests by their high diversity, and dominance of the Cortinariaceae.

Numerous DNA sequences and herbarium specimens from this study will provide a valuable resource for further molecular and morphological studies, and make a significant contribution to the advancement of our understanding in the diversity of southern hemisphere ECM fungi.

5.0 The ecology of ectomycorrhizal communities of declining temperate *E. delegatensis* forest

5.1 Introduction

Eucalypt forests dominate much of the temperate region of Australia, typically growing in high rainfall areas (>1000 mm) on infertile soils where nitrogen and phosphorus are limiting to plant growth (Adams & Attiwill 1986; Adams 1996; Richardson, Hocking *et al.* 2009). To enhance the uptake of limiting nutrients, many Australian eucalypt forest plants have adopted specialised nutrient acquisition strategies that enable them to survive in environments of low fertility (Lamont 1983; Brundrett & Abbott 1991; Pate 1994; Lambers, Raven *et al.* 2008; Brundrett 2009). These strategies are facilitated by a range of specialised root structures such as extremely dense fine root mats (cluster roots) that develop in surface soils by plants of the family Proteaceae among others, which aide re-absorption of phosphorus and micro nutrients from decaying litter (Sousa, Facanha *et al.* 2007 and references therein). Leguminous nodules are another specialisation which allow nitrogen-fixation by symbiotic rhizobia bacteria within specialised root structures of members of the Fabaceae *sensu lato* such as *Acacia* (Lafay & Burdon 1998; Thrall, Slattery *et al.* 2007). Nutrient acquisition strategies also include plant-fungus symbioses such as mycorrhizal associations that facilitate the uptake of phosphorus, nitrogen and immobile nutrients (Bolan 1991; Read 1992; Marschner & Dell 1994; Read 1996; Jones, Durall *et al.* 1998; Aerts 2002; Müller, Avolio *et al.* 2007; Alvarez, Huygens *et al.* 2009; Brundrett 2009; Jones, Grenon *et al.* 2009). These specialised strategies are important for the process of nutrient cycling within eucalypt forest ecosystems by aiding the release and transfer of nutrients that would otherwise be unavailable to plants (Adams & Attiwill 1986; Tommerup & Bougher 1999; Sousa, Facanha *et al.* 2007; Craine, Elmore *et al.* 2009).

Another component of Australian eucalypt forests that is important for the ecosystem process of nutrient cycling is fire. Fire combusts organic material, mobilising nutrients that have been locked in plant biomass, and heats the soil, modifying soil chemistry and microclimate (Raison

1980; Ellis, Lowry *et al.* 1982; Grove, O'Connell *et al.* 1986; Attiwill & Leeper 1987; Attiwill, Polgase *et al.* 1996; Certini 2005; McIntosh, Laffan *et al.* 2005; Turner, Lambert *et al.* 2008). Fire improves the availability of nutrients to plants but also causes loss of nutrients from the system through volatilisation, leaching and erosion (Raison 1980; McIntosh, Laffan *et al.* 2005). In Tasmanian mixed eucalypt/rainforest following fire, carbon, nitrogen and phosphorus are released and soil pH, available calcium, magnesium and zinc all increase (Ellis & Graley 1983). The impact of fire on the mobilisation of nutrients and the regeneration of the biota will depend on properties of the fire, of which intensity is most important (Certini 2005). Many Australian plants are adapted to particular fire regimes, having mechanisms to resist, survive or regenerate following fire (Gill, Grove *et al.* 1981; Buhk, Meyn *et al.* 2007), such as the lignotubers and epicormic buds found in members of the Myrtaceae (Burrows 2008). Some plants even require fire for successful reproduction (Gill, Grove *et al.* 1981; Burrows & Wardell-Johnson 2003). Furthermore, particular fire regimes are required for the maintenance of floristic and structural diversity of many Australian vegetation communities (Williams, Whelan *et al.* 1994; Burrows & Wardell-Johnson 2003; Russell-Smith, Stanton *et al.* 2004; Fisher, Loneragan *et al.* 2009; Pyke, Brooks *et al.* 2010).

High altitude *Eucalyptus delegatensis* forest is widespread and economically important in Tasmania (Ellis, Ratkowsky *et al.* 1987; Ellis & Lockett 1991; Ellis 1995; Neyland & Cunningham 2004, see Chapter 1 for further discussion of Tasmanian E. delegatensis forest). In *E. delegatensis* forest managed for timber production, fast growth is a highly desirable trait and is achieved where tree crowns are healthy (Ellis, Ratkowsky *et al.* 1987; Neyland & Cunningham 2004)). However, the phenomenon of tree decline is common in these *E. delegatensis* high altitude forests, dramatically reducing forest productivity (Ellis & Lockett 1991). Eucalypt decline is widespread within Australia, affecting many different species over a large geographic range in rural landscapes and in native forest (see Chapter 1). Eucalypt declines in native forest (as distinct from farm land) have complex aetiologies which are poorly understood (see Chapter 1).

Altered fire regimes since European settlement have been proposed as one of the possible causes of eucalypt declines in temperate Australian forests (Jurskis & Turner 2002; Jurskis 2005a; Close, Davidson *et al.* 2009). Close, Davidson *et al.* (2009) propose that in temperate

eucalypt ecosystems that have adapted to a particular fire regime, the absence of fire causes a cascade of changes with numerous feedbacks (Figure 5.1).

E. delegatensis decline, also known as high altitude dieback, has been specifically linked to altered fire regimes (Ellis, Mount *et al.* 1980). When fire is excluded in *E. delegatensis* forest, understorey vegetation composition changes from a sclerophyllous understorey to a mesic rainforest understorey (Ellis 1985), which is the first step of the eucalypt decline model (Figure 5.1). These vegetation changes can cause water stress in *E. delegatensis* trees through increased competition with the understorey (Turner & Lambert 2005, step 2 of the decline model, Figure 5.1). Altered soil chemistry, especially an increase in mineral nitrogen (Turner & Lambert 2005; Turner, Lambert *et al.* 2008) and a decrease in soil pH (Ellis & Graley 1987), also occurs (steps 3 and 4 of the decline model, Figure 5.1). Changes in soil microclimate include a decrease in soil temperature by as much as 7 °C (Ellis 1971). Changes in soil chemistry can occur by depletion (uptake and immobilisation in biomass), or through atmospheric inputs and increases in availability (Ellis & Pennington 1989; Turner & Lambert 2005; Turner, Lambert *et al.* 2008). Ammonification and nitrification are known to occur at higher rates in *E. delegatensis* forests that are further along the successional progression from sclerophyll to rainforest (Ellis & Pennington 1989; Turner & Lambert 2005; Turner, Lambert *et al.* 2008). *E. delegatensis* root morphology along with mycorrhizal development and fungal associates are altered in response to chemical and other changes within the forest (Ellis & Pennington 1992; Turner & Lambert 2005). At this stage, decline symptoms are evident and secondary feedbacks become important once the above changes are established (at step 4 of the model). Altered soil chemistry may lead to biochemical and nutrient imbalances in the foliage of *E. delegatensis*, including an increase in amino acids, resulting in foliage becoming more attractive to herbivores (Turner & Lambert 2005). This increase in susceptibility to herbivores and pathogens may further increase stress, and reduce productivity (Figure 5.1). In *E. delegatensis* forest, the decline process clearly culminates in reduced growth and vigour of the dominant eucalypts, and their premature death (Ellis 1964; Turner & Lambert 2005).

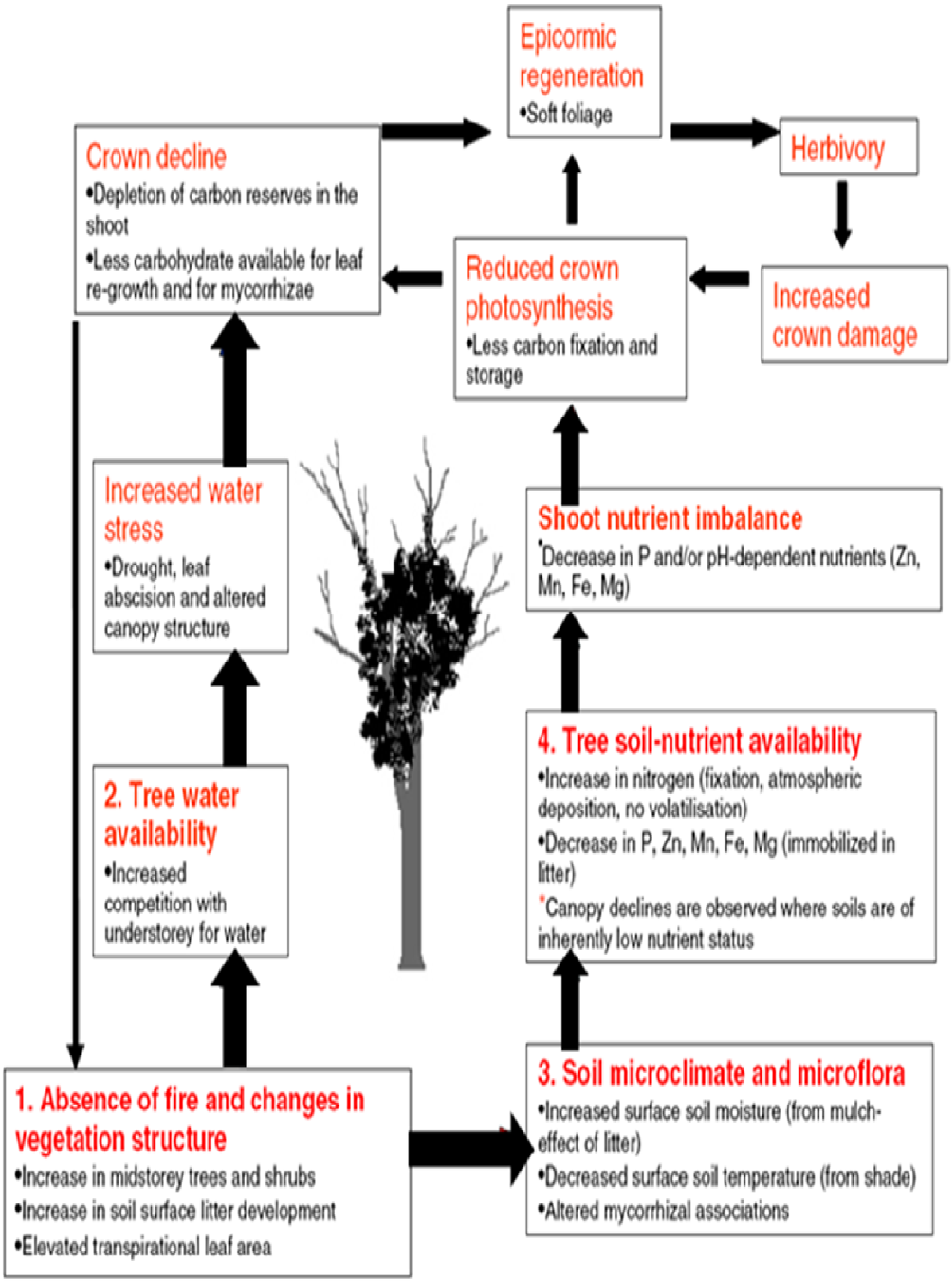

Figure 5.1. A model of premature decline of overstorey eucalypts in temperature Australian eucalypt forests (Close, Davidson *et al.* 2009).

The use of prescribed fire in forests suffering from decline may return the forest to a healthy state having a soil chemistry more conducive to maintaining healthier eucalypts (Ellis 1964; Ellis, Mount *et al.* 1980; Jurskis 2005b; Jurskis 2005a; Turner & Lambert 2005; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). In the case of *E. delegatensis* forest, burning of the understorey (but not just mechanical removal of the understorey) was able to halt the development of decline and improve growth (Ellis, Mount *et al.* 1980). Repeatedly burnt eucalypt forest in NSW also had healthier overstorey crowns than unburnt forest (Turner, Lambert *et al.* 2008). Fire releases bound nutrients such as nitrogen, through volatisation and transformation from organic forms to inorganic forms which are leached if not taken up by plants. This process reduces nitrogen levels that may have reached saturation. Limited nutrients such as phosphorus, are made more available following fire through altering soil pH and conversion of organic phosphorus to inorganic phosphorus which is readily available for plant uptake (Raison 1980; Attiwill, Polgase *et al.* 1996; Certini 2005).

The driving mechanisms of both eucalypt decline and the ameliorative effect of fire on decline, undoubtedly involves ectomycorrhizal (ECM) associations (Close, Davidson *et al.* 2009). The ECM community has the potential to affect forest composition and nutrient cycling and both the presence of different plant species, and the availability of various nutrients, shape the ECM community (Francis & Read 1994; Tommerup & Bougher 1999; Aerts 2002; Brundrett & Cairney 2002; Reynolds, Packer *et al.* 2003; Anderson & Cairney 2004; Booth 2004; Simard & Durall 2004; Peay, Kennedy *et al.* 2008b; Tedersoo, Jairus *et al.* 2008; van der Heijden, Bardgett *et al.* 2008; Simard 2009; van der Heijden & Horton 2009). For example, increases in the availability of soil nitrogen are known to influence ECM community structure and composition (Lilleskov, Fahey *et al.* 2001; Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003). A model conceptualising the various influences that potentially affect the ECM community and feed into the decline process is presented in Figure 5.2.

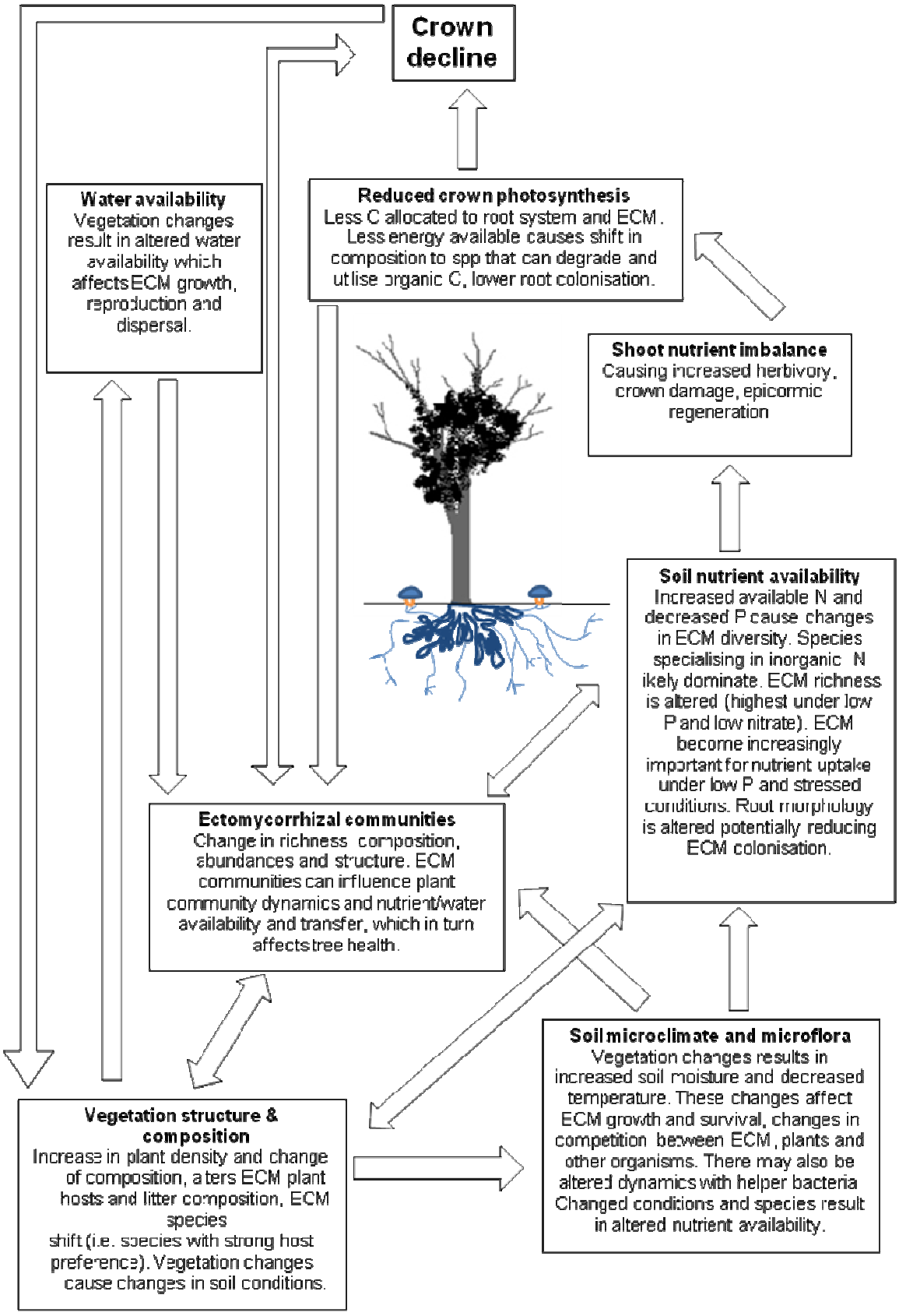
Crown decline
Water availability
Vegetation changes result in altered water availability which affects ECM growth, reproduction and dispersal.
Reduced crown photosynthesis
Less C allocated to root system and ECM. Less energy available causes shift in composition to spp that can degrade and utlise organic C, lower root colonisation.
Shoot nutrient imbalance
Causing increased herbivory, crown damage, epicormic regeneration
Soil nutrient availability
Increased available N and decreased P cause changes in ECM diversity. Species specialising in inorganic N likely dominate. ECM richness is altered (highest under low P and low nitrate). ECM become increasingly important for nutrient uptake under low P and stressed conditions. Root morphology is altered potentially reducing ECM colonisation.
Ectomycorrhizal communities
Change in richness composition, abundances and structure. ECM communities can influence plant community dynamics and nutrient/water availability and transfer, which in turn affects tree health.
Soil microclimate and microflora
Vegetation changes results in increased soil moisture and decreased temperature. These changes affect ECM growth and survival, changes in competition between ECM, plants and other organisms. There may also be altered dynamics with helper bacteria Changed conditions and species result in altered nutrient availability.
Vegetation structure & composition
Increase in plant density and change of composition, alters ECM plant hosts and litter composition, ECM species shift (i.e. species with strong host preference). Vegetation changes cause changes in soil conditions.

Figure 5.2 A conceptual model of the process of eucalypt decline showing how decline affects the ectomycorrhizal community, and feedbacks from the ectomycorrhizal community into the decline process. Changes in both vegetation dynamics in the understorey and overstorey, affect the ECM community through changed host species and densities, competition for nutrients and water, and changed litter. Vegetation changes contribute to a changed soil environment, especially in type and availability of nutrients, in which ECM function, community structure and composition are tightly linked. The ECM community also both responds and contributes to changes in soil nutrients. These changes cause a cascade of changes through the ecosystem, altering ECM communities and ultimately leading to tree decline, which then feeds back to ECM community structure and function.

A change in the ECM community means the gain and loss of species with different abilities to perform functions in water and nutrient uptake and transfer. The presence of species within the community that are capable of optimal functioning under the set of environmental conditions imposed, (i.e. prefer and uptake nitrate when soil nitrogen is saturated) is therefore critical for the healthy functioning of the ecosystem. Fire is known to affect the ECM community in a number of ways, especially by initially decreasing ECM biomass and causing shifts in community composition and structure through the process of succession (Warcup 1990; Visser 1995; Launonen, Ashton *et al.* 1999; Stendell, Horton *et al.* 1999; Chen & Cairney 2002; McMullan-Fisher, May *et al.* 2002; Bastias, Xu *et al.* 2006). The ameliorative affect of fire on eucalypt decline may act to reinstate ECM assemblages that are important for the maintenance of eucalypt health (Ellis & Pennington 1992; Turner & Lambert 2005).

The process of *E. delegatensis* forest decline is thus ecologically complex, involving many parts of the forest ecosystem. A study of the ECM fungi of declining eucalypt forest is thus essential to our understanding of eucalypt forest decline, as ECM fungal communities have the potential to influence many aspects of the decline process, being both affected by, and contributing to these changes (Figure 5.2).

This study is the first to explore the relationship of ECM fungal communities of declining *E. delegatensis* forest to a number of factors that are associated with the decline of temperate Australian eucalypt forests. The aims of this study were therefore to: determine if soil and foliage chemistry, especially nitrogen and phosphorus, influence ECM fungal community composition, structure and richness; explore the influence of forest type, considering the understorey and overstorey vegetation, and region, on ECM fungal species richness and community composition; and investigate the direct relationship between ECM fungal community composition and structure, and eucalypt forest health within Tasmanian *E. delegatensis* forest. The hypotheses that the ECM communities of *E. delegatensis* forest change as the forest succeeds from a sclerophyll understorey to a rainforest understorey, and that these changes are accompanied by a progressive decline in health of the overstorey eucalypts is proposed. Changes in the ECM communities of these forests are also hypothesised to be tightly linked to altered soil conditions that occur as a part of the eucalypt decline process.

5.2 Methods

5.2.1 Study sites

Twelve 0.25 ha plots with buffers were established in *E. delegatensis* forest in northern Tasmania, eight in the north-east and four in the north-west (Appendix 15). These plots are described in Chapter 4 section 4.2.1, and the main characteristics of the plots are summarised below (Tables 5.1 and 5.2).

5.2.2 Vegetation surveys

Vegetation surveys of each of the 12 plots are described in Chapter 4 section 4.2.2.

5.2.3 Nutrient sampling

Four soil samples (approximately 5 x 5 x 5 cm) taken approximately 2 m from the trunk equally spaced in a full circle around the tree (90° apart), were collected from five randomly located eucalypt trees per study plot. Soil samples were pooled per study plot. Resin bags consisting of 5 g of either a cation- or anion-exchange resin (Amberlite, Sigma-Aldrich) contained in pre-washed bags made of stocking nylon were used to sample available nitrogen and phosphorus within the soil solution (Giblin, Laundre *et al.* 1994). Four sets of resin bags were buried in pairs at 5 cm depth approximately 1 m apart under the drip-line of three randomly selected trees per site, and were collected approximately six months later.

Due to the height of the trees, eucalypt foliage was collected by Forestry Tasmania staff using a rifle to shoot off branches during August 2007. Whole branches from individual trees exhibiting a range of health were shot down from the outer and lower part of the crown on a northerly aspect. These trees were the same trees that were sampled for soil nutrients (five per plot), from each plot in July 2007. Foliage samples consisted of 30 - 40 young fully expanded leaves, free of damage, from each whole branch. Foliage samples were dried at 40 °C before sub-sampling for nutrient analysis.

Table 5.1. Summary of the main shared characteristics of the 0.25 ha study plots. NE means north-east and NW is north-west.

Characteristic	**NE plots**	**NW plots**
Dominant overstorey species	*E. delegatensis*	*E. delegatensis* & *E. dalrympleana*
Elevation (m)	850-900	800-900
Rainfall (mean mm)	1320	1564
Temperature (mean °C)	9.1	9.3
Parent material	Devonian granodiorite	Tertiary Basalt
Soil type	brown dermasols	dark brown ferasols
Phosphorus content	high	high
Nitrogen content	high	moderate
Organic material	medium	high
Productivity	medium but lower at altitude	high
Time since timber harvest (years)	>100	c. 30

Table 5.2. Summary of the unique characteristics of the 0.25 ha study plots. NE means north-east and NW is north-west. RF stands for rainforest and SC for sclerophyll.

Plot	**Region**	**Understorey type**	**No. of eucalypt trees per plot**	**Time since fire (years)**
1	NE	RF	>10	46
2				
3			6	126
4			9	
5		SC	>10	65
6				
7			>10	33
8			>10	
9	NW	RF	>10	>100
10			5	
11		SC	>10	>100
12				

5.2.4 Nutrient analysis

All nutrient analyses were conducted by a private sector laboratory services provider, CSBP Limited (Perth, Australia) during 2008-2009, using the following methods. Available soil phosphorus (P) was measured using the Colwell method (Rayment & Higginson 1992). Soil ammonium (NH_4^+) and nitrate (NO_3^-) nitrogen were measured simultaneously using a Lachat Flow Injection Analyser according to the method described by Searle (1984). Organic carbon (C) was estimated using the Walkley-Black method (Walkley & Black 1934). For the measurement of total nitrogen (N), soil samples were combusted at 950°C in oxygen using a Leco FP-428 Nitrogen Analyser. For total P, soils were digested with sulphuric acid-potassium-copper sulphate and the P concentration measured colormetrically at 880 nm (Allen & Jeffery 1990). Soil pH was determined using the method described by Rayment and Higginson (1992). NH_4^+, and NO_3^- and available P were extracted and measured from resin bags as described by Giblin *et al.* (1994).

Leaf material was ground using a Cyclotec mill (Foss Tecator, North Ryde, NSW, Australia). For nitrogen analysis, the finely ground leaf material was combusted at 950 °C in oxygen using a Leco FP-428 Nitrogen Analyser (Sweeney & Rexroad 1987). For P analysis, plant material was digested in nitric acid by heating using a Milestone microwave (McQuaker, Brown *et al.* 1979). P was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES - Varian Vista axial spectrometer, Palo Alto, CA, USA).

5.2.5 Crown health assessments

Crown health was used as an indicator of overall tree health (Manion 1991; Stone & Haywood 2006). Primary crown dieback defined as the proportion of primary branches that have died back from the terminal (Wardlaw 1989), and was assessed on a scale of 0 to 100, where 100 represents full health. This method was selected as it was determined to be an efficient and effective measure of eucalypt crown dieback (see Chapter 2). Ten randomly selected *E. delegatensis* trees per plot were assessed by a single observer for crown health using a single crown attribute. Three plots (plots 3, 4 and 10) did not have 10 eucalypt trees and all *E. delegatensis* trees present within the plots were assessed (Appendix 14). These trees were

the same trees used to determine the most appropriate measure of crown condition in Chapter 2. Examples of different crown conditions are shown in Appendix 2. Scores were averaged per study plot to give an overall eucalypt health score for each plot. Plots were allocated to one of three health categories based on Podger, Kile *et al.* (1980). Severe decline was defined as a health score of < 60 %, moderate decline was a health score of 60 – 80 % and healthy plots had a score > 80 %. These categories consider natural fluxes in crown health within healthy trees (> 80%) as physiological effects associated with various levels of dieback. Wardlaw (1989) found that eucalypt trees with dieback affecting > 40% of their crown (health score < 60%) exhibit reduced growth in girth, from which they were unable to recover.

5.2.6 Ectomycorrhizal fungal sampling and identification

Sporocarp and root tip samples were sampled from each of the plots as described in Chapter 4. Fungi present in samples were identified by PCR amplification of the rDNA ITS region and DNA sequencing as detailed in Chapter 4 methods (sections 4.2.5 – 4.2.8).

5.2.7 Statistical analyses

One-way analyses of variance (ANOVA) were performed in GenStat v7.1 (VSN International Ltd 2003) to test for the difference in ECM fungal community richness (total, and for the most OTU rich or dominant families) and proportional composition (the percentage richness of each ECM family within each plot) between understorey type, and between forests of different health category (moderately declining or severely declining), using the combined presence-absence data from the root tip and sporocarp surveys and family percentages (Appendices 26 and 27). Richness of each ECM family as a percentage of the total plot richness was angular transformed prior to inclusion in analyses. Where ANOVA's showed multiple comparisons a Bonferroni correction was applied to eliminate putatively significant results. This is indicated in the appropriate results tables. A one-way ANOVA to test between groups based on health and understorey (giving four groups) to minimise within group variation caused by strong effects of understorey type. Tukey's test was used as a post hoc comparison to determine which groups differed when the overall model was significant.

The Bray-Curtis similarity measure: $$Similarity^{\text{Bray-Curtis}} = \frac{2A}{2A + B + C}$$

was used to construct similarity matrices in PRIMER v6 (Anderson, Gorley *et al.* 2008) based on the combined sporocarp and root tip data (Appendix 28) and for proportional composition (Appendix 29), which were then used in statistical analyses. The combined data set was used as the primary data set as sporocarps and root tips sampled complementary assemblages and provided a more complete representation of the ECM community (see Chapter 4) that using either data set separately. In some cases, relationships between ECM assemblages and their environment were further explored using similarity matrices containing only the sporocarp data or root tip data (Appendix 29). All matrices based on fungal OTU community composition contained only the OTUs that were recorded in more than one plot, as single records were not informative for analyses in this study. To assess differences in ECM proportional composition a matrix consisting of the percentage richness of each ECM family within each plot was constructed from the transformed data (Appendix 29).

Unconstrained non-metric multi-dimensional scaling (MDS) analysis was used to explore similarities in ECM community composition and proportional composition between plots and was performed using the statistical program PRIMER v6 with PERMANOVA + (Anderson, Gorley *et al.* 2008). Both MDS analyses were based on a minimum Kruskal stress of 0.01 with 25 re-starts. Non-metric multi-dimensional scaling represents each sample (in this case the sample is a plot) as a point in two or three dimensional space so that the relative distances between all points are in the same rank order as the relative dissimilarities of the points measured by a resemblance matrix, in this case a Bray-Curtis similarity matrix. Therefore, points that are close together represent samples that are very similar in ECM community composition or proportional composition, and points that are far apart have different community composition or structure. MDS is useful to explore similarities and differences of observed ECM richness between each of the plots as, unlike other ordination techniques MDS does not make many assumptions regarding the nature of the data.

Canonical analyses of principal co-ordinates (CAP) (Anderson & Willis 2003) were performed using the statistical program PRIMER v6 with PERMANOVA + (Anderson, Gorley *et al.* 2008). A detailed discussion of CAP as a statistical tool is given in Chapter 3 (section 3.2.13). CAP analyses were done to assess differences in fungal community composition between: 1)

understorey type and region; 2) health categories (moderately or severely declining) and; 3) understorey type and health category (i.e. if fungal composition of moderately declining forest with sclerophyll understorey was different from moderately declining forest with rainforest understorey). All CAP analyses were tested by permutation, using 9999 permutations. Spearman's rank correlations were used for the vector overlays (rather than Pearson correlations) as this type of correlation was more appropriate for the non-parametric analyses performed on the multivariate data-set. The vector overlays are primarily an exploratory tool to assist in understanding the variables that are important in structuring the multivariate variation. These vectors are limited when ordinations have a more complex pattern or gradient, and may not provide a simple interpretation of the ordination (Anderson, Gorley *et al.* 2008).

Canonical correlations analysis (CCorA) was used to determine correlations between ECM fungal community similarities (composition and structure) and eucalypt crown health using the program PRIMER v6 with PERMANOVA + (Anderson, Gorley *et al.* 2008). CCorA is a tool for exploring how well multivariate data can predict the positions of samples along a gradient (in this case uncategorised crown health) (Anderson, Gorley *et al.* 2008). In this analysis the leave-one-out residual sum of squares is used to determine the number of axes (*m*) used in the analysis. The value of the canonical correlation reveals the strength of the model (i.e. a high value indicates a good model fit).

Draftsman's plots were used to test for normal distribution and indicated some variables were skewed. Nutrient data was log(x+1) transformed in PRIMER v6 (Anderson, Gorley *et al.* 2008) prior to inclusion in statistical analyses. Richness of each ECM family as a percentage of the total plot richness was angular transformed prior to inclusion in analyses.

A multiple linear regression was used to model ECM OTU richness on the basis of soil and foliage nutrients (soil pH, total soil N, total soil P, soil organic C, soil NO_3^-, soil NH_4^+, available NO_3^-, available NH_4^+, available P, foliage P and foliage N) and was performed using the statistical program SAS.

Distance-based multiple linear regression was used as a tool to explore the relationship of the ECM fungal community to soil and foliage nutrients. These analyses were used to test the hypothesis that plots with higher or lower soil nutrient concentrations, particularly of N and P,

and different eucalypt crown health, supported different ECM communities. Distance-based linear modelling is a non-parametric, permutation procedure for the analysis for variance which fits models between a multivariate data cloud and one or more explanatory variables (Anderson 2004).This type of statistical analysis is used for multivariate non-parametric data which cannot be modelled in the traditional manner of linear regressions. It differs from canonical correlations analysis in that distance-based linear-regression and redundancy analysis find linear combinations of the predictor variables that are best at explaining/predicting the response variables, whereas CCorA treats both sets of variables (predictor and response) as symmetrical (Anderson, Gorley *et al.* 2008). Distance-based linear regression has been used in a number of ecological studies where data are non-parametric and multivariate (i.e. there are a number of response variables) (see Jansen & Healey 2003; Brown, Smith *et al.* 2008; Chong, Pearce *et al.* 2009; Munaria, Tessarib *et al.* 2010). For example it has been used to test the hypothesis that high fire severity would result in high levels of plant canopy cover and exotic plant richness (Kuenzi, Fulé *et al.* 2008).

Distance-based multivariate multiple linear regressions were performed using the statistical program DISTLM available within PRIMER v6 with PERMANOVA + (Anderson, Gorley *et al.* 2008). Distance-based redundancy analysis was used for ordination of the fitted values from the models. Akaike's Information Criterion (AIC) was used as the goodness of fit measure (Burnham & Anderson 2002) and all models were run using 9999 permutations. Models were constructed for ECM OTU composition and for ECM proportional composition (relative richness of each family within a plot not including unknowns) using all available nutrient predictor variables (soil pH, total soil N, total soil P, soil PO_4^{3-}, soil organic C, soil NO_3^-, soil NH_4^+, available NO_3^-, available NH_4^+, available P, foliage P and foliage N) and crown health, which were added forward step-wise. The final models included only those predictor variables that were significant.

Another model used woody plant abundance of eight species (*Acacia dealbata*, *A. melanoxylon*, *E. delegatensis*, *E. dalrympleana*, *Leptospermum lanigerum*, *Melaleuca squarrosa*, *Nothofagus cunninghamii*, and *Pultenaea juniperina*) as the predictor variables for ECM OTU community composition, as predictor variables (abundances of these species are shown in Appendix 18). Abundances were angular transformed prior to inclusion in the model. The final model included only those predictor variables that were significant.

A final summary table of trends was determined by comparing mean values for a variety of variables for each group mean (understorey – rainforest and sclerophyll), region (north-east and north-west) and eucalypt crown health (moderate or severe decline), were compared to the overall means. The difference between the group mean and the overall mean were indicated with arrows, with ≥ 5 % difference in the group and overall mean represented by one arrow, and ≥ 25% difference in the group and overall mean represented by two arrows. Any statistically significant differences were indicated by a red arrow, and blue arrows indicate expected trends determined from the literature.

5.3 Results

5.3.1 Summary of ectomycorrhizal community species richness and composition

A total of 259 presumably ECM OTUs were recorded from the study sites using the combined sporocarp and root tip data (Appendices 20 and 26). ECM species richness, community composition and structure are discussed in detail in Chapter 4. Ninety-five of these OTUs were recorded from more than one plot and these were used for the statistical analyses (Appendix 12).

5.3.2 Eucalypt crown health

Eucalypt crown health varied greatly among plots ranging from 0.08 in a north-east rainforest plot to 0.8 in a north-west rainforest plot (Table 5.3). Full details of crown health scores are provided in Appendix 14. Five plots had crown health scores within the range of moderate decline (0.6 - 0.8 crown health score), and the remaining seven plots fell within the range of severe decline (<0.6 crown score) (Figure 5.3). None of the plots fell within the healthiest category (>0.8 crown score). The mean crown health score across plots with moderately declining eucalypts was 0.71 (*n*=5), for severely declining eucalypts was 0.36 (*n*=7), and the median crown health score across all plots was 0.52 (*n*=12). The five plots that suffered from moderate decline included two north-east sclerophyll plots, the two north-west sclerophyll plots and one north-west rainforest plot. The plots that had severely declining eucalypts were one north-west rainforest plots, all four north-east rainforest plots, and two north-east

sclerophyll plots (Figure 5.3). Eucalypt crown health was significantly lower in rainforest plots than sclerophyll plots (0.42 (*n*=50 SD=0.35) v. 0.63 (*n*=60 SD=0.30), $F_{1,10} = 11.41$, p = 0.001), and was also significantly lower in the north-east compared with the north-west (0.47 (*n*=75 SD 0.33) v. 0.67 (*n*=35 SD=0.31), $F_{1,10} = 8.59$, p = 0.004).

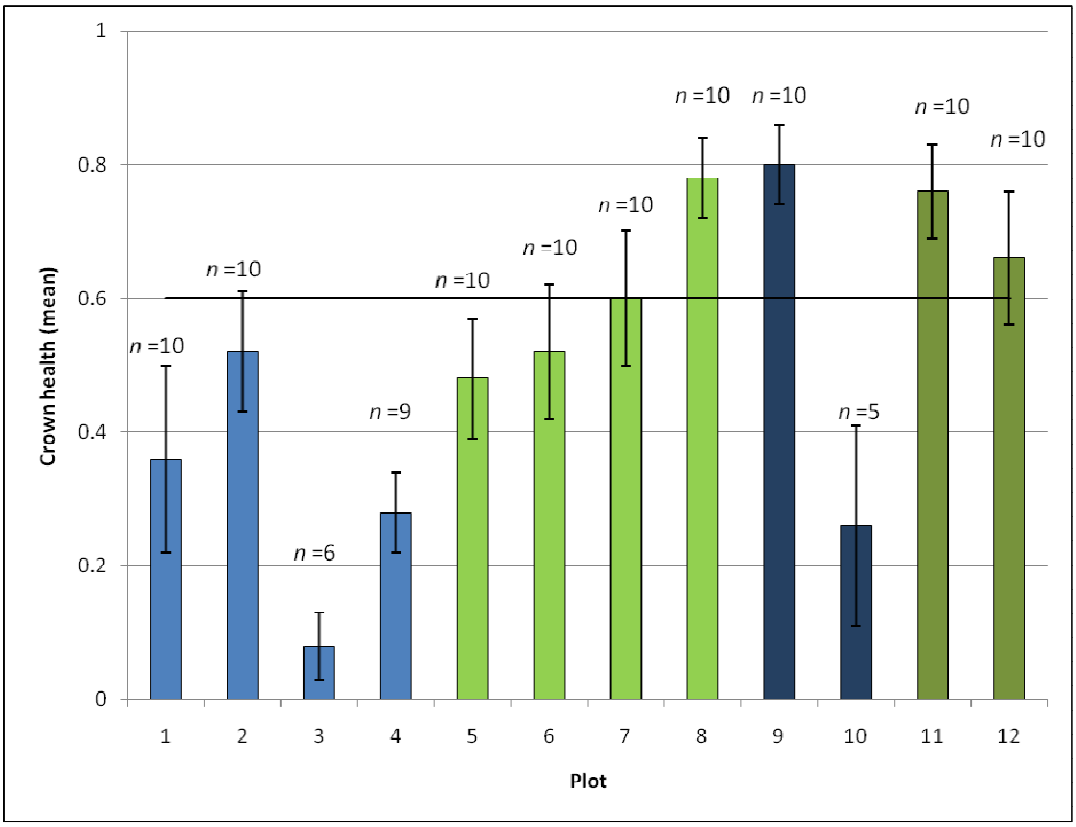

Figure 5.3. Mean crown health of eucalypts from within each of the plots. Bars indicate standard error. Rainforest plots are shown in blue and sclerophyll plots are shown in green. North-east plots are lighter coloured (plot 1-8) and north-west plots are darker coloured (plot 9-12). The black line indicates the boundary between moderate decline (above to 0.6) and severe decline (below). *n* is the number of eucalypt trees assessed in each plot. Paired plots are next to one another i.e. Plots 1 and 2, Plots 3 and 4, etc.

5.3.3 Soil and foliage nutrients of moderately and severely declining forest

Paired plots (I.e. plots 1 and 2, 3 and 4, etc.) were similar to one another for some of the variables, for example pH. For many other variables (for example available NO_3^-) most of the

paired plots were not very similar, indicating that these variables can change over smaller spatial scales than at the site level (Table 5.3). Overall, soil and foliage nutrients varied from plot to plot and some of the plots were outliers for particular variables (Table 5.3). Outliers may possibly cause larger than expected variance among plots. Statistically, this plot to plot variation, means large variation within groups (i.e. forest type or health status), and less variation among groups, often leading to statistical insignificance. Some of the interesting variables were foliage N which was very similar for all of the plots, and soil organic carbon, which was at the same percentage for five of the plots (Table 5.3). Also soil NO_3^- had a very high concentration for plot 2 and soil NH_4^+ was very low for plot 3, when compared to the other plots (Table 5.3).

Differences in soil and foliage chemistry between moderately and severely declining forest were tested by analyses of variance and also comparisons against the median, which allows a comparison of values within a skewed data set.

Means of the total pools of soil N, soil NO_3^-, and soil NH_4^+ were lower in moderately declining forest compared to severely declining plots, although these differences were not significant (for $p < 0.05$) because of substantial variation among plots (Table 5.4).

Mean values of total soil N of moderately declining plots were very near to the medians whereas in severely declining forest, total soil N was higher than the median (Table 5.4).

For available NO_3^- the mean was lower than the median in moderate declining forest but much higher than the median in severely declining forest (Table 5.4).

The median value of soil NH_4^+, in contrast to available NO_3^-, was slightly higher than the mean value of severely declining forest, and also higher than the mean value of moderately declining forest (Table 5.4). This differs from the mean of available NH_4^+ which was lower than the median in moderately declining forest but more than twice the median value in severely declining forest (Table 5.4).

Both total soil P and available P had greater mean concentrations than the median in moderately declining forest (Table 5.4) and soil P (both total and available) and were higher in moderately declining forest compared to severely declining forest plots.

Mean values of foliage N were lower than the median in moderately declining forest, whereas means of foliage P were higher than the median in both moderately and severely declining forest (Table 5.4).

Mean values of other variables, such as soil organic carbon, the soil C: N ratio and pH, were very similar for both moderately and severely declining forest (Table 5.4).

Soil chemical differences between moderately and severely declining forest were also tested only within the north-east region, as this area had more severe decline compared with the north-west (see section 5.3.2). This analysis (Table 5.5) indicated that both available P and foliage P were significantly lower in severely declining forest, but using Bonferroni's correction none of the variables are statistically significant in comparison with other tests.

Supplementary analyses to test the difference in soil and foliage chemistry between the two health categories considering the influence of understorey, between understorey types and between regions were also conducted (Appendix 30). The main findings from these analyses were that when considering understory type of the two health categories, soil pH, soil N:P and soil NH_4^+, were significantly different among groups (Appendix 30.1). Tukey's test indicated that these differences were mainly based on difference in understorey (appendix 30.1). When considering understorey type, soil pH and total soil P, available P, foliage P and foliage N:P ratio were significantly higher in sclerophyll forest than rainforest (Appendices 30.2). When these analyses were performed using only the north-east data, total soil P and foliage P were significantly higher in sclerophyll forest, whereas total soil N was significantly higher in rainforest (Appendix 30.3). Comparisons between the north-east and north-west did not reveal any statistical differences between the soil and foliage nutrients.

Table 5.3. Understorey type (SC sclerophyll and RF rainforest), region (NE north-east, NW north-west), mean crown health (± SE), crown health category (sd severe decline and md moderate decline) and pooled soil and foliar nutritional characteristics of the 12 plots. Plots with severe decline are shaded for easy reference.

Site Attribute	**Study site**												**Mean**	**Median**
Plot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**		
Region	NE								NW					
Understorey	RF				SC				RF		SC			
Crown health score	0.36 ± 0.14	0.52 ± 0.09	0.08 ± 0.05	0.28 ± 0.06	0.48 ± 0.09	0.52 ± 0.1	0.6 ± 0.1	0.78 ± 0.06	0.8 ± 0.06	0.26 ± 0.15	0.76 ± 0.07	0.66 ± 0.1	0.51 ± 0.07	0.52
No. eucalypt trees	10	10	6	9	10	10	10	10	10	5	10	10		
Crown health rank	9	6	12	10	8	6	5	2	1	11	3	4		
Crown health category	sd	sd	sd	sd	sd	sd	md	md	md	sd	md	md		
Soil type	brown dermasols derived from Devonian granodiorite								dark brown ferasols derived from Tertiary Basalt					
Soil pH	4.5	4.2	4.3	4.4	5.5	5.4	5.1	5.1	3.7	3.7	4.3	4.3	4.54 ± 0.17	4.4
Soil NO_3^- (mg/kg)	4	19	1	1	1	1	1	1	1	3	1	1	2.92 ± 1.49	1
Soil NH_4^+ (mg/kg)	111	143	12	141	108	89	66	93	49	114	120	102	95.67 ± 10.93	105
Total soil N (%)	0.62	1.34	1.06	1.04	0.58	0.46	0.49	0.63	1.17	1.8	0.82	0.6	0.88 ± 0.12	0.73
Total soil P (mg/kg)	202	499	254	349	712	540	672	743	436	525	402	340	472.83 ± 50.63	468

Site Attribute	Study site												Mean	Median
Plot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**		
Soil N:P	3.07	2.69	4.17	2.98	0.82	0.85	0.73	0.85	2.68	3.43	2.04	1.77	2.17 ± 0.34	2.36
Soil organic C (%)	10.00	10.00	10.00	10.00	6.92	9.96	5.28	9.61	5.14	5.11	10	8.11	8.34 ± 0.62	9.79
Soil C: N	16.13	7.46	9.43	9.62	11.93	21.65	10.78	15.25	4.39	2.84	12.2	13.52	11.27 ± 1.49	11.35
Foliage N (%)	1.47	1.51	1.52	1.4	1.54	1.35	1.41	1.34	1.12	1.09	1.58	1.31	1.39 ± 0.05	1.41
Foliage P (%)	0.08	0.1	0.09	0.09	0.26	0.16	0.24	0.3	0.1	0.11	0.11	0.16	0.15 ± 0.02	0.11
Foliage N:P	17.92	15.4	17.74	16.03	6.05	8.38	5.91	4.46	11.13	10.36	14.75	8.32	11.37 ± 1.4	10.75
Available NH_4^+ (mg/L)	1084.53	3315.2	590.16	1444	505.07	298.45	354.67	359.39	282.19	712	404.69	173.49	793.65 ± 252.58	454.88
Available NO_3^- (mg/L)	769.07	1020.27	124.37	253.55	21.15	230.16	163.71	85.33	14.08	155.33	2.56	2.96	236.88 ± 93.37	139.85
Available P (ppm)	1.3	2.9	1.8	0.75	11.3	1.85	20.2	9.2	1.1	1.75	1.05	8.5	5.14 ± 1.74	1.83

Table 5.4 One-way ANOVA results for differences in soil and foliage variables between severely declining and moderately declining eucalypt forest. Bonferroni correction $p \leq 0.0036$ indicates significance.

Variable	Median	Moderate decline (mean N=5)	Severe decline (mean N=7)	$F_{1,10}$	P-value
pH	4.4	4.50	4.57	0.04	0.85
Soil NO_3^- (mg/kg)	1.0	1.0	4.29	1.20	0.3
Soil NH_4^+ (mg/kg)	105	86.00	102.57	0.54	0.48
Total soil N (%)	0.73	0.74	0.99	1.05	0.33
Total soil P (mg/kg)	468	518.60	440.14	0.56	0.47
Soil N:P	2.36	1.61	2.43	1.41	0.27
Soil organic C (%)	9.79	7.63	8.86	0.96	0.35
Soil C: N	11.35	11.22	11.29	0.01	0.98
Foliage N (%)	1.41	1.35	1.41	0.38	0.55
Foliage P (%)	0.11	0.18	0.13	1.70	0.22
Foliage N:P	10.75	8.91	13.12	2.51	0.14
Available NH_4^+ (mg/L)	454.88	314.89	1135.63	3.04	0.11
Available NO_3^- (mg/L)	139.85	53.73	367.70	3.33	0.1
Available P (ppm)	1.83	8.01	3.09	2.15	0.17

Table 5.5 One-way ANOVA results for differences in soil and foliage variables between severely declining and moderately declining north-east eucalypt forest. Bonferroni correction $p \leq 0.0036$ indicates significance (in bold).

Variable	Median	Moderate decline (mean N=2)	Severe decline (mean N=6)	$F_{1,6}$	P-value
pH	4.4	5.10	4.70	0.79	0.41
Soil NO_3^- (mg/kg)	1.0	1.00	4.50	0.42	0.54
Soil NH_4^+ (mg/kg)	105	79.50	100.4	0.34	0.58
Total soil N (%)	0.73	0.56	0.85	1.24	0.31
Total soil P (mg/kg)	468	426.00	707.50	3.79	0.10
Soil N:P	2.36	0.79	2.43	2.71	0.15
Soil organic C (%)	9.79	7.45	9.48	2.16	0.19
Soil C: N	11.35	13.01	12.70	0.01	0.94
Foliage N (%)	1.41	1.38	1.46	2.18	0.19
Foliage P (%)	0.11	2.7	0.13	7.12	0.04
Foliage N:P	10.75	5.18	13.58	4.88	0.07
Available NH_4^+ (mg/L)	454.88	357.03	1206.24	1.04	0.35
Available NO_3^- (mg/L)	139.85	124.52	403.09	0.88	0.38
Available P (ppm)	1.83	13.7	3.32	8.36	0.03

5.3.4 The ectomycorrhizal community of moderately and severely declining forest

5.3.4.1 Ectomycorrhizal community OTU richness

Overall ECM richness was greater in moderately declining forest, but when examining the root tip and sporocarp data separately, OTU richness detected from root tips was slightly higher in severely declining forest than moderately declining forest, though these differences were not statistically significant (Table 5.6). Although the Cortinariaceae was richer in moderately declining forest, the large variation among plots caused this difference to be statistically not significant (Table 5.7). The Russulaceae was slightly richer in severely declining forest but again, this was not statistically significant (Table 5.7). Richness of the Thelephoraceae was similar in both moderately and severely declining forest (Table 5.6). More Cortinariaceae OTUs were unique to moderately declining plots than to severely declining plots with 26% of OTUs found in both (Figure 5.4). Although species richness of the Russulaceae was similar between the two different health categories (Table 5.7), severely declining plots contained a larger number of OTUs that were not found in moderately declining plots and 52% of OTUs were found in forests of both health categories (Figure 5.4). A greater number of Thelephoraceae were also unique to severely declining plots with a 25% overlap of OTUs between moderately and severely declining plots (Figure 5.4).

Table 5.6 One-way ANOVA results testing difference in ectomycorrhizal OTU richness (observed) between the two eucalypt health categories. Bonferonni correction $p \leq 0.016$.

Data	Severe decline (mean)	Moderate decline (mean)	$F_{1,10}$	P-value
Combined sporocarp & root tip	38	44	0.72	0.42
Sporocarp	24	32	1.87	0.20
Root tip	18	15	0.45	0.52

Table 5.7 One-way ANOVA results testing difference in observed richness of ECM families between eucalypt health categories.

Family	Severe decline (mean)	Moderate decline (mean)	$F_{1,10}$	P-value
Cortinariaceae	12	22	3.34	0.10
Russulaceae	4	3	0.37	0.56
Thelephoraceae	2	1	1.46	0.25

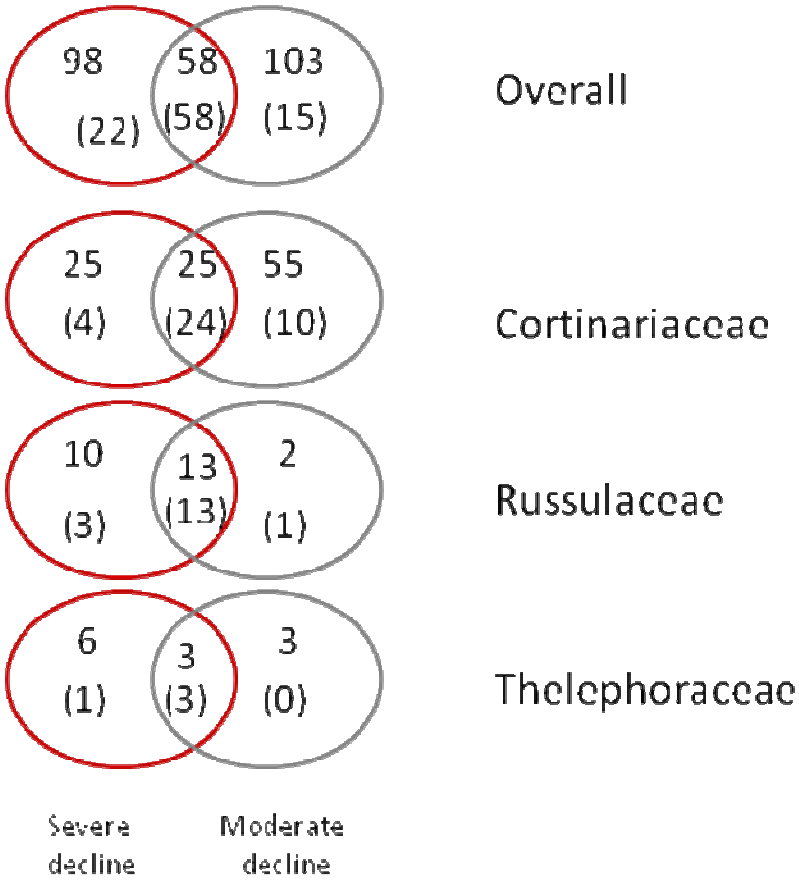

Figure 5.4 Venn diagrams showing the number of ectomycorrhizal OTUs overall and from the three most species rich ectomycorrhizal families found in forest with moderately declining eucalypts (grey), severely declining eucalypts (red), or in forest of both health status' (overlap). Numbers in brackets indicate the number of OTUs that were recorded from more than one plot within a health category.

5.3.4.2 Ectomycorrhizal community composition

The ECM fungal community composition of moderately and severely declining eucalypt plots was significantly different (CAP p = 0.04, m = 9 and misclassification = 25%). Boletaceae sp. 2, *Laccaria* sp. 4, *Cortinarius* sp. 67, *Cortinarius* aff. *sclerophyllarum*, and *Sebacina* sp. 2 were associated with severe decline whereas no OTUs were present in greater than three moderately declining plots and absent from severely declining plots. Basidiomycete sp. 8 was present in two moderately declining plots.

Canonical correlations analysis (CCorA) indicated that ECM community composition similarities were related to crown health (plots with similar ECM community species composition had crown health scores that were more similar than plots with dissimilar species composition) but not significantly so (Figure 5.5).

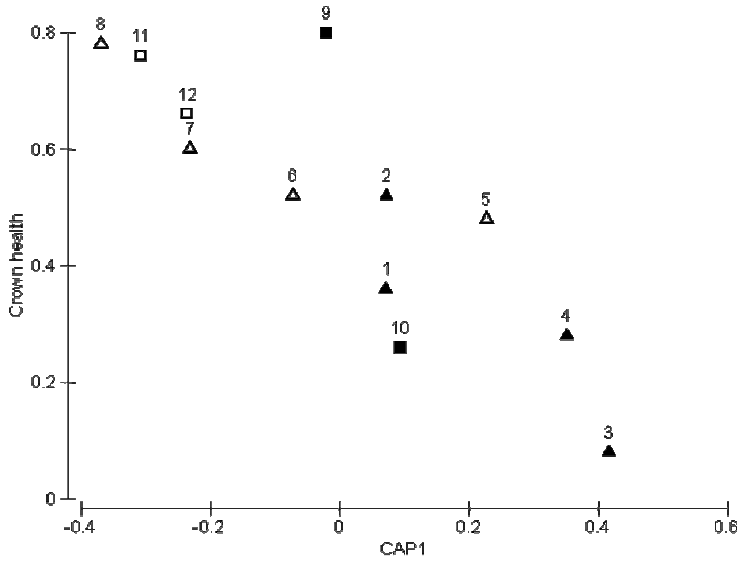

Figure 5.5 Canonical correlations analysis of ectomycorrhizal community composition with eucalypt crown health. Correlation squared = 0.71, p = 0.21. Filled markers are rainforest plots, open markers are sclerophyll plots. Squares are north-west plots and triangles are north-east plots. Most of the rainforest plots have poor crown health but are similar in ECM OTU community composition, and are located in the positive region of the CAP1 axis, but below 0.6 on the crown health axis (plot 9 is the exception). Similarly, plots with a sclerophyll understorey were generally healthier, lying between 0.5 and 0.8 on the crown health axis and below zero on the CAP1 axis.

5.3.4.3 Ectomycorrhizal community proportional composition

ECM proportional composition was not significantly different (at a significance level of $p \leq 0.05$) between plots of different health (CAP m= 4, p = 0.24 misclassification = 25%). Families associated with moderate decline were the Cortinariaceae, whereas the Amanitaceae, Russulaceae and Thelephoraceae were associated with severe decline.

One-way ANOVAs testing the difference in the proportions of each family between the two health categories showed that the proportion of Cortinariaceae was significantly higher in plots that suffered from moderate decline compared to plots with severe decline (Table 5.8). In contrast, the proportion of Thelephoraceae was higher in severely declining plots, and while the percentage of Russulaceae in the ECM community was greater in severely declining plots, neither of these differences were significant (Table 5.8).

Table 5.8 One-way ANOVA results testing difference in the proportion of three ectomycorrhizal families (angular transformed) in plots with moderate and severe declining eucalypts.

Family	Severe decline (mean)	Moderate decline (mean)	$F_{1,10}$	P-value
Cortinariaceae	0.30	0.46	2.38	**0.04**
Russulaceae	0.19	0.14	-0.6	0.56
Thelephoraceae	0.05	0.02	-1.6	0.15

A canonical correlations analysis between crown health and ECM proportional composition indicated no significant correlation (Figure 5.6).

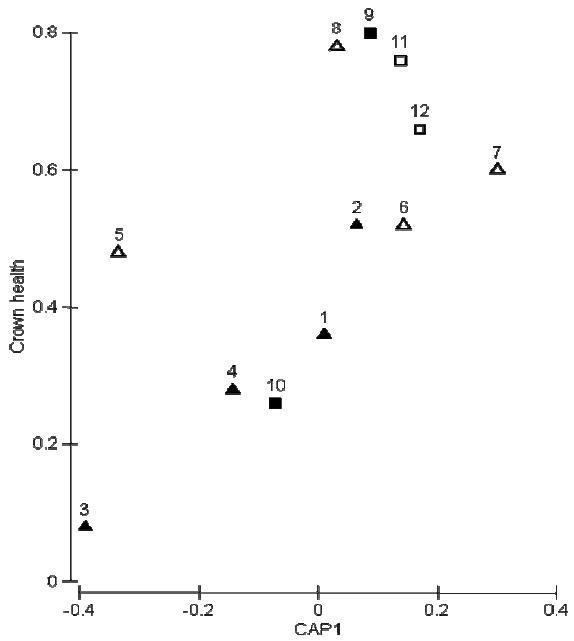

Figure 5.6 Canonical correlations analysis of ectomycorrhizal proportional composition with eucalypt crown health. Correlation square value = 0.46, p = 0.16. Filled markers are rainforest plots, open markers are sclerophyll plots. Squares are north-west plots and triangles are north-east plots. Similar to the relationship between ECM composition and crown health, there is some separation between the rainforest and sclerophyll understorey plots, with some exceptions (again plots 5 and 9).

5.3.5 The ectomycorrhizal community, forest types, region and eucalypt crown health

5.3.5.1 Ectomycorrhizal community OTU richness

As seen in Chapter 4, ECM fungal species richness did not significantly differ between rainforest understorey plots and sclerophyll understorey plots, for the complete data set (43 vs. 38, $F_{1,10}$=0.39, P=0.54) or for the data set excluding singletons (29 vs. 25, $F_{1,10}$=1.67, P=0.23). When comparing species richness (including singletons) of the three richest families, the Cortinariaceae, the Russulaceae and the Thelephoraceae, differences between understorey-types were revealed (Table 5.9). The Cortinariaceae and Thelephoraceae had very similar species richness between the two understorey types. There were more species of Russulaceae

in the sclerophyll plots than the rainforest plots although this only reached significance in the north-west plots (Table 5.9).

Table 5.9 One-way ANOVA results testing the difference in observed species richness of ectomycorrhizal families between understorey type using all 12 plots. Significant P values are in bold.

Family	Rainforest (mean)	Sclerophyll (mean)	$F_{1,10}$	P-value
Cortinariaceae (all plots)	17	15	0.06	0.81
Cortinariaceae (north-east)	11	9	0.78	0.41
Cortinariaceae (north-west)	28	29	0.02	0.90
Russulaceae (all plots)	3	6	4.58	0.06
Russulaceae (north-east)	3	6	1.66	0.25
Russulaceae (north-west)	2	5	49	**0.02**
Thelephoraceae (all plots)	2	1	2.57	0.14
Thelephoraceae (north-east)	2	1	2.14	1.19
Thelephoraceae (north-west)	3	2	1	0.42

Although species richness was similar for the Cortinariaceae in the two understorey types only 15% of OTUs were found in both rainforest and sclerophyll understorey plots. Thirty six percent of Russulaceae OTUs and 8% of Thelephoraceae OTUs were shared between understorey types (Figure 5.7). When considering the species richness of these families within a region, the Russulaceae were found to have a significantly greater richness in north-western sclerophyll plots than north-western rainforest plots (Table 5.9). Neither the Cortinariaceae nor

the Thelephoraceae showed any significant differences in ECM richness associated with understorey type within a region (Table 5.9). Again, only a small number of OTUs from each family were shared across the north-east and north-west (Figure 5.8), and as seen in Chapter 4, ECM richness was significantly greater in the north-west compared to the north-east (Table 5.10). The Cortinariaceae were significantly richer in the north-west, but there was no difference in the richness of the Russulaceae or Thelephoraceae between regions (Table 5.9).

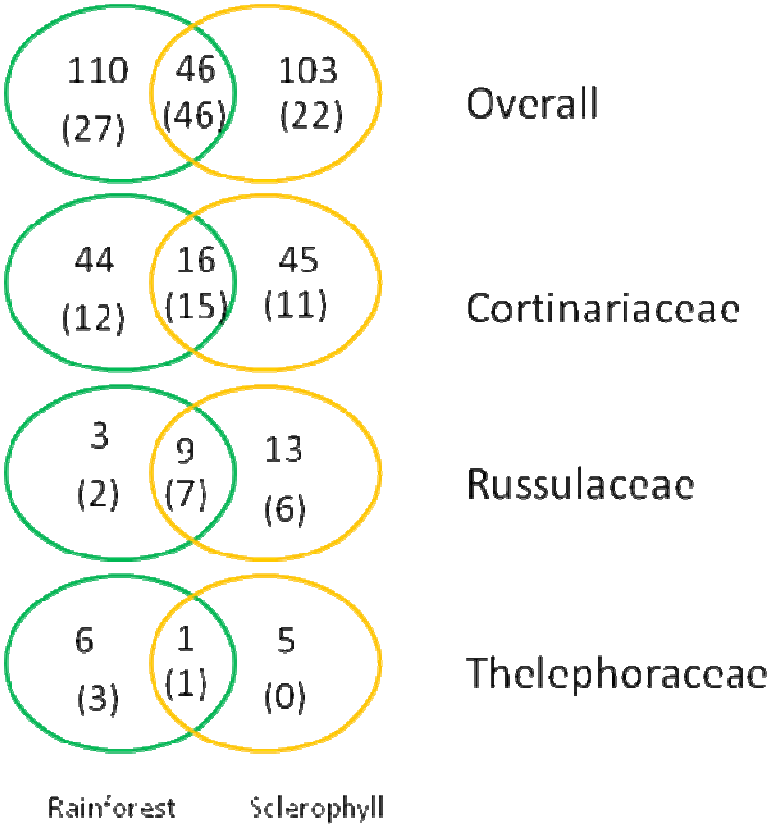

Figure 5.7 Venn diagrams showing the number of ectomycorrhizal OTUs found in forest with a rainforest understorey (green), sclerophyll understorey (orange), or in both forest-types (overlap), from the three most species rich families and overall. Numbers in brackets indicates the number of OTUs that were recorded from more than one plot from the two forest types.

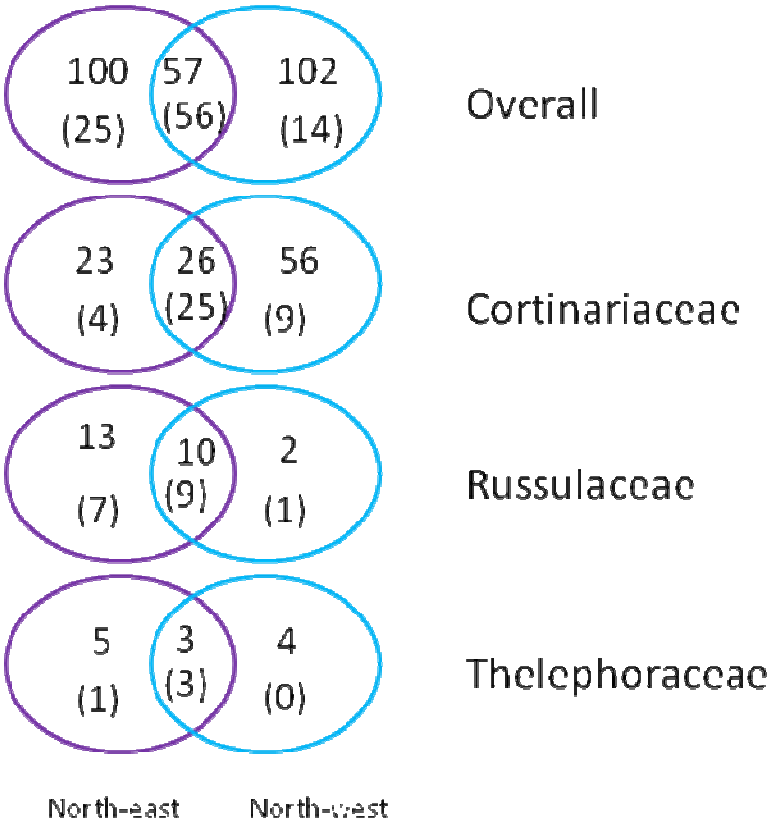

Figure 5.8 Venn diagrams showing the number of ectomycorrhizal OTUs found in forest located in the north-east (purple), or north-west (blue), or in both regions (overlap), from the three most species rich families and overall. Numbers in brackets indicate the number of OTUs that were recorded from more than one plot.

Table 5.10 One-way ANOVA results testing the difference in observed species richness of ectomycorrhizal families between the two regions. Significant P values are in bold.

Family	North-east (mean N=8)	North-west (mean N=4)	$F_{1,10}$	P-value
Overall	34	54	13.42	**0.004**
Cortinariaceae	9	28	38.28	**0.0001**
Russulaceae	4	3	0.41	0.53
Thelephoraceae	2	2	0.9	0.37

5.3.5.2 Ectomycorrhizal community composition

Multi-dimensional scaling (MDS) was performed on the combined sporocarp and root tip data (excluding singletons), to determine if there were any natural groups within the data (i.e. groups were not set prior to the analysis). The ordination of the MDS plots shows that the plots have clustered slightly on the basis of understorey and region. There are three natural groups; the north-east sclerophyll plots, the north-west sclerophyll plots and the rainforest plots (Figure 5.9).

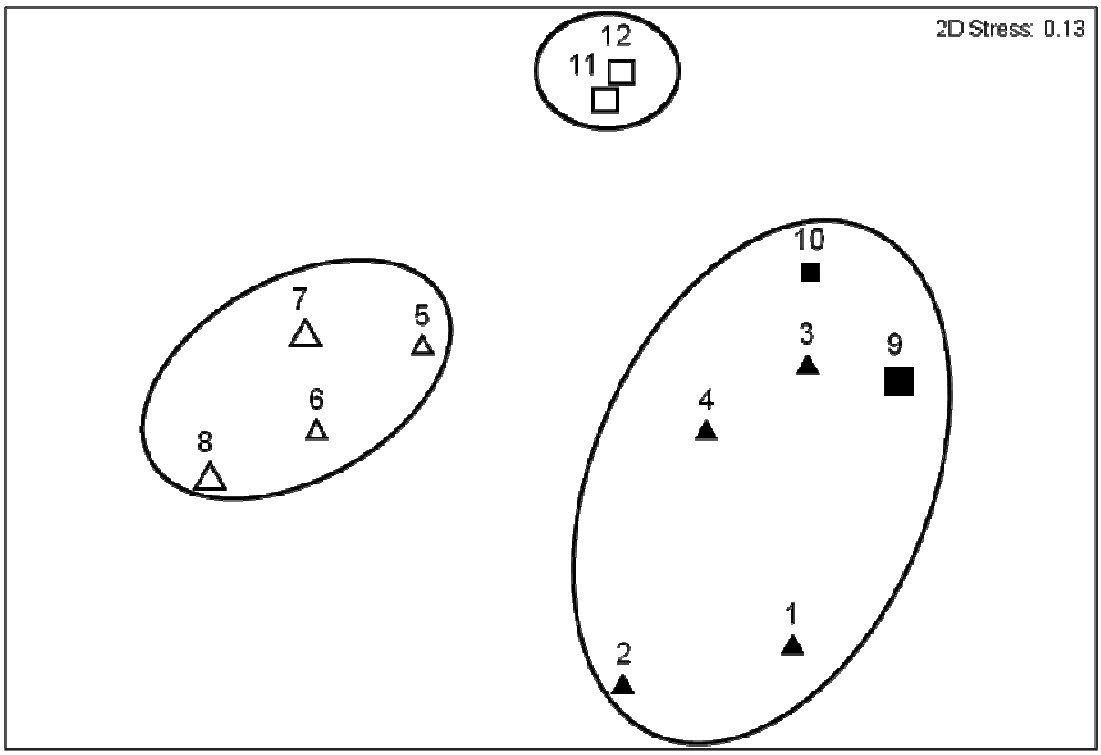

Figure 5.9 Ordination of the multi-dimensional scaling analysis of ectomycorrhizal community composition. Stress is 0.13 which is reasonable although this indicates that this model is not an excellent fit (<0.1 is regarded as an excellent model fit, and >0.15 is regarded as unacceptable (Anderson, Gorley *et al.* 2008). Rainforest plots are represented by filled markers, sclerophyll plots by open markers, squares are north-west plots and triangles are north-east plots. Large markers represent those plots with moderately declining forest, while small markers represent severely declining forest plots.

A CAP based on the ECM community OTU composition similarity matrix excluding singletons (Appendix 28) was used to test *a priori* differences of ECM OTU community composition with understorey type within the two regions. This analysis revealed three distinct ECM fungal communities that were significantly different to one another (Figure 5.10). These distinct groups were influenced by understorey and region with all six rainforest plots grouping together to form a distinct ECM community. The second group contained all the north-east sclerophyll plots and the third group consisted of the north-west sclerophyll plots (Figure 5.10). These groups remained the same when using only the root tip data or the sporocarp data (sporocarp p=0.02 misclassification = 25%, root tip p= 0.21, misclassification = 50%). Paired plots were not always most similar to one another, as demonstrated by their lack of proximity in the ordination (Figure 5.10).

Some OTUs were associated with a particular understorey/region group. *Cortinarius aff. tasmacamphoratus* was associated with rainforest and *Descomyces* aff. *albus* was associated with north-eastern sclerophyll forest. Some OTUs were also only found in a particular region or understorey (Figure 5.10 and Appendix 26). Cantharellales sp. 1 was only recorded in the north-west, and *Zelleromyces* sp. 1 and 2 were only recorded in the north-east. *Dermocybe* sp. 4 was associated with sclerophyll understorey, while *Cortinarius* aff. *sclerophyllarum* and *Cortinarius* aff. *tasmacamphoratus* were found only in forest with rainforest understorey.

A distance-based multiple linear regression model using abundance of dominant woody vegetation (angular (arcsine) transformed) (see Appendix 18 for plant species abundances) as predictor variables indicated that similarities in fungal assemblages of the plots could be predicted by the abundance of dominant woody plant species. Fifty three percent of the variation seen in ECM community composition was explained by three axes (Table 5.11). One species, *Acacia melanoxylon,* was significant in predicting of ECM fungal assemblage similarity among plots (Table 5.12). Some clustering on the basis of understorey type and region is evident most apparent between sclerophyll and rainforest plots (Figure 5.11). *E. delegatensis* and *Pultenaea juniperina* and were associated with the north-east sclerophyll plots, *A. melanoxylon* and *E. dalrympleana* were associated with the north-west sclerophyll plots, *A. dealbata* was associated with the north-eastern plots, and *Nothofagus cunninghamii*,

Melaleuca squarrosa and *Leptospermum lanigerum* were associated with rainforest (Figure 5.11).

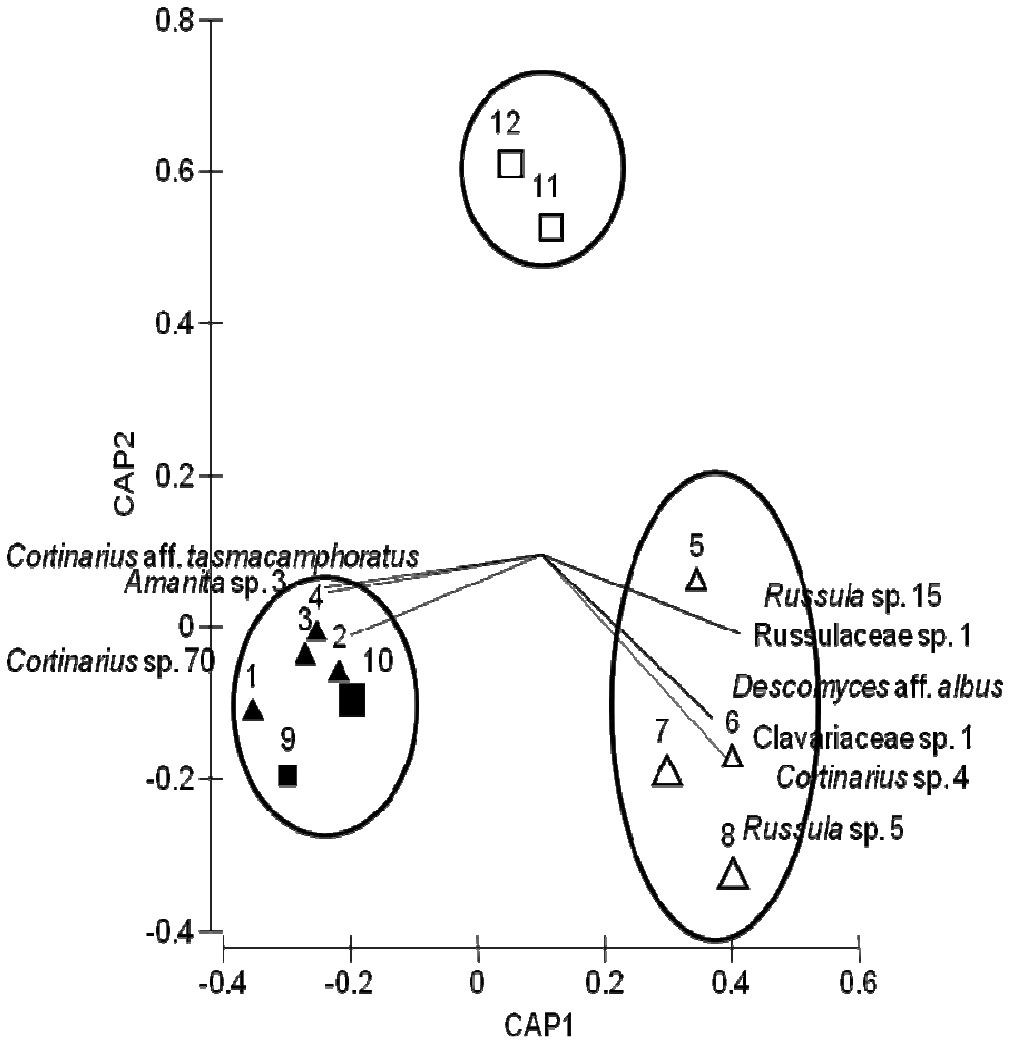

Figure 5.10 CAP analysis testing the differences in ectomycorrhizal community composition based on understorey and region (m = 3, misclassification = 8%, p = 0.004). Spearman's Rho correlations of species with CAP axes are shown as vectors for values > 0.75. Triangles indicate north-east plots and squares indicate north-west plots. Filled markers are rainforest plots and open markers are sclerophyll plots. Larger markers represent moderately declining forest and smaller markers represent severely declining forest. The CAP1 axis separates the plots on the basis of understorey type, with the sclerophyll plots lying above zero and rainforest plots lying in the negative region on the axis.

Table 5.11 Percentage of variation the ectomycorrhizal community composition explained by the individual axes in the distance-based multiple linear regression model using the abundance of dominant woody vegetation as predictor variables.

Axis	% explained variation out of fitted model		% explained variation out of total variation	
	Individual	Cumulative	Individual	Cumulative
1	47.74	47.74	25.35	25.35
2	31.73	79.47	16.85	42.19
3	20.53	100.00	10.90	53.10

Table 5.12 Summary of the sequential test of each of the woody vegetation predictor variables included in the distance-based multiple linear model on ectomycorrhizal community composition. AIC is Akaike Information Criterion, SS is the sum of squares and P is the significance value. Significant P values are in bold.

Variable	AIC	SS(trace)	Pseudo-F	P	Proportion of variation explained
Nothofagus cunninghamii	93.14	4912.8	2.43	0.06	0.20
Acacia melanoxylon	92.18	4416.6	2.52	**0.03**	0.18
Leptospermum lanigerum	90.67	3999.8	2.72	0.10	0.16

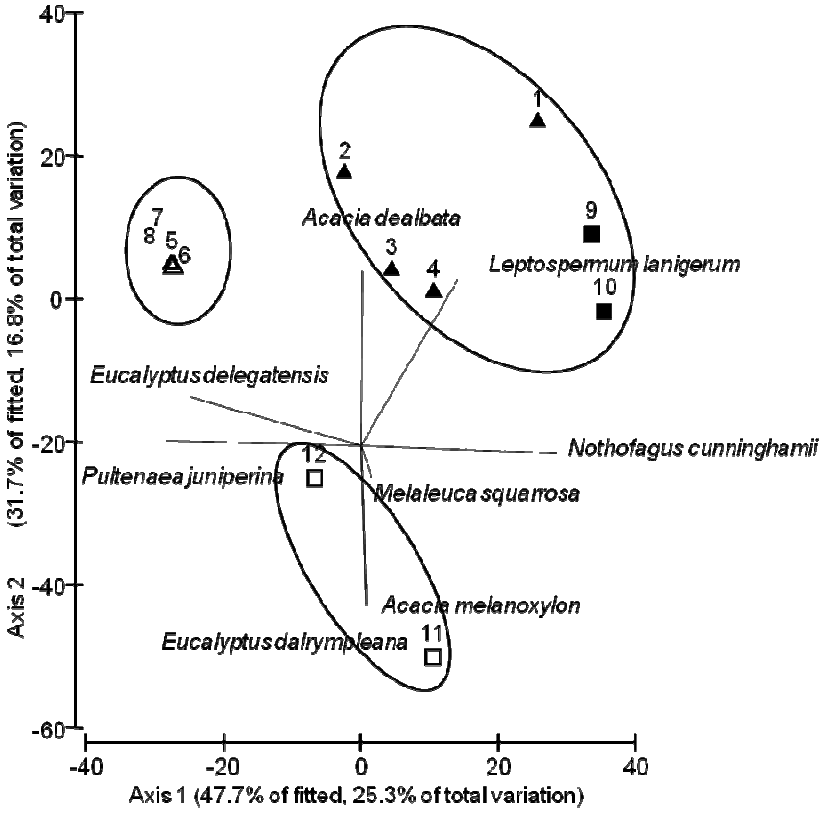

Figure 5.11 Distance-based redundancy analysis of ectomycorrhizal community composition using the abundance of dominant woody plant species as predictor variables. Model is based on step-wise regression. AIC= 90.67 and R^2= 0.53. Filled markers are rainforest plots, open markers are sclerophyll plots. Squares are north-west plots and triangles are north-east plots. Large markers represent moderately declining forest and smaller markers represent severely declining forest. Markers are labeled with plot numbers. All variables available for inclusion in the model are shown.

When the *a priori* groups are changed from understorey and region, to understorey and eucalypt crown health, there is no significant relationship (m= 3, misclassification = 16.67%, p = 0.07, m=9). Plots cluster on the basis of crown health and also separate between understorey type (Figure 5.12). These groups were not as separate or as strong when analysing the ECM community recorded as only root tips or sporocarps (root tips p = 0.29 misclassification = 58%, sporocarps p = 0.03 and misclassification = 33%).

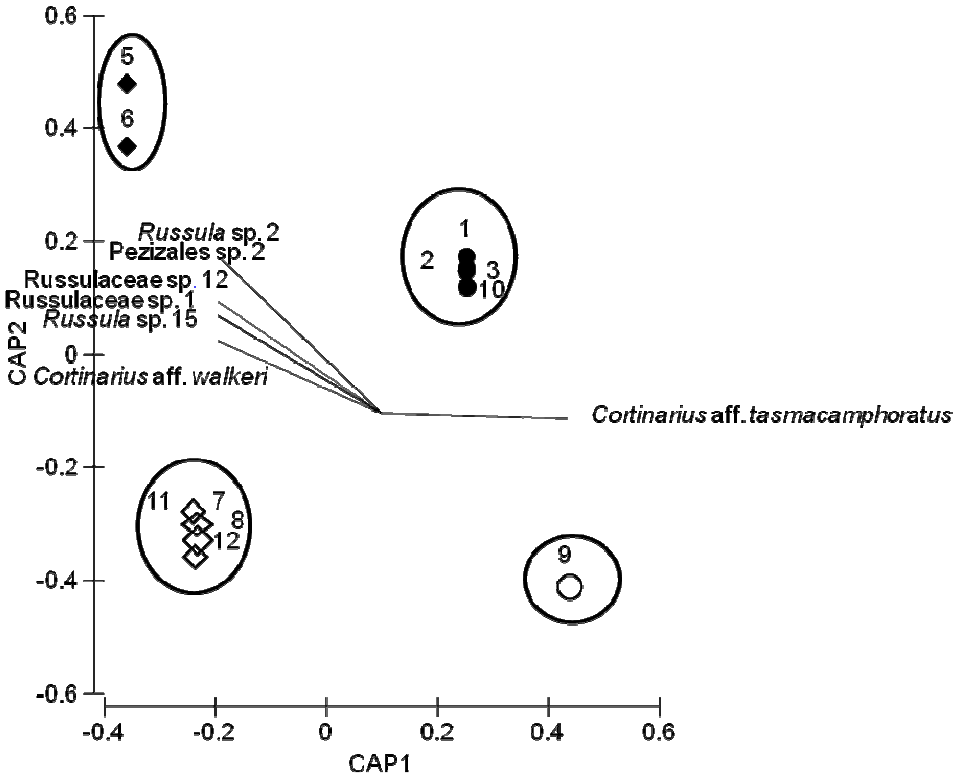

Figure 5.12 CAP analysis testing the difference in ectomycorrhizal community composition (using the combined root tip and sporocarp data) between understorey and crown health. Misclassification was 16.67% and p = 0.07 for m = 9. Open markers are plots that have moderate decline and closed markers indicate severe decline. Circles indicate rainforest understorey and diamonds indicate sclerophyll understorey. Large markers are north-west plots and smaller markers are north-east points. Plot numbers are labelled above markers. Vectors indicate Spearman's Rho correlation of fungal species, with length indicating value. Only correlations > 0.75 are shown. There is relatively little separation between region and health.

5.3.5.3 Ectomycorrhizal community proportional composition

Unconstrained multi-dimensional scaling was performed on the ECM proportional composition data (the percentage of each family within each plot) to determine if there were any natural groups within the data (i.e. groups were not set prior to the analysis). The ordination of the MDS on ECM community proportional composition shows that the plots do not show any clustering (Figure 5.13).

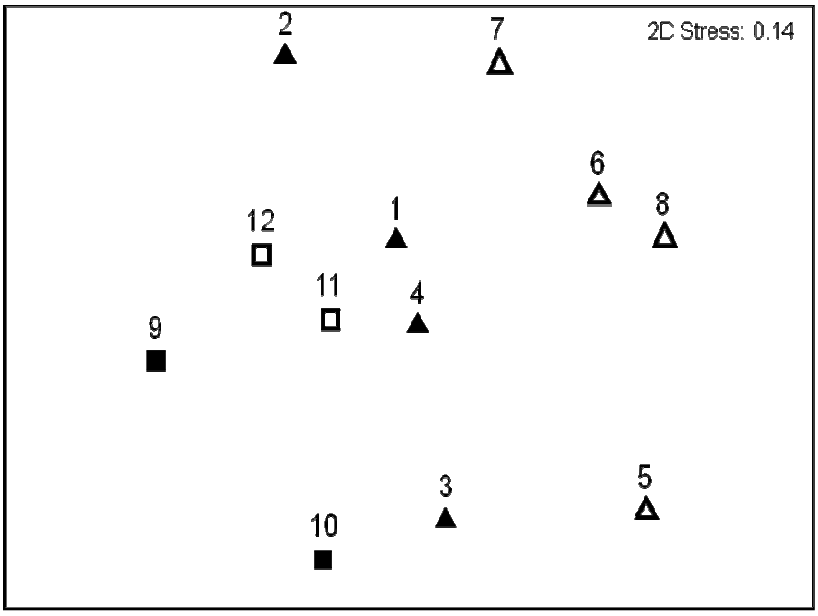

Figure 5.13 Ordination of the multi-dimensional scaling analysis of ectomycorrhizal community proportional composition. Stress is 0.14 which is reasonable although this indicates that this model is not an excellent fit (<0.1 is regarded as an excellent model fit, and >0.15 is regarded as unacceptable (Anderson, Gorley *et al.* 2008)). Rainforest plots are represented by filled markers, sclerophyll plots by open markers, squares are north-west plots and triangles are north-east plots. Larger markers represent those plots that had moderately declining forest, while smaller markers represent severely declining forest. There is no natural grouping of plots based on the percentage of different ECM families within each community.

One-way ANOVA testing the difference in the percentage of each family within the ECM community of each plot showed that the percentage of Cortinariaceae species in rainforest and sclerophyll plots was very similar, but the percentage of Russulaceae was significantly lower in rainforest plots compared to sclerophyll plots, and while the Thelephoraceae made up slightly more of rainforest ECM communities than sclerophyll communities this was not significant (Table 5.13). When considering only region, the Cortinariaceae made up a significantly greater percentage of the ECM community in the north-west than the north-east and the Russulaceae made up a significantly greater proportion of north-eastern ECM communities (Table 5.14). There was no difference in the percentage of Thelephoraceae in ECM communities of the north-east and north-west (Table 5.14).

Table 5.13 One-way ANOVA results testing the differences in the proportion (angular transformed) of the three richest families within the ectomycorrhizal communities between understorey type. Significant P values are in bold.

Family	Rainforest (mean N=6)	Sclerophyll (mean N=6)	$F_{1,10}$	P-value
Cortinariaceae	0.65	0.64	0.02	0.90
Russulaceae	0.32	0.49	5.97	**0.03**
Thelephoraceae	0.23	0.11	4.43	0.06

Table 5.14 One-way ANOVA results testing the differences in the proportion of the three richest families (angular transformed) within the ectomycorrhizal communities between regions. Significant P values are in bold.

Family	North-east (mean N=8)	North-west (mean N=4)	$F_{1,10}$	P-value
Cortinariaceae	0.56	0.81	22.61	**0.0008**
Russulaceae	0.46	0.29	6.09	**0.03**
Thelephoraceae	0.15	0.21	0.78	0.40

A CAP analysis of ECM fungal community proportional composition (i.e. the relative species richness of ECM families within a plot) with *a priori* groups based on understorey and region, showed a significant difference in ECM community proportional composition among groups. Similar to the CAP analysis of ECM species composition (Figure 5.10), the ordination of the CAP analysis of ECM community proportional composition as defined by understorey and region depicts the same number of groups, although members of the groups differ (Figure 5.14). Region influences ECM community proportional composition indicated by the north-west rainforest and sclerophyll plots clustering together forming a single group. This is because of a high level of misclassification of the north-west rainforest plots (misclassification 100%) resulting in them clustering with the other north-west plots, rather than the rainforest plots (Figure 5.14), as was seen with ECM community composition (Figure 5.10). The north-east plots separate based on understorey-type. All four north-west plots are spatially close to one another, as are the four north-east sclerophyll plots. Each group was associated with a

particular ECM family, with north-west plots associated with a higher proportion of Cortinariaceae, the north-east sclerophyll plots associated with a higher proportion of Russulaceae and the north-east rainforest plots associated with a higher proportion of Boletaceae within the ECM community (Figure 5.14).

When testing if ECM community proportional composition differed among groups based on understorey and crown health, there was no significant difference (p = 0.16 misclassification = 58%) and little separation between moderately and severely declining plots (Figure 5.15).

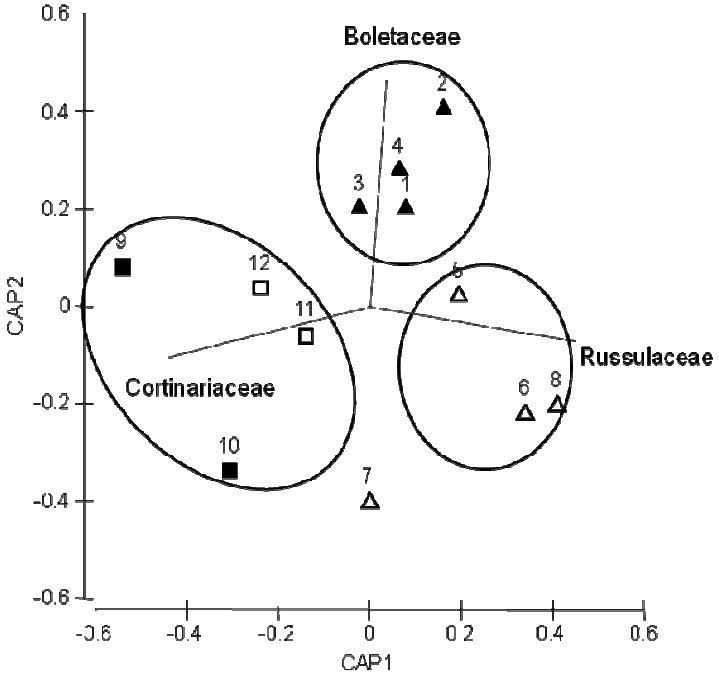

Figure 5.14 CAP analysis testing the differences in ectomycorrhizal community proportional composition based on understorey and region (m = 3, misclassification = 42%, p = 0.07). Spearman's Rho correlations of ECM families with CAP axes are shown for values > 0.7. Triangles indicate north-east plots and squares indicate north-west plots. Filled markers are rainforest plots and open markers are sclerophyll plots. Large markers are plots located in the north-west and smaller markers represent plots located in the north-east. Plot numbers are labelled above each marker. The Russulaceae and Cortinariaceae were correlated to the horizontal CAP1 axis. Moving from negative to positive, plots increase in the percentage of Russulaceae in the community and the percentage of Cortinariaceae decreases. The Boletaceae was correlated to the CAP2 axis, and there is a decrease in the percentage of Boletaceae moving from negative to positive.

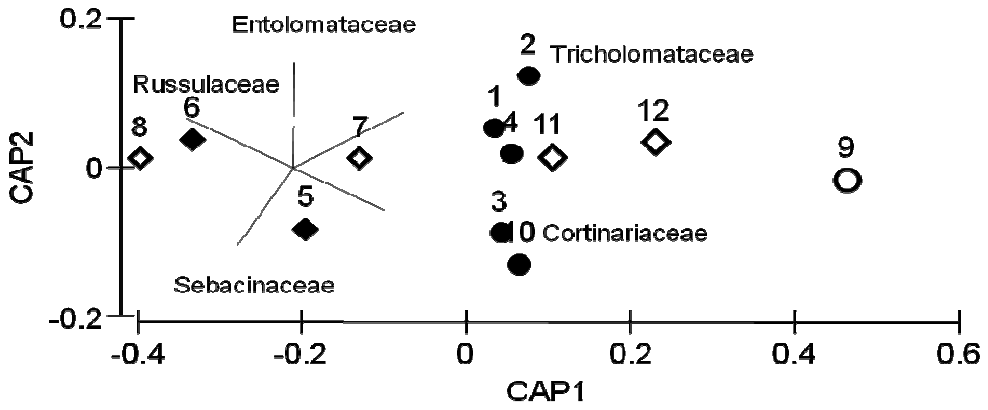

Figure 5.15 CAP analysis testing the difference in ectomycorrhizal community proportional composition among plots differing in understorey and crown health. Misclassification was 58% and p =0.16 for m = 2. Open markers are plots that have moderate decline and closed markers indicate severe decline. Circles indicate rainforest understorey and diamonds indicate sclerophyll understorey. Large markers are north-west plots and smaller markers are north-east plots. Plot numbers are shown above the markers. Vectors indicate Spearman's Rho correlation, with length indicating value. Only correlations > 0.75 are shown.

5.3.6 The ectomycorrhizal community, nutrients and eucalypt health

5.3.6.1 Ectomycorrhizal community OTU richness

A stepwise linear regression involving two predictors, available P and available NO_3^-, explained 80% (R^2=0.83) of the variation in ECM richness. The model is:

$$\text{ECM richness} = 95.28 - 24.13 * \text{available P} - 21.22 * \text{available } NO_3^-$$

This model shows that an increase in available P or NO_3^- results in a reduction in ECM OTU richness, or conversely, ECM richness is greatest at low levels of available P or NO_3^-. None of the other soil or foliage nutrient variables that were available for model selection (soil pH, soil NO_3^-, soil NH_4^+, total soil N, total soil P, foliage N, foliage P, and available NH_4^+) were significant in predicting ECM OTU richness and were not included in the model. A plot of observed ECM richness against predicted ECM richness shows the fit of the data to the model (Figure 5.16).

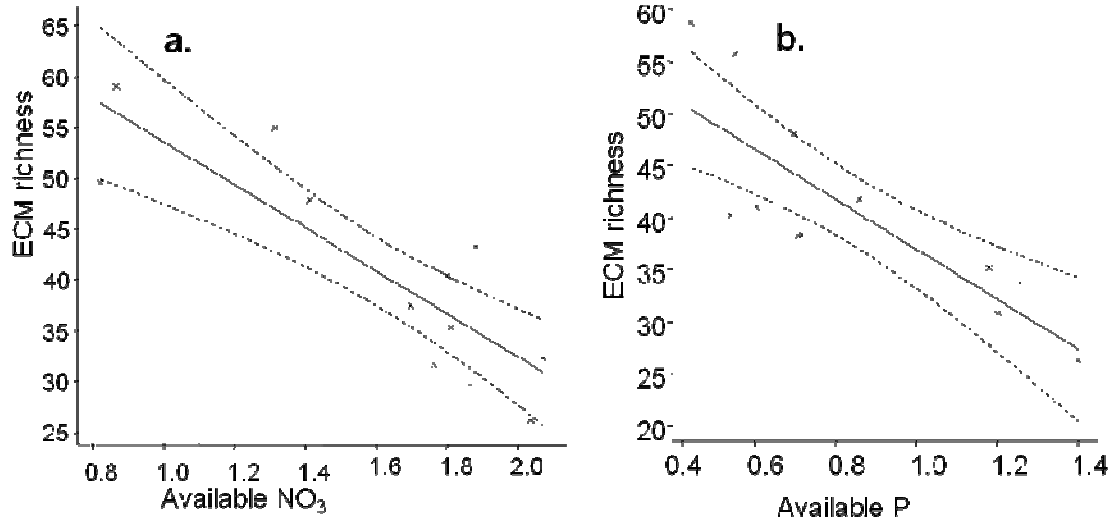

Figure 5.16 Observed and predicted ectomycorrhizal fungal species richness based on a linear regression model. Crosses represent observed values, solid line represents the predicted values, and dashed lines indicate 95% confidence intervals. a. ECM richness and exchangeable soil NO_3^-. B. ECM richness and exchangeable soil P.

5.2.6.2 Ectomycorrhizal community composition

Using a distance-based (Bray-Curtis resemblance) multiple linear model that included nutrient variables as well as crown health as predictor variables of ECM community composition, 72% percent of the variation in the data set was explained over five axes (data not shown). Soil pH ($p= 0.001$) and exchangeable NO_3^- ($p=0.002$) were highly significant in the model while soil P ($p=0.037$), soil NO_3^-($p=0.042$) and crown health ($p=0.046$) were marginally significant in predicting ECM community OTU composition. Only soil pH, exchangeable NO_3^- and soil P were included in the final model which explains 52% of the total variation in the data (Tables 5.15 and 5.16).

Table 5.15 Percentage of variation in ectomycorrhizal community composition explained by the individual axes in the distance-based multiple linear regression model using nutrient and crown health predictor variables.

	% explained variation out of fitted model		% explained variation out of total variation	
Axis	**Individual**	**Cumulative**	**Individual**	**Cumulative**
1	54.27	54.27	28.20	28.20
2	30.94	85.22	16.08	44.28
3	14.78	100.00	7.68	51.96

Table 5.16 Summary of the sequential test of each of the nutrient and crown health predictor variables included in the distance-based multiple linear model on ectomycorrhizal community composition. AIC is Akaike Information Criterion, SS is the sum of squares, P is the significance value, and d.f. is the degrees of freedom.

Variable	**AIC**	**SS(trace)**	**Pseudo-F**	**P**	**Proportion of variation explained**	**Cumulative variation explained**	**res.df**
Soil pH	92.33	6222.8	3.30	0.0005	0.25	0.25	10
Available NO_3^-	91.45	4031.3	2.44	0.0022	0.16	0.41	9
Soil P	90.95	2789.3	1.85	0.04	0.11	0.52	8

There is no separation of moderately and severely declining plots according to ECM similarities predicted by soil nutrients and crown health. Instead the ordination of the model shows that plots group according to understorey type and region, with the north-east sclerophyll plots lying near each other, the north-west sclerophyll plots near one another, and the rainforest plots clustering together (Figure 5.17). Plots in the positive region of Axis 1 had less acidic soils and higher concentrations of soil P (the north-east sclerophyll plots), while those in the negative region of Axis 2 had higher concentrations of available soil nitrate i.e. the rainforest plots, and the north-west plots had lower available soil nitrate and lower soil P (Figure 5.17).

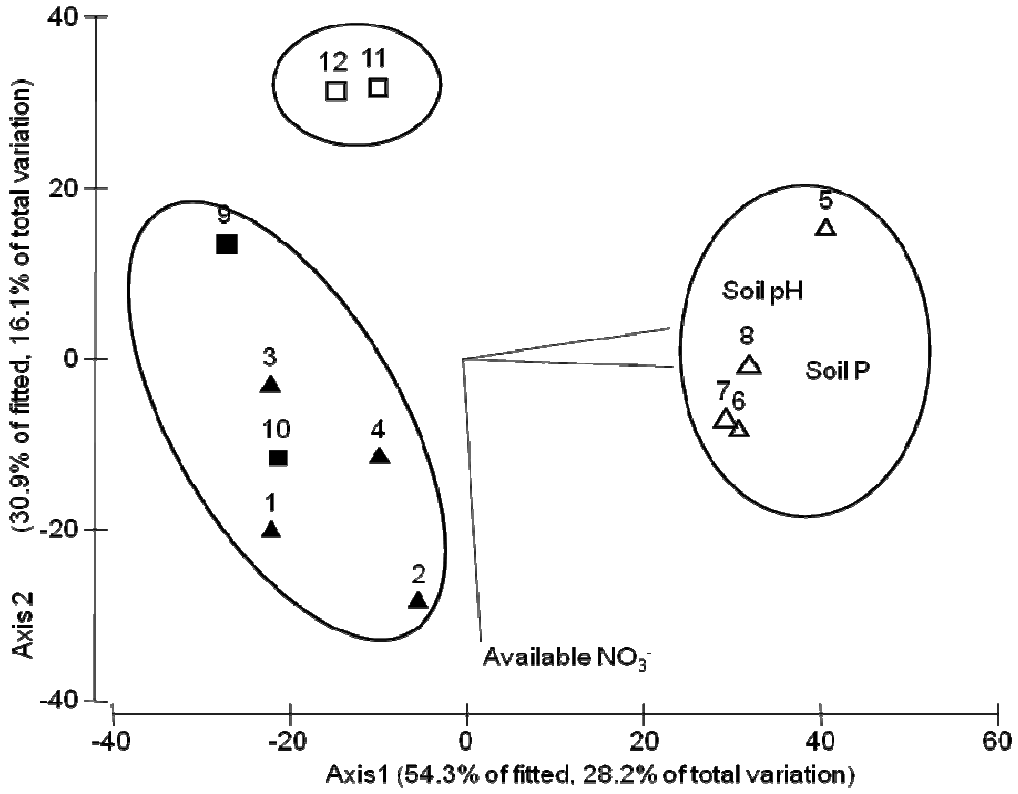

Figure 5.17 Distance-based redundancy analysis of ectomycorrhizal community composition using Bray-Curtis similarity matrix and all foliage and soil nutrient concentrations, plus crown health, as predictor variables. The model is based on step-wise regression. AIC= 90.95 and R^2= 0.52. Spearman's Rho correlations of species with axes 1 and 2 are shown as vectors for values > 0.75. Filled markers are rainforest plots, open markers are sclerophyll plots. Squares are north-west plots and triangles are north-east plots. Large markers represent moderately declining forest and small markers represent severely declining forest. Markers are labeled with plot numbers.

A canonical correlations analysis testing the correlations between available soil NO_3^-, and ECM community composition similarities and soil pH and ECM community composition similarities showed strong correlations between these two variables and the ECM communities. For the correlation analysis with available soil NO_3^-, the canonical correlations square = 0.85 and p = 0.007, and for the analysis with soil pH, the correlation square = 0.99 and p = 0.001. There are clear trends between these two soil variables with ECM families. As available soil NO_3^- increases, the relative richness of the Cortinariaceae within each plot decreases (Figure 5.18),

and as soil pH increases, the relative richness of the Russulaceae in each plot increases (Figure 5.19).

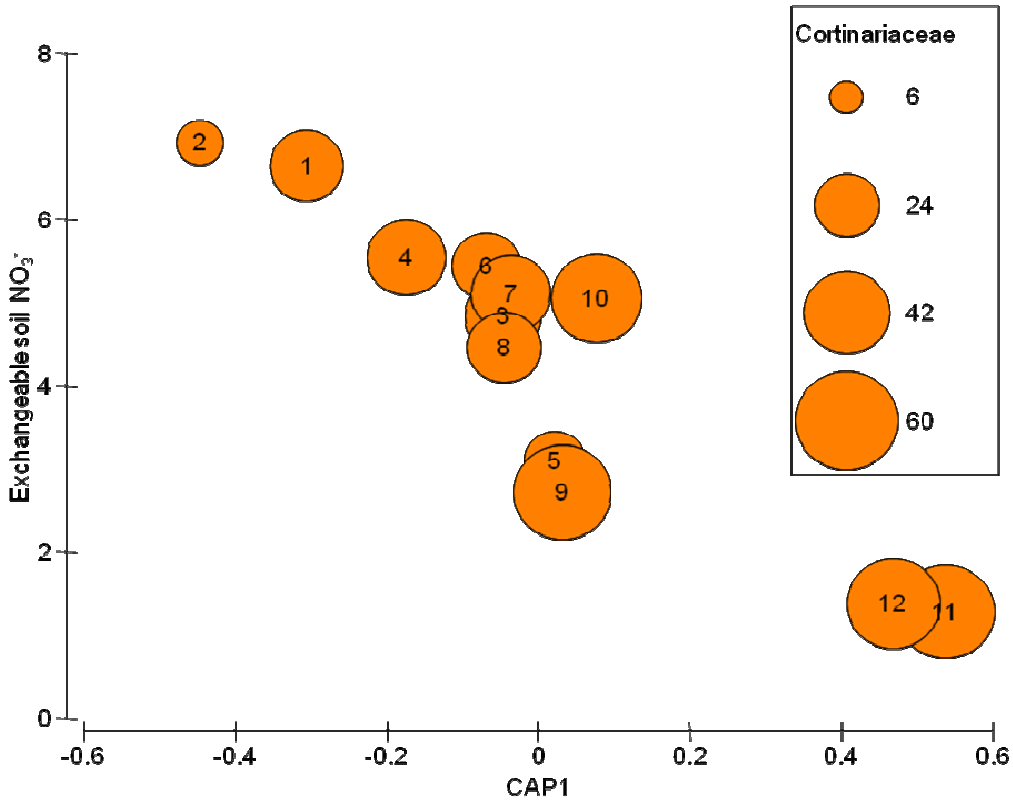

Figure 5.18 The correlation between available soil NO_3^- of *E. delegatensis* forest plots and ectomycorrhizal community composition. Plots are marked with plot number. The relationship between soil NO_3^- and the richness of the Cortinariaceae of each plot is shown with the orange circle overlays, with size of the circle an indication of the richness of the Cortinariaceae. In general there is an inverse relationship between the richness of the Cortinariaceae and available soil NO_3^-.

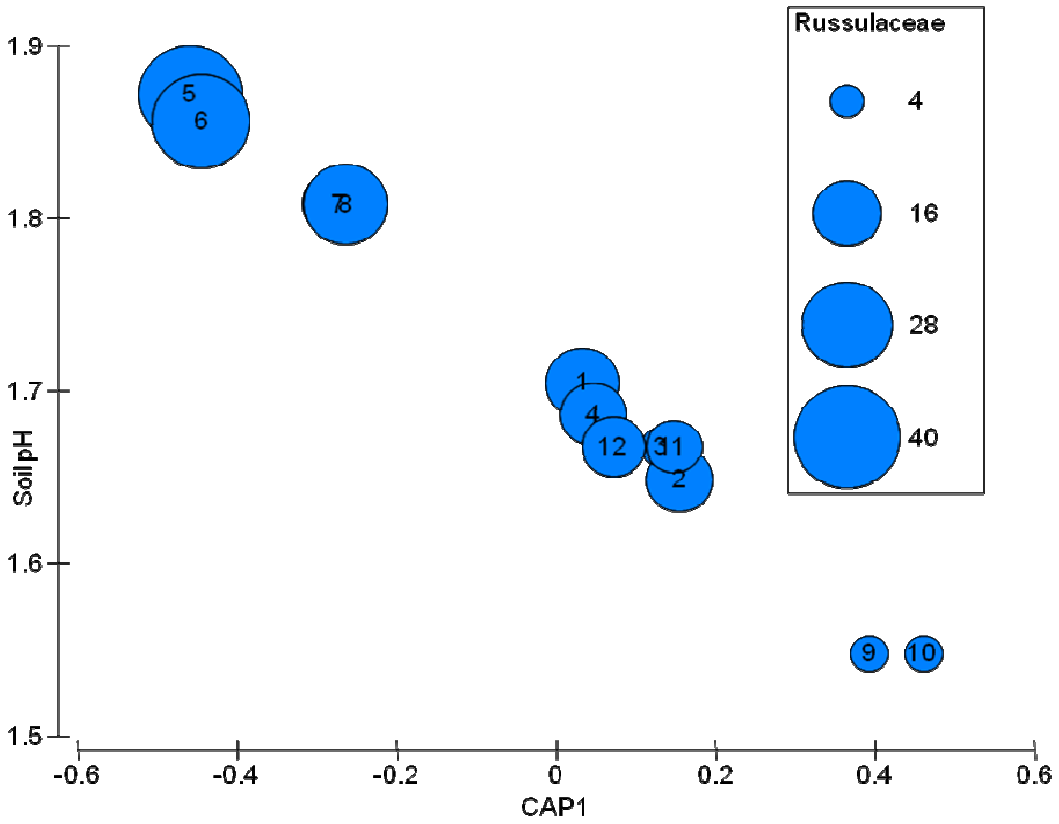

Figure 5.19 The correlation between soil pH of *E. delegatensis* forest plots and ectomycorrhizal community composition. Plots are marked with plot number. The relationship between soil pH and the richness of the Russulaceae of each plot is shown with the blue circle overlays, with size of the circle an indication of the richness of the Russulaceae. There is a positive correlation between the richness of the Russulaceae and increasing soil pH.

5.3.6.3 Ectomycorrhizal community proportional composition

The multivariate linear model based on a similarity matrix of ECM community proportional composition indicated that soil pH was highly significant and soil organic carbon was significant in predicting ECM community proportional composition (Table 5.17). These variables were the only variables included in the final model that explained 44% of the variation in ECM community proportional composition (Table 5.18).

Differences in the predictor variables among plots meant that plots did not cluster on the basis of health status but did differentiate on the basis of site suggesting that each site had similar soil conditions and more similar ECM communities (Figure 5.20). Plots located in the positive region of Axis 1 had higher soil pH while plots located in the positive area of Axis 2 had greater concentrations of soil organic carbon (Figure 5.20).

Table 5.17 Summary of the sequential test of each of the nutrient and crown health predictor variables included in the distance-based multiple linear model on ectomycorrhizal community proportional composition. AIC is Akaike Information Criterion, SS is the sum of squares, P is the significance value, and d.f. is the degrees of freedom.

Variable	AIC	SS (trace)	Pseudo-F	P	Proportion of explained variation	Cumulative variation explained	Residual d.f.
Soil pH	77.62	2147.4	3.8743	0.0006	0.28	0.28	10
Soil Organic C	76.65	2147.4	2.5304	0.02	0.16	0.44	9

Table 5.18 Percentage of variation in the ectomycorrhizal community proportional composition explained by the individual axes in the distance-based multiple linear regression model using nutrient and crown health predictor variables.

	% explained variation out of fitted model		% explained variation out of total variation	
Axis	Individual	Cumulative	Individual	Cumulative
1	65.63	65.63	28.71	28.71
2	34.37	100.00	15.03	43.74

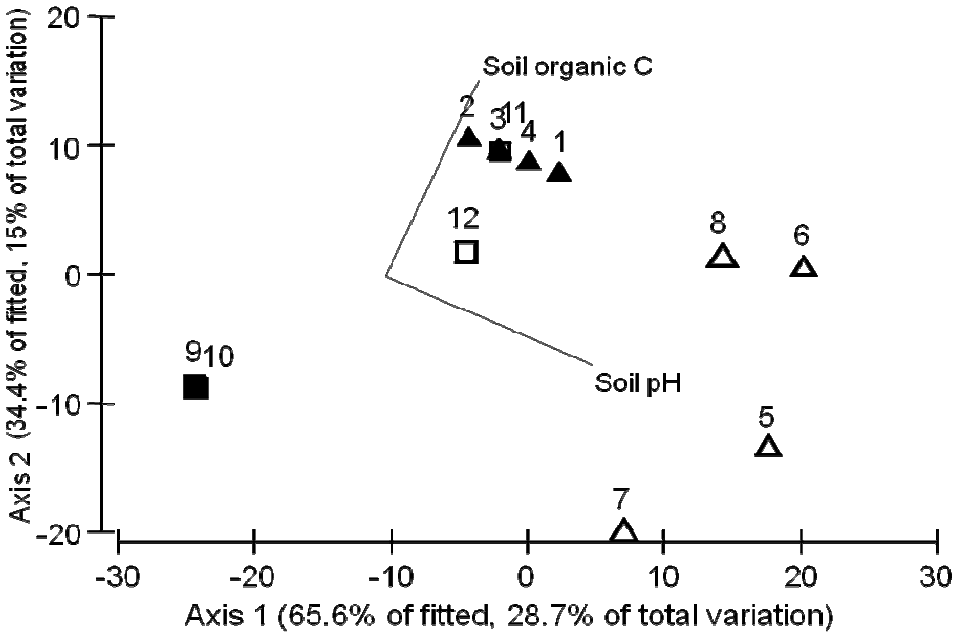

Figure 5.20 Distance-based redundancy analysis of ectomycorrhizal community proportional composition using Bray-Curtis similarity matrix and all foliage and soil nutrient concentrations, plus crown health, as predictor variables. Model is based on step-wise regression. AIC= 76.65 and R^2= 0.44. Spearman's Rho correlations of species with axes 1 and 2 are shown as vectors for values > 0.75. Filled markers are rainforest plots, open markers are sclerophyll plots. Squares are north-west plots and triangles are north-east plots. Large markers represent moderately declining forest and small markers represent severely declining forest. Markers are labeled with plot numbers.

5.3.7 Summary of results

All of the data and analyses provided in Chapter 5, along with those in Chapter 4 and additional information provided in the appendices are summarised in Table 5.19. Table 5.19 shows the main trends in all of the fungal, vegetation, and nutrient variables measured that were associated with health status, forest type, and region.

Table 5.19 A summary of the trend of different health, nutrient and community variables between region, understorey type and health status. Trend is represented by arrows where strength of the trend is indicated by either one (trend) or two arrows (strong trend). Direction of arrow indicates if the variables were higher (up arrow) or lower (down arrow) compared to variable means. Shading indicates a significant ANOVA result. Blue arrows indicate the expected trend established from the literature and observed trend are the same. Green arrows indicate the observed trend is opposite to the expected trend from the literature. Black arrows indicate the observed trend where expected trend is not known. A dash means there was no significant correlation or difference.

Comparisons		**Region**		**Understorey type**		**Health status**	
		North-east	North-west	Sclerophyll	Rainforest	Moderate decline	Severe decline
Soil	Fertility	⇩	⇧	⇩	⇧	⇧	⇩
	pH	⇧	⇩	⇧	⇩	—	—
	Total soil N	⇩	⇧⇧	⇩⇩	⇧⇧	⇩	⇧
	Soil NO_3^-	⇧	⇩⇩	⇩⇩	⇧⇧	⇩⇩	⇧⇧
	Available NO_3^-	⇧⇧	⇩⇩	⇩⇩	⇧⇧	⇩⇩	⇧⇧
	Soil NH_4^+	—	—	—	⇧	⇩	⇧
	Available NH_4^+	⇧	⇩⇩	⇩⇩	⇧⇧	⇩⇩	⇧⇧
	Total soil P		⇩	⇧	⇩	⇧	⇩

Comparisons		Region		Understorey type		Health status	
		North-east	North-west	Sclerophyll	Rainforest	Moderate decline	Severe decline
Soil	Available P	⇧	⇩⇩	⇧⇧	⇩⇩	⇧⇧	⇩⇩
	Organic C	⇧	⇩	—	—	⇩	⇩
	C: N ratio	⇧	⇩⇩	—	⇩	—	⇩
	N: P ratio	⇩	⇧	⇧⇧	⇩⇩	⇩⇩	⇧
Plants	Crown Health	⇩	⇧⇧	⇧	⇩	⇧⇧	⇩⇩
	Foliage P	⇧	⇩	⇧⇧	⇩⇩	⇧	⇩⇧
	Foliage N	—	⇩	—	—	—	⇧
	Foliage N: P	—	—	⇧⇧	⇩⇩	⇩	⇧
	Plant species richness	⇧	⇩	⇧⇧	⇩	⇧	⇩
	Acacia melanoxylon abundance	⇩⇩	⇧⇧	⇧⇧	⇩⇩	⇧⇧	⇩⇩
	E. delegatensis abundance	⇧	⇩⇩	⇧⇧	⇩⇩	⇩	–
	Leptospermum lanigerum abundance	⇧⇧	⇩⇩	⇩⇩	⇧⇧	⇩⇩	⇧⇧
	Nothofagus cunninghamii abundance	⇩⇩	⇧⇧	⇩⇩	⇧⇧	⇩⇩	⇧⇧
Fungi	Combined ECM species richness	⇩	⇧⇧	—	⇧	⇧	—
	Sporocarp species richness	⇩	⇧	—	—	⇧	⇩
	Root tip species richness	⇩	⇧⇧	⇩	⇧	⇩	⇩
	Cortinariaceae species richness	⇩⇩	⇧⇧	⇩	⇧	⇧⇧	⇩⇩
	Cortinariaceae proportional composition	⇩	⇧⇧	—	—	⇧⇧	⇩
	Russulaceae species richness	⇧	⇩	⇧⇧	⇩⇩	⇩	⇧
	Russulaceae proportional composition	⇧	⇩⇩	⇧⇧	⇩⇩	⇩	⇧
	Thelephoraceae species richness	—	—	⇩⇩	-	⇩⇩	⇧
	Thelephoraceae proportional composition	—	—	⇩⇩	⇧⇧	⇩⇩	⇧⇧

5.6 Discussion

5.6.1 The ectomycorrhizal fungal community of *E. delegatensis* forest is linked to eucalypt decline

This is the first study of temperate forest decline which investigated the link between ECM community attributes and health of the forest trees. This study was novel in its approach utilising molecular characterisation of the ECM communities and developed a robust method to measure eucalypt decline in the field. The work presented here builds on the current knowledge of eucalypt decline and dieback throughout Australia, as well as that of ECM ecology. The findings from this study have important implications for understanding ecological processes within eucalypt forest, for ensuring sustainable eucalypt forest management, as well as advancing our understanding of Australian fungal ecology within eucalypt forests.

The correlations between the ECM communities and crown health of *E. delegatensis*, along with the differences in composition and proportional composition of moderately and severely declining forest support the hypothesis that ECM communities differ in forests at different stages of decline. A similar relationship between ECM communities and forest decline was found by Peter, Ayer *et al.* (2008) who studied the effect of pollution on forest health and found that forests that differed in their level of decline, differed in ECM community compositions (Peter, Ayer *et al.* 2008).

Differences between forests of different health status in addition to ECM assemblages include ECM richness and OTUs that are unique to forests containing trees of a particular health status. Peter, Ayer *et al.* (2008) found that both ECM root tip and sporocarp richness was lower in declining Spruce forest, compared to healthier forest. Furthermore, declining oak trees were found to have a lower proportion of vital ECM root tips than healthier trees (Mosca, Montecchio *et al.* 2007). In *E. delegatensis* forest, sporocarp richness was slightly lower in severely declining forest but root tip richness was slightly higher in severely declining forest, although neither was statistically significant. Even so it is clear that forests with different health differ in their ECM communities as only 22% of OTUs were shared between moderately and severely declining forest. The majority of OTUs were associated with either severely or moderately declining eucalypts but further research would be required to determine if these OTUs were true indicators of forest health or decline.

Additional support for the association between ECM communities and eucalypt health comes from the significance of the crown health variable score in predicting ECM community compositional and structural similarities in the multivariate linear models, and the moderately strong correlation of ECM species composition and community proportional composition with crown health in the canonical correlations analyses. Determining other changes to the ECM community, such as changes in the abundance of ECM species was outside the scope of this study, but would shed more light on the relationship of the ECM community and eucalypt decline.

The conceptual model presented in the introduction of this chapter proposes that the 'ECM community' may influence 'Crown decline' (Figure 5.2). A lower richness of ECM communities in declining eucalypt forest may contribute to the decline process through reduced forest stability and resilience (Tommerup & Bougher 1999). Less diverse communities may be less able to cope with stress because of lower functional diversity resulting in inefficient nutrient acquisition and resource utilisation (Brundrett & Cairney 2002; Dickie & Moyersoen 2008; Courty, Buée *et al.* 2010). There may also be less functional redundancy within less diverse ECM communities i.e. if species with specialised functions are lost, and there are no species with the same function to replace them, the ecosystem function is diminished.

In the severe stage of decline not only is the ECM community composition altered, but as a consequence, the functioning of the ECM community may also be altered, which in turn could affect the health of the eucalypt trees. Declining forest trees tend to have fewer ECM roots than healthy trees (Perrin & Estivalet 1990; Power & Ashmore 1996). *E. delegatensis* seedlings grown in soil collected from Tasmanian eucalypt forest with secondary rainforest vegetation formed sparse mycorrhizas (Ellis & Pennington 1992). Seedlings grown in soil from beneath declining Tasmanian eucalypt forest, and inoculated with as little as 10% of soil from beneath healthy eucalypt forest, formed prolific mycorrhizas with fungi of the Basidiomycota, similar to seedlings that were grown only in soil collected from beneath healthy trees (Ellis & Pennington 1992). This work was supported by Harvest, Davidson *et al.* (2008) who showed differences in eucalypt seedling growth when exposed to the solutions extracted from soils taken from under healthy and unhealthy eucalypts. These two studies suggest either a deficiency of ECM fungi inoculum in forest soils under declining eucalypts, or that an inhibitory factor was overcome by the addition of healthy soil allowing the formation of mycorrhizas.

Shifts in the ECM community and colonisation may result in fewer benefits for the host tree, for example through reduced nutrient uptake. Despite reduced nutrient uptake, the host tree may still provide carbon to the mycorrhizal partners, which could increase the stress of the tree (Corrêa, Strasser *et al.* 2008), but simulations based on biological market-models indicate that to maintain diverse mycorrhizal communities, the host is more likely to use a selective carbon-allocation strategy wherein the plant adjusts the carbon allocation to individual root tips based on the cost of nutrients (Cowden & Petersen 2009).

The similarities and differences in the ECM communities of moderately and severely declining eucalypt forests are likely due to a number of factors of which soil chemistry and vegetation dynamics play an important role. These factors were conceptualised in the introduction (Figure 5.2) and are explored more fully below.

5.6.2 The effect of vegetation structure and composition on ectomycorrhizal communities and eucalypt decline

In the system of the current study, decline is more likely to occur in *E. delegatensis* trees with rainforest understorey than trees with sclerophyll understorey (the majority of the severely declining plots were rainforest and only one rainforest plot was not in severe decline). Eucalypt decline has been associated with the development of mesic understorey in a number of other studies (Ellis 1964; Jurskis & Turner 2002; Turner & Lambert 2005). This process differs from the model of natural succession in the absence of disturbance, prepared by Ellis (1985). The process of decline involves encroachment of an understorey of few species, either rainforest or sclerophyll species, that tend to shade the soil surface, which is followed by premature decline and death of the overstorey eucalypts. Understorey plants on sites with declining eucalypts tend to have greater N and P foliage concentrations, and once these nutrients are released by decomposition, changes in the soil chemical environment can occur (Turner & Lambert 2005). As such, the rate of decline appears to different with understorey type, occurring faster where there are rainforest elements present.

The *E. delegatensis* plots with rainforest understorey had different soil chemistry from plots with a sclerophyll understorey. Rainforest had higher N, lower P, and lower pH than sclerophyll plots. These results are consistent with previous studies of soil chemistry within *E. delegatensis* forests (Ellis & Graley 1987; Ellis & Pennington 1989). Different forest types

vary in their rate of production of mineral N and nitrification (Ellis & Pennington 1989; Turner & Lambert 2005). Soils under rainforest and eucalypt forests with developing rainforest understoreys have higher rates of nitrification and higher levels of mineral N than eucalypt forests lacking rainforest elements, and levels of N generally increase with forest succession (Lamb 1980; Ellis & Pennington 1989; Turner & Lambert 2005). Research on highland eucalypt forest in Tasmania has shown that total and mineralisable N increases, and pH and total P decreases along a successional gradient from grassland, to sclerophyll eucalypt forest to rainforest (Ellis & Graley 1987). These trends are also seen in this study, with forest earlier in the succession (sclerophyll understorey) having lower soil N, higher pH and higher soil P, than later successional forest (rainforest understorey). As these changes in soil chemistry occur they may act to favour the establishment of rainforest elements such as *Nothofagus*. Alternatively, the differences in soil chemistry between forests with different understorey vegetation likely relates to the vegetation present at the site (Ellis and Graley 1987).

ECM fungal communities are known to be shaped by the vegetation community including composition, forest structure and dominant tree species (Nantel & Neumann 1992; Aerts 2002; Debellis, Kernaghan *et al.* 2006; Twieg, Durall *et al.* 2007; Tedersoo, Jairus *et al.* 2008). In the conceptual model, a change in 'Vegetation Structure and Composition' directly affects the 'ECM community' (Figure 5.2) and the results of this chapter support this relationship. In this study, understorey and overstorey vegetation, along with geographic location, were found to significantly influence the ECM fungal communities of *E. delegatensis* forest. The influence of region is highlighted by the difference in ECM community composition of the north-east and north-west sclerophyll plots, despite all plots having a similar understorey. Within these groups, paired plots, although spatially close to one another did not necessarily have the most similar ECM communities.

Certain combinations of ECM species within the communities may therefore result in differential abilities to take up various forms of N (Aerts 2002). The ECM-mediated nutrient processes may be important mechanisms in plant competition (Aerts 2002). It is also possible that ECM communities with greater species richness and diversity (such as those of the north-west forests) have greater functional diversity allowing adaption to changed conditions more effectively than forests with a lower diversity of ECM fungi. Greater species richness and diversity can act to provide a greater resilience to disturbances and may help to stabilise ecosystem processes (Tommerup & Bougher 1999; Brundrett &

Cairney 2002; Dickie & Moyersoen 2008; Courty, Buée *et al.* 2010). This may help to explain why the north-west forests are less affected by decline than the north-east forests.

Another explanation for this separation between the north-east and north-west sclerophyll plots is the difference in overstorey vegetation between the regions. Distinct ECM communities are known to occur in association with particular overstorey tree compositions and richness (Nantel & Neumann 1992; Kernaghan, Widden *et al.* 2003; Debellis, Kernaghan *et al.* 2006; Kennedy & Hill 2010), and tree species composition, is known to influence understorey composition (Barbier, Gosselin *et al.* 2008). Furthermore ECM community compositions can be related to plant genetic differences (Sthultz, Whitham *et al.* 2009). In this study, *E. dalrympleana* is present as a co-dominant with *E. delegatensis* in the north-west, but absent from the north-east plots. In contrast, all of the rainforest understorey plots had a similar ECM composition irrespective of region, and all rainforest understorey plots had a very similar vegetation composition, with the same overstorey plants present.

Fungal host preference may be playing a role in defining the composition of these different forest types and has been reported for a number of fungal species (Molina, Massicotte *et al.* 1992; Bougher 1995; Ishida, Nara *et al.* 2007). Fungal species host preference has been demonstrated in Tasmanian wet sclerophyll forest with some species more frequently found as associates of *Eucalyptus* than of *Nothofagus* and vice-versa (Tedersoo, Jairus *et al.* 2008). Although Pryor (1976) suggested that the sub-family Monocalytpus, to which *E. delegatensis* belongs, has greater requirements for mycorrhizal associates than Symphyomyrtus, to which *E. dalrympleana* belongs, fungal host preference is generally regarded as not being significant within the genus *Eucalyptus* (Malajczuk, Molina *et al.* 1982). Host specificity and the requirements of the eucalypts for ECM associations may be important in determining the ECM community.

The richness of host plant species may also be an important determinant of ECM community composition and richness. Dickie (2007) analysed data from a number of recent ECM studies and found a positive linear relationship between ECM species richness and the number of host species present at the study site. This may explain some of the differences in *E. delegatensis* forests as the north-east sclerophyll plots had the lowest number and abundance of ECM plants, excluding *E. delegatensis* and had the lowest ECM species richness. Furthermore, the number of individual host trees may act to regulate the ECM

community, providing only a finite number of roots, or host plants. Host tree density was found to play a role in determining ECM community structure of northern hemisphere *Alnus* forest (Kennedy & Hill 2010). Forest with a lower density of host trees, such as eucalypts, may support a different ECM community than stands containing a greater density of host trees. Where there are fewer individual hosts, ECM species that are highly competitive may be favoured (Peay, Kennedy *et al.* 2008b; Peay, Bidartondo *et al.* 2010).

Other than overstorey vegetation, the presence of other ECM plants, such as members of the Fabaceae and Myrtaceae, along with associated fungal host specificity, may help explain similarities in ECM community composition. The north-west sclerophyll plots were associated with the N fixing shrub *Acacia melanoxylon*. A key species for the north-east sclerophyll plots was *Pultenaea juniperina*, also from the N fixing family (Fabaceae), and *Pultenaea* is a also known ECM genus (Warcup 1980; Kope & Warcup 1986). One of the distinct vegetation components of all the rainforest plots was the presence in high abundance of *Nothofagus cunninghamii*, another ECM host species (Bougher, Fuhrer *et al.* 1994; Tedersoo, Jairus *et al.* 2008; Alvarez, Huygens *et al.* 2009; Dickie 2009; Tedersoo, Gates *et al.* 2009). Rainforest plots also tended to have other Myrtaceous ECM shrub species, such as *Leptospermum lanigerum* and *Melaleuca squarrosa* (Warcup 1980). Both *N. cunninghamii* and *L. lanigerum* were identified as species associated with eucalypt decline (Ellis, Mount *et al.* 1980), and were significant in predicting ECM community composition in the multivariate model based on plant species cover performed in this study. Other than those plant species mentioned here (and noted in Appendix 18), there were no other known ECM plant species recorded in the forest plots.

The ECM community may also play a role in shaping the vegetation community by influencing plant biomass, nutrient status, relative abundance of plants, species richness and evenness, competitive ability and successional processes (Francis & Read 1994; Aerts 2002; Brundrett & Cairney 2002; Reynolds, Packer *et al.* 2003; Booth 2004; Wardle, Bardgett *et al.* 2004). Arbuscular mycorrhizal community diversity influences all of these factors (van der Heijden, Klironomos *et al.* 1998). Also, plant species diversity and productivity are positively correlated to mycorrhizal diversity (Kernaghan 2005). The presence of particular ECM fungi may be required for rainforest plants to colonise sclerophyll communities (i.e. if the plant has a strong preference for certain ECM fungi) and so ECM communities may be influencing the successional process of the forest vegetation from sclerophyll to rainforest.

The conceptual model (Figure 5.2) shows that ECM communities can be affected vegetation dynamics, but also that vegetation structure and composition can be influenced by the ECM community (double ended arrow). Mycorrhizal networks are known to transfer and allocate resources between both overstorey and understorey plants, and can thus influence competition between the overstorey and understorey (Amaranthus & Perry 1994; Kennedy, Izzo *et al.* 2003; Booth 2004; Courty, Buée *et al.* 2010). This may have important implications in declining eucalypt forests where competition for resources, especially water, between overstorey and understorey vegetation is thought to contribute to the decline process. Another mechanism by which vegetation composition and diversity may influence the ECM community is through the variety and chemical composition of the leaf litter that is returned to the soil, and the influence of this on decomposition and nutrient cycling (Figure 5.2). Combinations of differing quality and quantities of plant litter can potentially create different resources within a forest which would affect resource partitioning of ECM fungi and other soil biota (Reddell & Malajczuk 1984; Aerts 2002; Wardle, Bardgett *et al.* 2004; Wardle 2006). Also, secondary compounds that occur as bi-products of the litter decomposition process may influence the function of ECM fungi and uptake of nutrients (Aerts 2002). ECM species that are unable to utilise the available resources will be lost from the system, while others that are capable of utilising the new niches, will thrive. Changes in litter composition may raise levels of soil compounds such as tannins and lipids which can impede eucalypt seedling establishment and growth and alter soil microbial communities (Ashton & Willis 1982; Suoto, Bolano *et al.* 2001). Furthermore, host plants may be able to control the direct transfer of sugars to their fungal partners, influencing the abundance and diversity of ECM communities.

The changes in vegetation seen in declining eucalypt forests are also accompanied by a number of changes in the soil environment and the conceptual model (Figure 5.2) shows that vegetation structure and composition, and ECM communities can both influence and be affected by changes in soil microclimate. Changes in soil moisture and temperature may affect the growth and survival of ECM species, with some ECM species better able to cope under the modified conditions, while some may be lost (Parke, Trappe *et al.* 1983; Erland & Finlay 1992; Sanchez, Honrubia *et al.* 2001). This would also affect the ability of different fungal species to form ectomycorrhizas, and cause shifts in ECM community composition and structure. The relationship between the ECM community and soil conditions are discussed to a greater degree in the following section.

5.6.3 The influence of soil chemistry on ectomycorrhizal communities and eucalypt decline

5.6.3.1 Soil nitrogen

The accumulation of mineral N in the soil, which occurs in the absence of fire, has been proposed as a cause of eucalypt decline by a number of authors (Jurskis & Turner 2002; Turner & Lambert 2005; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). In the absence of fire, N inputs from the atmosphere are locked into biomass, and concentrations in an ecosystem slowly increase over time. Fire acts to volatize this N from biomass.

This study lends further evidence for a role of N accumulation in the process of eucalypt decline. Of all the soil nutrients measured, all of the N variables were correlated to some extent to decline with higher levels of mineral N associated with more severe decline. All of the moderately declining sites had total available mineral N contents (NO_3^- and NH_4^+) less than 500 mg/L, whereas severely declining forest was associated with much higher levels of mineral N. The presence of soil NO_3^- in all of the plots indicates that nitrification is occurring, although NO_3^- levels varied from plot to plot. In general, nitrification is thought to increase with temperate forest decline leading to leaching of NO_3^- (Aber, McDowell *et al.* 1998). In this study low NO_3^- levels (< 20mg/L) were generally found in plots with healthier eucalypts, with lowest concentrations in the north-west sclerophyll plots and highest levels in the two north-east rainforest plots that were burnt in the 1960s. North-eastern Tasmanian forests are known to have relatively low rates of nitrification in healthy forests and nitrification is influenced by plant species composition and disturbance history (Adams & Attiwill 1982; Ellis & Pennington 1989). One of the north-west rainforest plots also had low levels of NO_3^- (< 20mg/L) and was also of moderate health. Overall, these findings support the work of Turner, Lambert *et al.* (2008) who found that repeatedly burnt forest plots had lower extractable mineral N than unburnt plots.

In comparison to other eucalypt forest in Australia, both the north-east and north-west study sites had relatively high levels of total soil N (Laffan, Grant *et al.* 1998; Turner & Lambert 2005). In Australia, N in forest and cultivated soil are > 0.2%, whereas rangelands tend to be < 0.1%. The means of total soil N of the moderately and severely declining forest are high in comparison to other eucalypt forest sites, with severely declining plots approaching concentrations of N seen in highly fertile soils (Laffan, Grant *et al.* 1998; Turner

& Lambert 2005). Large increases in mineral nitrogen in the soil leads to N saturation and leaching of NO_3^- (Turner & Lambert 2005) causing a reduction in the soil C: N ratio to less than 20 (Polglase, Attiwill *et al.* 1986). In general the C : N ratio of topsoil from under dry eucalypt forest in Tasmania is 29, and 15 for soil under wet forest (McIntosh, Laffan *et al.* 2005). Turner, Lambert *et al.* (2008) found that organic carbon, total N and the C : N of eucalypt forest soils was significantly correlated to time since fire, with an accumulation of total nitrogen and reduction of the C: N ratio with increasing time since fire. In *E. delegatensis* forest the C: N was less than 20 in all plots bar one (and this was only slightly greater than 20). Although the analyses did not show statistically significant differences in the C: N ratio of forests with the different health status, this is likely caused by one severely declining plot having a higher C: N ratio, causing the mean and variance of soil C: N to be greater than expected. The low C: N ratios of the *E. delegatensis* plots in this study are therefore consistent with forests suffering from decline in the absence of fire. Woodland remnants with healthy eucalypts were shown to have C: N ratios greater than 20, while remnants in decline had C: N ratios between 10 and 13 (Close, Davidson *et al.* 2008). Turner *et al.* (2008) propose that in order to maintain healthy eucalypt forests a stable C: N ratio above 25 – 30 with low mineral N production (where N inputs and losses are equivalent) is required and can be achieved by periodic burning.

In general, eucalypt decline is less marked on soils with high natural fertility (Turner & Lambert 2005; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). Soils that are naturally low in N will reach saturation rapidly, whereas fertile soils (such as those on Basalt) are buffered and many plant species have adjusted to utilise high mineral N (Turner & Lambert 2005). Forests that are able to tolerate or utilise the mineral N (particularly NO_3^-) do not reach severe decline stages, but those that are unable to cope with these conditions degenerate (Turner & Lambert 2005). The differences in soil fertility between the north-west and north-east regions may partly explain differences in tree health between the regions (eucalypts in north-west plots were healthier than those in north-east plots). The north-west forests grow on higher fertility soils and are possibly better able to cope with increases in N by utilising mineral N (which may explain lower levels of NO_3^- due to their utilisation) than those forests of the north-east that grow on soils of low fertility. Differences in local soil conditions may play an important role in distinguishing the eastern and western ECM communities (Brundrett & Cairney 2002), as seen by differences in ECM community composition and richness. The western plots have higher soil fertility than the

eastern plots and this may contribute to the differences in the ECM community seen, with western species adapted to higher levels of soil nutrients.

Ectomycorrhizal community composition and structure are known to be influenced by soil chemistry, especially available N (Wallenda & Kottke 1998; Lilleskov & Bruns 2001; Peter, Ayer *et al.* 2001a; Peter, Ayer *et al.* 2001b; Avis, McLaughlin *et al.* 2003; Avis, Mueller *et al.* 2008). In general, high N results in a loss of particular species and a shift in dominance (Lilleskov & Bruns 2001; Lilleskov, Fahey *et al.* 2001; Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003). It is likely that differences in ECM fungal communities of *E. delegatensis* forests with different health status are related to levels of soil mineral N as there are clear trends showing a correlation between soil mineral N and ECM community similarities. Soil NO_3^- (either available NO_3^- as indicated by available NO_3^- levels, or total pools of soil NO_3^-) significantly predicted ECM species richness, ECM community composition and community structure (the percentage of OTUs from each family). Available soil NO_3^- was also significantly correlated to ECM community compositional similarities among plots (Figure 5.18), with the percentage of Cortinariaceae within plots varying with soil NO_3^- (the percentage of Cortinariaceae within a plot decreased with increasing soil NO_3^-). These findings support the close ties between the 'ECM community' and 'Soil nutrient availability' in the conceptual model (Figure 5.2).

Ectomycorrhizal fungal sporocarp production and diversity are known to decrease under high N conditions, and root tip colonisation and community richness are also reduced under high soil N concentrations (Ashton 1976; Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003; Treseder 2004). Nitrogen addition was shown to decrease detected sporocarp richness in a northern hemisphere Norway spruce forest (Peter, Ayer *et al.* 2001b). Sporocarp species richness was also reduced in a temperate oak savannah with long term N addition (Avis, McLaughlin *et al.* 2003). Shifts in community dominance have also been observed, with some families increasing in diversity and abundance with increasing N levels, while others decrease (Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003). For example, sporocarp production of *Amanita*, *Boletus* L. and *Cortinarius* decreased under fertilisation while *Russula* sporocarp production increased (Avis, McLaughlin *et al.* 2003). In another study that measured sporocarp abundance and species richness along a N gradient, *Cortinarius*, *Russula*, *Tricholoma*, *Lactarius* and *Hebeloma* (Fr.) P. Kumm. declined in species richness or abundance with increasing mineral N and were described as nitrophobic, whereas

sporocarp abundance of *Lactarius theiogalus* (Bull.) Gray*, Laccaria, Paxillus involutus* (Batsch) Fr.*,* and *Hygrophorus olivaceoalbus* (Fr.) Fr. was either not affected or slightly positively correlated with organic horizon N availability (Lilleskov, Fahey *et al.* 2001). In this study, sporocarp richness of *Cortinarius* and *Tricholoma* generally decreased with an increase in available NO_3^- and the sporocarp richness of *Russula* and *Laccaria* OTUs generally increased with increasing available NO_3^-.

Sporocarp richness is not the only aspect of the ECM ecology that is affected by N concentrations. Levels of soil N are also known to influence the ECM root-tip community. ECM root colonisation and species richness is lower under high inorganic N concentrations (Ashton 1976; Aerts 2002; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003; Avis, Mueller *et al.* 2008). Shifts in ECM root-tip community composition have also been documented with increasing soil N. In a Norway spruce forest community dominance shifted from *Russula* spp. under low N conditions to species from the Thelephoraceae and Corticiaceae under higher soil N conditions (Peter, Ayer *et al.* 2001b). In an oak savannah *Cortinarius* spp. were dominant in plots not fertilised with N and *Russula* spp. dominant in the highly fertilised plots (Avis, McLaughlin *et al.* 2003). Similar results were obtained by Lilleskov, Fahey *et al.* (2002) who found that ECM root tip community richness was reduced in high N sites. *Piloderma* spp., *Amphinema byssoides* (Pers.) J. Erikss.*, Cortinarius* spp. and *Tomentella* spp. were dominant in low N sites but were absent from high N sites where *Lactarius theiogalus, Paxillus involutus, Tylospora fibrillosa* (Burt) Donk*, Tomentella sublilacina* (Ellis & Howw.) Wakef.*, and Thelephora terrestris* Ehrh. predominated (Lilleskov, Fahey *et al.* 2002). In *E. delegatensis* forest, ECM root tip richness of *Cortinarius* decreased with increasing available soil NO_3^-, similarly to sporocarp richness of *Cortinarius*. Thelephoraceae also followed this general trend.

When considering the combined data from this study, ECM species richness decreased with increased availability of mineral N. The same result was seen in a study conducted by Parrent, Morris *et al.* (2006) in northern hemisphere *Pinus taeda* L. forest. The three *E. delegatensis* plots with the lowest concentrations of available NO_3^- had the highest ECM fungal species richness and also had a very high diversity of the Cortinariaceae. Both *Lactarius* and *Laccaria* species are reported to be generalists and tolerate a range of conditions including high N conditions (Wallenda & Kottke 1998). In *E. delegatensis* forest, *Laccaria* spp. and *Lactarius* spp. were among the most widely distributed and frequently sampled over all forest plots irrespective of soil conditions.

Ectomycorrhizal fungi are known to be important for plant N uptake in temperate forests (Aerts 2002) and species may have differential abilities in nutrient uptake (Jones, Grenon *et al.* 2009). Shifts in ECM community composition and structure may relate to functional attributes of different fungal species, such as preference and use of different forms of N (Lilleskov, Hobbie *et al.* 2002). For example eastern Australian *Amanita* species are able to utilise a range of N sources (Sawyer, Chambers *et al.* 2003). ECM species may be specialised for N uptake in either low or high N environments (Lilleskov, Fahey *et al.* 2002). Furthermore different ECM species may prefer a specific form of N. As inorganic N increases, ECM species that utilise organic N have been demonstrated to decrease (Lilleskov, Fahey *et al.* 2002). This indicates that species present in low N environments (in the Lilleskov, Fahey *et al.* 2002 study, low nitrogen sites had bulk N 0.27 ± 0.06 kg/ha, available NO_3^- 1.81 ± 1.15 mg/kg, and available NH_4^+ 29 ± 4.3 mg/kg, and high N sites had bulk N 2.44 ± 0.2 kg/ha, available NO_3^- 49.7 ± 4.0 mg/kg, and available NH_4^+ 134 ± 6 mg.kg), preferentially acquire organic N over mineral N (Lilleskov, Fahey *et al.* 2002; Lilleskov, Hobbie *et al.* 2002). Attempts to generalise the N preferences of species that were recorded in this study are difficult as the ability of species to utilise N varies among species from the same genus (Finlay, Frostegard *et al.* 1992; Wallenda & Kottke 1998), and high levels variation in use of different resources such as N, are also likely to influence intra-specific differences in nutritional preferences and uptake (Wallenda & Kottke 1998; Cairney 1999; Guidot, Verner *et al.* 2005). In *E. delegatensis* forest soils with low levels of mineral N, the Cortinariaceae are richer in species, while species of the Hydnangiaceae, Russulaceae and Thelephoraceae are more likely to be present under conditions of higher concentrations of available mineral N (declining forest soils).

Rare ECM species, have been found to contribute disproportionally to enzymatic activity indicating that not all species are equally as important for different functions (Bruns & Kennedy 2009). Under the soil conditions found in declining eucalypt forest, the presence and healthy functioning of ectomycorrhizas that take up and transfer mineral N, and access immobilised P, would be of utmost importance for the health of the trees. If root colonisation and ECM function are reduced under high N conditions (Treseder 2004), the competitive advantage that the ECM plants have over non ECM plants, such as the understorey vegetation, may be lost (Aerts 2002). The presence of ECM species that are adapted to high nutrient environments such as those found in severely declining forest, may help in buffering symptoms of dieback in the canopy by maintaining nutrient cycling processes that are comparable to healthy forests.

Sensitivity of mycelium to N additions are likely to strongly influence the competitiveness of ECM fungi (Wallenda & Kottke 1998; Brundrett & Cairney 2002) and it is possible that generalist species are less affected by increases in N availability than species that have a narrow host specificity (Arnolds 1991; Wallenda & Kottke 1998; Peter, Ayer *et al.* 2001b). Peter *et al.* (2001) propose that under high N conditions carbon supply to the fungus is reduced which leads to reduced fungal growth and colonisation potential. Species that are able to gain carbon from sources other than their host, such as through decomposition (Bougher & Tommerup 1996; Read & Perez-Moreno 2003; Courty, Buée *et al.* 2010), may have a competitive advantage over other ECM fungi under high N conditions as they would not be so reliant on host carbon allocation, which may be restricted under such conditions. At present, only 50 or so species have been studied for their ability to mobilise energy and nutrients from organic matter, and many genera, such as *Cortinarius* and *Russula*, which are difficult to culture have not been studied for their enzymatic capabilities (Courty, Buée *et al.* 2010).

A number of studies have found that eucalypt root growth is lower when seedlings are grown in soil taken from under eucalypts in decline, rather than in soil from beneath healthy eucalypts (Ellis & Pennington 1992; Harvest, Davidson *et al.* 2008) and that root morphology of declining *E. delegatensis* differs to that in healthy trees (Turner & Lambert 2005). In forest under N deposition, fine root biomass will decrease, and if there are chronic increases in N then fine root turnover and production may also decrease (Aber, McDowell *et al.* 1998; Nadelhoffer 2000). A reduction in fine root biomass and production could mean that there are less available roots for ECM fungi to colonise, leading to reduced nutrient uptake by the host tree (Wallenda & Kottke 1998). Also, a reduction in fine roots may result in greater competition of ECM fungi for colonisation space, which would alter ECM community dynamics, perhaps causing the loss of some species, and an increase in other species that are more able to compete (Bruns 1995; Peay, Kennedy *et al.* 2008b).

5.6.3.2 Soil phosphorus

Labile P is known to be important for forest productivity and microbial biomass in north-eastern Tasmanian eucalypt forests (Adams, Attiwill *et al.* 1989). Phosphorus content of most Australian soils is generally low (0.02%) but varies according to parent material. Basalt areas will have higher levels of soil P (so north-west Tasmanian sites have naturally higher soil P, > 0.05%, whereas the northeast will have lower soil P < 0.02%). Increasing P

limitation in declining forests has been discussed by a number of authors (Wardle, Walker *et al.* 2004; Close, Davidson *et al.* 2009) and this process of increasing P demand and lower P availability, is evident in this study as well as other *E. delegatensis* forest (Jones & Davidson 2009). In contrast to this study, another study of declining high altitude forest found that soil P was higher in unhealthy rainforest sites (Harvest, Davidson *et al.* 2008), which is supported by the results of Turner, Lambert *et al.* (2008), who found that the accumulation of soil N with increasing time since fire was related to total pools of soil P, with greater N levels associated with higher soil P. In this study, the accumulation of N and decline of pH leads to a reduction in P availability (but not to pools of P), and reduced P uptake. The conflicting relationship of P and decline suggests that altered P cycling may contribute to decline in specific circumstances, but is not a general cause. This study suggests that P limitation does occur in declining *E. delegatensis* forests and altered P cycling may contribute to the process of decline.

Ectomycorrhizal fungi are known to be important for plant P uptake, especially in situations of low P availability (Bolan 1991; Richardson, Hocking *et al.* 2009) and this relationship is shown in the conceptual model (Figure 5.2) through the link between 'ECM community' and 'Soil nutrient availability'. A meta-analysis showed that P fertilisation resulted in an average 32% (range 10 – 50%) decrease in mycorrhizal colonisation (Treseder 2004). Soil P was significant in predicting ECM species richness, and community composition suggesting that P is an important determinant of the ECM community. This study showed a negative relationship between ECM species richness and available soil P and this result was also seen in a study by Tweig *et al.* (2009) who found that mineral soil P was negatively correlated to ECM species richness in a Douglas-fir forest. Tweig *et al.* (2009) also found that forest floor organic P (which was not explored in this study) was positively correlated to ECM richness. The relationship between ECM richness and soil P in this study is expected from trends reported in the literature, with more ECM species detected under conditions of lower P. Also, under decline conditions where there is an accumulation of N, but a decline in available P, shifts in the ECM community leading to altered/lower mycorrhizal activity may explain the reduced P uptake evident in severely declining *E. delegatensis* forest.

5.6.3.3 Soil pH

In this study, severely declining forest had slightly lower pH than moderately declining forest within the north-east (pH was similar for health status among all plots), and soil pH

significantly predicted ECM fungal community composition and community structure. Soil pH explained the highest amount of variation in these models. Soil pH was also significant in predicting the relative abundance of ECM taxa in *Picea glauca* forest (Lilleskov, Fahey *et al.* 2002). Changes in mycorrhizal assemblages have been demonstrated following acidification (Coughlan, Dalpe *et al.* 2000; Brundrett & Cairney 2002) and in an Australian eucalypt plantation a strong inverse relationship between soil pH and sporocarp richness was found (Lu, Malajczuk *et al.* 1999). In *Acer* dominated forest, arbuscular mycorrhizal colonisation increased with pH in both declining and healthy forest (Coughlan, Dalpe *et al.* 2000), and acid deposition is known to decrease root length and biomass (Cudlin, Kieliszewska-Rokicka *et al.* 2008), which could influence mycorrhizal colonisation. Soil pH has also been implicated in the decline of Norway spruce (Estivalet, Perrin *et al.* 1990). In this study, ECM community composition and proportional composition changed with soil pH, further verifying the links between the 'ECM community' 'Soil nutrient availability' and 'Soil microclimate and microflora' (Figure 5.2). The Cortinariaceae made up a greater percentage of the ECM community under more acidic soil conditions, whereas the Russulaceae made up a greater percentage of the community under slightly less acidic soil conditions.

Soil pH affects the function of ECM fungi as phosphatases are known to have optimum pH for P uptake. Temperature also controls phosphomonoesterase production and activity (Courty, Buée *et al.* 2010). Soil pH also controls the availability of many nutrients and low soil pH can limit nitrification by inhibiting soil microbial activity. pH may also influence the rate of nitrification in eucalypt forests (Ellis & Pennington 1989). The processes of mineralisation and nitrification do not appear to be affected in *E. delegatensis* forest in this study (there is some uncertainty as these processes were not measured directly) at the measured pH, both NH_4^+ and NO_3^- were detected in forest soil irrespective of forest health status. Even so, conditions of low available soil N were generally associated with lower pH.

Available soil P is also controlled by soil pH with the highest P availability at mid levels of pH (5-6). Altered soil pH (acidity) within declining soils may therefore be acting to reduce soil P availability. Potassium, sulphur and magnesium have low availability in acidic soils whereas micronutrients (i. e. manganese, iron, boron, zinc and copper) become more available as pH decreases. Toxic metal concentrations, such as aluminium and manganese, are also influenced by pH, and in general have higher concentrations and availability at lower soil pH. A liming experiment found that the ECM community responded to liming as indicated

by altered enzymatic profile which facilitated the mobilization of mineral nutrients (Rineau & Garbaye 2009). It is also possible that soil pH represents a range of differences in soil chemistry among the plots that can influence the ECM community.

5.6.3.4 Organic carbon

Many species of ECM fungi occupy the organic soil and litter layers at the top of the soil profiles, and the level of organic carbon may be important for habitat selection by ECM fungi. Some species of ECM fungi are considered to be capable of actively decomposing organic material and extracting organic C directly from the soil (Read & Perez-Moreno 2003; Courty, Pritsch *et al.* 2005), and so organic C concentrations may influence the presence of species with this ability. Levels of organic carbon are correlated with soil N (Attiwill & Leeper 1987) and so levels of organic C may be a reflection of other soil conditions that are influencing the presence of ECM taxa. The importance of organic C for ECM fungi is illustrated in its significance in predicting ECM community proportional composition.

5.6.4 The influence of foliage chemistry on ectomycorrhizal communities and eucalypt decline

Foliage P was higher than expected overall, decline of *E. delegatensis* in this system was associated with an increase in foliage N accompanied with a decrease in foliage P (severely declining trees had lower foliage P than moderately declining trees). Mean percentage of foliage N and P for the subgenus Monocalytpus to which *E. delegatensis* belongs, are N 1.19% ± 0.04, P 0.071% ± 0.003 (Judd, Attiwill *et al.* 1996) whereas in this study mean foliage N and P for *E. delegatensis* were higher (N 1.39% ± 0.05 and P 0.15% ± 0.02). Granger *et al.* (1994) also found that declining *E. ovata* Labill. and *E. camphora* R.T. Baker forests of NSW were associated with higher foliage N concentrations and increased levels of foliage N have also been associated with northern hemisphere temperate forest decline (Aber, McDowell *et al.* 1998). The majority of the *E. delegatensis* trees researched had foliage N:P ratios lower than 15, which is optimal for a range of eucalypt species (Judd, Attiwill *et al.* 1996). This is mostly because of the high overall foliage P levels.

Foliage nutrient concentrations are known to be responsive to changes in soil fertility (Judd, Attiwill *et al.* 1996) and this direct link is shown in the conceptual model of eucalypt decline

and the ECM community (Figure 5.2). In this study there was a positive correlation between total soil P and foliage P (approximately 0.7). No strong trend between total N from soil, soil mineral N or foliage N, was detected in this study which was expected as available N is generally used for increased growth. It is possible that the accumulation of N in the soil is translating into increased plant available N leading to increased uptake by eucalypt trees, especially via ECM fungi. In general foliar N:P < 14 indicates N limitation, N:P between 14 and 16 suggests co-limitation of both N and P, while foliar N:P > 16 indicates P limitation. Premature decline of *E. delegatensis* forests is associated with high P demand indicated by decreasing soil and foliage P and increasing foliage N:P ratios (Jones & Davidson 2009). This response is seen in severely declining forest in this study where the majority of the severely declining forest plots exhibited high foliar N:P ratios indicting possible P limitation.

ECM fungi are important for nutrient uptake in Australia, where soils are often impoverished of nutrients, especially P (Tommerup & Bougher 1999). ECM fungi have a greater effect on plant growth under low nutrient concentrations, especially low P (Bougher, Grove *et al.* 1990). It is possible that ECM richness and composition may affect the resulting P assimilated in tree foliage, shown by the connection of the boxes 'ECM community', 'Soil nutrient availability' and 'Shoot nutrient imbalance' in the conceptual model (Figure 5.2). Although possible links between taxonomic diversity and improved function has been proposed, this has yet to be proven in Australian forests. In *E. delegatensis* forest in this study, high ECM richness was predicted to occur under conditions of low available P and NO_3^-. Forest that had greater richness of ECM fungi did not necessarily have greater foliage nutrients and so higher ECM richness, which was more likely to occur in moderately declining plots, does not necessarily imply improved nutrient uptake (Schwartz, Brigham *et al.* 2000). In severely declining forest where available soil P is lower and there is greater P demand, ECM fungi may be even more important for nutrient uptake than in healthy forest. Reduced P uptake via ECM fungi, as indicated by high foliar N:P ratios, may therefore be a contributor to the process of eucalypt decline, as suggested by the conceptual model.

ECM fungi may also be playing a role in deterring herbivores (Gehring & Whitham 1994) and changes in the ECM community may affect their hosts ability to cope with herbivory. Also, increased herbivory may result in a loss of important ECM species that aid the tree in resistance (Gehring & Whitham 2002) leading to a positive feedback resulting in greater herbivory and lower tree resistance.

Herbivory can further influence the ecology of *E. delegatensis* forest by altering plant litter input by affecting the chemical composition and nutrients present in the foliage, which are then released in the decomposition process, or through selectively using particular species influencing plant community composition (Bardgett, Wardle *et al.* 1998).

5.6.5 Other influences on the ectomycorrhizal community and their effect on the decline process

Geographic region and spatial proximity of plots may also help to explain differences in the ECM community. Lilleskov *et al.* (2004) found that for half of their study plots ECM community similarity decreased with distance. Plot and site similarities can also be interpreted in terms of past management history and disturbance events. Management practices and disturbances are known to influence ECM community composition, with a succession in the ECM community following large scale disturbances such as fire and timber harvesting (Visser 1995; Chen & Cairney 2002; Jones, Durall *et al.* 2003; Bastias, Xu *et al.* 2006). It is likely that past disturbances to the plots have helped to shape the ECM community. Evidence for this comes from the high similarity of the ECM communities of paired plots which have the same management history. Disturbance, or lack thereof, may act to remove or encourage certain species of ECM fungi, which may be better able to function under the current forest conditions.

Dispersal limitation is also thought to play a role in maintaining ECM communities (Brundrett & Cairney 2002; Peay, Bruns *et al.* 2007) and may play a role in these forests. Some species, such as hypogeous species which rely on mycophagous animals for dispersal, may be unable to disperse their propagules over long distances, or maintain viability during dispersal (Brundrett & Cairney 2002). These factors may become significant over large distances such as those between north-east and north-west Tasmania but may also play a role over smaller distances i.e. between the north-east sclerophyll and rainforest plots that were six km apart.

ECM fungi and other mycorrhizal fungi are known to be involved in water transport and uptake, aiding their host plant in water uptake, for example by accessing deep water (Smith & Read 1997; Plamboeck, Dawson *et al.* 2007; Lilleskov, Bruns *et al.* 2009). Pinyon pine forest stands that experience lower mortality caused by water stress have been found to have higher ECM colonisation with different mycorrhizal community compositions than

stands that experienced higher mortality (Swaty, Deckert *et al.* 2004) and in Chilean *Nothofagus* forests, some ECM species were shown to increase plant growth under drought conditions (Alvarez, Huygens *et al.* 2009). This relationship with water availability is also seen in a study on *Eucalyptus diversicolor* seedlings where increasing soil moisture correlated to reduced mycorrhizal formation. The reduced formation of ectomycorrhizas and potential absence of ECM fungi that function in water uptake may contribute to the increasing water stress experienced by the declining eucalypt trees through a restriction in access to water which would normally be exploited through ECM mycelium.

Antagonistic elements that are present in declining, but not healthy forests may also play a role in the decline process. These elements may be chemical, bacterial or fungal and negatively affect eucalypt health, or affect beneficial ECM fungi. Soil fauna and other soil microbes compete with mycorrhizal fungi for root exudes and carbon sources and can potentially influence the dynamics of the mycorrhizal community (Bonkowski, Villenave *et al.* 2009). The results of Harvest *et al.* (2008) linseed bioassays suggest there are bacterial and/or fungal antagonists present in unhealthy *E. delegatensis* forest soil. In a northern hemisphere study, Norway spruce decline was linked to the presence of a noxious soil element that involved poor mycorrhizal colonisation and tree nutrient status (Estivalet, Perrin *et al.* 1990). Antagonistic biota may occur because of altered conditions within unhealthy forests, such as lack of competition by other biota, or more optimal soil conditions such as temperature, allowing their survival.

Furthermore, rhizosphere biota may be important for maintaining eucalypt health or in ameliorating antagonistic effects that cause decline or inhibit the regeneration of eucalypts in declining forest (Ashton & Willis 1982; Cai, Barber *et al.* 2010). For example, changes in the rhizosphere bacteria community may affect plant nutrient uptake through the modification of helper-bacteria that promote ECM associations (Fitter & Garbaye 1994; Garbaye 1994). One recent study found that soil bacterial richness increased and bacterial community composition was altered in enriched N sites (Krumins, Dighton *et al.* 2009). The ratio of fungi to bacteria was reduced under N fertilisation and these changes in the microbial community were associated with the enhancement of plant-derived chemicals (Xiaojuan, Andre *et al.* 2009). In *E. gomphocephala* forest a decrease in the utilisation of carbohydrates, carboxylic acid, amino acids and amines by the soil bacterial community was correlated to poor eucalypt crown health indicating a strong relationship between the bacterial community and tuart eucalypt decline (Cai, Barber *et al.* 2010).

Alternatively, there may be species of bacteria and fungi present in declining forest that directly negatively affect eucalypt growth and health by pathogenic attack, or by the production of toxins. Mycorrhizal fungi are known to aid in resistance to pathogens and may help the tree to defend itself from secondary effects of decline (Chakravarty & Unestam 1987; Fitter & Garbaye 1994; Pozo, Verhage *et al.* 2009). It s possible that a disturbance in the balance of pathogenic fungi, such as *Rhizoctonia solani* J.G. Kühn, could reduce the health of trees roots, leading to an overall reduction in tree health (Ashton & Willis 1982; Perrin 1990; Qian, El-Ashker *et al.* 1998; Tang, Zhang *et al.* 2008; Tripathi, Kamal *et al.* 2008). In contrast *Phytophthora cambivora* (Petri) Buisman diseased chestnut forest had higher ECM richness than healthy forest (Blom, Vannini *et al.* 2009), but as discussed above, ECM richness does not always correlate directly to plant health.

5.7 Conclusion

The causes of eucalypt decline are complex and interrelated. The results of this study support the model of eucalypt decline in the absence of fire whereby N accumulates in the soil, leading to reduced soil C: N ratio, increased nitrogen mineralisation and a reduction of soil pH. The decline model proposed by Close, Davidson *et al.* (2009) involves changes to soil chemistry, especially an increase in available nitrogen, and a decrease in soil pH and other nutrients, such as P that become increasingly immobilised (Figure 5.1). The results of this study further support these relationships. *E. delegatensis* forests on soil of low fertility, and forests that have progressed further along a successional sequence toward a rainforest climax community are especially predisposed to decline.

The ECM community has the potential to affect and be affected by eucalypt decline by a number of pathways, which are proposed in the conceptual model presented in the introduction (Figure 5.2). The link between the ECM fungal community and eucalypt decline is likely to be mediated through soil chemistry and vegetation dynamics. During succession in the absence of fire, N accumulates to a level where nitrification occurs, and pH decreases. Under these condition P availability is reduced along with a substantial reduction in P uptake, potentially due to shifts in the mycorrhizal community, which is reflected in the higher foliar N:P ratios. Under these conditions (a shift to P limitation from N limitation) eucalypts may lose their competitive advantage and are replaced by more competitive species, such as AM plants. The rate of change is more likely to be faster where rainforest understory is present.

Forests at different successional stages (i.e. those with a sclerophyll or rainforest understorey) support different ECM communities. Soil pH, available and total soil NO_3^- and P, are all important determinants of ECM community diversity (richness, composition and proportional composition) in *E. delegatensis* forest, and changes in the soil environment as eucalypt forest decline progresses are likely to cause changes in the ECM community. The ECM community both responds to changing soil conditions (increased N and declining pH) and also causes further effects on plant performance (reduced P uptake).

Northern hemisphere studies that have found that ECM diversity and community composition change with differing soil chemistry, especially soil N, corroborate the likely influence of soil N, and especially available NO_3^-, on the ECM community of *E. delegatensis* forest. Furthermore, the progress of eucalypt decline to the more severe stages is accompanied by increases in soil N and divergent changes in soil P, which becomes limiting.

Although the ECM community appears to have an indirect role in eucalypt decline, it is probable that certain species and assemblages of ECM species differ in their ability to cope and adapt to ecosystem changes associated with eucalypt decline. Certain families of ECM fungi such as the Cortinariaceae had greater richness in healthier forest as opposed to declining forest whilst the Russulaceae and Thelephoraceae were less species rich. The challenge is to determine the functional role of ECM species, such as nutrient preferences and uptake pathways that would help to disentangle the complexity of the relationship between the ECM community and eucalypt decline. Altered ECM communities are likely contributing to reduced uptake of nutrients to trees in severely declining forest. Although there are clear trends indicating links between the ECM fungal communities and soil chemistry, vegetation and tree health, cause and effect cannot be determined from this study, and further manipulative research would aid in clarifying these links.

A comparison of the ECM communities and soil chemistry of healthy *E. delegatensis* forest and those that have begun to decline would help to characterise ECM communities according to the health of the forest. Future research should focus on soil chemistry, the ECM community and eucalypt health following fire in a declining *E. delegatensis* forest. An understanding of changes within completely healthy and declining eucalypt forests as the forest develops following disturbance would greatly improve our understanding of eucalypt decline, and the role that fire might play in retarding the process of forest decline.

6.0 General discussion

6.1 Introduction

Forest decline occurs across the globe, including temperate Australian eucalypt forest (Mueller-Dombois 1988; Ciesla and Donaubauer 1994; Wardle, Walker *et al.* 2004 Ellis 1964; Bird, Kile *et al.* 1974; Podger, Kile *et al.* 1980; Heatwole and Lowman 1986; Wardlaw 1989; Lowman and Heatwole 1992; Granger, Kasel *et al.* 1994; Davidson, Close *et al.* 2007). Eucalypt decline has been attributed to altered management practices which affects soil and vegetation dynamics, causing a cascade of changes through the ecosystem (Mueller-Dombois 1988; Close, Davidson *et al.* 2009). It has been postulated that because of their importance in ecosystem process such as nutrient cycling, ectomycorrhizal (ECM) fungi are likely to be linked to the decline of Australian eucalypt forest trees.

Relatively little is known about Australian ECM fungi and their ecology, and this thesis builds on current knowledge. The characterisation of the ECM fungal community of temperate *E. delegatensis* forest presented in this thesis is one of the first in Australia to use both root tips and sporocarps, utilising DNA sequencing to do so. The Australian fungal sequences will make a valuable contribute to public sequence databases that currently have only a small proportion of southern hemisphere ECM fungal sequences.

This thesis is also the first in Australia, and one of the few studies worldwide, to focus on the relationships between eucalypt decline and the ECM community. The ECM communities of moderately healthy forest were more similar in composition and proportional composition than communities from forest with trees in severe decline. Furthermore, the ECM community was shown to be correlated with a number of ecosystem components that are related to the process of eucalypt forest decline. The influences and impacts of eucalypt decline, and the importance of the ECM community in this process are highlighted.

6.2 Is the biodiversity and ecology of *Eucalyptus delegatensis* forest ectomycorrhizal fungal communities unique to Australia?

Tasmanian *E. delegatensis* forest supports diverse ECM communities that are rich in species derived from a range of ECM lineages (Chapter 4). Many of these lineages are common components of ECM communities around the world (Peintner, Moncalvo *et al.* 2004; Tedersoo 2007; Dickie & Moyersoen 2008). Although lineages were of global distribution many of the operational taxonomic units (OTUS are a proxy for species) recorded in *E. delegatensis* forest are likely to be novel, as they did not have a good match to identified DNA sequences from public databases (i.e. GenBank, UNITE).

Members of the Cortinariaceae were a particularly important component of the ECM communities of *E. delegatensis* forest and dominated both above- and below-ground communities, in the majority of the forest plots (Chapter 4). The dominance of the Cortinariaceae is a characteristic of Australian eucalypt forests (Bougher 1995; Gasparini 2004; Ratkowsky & Gates 2005; Gasparini 2007; Gasparini & Soop 2008; Tedersoo, Jairus *et al.* 2008; Gates 2009) suggesting they are important for the functioning of these forests. Their prevalence in Australian forests may be due to biogeography or perhaps is an adaptation to the often dry and nutrient poor environments of Australia. Australian eucalypt forests differ from northern hemisphere ECM forests, which tend to be dominated by the Thelephoraceae and Corticiaceae (Stendell, Horton *et al.* 1999; Dahlberg 2001).

6.3 What are the links between ectomycorrhizal fungi and the process of eucalypt forest decline?

Ectomycorrhizal community composition, diversity and structure are known to differ in healthy northern hemisphere forests compared to forests undergoing decline (Perrin & Estivalet 1990; Arnolds 1991; Power & Ashmore 1996; Vinceti, Paoletti *et al.* 1998; Peter, Ayer *et al.* 2008). The research presented here is the first to show that ECM community composition and proportional composition are directly linked to the health of eucalypt trees (Chapter 5). ECM community composition differed significantly between moderately and severely declining forest and ECM communities were correlated to crown health. *E. delegatensis* forest suffering from moderate decline was associated with a rich assemblage of the Cortinariaceae, whereas forest in severe decline was associated with the

Boletaceae, Russulaceae and Thelephoraceae (Chapter 5). The various pathways and links between the ECM community and eucalypt trees that can affect the decline process are shown in the conceptual model developed in Chapter 5. The research presented here suggests that the differences in ECM communities of moderately and severely declining *E. delegatensis* forests are likely to be mediated through changes in soil chemistry and vegetation dynamics that occur throughout the decline process.

Soil chemistry played an important role in determining ECM community richness, composition and proportional composition, and differed between healthier and declining forest. ECM colonisation is known to be lower under conditions of high soil P concentrations (Treseder 2004; Twieg, Durall *et al.* 2009). In *E. delegatensis* forest ECM richness was lower under higher levels of either available P and/or NO_3^- (Chapter 5). Available soil NO_3^-, soil pH, and organic C, also significantly predicted similarities in the ECM communities of the *E. delegatensis* forest plots (Chapter 5). The importance of soil chemistry, especially nitrogen, in shaping northern hemisphere ECM communities is well established (Peter, Ayer *et al.* 2001b; Lilleskov, Fahey *et al.* 2002; Avis, McLaughlin *et al.* 2003; Twieg, Durall *et al.* 2009) and the research presented in this thesis suggests this is also true for southern temperate forests .

Ectomycorrhizal species preferences for different nitrogen forms, and their ability to utilise different forms of nutrients are beginning to become apparent (Abuzinadah & Read 1986; Lilleskov, Fahey *et al.* 2002; Lilleskov, Hobbie *et al.* 2002; Courty, Buée *et al.* 2010). Evidence of ECM functional groups specialised in utilising different forms of nitrogen is minimal but this study found the richness of the Cortinariaceae was negatively correlated with available soil mineral nitrogen concentrations, which suggests the Cortinariaceae may have a preference for organic nitrogen (Chapter 5). The ECM community could therefore be responding to changes in soil chemistry, such as increases in mineral nitrogen, that are associated with development of decline (Ellis & Pennington 1989; Turner & Lambert 2005; Turner, Lambert *et al.* 2008; Close, Davidson *et al.* 2009). ECM species present in declining forest communities may function differently than those of healthy forests, and this may lead to altered nutrient uptake for their hosts, affecting tree health. Some evidence of these changes is seen in differences in foliar nutrition of healthier and declining forests, suggesting that as succession proceeds, a shift from N limitation to P limitation occurs in these ecosystems, due to a reduction in P availability and uptake. Changes in ECM

communities accompany these shifts and may also be a further causal factor in eucalypt decline.

Vegetation community characteristics influence ECM communities (Nantel & Neumann 1992; Kernaghan, Widden *et al.* 2003; Debellis, Kernaghan *et al.* 2006; Kennedy & Hill 2010) and like soil chemistry differ between healthy and declining eucalypt forests. Eucalypt forest in decline tends to have a thick understorey comprising mesic plants which differ in their foliage chemistry/nutrition (Ellis 1985; Close, Davidson *et al.* 2009). *E. delegatensis* forest with a mesic rainforest understorey supported ECM communities that differed in composition and proportional composition from forest with a dry sclerophyll understorey (Chapter 5). Forest with sclerophyll understorey supported a richer complement of Russulaceae than forest with rainforest understorey (Chapter 5). The abundance of particular overstorey and understorey plant species, such as *E. delegatensis*, *N. cunninghamii*, *L. lanigerum*, and *A. melanoxylon,* also played a role in shaping *E. delegatensis* forest ECM communities (Chapter 5). The ECM community may be both responding to changes in the vegetation assemblage within declining forests, and influencing these changes.

Overall, the results from this thesis indicate that ECM fungal communities are tightly linked to eucalypt health status and are likely to play an important role in the maintenance of healthy eucalypt forest through influencing and being affected by other ecosystem components. Thus, the research presented throughout this thesis is consistent with the hypotheses that the ECM community is influenced by vegetation, soil chemistry and tree health. Furthermore, the conceptual model presented in Chapter 5 indicates numerous feedbacks which are also likely to exist and suggests that the ECM communities are a key factor influencing vegetation community structure and composition, soil nutrient availability and tree health.

6.4 Can ectomycorrhizal fungi be used as indicator species of *E. delegatensis* forest decline?

Species that control critical ecosystem functions (keystone species), are more valuable indicators than species richness or diversity. Functional groups, such as ECM fungi, are possible indicators for forest health as they form part of the biota that drives ecosystem function.

Fungal indicators have been used for forest monitoring (Fellner 1990; Folke & Knudsen 1994; Lagana, Salerni *et al.* 2002) and although some ECM OTUs were associated with health status (i. e. Boletaceae sp. 2, *Laccaria* sp. 4, *Cortinarius* sp. 67, *Cortinarius* aff. *sclerophyllarum*, and *Sebacina* sp. 2 were associated with severe decline, Chapter 5) their use for early detection of eucalypt decline is not feasible.

Indicator species need to be independent, be present only at the site that displays the variable of interest (i.e. high soil nitrogen levels or severe crown decline), and have high frequency and abundance at those sites. To determine if a particular species of ECM fungus is present at any site, a large amount of sampling would need to take place to ensure that the species is detected if present. Also, ECM communities are structured in such a way that there are very few abundant, widespread taxa (Dahlberg 2001; Anderson & Cairney 2004) and those that are, tend to be generalists and common to all types of forest. Ideally, indicators of eucalypt forest health would allow the early detection of decline, before other more obvious changes (such as dieback of the crown) occur. Species sensitive to the changes in soil chemistry that are associated with decline may offer the greatest potential, but the large expense required to monitor these species is prohibitive (high molecular and labour costs, and high sampling effort).

To be able to use any potential indicator of eucalypt decline, further work is required to verify that each OTU is consistently associated with forest of certain health status. With increased knowledge of ECM fungal taxonomy, function, ecology and distribution, and more efficient and less expensive sampling and identification techniques, ECM fungal indicators may be able to be identified and utilised in the future. Alternative indicators of forest health or decline include functional group diversity, which would also require development and testing, or simply soil chemical indicators, such as concentrations of available nitrogen.

6.5 Was the methodology employed suitable for answering the hypotheses posed?

Eucalypt forest decline is considered to occur at the ecosystem or landscape level as presented in the two conceptual models (Chapter 5). In an attempt to capture broader ecological information forest decline was studied at the stand level at multiple places across the landscape, and also at the individual tree level. Eucalypt health was measured

for individuals but was averaged for a stand and was found to differ not only between stands but within a stand between individual trees (Chapter 5). This variation within a stand caused some non-significant relationships between relevant soil and fungal parameters and tree health. Even so, average crown scores for stands indicated that stands were of poor health across the range of the study. Healthy stands were not included in the study as healthy forest with a known disturbance history that met the study criteria was not found. The ability of individual trees to cope with stresses such as community interactions, fire and nutrient changes will vary according to both landscape factors such as management and land-use, topography, geology, and localised factors such as individual genetics and microclimate. Although the individual tree response may differ with these factors, the causal factors behind tree and forest decline are likely to be universal over large areas. Studying the elements of eucalypt decline at the individual, stand and landscape levels allowed an exploration of the hypotheses posed within the thesis and presented in the conceptual models in Chapter 5. The outcome was a better understanding of the processes involved in eucalypt decline."

Molecular techniques, such as PCR, DNA sequencing, PCR-RFLP and cloning, are widely used for the study of ECM communities and were successfully employed for the identification of fungal OTUs in this study. PCR successfully amplified the majority of root tip and sporocarp DNA, and was also successful in amplifying fungal DNA, including mycorrhizal fungal DNA, directly from soil (Chapters 3 and 4). Although some DNA sequences were of poor quality, most sequences were of high quality allowing searches within public databases. The majority of sequences did not have a good sequence match to identified sequences in these databases, restricting identification to genus or family in many cases (Chapters 3 and 4). The low matches of samples to known sequences highlights the importance of sequencing and making available more species from the southern hemisphere, as many databases are biased toward northern hemisphere species.

Sequence alignments and phylogenetic trees also proved to be useful tools for grouping sequences into OTUs (Chapter 4). The main problem in determining OTUs is identifying the appropriate cut-off level of sequence similarity between OTUs because some have high intra-specific variation and some have high inter-specific variation. Another problem encountered was misidentification of public database sequences.

Sample design and methodology are important determinants of successful and meaningful research and is the subject of evaluation in this thesis (Chapters 2, 3 and 4). ECM community studies often sample root tips or sporocarps, rarely both, and rarely soil, and comparison of different substrates are few (Peter, Ayer *et al.* 2001a; Landeweert, Leeflang *et al.* 2003; Landeweert, Leeflang *et al.* 2005; Porter, Skillman *et al.* 2008). Soil from *E. delegatensis* forest provides a similar amount of information on the ECM community (the percentage of OTUs from functional groups, and from different taxonomic groups) to that obtained from sampling root tips or sporocarps (Chapters 3 and 4), and using molecular techniques offers an alternative method to screen soil ECM communities that is both time efficient and of similar cost.

Despite the usefulness of soil, there were differences in the information obtained from sampling the different fungal material (Chapters 3 and 4). The differences seen between the ECM community composition as sampled by sporocarps, root tips and soil may reflect the differences in reproductive strategies of various species (some species do not produce sporocarps, or produce inconspicuous sporocarps) (Peter, Ayer *et al.* 2001a), or may reflect unequal and insufficient sampling strategies that failed to detect the presence of species (Taylor 2002; Anderson & Cairney 2007). Root tip and sporocarp sampling were more comprehensive and congruent than soil sampling, and as such resources should be directed to increasing sampling effort of these materials (Chapter 4). When complete characterisation of the ECM community is required, root tips, sporocarps and soil should all be sampled as each provides a complementary view of the ECM community (Chapter 3 and 4). This would be costly but the advent and lowering expense of new techniques, such as 454-sequencing, may allow a more complete characterisation of the ECM community in the future.

Insufficient sampling occurs in many ECM studies as ECM communities are normally diverse, are spatially heterogeneous, and have high temporal variability in fungal structures such as mycelia, root tips and sporocarps (O'Brien, Parrent *et al.* 2005; Peay, Bruns *et al.* 2007; Taylor 2008). The very high variation of *E. delegatensis* ECM communities, along with low replication, resulted in no significant correlation between soil fungal richness and sample distribution (Chapter 3). The high spatial variability in fungal species compositions means that despite similar observed OTU richness, fungal assemblages may change dramatically over short distances (Chapter 3). Due to OTU richness increasing more sharply with increases in area compared to increases in distribution, increasing the number of

samples within a site, rather than increasing the area over which the samples are collected, is a more efficient way of getting an accurate estimate of species richness.

For the purposes of this research, the development of a method to assess eucalypt dieback, consistently across plots, was imperative to allow eucalypt health to be linked the ECM fungal community. The attribute 'primary crown dieback' was identified as the most efficient, accurate and precise technique for the measurement of crown health (Chapter 2). Using this attribute it was possible to identify differences in health of *E. delegatensis* trees among plots, and to correlate these differences to ECM fungal community characteristics (Chapter 5). The attribute 'primary crown dieback' was also shown (out of a broad suite of characters) to be suitable for assessing eucalypt crown health, and could provide a standard for forest health monitoring (Chapter 2).

6.6 How should *E. delegatensis* forest be managed to avoid premature decline?

A large proportion of subalpine *E. delegatensis* forest is prone to tree decline (Ellis & Lockett 1991) and management is required to arrest the development of decline if eucalypt forests are to be utilised for wood production or conserved (Ellis & Lockett 1991; Close, Davidson *et al.* 2009; Archibald, Bradshaw *et al.* 2010). Many of the changes associated with eucalypt decline possibly occur as a result of exclusion of fire from forests that have adapted to a particular fire regime.

Fire results in altered soil chemistry and vegetation dynamics (Raison 1980; Ellis & Graley 1983; Adams & Attiwill 1986; Bauhus, Khanna *et al.* 1993; Certini 2005; McIntosh, Laffan *et al.* 2005). Fire also affects the ECM community in a number of ways including directly through heat penetration and combustion of litter, where many ECM fungi are abundant, resulting in the mortality or reduced abundance of ECM species (Stendell, Horton *et al.* 1999; Hart, DeLuca *et al.* 2005; Cairney & Bastias 2007).

The process of succession following fire is determined by a number of number of factors including soil pH, nutrient levels and availability, species survival and regeneration potential, and competition (Gill, Grove *et al.* 1981; Ellis & Graley 1987; Blasius & Oberwinkler 1989; Amaranthus & Perry 1994; Bruns 1995; Hart, DeLuca *et al.* 2005; Kernaghan 2005; Twieg, Durall *et al.* 2007; Turner, Lambert *et al.* 2008). Eucalypt

establishment is possible under conditions of low soil N and higher soil P which are present following fire (conditions of N limitation as most N is lost through volatisation or leaching). Variation in fire regimes can influence composition, structure and function of an ecosystem. Differences in fire frequency are known to result in very different ECM communities (Glen 2001; Chen & Cairney 2002; McMullan-Fisher, May *et al.* 2002; Bastias, Xu *et al.* 2006; Anderson, Bastias *et al.* 2007; Robinson, Mellican *et al.* 2008) and vegetation communities (Gill, Grove *et al.* 1981; Ellis 1985).Thus succession following fire may lead to an ECM community that functions differently to that present in declining eucalypt forest. The application of fire to declining eucalypt forests may improve forest health by removing detrimental factors and reinstating healthy ecosystem processes.

Although fire may act to reinstate healthy ecosystem functioning, there are many unknowns surrounding the application of fire for the management of eucalypt decline. Management histories of many eucalypt forests have not been documented and often there is no continuous record of fire history. Thus historic data is not available to inform an appropriate fire regime. Eucalypt health is probably not determined by time since last fire but by the fire regime comprising a particular fire frequency, intensity, and seasonality. Fire regimes are also likely to be specific to different forest types and would need to be determined individually for each. Further research on forests with different fire histories is required to determine what the appropriate fire regime is for *E. delegatensis* forest. Monitoring changes in eucalypt health following fire, and the corresponding changes in the ECM community, may determine the extent of changes in the ECM community that can take place without eucalypt decline occurring.

Other disturbance events, including forestry operations such as harvesting, are also likely to influence ecosystem components that are important in the decline process, and may also aid in the management of eucalypt decline (Ellis & Lockett 1991; Neyland & Cunningham 2004). This is suggested by the historic forestry disturbances of the north-western plots which had been disturbed most recently (by forestry) and contained the healthiest forest (Chapter 5). Again, rigorous scientific studies would need to determine the exact frequency and intensity of these events.

6.7 How should *E. delegatensis* forest be managed to conserve ectomycorrhizal fungi?

Fungi are rarely considered in conservation management in Australia. This is largely because of an incomplete knowledge of taxonomy, distribution and ecology of fungal species. The importance of ECM fungi in nutrient cycling and plant nutrient acquisition (Tommerup & Bougher 1999; Cairney 2005; Courty, Pritsch *et al.* 2005; Anderson & Cairney 2007; Courty, Buée *et al.* 2010) and their integral role in the maintenance of eucalypt forest health (Chapter 5) warrants their conservation.

In *E. delegatensis* forest, ECM OTUs varied greatly in distribution and occurrence. Some OTUs were common to the majority of plots, and these OTUs are most likely to be generalist species capable of living under a wider range of environmental conditions than specialist species (Chapter 4). Common species are likely to be adequately conserved through reservation of broad forest habitats that are protected for their landscape, flora, fauna, geomorphological or cultural values (McMullan-Fisher, Kirkpatrick *et al.* 2010). Many OTUs in this study were recorded in one location only, and each of the ECM communities of the forest plots had unique components (Chapter 4). Conservation of rare species is unlikely to occur through surrogate conservation and specific efforts will need to be made in order to conserve rare species and unique communities (McMullan-Fisher, Kirkpatrick *et al.* 2010).

In order to conserve a range of different ECM species, a range of habitats should be conserved that meet the various habitat needs of the ECM communities. These habitats need to differ in their vegetation characteristics (i.e. host plants and understorey composition), disturbance histories (i.e. fire histories), soil type and chemistry, and geographic aspects (i.e. range of regions and topographic locations).

Conservation of ECM communities has a number of beneficial outcomes. Diverse ECM communities probably represent diverse functionality (Tommerup & Bougher 1999; Leake 2001; Dickie & Moyersoen 2008), and thus preservation of these communities allows the preservation of ecosystem function. Diverse ECM communities may also be important for maintaining ecosystem stability and resilience(Molina, Pilz *et al.* 2001; Gates 2009), which may be particularly important for forests managed for wood production. Loss of ECM

diversity may also render ecosystems less able to cope, adapt and persist following any disturbance events or following environmental changes, such as climate change.

6.8 Future research directions

There are many aspects of the relationship between eucalypt decline and the ECM community that are unknown. A study of healthy (not affected by decline) *E. delegatensis* forest would provide base-line information on the soil chemistry and ECM fungal community of forests that represent well-managed healthy forests. This would allow a comparison of unhealthy and healthy forests and help to determine the major differences between the two.

Measuring the ECM community and soil chemistry following a fire would greatly improve our understanding of the changes that take place in *E. delegatensis* forest as succession occurs. Two of the study plots in the north-west, one sclerophyll and one rainforest understorey plot, were burnt in January 2009. The four plots in the north-west, with a burnt and un-burnt plot for each understorey type, provide a valuable resource for a long-term study of the post-fire changes in *E. delegatensis* forest. Measurements of the organic N component of soil, and how this compares to the inorganic N component of healthy and declining forest soils may assist in understanding ECM species preferences, such as those of the Cortinariaceae which have been hypothesised to prefer organic N.

Quantifying the carbon transfer between ECM hosts and ECM species would also be interesting to explore. Altered foliage nutrition that occurs as a result of decline can increase herbivory which may reduce tree productivity (Keith 1997). Foliage loss and decreased productivity may reduce carbon transfer to ECM fungal partners which can then affect ECM fungal growth, colonisation and function (Harley & Smith 1983; Gehring & Whitham 1991; Bardgett, Wardle *et al.* 1998; Cullings, Vogler *et al.* 2001; Gehring & Whitham 2002; Markkola, Kuikka *et al.* 2004; Peter, Ayer *et al.* 2008). This may lead to a feedback effect of reduced nutrient transfer to the host tree further decreasing the productivity of the tree. Determining any correlation between carbon transfer and ECM community characteristics such as diversity and structure, within healthy and declining forests would help to clarify the model of eucalypt decline and the role of the ECM community. Reduced carbon allocation of declining trees to their ECM associates, through

stresses on productivity to which herbivores contribute, may explain some of the differences in the ECM community of *E. delegatensis* forest.

Distinguishing the functional groups within the fungal assemblages present in healthy and declining forests would also help determine specific processes that are altered with the development of eucalypt decline. For example, determining species preferences for various forms of nitrogen, their ability to transfer nutrients to host eucalypts, and how these change under different soil conditions would be invaluable. The advent and development of metabolic and enzymatic tests can now be used to elucidate these preferences and the functional roles of ECM species and higher taxonomic groups. Genetic studies may also be important in revealing genes that are associated with specific functions. Field studies using radio-labelled N and C to determine the exact pathway of nitrogen and carbon movement through fungal communities and into trees in healthy and declining forests may also help to pinpoint feedbacks and links between ECM communities and eucalypt forest decline.

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