

The mycorrhizas of Europe's pine forests in the
context of nitrogen pollution

Filipa Cox

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Imperial College London
Department of Life Sciences

DECLARATION

I declare that this thesis is my own work. Where others have contributed data, or where information comes from other sources, this has been clearly acknowledged and referenced in the text.

Filipa Cox

ABSTRACT

Most trees in temperate and boreal ecosystems form obligate associations with ectomycorrhizal (ECM) fungi, but environmental change may alter ECM communities, potentially having major impacts on forest ecosystem functioning. Understanding the potential consequences of environmental change requires knowledge about current ECM fungal distributions, both to provide a baseline against which to measure changes in communities, and to establish the effects of current broad-scale environmental gradients on mycorrhizal biogeography. This study aims to assess the feasibility of a continental-scale survey of ECM fungal communities across a network of long-term forest monitoring plots, and provide insights into the community ecology of these important organisms. Using a spatially hierarchical sampling strategy and molecular identification of the fungi forming ECM root tips, the ECM fungal communities within 12 plots in the UK and Germany were characterised. In combination with measurements of a range of environmental variables at the 12 plots, the data indicate that nitrogen availability is a potentially important determinant of ECM fungal composition and richness across large spatial scales, but that other environmental variables may also act at large scales to influence ECM fungal communities. Evidence is also found to support a role of interspecific competition structuring ECM fungal communities within, but not across, plots, and a complex interplay between environmental gradients, co-occurrence patterns and phylogenetic relatedness is revealed. Within plots, microsatellite analysis indicates that pine roots from adjacent trees overlap, and do not have exclusive below-ground territories. Re-sampling plots, and reciprocal transplantation of pine seedlings between plots, indicates that pine mycorrhizas are stable both over time and on transplanted seedlings. This work increases our knowledge of the belowground ecology of ECM fungi, and highlights the value of identifying mycorrhizal abundance and distribution patterns - effects of global environmental change may already be significantly altering communities of this functionally critical group.

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Chapter One – General Introduction

Adapted from a paper published in Annals of Forest Science, 2010. Cox, F., Barsoum, N., Bidartondo, M.I., Børja, I., Lilleskov, E., Nilsson, L.O., Rautio, P., Tubby, K., Vesterdal L. 67, 200.

Ectomycorrhizal (ECM) fungi form intimate symbioses with most temperate and boreal tree species, playing a pivotal functional role in the uptake of nutrients, as well as providing protection against pathogens and drought (Baxter & Dighton, 2005). In exchange, the tree host supplies *ca.* 15% of its current photoassimilate to the fungi (Hobbie & Hobbie, 2006; Smith & Read, 2008). Ectomycorrhizal fungal diversity is high, with estimates of over 7,000 species worldwide, associating with approximately 8,000 plant species (Wang & Qiu, 2006; Rinaldi *et al.*, 2008). This high species richness has resulted in research efforts to establish the degree of functional diversity within ECM fungi. It is now known that different species of ECM fungi can access alternative nitrogen sources (Lilleskov *et al.*, 2002b), and that certain ECM species may play a role in the decomposition of organic carbon (Chapela *et al.*, 2001); some fungi confer more benefit to their host than others under certain environmental conditions (Johnson *et al.*, 1997; Gorissen & Kuyper, 2000), and different species of fungi can have differing carbon requirements from the host (Bidartondo *et al.*, 2001; Fransson *et al.*, 2007). Therefore, the biodiversity of ECM fungi in any given forest is likely to have direct implications for host tree health, carbon and nitrogen cycling, and the resiliency of forest functional biodiversity. Because forests are major players in the sequestration of atmospheric carbon (Canadell & Raupach, 2008; Read *et al.*, 2009), it is critical to understand the processes acting to maintain forest ecosystem function. To gain an understanding of factors that influence the mycorrhizal communities of forests, studies have used both natural gradients and manipulative experiments, and have demonstrated that pH, soil type, moisture, host tree species and nitrogen availability, amongst others, are determinants of mycorrhizal communities at local scales (e.g. Gehring *et al.*, 1998; Lilleskov *et al.*, 2002a; Børja & Nilsen, 2009).

The need to scale-up ECM research

Given the observed responsiveness of mycorrhizal fungi to shifts in environmental variables and global environmental change (Cudlin *et al.*, 2007; Kauserud *et al.*, 2008), it is of

immediate concern to know how mycorrhizal communities will respond to environmental shifts at regional and continental scales, and what effects such shifts will have on forest health and function (Lilleskov & Parrent, 2007). This task requires knowledge about current mycorrhizal distributions, both to provide a baseline against which to measure changes in communities, and to understand the effect of broad-scale environmental conditions on mycorrhizal biogeography. Whilst it would be convenient to extract these data from the numerous investigations of local ECM fungal communities carried out so far, the diversity of sampling methodologies employed makes this an impossible task. A primary disparity is that much research has relied upon morphology of reproductive structures (fruitbodies) and/or mycorrhizal roots to establish fungal identity, whilst more recent studies tend to apply various DNA techniques to mycorrhizas to identify fungi. Identification of mycorrhizal fungi using “morphotyping” of ECM roots and/or fruit bodies, formed the basis of many important early studies into the community dynamics of ECM fungal communities. Insights were gained into successional patterns in ECM fungal communities, both at the level of individual roots or root systems (Wilcox 1968; Fleming 1985, Danielson & Visser 1989; Gibson & Deacon 1990) and within whole communities (Fleming 1983, 1984; Fox 1986; Visser 1995). In addition, these studies first highlighted the differences in community structure observed between above-ground sporocarps and belowground ectomycorrhizas (Taylor & Alexander, 1990; Visser, 1995). The advent of molecular techniques, however, has given researchers the opportunity to develop a more robust, reproducible and rapid approach to ECM fungal research (Smith & Read 2008), which should facilitate studies over greater geographic scales. Unfortunately, making direct comparisons between studies in which communities have been described using morphological techniques with studies in which molecular tools have been employed can prove extremely difficult (Gardes & Bruns, 1996; Horton & Bruns, 2001). Furthermore, irrespective of the methods used to identify mycorrhizal fungi, most studies do not collect environmental data in a consistent fashion, making incorporation of data from multiple studies into a single predictive model extremely challenging.

Methodological inconsistencies aside, the responses of mycorrhizal communities to changes in environmental conditions appear to be complex, and it is difficult to confidently identify trends across studies. This is probably due to the localized nature and constrained time-scale at which most studies have been carried out, making results highly context-dependent. Particular site characteristics, pre-existing ECM fungi, and the scale of environmental change being studied, may all play a part in generating the complex picture of ECM fungal

responses to specific environmental changes we see when comparing studies. It was in this context that Lilleskov and Parrent (2007) recently called for the development of a large-scale, unified approach to ectomycorrhizal research, which would allow the distributions of individual species of ECM fungi to be mapped.

A large-scale survey of ECM fungi would allow researchers to ascertain the distribution of dominant species, and identify the environmental variables that are the most significant determinants of ECM fungal communities across broad spatial scales. Whilst the potential benefits of such research are clear, the costs and time associated with characterising both the ECM fungal community and environmental parameters could prohibit the number of sites that could feasibly be included. The use of forest monitoring plots, which would enable ECM fungal data to be linked to long-term, accurate data-sets available for individual plots, could provide an opportunity for increasing sample size by removing the need to carry out environmental monitoring of plots.

ICP Forests – an invaluable platform for large-scale research

The International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests, www.icp-forests.org) was launched in 1985. The programme is operated under the United Nations Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution that is ratified by 51 parties. The over-arching aim of ICP Forests is to understand the links between forest health, air pollution, climate change and biodiversity, through the establishment and monitoring of two complementary networks of forest plots. The first of these networks (called Level I) includes over 6,000 forest plots, set-up on a 16 x 16km grid across Europe, each monitored for tree crown condition (defoliation, discoloration and visible damage), soil condition and foliar nutrient levels. Many Level I plots have been extended to undergo more extensive monitoring as part of the BioSoil program, which aims to develop a common baseline of European forest soils, as well as assessing forest biodiversity. A further subset of 800 strategically located plots (Level II) forms an intensive continual monitoring system. Here, additional measurements include tree growth and phenology, ground vegetation, atmospheric deposition, soil chemistry, soil solution chemistry, and water balances. Expert panels are in place to ensure the harmonisation of methods used in the field, and quality assurance and control in laboratories. These networks cover the major tree species in Europe, and involve the participation of 41 countries.

The ICP Forests network provides mycorrhizal researchers with a huge and largely untapped resource on which to conduct a large-scale assessment of distributions, measure responses to environmental gradients, and link community composition directly to forest ecosystem function. The intensively monitored Level II plots of the ICP Forests programme confer a number of advantages: i) pre-established and maintained plots enable and facilitate project design, ii) access to an existing, large, and harmonized forest monitoring dataset, iii) access to information on site history, management practices and disturbances, allowing individual studies to control for, or take into account, specific inter-site variables, and iv) a long-term perspective to detect, and validate predictions for, future change.

Potential determinants of large-scale distribution patterns

Large-scale distributions of ECM fungal communities are likely to be strongly determined by dispersal constraints acting on individual species (Peay *et al.*, 2007), but environmental variables undoubtedly play an additional role. As some host tree species are known to associate with differential subsets of the ECM fungal community (Ishida *et al.*, 2007; Morris *et al.*, 2008; Tedersoo *et al.*, 2008), large-scale distributions of more host-specific ECM fungi are likely to be linked to the distributions of their host trees. Recent work has also suggested that climate may influence regional-scale macrofungal distributions (Wollan *et al.*, 2008), fruiting time (Gange *et al.*, 2007; Kauserud *et al.*, 2008) and differentially affect ECM species respiration rates (Malcolm *et al.*, 2008). It isn't yet known how climate may influence the distribution of below-ground ECM fungal communities, or what effect climate change will have on these distributions.

As ECM symbioses evolved in ecosystems where nitrogen (N) was the primary limiting nutrient (Smith & Read, 2008), it is likely that N availability may also act as a determinant of ECM fungal communities. Anthropogenic consumption of fertilisers and fossil fuels, and increased biomass burning, has resulted in a ~10 fold increase in the production of reactive N since the late 19th Century (Crutzen & Andreae, 1990; Smil, 1999; Galloway *et al.*, 2004). The resultant increase in terrestrial N availability has numerous ecological consequences such as altering nutrient cycles, leaching of base cations, acidification of soils, and alterations of plant and microbial communities (Vitousek *et al.*, 1997; Chung *et al.*, 2007; Galloway *et al.*, 2008).

Studies have established physiological mechanisms by which increased N availability may influence ECM fungi colonising the roots (Wallenda & Kotke 1998). High inorganic N supply to host plants is known to result in reduced carbon allocation to the roots (Vogt *et al.*, 1993) and a change in their carbohydrate composition (Wallenda *et al.*, 1996). As production of mycelia (Söderström & Read, 1987), fruit-bodies (Lamhamedi *et al.*, 1994) and formation and maintenance of ectomycorrhizas (Ineichen *et al.*, 1995; Wallenda & Kotke 1998) are dependent on the supply of current photoassimilate from the host, it seems probable that increased N availability can affect mycorrhizal physiology, as well as potentially influencing communities.

Local scale studies have indicated that the diversity and composition of ECM fungal fruit bodies respond to increased N (Wiklund *et al.*, 1995; Brandrud 1995). More recent studies have found that below-ground ECM fungal communities also show a reduction in diversity and change in community composition after increases in N availability at local scales (Lilleskov *et al.*, 2002a; Avis *et al.*, 2003; Parrent *et al.*, 2006). Increased N deposition has also been implicated as the cause of reduced fungal fruiting and species richness of ECM root-tip morphotypes observed over regional-continental scales (Arnolds, 1991; Taylor *et al.*, 2000). To date however, the impact of N availability on below-ground ECM communities at large spatial scales has not been assessed with molecular techniques. With N deposition patterns becoming increasingly global and continuing to rise (Matson *et al.*, 2002; Galloway *et al.*, 2008), it is important to address how N may influence below-ground ECM fungal communities across continents.

Aims of this study

The work in this thesis aims to assess the viability of utilising ICP Forests Level II plots for a continental-scale survey of ECM fungal communities, and generate data imperative to the successful design and implementation of such a large project. In meeting this end, the first unified data is gathered on below-ground ECM fungal communities across broad spatial scales, by sampling 12 level II plots in England, Scotland and Germany. The use of ICP forests long-term ecological data-sets enables an assessment of the role that increased N availability may play in structuring ECM fungal communities at large spatial scales, and an exploration of which other environmental variables may be important in structuring ECM fungal communities. As competitive interactions may also influence ECM fungal community structure, patterns of co-occurrence within and across plots are assessed for

evidence of ECM fungal segregation or aggregation, and the interactions between co-occurrence patterns, environmental gradients and ECM fungal relatedness are investigated. In addition to the focus on environmental and biotic determinants of ECM fungal communities within and across plots, a spatially explicit sampling design within individual plots is employed to ascertain optimal sampling strategies to fully capture ECM fungal diversity, and to test whether the ECM fungi associating with the roots of specific trees within level II plots, for which detailed health parameters are recorded, can be sampled by proximity to the tree. Finally, the degree of stability exhibited by ECM fungal communities over time, and on seedlings transplanted between two plots, is assessed to provide an insight into the resilience of ECM fungal communities.

ABSTRACT

Ectomycorrhizal (ECM) fungi are known to respond to changes in environmental conditions, but little is known about how rapidly below-ground ECM fungal communities respond to change, or whether communities change over time in the absence of environmental shifts or disturbance. Two approaches were used to assess these aspects of ECM fungal stability. Firstly, repeat-sampling of two forest stands was carried out to establish the degree of stability in below-ground ECM fungal communities over time. Secondly, the response of ECM fungal communities to rapid changes in environmental conditions was assessed through transplantation of *Pinus sylvestris* seedlings between the same two forest plots which differ substantially in a number of environmental conditions. Results indicate that the abundance of dominant ECM fungi remains stable in plots across years, whilst rarer fungi can be more variable. The ECM fungi on transplanted seedlings show a high degree of resilience to dramatic shifts in environmental conditions during the time-span of this study. A single sampling visit should therefore be sufficient to describe the ECM fungal community of a forest plot, providing sampling is of sufficient intensity to fully capture species diversity. Furthermore, these results suggest that short-term experimental manipulation of the environment may not induce immediate shifts in the ECM fungal community and therefore, might not provide a good proxy for longer-term exposure to environmental change.

INTRODUCTION

Ectomycorrhizal (ECM) fungi are known to respond to changes in a number of environmental conditions including soil pH, nutrient availability, CO₂ and moisture levels (Cudlin *et al.*, 2007). Alterations to ECM diversity and community composition could have significant consequences for forest ecosystem functioning, and potentially impact upon carbon and nutrient cycling, plant biodiversity, tree vitality and below-ground food webs and processes (van der Heijden *et al.*, 1998; Högborg *et al.*, 2001; Lilleskov *et al.*, 2002b; Baxter & Dighton, 2005). As global climate change and pollution are altering environments (Parmesan, 2006; Galloway *et al.*, 2008), it is critical to understand how ECM fungal communities respond to environmental disturbance. Recently, molecular tools have enabled us to characterise and monitor belowground ECM fungal communities, revolutionising the field of mycorrhizal ecology (Horton & Bruns, 2001). However, there are still gaps in our knowledge which hinder our ability to assess changes in ECM fungal diversity - if such monitoring is to be used to track changes in response to anthropogenic environmental change, it is necessary to consider how these communities change over time in the absence of disturbance or environmental change.

Attempting to understand the dynamics of diverse, predominantly below-ground communities is inherently difficult, and although there have been a number of recent studies we still lack a clear idea of how stable ECM communities are over time. Previous work has indicated that ECM fungi present on roots, or as hyphae in soil, can be temporally dynamic and influenced by seasonal changes (Izzo *et al.*, 2005; Koide *et al.*, 2007; Courty *et al.*, 2008; Walker *et al.*, 2008; Pickles *et al.*, 2010). These observations of a rapid turnover of ECM fungal species appear to be in contrast to studies demonstrating sizeable and persistent individual fungal genets that can occupy areas of hundreds of m² over tens of years (Bonello *et al.*, 1998; Kretzer *et al.*, 2004; Lian *et al.*, 2006). This could be because studies addressing temporal stability of ECM fungal communities have often been carried out in diverse forest systems, where recording the full suite of species is difficult (Izzo *et al.*, 2005; Courty *et al.*, 2008; Walker *et al.*, 2008). This can make comparing results from different sampling events problematic, as rare fungi may be 'missed', resulting in an artificially high observed temporal turnover of ECM fungal species.

From the perspective of implementing surveys of ECM fungi across large geographic regions, gaining insight into the stability of ECM fungal communities is essential to inform

fieldwork which may need to be conducted over several seasons and across different years. If fungal communities show high temporal dynamism, then characterising the ECM fungal community of a plot on the basis of a single sampling visit may lead to skewed community descriptions dependent on sampling time or season (Walker *et al.*, 2008). At worst, if ECM fungal communities change dramatically over time, comparing communities between different plots sampled on a single visit could be essentially meaningless. In this study, the extent to which a single sampling campaign can accurately describe the ECM fungal community of a plot is assessed. Repeat-sampling of two forest stands, where the aim is to saturate mycorrhizal accumulation curves, is carried out to test the hypotheses that ECM fungal communities are stable within plots, transects and cores over time.

Previous work to assess the impact of environmental change on ECM fungal communities has seldom addressed the speed of community responses to shifts in environmental conditions. There is evidence that above-ground ECM communities can display a rapid response to changes in nutrient availability (reviewed in Wallenda & Kottke, 1998), but below-ground responses are less clear. In many short-term fertilisation experiments there have not been measurable changes to below-ground ECM community composition and/or diversity following N addition (Brandrud & Timmermann, 1998; Jonsson *et al.*, 2000; Peter *et al.*, 2001), whilst effects of N after long-term fertilisation or deposition tend to be more marked (Lilleskov *et al.*, 2002a; Avis *et al.*, 2003). This suggests that below-ground, ECM fungal communities may display short-term stability to changes in environmental conditions. Here, the response of ECM fungal communities to rapid changes in environmental conditions is assessed, through transplantation of *P. sylvestris* seedlings between two forest plots which differ substantially in N availability. The hypothesis that the ECM fungal community on seedlings transplanted between plots will display short-term stability, despite a dramatic change in environmental conditions, is tested.

Finally, the study system also provides an opportunity to investigate other related aspects of mycorrhizal ecology. Firstly, which ECM fungal species are able to colonise outplanted seedlings and the similarity between the ECM fungal communities of seedlings and mature trees is assessed. Additionally, the hypothesis that mycorrhizal seedlings have greater biomass than seedlings that remain non-mycorrhizal in the field is tested.

METHODS

Study plots:

Two traditionally managed *Pinus sylvestris* L. stands in the UK were included in this study; Thetford (East Anglia, England) and Rannoch (Perthshire, Scotland). Both forest plots are part of the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests); a network of *ca.* 800 long-term intensively monitored plots (also known as “Level II” plots). The 0.3 ha forest plots are both managed, even-aged stands of mature *P. sylvestris*, planted in the mid 1960’s. The two plots differ considerably in N deposition, precipitation, soil type, depth of humus and litter layers, altitude and latitude (Table 2.1).

Sampling of adult P.sylvestris mycorrhizas:

Initial sampling of ECM roots took place in the autumn of 2006. In both plots, 10 trees were randomly selected and a transect laid out to the nearest neighbouring tree. Average transect lengths were 2.63m ($\pm$ 0.81 S.D) and 1.52m ($\pm$ 0.54 S.D) in Thetford and Rannoch respectively. Along each transect, four pairs of soil cores, each 30cm deep and 2cm in diameter, were removed at evenly spaced intervals. Soil cores in a pair were located 10cm apart, 5cm to the left and right of the transect. A total of 80 soil cores were removed at each plot, and all cores were analysed for ECM community composition. Live roots were maintained at 4°C and processed within 10 days of collection to minimise degradation. Soil was removed from roots by washing in 500 μ m sieves. Using a dissecting microscope, the first live ectomycorrhiza encountered at the end of a severed root, from each of the four largest root fragments in each soil core, was selected to maximise independence and minimise observer bias. From each plot, 320 root tips were therefore selected for molecular analysis. In 2006, these ectomycorrhizas underwent CTAB DNA extraction, following the protocol described in Gardes & Bruns (1993). Thetford was re-sampled in late spring 2008, and Rannoch was re-sampled in autumn 2008. Plots were re-sampled following the protocol above, and new soil cores were removed 5cm away from the original core position. In 2008, root-tips underwent DNA extraction using 8 μ l Extract-N-Amp, following the manufacturer’s protocol (Sigma, Dorset, UK).

Seedling transplantation experiment:

A total of 288 *P. sylvestris* seedlings were grown from surface-sterilised seed (Forestart, Shropshire UK) in growth chambers set to 16 hours daylight at 21°C, eight hours darkness

at 18°C, and a relative humidity of 70%. Half of these seedlings were grown in soil collected from Thetford, and the other half in soil collected from Rannoch. To eliminate mycorrhizal propagules, soil was microwaved for 40 mins (until the temperature of the soil exceeded 95°C), left to stand for 24 hours, and then microwaved a second time. Each seedling was grown in a 50µm nylon mesh “sock” (Plastok, Merseyside UK) to prevent outgrowth of roots whilst allowing water and fungal hyphae to pass through once outplanted. Each sock was inserted in a 49mL RLC-3UV Conetainer (Stuewe & Sons Inc., Oregon USA). Three month old seedlings were out-planted at the end of August 2007; seedlings growing in Thetford soil were outplanted in Thetford, whilst those grown in Rannoch soil were planted in the Rannoch plot. Seedlings in each plot were randomly allocated to one of four ‘seedling-beds’ created within the plots. Seedling beds were 3.5 x 3.0 m in size, and seedlings were placed 50cm apart from one-another on a grid within the beds. Individual seedlings were protected from herbivory with the use of a clear plastic tube (8.0 x 5.0cm) held down with a tent peg.

At the beginning of October, five weeks after out-planting, 48 seedlings from Rannoch were transplanted to the Thetford plot and *vice versa*. Within each plot, 48 seedlings were also replanted at a different location within the plot in order to control for the effects of transplanting upon ECM communities and seedlings in the absence of a significant environmental change. In addition, a further 48 seedlings in each plot were left in their original position without disturbance. Each seedling bed contributed an equal number of seedlings to different treatments, which were randomly selected from the bed. In mid November, after a further period of five weeks in each plot, all seedlings were harvested. Seedlings were carefully washed to remove soil adhering to roots in 500µm sieves. The total number of root tips and ectomycorrhizas were counted under a dissecting microscope, and the root and shoot dry weights (40°C for one week) were recorded. A representative root tip from each ectomycorrhizal morphotype found on each mycorrhizal seedling was retained for DNA extraction with 8µl Extract-N-Amp, as described above for mature tree roots.

PCR and DNA sequencing:

DNA extracted from ectomycorrhizas of both adult trees and seedlings underwent identical downstream processing. Polymerase chain reaction (PCR) amplification was carried out using the fungal-specific primer combination ITS1f and ITS4. An aliquot of 0.5µl of extracted DNA was combined with 4µl of Extract-N-Amp PCR solution/ JumpStart™

ReadyMix (Sigma, St Louis, MO, USA) in an 8µl reaction. Amplifications were performed with an initial denaturation at 94°C for 60 s, followed by 35 cycles of 94°C for 30 s, 53°C for 55 s and 72°C for 50 s, with a final extension of 72°C for 7 min (Extract-N-Amp). Alternatively, amplifications were carried out with an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 5 min (JumpStartTM ReadyMix). Successful PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA). Cycle sequencing was conducted using BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) and the resulting products were precipitated following the manufacturers' instructions for EDTA/ethanol. Sequences were analysed on an ABI Prism 3730 Genetic Analyser (Applied Biosystems) and edited with Sequencher (GeneCodes, Ann Arbor, MI, USA), before being preliminarily identified to family level using BLASTn searches on GenBank (<http://www.ncbi.nlm.nih.gov/blast>). Sequence alignments were subsequently generated for each family. Sequences were then assigned to a species-level grouping according to 97 or 98% sequence similarity for Basidiomycetes and Ascomycetes, respectively (Nilsson *et al.*, 2008), using the furthest neighbour algorithm in DOTUR (Schloss & Handelsman, 2005). Representative DNA sequences were later re-checked against the GenBank and UNITE sequence databases to assign a taxonomic name to each group, where possible.

Statistical analysis:

Temporal stability of ECM fungi on mature trees:

The ECM fungal richness of mature trees was compared across sampling dates. As final sample size differed due to variable DNA sequencing success rates, total richness was estimated using the non-parametric Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992), using the vegan package (Oksanen *et al.*, 2010) of the R statistical program (R Development Core Team, 2010). The relative abundance of ECM fungal species occurring on adult tree roots in a plot was calculated to allow comparison of ECM fungal composition across plots, whilst taking into account differences in sample size. This was calculated as the number of root-tips colonised by a fungus, divided by the number of successfully sequenced samples.

The proportion of fungi found in 2006 that were also found in the same transect in 2008 was observed. This proportion was re-calculated for 999 randomisations in which the 2008

transects, to be compared to the 2006 transects, were permuted to test whether re-sampled transects were significantly more similar across years to each other than to other transects in the plot. The proportion of fungi found in cores in 2006 that were also found in the same core in 2008 was also observed. This was compared first to 999 randomizations in which the 2008 cores were permuted across the whole plot. It was then also compared to randomizations in which cores within the transect were permuted.

Seedling transplant experiment:

The ACE estimator of total species richness was calculated to compare the expected ECM fungal richness of seedlings within each treatment group. To compare the composition of fungi on seedlings, whilst taking into account differences in sample size, the relative number of seedlings colonised by a fungus was calculated (number of seedlings colonised, divided by the number of mycorrhizal seedlings of a treatment).

Linear mixed effects models (LMM) with a Gaussian family and identity link were used to test for effects of treatment and mycorrhizal status on seedling weight (total, shoot and root weight) using the lme4 package (Bates & Maechler, 2010) of R. Treatment, original plot of outplanting, and mycorrhizal status were included as fixed factors, whilst original and final seedling bed were included as random factors to account for any influence of seedling beds on seedling weights. Models reported are the minimum adequate models; models were tested for potential simplification by testing for significant differences between models using ANOVA. The significance of fixed terms was calculated through Markov chain Monte-Carlo (MCMC) sampling to generate *P* values based on the posterior distribution using the languageR package (Baayen, 2010), because calculating the appropriate number of degrees of freedom to use when assessing statistical significance of terms in mixed-effects models is controversial (Baayen *et al.*, 2008).

Generalized linear mixed models (GLMM) with binomial errors were carried out to test for effects of treatment on the number of seedlings becoming mycorrhizal. Here, treatment was nested within plot of outplanting as a fixed factor, and original and final seedling beds were random factors. Significance of fixed factors was estimated using the Wald statistic, as MCMC sampling of GLMMs is not yet implemented in lme4.

To test for shifts in the ECM fungal communities of transplanted seedlings, permutational multivariate analysis of variance was carried out on distance matrices of seedlings using the function “adonis” in the vegan package (Oksanen *et al.*, 2010). Pairwise Bray-Curtis distances between the relative number of seedlings colonised by fungal species in each of the treatment groups was calculated. The significance of each distance was then tested against 999 permutations of the ECM fungal communities of seedlings between the two treatments being compared. As this procedure involved multiple pairwise tests, *P* values were adjusted to the appropriate Bonferroni level.

Fungi colonizing mature trees and seedlings:

To compare the ECM fungal communities of mature trees and seedlings, non-metric multidimensional scaling (NMDS) was performed using Bray-Curtis community dissimilarities between seedlings and plot transects (2006 data only), based on relative abundances of fungi or colonised seedlings to account for differences in sample size.

Table 2.1

Characteristics of the two study plots.

	Thetford	Rannoch
Soil type (FAO)	Ferralic arenosol	Gleyic podzol
Litter layer thickness (cm)	3	8
Planting year	1967	1965
Precipitation total (mm⁻¹ yr)	588.4	2351.6
Temperature mean (°C)	9.58 ± 5.38	5.74 ± 6.04
Altitude (m)	50	500
N deposition (kg⁻¹ha⁻¹yr)	12.49 ± 0.70	4.56 ± 1.33
Latitude/Longitude	52° 26' 00"/00° 52' 20"	56° 39' 10"/-4° 17' 10"

Standard deviations (±) are provided for the mean daily temperature across a 30 year period, and for mean annual throughfall nitrogen (N) deposition levels between 2001 and 2006. Soil types are named according to Food and Agriculture Organisation (FAO) classifications.

RESULTS

Temporal stability of ECM fungi on mature trees:

Mycorrhizal richness and community composition across plots and sampling dates:

A total of 1,280 adult *P. sylvestris* roots were analysed, generating 998 successful DNA sequences. These sequences represented an average success rate of 78% across sampling efforts, although success rate varied across plots and years, with 79 and 84% success from Rannoch in 2006 and 2008, respectively, and 84 and 65% success from Thetford in 2006 and 2008 respectively.

Observed fungal richness of plots was similar across plots and years. In 2006, 21 and 20 fungi were found in Thetford and Rannoch respectively. In 2008, both plots had a recorded richness of 21 fungi. Species accumulation curves indicated that sampling had captured most of the ECM diversity in plots (Fig. 2.1a). Estimates of expected total richness were similar across years in Rannoch, but differed in Thetford where an additional six fungi were predicted to occur in 2008 in comparison with 2006 (Table 2.2).

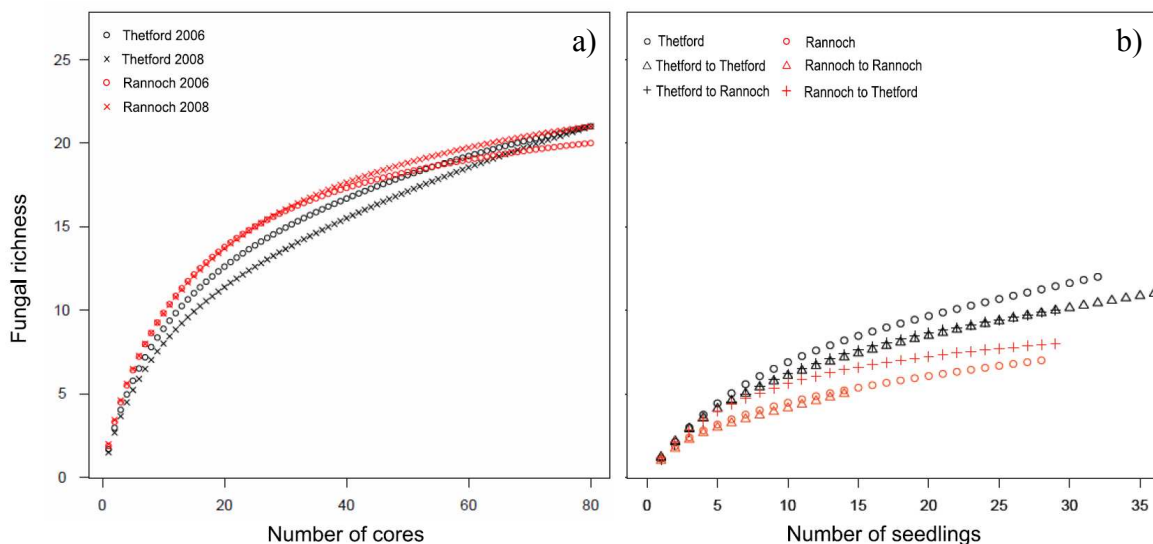


Figure 2.1

Species accumulation curves for ectomycorrhizal fungi occurring on a) adult trees with a soil core as an individual sampling unit and b) seedlings.

Table 2.2

Observed and expected (ACE) species richness of ectomycorrhizal fungi colonising adult and seedling *P. sylvestris*. T: untransplanted seedlings in Thetford, TT: seedlings transplanted within Thetford, TR: seedlings transplanted from Thetford to Rannoch, R: untransplanted seedlings in Rannoch, RR: seedlings transplanted within Rannoch, RT: seedlings transplanted from Rannoch to Thetford.

	Plot/seedling treatment	Observed	ACE ($\pm$ S.E)
Adults	Thetford 2006	21	23 ($\pm$ 2.26)
	Thetford 2008	21	29 ($\pm$ 2.83)
	Rannoch 2006	20	21 ($\pm$ 2.15)
	Rannoch 2008	21	23 ($\pm$ 2.34)
Seedlings	T	12	18 ($\pm$ 1.95)
	TT	11	17 ($\pm$ 2.09)
	TR	10	14 ($\pm$ 1.85)
	R	7	9 ($\pm$ 1.31)
	RR	5	9 ($\pm$ 1.35)
	RT	8	9 ($\pm$ 1.28)

Thetford and Rannoch have distinct fungal communities (Fig. 2.2). Overall, only five fungal species recorded on adult roots were common to both plots. The dominant fungus in Thetford was *Russula pectinatoides*. In Rannoch, an unknown Pezizalean fungus was dominant. Re-collection of live roots and sequencing of additional loci placed this fungus in a clade of Leotiomyces not formerly known to form ectomycorrhizas. Examination of vouchers identified the fungus as the morphologically characterised “*Piceirhiza sulfo-incrustata*” (Palfner *et al.*, 2005; Götz Palfner, personal communication). Both plots, on both sampling dates, show a highly similar pattern of a few very abundant fungi, and many rare fungi (Fig 2.2, Table 2.3).

At the plot level, abundances of more common fungi (relative abundances of $\geq 2\%$) are consistent over the 2006 and 2008 sampling dates at both plots (Fig. 2.2). All such common fungi were recorded at both sampling dates in a plot (Fig. 2.2). Rare fungi show a greater degree of temporal turnover, with 10 fungi in Thetford, and five fungi in Rannoch, being observed in just one of the two sampling years (Table 2.3). Overall, 62 and 78% of fungi were observed in both sampling years in Thetford and Rannoch respectively.

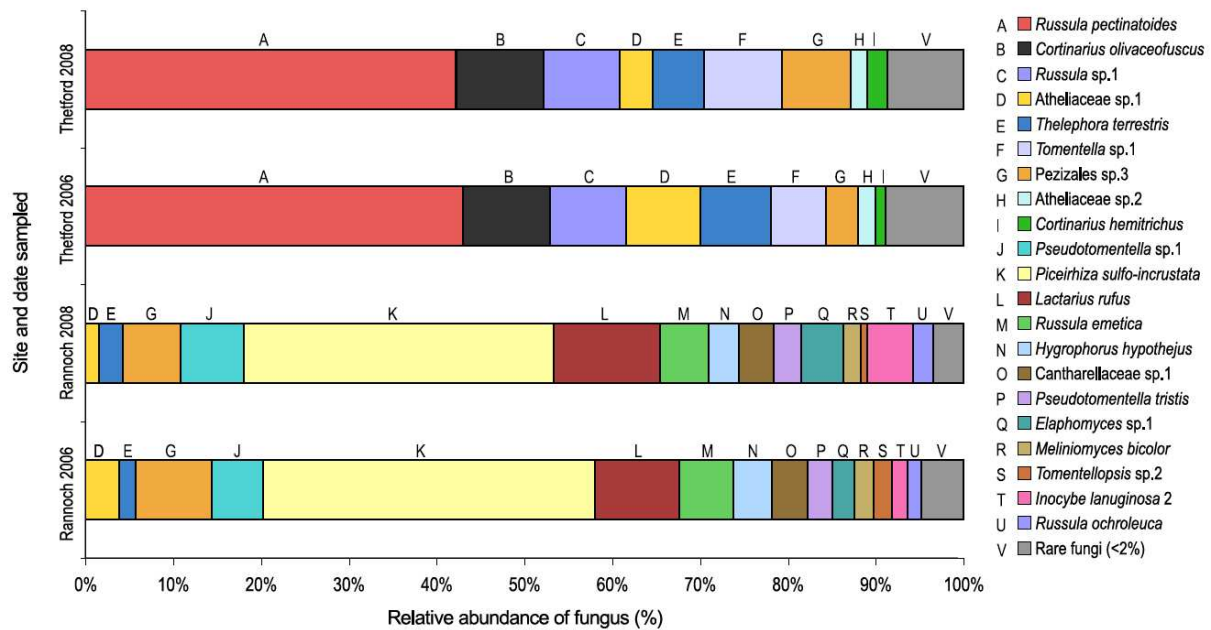


Figure 2.2

Percentage abundance of ectomycorrhizal fungi on mature trees in Thetford and Rannoch, across different sampling dates. Fungi with a relative abundance of less than 2% are grouped together as “rare fungi”.

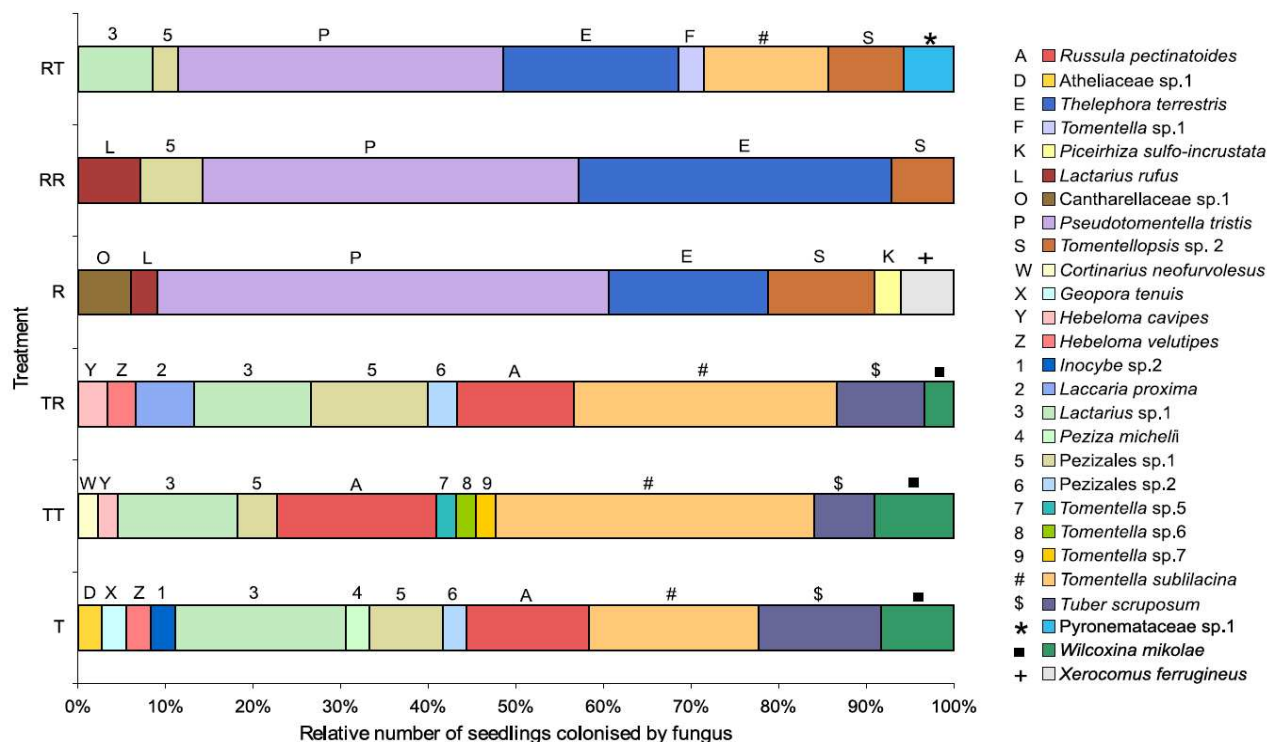


Figure 2.3

Percentage of mycorrhizal *P. sylvestris* seedlings that were colonized by particular ectomycorrhizal fungi, across different treatments. T: untransplanted seedlings in Thetford, TT: seedlings transplanted within Thetford, TR: seedling transplanted from Thetford to Rannoch, R: untransplanted seedlings in Rannoch, RR: seedlings transplanted within Rannoch, RT: seedlings transplanted from Rannoch to Thetford.

Table 2.3

Percentage abundance of rare (<2% relative abundance) ectomycorrhizal fungi found on adult *P. sylvestris* in Thetford and Rannoch.

Fungus	Rannoch		Thetford	
	2006	2008	2006	2008
<i>Tuber scruposum</i>	-	-	1.86	0.48
<i>Helotiales</i> sp.2	-	-	1.49	0.48
<i>Cortinarius</i> sp.2	-	-	1.49	1.93
<i>Tuber rufum</i>	-	-	1.49	1.45
<i>Pseudotomentella</i> sp.1	-	-	0.74	0.48
<i>Pezizales</i> sp.2	-	-	0.37	0.97
<i>Pezizales</i> sp.1	-	-	0.37	0.48
<i>Suillus granulatus</i>	-	-	0.74	0.00
<i>Inocybe</i> sp.2	-	-	0.74	0.00
<i>Tomentella</i> sp.3	-	-	0.37	0.00
<i>Tomentella</i> sp.2	-	-	0.74	0.00
<i>Wilcoxina</i> sp.1	-	-	0.37	0.00
<i>Tomentella</i> sp.4	-	-	0.00	0.97
<i>Hebeloma velutipes</i>	-	-	0.00	0.97
<i>Basidiomycete</i> sp.1	-	-	0.00	0.48
<i>Hebeloma cavipes</i>	-	-	0.00	0.48
<i>Tricholoma terreum</i>	-	-	0.00	0.48
<i>Tricholoma flavovirens</i>	1.97	0.75	-	-
<i>Lactarius quieticolor</i>	1.18	0.75	-	-
<i>Cortinarius aurantiobasis</i>	0.39	0.37	-	-
<i>Amanita muscaria</i>	0.79	0.00	-	-
<i>Wilcoxina mikolae</i>	0.39	0.00	-	-
<i>Xerocomus ferrugineus</i>	0.00	0.37	-	-
<i>Entoloma</i> sp.1	0.00	0.37	-	-
<i>Laccaria proxima</i>	0.00	0.37	-	-

Probability of observing the same fungi at re-sampled transects and cores:

Transects:

In Rannoch, there was an 80% chance of sampling the same ECM fungus found in 2006, if the same transect was re-sampled in 2008. This is a significantly greater chance than if random transects were sampled in 2008, where the average chance of sampling the same fungus is 46% ($P<0.001$).

In Thetford, there was a 69% chance of sampling the same ECM fungus if the same transect was re-sampled in 2008. This is a significantly higher probability than if random transects were sampled in the second campaign, where the average chance of sampling the same fungus was 35% ($P<0.001$).

Cores:

In Rannoch, there was a 55% chance of sampling the same ECM fungus when the same core location was re-sampled in 2008. This is a significantly higher probability than if a core was sampled randomly from within the plot, where the average chance was 29% ($P<0.001$), but was not significantly different to the probability of sampling the same fungus if cores were selected randomly from within the same transect, where the average chance would have been 50%.

In Thetford, there was a 55% chance of sampling the same ECM fungus when sampling the same core location in 2008 compared to 2006. This is a significantly higher probability than if a core was sampled randomly from the plot, where the average chance is 24% ($P<0.001$), and significantly higher than if cores were selected randomly from within the same transect, where the average chance of sampling the same fungus would have been 48% ($P=0.04$).

Table 2.4

Pairwise adonis r^2 values, testing the Bray-Curtis similarity of ectomycorrhizal communities on *P. sylvestris* seedlings subjected to different treatments. Values in bold are significant at the Bonferroni corrected value $P=0.015$. T: untransplanted seedlings in Thetford, TT: seedlings transplanted within Thetford, TR: seedlings transplanted from Thetford to Rannoch, R: untransplanted seedlings in Rannoch, RR: seedlings transplanted within Rannoch, RT: seedlings transplanted from Rannoch to Thetford.

	T	R	TT	RR	TR	RT
T	-					
R	0.17	-				
TT	0.02	0.21	-			
RR	0.11	0.02	0.15	-		
TR	0.01	0.17	0.01	0.12	-	
RT	0.09	0.03	0.12	0.02	0.09	-

Seedling transplant experiment:

Two seedlings died during the experiment and were excluded from analysis. Of the remaining 286 seedlings, 187 (65%) became mycorrhizal. Successful sequences were obtained from 169 (90%) mycorrhizal seedlings.

Effect of plot and treatment on mycorrhizal colonization and species richness:

Total expected richness of ECM fungi colonising seedlings indicate that seedlings initially outplanted in Thetford have a higher estimated total richness than seedlings that were initially planted into Rannoch (Table 2.2).

The number of seedlings that became mycorrhizal was not affected by the identity of the plot in which seedlings were originally planted (plot of outplanting). Seedlings transplanted within Rannoch were less likely to become mycorrhizal than seedlings with alternative treatments (GLMM; Est = -1.42, $z = -3.256$, $P = 0.001$). No other treatment had a significant effect on the number of seedlings becoming mycorrhizal.

The effects of mycorrhizal colonization, plot and treatment on seedling weight:

Mycorrhizal seedlings had significantly greater dry weights than non-mycorrhizal seedlings (LMM; Est = 9.54, $P < 0.001$). Mycorrhizal seedlings had greater dry root weight (LMM; Est = 5.74, $P < 0.001$) and shoot weight (LMM; Est = 4.02, $P = 0.02$) than non-mycorrhizal seedlings. Seedlings that were initially planted in Thetford had significantly smaller dry weights than seedlings initially planted in Rannoch (LMM; Est = -14.56, $P < 0.001$); this was caused by lighter roots of Thetford seedlings compared to Rannoch seedlings (LMM; Est = -11.81, $P = 0.002$), whilst there was no difference in seedling shoot weight between the two plots. Treatment had a significant impact on root weight of seedlings; seedlings transplanted from Rannoch to Thetford were lighter than seedlings that were untransplanted in Rannoch (LMM; Est = -5.04, $P = 0.012$). There were no significant effects of other treatments on root weight.

Response of ECM fungal communities to transplantation:

Seedlings that remained in either Thetford or Rannoch throughout the experimental period, had distinct fungal communities (Fig. 2.3). Only Pezizales sp.1 was present on seedlings that were un-transplanted or transplanted within both plots. Seedlings that were transplanted from Thetford to Rannoch did not show any evidence of colonization by fungi

that originated from Rannoch. Three seedlings that were transplanted from Rannoch to Thetford were colonized by *Lactarius* sp.1, and five seedlings were colonized by *Tomentella sublilacina*; both of these fungi likely originated from Thetford, as they were not recorded on seedlings that remained within Rannoch. Pairwise “adonis” tests revealed that seedlings transplanted between plots had ECM fungal communities that did not differ significantly from the ECM fungal communities of seedlings that had remained within the plot of outplanting (Table 2.4). Seedlings transplanted between plots had ECM communities that were significantly different to the ECM fungal communities of seedlings that had remained within the second plot (Table 2.4).

Fungi colonizing mature trees and seedlings:

Richness estimates of total diversity of seedlings and adults differed. Estimates of ECM fungal richness on seedlings were lower than estimates for adult trees in both plots (Table 2.2). The NMDS analysis (Fig. 2.4) groups the ECM fungal communities of seedlings with the adult ECM fungal communities of the plot of outplanting on the first axis, but separate from adult communities on the second axis, indicating that the observed ECM fungal communities of adult and seedling *P. sylvestris* differed. Comparing Figs 2.2 and 2.3 reveals large differences in the abundances of individual ECM fungal species on adults and seedlings. “*Piceirhiza sulfo-incrustata*”, a dominant on the roots of adult trees in Rannoch, was recorded on just one seedling, whilst *Pseudotomentella tristis* and *Thelephora terrestris* were dominant on seedlings but found on less than 3% of adult roots from Rannoch. Similarly, in Thetford *Tomentella sublilacina* was the dominant ECM fungus on seedlings, but it was not recorded on the roots of adult trees. Overall, 33% of the fungi recorded on seedlings were not recorded on the roots of adult trees in either plot.

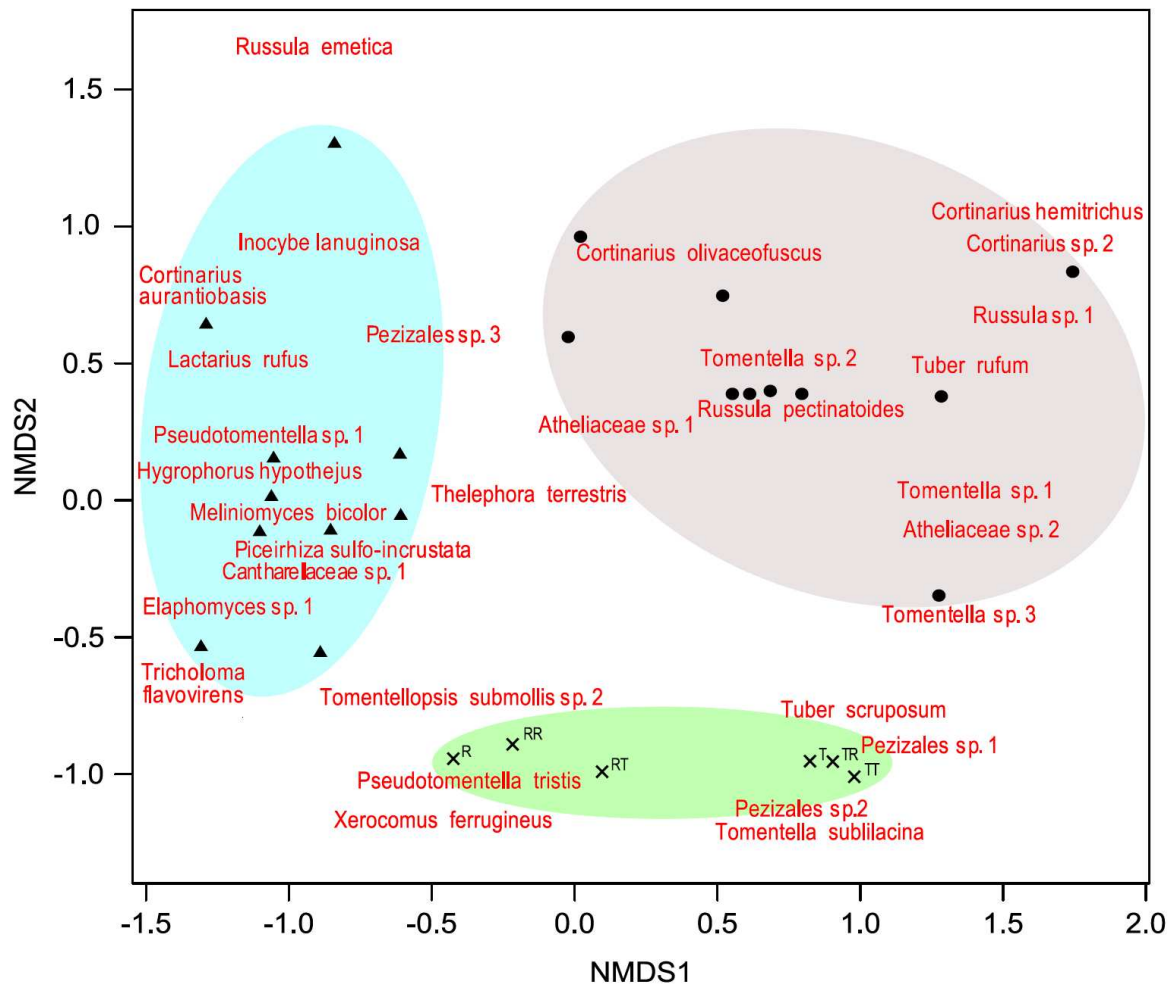


Figure 2.4

Non-metric multidimensional scaling plot of the ECM communities of transects taken between adult *P. sylvestris* in Rannoch (black triangles) and Thetford (black circles) in 2006, and on seedlings subjected to six different treatments (black crosses). Points that are located in close proximity to each other indicate a highly similar ECM fungal community composition, whilst points further apart have dissimilar communities. Points representing the ECM fungal community composition of seedlings and transects in Thetford and Rannoch, are grouped with ellipses to aid interpretation. T: untransplanted seedlings in Thetford, TT: seedlings transplanted within Thetford, TR: seedling transplanted from Thetford to Rannoch, R: untransplanted seedlings in Rannoch, RR: seedlings transplanted within Rannoch, RT: seedlings transplanted from Rannoch to Thetford.

DISCUSSION

Temporal stability of ECM fungi on mature trees:

The dominant ECM fungal communities of adult *P. sylvestris* trees are stable at both the plot and transect level across years, and the relative abundance of common fungi does not change substantially between years. This finding is in contrast to the results of some other studies which have found large differences in ECM community composition over similar time scales (Courty *et al.*, 2008; Walker *et al.*, 2008; Pickles *et al.*, 2010). Changes in the dominance of some common fungi were dramatic in these studies; for example, a *Russula* species was dominant during November to January, and rare in other times of the year (Courty *et al.*, 2008), and *Lactarius rufus* substantially declined in abundance between two sampling years (Pickles *et al.*, 2010). One possible reason behind these different findings could be the intense sampling of relatively species poor plots in the present study, allowing a reasonable description of the ECM fungal community. This is supported by Koide *et al.* (2007) who also found relative stability in the mycorrhizas of dominant ECM fungi in a species poor plot over a 13 month period. The high diversity of ECM fungi in the sampling plots of Courty *et al.* (2008) and Walker *et al.* (2008) (75 and 72 fungal types respectively) may have hindered the ability to fully capture species diversity, leading to skewed views of the composition and structure of plots over time. Alternatively, the broadleaf forests considered in these two studies may show greater temporal dynamism than coniferous systems. The dramatic decline in the abundance of *L. rufus* in the study plot of Pickles *et al.* (2010) may have been caused by drought in the second sampling year.

A higher proportion of fungi were recorded in only one sampling year in Thetford than in Rannoch. In both plots, these fungi always had relative abundances of less than 2%, making repeated detection difficult. A higher number of rare fungi, and the difficulty associated with detecting them, may explain the slightly higher turnover of fungi detected at Thetford. Alternatively, this difference could be the result of seasonal effects – following initial sampling of both plots in autumn, re-sampling of Thetford and Rannoch took place in spring and autumn respectively. Seasonal changes in belowground ECM fungal communities have been demonstrated via identification of hyphae (Koide *et al.*, 2007) and mycorrhizas (Courty *et al.*, 2008; Walker *et al.*, 2008).

The spatially explicit sampling methods in this study allow some speculation about the spatial scale of ECM fungal stability at the two forest plots. Re-sampled soil cores

demonstrated some spatial stability within transects at Thetford; fungi were more likely to be re-sampled in the same corresponding core position, rather than in a random core within the same transect -this was not the case at Rannoch. Pairs of cores were closer together at Rannoch (average distance 30cm) than at Thetford (average distance 53cm) – possibly indicating that fungi are less stable over time when considered at finer scales. These results are consistent with another study that assessed the spatiotemporal stability of ECM fungi, and found communities to be considerably less similar at small spatial scales (5cm) compared to larger (25-200m) spatial scales (Izzo *et al.*, 2005). The more ephemeral nature of ECM fungi at smaller scales may be due to a rapid turnover of fine roots, or because of expansion of individual fungal genets (Izzo *et al.*, 2005; Lian *et al.*, 2006). It should be noted, however, that it was not possible to re-sample the exact location of individual soil cores, instead sampling 5cm away from the original position in 2008. This, along with the physical disturbance created during coring in 2006, and the reduced sampling intensity of cores in comparison to transects, could influence the ability to detect the same fungal species in re-sampled cores in 2008.

Stability of ECM fungi on transplanted seedlings:

The ECM fungal communities of seedlings transplanted between plots remained stable during the time-scale of this experiment. It is impossible to rule out incorrectly ascribing fungal species to particular plots due to the possibility that a fungus was present but went undetected; however, the distinct communities of Thetford and Rannoch lends some confidence to the task of assessing the degree of any shift in ECM community towards the community of a particular plot. This is because the ECM fungal community of seedlings transplanted between plots can be compared to seedlings that have remained solely within a single plot. One-hundred percent of mycorrhizal seedlings transplanted from Thetford to Rannoch were colonized solely by fungi likely to have originated from Thetford, whilst 77% of seedlings transplanted from Rannoch to Thetford were colonised solely by fungi likely to have originated from Rannoch. The eight seedlings that were transplanted from Rannoch to Thetford, and associating with fungi from Thetford, were colonised by *Tomentella sublilacina* and *Lactarius* sp.1, which has close matches to *L. hepaticus* on GenBank. Both *T. sublilacina* and *L. hepaticus* are known to readily form mycorrhizal associations with seedlings in bioassays (Lilleskov & Bruns, 2003; Collier & Bidartondo, 2009), so it is unsurprising to find these fungi colonising the roots of transplanted seedlings.

The short-term stability of ECM fungi on seedlings transplanted between plots of contrasting N availability could explain why some shorter-term N addition experiments have not found measurable differences in below-ground ECM fungal diversity or community composition after N addition (Jonsson *et al.*, 2000). A notable exception to this was a two-year fertilization experiment conducted in temperate oak systems in which a loss of ECM fungal diversity was observed just one year after initiation of fertilisation (Avis *et al.*, 2008). Alongside the studies of Courty *et al.* (2008) and Walker *et al.* (2008), which indicate dramatic seasonal variations in below-ground ECM fungal communities in oak, there is some evidence to suggest that ECM fungal communities of broadleaf forests respond with greater rapidity to seasonal and environmental changes than those of coniferous forests. It would be useful to test whether the short-term stability displayed by ECM fungi on seedlings transplanted between plots in a coniferous system is mirrored in broadleaf systems.

The short duration of this transplantation experiment meant that 35% of seedlings did not become mycorrhizal, reducing the number of seedlings for comparison of ECM fungal community composition between treatments. A significantly lower proportion of seedlings transplanted within Rannoch became mycorrhizal when compared to seedlings in other treatment groups. The reason for this is unclear, as transplantation within Thetford, or between the two plots, did not have the same effect. Total species richness estimates for seedlings indicate that the suite of fungi able to colonise outplanted seedlings was lower in Rannoch than in Thetford. Rannoch appeared to have a lower number of fungi able to colonise seedlings over the duration of this experiment, despite similar numbers of ECM fungal species on mature trees. Perhaps the additional disturbance created by transplanting seedlings within Rannoch reduced further the pool of fungi able to colonise roots of seedlings transplanted within this plot, compared with those seedlings left undisturbed.

The larger root and shoot mass of mycorrhizal compared to non-mycorrhizal seedlings is likely due to enhanced ability to access nutrient and water supplies (Smith & Read, 2008). This is consistent with the findings of a meta-analysis of 36 studies exploring the impacts of mycorrhizal colonisation on seedling weight (Karst *et al.*, 2008). Root weights of seedlings initially planted into the two plots differed significantly, consistent with greater biomass allocation to roots where nutrient availability is limited (Ericsson, 1995). The lighter roots, but similar shoot-weight of seedlings transplanted from Rannoch to Thetford, compared to

seedlings remaining in Rannoch, might indicate that transplantation of seedlings to a nutrient-rich plot has caused a reduction in relative biomass allocation to roots.

There is a possibility that the soil present on seedling roots transplanted between plots could have hindered colonization of the fungal community of the second plot. However, studies on competitive interactions between fungal species have highlighted the importance of priority effects in determining competitive outcomes (Kennedy & Bruns, 2005; Kennedy *et al.*, 2009), which supports the observed resilience of the pre-existing ECM fungal community on transplanted seedlings in this study. The time span of this experiment allowed us a snap-shot view of the fungal communities immediately after exposure to shifts in environmental conditions, but a valuable extension to this work would be to characterize changes in community composition occurring on outplanted seedlings over time. This would allow an assessment of the time-scale over which fungi on transplanted seedlings remain stable, and whether fungi transplanted from the original plot can persist on seedling roots long-term. Previous studies have demonstrated that ECM fungi inoculated onto the roots of nursery trees survive long after outplanting in the field (Selosse *et al.*, 1998; Pruett *et al.*, 2008). Persistence of ECM fungi on transplanted seedlings has potentially serious implications for the introduction of exotic crop plants hosting alien fungal species (Schwartz *et al.*, 2006; Vellinga *et al.*, 2009). The presence of non-native ECM fungi could lead to detrimental effects on native ECM fungi, facilitate invasions of introduced plants and alter local soil nutrient cycling (Vellinga *et al.*, 2009).

Fungi colonizing mature trees and seedlings:

The ECM fungal communities of adults and seedlings differed, both in terms of diversity and abundance of fungal species. Seedling beds were not always located in the same positions as transects, making it possible that seedlings and the adult trees sampled were exposed to different fungal inoculum pools. However, “*Piceirhiza sulfo-incrustata*” was found in nine of 10 transects in Rannoch, and only on one root-tip of a single seedling, lending support to real differences in the fungal species able to colonise mature trees and outplanted seedlings in this study. Previous studies involving naturally re-generated seedlings have shown a high degree of similarity between ECM fungal communities of seedlings and surrounding mature trees (Jonsson *et al.*, 1999; Fransson *et al.*, 2000; Fransson *et al.*, 2007). The short time-span in which seedlings were exposed to inoculum in the present study, and the additional disturbance created during transplantation, may have biased the ECM fungal communities on seedlings towards those capable of fast colonization

in disturbed ground, akin to those frequently recovered in bioassay experiments. The fungal community composition of seedlings supports this assertion; fungi dominant on seedlings but not present or uncommon on adult roots such as *Tomentella subulilacina*, *Thelephora terrestris*, *Lactarius* sp.1 (possibly *L. hepaticus*), *Pseudotomentella tristis*, *Tuber scruposum* and *Wilcoxina mikolae* are all recognized as fungal species capable of colonizing seedlings in disturbed ground or in bioassays (Colpaert, 1999; Cline *et al.*, 2005; Collier & Bidartondo, 2009). In contrast, whilst attempting to characterize the novel ECM fungus “*Piceirhiza sulfo-incrustata*”, considerable effort to colonise seedlings using freshly collected “*Piceirhiza sulfo-incrustata*” root-tips in microcosms was fruitless (data not published). The presence of the mesh “sock” may have hindered colonisation by some fungal species, further influencing the ECM fungal community found on outplanted seedlings.

CONCLUSIONS

The ECM communities of both mature *P. sylvestris* trees over time, and seedlings exposed to short-term shifts in environmental conditions, have shown a high degree of stability in this study. The relative constancy of common ECM fungi in plots indicate that, from a sampling perspective, there should not be any serious concerns when comparing the composition of plots sampled across different years, providing sampling is of sufficient intensity to capture most of the fungal diversity. The resiliency of fungi on transplanted seedlings highlights that below-ground ECM fungal communities may not show immediate responses to environmental change, and studies investigating community responses to shifts in the environment may need to consider that short-term exposure to new environmental conditions may not cause change in below-ground ECM fungal communities. The ability of fungal species to persist on roots (at least in the short-term) when transplanted to a new environment raises concerns about the potential introduction of alien species when establishing exotic plants in native habitats. Well characterised plots will be a useful resource for future re-sampling efforts to investigate how ECM fungal communities change over longer time periods.

Chapter Three - The contrasting distribution patterns of fine roots and ectomycorrhizas in *Pinus sylvestris* stands.

ABSTRACT

The below-ground parts of plants and ECM fungi are inherently difficult to observe and measure, resulting in limited knowledge about their fine-scale distribution. Here, microsatellite markers are used to link the ECM fungal community to individual trees in two *Pinus sylvestris* plots, to determine the degree of fine root overlap between adjacent trees, and to test for non-random associations between individual trees and their ECM fungal communities. In addition, the degree and spatial scale of below-ground ECM fungal autocorrelation in three forest plots is established, so that future sampling efforts may reduce non-independent sampling of fungal communities. The analyses indicate that the fine root distributions of adjacent trees overlap substantially, although a decreasing proportion of an individual trees roots are found at increasing distance classes away. No evidence is found to support non-random host-fungal associations in the field, but a strong degree of spatial autocorrelation of ECM fungal communities at small distances (<2.5m) is detected. The results suggest that below-ground, tree roots have a strong potential for competitive interactions, and that sampling an individual trees roots by proximity to the trunk is not a feasible option. In addition, future sampling efforts should aim to retain spatially hierarchical sampling designs, but reduce the number of samples taken in close proximity.

INTRODUCTION

Below-ground processes and interactions are a vital part of ecosystem functioning, and may play a role in governing above-ground plant community dynamics (Wardle, 2002). The intrinsic difficulty with studying below-ground organisms or organs means that often basic ecological knowledge such as distribution or abundance is lacking. The canopies of individual trees compete for light and space such that conspecific trees essentially occupy an above-ground territory, excluding other individuals from that space. Below-ground, trees are expected to compete for space, nutrients and water, but studies to ascertain root distributions have been hindered by the difficulty of identifying roots from individual trees, especially fine roots and those of the same species (Schenk *et al.*, 1999). A variety of techniques have been used in an attempt to assess the spread and distribution of roots, including excavating root systems (Gehring *et al.*, 1998), uptake of isotopes (Gottlicher *et al.*, 2008) or nutrient analogues such as Rb, Cs, Li or Sr (Casper *et al.*, 2003) and molecular methods such as microsatellite analysis of roots and trees (Brunner *et al.*, 2004; Saari *et al.*, 2005; Lang *et al.*, 2010). Microsatellites are short repeated DNA sequences that generally evolve neutrally without selective constraints. Because they can be highly polymorphic in length, characterising the length of several of these repeated sequences provides a unique fingerprint pattern for each genetic individual (Zane *et al.*, 2002; Lian *et al.*, 2006). This approach allows roots to be linked to individual trees of the same species, providing insight into the degree of root overlap and how tree roots may compete for below-ground resources.

Researchers often characterise the below-ground ectomycorrhizal (ECM) fungal communities of forests by identifying the fungi forming mycorrhizal root-tips. Typically, it remains unknown which individual tree the sampled mycorrhizal root tip originates from, although experimental studies often assume that they belong to the nearest tree (e.g. Mosca *et al.*, 2007; Sthultz *et al.*, 2009). Microsatellite analysis of fine roots can allow each ECM root-tip to be directly linked with individual host trees (Lian *et al.*, 2006), offering several possibilities to researchers surveying ECM fungi. This technique may be particularly useful in ICP Forests' Level II plots, each of which contains several trees that undergo in-depth foliar chemistry, growth and health parameter analysis. The ability to link the ECM fungal community to one of these 'focal' trees would allow a direct link between the condition of the tree and the ECM fungal species associated with it. If microsatellite analysis reveals that tree roots occupy below-ground territories, then future studies may be able to confidently sample the roots of an individual tree by sampling a set distance away from the

trunk. Alternatively, if it is found that root tips can not be confidently assigned to an individual tree, then microsatellite markers would be needed to link mycorrhizas to focal trees in the future, and information on the identity and number of markers needed to genotype trees in Level II plots should prove useful.

Some tree species are known to show preferences for particular species of ECM fungi even when growing with other host species in mixed woodland (Ishida *et al.*, 2007; Morris *et al.*, 2008; Tedersoo *et al.*, 2008). There is some evidence that different genotypes of the same tree species may have preferences for particular ECM fungi or *vice versa* (Rosado *et al.*, 1994; Korkama *et al.*, 2006; Sthultz *et al.*, 2009; Leski *et al.*, 2010); such preferences could be the result of different secondary metabolites produced by particular hosts, as well as variable susceptibility to those metabolites by different fungal species (Saari *et al.*, 2005). To date, little work has been carried out to investigate this in the field. A study on five neighbouring *Pinus sylvestris* trees did not find evidence of non-random host-fungal associations, but limited sampling intensity of some trees meant it was not possible to rule out such a pattern (Saari *et al.*, 2005). Recently, *P. sylvestris* seedlings with differing geographic origins have been shown to harbour distinct ECM fungal communities when growing in a nursery (Leski *et al.*, 2010).

The distribution of fine roots and ECM fungi are intimately linked, but individual species of ECM fungi also face dispersal limitation and competitive interactions with other fungi, which probably contributes to the patchy distribution of ECM fungi observed in many previous studies (e.g. Lilleskov *et al.*, 2004; Peay *et al.*, 2010). Gaining an insight into the spatial scale of ECM fungal species turnover is useful for surveys of ECM fungi which aim to incorporate an optimal sampling design, and want to avoid spatially dependent samples.

In this study, molecular techniques were combined with a detailed sampling strategy to gain insight into the belowground distribution of mycorrhizas. Firstly, a spatially hierarchical sampling design was used to address the degree and size of fungal patchiness in three *P. sylvestris* level II plots. In addition, trees within two of the plots were genotyped using microsatellite analysis of foliar samples. Subsequent microsatellite analysis of roots allowed individual root-tips to be assigned to the genotyped trees, enabling the following questions to be addressed: 1) to what extent do the roots of adjacent trees overlap? 2) can the roots of an individual tree be confidently sampled by sampling near the trunk? and 3) do fungi exhibit non-random patterns of associations with individual trees?

METHODS

Study plots:

Three plots were included in this study; Thetford (East Anglia, England), Sherwood (Derbyshire, England) and Rannoch (Perthshire, Scotland). The three plots are part of the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests); a network of *ca.* 800 long-term intensively monitored plots (also known as “Level II” plots). Each forest plot is a managed, 0.3 ha, even-aged stand of mature *Pinus sylvestris* L. Further details about the Thetford and Rannoch plots can be found in Table 2.1 (Chapter Two). Briefly, Sherwood was planted in 1952, has a 30 year mean annual temperature of 8.85°C ($\pm$ 4.99 S.D), mean total precipitation of 860.9mm¹ yr, and lies at an altitude of 300m. The soil is a cambic podzol (Food and Agriculture Organisation classification), with a litter layer depth of 2.5cm. The plot lies approximately 53°24’50” by -01°45’20”.

Sampling of fine roots and identification of ECM fungi:

Sampling of Thetford and Rannoch was carried out as described in Chapter Two; Sherwood was sampled in an identical manner. Briefly, all three plots were sampled in the autumn of 2006. In each plot, 10 trees were randomly selected and a transect laid out to the nearest neighbouring tree. Average transect lengths were 2.63m ($\pm$ 0.81 S.D), 1.71m ($\pm$ 0.72 S.D) and 1.54m ($\pm$ 0.44 S.D) in Thetford, Sherwood and Rannoch respectively. Eight soil cores were removed from each transect (Fig 3.1) and four root-tips removed from each core, giving a total of 320 root-tips per plot for molecular analysis (see Chapter Two for details about the selection of root tips for sampling). Root-tips underwent CTAB DNA extraction prior to polymerase chain reaction (PCR) amplification using the primers ITS1F and ITS4, following the protocol of Gardes & Bruns (1993). Successful products were purified using ExoSAP-IT (USB, Cleveland, OH, USA), before cycle sequencing using BigDye v3.1 (Applied Biosystems Co., Foster City, CA, USA). Following EDTA/ethanol precipitation, sequences were analysed on an ABI PrismTM 3730 Genetic Analyser (Applied Biosystems) and edited with Sequencher (GeneCodes, Ann Arbor, MI, USA). Fungi were identified to species level where possible, through comparison of sequences via BLASTn matches on GenBank (<http://www.ncbi.nlm.nih.gov/blast>).

Tree genotyping:

Foliar samples were collected from all 20 trees in Thetford that were included in the 10 transects sampled, described above. In Sherwood, foliar material was obtained for 16 of the 20 trees (8 transects) included. Foliar material was collected for some trees in Rannoch; however, various problems encountered with samples from this plot (foliar material could not be gathered for some transects, loss of data from a sequencing plate, lack of sufficient DNA volumes for all three primer pairs for some samples) meant that this plot was excluded from microsatellite analysis. Although problems could undoubtedly be resolved with additional effort, it was felt that two plots would provide sufficient data to address the questions of interest.

Plant genomic DNA was extracted from buds using the CTAB protocol. Preliminary analysis of potential pine microsatellite primers (Soranzo *et al.*, 1998; Echt & Burns, 1999) identified three primer pairs (RPtest9, RPtest11, RPtest15) that could successfully distinguish between trees included in the survey, and consistently amplify sufficient product for unambiguous peak determination. The three primers were originally developed for *P. taeda*, but are known to successfully amplify microsatellite regions from *P. sylvestris*, as well as other *Pinus* species (Chagne *et al.*, 2004; Saari *et al.*, 2005). Forward primers were fluorescently end-labelled with FAM fluorophores. The PCR amplification was carried out using an aliquot of 0.5 µl of extracted DNA, combined with 5 µl of JumpStart™ ReadyMix (Sigma, St Louis, MO, USA) in a 10 µl reaction. Amplifications were performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 5 min. Amplification products were visualised on a 2% agarose gel to estimate optimal dilution of products (1:200 µl was generally sufficient). One µl of this diluted product was mixed with 10 µl Hi-Di™ (Applied Biosystems), and 0.16 µl ROX™ (Applied Biosystems), before being loaded on an ABI Prism™ 3100 in GeneScan® mode.

Host identification:

All root-tips from which the ECM fungus was successfully sequenced underwent microsatellite analysis. Amplification followed the protocol described for foliar samples, but the volume of extracted DNA used for PCR amplification was increased to 4 µl. The subsequent PCR products were of variable concentration, but were generally diluted 1:100 µl. Resultant chromatograms for root-tips were individually inspected and compared to chromatograms obtained for the two trees on the transect from which the root-tip originated.

Root-tips were then scored by eye as belonging to one of the two trees in the transect (A or B), or to another, unidentified tree (C).

Statistical analyses:

In order to calculate the proportion of roots identified as belonging to a tree at different distances within a transect, data were split into four distance classes. Class boundaries were chosen to allow approximately even replication of successfully identified root-tip numbers within each class. The percentage number of roots belonging to the proximal tree, the distal tree, or another, unidentified tree, were calculated as the number of roots belonging to each tree within the distance class, divided by the total number of roots in that distance class. This was calculated independently for Sherwood and Thetford plots, and for data from both plots combined.

Two approaches were taken to investigate whether tree genotype influences fungal communities. Firstly, at each site, a count was made of the number of times that roots from different trees occur in the same soil core but the different trees associate with unique fungi. If individual trees have a preference for different subsets of fungi, or *vice versa*, one would expect a high proportion of such occurrences. To test for significance, the count was compared to a distribution of 999 counts generated when fungal samples within a transect were randomly permuted. Significant departures from expected patterns would be apparent if the observed counts were higher or lower than 5% of the generated distributions. Secondly, to investigate whether some fungi were associated with particular trees more than would be expected by chance, for each fungus in a transect, a count was made of the number of root-tips that originated from each of the two trees. Significance was again tested by comparing observed counts to distributions of 999 counts generated when fungal samples within a transect were randomly permuted.

In order to address the scale at which fungal community samples (cores) become spatially independent, Mantel correlograms were calculated for each plot in the *vegan* package (Oksanen *et al.*, 2010) of the R statistical program (R Development Core Team, 2010). Mantel correlograms test the Bray-Curtis similarity of samples within a spatial distance class against the similarity of all samples within the plot. Autocorrelated samples are those that have a significant, positive Mantel r score. As the procedure involves multiple tests, P values were corrected using the global Bonferroni method. Distance boundaries were selected to ensure an even replication of comparisons within each distance class. Within

and between transect distances were included in the same analyses, but distance class boundaries were calculated separately as replication for between-transect comparisons was ten-fold higher.

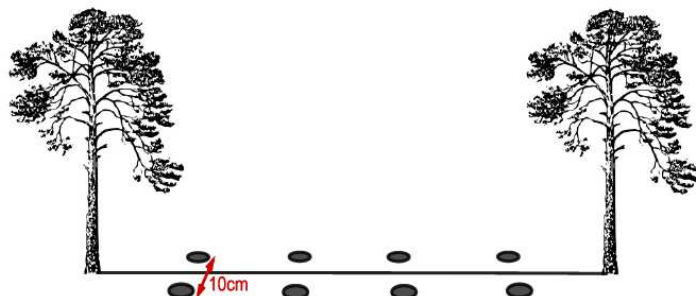


Figure 3.1

Sampling design of transects. Grey circles indicate soil core positions. Four pairs of cores were positioned evenly along the transect. Individual cores in a pair were located 10cm apart.

RESULTS

Of the 960 root tips collected for molecular analysis, 791 successful DNA sequences were generated, representing a sequencing success rate of 82% across the three plots. As described in Chapter Two, sequencing success rates for Thetford and Rannoch were 84% and 79% respectively. The corresponding success rate for Sherwood was also 84%, with 269 successful sequences.

Spatial structure of mycorrhizas:

Mantel correlograms indicate that as spatial scale between cores increases within each plot, the degree of fungal community similarity decreases (Fig 3.2). In Thetford and Sherwood, the ECM community of cores within-transects were significantly more similar to each other than to the community of cores found when fungi are permuted across the plot, but Mantel r tends to decrease with increasing distance. The same is true at Rannoch, although here the largest distance class within transects (0.64 to 1.52m) does not show significant autocorrelation. Between transects, there is no significant positive autocorrelation of ECM fungal communities at any of the plots, and significant negative correlation here appears to be the result of significant positive correlations within transects. The correlograms indicate

that spatial autocorrelation of ECM fungi is detectable at scales of 10 to 229cm in Thetford, 10 to 160cm in Sherwood, and 10 to 64cm in Rannoch.

Microsatellite analysis:

The three primer pairs employed in this study gave good amplification of DNA extracted from both foliar and root material, and all 36 trees from Thetford and Sherwood from which foliar samples were collected were successfully genotyped. Of the 499 root-tips included in the microsatellite analysis, 493 were genotyped, representing a 99% success rate. The three primer pairs could be used to successfully discriminate between the included trees; there were no instances where the pairs of trees in a transect could not be distinguished. Consistent with Saari *et al.* (2005), two primer pairs were generally sufficient to discriminate between trees, although a third primer pair was occasionally needed. Each primer amplifies multiple fragments but results were reproducible; repeat inclusion of samples in multiple runs was carried out to check reliability (data not shown).

Spatial structure of fine roots:

Microsatellite analysis of trees and root tips indicate a high degree of root overlap between neighbouring trees within a transect (Fig 3.3). Combined data for Thetford and Sherwood indicates that the proportion of roots originating from the proximal tree tend to decrease away from it (42% at 0.21- 0.52 m away, decreasing to 17% at 1.53 - 3.04 m away). Similarly, as distance from the proximal tree increases, the proportion of roots originating from the distal tree increases. A similar pattern of root distributions emerge when data sets from Thetford and Sherwood are considered separately, although there are some differences. In Sherwood, the highest proportion of fine roots derived from the proximal tree is at the second closest distance class (35% at 0.44 – 0.63 m away) whilst at Thetford, the highest proportion is found at the nearest distance class (52% at 0.3 – 0.74 m away). Similarly, in Thetford, the highest proportion of roots derived from the distal tree is at the second furthest distance class (48% at 1.2 – 1.68 m away), whilst at Sherwood the equivalent highest proportion is found at the furthest distance class (31% 1.04 – 3.08 m away). Roots originating from trees other than those included in the sampling (roots from trees outside of the transect) are common at all distance classes from the proximal tree, making up 42% of roots when both datasets are considered. Roots from these unsampled trees make up a higher proportion of all sampled roots at Sherwood (55%) in comparison with Thetford (32%).

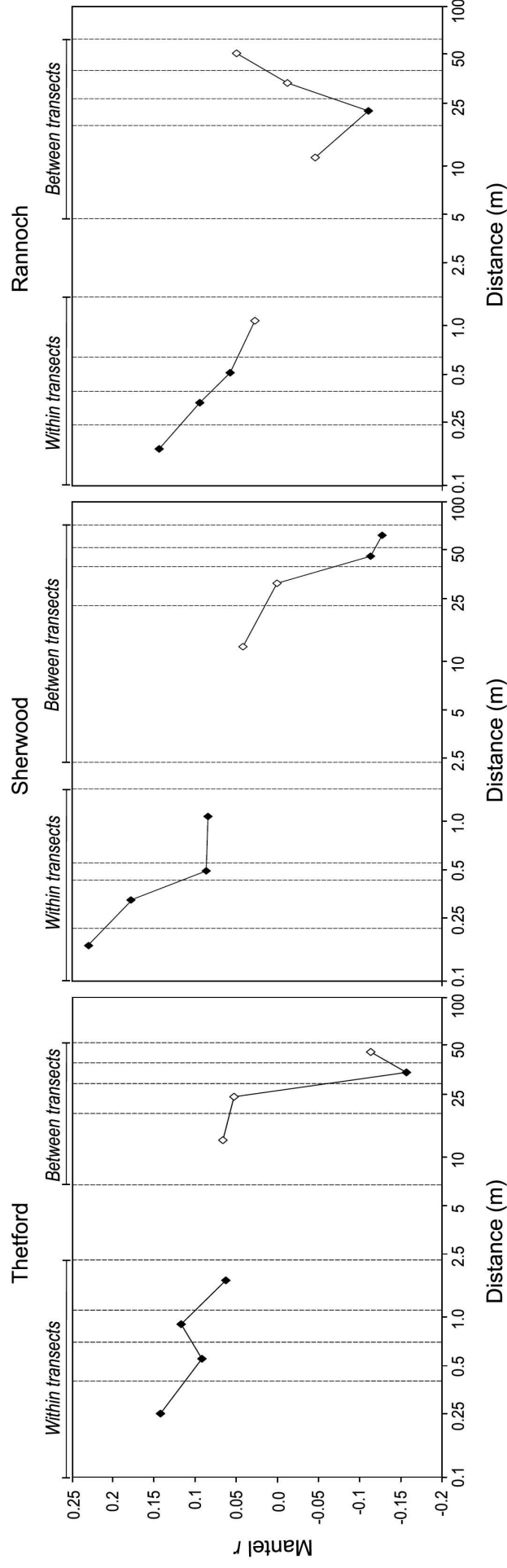


Figure 3.2

Mantel correlograms for the ectomycorrhizal communities at three plots, based on the Bray-Curtis dissimilarity coefficients. Significant ($P < 0.05$, Bonferroni-corrected) Mantel r values are indicated by a filled diamond. A significant, positive Mantel r value indicates significant spatial autocorrelation among community samples within the distance class boundaries indicated by the vertical dashed lines. Distance classes were designed to keep replicate numbers similar in all classes, but were calculated separately for within-transect distances and between-transect distances, as the number of comparisons in the latter group was 10 fold higher.

Testing for non-random host-fungal associations:

At Sherwood, out of 42 cores that contained the roots of multiple trees, there were eight occurrences where a particular tree was associated with a fungus different to the fungi of the other trees. At Thetford, there were 51 cores containing the roots of multiple trees, and 10 such occurrences. Neither of these observed counts differed significantly from distributions generated when the order of fungal samples within a transect was randomized.

Similarly, when each fungus within a transect was investigated, only two fungi, within two transects, were found to associate with an individual tree more or less than expected, compared to transects where the order of fungal samples was randomized. In Sherwood, *Russula ochroleuca* was found to occur on the roots of one of the two genotyped trees in a transect more than expected by chance ($P=0.001$). Similarly, in Thetford, *R. pectinatoides* was found on the roots of one of the two trees in a transect significantly more than expected by chance ($P=0.004$).

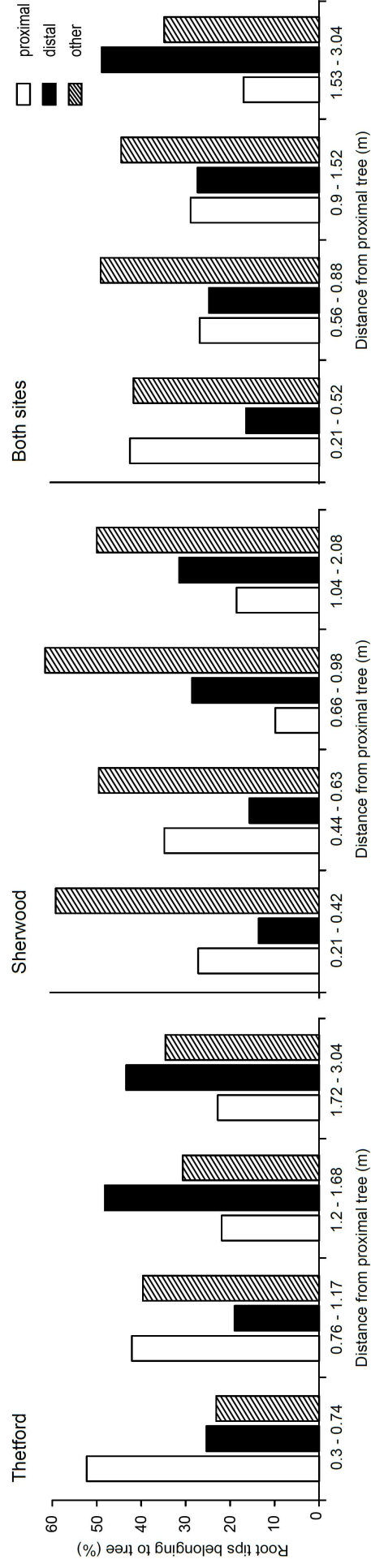


Figure 3.3

The percentage of roots belonging to proximal and distal trees as distance from the proximal tree increases, shown for Thetford, Sherwood and both plots combined. Distance classes were designed to keep replicate numbers similar in all classes.

DISCUSSION

In this study, the use of molecular techniques has provided insight into the below-ground distributions of fine roots and ectomycorrhizas. Microsatellite analysis of 36 trees in two plots allowed fine roots and ECM fungi to be linked to individual trees. This revealed that neighbouring trees have overlapping root systems and that when sampling an individual transect, the roots of multiple trees may be included in the sample. Whilst the fine roots of individual trees overlap, there is a trend of decreasing proportions of roots belonging to a tree with increasing distance. These findings are consistent with two other studies that have used microsatellite markers to assess the degree of root overlap between conspecific trees in stands of silver fir, *Abies alba* (Brunner *et al.*, 2004) and beech, *Fagus sylvatica* (Lang *et al.*, 2010). The current study indicates that *Pinus sylvestris* roots do not have below-ground territories, but is unable to rule out a vertical segregation of roots. By stratifying sampling by soil depth Brunner *et al.* were able to rule out vertical partitioning of beech roots; a similar sampling design is needed to test for this in *P. sylvestris*.

Root overlap between conspecific trees appears to be extensive in both coniferous and broadleaf systems, suggesting that sampling an individual tree's roots by proximity to that tree is not a feasible option. Although the number of roots belonging to a particular tree are greatest at near distance classes, the percentage of roots originating from the tree can be as little as 27% of roots just 0.21 to 0.42 m away. Instead, studies aiming to explore the fine roots or ECM communities of individual trees may consider employing microsatellite analysis of roots and trees, which was found to be a practicable option in this study. The protocol employed here could have been optimised further to allow multiplexing of PCR products in a single reaction tube for genetic analysis using different fluorophores; this would increase the speed and efficacy of the procedure and should be considered in future large-scale genotyping efforts. These findings should be particularly applicable to studies that seek to link ECM fungal communities to individual trees that have undergone experimental manipulation (e.g. Stultz *et al.*, 2009), to individual trees of different species (e.g. Morris *et al.*, 2008), or to trees of differing health status, as proposed for focal trees in Level II forest biomonitoring plots.

Linking ECM fungi to individual trees allowed an in-depth assessment of the potential role that individual, conspecific trees of the same age, may have in influencing ECM fungal community distributions in the field. There was no evidence to support the hypothesis that

individual host-trees were supporting differential subsets of fungi when this was assessed within individual cores. However, there were two instances where fungi were found on the roots of one of the two sampled trees more than expected by chance when this was assessed at the transect level. Closer inspection of these two transects revealed an unusually strong split between the roots of the two trees within each transect. The apparent pattern of non-random associations between trees and ECM fungi in these transects is therefore likely to be a result of overlap between fine roots and fungi, rather than a result of host-fungal preferences, supporting the findings of Lian *et al.* (2006), who found that a single fungal genet of *Tricholoma matsutake* was colonising the roots of up to seven individual trees, and consistent with the preliminary findings of Saari *et al.* (2005). This is in contrast to studies which have shown a potential influence of host genotype on ECM fungal assemblages (Rosado *et al.*, 1994; Korkama *et al.*, 2006; Sthultz *et al.*, 2009; Leski *et al.*, 2010). Many of these studies were not conducted in mature forest stands, where root mixing is high and common mycorrhizal networks are established, which may reduce any potential effects of genotypic influences on fungal assemblages. It is unknown how closely related the trees included in this study were; trees from populations spread over wide geographic areas may have a greater potential for ECM fungal community preferences than trees that originate from the same source (Leski *et al.*, 2010).

Spatial analysis of fungal distributions revealed a high degree of spatial autocorrelation in ECM fungal communities in all three plots, although this was restricted to samples located within small distances from one-another. Although it is not possible to determine the exact distance at which samples become spatially independent, due to the grouping of data into distance classes for Mantel correlogram analysis, it appears that across the three plots, samples >2.5m apart are not spatially autocorrelated. This result is surprisingly consistent with another study addressing the spatial scale of fungal patchiness across a range of forest types, which found that in 3 of 4 plots, autocorrelation of samples only occurred at distances <2.6m (Lilleskov *et al.*, 2004). In addition, other studies have found spatial autocorrelation of samples to occur at scales of <5m (Jonsson *et al.*, 2000; Izzo *et al.*, 2005; Tedersoo *et al.*, 2008; Peay *et al.*, 2010). This has implications for the design of optimal sampling strategies, and highlights the potential inefficiency of the sampling design employed in this study. In relatively species-poor plots such as those included in this study, the current sampling design is generally sufficient to capture most fungal species (see chapter 4), but in sites with as many as 100 ECM fungal types (e.g. Smith *et al.*, 2007), sampling with maximal efficiency becomes critical. Reducing the number of cores within transects (where

there is high positive autocorrelation), and increasing the number of transects (where there is no evidence of autocorrelation) should increase the chance of fully capturing species diversity, resulting in better characterised sites.

CONCLUSIONS

As the tree roots and fungi that form mycorrhizas grow below-ground, they are inherently difficult to track and identify. The application of PCR and DNA sequencing has revolutionised the field of mycorrhizal ecology, allowing rapid identification of fungal communities (for example, Chapter Two). This study has demonstrated how additional molecular techniques and analyses can provide further insight into below ground dynamics of the trees and fungi involved in ectomycorrhizal symbiosis. The extensive overlap of conspecific trees suggests a need to integrate the presence of fine roots originating from neighbouring plants in models of plants' below-ground zones of influence (Brunner *et al.*, 2004), and suggests that sampling roots of individual trees by proximity alone is not an option. Spatial turnover of ECM fungal species is high, but sampling within transects increases levels of autocorrelation between samples and should be reduced in future sampling designs, especially where ECM fungal diversity is expected to be high.

Chapter Four - Nitrogen availability is a primary determinant of conifer mycorrhizas across complex environmental gradients

Adapted from a paper published in Ecology Letters, 2010. Cox F., Barsoum N., Lilleskov E.A., Bidartondo M. 13:1103-1113. E. Lilleskov provided the root nitrogen data presented in this chapter.

ABSTRACT

Global environmental change has serious implications for functional biodiversity in temperate and boreal forests. Trees depend on mycorrhizal fungi for nutrient uptake, but predicted increases in nitrogen availability may alter fungal communities. To address a knowledge gap regarding the effects of nitrogen availability on mycorrhizal communities at large scales, the relationship between nitrogen and ectomycorrhizas was investigated in part of a European biomonitoring network of pine forest plots. The analyses indicate that increased nitrogen reduces fungal diversity and causes shifts in mycorrhizal community composition across plots, but there was no strong evidence that within-plot differences in nitrogen availability affect ectomycorrhizal communities. Exploratory analyses are also performed to determine the relative importance of other environmental variables in structuring mycorrhizal communities, and the potential use of indicator species to predict nitrogen-induced shifts in fungal communities is discussed.

INTRODUCTION

Ectomycorrhizal (ECM) fungi form symbioses with most temperate and boreal tree species, providing their hosts with soil nitrogen (N) and other nutrients, as well as increased resistance against pathogens and drought, in exchange for carbohydrates (Smith & Read, 2008). These symbioses predominate in forest ecosystems where N is a major limiting nutrient (Smith & Read, 2008). Nitrogen deposition from anthropogenic pollutants has increased dramatically from pre-industrialised rates in many parts of the world, and this has already had major ecological impacts, including altering nutrient cycles, leaching of base cations, acidification of soils, and alterations of plant and microbial communities (Vitousek *et al.*, 1997; Chung *et al.*, 2007; Galloway *et al.*, 2008). Even low-level N deposition can cause a significant loss of plant species diversity over several years (Clark & Tilman, 2008). Ectomycorrhizal fungal communities are known to respond to increased N availability at local scales (reviewed by Wallenda & Kottke, 1998). However, there is a growing awareness that the factors influencing patterns of biodiversity are strongly determined by scale, and that variables important at local scales may not necessarily be the primary drivers of diversity at larger spatial scales (Willis & Whittaker, 2002). While factors such as competition, disturbance and nutrients are likely to be important determinants of ECM fungal communities at local scales, factors such as climate and biogeographic constraints are hypothesised to become more significant at larger scales (Lilleskov & Parrent, 2007). The below-ground responses of ECM fungi to increased N availability at landscape, regional and continental scales are still unclear (Lilleskov & Parrent, 2007; Smith & Read, 2008).

Studies of above-ground fungal fruiting bodies have found evidence for a decrease in diversity and abundance of ECM reproductive structures over regional scale N availability gradients (Arnolds, 1991), but it has been demonstrated that extrapolating these trends to below-ground responses is inaccurate (Jonsson *et al.*, 2000; Peter *et al.*, 2001). Most studies that specifically investigate below-ground responses of ECM fungi to N availability have been conducted at local scales, often involving single stands, or sites within a few kilometres of one another (Table 4.1). Whilst this reduces the problem of complex gradients, which can make interpretation of results difficult, it does not address how below-ground ECM communities respond to N deposition at larger spatial scales. Local-scale studies have sometimes shown inconsistent findings (Table 4.1) and the different methodological approaches used make comparing these studies extremely difficult. For

example, the use of different morphological and molecular techniques for identifying fungi can lead to inconsistently defined communities (Lilleskov & Bruns, 2001). In addition, studies based on N fertilisation may differ from those based on long-term N deposition gradients due to the application of high levels of N over short periods of time and the absence of indirect effects such as acidification.

In this study, molecular techniques are used to assess the below-ground responses of ECM fungi to increased N availability over a geographic region of 300,000km², which lies somewhere between regional and continental scales, defined here as “intracontinental scale”. The hypotheses that increased N availability, assessed here through soil solution nitrate and plant tissue N concentrations, decreases ECM diversity and alters community composition both within and between forest plots is tested. The responses of individual ECM fungi to increased N availability across plots is explored to identify fungi that are indicators of high and low N availability. Finally, the relative importance of N in comparison to other environmental parameters hypothesised to drive ECM fungal communities at intracontinental scales is assessed. The results indicate that increased N reduces species richness and alters community composition between forest plots, but there is no strong evidence that N affects ECM communities within plots. Nitrogen availability appears to be a dominant variable influencing ECM community composition, and may be more significant than variables such as climate and soil type at the scale of this study.

Table 4.1

Reported effects of increasing nitrogen on below-ground ectomycorrhizal communities.

Diversity	Community composition	Study type	Identification	Geographic scale	Reference
no change	no change	short-term fertilisation	morphological	single stand	Brandrud & Timmermann 1998
no change	no change	short-term fertilisation	molecular	single stand	Jonsson <i>et al.</i> 2000
no change	altered	short-term fertilisation	molecular	single stand	Kårén & Nylund 1997
no change	altered	long-term fertilisation	morphological	single stand	Fransson <i>et al.</i> 2000
no change	altered	short-term fertilisation	molecular	single stand	Peter <i>et al.</i> 2001
decreased	altered	gradient	morphological	4+4 sites on ca. 2,000km transect	Taylor <i>et al.</i> 2000
decreased	altered	gradient	molecular	5 sites in 7km ²	Lilleskov <i>et al.</i> 2002
decreased	altered	long-term fertilisation	molecular	single stand	Avis <i>et al.</i> 2003
decreased	altered	gradient	morphological	3 sites on ca. 100km transect	Dighton <i>et al.</i> 2004
decreased	altered	long-term fertilisation	molecular	single stand	Edwards <i>et al.</i> 2004
decreased	altered	long-term fertilisation	molecular	single stand	Parrent <i>et al.</i> 2006
decreased	altered	long-term fertilisation	molecular	two stands, separated by 70km	Avis <i>et al.</i> 2008

METHODS

Plot descriptions:

Three plots in the UK and nine plots in Germany were included in this study (Fig. 4.1). All plots are part of the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests); a network of *ca.* 800 long-term intensively monitored plots (also known as “Level II” plots). The forest plots included in this study are all managed, 0.25-0.3 ha, even-aged stands of mature *Pinus sylvestris* L., subject to substantial differences in N deposition levels (Table 4.2). Data generated through ICP Forests monitoring activities on plot characteristics (mean temperature, precipitation, altitude, plot age, understory vegetation, throughfall N deposition), foliar chemistry (N, Ca^{2+} , Mg^{2+} , K^+ , P) and soil organic layer characteristics (pH, organic carbon concentration and C:N ratio) were used. Soil solution nitrate data were also available for 11 plots. Detailed methodology of data collection by ICP Forests is available online (<http://www.icp-forests.org>), where full information about the network, harmonisation and quality control of data collection are also available. Details on the data are included in Table 4.3.

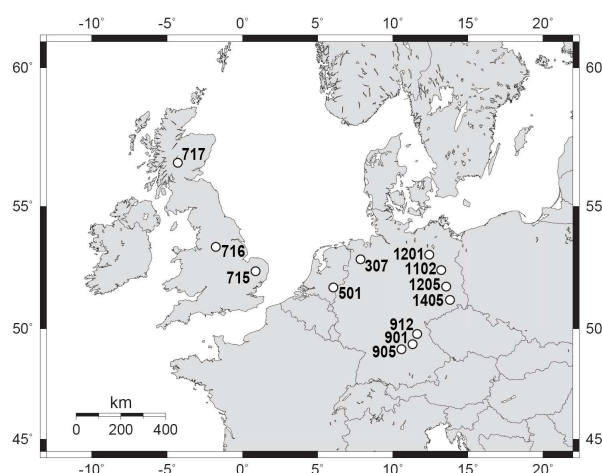


Figure 4.1

Locations of ICP Forest long-term monitoring plots included in the present study.

In addition to ICP Forests data, root N concentrations (% N content) were generated for each transect in a plot (see below). Mean un-weighted Ellenberg values were calculated for each plot as a measure of understory vegetation responses to N availability; each plant species has a value, ranging from one to nine, indicating the fertility conditions within

which they typically occur. Original Ellenberg values were used for German plots (Ellenberg *et al.*, 1992), whilst values calibrated for British vascular plants were assigned to plants in UK plots (Hill *et al.*, 1999).

Sampling of mycorrhizas:

Sampling of the three UK plots took place in autumn 2006, whilst the nine German plots were sampled in autumn 2008. Sampling of plots was carried out as described in Chapter Two. Briefly, in each plot, 10 trees were randomly selected and a transect laid out to the nearest neighbouring tree. The average transect length across plots was 2.53m, and ranged between 1.05m and 4.25m. Eight soil cores were removed from each transect, and four root-tips removed from each core, giving a total of 320 root-tips per plot for molecular analysis (see Chapter Two for details about the selection of root tips for sampling).

Sample processing and DNA sequencing:

A full description of downstream processing of samples is described in Chapter Two. Briefly, root-tips underwent DNA extraction with Extract-N-Amp (Sigma, Dorset, UK), prior to polymerase chain reaction (PCR) amplification using the primers ITS1F and ITS4. Successful products were purified using ExoSAP-IT (USB, Cleveland, OH, USA), before cycle sequencing using BigDye v3.1 (Applied Biosystems Co., Foster City, CA, USA). Following EDTA/ethanol precipitation, sequences were analysed on an ABI PrismTM 3730 Genetic Analyser (Applied Biosystems) and edited with Sequencher (GeneCodes, Ann Arbor, MI, USA). Fungi were identified to species level where possible, through comparison of sequences via BLASTn matches on GenBank (<http://www.ncbi.nlm.nih.gov/blast>).

Root N concentrations:

Roots not used for molecular analysis were freeze-dried, and non-ectomycorrhizal segments of 1-2mm in diameter were selected under a dissecting microscope, pooled according to transect, and ground to a fine powder in a Retsch MM301 mixer mill. Ground material was subsampled and analysed for carbon and nitrogen on a Fisons NA 1500 Elemental Analyzer.

Statistical analyses:

All analyses were carried out using R version 2.9.2. (R Development Core Team, 2010). Linear regression analyses was carried out to examine the relationship between soil solution

nitrate and plant tissue N concentration, and to predict soil nitrate levels in plot 501 where no soil solution data were available. Soil nitrate data were log-transformed prior to all analyses as values spanned several orders of magnitude. Relationships between N deposition levels, foliar and root N concentrations, soil nitrate, Ellenberg N and stand age were explored with Pearson's correlations, r , or when variables did not meet the normality assumptions of the test, the non-parametric Spearman's correlation coefficient, ρ .

To test whether N status influenced ECM richness, linear regressions were performed on the estimated richness of each plot against both plant tissue N concentrations and soil nitrate levels. As final sample size differed due to variable DNA sequencing success rates, total richness was estimated using the non-parametric Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992). The ACE values were log-transformed to linearise relationships with the predictor variables. Global Moran's I tests were carried out on the residuals of the three fitted models to assess whether residuals were autocorrelated and the assumptions of the test violated. As well as testing for intracontinental-scale effects, the above analysis was repeated to test for small-scale (within-plot) effects of root N concentration on the estimated richness of transects. Regressions were also carried out to test for between-plot effects of soil nitrate and foliar and root N concentrations on Pielou's estimate of community evenness (Pielou, 1966).

Partial Mantel tests, carried out in the *ecodist* package (Goslee & Urban, 2007), were used to test for a relationship between N availability and ECM fungal communities between plots, comparing a Bray-Curtis dissimilarity matrix of fungal community composition to Euclidean distance matrices of soil nitrate, foliar N concentrations and root N concentrations. To account for differences in sample size, the Bray-Curtis matrix was based on relative abundances of each fungus (the number of root tips occupied by each fungus in a plot, divided by the total number of samples from the plot). Because the 12 plots covered a large geographic area there is a strong possibility that, as a result of biogeographic patterns, plots located nearer to one another are more similar in ECM community composition than plots that are further away, i.e., spatially autocorrelated. In addition, stand age is thought to influence ECM communities (Termorshuizen, 1991; Visser, 1995). Therefore, variability caused by geographic distance and stand age were partialled out from the analysis using Euclidean distance matrices, so that the effects of N availability could be assessed independently. Mantel tests were performed to test for within-plot effects of root N concentration on the fungal community composition of transects. Distances between

transects were available for six plots, and here the effects of geographic distance were partialled out.

To identify the responses of different fungal taxa to N levels between plots, linear regressions were carried out between the log relative abundances of each fungus, and both soil nitrate and plant tissue N concentrations. As the possibility that a fungus was absent from a plot due to dispersal barriers rather than inappropriate habitat could not be excluded, only abundances from plots in which a particular fungus was found to occur at least once were taken into account. In addition, in order to provide greater statistical power and maximise the usefulness of potential indicators, this analysis was limited to only widespread fungi, i.e., those occurring in five or more plots. In order to consider non-linear relationships between ECM fungal taxa and N availability, Dufrêne-Legendre indicator species analysis (Dufrêne & Legendre, 1997) was carried out to detect taxa indicative of high or low N status. Here, analysis was again restricted to taxa occurring in five or more plots, but absence data was included. The above analyses were repeated for genus-level groups of ECM fungi.

To explore the relative importance of soil nitrate and plant tissue N concentration in structuring ECM fungal communities, unconstrained ordination with subsequent fitting of environmental variables was carried out. The function “envfit” from the Vegan library (Oksanen *et al.*, 2010) was used to identify variables that were significantly correlated with the community composition of ECM fungi. Eighteen variables relating to N availability, acidification, climate, soil type, stand age and geography were fitted as vectors or centroids (in the case of factors) to a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis community dissimilarities between plots. The significance of correlations was assessed by comparing the r^2 fit to r^2 values generated *via* 1,000 random permutations of the environmental variables. In order to assess the independent importance of matrices of related environmental variables with effects of other matrices removed, exploratory partial Mantel analyses were performed. This involved sequentially testing the importance of N availability (soil nitrate, foliar and root N concentrations), soil characteristics (soil-type, pH, organic carbon content), stand age, climate (mean temperature, total precipitation, altitude) and geographic distance (latitude, longitude) on ECM community composition once the effects of remaining matrices were partialled out from the analysis.

Table 4.2

Characteristics of the 12 study plots.

Plot	Country	Soil type	Planting year	Precipitation total (mm ⁻¹ yr)	Temperature mean (°C)	Altitude (m)	N deposition (kg ⁻¹ ha ⁻¹ yr)
307	Germany	Haplic podzol	1949-1969	768	8.76 ± 5.96	50	28.57 ± 2.51
501	Germany	Dystric cambisol	1934	776.7	9.48 ± 5.79	50	28.48 ± 2.89
1405	Germany	Cambric arenosol	1908	646.6	8.60 ± 7.14	200	18.04 ± 0.82
716	England	Cambric podzol	1952	860.9	8.85 ± 4.99	300	17.25 ± 5.12
901	Germany	Haplic podzol	1909-1929	715.3	7.92 ± 6.96	450	16.07 ± 2.57
905	Germany	Cambric arenosol	1889-1909	774.3	7.78 ± 6.86	500	14.80 ± 1.92
1102	Germany	Dystric cambisol	1951	598	8.51 ± 7.17	100	13.81 ± 2.49
715	England	Ferralic arenosol	1967	588.4	9.58 ± 5.38	50	12.49 ± 0.70
1201	Germany	Cambric arenosol	1927	584.4	8.33 ± 6.77	100	11.22 ± 2.83
1205	Germany	Dystric cambisol	1924	548.5	8.96 ± 7.19	100	10.64 ± 0.82
912	Germany	Haplic podzol	1929-1949	700.7	7.76 ± 8.86	450	8.37 ± 0.25
717	Scotland	Gleyic podzol	1965	2351.6	5.74 ± 6.04	500	4.56 ± 1.33

Standard deviations (±) are provided for the mean daily temperature across a 30 year period, and for mean annual throughfall nitrogen (N) deposition levels between 2001 and 2006.

Soil types are named according to Food and Agriculture Organisation classification.

Table 4.3

Environmental variables for the 12 study plots.

Data source	307 ± S.D	501 ± S.D	901 ± S.D	905 ± S.D	912 ± S.D	1102 ± S.D	1201 ± S.D	1205 ± S.D	1405 ± S.D	715 ± S.D	716 ± S.D	717 ± S.D
N foliar mg ⁻¹ g	20.44 ± 0.90	18.46 ± 1.86	15.35 ± 0.97	15.18 ± 1.60	15.45 ± 1.30	16.23 ± 0.33	14.78 ± 0.93	15.56 ± 1.31	16.62 ± 1.02	18.89 ± 0.67	18.28 ± 1.71	15.44 ± 0.95
CA foliar mg ⁻¹ g	2.61 ± 0.25	1.93 ± 0.29	2.54 ± 0.46	2.79 ± 0.86	2.72 ± 0.42	2.83 ± 0.51	2.33 ± 0.36	3.08 ± 0.69	2.73 ± 0.35	4.80 ± 1.46	2.16 ± 0.41	2.80 ± 0.63
MG foliar mg ⁻¹ g	0.84 ± 0.06	0.47 ± 0.13	0.78 ± 0.14	0.73 ± 0.29	1.06 ± 0.06	1.01 ± 0.15	0.88 ± 0.14	0.78 ± 0.07	0.86 ± 0.08	0.9 ± 0.95	0.67 ± 0.11	0.9 ± 0.7
K foliar mg ⁻¹ g	5.89 ± 0.29	5.37 ± 0.63	5.62 ± 0.54	5.75 ± 0.60	5.57 ± 1.28	5.37 ± 0.69	5.48 ± 0.43	5.56 ± 0.55	5.24 ± 0.26	5.59 ± 0.83	8.14 ± 1.00	6.41 ± 0.51
P foliar mg ⁻¹ g	1.67 ± 0.08	1.59 ± 0.14	1.40 ± 0.08	1.35 ± 0.08	1.61 ± 0.11	1.53 ± 0.09	1.49 ± 0.12	1.49 ± 0.06	1.35 ± 0.06	1.60 ± 0.20	1.59 ± 0.16	1.70 ± 0.14
Organic soil pH	3.1	3.1	2.3	2.8	2.8	3.5	2.8	3.2	3.2	4.1	3.8	4.4
Organic C conc g ⁻¹ kg	35.4	34.9	50.0	46.8	44.9	31.1	34.9	35.7	38.0	54.0	46.7	52.9
C:N ratio	23.9	25.2	32.3	34.4	30.3	22.1	26.2	27.3	23.8	55.3	23.6	32.7
Ellenberg N	5.41	4.00	2.00	2.67	2.40	4.55	4.00	4.69	3.71	5.62	3.76	3.13
Root N (%)	1.39 ± 0.18	1.36 ± 0.12	0.74 ± 0.09	0.73 ± 0.10	0.90 ± 0.08	1.34 ± 0.14	0.96 ± 0.14	1.02 ± 0.18	0.87 ± 0.10	1.38 ± 0.13	1.39 ± 0.14	1.23 ± 0.18
Soil solution NO₃-N	12.01 ± 5.05	NA	0.05 ± 0.05	0.02 ± 0.02	0.01 ± 0.01	0.15 ± 0.20	0.058 ± 0.04	0.14 ± 0.34	0.06 ± 0.00	18.29 ± 12.88	3.36 ± 3.73	0.02 ± 0.02

Standard deviations (±) are provided where this data was available.

Nitrogen deposition values are shown as means across the years 2001 to 2006.

Values relating to foliar chemistry are means across the years 1995-2005 for the nine German plots, and across 1995-2003 for the three UK plots.

Soil pH, organic C and C:N ratio are mean values from 1995 for all German plots except 901 and 912, which are from 1994; values for UK plots are from 1996.

Ellenberg N values were calculated from vegetation data gathered in 2006 in all German plots, except 905 and 912 that are from 2005, and 501 from 1998; vegetation data from the UK plots was from 2004.

Root N values were generated from roots collected in 2006 and 2008.

Soil solution NO₃-N were collected in various years between 1996 and 2006 at 10cm depths, except for plots 901, 912 and 1102, which were collected at 50cm. No soil solution NO₃-N data were available for plot 501.

Details on sample size for variables provided by ICP Forests can be found at <http://www.icp-forests.org>.

RESULTS

Mycorrhizal community structure:

A total of 3,840 roots were analysed, generating 3,114 successful DNA sequences. Of these, 48 sequenced roots (*ca.* 1.5%) yielded non-ECM fungi, and were excluded from further analyses; sequences were designated non-ECM when they matched known root endophytes or free-living soil fungi. The remaining 3,066 sequences represented an average success rate of 80% across all plots, although success rate varied from 69% at plot 1205 to 84% at both plots 716 and 715. Average ECM richness within plots was 24, but actual values varied considerably between 15 fungi at 716, to 34 at 1102. Fungal accumulation curves begin to reach an asymptote at most plots, indicating that sampling was generally of sufficient intensity to capture ECM diversity (Fig. 4.2). The ACE estimates of total richness predicted that the percentage of fungi sampled from the total pool ranged from 73% at plot 901, to 100 % at plots 501 and 716. Pielou's indices and rank abundance curves (data not shown) indicate a similar pattern of evenness across plots, with one or two fungi dominating, and many rare fungi. The most dominant in terms of sample number was *Russula ochroleuca*, representing 7.7% of all samples. Two fungi that form inconspicuous hypogeous fruiting bodies, *Piloderma* sp. 1 and *Elaphomyces granulatus* were the second and third most dominant, representing 6.8 and 6.3% of all samples respectively (see Appendix A for relative abundances of each fungus).

Relationships between soil and plant -based measures of N:

Regression analysis showed soil nitrate was positively related to both foliar and root N concentrations ($r^2=0.86$, $P<0.001$ and $r^2=0.60$, $P=0.005$ respectively). The two regressions predicted soil nitrate values for plot 501 of 3.6 and 6.3 mg/l respectively. As the regression between foliar N concentration and soil nitrate yielded the highest r^2 value, results are presented from analyses in which only the former value is employed; however, results are similar when the alternative predicted value is used.

Relationship between N deposition and N availability:

Nitrogen deposition was positively correlated with foliar N concentration ($r=0.72$, $P=0.008$) and soil nitrate ($r=0.64$, $P=0.026$), but not significantly correlated with fine root N concentration. Soil nitrate and foliar N concentration were not significantly related to stand age, but fine-root N concentration was negatively correlated with stand age ($\rho=-0.82$, $P=0.001$). A partial correlation between N deposition levels and root N concentration, in

which the effects of stand age are removed, found a strong correlation ($\rho=0.70$, $P=0.003$). Ellenberg N values for vascular understory plant communities were positively related to soil nitrate ($r=0.76$, $P=0.004$), foliar N concentration ($r=0.67$, $P=0.018$), and fine root N concentration ($\rho=0.71$, $P=0.009$).

Effect of N on mycorrhizal richness:

Across plots, there was a significant negative relationship between the total fungal richness estimate (ACE) and soil nitrate and plant tissue N concentrations (Fig. 4.3). Moran's I tests for autocorrelation in the residuals of fitted models were not significant, indicating that the assumption of independence of the residuals was met. There were no significant relationships between Pielou's evenness and soil nitrate, foliar or root N concentrations. Within plots, linear regression of ACE values for transects against root N concentration showed significant patterns of increasing estimated fungal richness with increasing root N concentrations in plot 717 ($r^2=0.72$, $P=0.016$). There were no significant relationships in the other 11 plots (Appendix B).

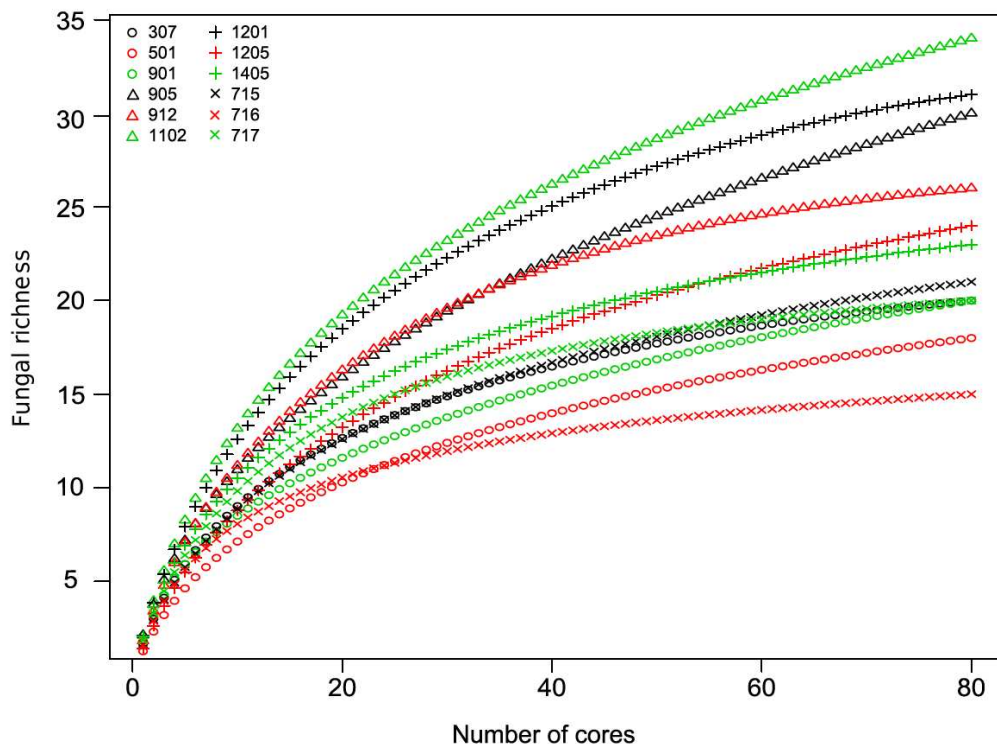


Figure 4.2

Species accumulation curves for 12 study plots, showing the accumulation of species as soil cores are randomly re-sampled. Standard deviations are not shown for ease of presentation.

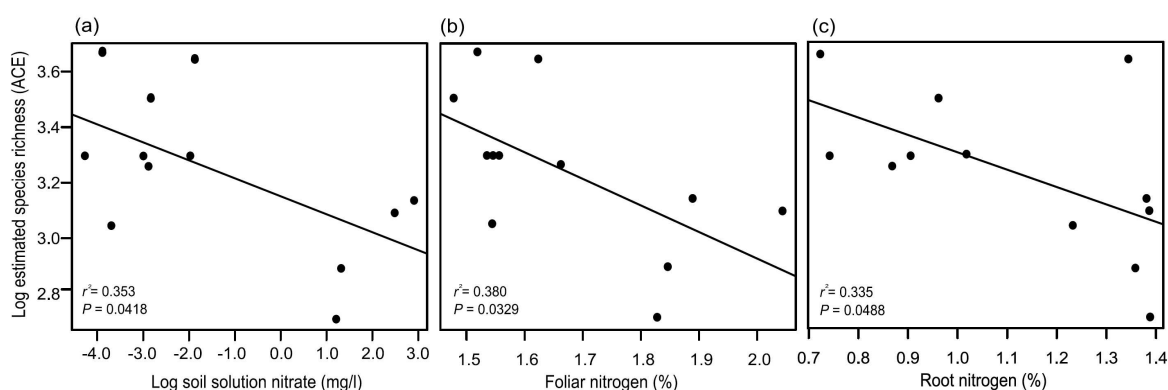


Figure 4.3

Regression analysis of the Abundance-based Coverage Estimates (ACE) of total fungal richness, against (a) log soil solution nitrate (mg/l), (b) foliar nitrogen (%), and (c) root nitrogen (%).

Effect of N on mycorrhizal communities and individual fungal taxa:

Across plots, partial Mantel tests found Euclidean distances of soil nitrate (Mantel $r=0.51$, $P=0.005$), root N concentration (Mantel $r=0.45$, $P=0.002$) and foliar N concentration (Mantel $r=0.44$, $P=0.004$) to be significantly correlated with Bray-Curtis community dissimilarity of ECM fungal communities when effects of stand age and geography were removed. Within plots, Mantel and partial Mantel tests (where spatial data were available) revealed no significant correlation between Bray-Curtis community dissimilarities and differences in root N concentration between transects (Appendix C).

The majority of fungal taxa were not individually tested as potential indicators because they occurred in fewer than five plots. Of the 35 included species/genera, 11 show significant responses to increases in N availability (Tables 4.4 and 4.5). *Russula ochroleuca*, *Thelephora terrestris*, *Pezizales* sp. 3, *Amanita*, *Thelephora/Tomentella* and *Lactarius* all responded positively to increasing N, whilst *Pseudotomentella tristis*, *Cantharellaceae* sp. 1, *Cenococcum* 3, *Piloderma* sp. 3 and *Piloderma* responded negatively to increasing N. *Russula ochroleuca*, *Piloderma*, and *Thelephora/Tomentella* displayed statistically significant responses in both linear regression and Dufrêne-Legendre indicator species analyses.

Table 4.4

Responses of mycorrhizal fungal abundance (log % abundances) to increasing nitrogen (N) availability.

Response	Taxa	Log soil solution nitrate		N roots		N foliar			
		r ²	P	r ²	P	r ²	P	d.f	
Positive	Species	<i>Russula ochroleuca</i>	0.891	0.016	0.979	0.0013	0.731	0.065	3
		<i>Thelephora terrestris</i>	0.481	0.038	0.131	0.339	0.462	0.044	7
		Pezizales sp. 3	0.116	0.307	0.424	0.0299	0.215	0.151	3
	Genus	<i>Amanita</i>	0.216	0.207	0.46	0.045	0.172	0.268	7
		<i>Thelephora/Tomentella</i>	0.646	0.003	0.402	0.036	0.368	0.048	9
		<i>Lactarius</i>	0.356	0.053	0.581	0.0064	0.251	0.117	9
negative	Species	<i>Pseudotomentella tristis</i>	0.168	0.493	0.0303	0.779	0.907	0.013	3
	Genus	<i>Piloderma</i>	0.52	0.044	0.628	0.019	0.225	0.235	6

Only taxa present in five or more plots were assessed as potential indicators.

Only taxa which display a significant relationship with N availability are shown. Values in bold are significant ($P < 0.05$).

Relative importance of N compared to other environmental variables:

Of the 18 variables fitted as vectors to the NMDS ordination of plot community composition, nine were significantly correlated (Fig. 4.4). Root N concentration was the variable most closely aligned with the primary axis, which corresponds with the highest variation between plots. In addition to the five included N availability variables, soil pH, stand age, mean annual temperature and altitude were also significantly related to ECM community composition (Fig. 4.4).

Partial Mantel tests, in which the independent effects of matrices of related variables were sequentially tested when effects of all other matrices were removed, indicated that N availability, stand age and geography were significantly related to ECM community composition (Table 4.6). Of these, N availability achieved the highest Mantel r score, and stand age the lowest. Climate and soil characteristics were not significantly related to ECM composition once the effects of other variables were removed. Effects of stand age appeared largely confounded with root N concentration; partial Mantel tests, where effects of age were removed, indicated a significant correlation between ECM fungal community dissimilarity and root N concentration (Mantel $r = 0.42$, $P = 0.003$), whilst the reverse (effects of stand age when root N concentration was removed) was non-significant.

Table 4.5

Fungi found to be significant indicators of high/low nitrogen (N) availability measures by Dufrêne-Legendre indicator analysis.

			Foliar N		Root N		Soil solution nitrate (log)	
Taxa			Indicator value	P	Indicator value	P	Indicator value	P
Indicator of high N	Species	Pezizales sp. 3	0.612	0.28	0.763	0.003	0.612	0.27
		<i>Russula ochroleuca</i>	0.707	0.025	0.833	0.012	0.707	0.021
	Genus	<i>Thelephora/tomentella</i>	0.719	0.05	0.687	0.102	0.719	0.048
Indicator of low N	Species	Cantharellaceae sp.1	0.853	0.028	0.74	0.07	0.853	0.033
		<i>Cenococcum</i> 3	0.75	0.048	0.807	0.02	0.75	0.045
		<i>Piloderma</i> sp. 3	0.593	0.204	0.812	0.034	0.593	0.201
	Genus	<i>Piloderma</i>	0.859	0.032	0.977	0.006	0.859	0.023

Only taxa present in five or more plots were assessed as potential indicators.

Only taxa which display a significant relationship with N availability are shown.

Values in bold are significant ($P < 0.05$).

Table 4.6

Partial Mantel test results, where each test relates to the independent effect of individual matrices of environmental variables on ECM community composition, when the effects of other included matrices are removed.

Matrix	Component variables	Mantel <i>r</i>	<i>P</i>	Matrices partialled out
N availability	foliar N/root N/soil solution nitrate	0.408	0.006	Soil, Stand age, Climate, Geography
Soil	soil type/pH/organic C content	0.116	0.172	N availability, Stand age, Climate, Geography
Stand age	stand age	0.275	0.033	N availability, Soil, Climate, Geography
Climate	temperature/precipitation/altitude	-0.017	0.478	N availability, Soil, Stand age, Geography
Geography	latitude/longitude	0.330	0.015	N availability, Soil, Stand age, Climate

Values in bold are significant ($P < 0.05$)

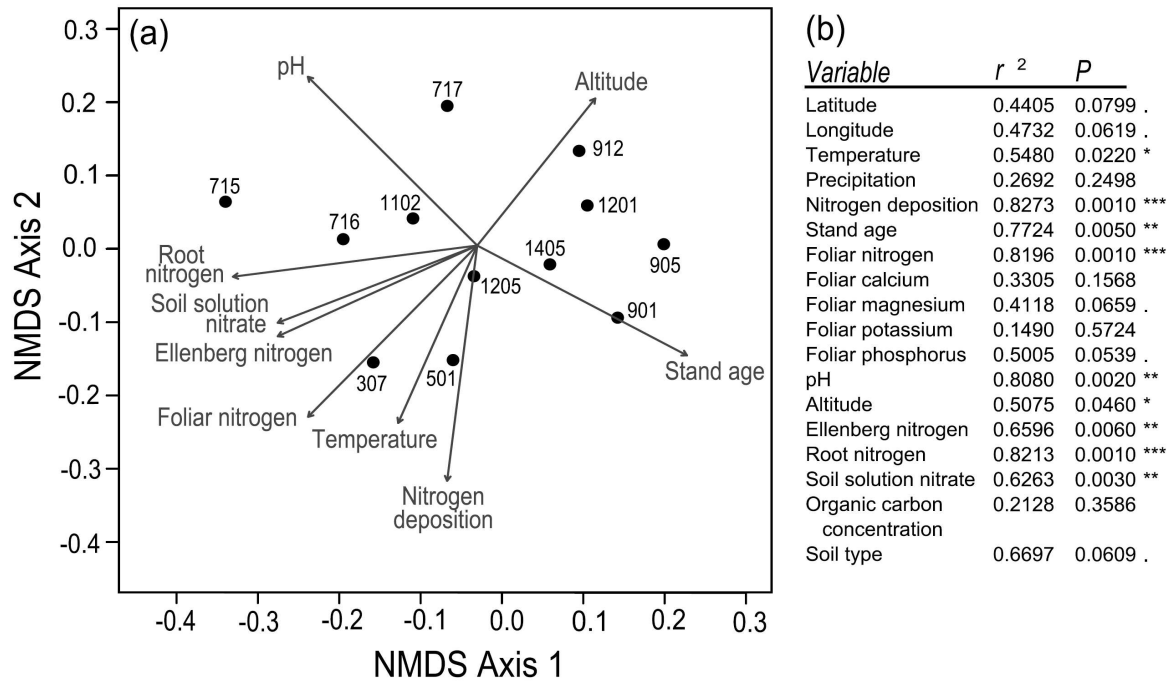


Figure 4.4

(a) Fungal community compositions of the 12 plots are displayed using non-metric multidimensional scaling (NMDS). Multiple random starts were performed to avoid local minima and the percentage stress of the ordination is 14.9. The ordination is rotated so that the greatest variation between plots lies on the x-axis (Oksanen *et al.*, 2010). Eighteen environmental variables were tested for significant correlation with community dissimilarities (b), and significantly correlated variables are shown in the plot as arrows. The length of the arrow is proportional to the strength of the correlation with the ordination, and vectors indicate the direction of the greatest change. (· : $0.1 > P > 0.05$; * : $0.05 > P > 0.01$; ** : $0.01 > P > 0.001$; *** : $0.001 > P$)

DISCUSSION

This study has confronted the challenge of assessing impacts of N availability on mycorrhizas at intracontinental scales. The negative effects of increased N on fungal diversity (Fig. 4.3), and its impacts on community composition across plots (Fig. 4.4), are in agreement with other studies of below-ground ECM fungal communities (Taylor *et al.*, 2000; Lilleskov *et al.*, 2002a; Avis *et al.*, 2003), but this study provides molecular-based evidence that N is a major factor influencing ECM communities across complex environmental gradients. These effects could have significant functional consequences at the ecosystem level; different species of ECM fungi can play important roles in acquiring N and P from distinct sources (Wallander *et al.*, 1997; Lilleskov *et al.*, 2002b). In addition, these transitions can have implications for below-ground food webs and processes, and they could alter carbon cycling and sequestration in forest soils - processes in which ECM fungi play a dominant role (Högberg *et al.*, 2001; Godbold *et al.*, 2006). Conversely, functional redundancy among ECM fungal species may buffer against significant functional changes; thus, testing for functional consequences of N-induced mycorrhizal shifts should be a priority for future research.

Only plot 717 showed a significant within-plot effect of root N concentration, but with an opposite trend to that observed across plots. This Scottish plot is noteworthy for having the lowest N deposition, greatest precipitation, lowest mean temperature and highest altitude (Table 4.1); this suggests that prior to a threshold level of background N availability being reached, small increases in N could increase the suite of fungi that can persist in a local area. Within the other 11 plots, there was no evidence that differences in N availability affect ECM communities, a result in agreement with previous N fertilisation studies in oak forests (Avis *et al.*, 2003; Avis *et al.*, 2008) which found that increased N only affected ECM communities at scales $>500\text{m}^2$. Within-plot N gradients were roughly two-fold smaller than between-plot gradients, and they may not have been strong enough to influence ECM communities at within-plot scales where processes such as competition may be more significant (Koide *et al.*, 2005). However, it should be noted that the inability to detect a correlation does not discount the possibility that within-plot N gradients affect ECM communities; only that there was no evidence for a relation at the within-plot scale examined in this study. As well as the scale of sampling, reduced sample size and plant-based measurement of N within plots could influence the ability to detect fine-scale effects of N, and the presence of a relationship at one plot out of 12 could be due to chance alone.

Replicated experimental studies should now be used to confirm whether N availability is a less important determinant of mycorrhizal diversity at within-plot scales.

No single fungus was detected in all 12 plots, indicative of the high spatial variability of ECM fungal communities. Over a broad geographic area, this makes the task of identifying indicator species difficult, as absence from a plot could be a result of unsuitable habitat, or dispersal limitation. Nevertheless, a number of taxa show significant responses to differences in N availability. *Thelephora/Tomentella* spp., *Lactarius* spp. and *Piloderma* spp. display analogous responses to increasing N at both local (Lilleskov *et al.*, 2002a; Lilleskov *et al.*, 2008) and intracontinental scales (Tables 4.4 and 4.5), highlighting them as strong candidates for use as indicator species. In contrast, although *Russula* and *Cortinarius* have previously been suggested as nitrophobic genera (Brandrud, 1995; Peter *et al.*, 2001; Lilleskov *et al.*, 2002a), the results from this study do not support this assertion. Whereas some *Russula* species, such as *R. paludosa*, appeared to decline in abundance with increasing N levels (Appendix A), *R. ochroleuca* increased in abundance, consistent with previous reports of N tolerance of its sporocarp production (Arnolds, 1991) and in a similar manner to *R. amoenolens* in a fertilisation experiment (Avis *et al.*, 2003). *Cortinarius* showed no significant relationship with N availability metrics, but had low abundances across all plots, possibly reflecting the generally high levels of N availability. *Cortinarius olivaceofuscus* was most abundant at high N plot 715, but this plot is base rich, and this species is known to be calciphilous (Hansen & Knutsen, 1992); *C. olivaceofuscus* may therefore be less sensitive to elevated N, or be buffered against the acidification effects of N deposition. Responses can clearly vary between species of the same genus, highlighting the need to examine species-specific responses to elevated N. Among the more common fungi that were tested, roughly equal numbers responded positively or negatively to increased N availability (Tables 4.4 and 4.5). The overall pattern of reduced fungal diversity (Fig. 4.3) therefore suggests that rarer taxa may be impacted most heavily by increased N, in agreement with a fertilisation study of oak forest mycorrhizas (Avis *et al.*, 2008).

Unusually for surveys of below-ground fungal diversity, accumulation curves of ECM fungi were beginning to plateau at the majority of the study plots (Fig. 4.2), suggesting that these pine plots are relatively species-poor, especially when compared to deciduous woodlands where upwards of 100 ECM fungi have been recorded (Richard *et al.*, 2005; Avis *et al.*, 2008). Different forest types may respond differently to increased N availability, but without additional large-scale surveys of deciduous forests, the effect of forest type on

fungal responses remain unknown. The low richness of the plots included in this study could reflect suppression of the fungal community because of cumulative N deposition effects. Alternatively, employing direct sequencing without pooling or morphotyping roots, may have avoided some of the analytical uncertainties currently inherent within other molecular methods, which could lead to over-estimation of species richness (Avis *et al.*, 2008; Buée *et al.*, 2009). High-throughput sequencing is likely to play an important part in future surveys of ECM fungi, but validation studies are needed (Avis *et al.*, 2010); the presence of abundant sclerotia or dominant unknown ECM fungi, as was the case in this study, can strongly bias community descriptions from pooled samples. Whilst roots were collected from the entire soil core and sampling was not stratified by soil horizon, future studies should also consider sampling roots within individual horizons because different ECM fungi can be distributed in different horizons (Lindahl *et al.*, 2007), and depths of horizons can vary across plots.

Ellenberg N values indicate that predictable shifts in understory plant species are occurring as a result of increased N, and that this is mirrored by below-ground changes in ECM fungal communities (Fig. 4.4); this could represent a useful warning tool for site managers. It is unlikely that shifts in understory plant communities directly cause the observed differences in ECM fungal communities, as the sampled host plant was uniform across study plots, and the majority of understory plants associate with distinct arbuscular or ericoid mycorrhizal fungi. However, it is not possible to rule out potential indirect effects of changes in competitive interactions between ECM and arbuscular or ericoid mycorrhizal fungi, which could hypothetically be altered as N availability or plant communities shift.

We utilised three measures of N availability, and they all showed the same relationship with fungal communities at intracontinental scales. Soil solution nitrate data was available for only 11 of the 12 plots, and was measured at inconsistent depths across plots (Table 4.3), which could influence results. Foliar and root N concentrations were collected consistently; however, plant-based measures could hypothetically be influenced by differential N uptake of particular ECM fungi associated with each root system, possibly giving rise to a circular relationship between plant N concentrations and ECM communities. Nevertheless, the consistent pattern displayed by both plant and soil measures of N availability supports the finding that changes in N impact ECM fungal communities at intracontinental scales.

Given more time, it would be informative to assess the impacts of ammonium, as well as nitrate, on ECM fungal communities. In most ecosystems, ammonia is converted into nitrate by bacteria in a process called nitrification. However, nitrification processes are likely to only occur at low rates in the acidic conditions prevailing in most coniferous forests, so that ammonia is likely to be the predominant mineral N ion (Keeney 1980). Furthermore, up to half of the anthropogenically derived N deposited onto the study plots consisted of ammonium (Fischer *et al.*, 2010). Given that ammonia is the preferred mineral N source for many ECM fungi (France & Reid 1984; Finlay *et al.*, 1992; Sawyer *et al.*, 2003), it would be useful to test how fungi and fungal communities respond to changes in ammonia as well as nitrate levels.

Variables other than N availability are also undoubtedly important determinants of fungal communities (Fig. 4.4). There was evidence that stand age was influencing ECM communities across plots (Table 4.6), but its effects appeared largely confounded with root N concentration - previous studies have shown that N availability can decrease as stands age (Prescott, 1997; Bradley *et al.*, 2002) and this appeared to be the case here. It is often difficult to disentangle the relative importance of direct eutrophication effects of N deposition, and indirect effects such as acidification and leaching of base cations (Lilleskov *et al.*, 2002a). Soil pH was identified as a potentially important variable (Fig. 4.4), but neither pH nor foliar Ca^{2+} levels have the expected negative correlation with N availability (Table 4.3). This is probably due to the inclusion of a variety of soil types, which can vary in buffering capacity (Aber *et al.*, 1998), leading us to conclude that acidification effects of N were unlikely to be responsible for the observed relationship between N and ECM fungal communities. Nitrogen could also indirectly affect fungal communities *via* an influence on stand productivity, which is also likely to be influenced by stand age and geographic variables. Thus, the potential effects of stand productivity warrant further study.

Climate has been hypothesised as a potentially important variable structuring ECM communities across broad spatial scales (Lilleskov & Parrent, 2007), and could alter edaphic factors or carbon supply from the host (Clemmensen *et al.*, 2006; Craine *et al.*, 2009), or differentially affect respiration rates of different ECM fungi (Malcolm *et al.*, 2008). To date, limited research has been conducted on the effects of climate on ECM fungal communities (but see Gange *et al.*, 2007). There was some evidence that temperature and altitude were related to ECM community composition (Fig. 4.4), although the independent effect of climate was non-significant (Table 4.6). This could mean that the

correlation with other removed variables masked real climatic effects, or that combining groups of related variables into a single predictive climate matrix masks the effects of individual variables.

CONCLUSIONS

These findings indicate that increased N is altering ECM fungal communities at intracontinental scales, and suggest that changes in N could be altering large-scale distribution patterns of individual species. Studies to uncover baseline distributions of ECM fungi before patterns are radically altered are urgently required, and the vast network of ICP Forest plots would provide an excellent platform for this (Chapter One). This would allow future changes in ECM fungal communities to be measured and predicted, and inform ecologically relevant manipulative experiments designed to gain a mechanistic understanding of the relationship between N and changes in ECM fungal communities, and the consequences of these changes to forest ecosystem vitality.

Chapter Five - Evidence for a role of competition structuring ectomycorrhizal fungal communities over a nitrogen availability gradient

ABSTRACT

Competitive interactions and habitat filtering are important processes thought to structure many plant, animal and microbial communities. However, the role that these two forces play in determining ectomycorrhizal fungal communities is not known. Here, the interaction between environmental gradients, interspecific competition and ECM fungal relatedness is assessed at three spatial scales across 12 ECM fungal communities. The results provide evidence to suggest that competitive interactions may be important in structuring ECM fungal communities within soil cores and tree-tree transects, but not across plots. In addition, analysis of the extent of community segregation along an N availability gradient indicates that at the scale of cores, ECM fungal communities display increasing segregation as N availability increases. This pattern is not evident at the scale of transects. Finally, the analyses do not find evidence that closely related fungi are more, or less, likely to co-occur within plots, suggesting that important functional traits may not always be conserved within fungal lineages. Similarly, closely related species do not appear to always share preferences to environmental conditions across plots. This study provides an insight into the complex and scale dependent interplay between environment and competition, which may simultaneously act to drive the community structure of ECM fungi.

INTRODUCTION

The structure and composition of diverse communities of ectomycorrhizal (ECM) fungi are known to be influenced by environmental conditions (Chapter Four; Cudlin *et al.*, 2007), host plant species (Ishida *et al.*, 2007; Morris *et al.*, 2008; Tedersoo *et al.*, 2008) and geography (Peay *et al.*, 2007), but there is also evidence that interactions between individual ECM fungi can affect community assembly (Kennedy, 2010). For example, microcosm studies have shown that interspecific competition can play a role in determining the extent of colonization or biomass of different ECM fungi (Wu *et al.*, 1999; Landeweert *et al.*, 2003; Kennedy & Bruns, 2005).

Experimental field studies to address the role that competitive interactions play in structuring ECM fungal communities are lacking as a result of the inherent difficulty associated with the study of below-ground communities. However, inferences based on fine-scale ECM fungal distributions and co-occurrence patterns suggest that competition has the potential to influence the distributions of ECM fungi (Dahlberg *et al.*, 1997; Agerer *et al.*, 2002; Koide *et al.*, 2005; Pickles *et al.*, 2010). Most studies have aimed to assess the degree to which particular fungal taxa co-occur, i.e., when particular fungal pairs are found to rarely or never co-occur this is thought to indicate competitive interactions between such fungi. Although such co-occurrence patterns can also be generated through alternative ecological and evolutionary processes, such as dispersal limitation or habitat preferences (Gotelli & McCabe, 2002), assessment of co-occurrence patterns can at least give an indication of the potential role and ecological relevance of competition in complex communities. This work can provide data to inform subsequent experimental studies aimed at confirming the presence of competition between identified fungal taxa (Kennedy, 2010).

The outcome of interspecific interactions can be largely dependent on the particular environmental conditions to which fungi are exposed (Erland & Finlay, 1992; Mahmood, 2003). Interactions between species are hypothesised to change over environmental gradients, with decreasing competition and greater facilitation as environmental stress increases (Callaway & Walker, 1997). Nitrogen (N) availability gradients are associated with major changes in fungal community composition (Chapter Four), but it is not known how increased N affects community-level interactions. The principle that environmental gradients will alter the degree of competition within communities suggests that N availability will have a strong affect on patterns of co-occurrence, although it is difficult to

make predictions about whether increasing N should increase or reduce competition, due to uncertainty over whether high or low N availability represents a high stress environment to ECM fungal communities. There is some evidence that ECM fungal communities become less segregated under high N conditions (Koide *et al.*, 2005). However, this result was based on comparing fertilised and unfertilised plots; it is unknown how co-occurrence patterns of ECM fungi might change over a natural N gradient.

It is unclear how patterns of co-occurrence amongst ECM fungi are related to possession of functional traits or phylogenetic relatedness. Community ecology predicts that, in some habitats, only individuals which possess a particular trait can persist; if this habitat filtering is a dominant process, co-occurrence among taxa possessing the trait is likely to be high (Keddy, 1992). Alternatively, if competition is the dominant process, sharing a trait may result in a pattern of segregation between taxa as they compete for similar resources, resulting in exclusion of the weaker competitors (Diamond, 1975). These opposing forces of habitat filtering and competition can operate simultaneously, and often at different spatial scales. The possession of individual traits can often be linked to phylogenetic relatedness – some traits are conserved amongst related taxa, whilst others may evolve through convergence and be shared by distantly related taxa (Prinzing *et al.*, 2001). Therefore, based on the distribution of these traits and their effects on co-occurrence, a number of predictions can be made about the likelihood of closely related taxa to co-occur within communities or not (Table 5.1; Webb *et al.*, 2002).

The complex interplay between phylogenetic relatedness, co-occurrence, environment and scale, has been well studied in many other organisms (Cavender-Bares *et al.*, 2006; Silvertown *et al.*, 2006; Helmus *et al.*, 2007; Bowker *et al.*, 2010), but little work has focused on the role these processes may play in structuring ECM fungal communities. The aim of this study is to provide insight into the relative importance that competition, environment and phylogenetic relatedness may have in structuring ECM fungal communities across different spatial scales. Firstly, the ECM fungal communities of 12 ICP Forests biomonitoring plots are analysed to test whether co-occurrence patterns are consistent with a role for interspecific competition in structuring community composition within and across plots. Next, to assess whether competitive processes are altered by changes in environmental conditions, a relationship between patterns of fungal co-occurrence and the availability of nitrogen is investigated. Finally, the interaction between phylogenetic relatedness and the processes of competition and habitat filtering is

investigated by testing whether closely related ECM fungi share similar ecological requirements, and whether they show higher or lower co-occurrence than expected.

Table 5.1

The interaction between dominant ecological processes and the evolution of functional traits gives rise to predictions about the phylogenetic structure of a community.

	Trait evolution	
	Conserved	Convergent
Ecological process		
Habitat filtering	Clustered	Over-dispersed
Competition	Over-dispersed	Random

Table adapted from Webb *et al.* (2002)

METHODS

Data:

The environmental and ECM fungal community data generated in Chapter Four was used for all of the analyses presented here. This data-set of 3,066 ECM fungal sequences from ectomycorrhizal roots, represents 111 fungal taxa across 12 plots in the UK and Germany. Within each plot, 10 randomly placed transects were sampled, each of which contained four pairs of equidistant soil cores (see Fig. 3.1). Average within-plot richness of ECM fungi is 24, but ranges between 15 and 34. Environmental data gathered from ICP Forests monitoring plots includes foliar N, soil solution nitrate, N deposition, altitude, age of stand, soil pH and mean temperature. Additional data for root N concentrations and Ellenberg N values for vascular plants were generated in Chapter Four. All analyses were carried out using R version 2.11.1 (R Development Core Team, 2010), unless otherwise stated.

Statistical analyses:

To examine patterns of co-occurrence between ECM fungi across plots, Monte Carlo null model analysis was carried out using EcoSim 7.22 (Gotelli & Entsminger, 2007). A matrix of ECM fungal presence/absence in plots was used to quantify co-occurrence patterns across the 12 plots. The C-score index of co-occurrence (Stone & Roberts, 1990) was calculated. This index is based on the average number of checkerboard units between all possible fungal pairs present in the matrix. A C-score that is significantly larger than the C-scores of the null distribution indicates that a community is more segregated than expected

by chance (i.e., some fungi co-occur less than expected). Conversely, a C-score that is lower than that produced by the null distribution indicates aggregation (i.e., some fungi co-occur more than expected by chance). The 10,000 null communities were generated using sequential swapping with fixed row and column totals, meaning that both the abundances of individual taxa, and the number of taxa detected within plots, were held constant. This algorithm has been shown to have desirable statistical properties when used in conjunction with the C-score (i.e., appropriate type I and type II error rates) (Gotelli, 2000). The above analysis was then repeated to assess co-occurrence patterns across transects and cores for each plot. The latter analysis only included soil cores from which all four ECM fungal DNA sequences were generated to avoid the potential influence of differences in within-core sample size on the analysis. To facilitate comparison of C-scores generated from transects and cores, the standardised effect score (SES) was calculated as $(I_{\text{obs}} - I_{\text{sim}})/S_{\text{sim}}$, where I_{obs} is the observed C-score, and I_{sim} and S_{sim} are the means and standard deviations of the C-score obtained from the null communities, respectively (Gotelli & Entsminger, 2007). The SES for plots based on co-occurrence patterns across cores and transects are labelled as SES_c and SES_t, respectively.

Linear regressions were used to explore the relationships between fungal richness, fungal co-occurrence patterns and N availability. To test whether N availability is associated with a change in ECM fungal co-occurrence patterns across plots, linear regressions were carried out between the SES of plots and foliar N, root N, and log soil solution nitrate. Linear regressions were also carried out between the average richness of soil cores (only those where all four mycorrhizas were successfully sequenced) and transects within a plot, and foliar N, root N, and log soil solution nitrate. Finally, the Pearson correlation coefficient (r) was calculated between the SES and the estimated total richness (ACE, see Chapter Four) of plots.

To explore the co-occurrence patterns of individual ECM fungal pairs within plots, the Spearman rank correlation coefficient (ρ) was calculated between the within-core abundances of all fungal pairs. A positive correlation would indicate that those fungi co-occur, while a negative correlation would indicate that those fungi do not co-occur. The high number of pairwise comparisons necessitated Bonferroni correction of P values to reduce the chances of falsely rejecting the null hypothesis of no correlation between fungal pairs. The analysis was limited to include only those fungi that occurred in three or more

soil cores per plot, as correlations based on two or fewer occurrences were not deemed meaningful.

If closely related fungi respond similarly to environmental variation, their co-occurrence is likely to be impacted by processes of competition or habitat filtering. Habitat filtering will lead to high levels of co-occurrence, whilst competition will lead to low levels of co-occurrence. To identify environmental variables that might influence the co-occurrence of phylogenetically related fungi, Spearman correlation coefficients were calculated between the abundances of all fungi that occurred in at least three plots, with nine environmental variables previously identified in Chapter Four as potentially important determinants of ECM fungal community composition (foliar and root N, soil solution nitrate, Ellenberg N, N deposition, altitude, age of stand, soil pH and temperature). The difference in correlation coefficient between closely related fungi (defined as those with 85% or higher ITS sequence similarity) was then compared to the difference in correlation coefficient between random pairs of fungi (999 permutations) – a lower difference would indicate that related fungi respond similarly to that environmental variable; a higher difference would indicate that related fungi have very different responses to that environmental variable. Ideally, phylogenetic distance between all pairs of fungi could be correlated against pairwise co-occurrence. However, the ITS region used to identify fungi in this study is too variable for this kind of phylogenetic analysis, and is only suitable for identifying relatedness between closely related fungi such as those within the same genus/family.

To look at the responses to the environment of particular pairs of related fungi, the differences in the nine Spearman rho values for each pair of related fungi (with 85% or higher sequence similarity) were summed, and this value was compared to the sum of the nine differences in rho values for random pairs of fungi (999 permutations). A significantly low value would indicate that a particular pair of related fungi shares a similar response to environmental variation; a significantly high value would indicate a pair of related fungi with very different responses to environmental variation.

To test for effects of phylogenetic relatedness on fungal community composition, the co-occurrence of pairs of closely related fungi was compared to the co-occurrence of random pairs of fungi. Bray-Curtis distances were calculated between all pairs of fungi based on their abundances across the 12 sampled plots. The number of related fungi that often co-occur (Bray-Curtis distance lower than 0.5), sometimes co-occur (Bray-Curtis distance

lower than 1), and never co-occur (Bray-Curtis distance of 1), was recorded. To test for significant differences to random pairs of fungi, these values were compared to distributions of values generated when the matrix of Bray-Curtis distances was randomly permuted 999 times. This process was repeated to test for an effect of phylogenetic relatedness on co-occurrence within plots, using Bray-Curtis distances based on abundances within transects, and within cores. To check for sensitivity to the definition of closely related fungi, the analyses were repeated, defining related fungi as those with 90% and 95% ITS sequence similarity cut-offs.

RESULTS

Patterns of ECM fungal co-occurrence and the influence of N availability:

Co-occurrence within plots:

The 12 plots showed an overall C-score that was not significantly different from the randomly generated distribution of scores (observed C-score=2.58, average expected C-score($\pm$ SD)=2.56($\pm$ 0.02), $P=0.19$), indicating that fungi do not occur together more, or less, than expected by chance at the scale of plots.

Table 5.2

C-scores and standardised effect scores (SES) for plots based on co-occurrence patterns of ECM fungi in cores and transects. The observed (obs) C-scores are provided, along with the expected (exp) C-score based on 10,000 permutations of the original data. P values which are significant at the 0.05 level are highlighted in bold.

Plot	Cores					Transects				
	C-score		P value		SES	C-score		P value		SES
	obs	exp ($\pm$ SD)	obs>exp	obs<exp		obs	exp ($\pm$ SD)	obs>exp	obs<exp	
307	14.03	13.37 ($\pm$ 0.27)	0.013	0.989	2.419	2.72	2.80 ($\pm$ 0.06)	0.925	0.091	-1.288
501	5.88	5.61 ($\pm$ 0.10)	0.001	0.999	2.916	2.49	2.29 ($\pm$ 0.07)	0.014	0.989	2.686
901	20.51	20.18 ($\pm$ 0.18)	0.041	0.962	1.778	2.54	2.43 ($\pm$ 0.06)	0.050	0.957	1.872
905	8.99	8.95 ($\pm$ 0.12)	0.325	0.681	0.361	2.09	1.97 ($\pm$ 0.05)	0.025	0.976	2.388
912	11.7	11.58 ($\pm$ 0.11)	0.152	0.855	1.065	2.93	2.93 ($\pm$ 0.04)	0.491	0.536	-0.016
1102	10.12	10.19 ($\pm$ 0.09)	0.770	0.238	-0.778	2.24	2.27 ($\pm$ 0.04)	0.714	0.304	-0.615
1201	9.22	9.20 ($\pm$ 0.10)	0.418	0.593	0.205	2.62	2.60 ($\pm$ 0.03)	0.212	0.811	0.801
1205	4.94	4.84 ($\pm$ 0.10)	0.173	0.841	0.977	2.46	2.42 ($\pm$ 0.05)	0.228	0.795	0.753
1405	12.54	11.91 ($\pm$ 0.22)	0.007	0.994	2.790	3.07	3.04 ($\pm$ 0.05)	0.254	0.768	0.613
715	12.67	11.26 ($\pm$ 0.29)	0.000	1.000	4.846	2.95	2.74 ($\pm$ 0.06)	0.003	0.997	3.647
716	26.29	23.28 ($\pm$ 0.47)	0.000	1.000	6.364	2.92	3.00 ($\pm$ 0.08)	0.830	0.208	-0.868
717	11.74	11.38 ($\pm$ 0.23)	0.079	0.925	1.559	2.92	2.91 ($\pm$ 0.06)	0.440	0.595	0.063

Co-occurrence within transects:

Four plots showed a C-score that was significantly greater than the C-score obtained from randomly generated distributions, indicating that there is less co-occurrence of ECM fungi within transects in these four plots than expected by chance (Table 5.2).

Co-occurrence within cores:

Six plots showed a C-score that was significantly greater than the C-score obtained from randomly generated distributions, indicating that there is less co-occurrence of ECM fungi within cores in these plots than expected by chance (Table 5.2). Only plot 1102 had an observed C-score that was less than the expected C-score, but this difference was not significant.

The influence of N availability on co-occurrence patterns:

The SESt of plots was not related to log soil solution nitrate values ($r^2 = 0.011$, $P > 0.05$), foliar N ($r^2 = 0.005$, $P > 0.05$), or root N ($r^2 = 0.051$, $P > 0.05$). The SESt of plots was also not correlated with estimated total richness of plots ($r = 0.08$, $P > 0.05$). The SESc of plots was significantly related to log soil solution nitrate values ($r^2 = 0.456$, $P = 0.016$), and foliar N ($r^2 = 0.433$, $P = 0.020$) (Fig. 5.1), but was not significantly related to root N ($r^2 = 0.215$, $P > 0.05$). The SESc of plots was also significantly negatively correlated with the estimated total richness of plots ($r = -0.814$, $P = 0.001$, Fig. 5.2).

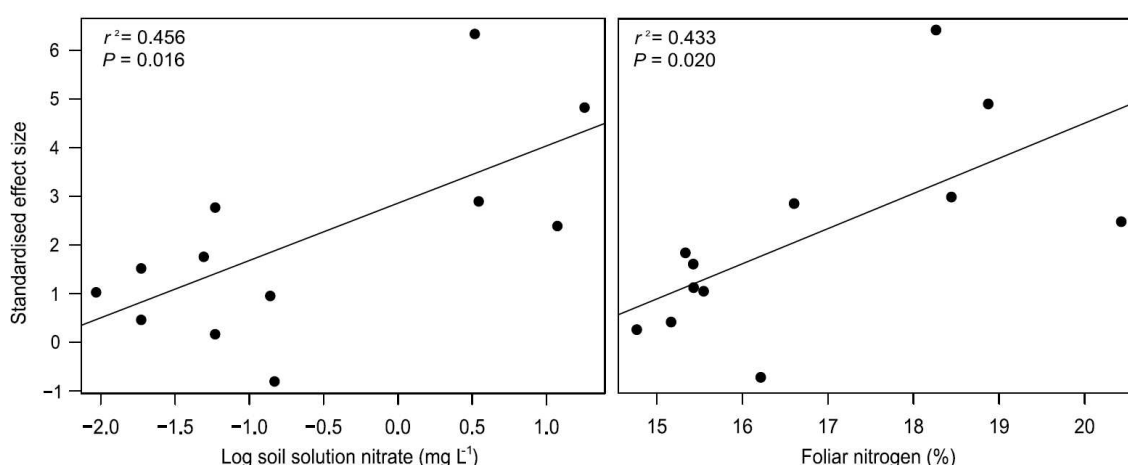


Figure 5.1

Linear regressions between soil solution nitrate (log) and foliar nitrogen concentration, against the standardised effect size of plots, based on fungal co-occurrence patterns across cores.

Standardised effect sizes are centred around zero, with positive values indicating segregation of fungi across cores, and negative values indicating aggregation of fungi within cores.

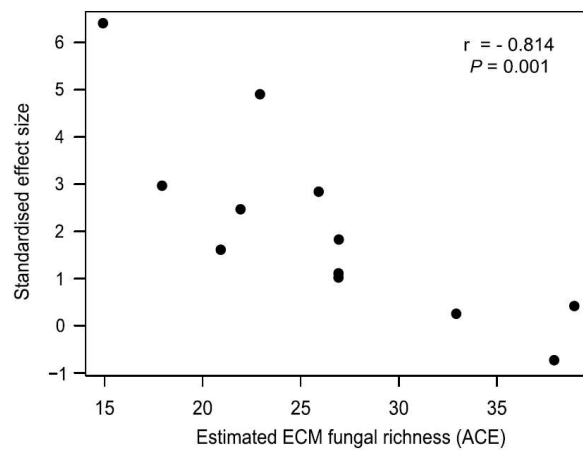


Figure 5.2

Pearson's correlation between the ACE estimated total ectomycorrhizal fungal richness of plots, and the standardised effect size of plots, based on fungal co-occurrence patterns across cores.

Table 5.3

Results of linear regression between the mean ectomycorrhizal fungal richness of cores and transects of plots, against three measures of nitrogen availability.

	Mean core richness		Mean transect richness	
	r^2	P	r^2	P
Log soil solution nitrate (mg L ⁻¹)	0.511	0.009	0.51	0.009
Foliar nitrogen (%)	0.429	0.021	0.475	0.013
Root nitrogen (%)	0.345	0.045	0.292	0.07

P values significant at 0.05 are highlighted in bold.

Effect of N on fungal richness of transects and cores:

There was a significant negative relationship between the average richness of cores within a plot and all three measures of N availability, whilst there was a significant negative relationship between average richness of transects and soil solution nitrate and foliar N, but not with root N concentrations (Table 5.3).

Co-occurrence patterns of individual fungi within cores:

Correlations of fungal abundances within cores found 57 pairs of ECM fungi with significant correlations across all 12 plots. However, because of the high number of pairwise comparisons within plots, only the fungal pairs with a significant correlation after

Bonferroni correction are shown (Table 5.4). Of these 10 pairs, six show significant negative associations (i.e., they rarely co-occur in the same core), whilst four show significant positive associations (i.e., they are frequently found in the same core). Ectomycorrhizal fungal taxa within Russulaceae are involved in eight of the 10 significant correlations; six of these are negative associations and *Lactarius necator* is the only member of the Russulaceae with positive associations with other fungi.

Table 5.4

Spearman rank correlation coefficients, rho, for abundances of pairs of fungi occurring within cores. Only fungi significant after Bonferroni correction of *P* values are shown, and *P* values indicated are adjusted to the appropriate significance level. Positive correlations (rho) indicate that the fungus pairs show high co-occurrence within cores, whilst negative correlations indicate that the fungus pairs show high segregation across cores.

Plot	Fungus pairs	rho	<i>P</i> (Bonferroni)
307	<i>Lactarius</i> sp. 1		
	<i>Russula ochroleuca</i>	-0.487	0.000
501	<i>Cenococcum</i> 1		
	<i>Cenococcum</i> 2	0.398	0.009
901	<i>Elaphomyces granulatus</i>		
	<i>Russula paludosa</i>	-0.367	0.036
	<i>Russula paludosa</i>		
905	<i>Russula decolorans</i>	-0.390	0.016
	<i>Russula paludosa</i>		
	<i>Piloderma</i> sp. 1	-0.402	0.020
715	<i>Russula pectinatoides</i>		
	<i>Tomentella</i> sp. 1	-0.418	0.006
716	Atheliaceae sp. 1		
	<i>Lactarius necator</i>	0.354	0.036
	Cantharellaceae sp. 1		
	<i>Lactarius necator</i>	0.391	0.009
	<i>Russula ochroleuca</i>		
717	<i>Lactarius rufus</i>	-0.387	0.011
	<i>Pseudotomentella</i> sp. 1		
	<i>Hygrophorus hypothejus</i>	0.389	0.028

Patterns of co-occurrence and responses by closely related ECM fungi:

Co-occurrence patterns of related fungi:

No significant differences were found in the co-occurrence patterns of pairs of closely related fungi compared to randomly paired fungi across the 12 plots. Similarly, closely related fungal pairs were not found to co-occur more, or less, than randomly generated fungal pairs within transects or cores. These results were not sensitive to the cut-off used to define closely related fungi (85%, 90% or 95% ITS sequence similarity).

Responses of related fungi to environmental variables:

As a group, closely related pairs of fungi were not found to respond to the nine environmental variables any more or less similarly than random pairs of fungi (16 pairs of fungi, $P > 0.05$). However, when looking at individual pairs of closely related ECM fungi, several were found to have more, or less, similar responses to the suite of environmental variables than randomly paired ECM fungi (Table 5.5). Three pairs of closely related ECM fungi displayed highly similar responses to the environmental variables, whilst three closely related pairs differed in their responses to the environmental variables.

Table 5.5

Similarity of responses to nine environmental variables by closely related pairs of ECM fungi. The nine environmental variables included were foliar and root nitrogen (N), soil solution nitrate, Ellenberg N, N deposition, altitude, age of stand, soil pH and temperature. All fungal pairs showing a significant response are shown, but only those values highlighted in bold are significant at 0.05 after Bonferroni correction for 16 pairwise tests. The DNA sequence similarity of each pair of fungi is also indicated.

Related pairs	Sequence similarity(%)	Response	<i>P</i>
<i>Thelephora terrestris</i> <i>Tomentella sublilacina</i>	95	Different	<0.001
<i>Tylopilus felleus</i> 2 <i>Xerocomus badius</i>	85	Similar	0.042
<i>Cortinarius</i> sp. 1 <i>Cortinarius aurantiobasis</i>	87	Different	0.014
<i>Russula paludosa</i> <i>Russula amethystina</i>	87	Similar	0.048
<i>Tomentella sublilacina</i> <i>Tomentella</i> sp. 1	85	Different	<0.001
<i>Thelephora terrestris</i> <i>Tomentella</i> sp. 1	85	Similar	0.036

DISCUSSION

Analysis of the fungal community composition of 12 forest plots found evidence that ECM fungi co-occur in transects and cores less than expected by chance within some plots, but that patterns of co-occurrence did not significantly differ from random across plots. These results, alongside those of Dahlberg *et al.* (1997), Agerer *et al.* (2002), Koide *et al.* (2005) and Pickles *et al.* (2010), demonstrate that segregation of ECM fungal communities within plots is widespread, akin to patterns frequently observed in plant, animal and microbial communities (Gotelli & McCabe, 2002; Horner-Devine *et al.*, 2007). Less co-occurrence than expected by chance is often believed to be indicative of a community that is structured by competitive interactions (Diamond, 1975), leading to a segregation of taxa. As alternative processes, such as habitat preferences amongst taxa, can result in a similar pattern of community segregation (Gotelli & McCabe, 2002), it is not possible to attribute competition as the causal mechanism driving the observed co-occurrence patterns. Laboratory studies are, however, providing increasing evidence that competitive interactions between ECM fungi may play an important role in structuring ECM fungal communities (Wu *et al.*, 1999; Landeweert *et al.*, 2003; Kennedy & Bruns, 2005) and it therefore seems likely that interspecific competition is at least in part driving the patterns of co-occurrence seen in this study. Experimental manipulations targeted at particular species could be used to confirm competitive interactions between ECM fungi.

We found a pattern of increasing segregation of ECM fungi across cores as N availability across plots increases, but no relationship between increasing N and ECM fungal co-occurrence patterns across transects. The stress gradient hypothesis (Bertness & Callaway, 1994), predicts a pattern of decreasing segregation amongst taxa with increasing environmental stress. Low N availability is usually regarded as an environmental stress, so increasing N availability should result in an increase in competitive interactions (Callaway & Walker, 1997), consistent with the pattern found here. Of course, it may not be appropriate to consider high N conditions as low stress for a community of symbionts which evolved in low N environments (Smith & Read, 2008). Nevertheless, these results suggest that under conditions of high N availability, there are an increasing number of soil cores dominated by a single ECM fungus, whilst in transects, there is no effect of increasing N on the co-occurrence patterns of ECM fungi, despite a negative effect on fungal richness within transects.

These co-occurrence patterns might indicate that increasing N availability is altering the competitive interactions between ECM fungi at very small spatial scales, resulting in fine-scale dominance of N-tolerant taxa, and loss of rare fungi found infrequently within cores. The strong relationship between the degree of community segregation across cores and total estimated fungal richness of plots could arise as a result of these competitive interactions, and this is a potential mechanism driving the relationship between increasing N availability and the observed reduction in ECM fungal richness. However, it is not possible to rule out other N effects, such a direct negative impact on certain ECM fungi, that could also result in a loss of fungal richness and which could, in turn, influence the degree of segregation between ECM fungal taxa. It is worth noting that SES is not necessarily related to fungal richness in this study; although increasing N availability reduces the mean richness of transects, there is no relationship between the degree of segregation across transects and N availability.

It is interesting to note that in an N addition trial, Koide *et al.* (2005) observed a decrease in the segregation of an ECM fungal community; an opposite trend to the pattern found here. This could be due to differences between the two studies; whilst we have examined the effects of a natural N availability gradient, Koide *et al.* compared co-occurrence patterns of ECM fungi between control (low N) and treatment (high N) plots within a single forest stand, where additional N was added for one year prior to sampling. In addition, Koide *et al.* observed the influence of increased N on the co-occurrence patterns of ECM fungal hyphae, whilst we have examined ECM root-tips.

The large number of fungi included in this study, and the high turnover of taxa across plots, make it difficult to identify individual fungal pairs which co-occur more, or less, than expected. Whilst the ECM fungi presented in Table 5.4 are restricted to only those fungal pairs demonstrating extremely significant correlations, due to the high number of pairwise tests, it does provide an indication as to which fungi may be most important in driving the observed co-occurrence patterns within plots. It is interesting that despite the strong overall segregation of ECM fungal communities in six plots, some fungal pairs do show higher than expected co-occurrence, consistent with the findings of previous studies looking at co-occurrence patterns of mycorrhizas (Koide *et al.*, 2005; Pickles *et al.*, 2010). *Cenococcum* 1 and 2 are found in the same core more than expected by chance in this study, which could indicate that these fungi share resource use and co-occur where that resource is not limited, or that close proximity is beneficial to one or both fungi. Members of the Russulaceae

generally appeared to be negatively associated with other ECM fungi in this study, although *Lactarius necator* co-occurs with two other fungi more than expected by chance. We found that *L. rufus* co-occurred with *R. ochroleuca* less than expected by chance, whilst Pickles *et al.* found that *L. rufus* was only positively associated with other fungal taxa. This highlights the context-specific nature of competitive interactions between ECM fungi, as has been highlighted in laboratory studies (Mahmood, 2003; Kennedy *et al.*, 2007).

Closely related taxa are expected to share ecological requirements, resulting in a high degree of competition and preventing their co-occurrence (Darwin, 1859). However, sharing requirements might also lead related taxa to co-occur in communities where the requirements are met (Keddy, 1992). In this way, the distribution of important functional traits interacts with processes of competition and habitat filtering to determine the phylogenetic structure of a community (Webb *et al.*, 2002). In this study no evidence was found that closely related ECM fungi co-occur more or less than expected by chance at the scale of plots, transects, or cores. As there is evidence consistent with a role of competition driving co-occurrence patterns within plots, this result may indicate that competitive interactions within plots are no stronger or weaker between closely related fungi than between random pairs of fungi. This pattern could arise if traits allowing ECM fungi to occur within a habitat are not always conserved between closely related fungi (Table 5.1). Alternatively, phylogenetic structure in the community may only be apparent at sampling scales not included in this study, and consequently went undetected.

No evidence was found that closely related ECM fungi respond to environmental gradients more similarly than randomly paired fungi in this study. However, six of the 16 (38%) closely related fungal pairs responded to the suite of environmental conditions in a more, or less, similar way than expected by chance. The differential similarity of responses to environmental variables between pairs of closely related ECM fungi may hinder our ability to find a significant overall pattern in the way closely related fungi respond to environmental gradients. Given the wide phylogenetic breadth of ECM fungi it may be likely that functional traits are conserved within some fungal groups, but evolve convergently in other groups.

In contrast to the results here, a recent study by Peay *et al.* (2010) assessed the influence of phylogenetic relatedness on ECM fungal community structure, and found that fungi were phylogenetically clustered within two soil/vegetation types. However, in the study system

of Peay *et al.*, a shift in soil type was mirrored by a shift in host plant community composition; traits determining host plant preferences may show different patterns of conservatism amongst ECM fungi than traits involved in determining preferences to abiotic conditions. Alternatively, the relatively small number of closely related fungi that occurred in multiple plots in the present study may have reduced our power to detect a difference in the response to environmental variables by closely related, as opposed to random, fungal pairings. A larger study, encompassing a greater number of sites and combined with a full phylogenetic analysis of communities via sequencing of the more conserved nuclear ribosomal 28s region would provide a better test of the influence that phylogeny may play in driving ECM fungal community ecology.

CONCLUSIONS

This study provides a first insight into how competition, environmental gradients and relatedness may interact to influence ECM fungal community structure and richness both within and across pine forest plots in Scotland, England and Germany. The results indicate that competitive interactions may act as a dominant process structuring communities within plots. In addition, there is evidence to suggest that increasing N availability may alter the degree of segregation, and possibly competitive interactions, within plots. In contrast, no evidence was found that closely related fungi are more, or less, likely to co-occur within plots, suggesting that important functional traits may not always be conserved within fungal lineages. Similarly, closely related fungi do not appear to always share preferences to environmental conditions across plots. These findings reveal a complex picture of interacting processes which are both scale and context-dependent. A greater number of sites, in addition to a more powerful phylogenetic analysis of ECM fungal communities, would help to clarify the importance of phylogenetic relatedness in structuring ECM fungal communities, whilst experimental manipulation may help to disentangle the relative roles of habitat filtering and competition.

Chapter Six – General Conclusion

Adapted from a paper published in Annals of Forest Science, 2010. Cox, F., Barsoum, N., Bidartondo, M.I., Børja, I., Lilleskov, E., Nilsson, L.O., Rautio, P., Tubby, K., Vesterdal L. 67, 200.

Environmental change is likely to influence the structure of ECM fungal communities, in combination with dispersal constraints, competitive interactions, and fungal habitat preferences. If we wish to understand the extent and impact of changes in ECM communities it will be necessary to increase the scale of ECM fungal research from local-scale studies, to studies carried out at continental scales. Although this is a daunting task, the results in this thesis demonstrate that large-scale surveying of ECM fungi is feasible, and provide a first indication as to which environmental variables may be most important in structuring ECM fungal communities at continental scales (Chapter Four). This data can be used to inform further European-scale sampling of ECM fungi, where plot selection may be aimed at stratifying across the variables of interest (Lilleskov & Parrent, 2007). The use of ICP Forests network of monitoring plots can drastically reduce the time and cost associated with collection of environmental parameters, and provides long-term data that is of greater benefit than measurements from a single occasion.

Across-site comparisons of fungal communities are more informative when sampling has been of sufficient intensity to fully capture species diversity (Taylor, 2002; Peay *et al.*, 2008). These studies provide preliminary data aimed at informing an optimised sampling strategy in a large-scale survey. The spatially explicit sampling design allowed an assessment of the role that spatial scale had in influencing temporal stability (Chapter Two), the degree of fine-root overlap between adjacent trees (Chapter Three), effects of environmental conditions (Chapter Four) and the importance of competitive interactions (Chapter Five), and a similar sampling strategy would be strongly recommended for future surveys. However, as the ECM fungal communities of cores within transects are highly auto-correlated (Chapter Three), reducing the number of cores within transects, and increasing the number of transects per plot, would give better coverage of ECM fungal diversity and increase sampling efficacy.

Below-ground ECM fungal communities appear to display short-term resilience to changes in environmental conditions, including dramatic shifts in N availability (Chapter Two), but are influenced by longer term shifts in N availability at both local (e.g. Lilleskov *et al.*, 2002a) and intracontinental scales (Chapter Four). This raises doubt over the usefulness of short-term fertilisation studies aimed at replicating impacts of natural N gradients, and further highlights the potentially detrimental impact of long term increases in N deposition on both above and below-ground communities. The short-term resilience of ECM fungi to rapid changes in the environment highlights the potential for introduced ECM fungal species to persist in new habitats.

Future Directions

This study has provided preliminary data which can facilitate a continental-scale survey of ECM fungi. However, this pilot study was carried out using Sanger sequencing; the recent development of ultra-high throughput sequencing technologies may help increase the number of sites that can be rapidly characterised, and its use should therefore be considered in a continental scale assessment of ECM fungal communities. Validation studies will be needed to ensure that these novel methodologies result in accurate and precise community data (Avis *et al.*, 2010) and there were two specific situations encountered during this study which might pose problems for high-throughput technologies. In several German plots, *Cenococcum* sclerotia were found in great abundance despite infrequent occurrence of this fungus as mycorrhizas, raising concerns over how these technologies could potentially bias community descriptions of ECM fungi from soil samples (Chapter Four). Secondly, molecular assessment can potentially lead to erroneous removal of samples thought to be non-mycorrhizal. Sampling of individual ECM, rather than bulking roots or soil, allows for in-depth queries relating to the mycorrhizal status of unknown fungi, such as the unknown Pezizalean fungus found in the Scottish plot (717), and later identified as the morphologically characterised “*Piceirhiza sulfo-incrustata*” (Chapter Two). In addition, there is a need to further develop bioinformatics technology to cope with the large volumes of sequence data. Nevertheless, these ultra-high throughput technologies could offer the possibility of rapid characterisation of ECM fungal communities, with the benefit of increasing the number of plots included in the survey, and the potential to saturate species accumulation curves. More preliminary work using this technology would be required prior to launching into a large-scale survey.

The benefits of a continental-scale assessment of ECM fungal communities

A continental-scale survey of ECM fungi would provide the baseline data on below-ground ECM fungal distributions that is required to improve our understanding of factors which drive the function and biodiversity of mycorrhizal trees and fungi (Fig. 6.1, phase 1).

The primary benefit of large-scale distribution data would be the ability to identify patterns in ECM community composition in forests across geographic regions, and link this to broad environmental variables recorded at each sampling location. With these data, it should be possible to predict mycorrhizal communities in un-sampled European forests as a result of site characteristics *via* GIS layers, enabling the mapping of current fungal distributions across the continent. Whilst predicting the distributions of infrequent species that constitute the major part of fungal diversity will prove challenging, realistic predictions for dominant taxa should be achievable, and represent a test of the power of predictive mapping. A baseline map, combined with information about dynamic responses to changing environmental conditions, can also allow the prediction of future distributions of dominant species as a result of changes in climate, atmospheric pollution and land use (Ellis *et al.*, 2007).

Currently, mycorrhizal species ranges based on fruitbody collections have been described for some ECM fungal species at national scales (e.g. Courtecuisse *et al.*, 2008). However, these data are lacking for the majority of species, do not take into account below-ground presence, and are poorly harmonizable over the continental scale due to differing morphospecies concepts. Through DNA sequence based distribution maps, the extent to which geographic dispersal constraints act on mycorrhizal fungal species distributions, and how these differ among species, higher taxonomic levels, and functional guilds, could be established. Continental scale distribution data would also allow the identification of the most abundant and widespread mycorrhizal fungi across Europe, providing a prioritized list of fungal species to target as models for future research (e.g., genetics, ecology, physiology, genomics), increasing its applicability and relevance.

Linking environmental and mycorrhizal community data would allow the effects of variables such as temperature, rainfall, and soil type on the community assemblage of ECM fungi to be tested. These data would enable identification of broad niches for individual species (host type, soil type, climatic constraints). In particular, we would be able to extend

and test existing knowledge on mycorrhizal specialists and generalists (e.g. Molina *et al.*, 1992) and edaphic endemics. In addition, we would test and identify fungi sensitive to particular agents of pollution, such as atmospheric N deposition and oxidants. It may also be possible for mycorrhizal community data to be linked directly to data on the composition of ground flora and environmental characteristics. Although such inferences would be limited by the power of regression and correlation analyses, they can still point the way forward for experiments designed to provide a mechanistic understanding of fungus-environment interactions.

Analysis of mycorrhizal diversity across large scales would allow us to identify geographic areas with the greatest diversity, as well as areas of endemism. These mycorrhizal ‘hotspots’ can then be the target for habitat conservation action to maintain soil biodiversity. In combination with environmental and geographic data, we should be able to model the factors that are likely contributors to hotspot generation, leading to predictions of hotspots elsewhere.

Although mycorrhizal fungi are little understood compared to other large perennial organisms, there is nevertheless evidence of a sharp decline in the reproductive output of some mycorrhizal fungi in polluted regions of Europe (Arnolds, 1991). Currently, declining, ‘Red list’ and locally extinct mycorrhizal fungi are assessed and monitored only through their sporadic and ephemeral reproductive structure production. This information does, however, constitute a warning signal to uncover their distribution and source/sink dynamics belowground at the continental scale. Reliable large-scale distribution data would allow the conservation status of individual species to be assessed – species not previously considered rare may be identified as targets for conservation activities, and *vice versa*. Sampling a large geographic area belowground would provide the opportunity to improve currently imprecise estimates of the total number of ECM fungal species (Rinaldi *et al.*, 2008), and discover what proportion have not been taxonomically described to date.

In addition to the broad questions that would be addressed through a large-scale survey of ECM fungi, there are additional local-scale studies that would be directly informed by baseline continental survey data (Fig 6.1, phase 2).

The identification of dominant mycorrhizal species by indicator species analyses would be a first step for many potential future studies. The development of species-specific primers

would permit the quantification of ECM in both roots and soils of plots known to contain the mycorrhizal species of interest. Dominant fungal taxa could also be targeted for the next sequencing programs of fungal genomes, which would provide genetics markers to gain some of the first insights into the population structure, recombination levels, genet size and overlap, as well as the extent and direction of gene-flow and dispersal between geographically separated populations (e.g. Kretzer *et al.*, 2004).

The importance of mycorrhizal fungi as carbon sinks in forest ecosystems (Högberg *et al.*, 2001; Smith & Read, 2008), and the potential for carbon demand to change due to elevated CO₂ and nitrogen availability (Bidartondo *et al.*, 2001; Alberton & Kuyper, 2009), makes it necessary to understand the role of mycorrhizal fungi in global carbon and nitrogen dynamics. A critical area for research will be whether loss of fungal richness, replacement of dominants, and shifts in evenness as a result of environmental change, will alter the carbon sink potential of forest ecosystems.

Pre-characterised mycorrhizal communities in plots would provide an unrivalled platform for experimental manipulations, such as nitrogen fertilisation. By selecting sites with similar initial mycorrhizal species composition, treating a subset of these sites, and then comparing the degree and direction of any changes in the mycorrhizal communities between treatment and control groups, the effects of elevated N availability could be ascertained at a larger scale, and in a more informed, replicated and controlled way, than has ever been possible.

Finally, assessing micro-scale habitats for a range of factors (e.g., soil pH, moisture, organic matter, coarse woody debris, nutrient availability), should allow correlations between the presence and/or abundance of mycorrhizal species and specific environmental conditions. When conducted at several sites, robust inferences about niches of individual fungi can be made, with implications for forest management practices (e.g., removal of brash and other deadwood) that alter the availability of micro-niche sites.

The functionally critical role of mycorrhizal fungi in forest ecosystems, and the imminent threat of global environmental change that may act to alter mycorrhizal functional biodiversity, means there is an urgent need for a regional to continental-scale assessment of mycorrhizal distributions. A large-scale survey of ICP Forests plots would be only the first stage in answering many of the questions outlined above, but it is essential if future studies

are going to address these questions with hypothesis-driven research in a cohesive manner, rather than remain independent for lack of a unified approach. The chance to utilise the vast network of biomonitoring plots at this time is a remarkable opportunity because it minimises the logistics and costs associated with achieving such an enormous effort and provides a rare stable - past and future - ground for forest ecosystem scientific investigation. In the face of rapid global change, we finally have an opportunity to accurately integrate mycorrhizal distribution data with long-term environmental monitoring, providing a basic understanding of functionally crucial organisms, and at the same time creating an invaluable resource for future research.

Phase 1

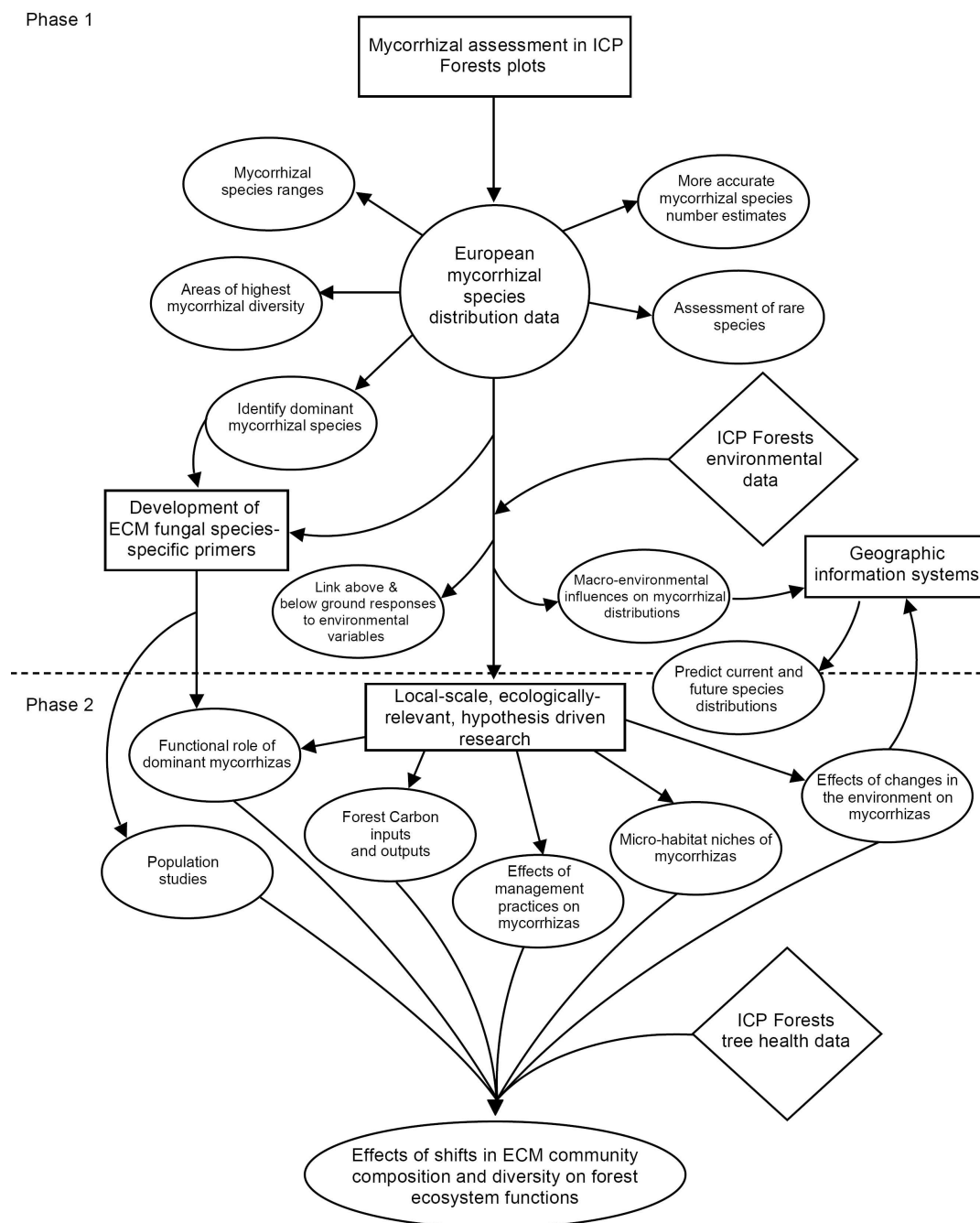


Figure 6.1

The potential research outputs of a European-wide survey of mycorrhizal fungi in biomonitoring plots. Rectangles represent a research effort, ovals indicate a research output and diamonds indicate an instance when data gathered from ICP Forest plots would enhance research efforts.

Phase 1: Whilst the primary research achievement would be European-wide distributional data for mycorrhizal fungi, a number of immediate secondary research outputs would also be generated. Combining the mycorrhizal fungal survey data with environmental data would be critical to the development of Phase 2, which focuses on ecologically-relevant and hypothesis-driven research at smaller scales. Combining these data with tree health and environmental data can pinpoint key links between shifts in mycorrhizal fungal community composition and forest ecosystem health. ICP: The International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests.

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Appendix A

Relative abundances (%) of 3066 ectomycorrhizal fungi detected in 12 level II plots, with Genbank accession numbers.

Family/Order	Fungus	High N ← → Low N												Accession
		307	715	716	501	1102	1205	1405	717	912	1201	901	905	
Amanitaceae	<i>Amanita citrina</i>	0.0	0.0	0.0	0.0	3.8	0.5	0.0	0.0	0.0	0.0	0.0	0.0	HM146787
	<i>Amanita muscaria</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.8	0.0	0.0	0.0	0.0	HM146788
	<i>Amanita pantherina</i> 1	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146790
	<i>Amanita pantherina</i> 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	1.5	HM146791
	<i>Amanita porphyria</i>	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	HM146792
	<i>Amanita rubescens</i>	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146793
	<i>Amanita spissa</i>	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	1.5	0.0	0.4	0.4	HM146794
	<i>Amanita stranelia</i>	0.0	0.0	0.0	0.0	11.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146789
Atheliaceae	<i>Atheliaceae</i> sp. 1	1.5	7.4	3.0	0.0	8.0	1.8	5.2	3.9	1.9	0.0	1.2	2.3	HM146795
	<i>Atheliaceae</i> sp. 2	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146796
	<i>Piloderma</i> sp. 1	0.0	0.0	0.0	0.0	0.0	0.0	37.3	0.0	9.2	0.0	7.8	27.5	HM146845
	<i>Piloderma</i> sp. 2	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146890
	<i>Piloderma</i> sp. 3	0.0	0.0	0.0	1.3	0.0	0.9	5.6	0.0	0.0	21.4	8.6	12.1	HM146891
	<i>Piloderma</i> sp. 4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	0.0	0.0	0.0	HM146892
	<i>Tylospora asterophora</i>	1.5	0.0	0.0	0.0	0.0	0.0	1.2	0.0	1.2	0.0	0.0	0.8	HM146888
	<i>Tylospora fibrillosa</i>	0.0	0.0	0.0	0.0	0.0	0.0	3.6	0.0	12.7	0.0	0.0	0.4	HM146889
Boletaceae	<i>Boletus erythropus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	HM146797
	<i>Tylopilus felleus</i> 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	HM146886
	<i>Tylopilus felleus</i> 2	0.0	0.0	0.0	0.0	1.1	3.2	0.8	0.0	0.0	3.1	0.0	0.0	HM146887
	<i>Xerocomus badius</i>	4.5	0.0	0.0	1.7	1.5	16.8	2.8	0.0	1.2	10.7	4.3	1.1	HM146895
	<i>Xerocomus ferrugineus</i>	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146896
	<i>Xerocomus</i> sp. 1	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146897
	<i>Cantharellaceae</i> sp. 1	0.0	0.0	2.2	0.0	0.8	0.5	1.6	4.3	9.2	8.4	0.8	0.4	HM146798
	<i>Craterellus tubaeformis</i>	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	5.4	0.0	0.0	0.8	HM146810
Ceratobasidiaceae	<i>Ceratobasidiaceae</i> sp. 1	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	1.9	0.0	0.0	HM146802
Clavulinaceae	<i>Clavulina amethystine</i>	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146803
Cortinariaceae	<i>Cortinarius alboviolaceus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8	HM146808
	<i>Cortinarius aurantiobasis</i>	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.4	0.0	3.8	2.0	0.0	HM146811
	<i>Cortinarius hemitrichus</i>	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146807
	<i>Cortinarius olivaceofuscus</i>	0.0	8.2	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146809
	<i>Cortinarius semisanguineus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.4	0.0	5.3	HM146812
	<i>Cortinarius</i> sp. 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.8	0.8	HM146804
	<i>Cortinarius</i> sp. 2	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146806
	<i>Cortinarius</i> sp. 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	HM146805
	<i>Hebeloma</i> sp. 1	8.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	1.5	HM146817
	<i>Inocybe lacera</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	HM146824
	<i>Inocybe lanuginosa</i> 1	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	HM146825
	<i>Inocybe lanuginosa</i> 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	HM146823
	<i>Inocybe soluta</i>	0.0	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	HM146827
	<i>Inocybe stellatospora</i>	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146829
	<i>Inocybe</i> sp. 1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146826
	<i>Inocybe</i> sp. 2	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146828
	<i>Inocybe</i> sp. 3	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146830
Discinaceae	<i>Hydnотrya michaelis</i>	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146816
	<i>Hydnотrya tulasnei</i>	9.7	0.0	0.7	0.0	1.9	0.5	0.0	0.0	0.0	0.8	0.0	0.0	HM146820
Dothidiomycete	<i>Cenococcum</i> 1	2.2	0.0	0.0	1.3	14.8	4.1	12.3	0.0	0.4	0.8	0.0	0.0	HM146799
	<i>Cenococcum</i> 2	0.0	0.0	0.0	7.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146800
	<i>Cenococcum</i> 3	0.0	0.0	0.0	0.0	0.4	3.2	4.0	0.0	1.2	1.1	0.0	2.3	HM146801
Elaphomycetaceae	<i>Elaphomyces granulatus</i>	1.5	0.0	0.0	27.4	0.4	9.5	6.7	0.0	1.9	6.1	20.7	4.5	HM146813
	<i>Elaphomyces muricatus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	HM146815
	<i>Elaphomyces</i> sp. 1	0.0	0.0	0.0	0.0	0.0	4.5	2.4	2.8	0.0	0.0	0.8	0.0	HM146814
Helotiales	<i>Meliniomyces bicolor</i>	0.0	0.0	0.0	0.0	0.8	0.0	0.0	2.4	3.8	0.0	0.4	0.0	HM146839
	<i>Piceirhiza sulfo-incrustata</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	38.6	0.0	0.0	0.0	0.0	HM146841
	<i>Helotiales</i> sp. 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.4	HM146818
	<i>Helotiales</i> sp. 2	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146819
Hydnaceae	<i>Hydnum rufescens</i>	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146821
Hygrophoraceae	<i>Hygrophorus hypothejus</i>	0.0	0.0	0.7	0.0	1.5	0.0	0.0	4.3	0.0	0.0	0.0	0.0	HM146822
Paxillaceae	<i>Paxillus involutus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	1.5	1.2	0.0	HM146842

Pezizales	Pezizales sp. 1	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146843
	Pezizales sp. 2	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146844
	Pezizales sp. 3	3.7	3.0	6.3	3.5	3.8	2.3	2.8	8.3	2.7	0.4	0.0	0.8	0.0	0.0	HM146849
Pyronemataceae	<i>Otidea cochleata</i>	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146840
	<i>Wilcoxina mikolae</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	HM146893
	<i>Wilcoxina</i> sp. 1	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146894
Russulaceae	<i>Lactarius camphoratus</i>	0.0	0.0	0.0	0.0	0.0	1.8	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146832
	<i>Lactarius helvus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.8	0.0	0.0	HM146833
	<i>Lactarius necator</i>	0.0	0.0	6.7	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146835
	<i>Lactarius quieticolor</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	HM146837
	<i>Lactarius rufus</i>	0.0	0.0	21.6	0.9	3.8	0.9	0.4	9.1	6.2	5.7	0.4	0.4	0.0	0.0	HM146836
	<i>Lactarius vellereus</i>	0.0	0.0	0.0	0.0	1.1	28.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146838
	<i>Lactarius</i> sp. 1	20.6	0.0	0.0	17.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146834
	<i>Russula aeruginea</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	HM146850
	<i>Russula amethystina</i>	0.0	0.0	0.0	0.0	0.8	0.0	6.3	0.0	0.0	1.9	0.0	0.0	0.0	0.0	HM146851
	<i>Russula amoenolens</i>	0.0	0.0	0.0	0.0	7.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146852
	<i>Russula decolorans</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	20.3	0.0	0.0	0.0	HM146853
	<i>Russula emetica</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	0.0	0.0	0.0	0.4	0.0	0.0	HM146854
	<i>Russula grisea</i>	0.0	0.0	0.0	0.0	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146855
	<i>Russula nigricans</i>	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146856
	<i>Russula ochroleuca</i>	29.6	0.0	34.0	17.8	8.4	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	HM146857
	<i>Russula paludosa</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.3	8.4	23.4	15.5	0.0	0.0	HM146858
	<i>Russula pectinatoides</i>	0.0	43.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146859
	<i>Russula puellaris</i>	0.0	0.0	0.0	0.0	0.4	4.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146860
	<i>Russula tenuiceps</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	HM146862
	<i>Russula vesca</i>	0.0	0.0	0.0	0.0	1.5	0.0	0.8	0.0	0.0	0.0	0.0	0.8	0.0	0.0	HM146863
	<i>Russula</i> sp.1	0.0	8.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146861
Sclerodermataceae	<i>Scleroderma citrinum</i>	0.0	0.0	0.0	2.6	2.7	0.0	1.2	0.0	0.0	0.0	0.4	1.5	0.0	0.0	HM146864
Sebacinaceae	<i>Sebacina</i> sp. 1	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146865
	<i>Sebacina</i> sp. 2	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146866
	<i>Sebacina</i> sp. 3	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146867
	<i>Sebacina</i> sp. 4	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146868
Suillaceae	<i>Suillus granulatus</i>	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146869
	<i>Suillus variegatus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.0	2.3	10.2	0.0	0.0	HM146870
Thelephoraceae	<i>Pseudotomentella tristis</i>	0.0	0.0	0.0	0.0	0.8	1.4	0.4	2.8	0.0	3.1	0.0	0.0	0.0	0.0	HM146847
	<i>Pseudotomentella vepallidospora</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	HM146848
	<i>Pseudotomentella</i> sp. 1	0.0	0.7	10.4	0.0	0.0	0.5	0.8	5.9	0.0	0.0	0.0	0.0	0.0	0.0	HM146846
	<i>Thelephora terrestris</i>	0.0	8.2	7.8	5.7	1.1	8.6	0.0	2.0	0.0	0.8	3.5	1.5	0.0	0.0	HM146871
	<i>Tomentella stiposa</i>	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146876
	<i>Tomentella sublilacina</i>	0.4	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	HM146877
	<i>Tomentella</i> sp. 1	0.7	6.7	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146872
	<i>Tomentella</i> sp. 2	2.2	0.4	0.0	0.0	2.7	0.5	0.0	0.0	1.2	0.8	0.0	0.0	0.0	0.0	HM146873
	<i>Tomentella</i> sp. 3	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146874
	<i>Tomentella</i> sp. 4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	HM146875
	<i>Tomentellopsis</i> sp. 1	0.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0	5.7	0.0	0.4	0.0	0.0	HM146878
	<i>Tomentellopsis</i> sp. 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.8	1.9	0.0	1.5	0.0	0.0	HM146879
Tricholomataceae	<i>Laccaria proxima</i>	0.0	0.0	0.0	5.2	0.4	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	HM146831
	<i>Tricholoma flavovirens</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146880
	<i>Tricholoma saponaceum</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	HM146882
	<i>Tricholoma</i> sp.1	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146881
Tuberaceae	<i>Tuber rufum</i>	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146884
	<i>Tuber scruposum</i>	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146885
	<i>Tuber</i> sp. 1	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	HM146883

Appendix B

Linear regressions of log ACE fungal richness estimates of transects against mean root nitrogen concentration (%) for 12 study plots.

Plot	r^2	P
307	0.053	0.522
501	0.003	0.881
901	0.003	0.873
905	0.048	0.545
912	0.050	0.537
1102	0.263	0.130
1201	0.002	0.898
1205	0.122	0.322
1405	0.109	0.750
715	0.159	0.254
716	0.013	0.751
717	0.720	0.016

n=10 in all plots except 717, where n=8, as root N concentration could not be calculated for two transects.

Values in bold are significant at $P<0.05$.

Appendix C

Within-plot Mantel's tests, between Bray-Curtis similarity of ectomycorrhizal fungal communities of transects against mean root nitrogen concentrations (%).

Plot	Space partialled?	Mantel <i>r</i>	<i>P</i>
307	No	-0.390	0.987
501	No	-0.097	0.665
901	Yes	-0.139	0.783
905	No	-0.227	0.886
912	Yes	0.210	0.095
1102	No	-0.043	0.485
1201	No	0.100	0.284
1205	No	0.184	0.113
1405	Yes	-0.100	0.585
715	Yes	-0.076	0.584
716	Yes	-0.030	0.579
717	Yes	-0.101	0.62

Distance between transects is partialled out where within-plot spatial data was available.

The Bray-Curtis dissimilarity matrix is based on relative abundances of each fungus (the number of root-tips occupied by each fungus in a transect, divided by the total number of samples from the transect), in order to account for differences in sample size.

n=10 in all plots except 717, where n=8, as root N concentration could not be calculated for two transects.