DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

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ECTOMYCORRHIZAL FUNGI: DIVERSITY AND COMMUNITY STRUCTURE IN ESTONIA, SEYCHELLES AND AUSTRALIA

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1. LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications that are referred in the further text by their Roman numerals:

I. **Tedersoo L**, Kõljalg U, Hallenberg N, Larsson K-H. 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytologist 159: 153–165.

II. **Tedersoo L**, Suvi T, Larsson E, Kõljalg U. 2006. Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. Mycological Research 110: 734–748.

III. **Tedersoo L**, Hansen K, Perry BA, Kjøller R. 2006. Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytologist 170: 581–596.

IV. **Tedersoo L**, Pellet P, Kõljalg U, Selosse M-A. 2007. Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. Oecologia 151: 206–217.

V. **Tedersoo L**, Suvi T, Beaver K, Kõljalg U. 2007. Ectomycorrhizal fungi of the Seychelles: diversity patterns and host shifts from the native *Vateriopsis seychellarum* (Dipterocarpaceae) and *Intsia bijuga* (Caesalpiniaceae) to the introduced *Eucalyptus robusta* (Myrtaceae), but not *Pinus caribea* (Pinaceae). New Phytologist. In press. doi: 10.1111/j.1469–8137.2007.02104.x

VI. **Tedersoo L**, Suvi T, Beaver K, Saar I. 2007. Ectomycorrhizas of *Coltricia* and *Coltriciella* (Hymenochaetales, Basidiomycota) on Caesalpiniaceae, Dipterocarpaceae and Myrtaceae in Seychelles. Mycological Progress. In press. doi: 10.1007/s11557-007-0530-4

VII. **Tedersoo L**, Suvi T, Kõljalg U. Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of *Picea abies* and *Betula pendula*. Unpublished.

VIII. **Tedersoo L**, Jairus T, Horton B, Glen M, Kõljalg U. Ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest. Unpublished.

IX. **Tedersoo L**, Dunk C, Gates G, Jairus T, Lebel T, May T, Kõljalg U. Establishment of ectomycorrhizal fungi on *Nothofagus cunninghamii* seedlings on dead wood in Australian temperate wet sclerophyll forests. Unpublished.

Table 1. Author's contribution to each paper (%)

	I	II	III	IV	V	VI	VII	VIII	IX
Idea and design	50	40	40	50	80	90	90	80	60
Sampling	100	40	n.a.*	100	90	80	100	70	n.a.
Morpho/anatomotyping	100	50	n.a.	100	100	100	100	100	n.a.
Molecular analyses	50	50	n.a.	10	50	70	60	50	n.a.
Data analysis, statistics	90	90	0	80	100	50	90	90	n.a.
Writing	80	80	40	40	80	80	n.a.	n.a.	n.a.

* n.a., not applicable.

2. INTRODUCTION

2.1. Theoretical background

Symbiosis between plant roots and fungi is termed 'mycorrhiza' (Frank 2005). In this thesis, mycorrhizal symbiosis is viewed as reciprocal parasitism that results in mutualistic to parasitic outcomes depending on genetic and environmental constraints (Egger & Hibbett 2004). Based on anatomical and morphological features, there are four major mycorrhiza types that possess different evolutionary backgrounds and ecological roles (Smith & Read 1997). Arbuscular mycorrhiza (AM), ectomycorrhiza (EcM), ericoid mycorrhiza (ErM) and orchid mycorrhiza are ecologically the most important, independently evolved types. Other mycorrhiza types (ectendomycorrhiza, arbutoid mycorrhiza) are considered structural derivatives of EcM.

AM-forming fungi from the monophyletic phylum Glomeromycota probably facilitated land colonization by early plants in the Ordovician (Pirozynski & Malloch 1975; Selosse & Le Tacon 1998). AM still dominates in most major terrestrial plant lineages (Brundrett 2002; Wang & Qiu 2006). Based on dated evolution of host plants, EcM and orchid mycorrhizal as well as non-mycorrhizal plants have evolved more recently from AM-forming ancestors (Bruns & Shefferson 2004; Wang & Qiu 2006). ErM plants, in turn, evolved from EcM plants (Cullings 1996), but switched to new fungal partners with more efficient nutrient uptake from recalcitrant complex organic compounds (Read *et al.* 2004). Many common ascomycetous and sebacinoid (heterobasidiomycetes) root endophytes enter ErM with Ericaceae (Bergero *et al.* 2000; Selosse *et al.* 2007) and form similar associations with certain hepatics (Read *et al.* 2000; Selosse 2005). Thus, ErM can be alternatively viewed as a differentiated endophytic interaction.

In all types of mycorrhizal associations, autotrophic plants provide carbon to their fungi and receive dissolved nutrients in return. In fully or partially mycoheterotrophic (MH) plants the carbon flow is reversed and mycorrhizal plants function as parasites on fungi and overstorey trees, although they may provide additional benefits to their 'victims' (Bidartondo *et al.* 2000). Plant roots provide their symbiotic fungi a relatively stable habitat in soil that is infested with fungi- and detritivorous micro- and mesofauna. In addition to mineral nutrition, symbiotic fungi may enhance plant tolerance to environmental stress caused by low soil water potentials, toxic heavy metals, salinity, herbivores and root pathogens. Costs and benefits largely depend on mycorrhiza types, environmental conditions, species and genotypes involved (Smith & Read 1997).

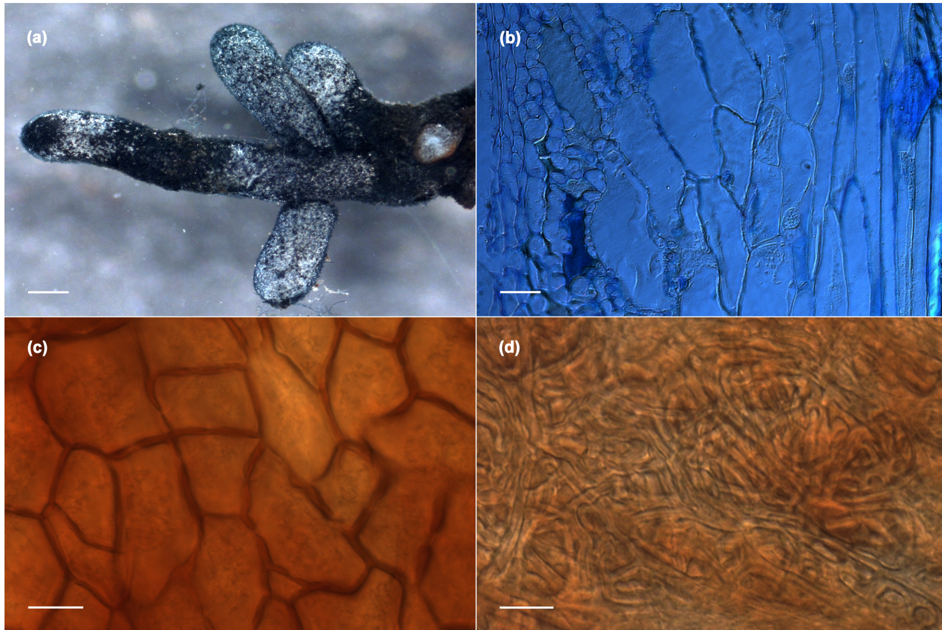

Figure 1. Ectomycorrhizas. (a) Plan view of ectomycorrhizal root tips (*Tomentella* sp. on *Quercus robur*); (b) Longitudinal section of an ectomycorrhiza formed by *Picea abies* and a mycobiont identified as *Ceratobasidium* sp. indicating fungal mantle, Hartig net and host root cells; (c) Structure of outer mantle layer of *Genea* sp.-*P. abies* ectomycorrhiza; (d) Middle mantle layer of *Endogone* sp.-*Pomaderris apetala* ectomycorrhiza. Bar, 0.2 mm (a) or 10 µm (b-d).

Ectomycorrhiza is the most conspicuous mycorrhiza type due to the presence of a fungal mantle that covers epidermal cells of host root tips (Fig. 1a). External mycelium takes up nutrients from soil solution and transports these *via* rhizomorphs (if present) through the fungal mantle to Hartig net, where nutrient exchange with the host occurs. Therefore, Hartig net has a highly differentiated structure with substantially increased contact area (Fig. 1b). Hartig net is often poorly developed or lacking, depending on both species and genotypes of host plants and fungi, and environmental conditions, particularly nutrition (Mikola 1965; Chilvers 1968; Ashford & Allaway 1982; Reddell & Milnes 1992; Smith & Read 1997). This indicates that the presence of a Hartig net is not an exclusive criterion of EcM symbiosis.

EcM symbiosis involves an estimated number of 8,000 species of higher plants and 7,000–10,000 fungal species worldwide (Taylor & Alexander 2005). EcM symbiosis is prevalent in boreal and temperate forests of the Northern Hemisphere involving the most important plant families, in particular Pinaceae, Betulaceae, Fagaceae, Salicaceae, *etc.* (Malloch *et al.* 1980; Brundrett 2002; Bruns & Shefferson 2004). Most Australian members of Myrtaceae, Rham-

naceae, Nothofagaceae and Leguminosae *p. parte* (in particular, *Acacia* and the Mirbelioideae) also form EcM that plays an important role in nutrient cycling in many ecosystems (Warcup 1980; Ashford & Allaway 1982; Reddell & Milnes 1992; Tommerup & Bougher 1999). In Africa, EcM-forming Dipterocarpaceae, Caesalpiniaceae and Uapacaceae form monodominant patches in the AM-dominated forests (Alexander & Lee 2005). Dipterocarpacae and Fagaceae also prevail in SE Asia. In neotropics, EcM Leguminosae, Gnetaceae, Nyctaginaceae, Polygonaceae and Dipterocarpaceae are concentrated mainly on poor sandy soils in the Rio Negro delta (NW Brazil) and Pakaraima mountains (Guyana and Venezuela; Moyersoen 1993, 2006; ter Seege *et al.* 2006). In boreal forests, EcM fungi deliver recent plant photosynthates to other soil microbes (Högberg & Read 2006) and comprise up to one third of soil microbial biomass (Högberg & Högberg 2002). EcM fungi most probably function similarly in tropical ectotrophic forests, where they are suggested to maintain the monodominance of EcM plants through effective nutrient capture from poor soils and recalcitrant litter, regulation of mast fruiting of their hosts and positive feedback to seedling establishment (Newbery *et al.* 1997, 2000, 2006).

Three phyla of fungi, Basidiomycota, Ascomycota and Zygomycota include EcM-forming lineages. Based on molecular phylogenies, approximately 32 lineages from most of the major orders of Basidiomycota (*sensu* Hibbett *et al.* 2007) have evolved EcM symbiosis independently from saprotrophic ancestors (Appendix 1 and references therein). Integrating fine-tuning of phylogenies and molecular belowground community studies will likely reveal additional independent lineages. Reversal to saprotrophic conditions has been demonstrated in EcM basidiomycetes (Hibbett et al. 2000), but unfortunately these analyses were based on biased taxa sampling (many important saprotrophic lineages were absent), incorrect assignment of trophic status (for example, *Leucopaxillus*) and questionable weighting of characters (Bruns & Shefferson 2004; Matheny *et al.* 2006). Moreover, Bruns & Shefferson (2004) suggested that saprotrophs lacked vacant niches, whereas EcM plants and fungi rapidly spread and radiated. Thus, evolution of other types of biotrophic interactions (e.g. lichenization, associations with insects and other soil microbes) from EcM symbiosis seem more probable than reversal to saprotrophic habit, although at present such evidence is restricted to the Boletales (Binder & Hibbett 2006). In Ascomycota, EcM associations have evolved in at least five classes (Pezizomycetes, Leotiomycetes, Loculoascomycetes, Eurotiomycetes and Sordariomycetes; LoBuglio *et al.* 1996; Vrålstad *et al.* 2000; Trowbridge & Jumpponen 2004). Most likely, several lineages of Pezizales and Helotiales have gained EcM habit independently (III; Appendix 1). Finally, a few species of *Densispora* (formerly included in *Glomus s.lato*) and *Endogone* (Endogonales) of the Zygomycota form EcM structures (Fassi *et al.* 1969; McGee 1996). Phylogenetic relations of these fungi, however, remain unknown. Due to abundant paraphyly and controversy in fungal taxonomy, I prefer

following phylogenetically supported EcM lineages instead of genera within orders (*sensu* Hibbett *et al.* 2007) in community structure analyses (Appendix 1).

Despite the wide taxonomic coverage of EcM lineages, only a few of these dominate in natural habitats. The *Russula-Lactarius* (Russulales) and *Tomentella-Thelephora* (Thelephorales) are particularly abundant both in terms of species richness and biomass on root tips in boreal and tropical forests (Kõljalg *et al.* 2000; Horton & Bruns 2001; Sirikantaramas *et al.* 2003; I, II, V; VIII). In temperate deciduous forests, *Sebacina* (Sebacinales) and members of Pezizales tend to co-dominate (Weiß *et al.* 2004; II, III; Smith *et al.* 2007), whereas the *Suillus-Rhizopogon* group of the Boletaceae-Sclrodermatacae (Boletales), *Amphinema-Tylospora*, *Piloderma* (Atheliales) and *Cortinarius* (Agaricales) form a substantial part of the boreal coniferous forest fungal community (Taylor *et al.* 2000; Horton & Bruns 2001; VII). Interestingly, MH orchids form obligate associations with a few species from the most common EcM fungal lineages (Taylor *et al.* 2002), indicating that high availability of potential symbionts might be selected.

Studies on ecology and physiology of EcM symbiosis are concentrated in Europe, North America and Australia (Smith & Read 1997). Early studies focused on mycorrhizal anatomy, distribution of mycorrhizal types among plant taxa, growth benefits and nutrition of seedlings following inoculation. Studies on nutrition and physiology of EcM fungi largely relied on pure cultures, which is a highly artificial and stressful state for EcM fungi. However, in my opinion, the most serious shortcoming of experimental studies was (and still is) the use of single strains of species (although in many replicates instead of replicating strains) and extending these results to species. Intraspecific variation in nearly all functions can be as high as interspecific variation (reviewed in Cairney 1999). It needs to be emphasized that much of this intraspecific variation is artefactual, resulting from poor taxonomic knowledge of physiologists and forestry researchers. Model species of EcM fungi, *Laccaria laccata*, *Paxillus involutus, Hebeloma crustuliniforme* and *Pisolithus tinctorius* comprise many functionally and ecologically different biological ('cryptic') species (Fries 1983, 1985; Aanen *et al.* 2000; Martin *et al.* 2002).

Since early 1990s, studies on the ecology and function of EcM fungi have been benefiting from the advent of molecular techniques utilizing DNA (Egger *et al.* 1991; Gardes *et al.* 1991; Henrion *et al.* 1992) and stable isotopes (Hög-berg 1990; Gebauer & Dietrich 1993; Taylor *et al.* 1997). These methods evol-ved rapidly to address fundamental questions about the community structure, host relations and nutritional mode of fungi and their autotrophic partners (see methods). In a few recent years, microarrays were introduced to microbial ecology, permitting large-scale studies on gene expression and detection of taxa from environmental samples (Martin 2001; Sessitsch *et al.* 2006).

2.2. Why study EcM fungi and their communities?

In temperate and boreal forests of the Northern Hemisphere, EcM fungi form diverse communities comprising hundreds of species in late-successional ecosystems (Richard *et al.* 2005; Walker *et al.* 2005; II; Ishida *et al.* 2007; Smith *et al.* 2007). Based on fruit-body surveys, certain Australian, Central African and SE Asian forests also support diverse communities of EcM fungi (Buyck *et al.* 1996; May & Simpson 1997; Watling *et al.* 2002; Riviere *et al.* 2007; Ramanankienana *et al.* 2007). All these species of EcM fungi are assumed to deliver the same basic benefits to their host plants. This raises a question, whether there is any difference if a plant is colonized by species A or species B, a single species or plenty of species – i.e. what is the level of redundancy, additivity and idiosyncracy (unpredictability) among EcM fungi? If we follow the concept of reciprocal parasitism, fungal species or even strains have differential positions in multidimensional mutualism-parasitism continuum (Johnson *et al.* 1997; Egger & Hibbett 2004). As shown in some recent studies, EcM fungi display different root colonization strategies and deliver species-specific costs and benefits to their host plants in terms of nutrition, protection, *etc.* (van der Heijden & Kuyper 2003). Moreover, species of EcM fungi differ in enzymatic activities in natural forest soils (Courty *et al.* 2005). Most likely, these differential functions have both anatomical (Agerer 2001) and phylogenetic background (hypothesized in I), further depending on host species and environment.

What might happen when such functionally different species are taken together? First of all, several species unsuited to a particular environment become extinct from the system due to competitive exclusion (in EcM fungi, Jonsson *et al.* 2001). In theory, if the remaining species functionally complement each other, there is an additive outcome, i.e. enhanced production, resistance to invasion, nutrient uptake, community level temporal stability and reduced losses *via* leaching, *etc.* (Tilman 1999; Loreau 2000). Indeed, ecological studies on various organisms provide strong evidence for such positive diversity effect on ecosystem functioning (e.g. Naeem *et al.* 1996; Tilman 1996; van der Heijden *et al.* 1998; Lyons & Schwartz 2001; Iason *et al.* 2005; Kiessling 2005). Compared to single species treatments, more diverse communities of EcM fungi enhance mycorrhizal root biomass (Baxter & Dighton 2001) and improve phosphorus nutrition particularly from complex organic compounds (Baxter & Dighton 2005). However, Jonsson *et al.* (2001) found idiosyncratic diversity effects of EcM fungal community that depend on both host species and soil type. In biodiversity-ecosystem function studies, the effect of diversity *per se* is masked behind 'sampling effect' – i.e. benefits of a diverse community depend on the presence of the most efficient member(s) (Wardle 1999; Loreau 2000). Whatever the actual mechanism is, diverse communities of EcM fungi in patchy soils have higher probability of including

the ‘best’ species in a species pool considering disturbance, next stages of succession and climate change. Thus, knowledge of the composition and function of EcM fungal communities provides essential information for understanding the effects of anthropogenic disturbance, climate change and nutrient dynamics at the ecosystem level.

2.3. Aims

The major aim of this thesis is to characterize of EcM fungal communities in natural ecosystems to provide background information for addressing anthropogenic impacts on natural communities in the future. So far, forestry-biased and phytocentric approaches and research funding, as well as technical ease, have resulted in much attention to forest nurseries and other artificial, monospecific plant communities that render inadequate for addressing the population dynamics and ecology of EcM fungi in natural ecosystems. This thesis also aims at developing fast, reliable and cost-effective methods for studying community structure, diversity and, as a future perspective, biogeography of EcM fungi. All case studies (I–IX) provided substance for iterative optimization of sampling, DNA extraction, PCR and sequencing protocols. Individual case studies complied in this thesis focus on the following basic aims:

- To establish the diversity and community structure of EcM fungi in natural or seminatural habitats in relation to soil variables and/or microsites, with emphasis on phylogenetic community composition (I, II, IV, V, VII–IX);
- To determine the importance of dead wood for EcM fungi (I, V, VII, IX);
- To discover new lineages of EcM fungi and to confirm the EcM status of fungal lineages that lack unambiguous information of trophic status (I, III, V, VI, VII);
- To determine the level of host specificity or host preference among EcM fungi (I, IV, V, VII, VIII);
- To uncover the trophic status and mycorrhizal partners of Pyroleae that possess many characters in common with MH plants (IV);
- To find belowground molecular evidence for biological invasions of EcM fungi (V).

3. METHODS: CONSTRAINTS AND IMPLICATIONS

3.1. Sampling

Depending on particular scientific hypotheses, all study sites were arbitrarily selected based on certain criteria (see I–IX). Root samples were usually taken using a sharp knife or flat, sharpened spade. Soil corers proved unapplicable due to the presence of coarse roots, stones, *etc*. Since 2003, our research group has been collecting soil samples of 15 x 15 cm to 5 cm depth (excluding litter layer), which includes both organic and upper mineral soil. Topsoil usually contains most of the roots and plays the most important role in nutrient cycling. Noteworthy, it is most unlikely that *preference* for deep soil horizons occurs among EcM fungi, because this likely impedes fruit body production (except in taxa possessing extensive rhizomorphs) and hence reduces reproductive success. Perhaps species 'preferentially' inhabiting deep mineral soil are competitively excluded from topsoil or acquire nutrients from both top and bottom layers. We prefer large cores over small ones for several reasons: i) reduced number of samples containing no roots and less heterogeneity in root biomass among samples (compare Yamada & Katsuya 2001; I); ii) greater choice of root tips from the same morphotype for further anatomotyping and molecular analyses; iii) more fungal species and more homogeneous species diversity resulting in less zeroes in data matrices and lower statistical variation. However, long-distance transportation from remote sites likely accounts for the major disadvantage of large samples.

Root samples are taken to a lab, cleaned from adhering soil and debris in buckets containing tap water, then cut into *ca.* 3-cm fragments or left intact, and transferred into Petri dishes with tap water. All roots (I, VIII, IX) or a random subsample of root fragments (n = 12–20, II, V, VII) are studied more carefully under a stereomicroscope. Subsampling usually reveals all EcM morphotypes within a core, except singletons that tend to be particularly numerous in diverse communities. Root tips are sorted into morphotypes based on colour, occurrence and abundance of cystidia, emanating hyphae and rhizomorphs (Agerer 1987–2002). Usually several root tips of each morphotype per root sample are anatomotyped following Agerer (1991a). The most important anatomical characters include the shape and size of cells in all 2–6 distinct mantle layers, the presence, shape and diameter of emanating hyphae, cystidia, clamp connections and rhizomorphs, and thickness of their cell walls. Features of cystidia and rhizomorphs are especially informative, providing reliable identification to the level of entire EcM lineage or a narrow group within a genus (Agerer 2006). Pale and brownish, more or less smooth morphotypes usually comprise several anatomotypes per sample (for example, *Tylospora* spp., *Thelephora* spp., *Laccaria* spp. and *Lactarius tabidus* in boreal coniferous forests; VII) that can be distinguished based on their anatomical characters. To

my experience, anatomotyping loses its value as an effective typing method when *ca.* 50 anatomotypes have accumulated, because closely related species look very similar. Nevertheless, anatomotyping provides hints for taxonomic affinities (Agerer 2006), which may be of importance for primer choice in molecular analyses (VIII; IX).

A single root tip from each anatomotype per sample is typically placed into 1.5 ml Eppendorf tube containing 0.1 ml CTAB DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% cetyl trimethyl ammonium bromide) and stored at room temperature, or frozen without liquids at –20ºC. The remaining root tips are usually stored at room temperature in tubes containing 0.5–1 ml CTAB buffer or 60% ethanol. Both substances retain the integrity of DNA, but distort the plan morphology (shape, colour) of EcM, especially in loose rhizomorphic types (e.g. *Cortinarius, Piloderma*). In tropical surveys, whole EcM root fragments can be preserved in 10 ml CTAB solution with no harm to DNA (V). However, morphotyping of CTAB-stored material is painstaking, because brown morphotypes fade in CTAB buffer and become indistinguishable from naturally pale types. In addition, CTAB is toxic and needs care when handled in large quantities.

Anatomotypes are compared between closely related samples (within a plot) and one or two representatives per plot are subjected to DNA extraction. Careful anatomotyping (but not morphotyping only!) is quite reliable on the scale of a few thousand square meters and molecular analyses confirm the results. However, on a larger scale, the same anatomotype most often comprises several closely related species. Based on anatomotyping, taxa possessing pseudoparenchymatous mantles (e.g. *Tomentella-Thelephora*, Pezizales) can be quite reliably separated to the species level. Conversely, species of the russuloid clade and taxa with plectenchymatous mantles (e.g. *Cortinarius, Hebeloma*; *Tomentellopsis*) are very difficult to differentiate.

3.2. Molecular techniques

Molecular PCR-based methods have strongly improved the understanding of mycorrhizal ecology, providing more reliable ecological results and correct identification of more species (reviewed in Horton & Bruns 2001). The substantial increase in the number of species recorded from mid- and late-successional ecosystems and a greater proportion of identified species compared to 'unknowns' certainly indicate the superiority of molecular methods in EcM community studies.

During the (r)evolution of molecular techniques, Restriction Fragment Length Polymorphism (RFLP) has been the most popular. Briefly, fungal DNA in EcM root tip is PCR-amplified and the product is further cleaved using two or more restriction enzymes. The resulting fragments are separated on an

agarose gel by fragment length. Each fragment (typically between one and four per restrictase) represents a single characteristic of the whole sequence that usually comprises 500–800 bp. However, agarose gels provide no resolution for fragments of less than 2–3% difference in length. This and the low total number of characters results in poor resolution among closely related species. On the other hand, a single nucleotide polymorphism or co-amplified 'contaminant' DNA potentially creates an additional RFLP type (Kåren *et al.*1997; Glen *et al.* 2001a). For example, in an EcM community study, Kennedy *et al.* (2003) obtained 168 distinct RFLP types. Of these, further sequencing revealed 48 artefactual types (from multiple PCR products) and only 56 distinct operational taxonomic units (OTUs).

More recently, terminal RFLP (T-RFLP) was introduced to EcM community studies (Zhou & Hogetsu 2002). In this technique, one or both of the PCR primers are labeled using a fluorescent marker. Cleaved PCR products are run on a sequencing gel and the position of marked end fragment(s) is automatically and more precisely recorded (detection limit ±1 base pair; Dickie *et al.* 2002; Avis *et al.* 2006; Dickie & FitzJohn 2007). The taxonomic resolution of T-RFLP is typically between 90 and 95% of sequence identity (Edwards & Turco 2005). Similarly to RFLP, T-RFLP disables identification of species unless precise matches to pre-identified fruit bodies are provided. Because agaricoid fruit bodies are easily found, whereas resupinate and hypogeous ones usually overlooked, only the former are potentially included as reference taxa. As fruit-body types are strongly determined by fungal lineage, such community fingerprinting approach may provide a strongly biased view of the community structure (similarly to the 'discrepancy' in fruit body and root tip surveys; Gardes & Bruns 1996).

Sequence analysis provides 50–200 times more characters compared to RFLP-based methods when utilizing the ITS region. If a DNA sample cannot be identified to species, phylogenetic analyses enable detection of its phylogenetic affiliations at higher taxonomic levels. Moreover, sequencing allows distinguishing true EcM fungi from contaminant fungi (Kennedy *et al.* 2003). Using T-RFLP, Dickie *et al.* (2002) suggested vertical niche differentiation among hyphae of 'EcM fungi', although only a few species were unambiguously identified as EcM. Moreover, the upper litter layer is known to harbour a diverse decomposer community (Lindahl *et al.* 2007). Extensive ITS sequence data from soil microeukaryotes (O´Brien *et al.* 2005) reveals that only around 10% of fungal OTUs derive from putative EcM fungi. Such large-scale sequencing analyses have become increasingly cost-effective due to falling prices and improved sequence quality. DNA sequences carry taxonomic and biogeographic information and provide high reproducibility, enabling comparisons of taxa between studies. Thus, sequencing of each RFLP type or anatomotype is highly informative in a long-term perspective.

Our research group has experimented several tube-based DNA extraction methods. A slightly modified protocol of a High Pure PCR Template Prepa-

ration Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, Indiana, USA; Appendix 2) has proven the most cost-effective, allowing amplification of >2000 bp of rDNA from fresh EcM root tips at nearly 100% success. In the future, we intend to skip the anatomotyping step and perform DNA extractions in 96-well plates.

One of the most important steps in molecular analyses is the choice of a suitable DNA region and primers. Suitable DNA region should be i) easily amplifiable (i.e. in several copies per cell and allow designing more or less universal primers); ii) variable enough to discriminate between closely related species and individuals; iii) conservative enough to allow broader scale phylogenetic and biogeographic analyses. The two latter criteria are contradictory, but are usually complemented in long DNA fragments comprising both encoding regions and introns. In particular, rDNA nuclear Internal Transcribed Spacer (ITS) region and flanking nuclear Large Subunit (nLSU; 26S rDNA gene) seem to fit all three criteria. Usually, the ITS region provides sufficient resolution to discriminate between sister species, whereas nLSU and 5.8S rDNA allow alignment of sequences from all fungal phyla. As an alternative to the ITS region, early studies employed mitochondrial rDNA Large Subunit (mtLSU; Gardes & Bruns 1996; Bruns *et al.* 1998). However, in many basidiomycete and ascomycete taxa, mtLSU cannot be amplified with the default ML5/ML6 primer set (Glen *et al.* 2001a; L.T. unpublished) and it provides poor resolution within a genus (except *Cortinarius*; Glen *et al.* 2001a). Barcoding of Life consortium suggested another mitochondrial region, Cytochrome c Oxidase 1 (CO1) for universal use. Both mitochondrial regions virtually lack well-annotated reference sequences of EcM fungi in public sequence databases.

Several universal and fungal specific primers have been developed for the ITS region that are widely used in fungal diversity studies (White *et al.* 1990; Gardes & Bruns 1993; Egger 1995; Glen *et al.* 2001b; Martin & Rygiewicz 2005). Because plants provide an important carbon source below ground, roots are a desireable habitat for many saprobes, parasites and endophytes. The DNA of these co-occurring organisms is often co-extracted and amplified during molecular analyses. Because success in amplification and sequencing is often taxonomically biased, restricted efforts in molecular identification likely result in biased view of the diversity and community structure as well. To reduce the risk of such biases, some additional taxon-specific primers were developed for problematic cases (short DNA fragments, mixed DNA of several fungal taxa, mismatching ‘universal’ primers) (Fig. 2). In nLSU, the region between 850 and 1150 base pairs is particularly suitable for taxon-specific primer design, because sequences within lineages are conserved, but tend to be differentiated among higher taxonomic levels. Of primers tested, I recommend a combination of a fungal-specific primer ITS1F (Garders & Bruns 1993) and universal primer TW13 (Taylor & Bruns 1999) for routine use on fresh root material. Moribound root tips and known basidiomyceteous EcM is best amplified using ITS1F and a

basidiomycete-specific primer, LB-W that amplifies all EcM basidiomycetes tested (contrary to ITS4B; Gardes & Bruns 1993)(Fig. 2; Appendix 3). LB-W excludes all ascomycetes, but amplifies Endogonales (Zygomycota). Because in certain taxa (some *Lactarius* spp., Sebacinales, Pezizales, Cantharellales), the ITS region cannot be amplified for unknown reasons (possibly due to large introns, secondary structure, polyploidization, ITS length polymorphism), their nLSU is amplified using a combination of universal primer Lr0R (Vilgalys & Hester 1990) and any fungal-specific primer, of which Lr5F and basidiomycete-specific LB-Y and LB-Z (Fig. 2; Appendix 3) perform best and retain specificity at all tested annealing temperatures between 52 and 58ºC.

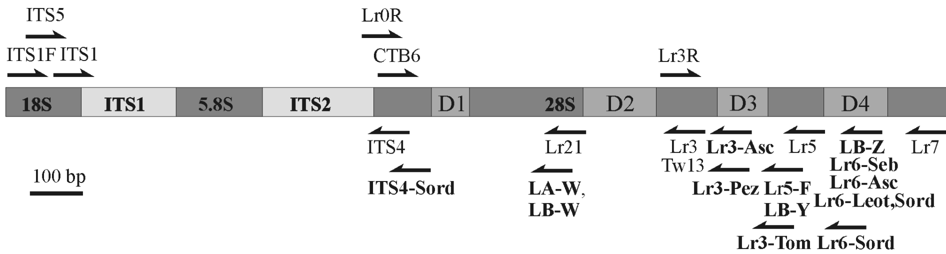

Figure 2. Map of rDNA primers used in this study. Primers in bold are newly designed. Major rDNA regions and domains are indicated.

Nevertheless, cloning of PCR products may be necessary from roots, soil and other environmental samples that contain multiple organisms or ITS copies (IV). PCR products of different sources are run on the gel, cut and cloned in a plasmid vector in *Escherichia coli*. Bacteria are propagated on agar media and colonies carrying inserts are detected by colour reactions. Then, DNA is re-extracted and re-amplified.

All single, more or less strong PCR products are purified using Exo-Sap enzymes (Sigma, St. Louis, Missouri, USA), which is one of the fastest methods with highest recoveries. In our lab, sequencing is routinely performed using primers ITS5 (White *et al.* 1990) instead of a more widely used ITS1, because the latter excludes *ca.* 10 base pairs of the ITS region in the sequencing chromatogram. ITS4 (White *et al.* 1990) and ctb6 (Taylor & Bruns 1999) are also used for sequencing the ITS region and nLSU, respectively. Sequencing is performed in Macrogen, Inc., Korea or MWG Biotech, Germany, with cost ranging from 3 to 10 EUR per reaction (in April 2007).

Raw sequences of typically 800–1000 base pairs are imported to Sequencher 4.7 software (GeneCodes Corp., Ann Arbor, Michigan, USA). Sequences are automatically aligned in contigs of 85–90% raw sequence identity and manually trimmed to exclude both the flanking 18S rDNA and low-quality 3' end. Unambiguous and false readings are detected by eye and edited manually based on the alignment. Poor quality sequences resulting from i) sequencing primer

mismatch; ii) mixed PCR products due to multiple fungal colonization of the root tips or air-borne contamination; iii) low purity DNA extract or PCR product are removed from sequence comparisons. Another DNA sample of the corresponding anatomotype is re-extracted, re-amplified and/or re-sequenced. Lack of any reliable signal from unsuccessfully sequenced samples is the major shortcoming in sequencing analyses, which is, again, taxonomically biased.

Sequences above certain level of identity are grouped and assigned into OTUs. This approach is termed 'DNA barcoding' (Floyd *et al.* 2002). The methodology has been used for a long time to define OTUs of bacteria. In bacteria, usually 97% sequence identity of the conservative 18S rDNA is used as a phylogenetic species criterion (barcoding treshold). Fungal species usually possess low local intraspecific ITS sequence variation (Kåren *et al.* 1997; Horton 2002). Understanding of this variation provides a basis for developing DNA barcoding thresholds (Will & Rubinoff 2004). On the other hand, barcoding itself may allow detection of cryptic species that often possess substantially different ITS regions. Subsequently, other methods can be used to reveal the biological meaning of such molecular diversification. The greatest problem of DNA barcoding lies in the unequal rate of evolution in the ITS (and any other) sequences in different fungal lineages. This has been attributed to the relative age of a lineage (Kåren *et al.* 1997), but additional information from other DNA loci may contradict such view (Glen *et al.* 2001a). Species of the *Cortinarius* and *Hebeloma-Alnicola* lineages possess highly similar ITS sequences, which also result in their poor taxonomic resolution (Aanen *et al.* 2000; Frøslev *et al.* 2005). In contrast, the *Inocybe, Genea-Humaria,* Boletaceae-Sclerodermataceae*, Cantharellus* and several others lineages possess a strongly divergent ITS region. Therefore, at least in theory, DNA barcoding tresholds should be generated separately for each EcM-forming lineage (II). Nevertheless, based on experimental sequence data, distinct, continuous OTUs usually display less than 2% ITS sequence variation, whereas sequence difference between the most closely related OTUs (the same morphological species) usually exceeds 4% (I, II, IV, V, VII, VIII) at a local scale. Thus, as a rule of thumb, 97±1% of sequence identity seems to fit the local sequence variation the best (see also Horton 2002). Similar DNA barcoding criteria are used in most other EcM community studies (Izzo *et al.* 2005; O´Brien *et al.* 2005; Parrent *et al.* 2006). However, Ishida *et al.* (2007) raised the value to 99% and demonstrated the presence of >200 OTUs in two mixed forests in Japan. Similarly to species concepts, barcoding thresholds are likely to blur when geographic distance and duration of isolation increases (Petersen & Hughes 1999; Sharon *et al.* 2006).

Analogous criteria are applied when comparing one's sequences to publicly available sequence data deposited in huge databases such as European Molecular Biology Laboratory (EMBL), National Centre of Biotechnology Information (NCBI), DNA Data Bank of Japan (DDBJ). These databases contain sequences from everything and everywhere and, again, biogeographic issues

arise. Sequence comparisons with public sequences can be performed fastest using BLASTn algorithm (Altschul *et al.* 1997) at NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST). However, sequences possessing less than *ca.* 90% identity to any other published sequence are not matched in their entire length using BLASTn searches. Similarly, BLAST algorithm removes 5' or 3' endings with slight sequence differences automatically from comparison. The report shows a match of the aligned region only (which may comprise only the extremely conservative 5.8S rDNA!), thus overestimating sequence similarity and potentially resulting in incorrect identification to species. As an alternative to BLASTn, similar queries using FASTA3 algorithm can be performed at EMBL homepage (http://www.ebi.ac.uk/fasta33/nucleotide.html). FASTA3 queries are strongly recommended, because they provide an alignment when sequences are >60% identical (e.g., when comparing sequences with poor-quality or from different orders), although it may take several minutes. In both algorithms, the goodness of a match depends on percent identity or similarity and the length of the aligned region. Thus, sequences that include both the ITS region and the more conserved, flanking nLSU are prone to match to another exceptionally long sequence (or a sequence comprising nLSU only) preferentially. Again, this may result in incorrect identification. Noteworthy, most fungal rDNA sequences in public databases comprise either ITS, partial nLSU or 18S rDNA.

Contamination by misidentified and chimeric sequences accounts for a major shortcoming of public sequence databases. Nilsson *et al.* (2006b) estimated that 10–20% of fungal sequences are poorly annotated and *ca*. 20% are probably misidentified. Invalid sequences tend to accumulate from taxonomically difficult taxa and result in further misnamed entries (e.g. the *Meliniomyces-Cadophora finlandica-Rhizoscyphus* complex; Hambleton & Sigler 2005). In biotechnology and food microbiology, such misidentifications may cause fatal outcomes. To overcome this problem in EcM research, Nordic-Baltic initiative created the UNITE database (http://unite.ut.ee/) that includes only well-annotated and vouchered specimens identified by taxonomists (Kõljalg *et al.* 2005). In April 2007, UNITE comprised 2511 ITS sequences from 1046 species of fungi (mostly EcM). The incorporated BLASTn algorithm takes a few seconds to provide results for a sequence query. Despite its relatively small size, UNITE has contributed 40% (II) to 90% (Clemmensen & Michelsen 2006) of the best sequence matches in recent Nordic EcM community studies. However, sequences from other continents are lacking in UNITE and are thus far better compared through EMBL (V; VIII; IX). Poor representation of saprobes and parasites forms another constraint of UNITE, because any queried sequence would result in EcM fungi as the best matches. This clearly aggravates the detection of 'contaminants'. Inclusion of well-annotated ITS sequences from saprobes, parasites, root- and soil-inhabiting fungi would alleviate these problems.

Unreliability in determining the relative abundance of species from environmental samples accounts for the major shortcoming of PCR-based molecular techniques. Species and genotypes differ in their ploidy level and copy number of genes; the DNA of certain species is preferentially amplified due to primer bias, differential sequence length and secondary structure (von Wintzingerode *et al.* 1997; see discussion in Kjøller 2006 for EcM fungi). Despite these biases, relative quantification of species is tempting. For example, Burke *et al.* (2006) compared careful root tip counts with T-RFLP peak area to quantify species' abundance. The authors reported significant linear regressions between the two methods and concluded that T-RFLP can be safely used for quantitative purposes. However, significant regressions were restricted to only three taxa out of around ten common taxa, where such comparisons were statistically reasonable. Even in these three taxa the slope of regression varied considerably (range, 0.44–0.77; Burke *et al.* 2006).

3.3. Stable isotopes

Utilization of stable isotopes, particularly ^{15}N and ^{13}C has strongly benefited the understanding of energy flow at the level of molecules to ecosystems (Dawson *et al.* 2002). Discrimination against heavier isotopes is common in many enzymatic, physiological and physical processes, e.g. photosynthesis, respiration and evaporation. EcM fungi are relatively enriched in ^{15}N and ^{13}C compared to saprobes and autotrophs (Gebauer & Dietrich 1993; Taylor *et al.* 1997) due to different N and C sources. Similarly, MH orchids and monotropes are enriched in ^{15}N and ^{13}C compared to autotrophic plants. Instead, the isotope concentrations of MH plants resemble these of their symbiotic fungi (Trudell *et al.* 2003; IV) that provide both C and N. Based on stable isotope concentrations and mixing models, heterotrophic contribution in hemiparasitic plants (reviewed in Press & Phoenix 2005) and orchids (Gebauer & Mayer 2003) can be estimated. We used these linear mixing models (Phillips & Gregg 2001) to uncover the trophic status of Pyroleae (for details, see IV). It needs to be emphasized that stable isotope concentrations are taxonomically biased within 'functional guilds' both in plants (Delwiche *et al.* 1978) and fungi (Taylor *et al.* 2003). This bias should be considered when choosing reference taxa and comparing across temporal and spatial scales (Taylor *et al.* 2003; IV). Ignoring these facts may lead to incorrect conclusions, especially when assigning trophic status to fungi.

3.4. Data analysis

Sequencing-based EcM community studies usually provide i) diversity (including richness, evenness and a plethora of indexes), ii) compositional (abundance, frequency or presence/absence of species) and iii) phylogenetic (sequence) data. Diversity data further enables species richness extrapolation and interpolation (see below). Diversity measures are usually compared using conventional statistics, e.g. regression, analysis of variance (ANOVA), *etc.* (Zar 1999). Studies that are solely based on anatomotyping and/or RFLP have produced quantities of such data and provided answers to the basic questions 'Is diversity of EcM fungi affected by…?'. Similarly, the quantitative or binary (presence/absence) data of most common species (or OTU) can be analyzed using conventional statistics, but usually transformation or use of nonparametric methods is inescapable due to the presence of informative zeroes. As pointed out above, the relative proportion of zeroes can be reduced by taking larger samples or by pooling small samples that result in less replication. Ironically, the more species one records, the less chance one has to obtain statistically significant results, because statistical corrections (e.g. Bonferroni correction) need to be introduced to reduce the familywise error rate associated with multiple testing. Statisticians have elaborated several more efficient, less conservative methods to reduce the amount of type II errors. For example, the *P*-value distribution-based sharpened Benjamini-Hochberg procedure both reduces familywise error rate and controls false discovery rate (Verhoeven *et al.* 2005), providing 2.5–3.7 times more significant results compared to classical Bonferroni correction in host preference analyses (VII; VIII). Yet, the possibility of committing type I error in all analyses equals α (Verhoeven *et al.* 2005). Unfortunately, such efficient procedures are rarely encountered in case studies published in peer-reviewed journals.

Interpolation (rarefaction) and extrapolation (minimal species richness estimates) facilitate comparisons of species richness as well as β- and γ-diversity in communities that are unequally sampled (Colwell & Coddington 1994). In both cases, individual-based and sample-based methods occur. EcM fungal communities are spatially strongly structured and infrequent species are difficult to detect on root tips and as mycelia (even when actually present in a sample; Burke *et al.* 2005). In addition, fungal *individuals* are difficult to determine (Taylor *et al.* 2002). Therefore, sample-based methods suit the best for EcM community studies (for contrasting opinion, see Taylor 2002) provided that these are taken from sufficient distance (at least 8 m apart; Lilleskov *et al.* 2004).

Rarefaction provides a powerful alternative for species richness comparisons between sites and studies when more or less similar sampling protocols are used. Rarefaction enables biodiversity comparisons by interpolating randomized species accumulation curves to the same sample size and provides support by

calculating confidence intervals (Gotelli & Colwell 2001; Taylor 2002; Colwell *et al.* 2004).

Extrapolation is defined as estimation of the unseen part of the community based on the observed diversity patterns. Extrapolation methods facilitate comparisons between sites and studies that employ different sampling schemes (Colwell & Coddington 1994; Gotelli & Colwell 2001; Bohannan & Hughes 2003). Nonparametric estimates such as Chao2, Jackknife2 and ACE consider the amount of rare species in the selection and usually provide more precise results compared to extrapolations from rarefaction curves and parametric estimates (Colwell & Coddington 1994; Melo & Froelich 2001; Walther & Moore 2005). Note that these methods have been elaborated based on animal and plant communities that can be relatively exhaustively sampled. In contrast, most of the species actually present have likely remained undetected in fungal communities of natural ecosystems. This likely results in further underestimates of the total species richness, because the reliability of estimates depends on sampling effort and accuracy (Colwell & Coddington 1994; Melo & Froelich 2001). In parts of this thesis, I used Chao2 and Jackknife2 estimates (II, V, VIII) and ACE (VIII) as implemented in EstimateS (Colwell 2006).

Compositional data is best analyzed using various ordination methods, the choice depending on hypotheses and software. For EcM fungal community ordination, data from several root samples usually require pooling (e.g. by plot; I; II; V; VII), because each sample comprises too small subset of the total community and includes abundant noise (species present, but undetected). Abundance and frequency data of EcM fungi, in spite of transformations, tend to perform poorly compared to binary data. This may at least partly stem from the underlying spatially clumped distribution of EcM fungi that, in turn, results in poor correlation between the observed and actual abundance (or frequency). Ordination results usually provide some implications whether the community composition as a whole changes and which factors account for most of the variation. Ordination itself proves nothing, because most methods lack relevant statistical testing and alternative ordination methods or distance algorithms may produce contrasting results. Similar problems apply to interpretations of treatment or soil variable effects on individual species that are scattered around the ordination diagram. Species' position relative to axes and factors provides a fertile ground for developing new hypotheses that could be subsequently experimentally tested. Based on some experience, Detrended Correspondence Analysis (DCA) and Canonical Correspondence Analysis (CCA) are among the most consistent and useful ordination methods for indirect and direct gradient analysis, respectively. I have used PC-ORD ver 4. (McCune & Mefford 1999) or CANOCO ver. 4.5 (ter Braak & Šmilauer 2002) throughout this thesis, although more sophisticated (and demanding) programs exist.

As stated above, only sequencing produces relatively unbiased compositional and phylogenetic data in EcM fungal communities. Sequences enable further complicated analyses on phylogenetic niche differentiation (sensu Webb

et al. 2002; Martin 2002; in fungi: Schadt *et al.* 2003) and biogeographic relations. Correct phylogenies rely on accurate sequence alignments. For primer design and phylogenetic analyses, our research group has routinely used automated sequence alignment as implemented in MAFFT ver. 5.861 (Katoh *et al.* 2005), followed by manual corrections. Neighbour-Joining and Parsimony-Bootstrap analyses are performed in PAUP 4.0 (Swofford 2002). Substitution models for Neighbour-joining and Bayesian analyses (Mr. Bayes 3.1.1; Ronquist & Huelsenbeck 2003) are inferred from Mr. Modeltest (Nylander 2004). Note that in this thesis, cladistic methods are used to infer phylogenetic placement of EcM fungal taxa rather than reconstructing the phylogeny.

4. RESULTS AND DISCUSSION

Results regarding optimization of sample preparation and molecular techniques are integrated to the methods section. The scientific results are discussed in detail in case studies (I–IX) and are briefly compiled below:

- **Forest microsites (decayed wood, windthrow mounds, pits and undisturbed forest floor) affect the community structure and frequency of individual species of EcM fungi (I, VII, IX, but no evidence in V).** Most microsites have developed due to disturbance and thus provide fertile ground for secondary succession. Therefore, species most easily spread and/or most tolerant to specific, stressful conditions can establish and survive competition. Decayed wood differs in nutrient concentrations and physical features compared to humus and mineral soil (Harvey *et al.* 1978), which probably alter the competitive balance of species and hence shape the community structure. **In addition, brown-rotted spruce wood and white-rotted birch wood differ in the fungal community composition**. The lower fungal diversity in both types of decayed wood compared to other microsites and greater dominance of certain resupinate-fruiting species leads to hypothesize either competitive superiority of resupinate fruit body type in dead wood, strong priority effects, competitive exclusion due to substrate preference or differential efficiency in spore dispersal. The three latter hypotheses most plausibly explain the observed pattern (VII; IX). Due to sampling design, low replication and neglection of humus horizon in study I were likely the greatest shortcomings, because humus and CWD form subsequent stages of forest floor development and both substrates share many fungal species (Goodman & Trofymow 1998).
- **Host preference rather than specificity is common among the dominant fungal species in mixed forest ecosystems (VII, VIII).** Many species may display host preference that can be attributed to genetic compatibility, preference for particular root exudates or soil conditions generated by stem flow or litter characteristics (II; VIII; Dickie 2007). Note that plants that host many *specific* fungi (*Alnus* spp.; *Pisonia grandis;* certain Pinaceae associated with suilloids) were not included in these studies. Nevertheless, previous research has documented little host preference in EcM fungal communities (Horton & Bruns 1998; Horton *et al.* 1999; Kennedy *et al.* 2003; Richard *et al.* 2005; Nara 2006; Ishida *et al.* 2007 (supplementary data re-analysed at the genus level using Fisher's Exact tests, but see alternative interpretation in Dickie 2007)). True specialists of other organisms are usually infrequent, S-selected taxa (Lomolino *et al.* 2006). Current small sample sizes aggravate addressing these questions for rare species. Experimental studies employing culturable members of the community provide a good alternative to address these questions. The finding of substantial host

preference in a Tasmanian wet sclerophyll forest (VIII) clearly deserves more research.

- **There is little evidence for the effect of soil nutrients on structuring the EcM fungal communities (II).** This result has to be, however, interpreted with caution, because quite a large spatial scale was studied, but nutrients are patchily distributed over smaller spatial scales. On the contrary, previous studies have demonstrated that particularly nitrogen gradient drives the community composition of EcM fungi both in polluted and natural eco-systems (Lilleskov *et al.* 2002; Agerer & Göttlein 2003; Avis *et al.* 2003; Toljander *et al.* 2006). However, care is needed with the interpretation, because many chemical and physical variables (both addressed and un-studied) can be strongly inter-related, rendering the true causal mechanisms uncertain.
- **Management of a wooded meadow alters community composition of EcM fungi (II).** Mowing and coppice cutting removes much of autotrophic biomass and exposes soils, thus altering chemical and physical soil conditions. The complex of these factors likely accounts for such influence.
- **Fungal genera such as *Membranomyces* (syn. *Clavulicium*, the *Clavulina* lineage, Cantharellales; I), *Humaria* (the *Genea-Humaria* lineage, Pezizles; I, III), *Tarzetta* (the *Tarzetta* lineage, Pezizales; III), *Tri-chophaea p. parte* (the *Sphaeosporella-Trichophaea woolhopeia* and *Wilcoxina* lineages, Pezizales; III), *Pachyphloeus* (the *Pachyphloeus-Amylascus* lineage, Pezizales; III), *Sarcosphaera* (the *Sarcosphaera-Hydnotryopsis* lineage, Pezizales; III) and *Coltriciella* (the *Coltricia-Coltriciella* lineage, Hymenochaetales; V, VI, IX) are demonstrated EcM for the first time. In addition, the genera *Clavulina* (the *Clavulina* lineage, Cantharellales; I, VII, VIII), *Boletellus* (the Boletaceae-Sclero-dermataceae lineage, Boletales; V), an unknown sordariomycete genus (Sordariales; V, IX), *Coltricia* (the *Coltricia-Coltriciella* lineage, Hymenochaetales; V, VI, VIII) and *Hydnobolites* (the *Hydnobolites* lineage, Pezizles; VIII) are confirmed to be EcM symbionts.** Similarly, recent molecular studies have also demonstrated the EcM lifestyle of *Sistotrema p. parte* (Nilsson *et al.* 2006a), *Otidea* (Toljander *et al.* 2006), *Hydnobolites*, *Marcelleina* and *Genabea* (Smith *et al.* 2007). Some reports solely based on stable isotope and radiocarbon signatures have proven erroneous or remained unproven (Hobbie *et al.* 2001, 2002), but nevertheless provide good working hypotheses for future *in situ* and experimental studies.
- **Pyroleae (Ericaceae) and orchids (Orchidaceae) comprise several partly MH (mixotrophic) species in Estonia. Their level of heterotrophy depends on a species and site (IV). Pyroleae spp. associate with many species of EcM and endophytic basidiomycetes and ascomycetes (IV).** Recently, supporting evidence for mixotrophy in Pyroleae was demonstrated in California and Germany (Zimmer at al. 2007). These authors observed

significant mixotrophy for nitrogen, which strongly contrasts with our results on carbon mixotrophy. The causal mechanisms for such discrepancy remain unknown, but may depend on time of sampling, sample storage and fungi involved. Similarly to Pyroleae, previous studies have indicated that several green orchids phylogenetically closely related to MH species are more or less mixotrophic (Gebauer & Meyer 2003; Julou *et al.* 2005; Abadie *et al.* 2006) and usually harbour many species of symbiotic fungi compared to a few closely related taxa in MH orchids (Taylor *et al.* 2002). The loss of photosynthesis in MH orchids coincides with tightened co-evolution with certain fungal taxa and development of host specificity, which potentially improves nutrient transfer from fungi to MH hosts (Bruns *et al.* 2002; IV).

- **Native EcM plants of Seychelles harbour a low diversity of symbionts and there is no evidence of fungal radiation among isolated stands and islands (V).** The low diversity and lack of radiation are attributable to the long-term isolation of Seychelles, formation of a continuous land mass during much of the Tertiary and/or recent deforestation. Alternatively, many of the symbionts may have gone extinct during the loss of habitat. The native EcM host trees were probably more widespread before settlement and intense deforestation (Fleischmann *et al.* 2003).
- **The introduced eucalypts can associate with native EcM fungi in Seychelles, whereas there is no such evidence for pines (V).** In contrast, Chen *et al.* (2007) reported no obvious host shifts of native fungi to the introduced eucalypts in South China. Compared to gymnosperms, eucalypts probably resemble other angiosperms physiologically more closely. The natural ranges of Myrtaceae, Dipterocarpaceae and Caesalpiniaceae overlap in lowland and submontane Southeast Asia, Indonesia and Papua New Guinea, whereas Pinaceae are restricted to montane habitats in Sumatra and SE Asia. The results may be artefactual, because pines formed symbiotic associations with host-specific taxa before the introduction as containerized seedlings and native fungi may have been competitively inferior on roots and acidic litter of conifers. Eucalypts, on the contrary, were germinated in Seychelles, suggesting low chances of fungal co-introduction.
- ***Coltricia* and *Coltriciella* spp. form EcM on various trees with distinct morphology, permitting their recognition without using molecular techniques (VI).** Thus far, these taxa are not reported in EcM community studies (but see Thoen & Ba 1989). *Coltricia and Coltriciella* are known to form imperforate parenthesomes, but none of the previously described morphotypes possessing imperforate parenthesomes match the descriptions of these genera (Haug & Oberwinkler 1987; Buscot & Kottke 1990)
- **Decayed wood provides a regeneration niche for the most common EcM fungal species of boreal forests (*Tylospora fibrillosa*, *Tomentella sublilacina* and *Amphinema byssoides*; VII).** As discussed above, it remains unclear whether their high abundance in decayed wood is attributable to the

competitive superiority of their mycelium, priority effects, more efficient spore dispersal or improved germination rates.

- **Tasmanian temperate wet sclerophyll forest harbous a high diversity of EcM fungi,** which is comparable to boreal and temperate forests of the Northern Hemisphere. **Most of the common fungal species were significantly more frequent on certain host species.** Such host preference probably contributes to the high species richness. **A monospecific old-growth forest of *Nothofagus cunninghamii* hosted a less diverse community in Victoria, Australia (IX).**
- **The *Tomentella-Thelephora*, *Russula-Lactarius*, *Cortinarius* and *Inocybe* are among the most species-rich lineages in most EcM fungal communities throughout the world (I; II; V; VII; VIII; IX)** corroborating the results from boreal and temperate forests of the Northern Hemisphere before 2001 (Horton & Bruns 2001) and thereafter (Lilleskov *et al.* 2002; Kennedy *et al.* Bruns 2003; Richard *et al.* 2005; Walker *et al.* 2005; Toljander *et al.* 2006; Ishida *et al.* 2007; Smith *et al.* 2007) and in tropical ecosystems (Sirikantaramas *et al.* 2003). Despite the large-scale phylogenetic similarity that probably results from the ancient origin of EcM lineages, certain lineages are pronouncedly over- or underrespresented in these ecosystems. For example, the *Descolea* lineage is particularly common in Australia (VIII; IX), but never observed in root tips surveys in the Northern Hemisphere. Replication of sites is urgently needed to prove these patterns, because the relative frequency of fungal lineages most probably depends on environmental variables in addition to biogeographical constraints.
- **Fungal taxa and lineages that are abundant in dead wood in the Northern Hemisphere (*Tomentella sublilacina* group; *Tylospora-Amphinema*) seem to be lacking or very rare in Australian temperate rain forest (VIII, IX).** This may explain the relatively higher diversity of fungi and different community composition on seedlings in dead wood in Australia (IX) compared to Estonia (VII).

5. CONCLUSIONS

5.1. Scientific conclusions and hypotheses

- Decayed wood provides an important substrate for root growth and a niche for certain EcM fungal taxa in boreal coniferous forests (I, VII). Similarly to EcM trees, decayed wood provides a safe site for regeneration of the dominant EcM fungi in boreal coniferous forests (VII).
- Management of a wooded meadow may alter the EcM fungal community composition (II), although confirmative studies and addressing direct causal agents are required.
- Pezizales (Ascomycota) comprise many EcM-forming lineages that were previously considered saprobic (III). These lineages may follow different ecological strategies. Several pezizalean lineages are particularly abundant in early successional ecosystems, especially after burning. EcM habit seems to be a precondition for the development of hypogeous fruiting and subsequent radiation of species both in ascomycetes and basidiomycetes (III).
- Molecular techniques provide evidence for the presence of additional EcM-forming taxa that may be especially abundant in poorly studied tropical ecosystems (V; VI).
- Fruiting habit on dead wood does not exclude a fungal species being EcM (I, III, V, VI).
- Members of Pyroleae are mixotrophic. The extent of heterotrophy depends on species and site (IV). Similarly to Arbutoideae, Pyroleae display low fungal specificity, but their functional compatibility should be addressed experimentally.
- Mixotrophy in Pyroleae and green orchids related to MH species suggests that this nutritional mode may be more common among forest understorey plants, particularly in tropics (IV).
- Seychelles support relatively low diversity of native EcM fungi (V), which is in agreement with the general island biogeography theory (Lomolino *et al.* 2006).
- The ability of association with indigenous fungi may enhance invasibility of eucalypts in exotic habitats (V).
- 'Preference' for forest microsites may be an important driver of EcM fungal community composition and overall species richness (I, VII, no evidence for microsite preference in V or for soil horizon preference in II). This likely depends on the importance and differentiation of microsites and soil horizons in particular ecosystems, and species pool of EcM fungi.
- Diversity of EcM fungi in Australian temperate rain forests resembles that of temperate and boreal regions of the Northern Hemisphere (VIII, IX).

- EcM fungi display substantial host preference in a Tasmanian wet sclerophyll forest.
- Australian wet sclerophyll forests comprise the same fungal lineages that are present in the Northern Hemisphere (with a few exceptions). However, the *Cortinarius*, *Tomentella-Thelephora*, *Descolea* and *Laccaria* lineages dominate in Australian wet sclerophyll forests. Marked compositional differences of other lineages between the Australian two sites suggest that replication of sites is needed in less studied ecosystems and continents.

5.2. Technical conclusions

- Morphotyping and anatomotyping integrated with sequencing is a powerful tool in EcM fungal community studies (I, II, V, VII, VIII, IX). Sequencing provides a phylogenetic position from 95.7% (I) to 100% (V) of anatomotypes observed and facilitates recognition of obvious contaminants (3.4% (II) to 5.4% (V) of species on root tips in these studies).
- DNA barcoding criteria need to be developed, preferably for each EcM fungal lineage separately, by molecular taxonomists. Appropriate software has been developed (e.g. Schloss & Handelsman 2005).
- Construction of identification microarrays (‘phylochips’) is desireable, but these likely cannot handle thousands of species present in the local species pool, most of which have remained undescribed.

6. SUMMARY IN ESTONIAN

Ektomükoriisat moodustavate seente liigirikkus ja koosluste struktuur Eestis, Seishelli saartel ning Austraalias.

Sümbioos seente ja taimejuurte vahel ehk mükoriisa on laialt levinud kogu maailmas. Ektomükoriisa on peamine mükoriisa ehk seenjuure tüüp põhja-poolkera okas- ja segametsades, Austraalia poolkuivades sklerofüllimetsades ning paiguti ka Aafrika ja Lõuna-Ameerika troopilistes vihmametsa- ja savannikooslustes. Teaduse poolt on kirjeldatud ligikaudu 75 000 seeneliiki. Ektomükoriisat moodustavaid seeni arvatakse olevat ligikaudu 7 000–10 000 liiki, kusjuures mõnetuhande ruutmeetrisel maa-alal võib koos esineda mitusada seeneliiki. Minu doktoritöö eesmärkideks oli tuvastada ektomükoriisat moodustavate seeneliikide koosluse struktuur ja liigirikkus Eesti, Seishelli saarte (India ookean) ja Austraalia valitud taimekooslustes. Iga uurimus (artiklid I–IX) täitis lokaalse koosluse tasandil püstitatud lisa-eesmärke. Eestis läbiviidud uurimustöödes püstitasin järgmised alternatiivsed hüpoteesid: 1) seenekoosluse struktuur ja liigirikkus sõltuvad metsa mullahorisontidest (I; II) ja häiringute põhjustatud mikrobiotoobist (VII); 2) Tagamõisa puisniidu majandamine mõjutab seenekoosluse liigirikkust ja liigilist koosseisu (II); 3) kottseente selts *Pezizales*, mida peeti enamjaolt saproobideks, sisaldab palju ektomükoriisat moodustavaid seeneliike (III); ning 4) uibulehelised (*Pyroleae, Ericaceae*) saavad osa süsinikenergiast mükoriisat moodustavate seente vahendusel metsapuudelt (IV). Pea täielikult hävinud loodusliku taimkattega Seishellidel uurisime pärismaiste peremeestaimede *Vateriopsis seychellarum* (*Dipterocarpaceae*) ja *Intsia bijuga* (*Caesalpiniaceae*) ning sissetoodud võõrliikide *Eucalyptus robusta* (*Myrtaceae*) ja *Pinus caribea* (*Pinaceae*) ektomükoriisat moodustavate seente kooslusi, et tuvastada võimalikke peremeestaimede vahetusi ja võõr-seeneliikide invasiooni (V). Austraalias kontrollisime järgmisi hüpoteese: 1) niisket tüüpi sklerofüllimetsa ektomükoriisat moodustavatel seentel puudub peremeestaime eelistus, sest arvatavasti on eukalüptid (perek. *Eucalyptus, Myrtaceae*) ja perekonna *Pomaderris* (*Rhamnaceae*) liigid omandanud ektomükoriissed seened lõunapöökidelt (perek. *Nothofagus*; *Nothofagaceae*) kui Austraalia manner oli sademeterohke ja lõunapöögid domineerisid metsakooslustes (VIII); 2) lagupuidul idanevad lõunapöögi seemikud on seotud ektomükoriisat moodustavate seentega, mis on fülogeneetiliselt lähedased Eestis lagupuidul esinevate seeneliikidega.

Kõik tööd põhinesid juureproovide morfo-anatoomiliste tunnuste ja molekulaarsete meetodite kombineeritud rakendamisel. Ektomükoriisat moodustavad ja/või endofüütsed seened määrati tuuma ribosomaalse DNA ITS regiooni nukleotiidse järjestuse järgi liigi või perekonnani. Käesoleval hetkel on see täpseim seente taimejuurtest määramise meetod. Uibuleheliste uuringus mõõtsime nii seentel kui taimedel stabiilsete süsinik- ja lämmastikisotoopide kontsentratsioonid, et tuvastada uibuleheliste orgaanilise süsiniku päritolu (IV).

Doktoritöös selgus, et ektomükoriisat moodustavate seente kooslused on nii Eestis kui ka Austraalias äärmiselt liigirikkad. Tagamõisa puisniit on seejuures seniuuritud seenekooslustest maailmas kõige liigirikkam, mida tõenäoliselt põhjustab paljude peremeestaimede olemasolu ja mitmekesised mullastiku- ning valgustingimused (II). Seishellide ektomükoriisat moodustavate seente kooslused olid seevastu üsna liigivaesed, mida võib seletada nii kauaaegse eraldatuse kui ka looduslike koosluste hävimisega (V). Huvipakkuvaimaks avastuseks kujunes kohalike seente assotsieerumine sissetoodud eukalüptide, ent mitte mändidega. Taimejuurte seondumine mitte-omaste seentega võib soodustada nii võõrpuuliikide kui ka eksootiliste seeneliikide invasiooni looduslikesse kooslustesse (V). Seishellidel esinevad vähesed seeneliigid ei olnud spetsialiseerunud metsa mikrobiotoopidele, samas kui Eesti ja Austraalia liigiliselt mitmekesistele metsadele oli iseloomulik teatud substraaditüüpide (lagupuit, mättad jms.) ja mullahorisontide eelistus (I; VII; IX). Seened seltsidest *Thelephorales*, *Atheliales* ja *Sebacinales* olid Eestis palju arvukamad lagupuidus kui mineraalmullas (I). Euroopa okasmetsade kõige sagedasemad ektomükoriisat moodustavad seeneliigid *Tomentella sublilacina* (*Thelephorales*), *Amphinema byssoides s. lato* ja *Tylospora fibrillosa* (mõl. *Atheliales*) domineerisid hariliku kuuse (*Picea abies*) ja arukase (*Betula pendula*) seemikute juurtel lagupuidus. Seejuures *A. byssoides s. lato* eelistas peremehena kasejuuri ning kasvusubstraadina kase lagupuitu (VII). Ülalnimetatud kolm seeneliiki moodustavad lamatüvede alaküljel kuni mõne millimeetri paksuse koorikja (resupinaatse) viljakeha. Arvatavasti on resupinaatseid viljakehi moodustavate seeneliikide domineerimine lagupuidus olevatel mükoriissetel puujuurtel siiski juhuslik, sest Austraalias olid lagupuidus ülekaalus hoopis kübarseened *Laccaria* sp. ja *Descolea* sp. seltsist *Agaricales* (IX). Ektomükoriisat moodustavad seened olid Tasmaania sklerofüllimetsas tugeva peremeestaime eelistusega. See erineb põhja-poolkerast kus peremehe-spetsiifilisus on dominantsetel seeneliikidel vähelevinud (I;VII;VIII). Seente taksonid *Tomentella-Thelephora*, *Russula-Lactarius*, *Cortinarius* ja *Inocybe* domineerivad ektomükoriisat moodustavate seente kooslustes kogu maailmas, kuigi erinevatel kontinentidel ja kliimavöötmetes esineb teatud eripärasid. Uurimustööde käigus tuvastasime teadusele seitse uut ektomükoriisat moodustavat seeneperekonda (*Membranomyces, Coltriciella, Tarzetta, Pachyphloeus, Sarcosphaera, Humaria* ja *Trichophaea*), kelle eluviis polnud varem teada või keda peeti saproobideks (I; III; V; VI; VIII; IX). Uibulehelised ja orhideed omastasid Värska ja Saaremaa männikutes 10–68% süsinikust ektomükoriisat moodustavate seente kaudu, mis sõltus nii taimeliigist kui kasvupaigast. Uuritud uibuleheliste juuri asustasid nii ektomükoriisat moodustavad kui ka endofüüütsed seened. Kõige sagedamini esinesid uibuleheliste juurtel seeneperekonna heinik (*Tricholoma; Agaricales*) liigid. Mükoheterotroofsete ja poolparasiitsete taimede rohkus teatud kasvukohtades võib põhjustada metsakooslustele kõrget stressi. Viimane uuring lubab oletada, et ka muu varjulembese metsa alustaimestiku seas võib leiduda seente abil orgaanilist süsinikku hankivaid taimeliike (IV).

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Appendix 1. Independently evolved lineages of ectomycorrhizal fungi based on recent phylogenies. Minor genera of especially hypogeous-fruiting fungi are excluded. To compare, see also Trappe (1962), de Roman *et al.* (2005) and Agerer *et al.* (2006).

Lineage	Inclusive major genera	Order (following Hibbett *et al.* 2007)	References for EcM habit	References for phylogeny
Basidiomycota				
Entoloma	*Entoloma s. str.*	Agaricales	Loree *et al.* 1989; Agerer 1997	Matheny *et al.* 2006
Hygrophorus	*Hygrophorus s. str.*	Agaricales	Kropp & Trappe 1982	Matheny *et al.* 2006
Phaeocollybia	*Phaeocollybia*[1]	Agaricales	Trudell *et al.* 2004[2]; common opinion (e.g. Matheny *et al.* 2006)	Matheny *et al.* 2006
Descolea	*Descolea, Descomyces, Setchelliogaster*	Agaricales	Bougher & Malajczuk 1985	Peintner *et al.* 2001; Matheny *et al.* 2006
Catathelasma	*Catathelasma*[1]	Agaricales	Common opinion (e.g. Matheny *et al.* 2006)	Matheny *et al.* 2006
Laccaria	*Laccaria, Hydnangium, Podohydnangium*	Agaricales	Trappe 1962 and references therein	Matheny *et al.* 2006
Cortinarius	*Cortinarius, Rozites, Dermocybe, Thaxterogaster, Hymenogaster p. parte*	Agaricales	Trappe 1962 and references therein	Peintner *et al.* 2001; Matheny *et al.* 2006
Inocybe	*Inocybe, Auritella*	Agaricales	Trappe 1962 and references therein	Matheny *et al.* 2006
Hebeloma	*Hebeloma, Alnicola, Anamika, Hymenogaster p. parte*	Agaricales	Trappe 1962 and references therein	Peintner *et al.* 2001; Matheny *et al.* 2006
Amanita	*Amanita, Torrendia*	Agaricales	Trappe 1962 and references therein	Matheny *et al.* 2006
Tricholoma	*Tricholoma*	Agaricales	Trappe 1962 and references therein	Matheny *et al.* 2006
Lyophyllum	*Lyophyllum p. parte*[1]	Agaricales	Kawai 1997; Agerer & Beenken 1998b	Matheny *et al.* 2006

Austropaxillus-Gymnopaxillus	*Austropaxillus, Gymnopaxillus*	Boletales	Palfner 2002	Binder *et al.* 2006
Boletaceae-Sclerodermataceae*	*Boletus, Xerocomus, Tylopilus, Suillus, Rhizopogon, Leccinum, Phylloporus, Boletellus, Strobilomyces, Alpova, Melanogaster, Paxillus, Gyrodon, Scleroderma, Pisolithus*	Boletales	Melin 1923[3]; Trappe *et al.* 1962 and references therein	Binder *et al.* 2006
*Amphinema-Tylospora**	*Amphinema, Tylospora*	Atheliales	Fassi & De Vecchi 1962; Taylor & Alexander 1991	affinities uncertain
Piloderma	*Piloderma*	Atheliales	Melin 1936[3]	affinities uncertain
Byssocorticium	*Byssocorticium*	Atheliales	Peyronel 1922[3]	affinities uncertain
Russula-Lactarius	*Russula, Lactarius, Gymnomyces, Zelleromyces, Macowanites*	Russulales	Trappe 1962 and references therein	Miller *et al.* 2006
Albatrellus	*Albatrellus, Polyporoletus, Byssoporia*	Russulales	Zak 1969	Miller *et al.* 2006
Coltricia-Coltriciella	*Coltricia, Coltriciella*	Hymenochaetales	Danielson 1984b; Thoen & Ba 1989; VI	Larsson *et al.* 2006
Tomentellopsis-Bankera	*Bankera, Boletopsis, Phellodon, Tomentellopsis*	Thelephorales	Agerer 1992; Kõljalg *et al.* 2002	U. Kõljalg *et al.* unpublished
Pseudotomentella	*Pseudotomentella, Polyozellus*[1]	Thelephorales	Agerer 1994	U. Kõljalg *et al.* unpublished
Tomentella-Thelephora	*Tomentella, Thelephora*	Thelephorales	Hacskaylo 1965; Danielson *et al.* 1984	U. Kõljalg *et al.* unpublished
Hydnellum-Sarcodon	*Hydnellum, Sarcodon*	Thelephorales	Agerer 1991b; Agerer 1993	U. Kõljalg *et al.* unpublished
Ramaria-Gautieria	*Ramaria p. parte, Gautieria, Gomphus, Turbinellus*	Gomphales	Agerer 1996; Dunabeitia *et al.* 1996	Hosaka *et al.* 2006
Geastrum-Radiigera	*Radiigera*[1], *Geastrum p. parte*[1]	Geastrales	Agerer & Beenken 1998a	Hosaka *et al.* 2006
Hysterangium	*Hysterangium, Mesophellia, Malajczukia, Nothocastoreum, Gummiglobus, Austrogauteria, Gallacea*	Hysterangiales	Ashton 1976; Molina & Trappe 1982	Hosaka *et al.* 2006

Cantharellus	*Cantharellus, Craterellus, Hydnum, Sistotrema p. parte*	Cantharellales	Danell *et al.* 1994; Nilsson *et al.* 2006a, Smith *et al.* 2007	Moncalvo *et al.* 2006
Clavulina	*Clavulina, Membranomyces*	Cantharellales	I; Buée *et al.* 2005	Moncalvo *et al.* 2006
Tulasnella	*Tulasnella p. parte*[1]	Cantharellales	Bidartondo *et al.* 2003	Moncalvo *et al.* 2006
Ceratobasidiaceae*	*Ceratobasidium p. parte*[1], *Thanatephorus p. parte*[1]	Ceratobasidiales/ Cantharellales (?)	Warcup 1991; Rosling *et al.* 2003; L.T., unpublished data	affinities uncertain
Sebacina	*Sebacina pp., Tremellodendron*	Sebacinales	Glen *et al.* 2002; Selosse *et al.* 2002; I	Weiβ *et al.* 2004
Ascomycota				
Otidea	*Otidea*	Pezizales	Toljander *et al.* 2006; Smith *et al.* 2007	Perry *et al.* 2007
Genea-Humaria	*Genea, Humaria hemisphaerica, Genabea, Gilkeya*	Pezizales	Fontana & Centrella 1967; Smith *et al.* 2006; III	Hansen & Pfister 2006; Smith *et al.* 2006; Perry *et al.* 2007
Wilcoxina	*Wilcoxina, Trichophaea hybrida (E-strain)*	Pezizales	Laiho 1965; Mikola 1965; III	Hansen & Pfister 2006; Perry *et al.* 2007
Sphaerosporella-Trichophaea woolhopeia	*Sphaerosporella brunnea, Trichophaea woolhopeia*	Pezizales	Danielson 1984b; III	Hansen & Pfister 2006; Perry *et al.* 2007
Geopora	*Geopora*	Pezizales	Fujimura *et al.* 2005; III	Hansen & Pfister 2006; Perry *et al.* 2007
Tarzetta	*Tarzetta*	Pezizales	III; Smith *et al.* 2007	Perry *et al.* 2007
Pulvinula	*Pulvinula tetraspora, P. constellatio*	Pezizales	Warcup 1990a; Amicucci *et al.* 2001	Perry *et al.* 2007
Tuber-Helvella	*Helvella, Tuber, Balsamia, Choiromyces, Labyrinthomyces, Barssia, Wynnella, Reddellomyces, Dingleya*	Pezizales	Palfner & Agerer 1998a; Weidemann 1998; Frank 2005; III; L.T. unpublished	O´Donnell *et al.* 1997;
Hydnotrya	*Hydnotrya*	Pezizales	I; III	Hansen & Pfister 2006
Leucangium	*Leucangium carthusianum*	Pezizales	Palfner & Agerer 1998b	Hansen & Pfister 2006

Pachyphloeus-Amylascus	*Boudiera, Pachyella, Scabropezia, Pachyphloeus, Amylascus*	Pezizales	Warcup 1990a; III	III
Peziza michelii-P. succosa	*Peziza succosa, P. succosella, P. michelii, P. infossa*	Pezizales	III; Smith *et al.* 2007	Hansen *et al.* 2005; III
Sarcosphaera-Hydnotryopsis	*Sarcosphaera, Hydnotryopsis*	Pezizales	III; Smith *et al.* 2007	Hansen *et al.* 2005; III
Marcelleina	*Marcelleina*	Pezizales	Smith *et al.* 2007	Hansen *et al.* 2005
Peziza-Terfezia	*Peziza whitei, P. depressa, Peziza badia group, Terfezia, Cazia, Tirmania, Ruhlandiella*	Pezizales	Warcup 1990a; Gutierrez *et al.* 2003; III	Hansen *et al.* 2005; III
Hydnobolites	*Hydnobolites*	Pezizales	Smith *et al.* 2007; VII	affinities uncertain
Elaphomyces	*Elaphomyces, Pseudotulostoma*	Eurotiales	Fontana & Centrella 1967; Henkel *et al.* 2006	LoBuglio *et al.* 1996; Henkel *et al.* 2006
Cenococcum	*Cenococcum*	uncertain	Lihnell 1942	LoBuglio *et al.* 1996
Meliniomyces bicolor	*Meliniomyces bicolor*	Helotiales	Vrålstad *et al.* 2000	Hambleton & Sigler 2005
Cadophora finlandica	*Cadophora finlandica*[1]	Helotiales	Wilcox & Wang 1987; Ursic & Peterson 1997	Hambleton & Sigler 2005
Unknown*	unnamed helotialean genera[1]	Helotiales	e.g. VII, VIII	affinities uncertain
unknown	unnamed sordarialean genus/genera	Sordariales	Trowbridge & Jumpponen 2004; Nara 2006; V; IX	V
Zygomycota				
Endogone	*Endogone, Sclerogone*	Endogonales	Fassi 1965; Warcup 1990b	affinities uncertain
Diversispora	*Diversispora*	uncertain	McGee 1996	affinities uncertain

* may comprise more than one independent EcM lineage;
[1] doubtful reports that require further evidence;
[2] based on isotope signatures;
[3] as cited in Cairney & Chambers (1999).

Appendix 2. Modified DNA extraction protocol using a High Pure PCR Template Preparation Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, Indiana, USA

- Frozen or fresh EcM root tips or fruit-body pieces are macerated in 1.5 ml Eppendorff tubes supplemented with 10 µl Tissue Lysis Buffer using bead beating for 2 min.
- 180 µl Tissue Lysis Buffer and 40 µl Proteinase K are added and mixed by shaking. The mixture is incubated at 55 ºC for 30–60 min.
- 190 µl Binding Buffer is added, mixed by shaking and incubated at 70 ºC for 10 min.
- 100 µl isopropanol is added.
- The solution is mixed by pipetting and transferred to High Pure Filtration tubes (placed in collector tubes) and centrifuged at 8000 rpm (4500 g) for 1 min.
- The collector tubes with flow-through are discarded. High Pure Filtration tubes are placed into clean collector tubes.
- 450 µl Inhibitor Removal Buffer is added and tubes are centrifuged at 8000 rpm for 1 min.
- The collector tubes with flow-through are discarded. High Pure Filtration tubes are placed into clean collector tubes.
- 500 µl Wash Buffer is added and centrifuged at 8000 rpm for 1 min.
- The flow-through from collector tube is discarded by decanting and 250 µl Wash Buffer is added, followed by centrifugation at 8000 rpm for 1 min and at 14 000 rpm for 10 sec.
- High Pure Filtration tubes are placed in 1.5 ml Eppendorff tubes and 200 µl preheated (70 ºC) Elution Buffer is added. Tubes are centrifuged at 8000 rpm for 1 min.

Appendix 3. Multiple alignments of published nLSU sequences for primer design. The alignment included available representatives from nearly all known EcM lineages (except Zygomycota) and most saprotrophic orders within basidiomycetes and ascomycetes. Note that sequences from heterobasidiomycetes, Saccharomycotina, Taphrinomycotina, lower fungi, animals and prokaryotes were not compared. Mostly AFTOL sequences were used due to length, accuracy and reliability considerations. Primer sequences are indicated in bold; * denotes matched sequences of ingroup and outgroup taxa (number of identical sequence types within taxa is shown in parentheses). Mismatched bases are indicated.

LB-W (calculated T_M = 60 ºC); when combined with ITS1F, specific to basidiomycetes at all tested annealing temperatures (52–58 ºC).

```
primer                                 5´-CTTTTCATCTTTCCCTCACGG-3´
Homobasidiomycetes, Sebacinales (92)      *********************
     Cantharellus cibarius (1)            ****************TG***
     Sistotrema confluens (1)             *****************G***
     Tulasnella (2)                       ******C**************
Ascomycota (101)                          *************GA****TC
Plants (6)                                *****************G***
```

LA-W (calculated T_M = 58 ºC); when combined with ITS1F, specific to ascomycetes at all tested annealing temperatures (52–58 ºC).

```
primer                                 5´-CTTTTCATCTTTCGATCACTC-3´
Ascomycota (98)                           *********************
     Pachyella (2)                        ***************T*****
Homobasidiomycetes, Sebacinales (92)      *************CC****GG
     Cantharellus cibarius (1)            *************CC*TG*GG
     Sistotrema confluens (1)             *************CC**G*GG
     Tulasnella (2)                       ******C******CC****GG
Plants (6)                                *************CC**G*GG
```

LB-Y (calculated T_M = 58º C); when combined with ITS1F, specific to basidiomycetes at all tested annealing temperatures (52–58 ºC).

```
primer                                 5´-TTTGCACGTCAGAATCGCTA-3´
Homobasidiomycetes, Sebacinales (61)      ********************
     Tulasnella pruinosa (1)              *C************C*****
Ascomycota (94)                           **************C****G
Pinaceae (2)                              ************T*******
Angiosperms (4)                           ************T******G
```

LB-Z (calculated T_M = 58 ºC); when combined with ITS1F, specific to basidiomycetes (incl. *Cantharellus*) at all tested annealing temperatures (52–58 ºC).

```
primer                                 5´-AAAAATGGCCCACTAGAAACT-3´
Homobasidiomycetes, Sebacinales (46)      *********************
     Cantharellus cibarius (1)            *G***C***********G***
     Craterellus (1)                      ****GC***********G***
     Amanita brunnescens (1)              ******************G**
     Calostoma cinnabarinum (1)           ******************G**
Ascomycota (35)                           ****************TGTTG
     Lecanoromycetes, Pezizales (29)      ****************T***G
     Trichoglossum, Geoglossum (2)        *****************AACG
     Plants (4)                           **************T*G*G**
```

LR3-Pez (calculated T_M = 62 ºC); specific to Pezizales and Lecanoromycetes; experimentally little tested.

```
primer                                     5´-CWTCRGGATCGGTCGATGG-3´
Pezizales (34)                                *A**A**************
     Scutellinia, Cheilymenia (2)             ********C*********T
     Morchella, Genea (7)                     *T**G**************
     Caloscypha (1)                           *G*****************
     Discinaceae (3)                          ********C**********
     Anthracobia (1)                          *G******C**********
Elaphomyces, Geoglossum (3)                   *C**A**************
Sordariomycetes, Helotiales (35)              ******************A
Lecanoromycetes (9)                           ******************R
Basidiomycota (62)                            RN**SRWTCW**G**YY*R
Plants (6)                                    GRTCAA*G****T**GCRR
```

LR5-Seb (calculated T_M = 60 ºC); specific to Sebacinales; experimentally little tested.

```
Primer                                     5´-ATTCGCTTTACCGCACAAGG-3´
Sebacinales (12)                              ********************
Basidiomycota (46)                            ******************AT
     Sistotrema, Hydnum, Clavulina (4)        ***************T***A
     Cantharellus, Craterellus (2)            ****************GCAA
Ascomycota (66)                               ******************AT
Pinaceae (2)                                  ************CG*T**AA
Angiosperms (4)                               ************CG*T*GAA
```

LR5-F (calculated T_M = 60 ºC); when combined with LR0r, specific to Asco- and Basidiomycota at all tested annealing temperatures (52–58 ºC).

```
Primer                                     5´-CGATCGATTTGCACGTCAGA-3´
Ascomycota, Basidiomycota (157)               ********************
     Tulasnella (2)                           ******G*C***********
Plants (6)                                    *******************T
```

LR3-Asc (calculated T_M = 57 ºC); when combined with ITS1F, specific to ascomycetes at all tested annealing temperatures (52-58 ºC); Often gives faint bands and may require removal of degenerative sites.

```
Primer                                     5´-CACYTACTCAAATCCWAGMG-3´
Ascomycota (74; incl Pezizales (26)           ***Y***********A**C*
     Pezizales (18)                           ***Y***********T**A*
Basidiomycota (62)                            ***TAC*K*NG*WS*G**RY
Pinaceae (2)                                  ***GC****G**C**T**GC
Angiosperms (4)                               ***W*****G*****T**TC
```

LR3-Tom (calculated TM = 60 ºC); specific to *Tomentella* and *Thelephora*; experimentally little tested.

```
primer                                     5´-CTACCGTAGAACCGTCTCC-3´
Tomentella, Thelephora (30)                   *******************
Tomentella fibrosa, T. crinalis               *******G***********
Thelephorales other (5)                       ******CG***********
Basidiomycota (62)                            ******CR**TW****A*A
Ascomycota (110)                              **CNTACTC**ATCCA**A
Plants (6)                                    *NCA*TCGA*C***T*S*A
```

ITS4-Sord (calculated T_M = 56 ºC); specific to Sordariomycetes; experimentally not tested.

primer	5´-**CCCGTTCCAGGGAACTC**-3´
Sordariomycetes (45)	*****************
Ascomycota (101)	**Y******A**R***T
Basidiomycota (95)	*********A*AR***T
Plants (6)	***C********G***T

LR6-Sord (calculated T_M = 58 ºC); specific to Sordariomycetes; experimentally not tested.

primer	5´-**GTTTGAGAATGGATGAAGGC**-3´
Sordariomycetes (40)	********************
Ascomycota (60)	**********A*G*T****W
Basidiomycota (70)	**********A*G*T****W
Plants (6)	**********A*G*CG***G

LR6-Leot,Sord (calculated T_M = 58 ºC); specific to Leotio- and Sordariomycetes; experimentally not tested.

primer	5´-**AAAATGGCCCACTAGTGTTG**-3´
Sordariomycetes, Leotiomycetes (60)	********************
Ascomycota (40)	****************AAC*
Basidiomycota (70)	***************A**CT
Plants (6)	*************T*G*G**

LR6-Asc (calculated T_M = 58 ºC); specific to Ascomycota, except Leotio- and Sordariomycetes; experimentally not tested.

primer	5´-**AAAATGGCCCACTAGTAACG**-3´
Ascomycota (40)	********************
Sordariomycetes (60)	****************GTT*
Basidiomycota (70)	***************A**CT
Plants (6)	*************T*G*G**

PUBLICATIONS

VII

Tedersoo L, Suvi T, Kõljalg U. Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of *Picea abies* and *Betula pendula*. Unpublished.

Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of *Picea abies* and *Betula pendula*

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ABSTRACT

Differential preference for forest microsites, soil horizons and host species are believed to contribute most to the high diversity of ectomycorrhizal fungi in boreal forests. However, little is known of ectomycorrhizal associates of seedlings establishing in mature forest ecosystems. This study aims at documenting the diversity and community composition of ectomycorrhizal fungi of Norway spruce (*Picea abies*) and silver birch (*Betula pendula*) seedlings in five dominant microsites in three Estonian old-growth forests. Undisturbed forest floor, windthrow mounds and pits harboured more species than brown- and white-rotted wood. Several species of ectomycorrhizal fungi were differentially represented on either hosts, microsites and sites. Generally, the most frequent species in dead wood were also common in forest floor soil. Ordination analyses suggested that decay type determined the composition of EcM fungal community in dead wood. Ingrowth of mature tree roots from below affected the occurrence of certain fungal species on seedlings in dead wood. This study demonstrates that ectomycorrhizal fungi differentially establish in certain forest microsites that probably depends on their dispersal and competitive abilities. Elevated microsites, especially decayed wood act as seed beds for both ectomycorrhizal forest trees and fungi, thus affecting the succession of boreal forest ecosystems.

INTRODUCTION

Seedling establishment and survival influence the continuity and succession of natural forest ecosystems (Gray & Spies 1997; Nakashizuka 2001; Christie & Armesto 2003). Most forest trees and shrubs germinate and establish preferentially in certain microsites, i.e. 'safe sites' that differ by plant species and depend on environmental conditions (Veblen 1989; St. Hilaire & Leopold 1995;

Gray & Spies 1997). Natural microtopography and disturbance, particularly windthrow and wild animal activities are responsible for the development of most forest microsites. Pits, root mounds and logs (termed 'coarse woody debris', CWD) emerge as a result of tree fall. Windthrow mounds and CWD are elevated above the forest floor and thus experience greater light availability and less litter accumulation, which prevents radicle penetration of small-seeded plant species. Moreover, disturbed microsites harbour sparse or no vegatation, resulting in reduced shoot and root competitition between tree seedlings, herbs and mosses, which account for greater seedling survival (Harmon & Franklin 1989).

CWD is considered the most important safe site in boreal and temperate forests (Harmon *et al.* 1986). CWD may harbour less fungal seed pathogens (O'Hanlon-Manners & Kotanen 2004) or damping-off fungi (Zhang & van der Kamp 1999) compared to forest floor soil. In addition, dead wood has higher moisture retaining capacity, temperature, softness and resistance to erosion (Harvey *et al.* 1978; DeLong *et al.* 1997) that improve root growth. In particular, higher moisture availability results in greater mycorrhizal root biomass in dead wood (Harvey *et al.* 1978). Due to high moisture content, nitrogen fixation occurs in well-decayed wood, depending on rot type, decay stage and fungal species involved (Larsen *et al.* 1978; Wicks *et al.* 2003). Recently, ectomycorrhizal (EcM) fungi were demonstrated to associate with N_2-fixing bacteria under decayed wood (Izumi *et al.* 2006). Thus, middle and later stages of CWD can be regarded as a long-term, slow-release nutrient source (Harmon et al. 1986).

CWD forms an important germination substrate particularly for EcM and ericoid mycorrhizal plants (McCullough 1948; Hofgaard 1993; Gray & Spies 1997), but also for certain rare orchids (Rasmussen & Whigham 1998) and liverworts (McCullough 1948; Kruys *et al.* 1998). This may be partly attributed to their mycorrhizal symbionts that are able to take up simple organic molecules (Read *et al.* 2004). Seedlings usually establish in moderately decayed wood, becoming rare with progressing decay due to litter accumulation (Christy & Mack 1984) and development of root competition with mature trees that reduces growth and survival of seedlings (Fleming 1984; Booth 2004). Mature EcM tree roots penetrate strongly decayed CWD from soil and form abundant EcM inside CWD (Harvey *et al.* 1978; Vogt *et al.* 1995; Tedersoo *et al.* 2003).

Community structure of EcM fungi on mature trees differs among forest microsites (Goodman & Trofymow 1998). In particular, CWD supports relatively high abundance of EcM fungi from the lineages *Piloderma, Amphinema-Tylospora* (Atheliales), *Sebacina* (Sebacinales) and *Tomentella-Thelephora* (Thelephorales; Goodman & Trofymow 1998; Tedersoo *et al.* 2003). Seedlings establishing on CWD are colonized by a few EcM fungi, especially an unidentified ,tan' morphotype and *Cenococcum geophilum* Fr. (Harvey *et al.* 1976, 1978, 1979; Christy *et al.* 1982; Kropp 1982).

This study aims at comparing the EcM fungal diversity and community composition on silver birch (*Betula pendula* L.) and Norway spruce (*Picea abies* (L.) H. Karst in disturbance-created microsites of three old-growth forests in Estonia. We hypothesize that EcM fungi display differential preferences for microsites and host species. The study further aims to establish the relative roles of dispersal and competition between EcM fungi in CWD. Using morphotyping and rDNA sequence analysis, we demonstrate that forest microsites differ in diversity and species composition of EcM fungi.

MATERIALS AND METHODS

Study sites and sampling

Three 10-ha sites were established in old-growth forests at Järvselja (geocode 58º16.8'N; 27º19.5'E), Rongu (58º01.9'N; 24º57.7'E) and Välgi (58º36.0'N; 26º50.1'E) in Estonia. These sites are influenced by storms that created abundant CWD, windthrow mounds and windthrow pits. All sites comprise *Vaccinium myrtillus*-type *P. abies* forests with occasional *B. pendula*, *Populus tremula* L. and *Pinus sylvestris* L. In addition, *Alnus glutinosa* L. and *Tilia cordata* L. form a subdominant component of the overstorey at Järvselja. Shrub layer consists of *Vaccinium myrtillus* L., *Oxalis acetosella* L. and *Rubus saxatilis* L. The dominant mosses include *Sphagnum* spp. and *Hylocomium splendens* (Hedw.) Schimp. Luvisol is the dominant soil type, with podzol affinities at Järvselja and Välgi. All sites experience mean annual rainfall of 550–600 mm and mean annual temperature of 5.5–6.0 ºC.

Seedlings of *P. abies* and *B. pendula* were observed most commonly on CWD, particularly brown-rotted spruce logs of decay classes III–V and white-rotted birch logs of decay classes IV–V (definition of decay classes follows Tedersoo *et al.* 2003). Mosses and liverworts had usually occupied these spruce logs, but not birch logs, because the latter possessed smooth, tough bark, which also seemed to prevent seed retention and root penetration from soil. Thus, tree seedlings were also occasionally found on birch logs. In contrast, seedlings of *P. abies, B. pendula, Sorbus aucuparia* L., *V. myrtillus* and *O. acetosella* were abundant on spruce logs. In addition, *T. cordata* and *A. glutinosa* frequently germinated on CWD at Järvselja. Windthrow mounds that supported tree seedlings were usually elevated 0.5–2 m above forest floor. Mounds comprised both humus and mineral soil that were densely covered by grasses, herbs and shrubs, especially *V. myrtillus, Urtica dioica* L. and *Rubus idaeus* L. Much of the soil was eroded from mounds when reaching *ca.* 7–10 years from disturbance, resulting in loss of most vegetation, including virtually all EcM tree seedlings. Windthrow pits were situated 10–30 cm below the forest floor surface on exposed mineral soil and rocks. Pits were usually waterlogged after rains and developed no vegetation for *ca.* 3 years from disturbance. Then,

mosses (*Sphagnum* and *Polytrichium* spp.), grasses, ruderal herbs (*U. dioica* and *R. idaeus*) and tree seedlings (especially the fast-growing *B. pendula*) emerged. In pits, seedlings of *P. abies* were scarce and occurred only when herbs were inabundant.

At each site, 2–4 seedlings of *P. abies* (2–6 years old) and *B. pendula* (1–4 y due to faster growth) were sampled from five forest microsites: i) undisturbed forest floor (control); ii) windthrow mounds; iii) windthrow pits; iv) white-rotted birch logs; and v) brown-rotted spruce logs. Large logs of other tree-decay type combinations were too infrequent to provide sufficient replication. To study the effect of root contact with mature trees to EcM fungi on seedlings, additional seedlings and roots of mature *P. abies* were sampled from CWD. In addition, roots of mature *P. abies* were sampled from CWD where seedlings were absent, to distinguish fungal species colonizing vegetatively from below. Seedlings were carefully pulled out of soil or CWD, whereas roots of mature trees were collected from samples of 15 x 15 cm to 5 cm depth.

Root samples were stored at 4 °C in plastic bags for up to five days until processed. Root systems were cut into 3-cm fragments. All root tips were assigned to morphotypes on 16–32 randomly selected fragments (depending on the size of a root system). Morphotypes were further separated into anatomotypes following Agerer (1991). Particular attention was paid to the occurrence of clamp to study the sexual state of mycelium in basidiomycetes. Clusters or single root tips of each morphotype were preserved in 60° ethanol and/or CTAB lysis buffer [100 mM TRIS–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB]. One or two root tips from each anatomotype per site were subjected to DNA extraction.

Molecular techniques

DNA extraction was performed using both a CTAB-based protocol (Gardes & Bruns 1996) and a High Pure PCR Template Preparation Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, Indiana, USA). The nuclear rDNA Internal Transcribed Spacer (ITS) and Large Subunit (nLSU) were amplified as described in Tedersoo *et al.* (2006b) using primers ITS1F (5' cttggtcatttagaggaagtaa 3') and TW13 (5' ggtccgtgtttcaagacg 3') or ITS4 (5' tcctccgcttattgatatgc 3'). PCR products were purified using Exo-Sap enzymes (Sigma, St. Louis, Missouri, USA). Sequencing was performed using primers ITS4 (5' tcctccgcttattgatatgc 3') and/or ITS5 (5' ggaagtaaaagtcg-taacaagg 3') for the ITS region and, in some cases, using ctb6 (5' gcatatcaataagcggagg 3') for the nLSU. A value of 97.0% ITS region identity (excluding flanking rDNA Small Subunit and nLSU sequences) was used as a molecular species criterion (barcoding treshold; Tedersoo *et al.* 2003). BLASTn and FASTA3 searches were performed against public sequence databases National Centre of Biotechnology Information (NCBI), European Molecular Biology Laboratory (EMBL) and UNITE to identify the EcM fungi and to

detect putative contaminants. All unique sequences retrieved from this study were submitted to the UNITE database (Kõljalg *et al.* 2005).

Statistical analyses

To compare species accumulation and richness estimates among microsites, rarefaction curves with 95% confidence intervals and minimal species richness estimates Jackknife2 and Chao2 were calculated using EstimateS ver. 8 (Colwell 2006). Fungal species data was binary-transformed and pooled by site and host species. Root systems of individual seedlings were used as sampling units and sampled randomly without replacement. In all analyses, three *Amphinema* species were pooled, because they were frequent, but anatomically indistinguishable.

Three-way mixed ANOVAs were performed to study the effects of host tree, site and microsite on species richness of EcM fungi (analysis 1). Host and microsite were used as fixed factors, site as a random factor, and age as a covariate. To test the effect of root connection with mature trees, all seedlings inhabiting CWD were subjected to a four-way ANOVA, where log type, host and root connection were defined as fixed factors, site as a random factor, and age as a covariate (analysis 2). Further, to test the effect of maturity (mature host *vs*. seedling), another four-way ANOVA was performed (analysis 3). Log type, maturity and root connection were used as fixed factors and site as a random factor. In analysis 3, seedlings of *P. abies* and *B. pendula* were pooled based on the results of previous analyses and the lack of mature *B. pendula* roots in CWD. In all three ANOVAs, interactions were initially included. Later, all non-significant interactions were successively removed to enhance statistical power. To determine competitive exclusion among EcM fungal species on seedlings in CWD, chi-square tests were performed. The observed frequency was compared to the expected frequency of co-occurrence in three most common fungal species. Fisher's Exact tests were used to determine the effect of microsite, host species, root connection, maturity and site on species frequency. Familywise error rate and false discovery rate were controlled as implemented in Verhoeven *et al.* (2005).

Using Canoco for Windows ver 4.53 (ter Braak & Šmilauer 2002), Detrended Correspondence Analysis (DCA) was applied to study the effects of microsites, host species and sites on seedling EcM fungal community composition. Factors were transformed to dummy variables to demonstrate the effect of each treatment level separately. Due to low species richness, all replicate seedlings of each factor combination were pooled and their relative frequency was used in the analyses. Using the same options, another DCA was used to study the effects of host maturity, host species, log type and site on EcM fungal comunities in CWD.

RESULTS

Anatomotyping combined with sequencing retrieved 86 species of EcM fungi from seedlings at three sites (Fig. 1.; Appendix). Eight of these species remained unamplified and were included based on their distinct anatomical characters. Sampling mature tree roots from CWD revealed 10 additional EcM fungal species.

Forest microsites differed in EcM fungal species composition (Fig. 1). *Cenococcum geophilum*, *Tomentella sublilacina*, *Amphinema byssoides s. lato* and *Meliniomyces bicolor* dominated the seedlings on forest floor. *Cenococcum geophilum, T. sublilacina, M. bicolor* and *Paxillus involutus* prevailed in windthrow mounds. Windthrow pits harboured *Lactarius tabidus* and *A. byssoides s. lato* most frequently. No other species occurred more than twice. *Amphinema byssoides s. lato*, *Tylospora fibrillosa*1 and *T. sublilacina* dominated the seedlings on white-rotted birch logs, whereas *T. sublilacina*, *T. fibrillosa1* and *L. tabidus* occurred most frequently on brown-rotted spruce logs. The frequency of eight out of 20 (40%) most common species was significantly biased to certain forest microsites (Fig. 1; Appendix). In addition, three out of 16 (19%) EcM fungal species occurred significantly more frequently in certain sites and five out of 12 (42%) species were significantly more frequent on either hosts (Fig. 1; Appendix). Of host-preferring species, *P. involutus* and *T. sublilacina* were more frequent on *B. pendula*, whereas *A. byssoides s. lato*, *T. fibrillosa*1 and *Trichophaea hybrida* were more common on *P. abies*. *Cenococcum geophilum* (Fisher's Exact test: $df = 1$; $P = 0.015$) and *Tomentella stuposa*2 ($df = 1$; $P = 0.029$) were significantly more frequent on roots of mature *P. abies* than its seedlings in CWD. *Tylospora asterophora* ($df = 1$; $P = 0.002$) and *T. stuposa*2 ($df = 1$; $P = 0.002$) were significantly more frequent on seedlings with root connection to mature trees compared to isolated seedlings in CWD. Conversely, the occurrence of root connection had no significant effect on EcM fungi colonizing mature host roots. Species pairs *T. sublilacina*-*A. byssoides s. lato* ($\chi^2 = 7.89$; $df = 1$; $P = 0.005$) and *T. sublilacina*-*T. fibrillosa*1 ($\chi^2 = 9.07$; $df = 1$; $P = 0.003$) co-occured significantly less frequently than expected in CWD. Except for inherently clampless taxa (e.g. *Piloderma, Russula, Lactarius, Sebacina, Tomentella p. parte*), basidiomycete species always formed clamp connections in all forest microsites, indicating the dikaryotic state of mycelium.

Based on DCA, forest microsite and host species affected the seedling EcM fungal community composition more than a site (Fig. 2a). The primary axis was strongly related to host tree effect. Nevertheless, the first two axes of DCA failed to separate the EcM fungal community based on pooled samples. When only CWD was included in the ordination, the primary and secondary axes separated fungal communities from white-rotted birch wood and brown-rotted spruce wood (Fig. 2b). The effects of site, host maturity and host species were of minor importance as judged from arrow length.

Cumulative species richness of EcM fungi was significantly lower on seedlings inhabiting CWD compared to other microsites (Fig. 3a). Similarly, the number of EcM fungal species per seedling was significantly lower in CWD (analysis 1: $F_{4,107} = 16.73$; $P < 0.001$; Fig. 4). There was no difference in cumulative species richness on seedlings of *P. abies* and *B. pendula* or between sites (not shown). Similarly, species richness per seedling did not differ among sites or host plants, but was significantly influenced by seedling age (analysis 1: $F_{1,107} = 0.002$; Table 1). Based on overlapping confidence intervals, root connection had no significant effect on cumulative species richness of EcM fungi in CWD (Fig. 3b). Conversely, individual seedlings connected to root systems of mature trees had approximately 60.2% more species of EcM fungi compared to isolated seedlings on CWD (analysis 2: $F_{1,70} < 0.001$). Cumulative species richness rarefied to 12 samples was respectively 2.48–3.37 and 1.15–1.59 times higher on mature tree roots compared to isolated seedlings and root-connected seedlings in CWD. The non-overlapping confidence intervals indicated that the cumulative species richness of EcM fungi was significantly higher on mature tree roots than isolated seedlings in spruce logs (Fig. 3b). Mature tree roots harboured significantly more species of EcM fungi per root sample/ seedling in CWD (analysis 3: $F_{1,103} = 31.4$; $P < 0.001$). There was also a highly significant interaction between root connection and host maturity (analysis 3: $F_{1,103} = 19.0$; $P < 0.001$; Fig. 5).

DISCUSSION

Forest microsites

The frequency of several common EcM fungal species differed among forest microsites, host species and sites on regenerating seedlings. The similar proportion of statistically significant differences suggests that the effects of microsite and host are comparable. On mature tree roots, species of EcM fungi ‘prefer’ certain forest microsites (Goodman & Trofymow 1998) and soil horizons in deep, strongly stratified soils (Rosling *et al.* 2003; Tedersoo *et al.* 2003). In open forests and woodlands, trees create soil nutrient patches or gradients *via* stem flow, litter fall, hydraulic lift and shading. These factors probably influence the EcM fungal community composition in relation to distance from mature trees (Deacon *et al.* 1983; Dickie *et al.* 2002 Cline *et al.* 2005; Dickie & Reich 2005). In addition, greater availability of mycorrhizal propagules and less stressful soil conditions account for higher fungal species richness and EcM colonization on seedlings establishing close to mature host trees (Alexander *et al.* 1992; Onguene 2000; Dickie *et al.* 2002; Cline *et al.* 2005; Dickie & Reich 2005). Thus, proximity, host age, host preference, soil horizons and disturbance-generated microsites form differential niches that

support the coexistence of hundreds of EcM fungal species (Bruns 1995; Dickie 2007).

The dominant EcM fungal species inhabiting various microsites in this study are among the most common members of EcM fungal communities in young and mature boreal coniferous forests in Northern Europe (e.g. Genney *et al.* 2006; Toljander *et al.* 2006; Korkama *et al.* 2007). Among these common species, *Cenococcum geophilum* and *Meliniomyces bicolor* occurred substantially more frequently on seedlings inhabiting forest floor and windthrow mounds compared to other microsites, and *C. geophilum* was more frequent on roots of mature *P. abies* than its seedlings. These two ascomycetes may have limited capacities to colonize elevated substrates, because they are hitherto known to spread only vegetatively. In windthrow mounds, however, both of these ascomycetes and the sclerotia-forming *Paxillus involutus* may have persisted as resistant propagules in soil or on root systems of dying hosts and seedlings that survived the windthrow.

Amphinema byssoides s. lato, *Tomentella sublilacina* and *Tylospora fibrillosa*1 were relatively frequent in all microsites, particularly in CWD. Noteworthy, *A. byssoides s. lato* dominated in white-rotted birch logs, whereas *T. sublilacina* prevailed in brown-rotted spruce logs, suggesting either differential preference or competitive exclusion. Indeed, these two species and *T. sublilacina-T. fibrillosa* co-occurred substantially less commonly than expected on seedlings in CWD. Interestingly, competitive exclusion was more pronounced among the distantly related *T. sublilacina* (Thelephorales) and species from the Atheliales (closely related genera, *Amphinema* and *Tylospora*) than among species of Atheliales. Low competition between *A. byssoides s. lato* and *T. fibrillosa*1 can be explained by their differential exploration type, i.e. abundance and extension of external hyphae and rhizomorphs (Agerer 2001). Such ‚avoidance' suggests either strong competitive interactions (Wu *et al.* 1999; Lilleskov & Bruns 2003) or colonization priority effects (*cf.* Kennedy & Bruns 2005) that are demonstrated in experimental microcosms. Direct outcompetition seems more plausible than colonization priority, because several EcM fungal species occurred in substantially biased frequency in different log types, whereas fungal spore banks are assumably similar in the two woody substrates. However, species of *Amphinema*, *Tylospora* and *Tomentella* form spatially overlapping fruit bodies with no obvious demarcation zones on the underside of CWD (U. Kõljalg, personal observation).

DCA ordination demonstrated that the EcM fungal communities differed between the two log types. Thus, properties of CWD affect EcM fungi at both species and community levels. In agreement with this study, relatively higher abundance of members of Atheliales, Thelephorales and/or Sebacinales were found in CWD compared to other forest microsites in boreal forests (Goodman & Trofymow 1998; Tedersoo *et al.* 2003). Most species of these orders form resupinate fruit bodies on the underside of dead wood. Resupinate fungi, as shown for *T. sublilacina* (Lilleskov & Bruns 2005), are dispersed by soil

microarthropodes that are especially abundant in litter and CWD. One could speculate that the higher frequency of resupinate fungi results from either more efficient dispersal or greater competitive abilities of their hyphae in CWD. While these effects may be important for fungal species inhabiting roots of mature host trees, they fail to explain the colonization pattern of isolated seedlings. Atheliales, Thelephorales, Sebacinales and many other resupinate taxa comprise tens to hundreds of species. Only a few of these frequently colonized seedlings on CWD at three distant sites, whereas many additional species from *Tomentella-Thelephora* and *Sebacina* lineages were observed on mature host roots in CWD (Appendix; Tedersoo *et al.* 2003). This suggests that other traits attributable to pioneer strategy, such as low critical spore concentrations or low carbon requirements (Newton 1992) may account for the dominance of these few fungal species on isolated seedlings in CWD. Indeed, *A. byssoides* and *T. fibrillosa* inhabit forest nurseries in Lithuania (Menkis *et al.* 2006), whereas *T. sublilacina* co-dominates on seedling roots in soil bioassays (Taylor & Bruns 1999). These three common species were also abundant on mature tree roots in CWD, but here it cannot be determined whether the symbionts were introduced from soil with ingrowing roots or acquired from germinating spores. The prevalence of *A. byssoides s. lato, T. sublilacina* and *T. fibrillosa*1 in both early and late successional habitats suggests a competitive strategy (*cf*. Grime 1977). EcM fungal species such as *Tylospora asterophora* and *Tomentella stuposa*2 were observed only on seedlings that had established root connections with mature trees. This phenomenon was initially described in a young *B. pendula* plantation and was ascribed to fungal successional stage (Fleming 1983, 1984). Thus, our results extend this phenomenon to old-growth forests and further suggest that elevated microsites provide a regeneration niche for species of both EcM trees and fungi.

The exclusive occurrence of clamp connections in dominant basidiomycete species in elevated microsites suggests that dikaryotic phase is a rule in EcM homobasidiomycetes in natural, disturbed habitats. These results corroborate similar observations from primary successional areas (Allen *et al.* 1992). However, the fact that all dominant homobasidiomycetes were in dikaryotic phase does not rule out EcM initiation by germinating haploid mycelium (Kropp *et al*. 1987; Debaud *et al.* 1988), rapid subsequent mating and dikaryotization. Nevertheless, dikaryotic mycelium and exclusive EcM colonization of all seedlings indicate the presence of abundant and viable spore bank in CWD and other elevated microsites.

Host preference

Several common EcM fungal species displayed host preference, although *specificity* was evident only in *Trichophaea hybrida* (Sowerby) T. Schumach. This ascomycete colonized exclusively spruce seedlings and is closely related to the pioneer *Wilcoxina* spp. that dominate conifer seedlings in forest nurseries

(Mikola 1965; Tedersoo *et al.* 2006a). Except for *Lactarius deterrimus*, no known host specific taxa were found among the rare fungal species. Similarly, Newton (1991) observed host preference rather than specificity among EcM fungi colonizing seedlings of *B. pendula* and *Quercus robur* L. It has been hypothesized that late successional ecosystems include more host-specific EcM symbionts compared to early stages (Horton *et al.* 2005; Ishida *et al.* 2007). Based on our results, this is clearly not the case in seedlings in old-growth forests.

Seedlings usually share their EcM symbionts with mature trees nearby (Alexander *et al.* 1992; Simard *et al.* 1997a; Jonsson *et al.* 1999, Matsuda & Hijii 2004). This enables the development of mycelial networks interconnecting plants from different species and developmental stages. Mycelial networks facilitate seedling establisment either *via* direct net carbon transfer (Simard *et al.* 1997b) or through mineral nutrition from the symbiotic network that is maintained by mature trees. Such mycelial networks drive forest succession by facilitating establishment of late successional plant species (Horton *et al.* 1999; Kennedy *et al.* 2003; Dickie *et al.* 2004; Nara & Hogetsu 2004; Richard *et al.* 2005). Seedlings incorporated in the common mycelial network have lower mortality (Booth 2004) or improved growth (Kranabetter 2005). Despite the presence of mycelial networks, root competition with mature trees is detrimental to seedlings (Booth 2004). In elevated microsites, root competition with mature trees is often lacking, but the extent of mycelial connection through exploring hyphae and rhizomorphs is not studied. Rhizomorphs of the fast-growing *Paxillus involutus* may extend at least two meters from nearest roots (Laiho 1970). Whether mycelium of EcM fungi proliferates inside CWD in the absence of roots remains unknown. If this were true, enhanced seedling establishment in elevated microsites could be ascribed at least partly to common mycelial networks and lack of direct root competition. The exploration of EcM mycelium and N_2 fixation in CWD clearly deserve further research to explain both seedling establishment and nutritional aspects of forest ecosystems.

In conclusion, seedlings on forest floor and elevated microsites are dominated by the most common EcM fungi of boreal forests. Differential competitive and dispersal abilities as well as host preference affect the distribution of EcM fungi on seedlings regenerating in forest microsites. White-rotted birch wood and brown-rotted spruce wood support different EcM fungal communities that probably results from chemical and physical differences influencing the competitive balance. Old-growth forests develop different forest microsites that, in turn, act as seed beds for both plants and their EcM symbionts, driving succession and maintaining the continuity of these ecosystems.

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TABLES AND FIGURES

Table 1. Probability values of main effects and statistically significant interactions of species richness of ectomycorrhizal fungi on seedlings.

Analysis/ effect	*df*	Value of a statistic	*P*-value
Analysis 1 (microsite)			
Site	n.a.*	$Z = 0.60$	0.276
Microsite	4	$F = 16.73$	<0.001
Host species	1	$F = 2.17$	0.144
Seedling age	1	$F = 10.50$	0.002
Analysis 2 (root connection)			
Site	n.a.	$Z = 0.50$	0.308
Log type	1	$F = 0.25$	0.617
Host species	1	$F = 1.96$	0.166
Root connection	1	$F = 13.86$	<0.001
Seedling age	1	$F = 4.63$	0.035
Analysis 3 (maturity)			
Site	n.a.	$Z = 0.56$	0.289
Log type	1	$F = 0.96$	0.407
Root connection	1	$F = 0.11$	0.745
Maturity	1	$F = 31.45$	<0.001
Maturity x root connection	1	$F = 18.98$	<0.001

*n.a., not applicable for random factors.

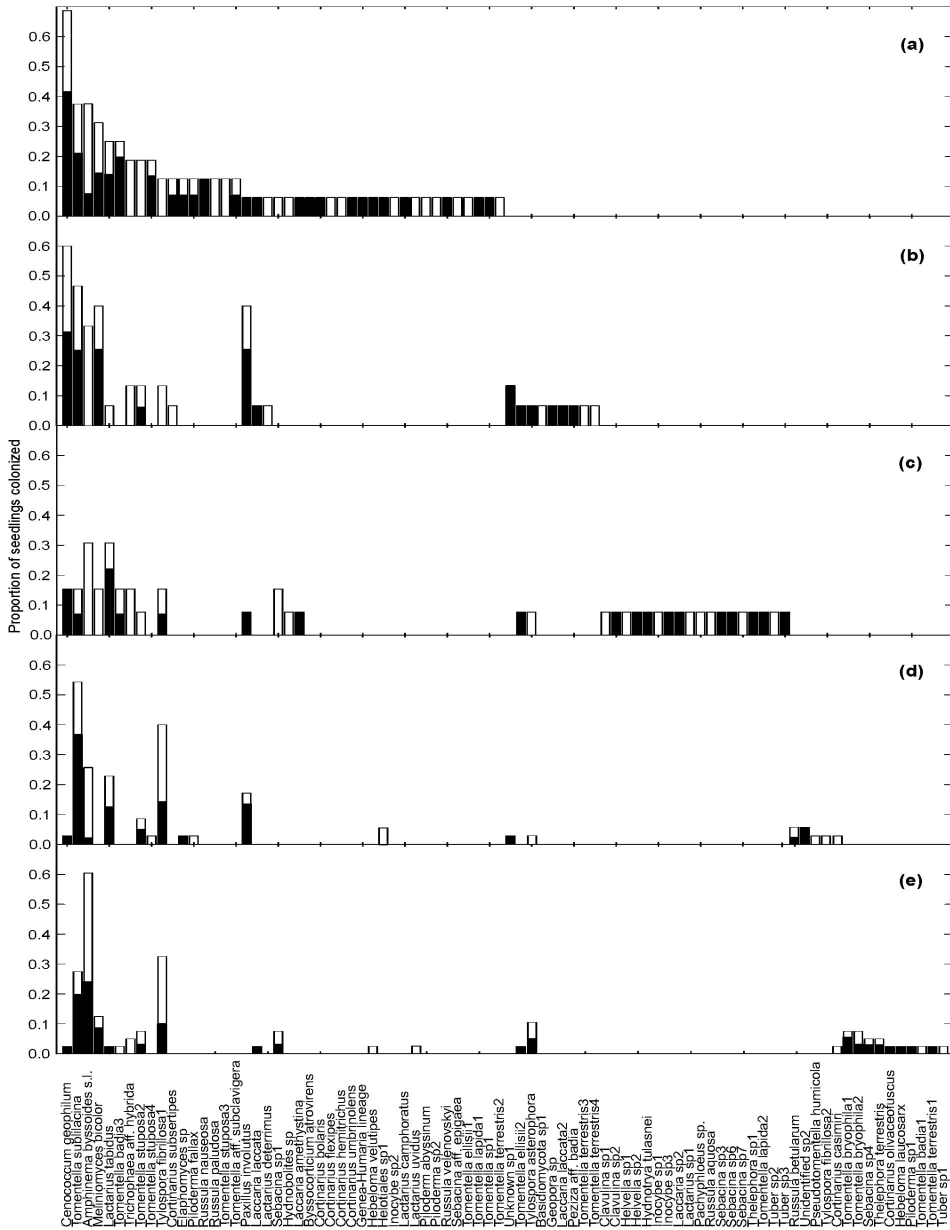

Figure 1. Relative frequency of ectomycorrhizal fungal species in forest microsites. (a) forest floor; (b) windthrow mounds; (c) windthrow pits; (d) brown-rotted spruce logs; (e) white-rotted birch logs. Open columns, weighted proportion of *Picea abies* seedlings colonized; filled colums, weighted proportion of *Betula pendula* seedlings colonized.

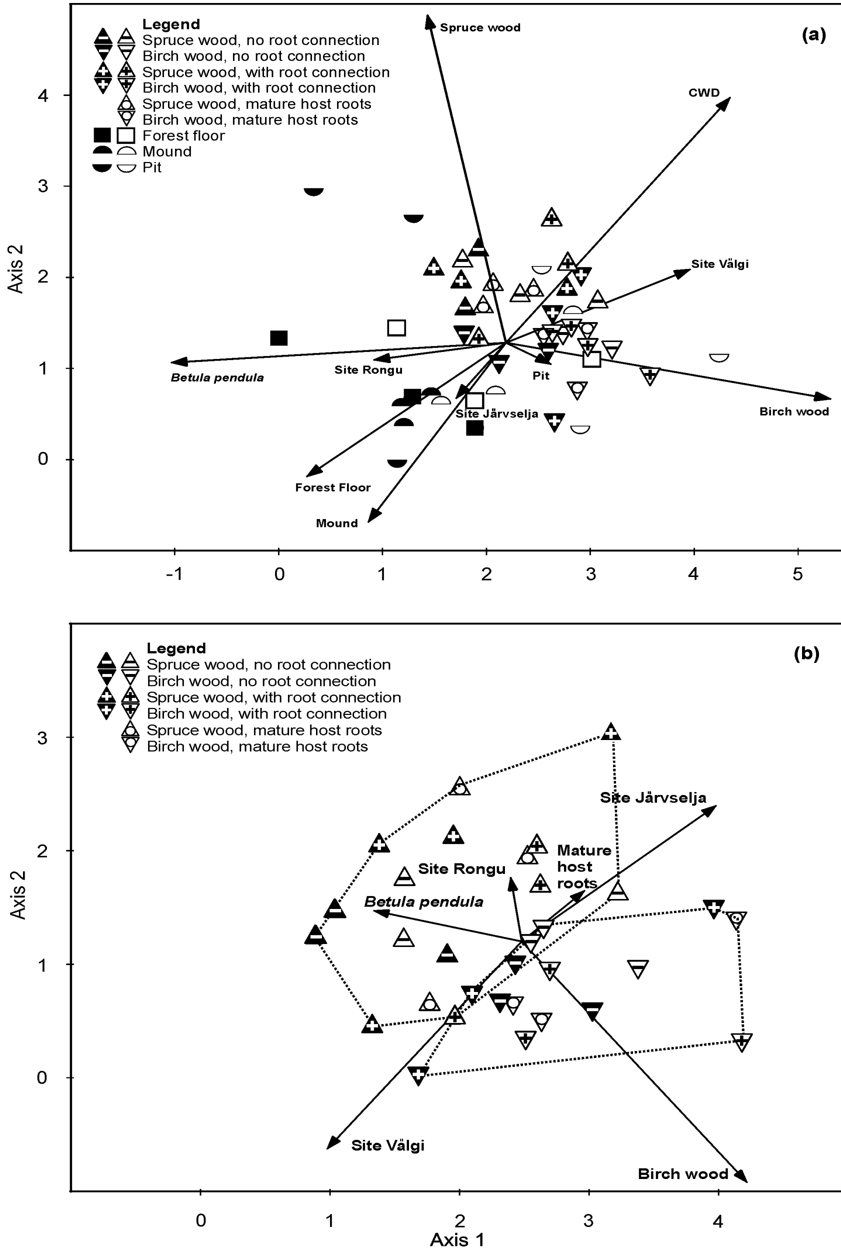

Figure 2. Detrended correspondence Analysis (DCA) demonstrating the relative effects of microsites, host species and age (arrows) on EcM fungal community composition. (a) seedlings in all microsites. Axes 1 and 2 explain 12.5% and 6.2% of variation in species data; (b) seedlings and mature trees on decayed wood. Axes 1 and 2 explain 10.0% and 7.0% of variation in species data. Pointed lines demonstrate separation of samples from white-rotted birch wood and brown-rotted spruce wood.

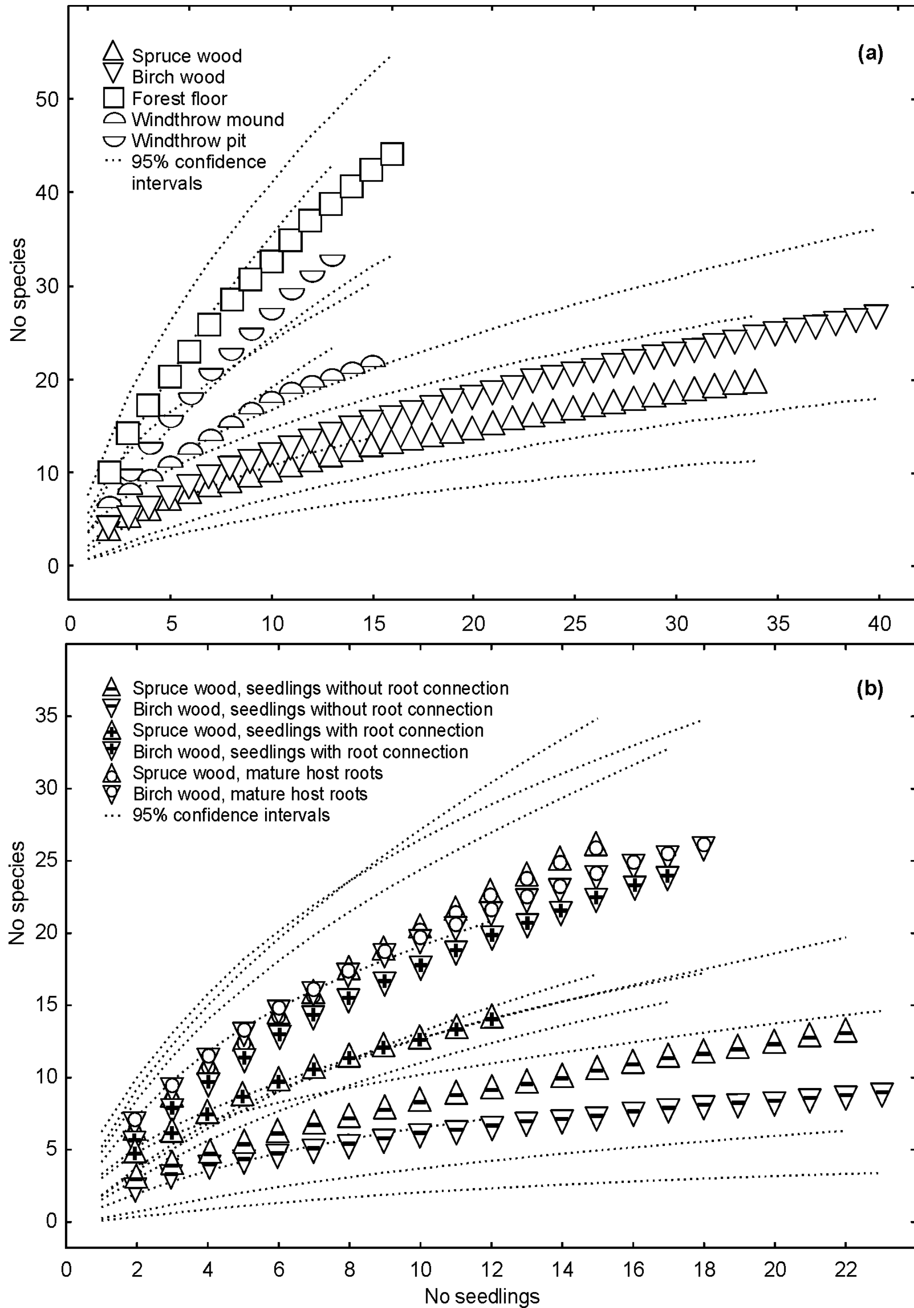

Figure 3. Species accumulation curves of ectomycorrhizal fungi and their 95% confidence intervals (pointed lines). (a) Seedlings in forest microsites. (b) Seedlings and mature host roots with or without root connection in CWD. Sites and seedlings of *Picea abies* and *Betula pendula* are pooled.

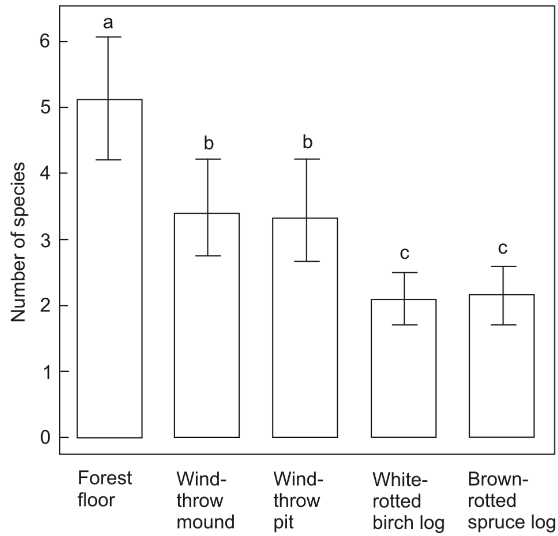

Figure 4. Species richness (mean ± 95% CI) of EcM fungi on seedlings in forest microsites. Sites and seedlings of *Picea abies* and *Betula pendula* are pooled. Letters above columns indicate statistically significant differences between groups.

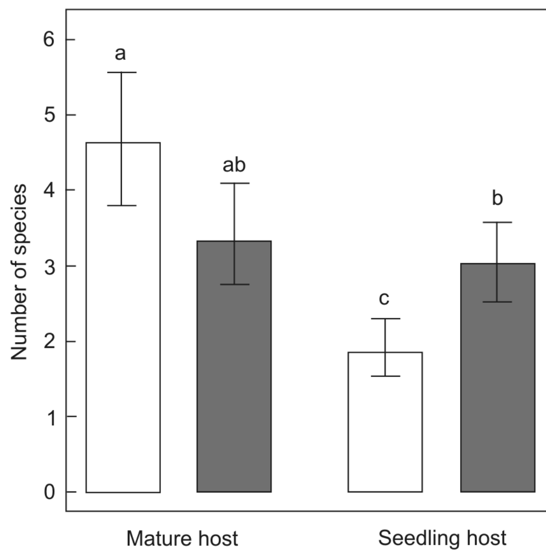

Figure 5. Species richness (mean ± 95% CI) of EcM fungi on roots of seedlings and mature trees in decayed wood depending on the occurrence of root connection. Sites and seedlings of *Picea abies* and *Betula pendula* are pooled. Open columns, no root connection; shaded colums, root connection present. Letters above columns indicate statistically significant differences between groups.

Appendix. Identification of ectomycorrhizal fungi from root tips of *Picea abies* and *Betula pendula* seedlings and mature trees. The distribution of fungal species on seedlings and mature *P. abies* in CWD are shown. *P*-values of Fisher's Exact test for microsite, host and site preference are indicated for the statistically analyzed species. Statistically significant *P*-values following Benjamini-Hochberg correction are indicated in bold.

Species	Best BLASTn/ FASTA3 match	Identity (%)	Percent of samples colonized in coarse woody debris		*P*-values		
			Seedling roots (n = 74)	Mature host roots (n = 33)	Micro-site (df = 4)	Host species (df = 1)	Site (df = 2)
Amphinema byssoides s. lato	n.a.*	n.a.	39.2	62.5	**0.001**	**<0.001**	0.100
Amphinema sp1	*Amphinema byssoides* AY219839	86.1					
Amphinema sp2	*Amphinema byssoides* AY838271	99.6					
Amphinema sp3	*Amphinema byssoides* AY219839	90.2					
Basidiomycota sp1	n.s.*	n.a.		3.1			
Basidiomycota sp2	n.s.	n.a.		3.1			
Byssocorticium atrovirens	*Byssocorticium atrovirens* AJ889936	82.8					
Cenococcum geophilum	n.s.	n.a.	2.7	15.6	**<0.001**	0.169	**0.006**
Clavulina sp1	*Clavulina* cf. *cristata* DQ974710	94.1					
Clavulina sp2	*Clavulina* sp. DQ202266	96.2					
Cortinarius olivaceofuscus	*Cortinarius olivaceofuscus* AY669585	98.0	1.4				
Cortinarius bolaris	*Cortinarius bolaris* AY669596	100.0					
Cortinarius casimiri	*Cortinarius casimiri* AJ889945	99.8	2.7	6.2			
Cortinarius flexipes	*Cortinarius flexipes* AY669678	99.4					
Cortinarius hemitrichus	*Cortinarius hemitrichus* DQ097870	99.6					
Cortinarius subsertipes	*Cortinarius subsertipes* AY669679	99.8			**0.037**		
Cortinarius umbrinolens	*Cortinarius umbrinolens* AY669658	100.0					
Elaphomyces sp.	*Elaphomyces* sp. UDB000043	99.0					
Entoloma sp	*Entoloma sinuatum* DQ486700	85.8		3.1			

Genea-Humaria lineage	n.s.	n.a.					
Geopora sp	*Geopora* cf. *cervina* DQ200831	93.6					
Hebeloma laucosarx	*Hebeloma leucosarx* UDB000022	99.5	1.4				
Hebeloma velutipes	*Hebeloma velutipes* AF124685	99.5					
Helotiales sp1	*Leptodontidium elatius* AY781230	85.6	2.7	6.2	0.392		0.042
Helvella sp1	*Helvella chinensis* AF046220	77.0					
Helvella sp2	n.s.	n.a.					
Hydnobolites sp	*Hydnobolites californicus* DQ974733	87.4					
Hydnotrya tulasnei	*Hydnotrya tulasnei* AM261222	100.0					
Inocybe sp1	*Inocybe calospora* AF325665	76.1	1.4				
Inocybe sp2	*Inocybe* sp. AY751958	78.3					
Inocybe sp3	*Inocybe nitidiuscula* AB244791	96.0					
Laccaria amethystina	*Laccaria amethystina* AM113955	100.0					
*Laccaria laccata*1	*Laccaria laccata* UDB000767	100.0	1.4	3.1	0.427		0.46
*Laccaria laccata*2	*Laccaria laccata* UDB000106	99.8					
Laccaria sp2	*Laccaria trichodermophora* DQ149855	97.7					
Lactarius camphoratus	*Lactarius camphoratus* AY606945	99.8					
Lactarius deterrimus	n.s.	n.a.		9.4			
Lactarius sp	*Lactarius lignyotus* AY631898¶	98.9					
Lactarius tabidus	*Lactarius tabidus* AY606956	100.0	10.8	25.0	**0.010**	0.435	0.646
Lactarius uvidus	*Lactarius uvidus* AY606957	99.8	1.4	3.1			
Meliniomyces bicolor	*Meliniomyces bicolor* AY394885	98.5	6.8	9.4	**<0.001**	1.000	0.020
Pachyphloeus-Amylascus lin.	*Pachyphloeus* sp. AY920528	75.5					
Paxillus involutus	*Paxillus involutus* AJ438984	99.9	8.1	6.2	**0.001**	**0.022**	0.814
Peziza aff. *badia*	*Peziza badia* DQ384574	95.7					
Piloderma byssinum	*Piloderma byssinum* AM084703	97.8					
Piloderma fallax	*Piloderma fallax* DQ365667	99.8					
Piloderma sp1	*Piloderma croceum* AJ438982	86.1					

Piloderma sp2	*Piloderma* sp. DQ179185	84.6	1.4	3.1			
Pseudotomentella humicola	*Pseudotomentella humicola* UDB000277	99.5	1.4	6.2			
Russula aquosa	*Russula aquosa* AY061657	99.6					
Russula betularum	*Russula betularum* AJ534937	100.0	2.7				
Russula decolorans	*Russula decolorans* DQ367913	99.5		3.1			
Russula nauseosa	*Russula nauseosa* AY061733	99.0	1.4	3.1	0.114		
Russula paludosa	*Russula paludosa* AJ971402	99.8					
Russula sphagnophila	*Russula sphagnophila* AY061719	99.8		3.1			
Russula velenovskyi	*Russula velenovskyi* UDB000921	100.0		3.1			
Russula vinosa	*Russula vinosa* UDB000902	100.0		3.1			
Sebacina aff. *epigaea*	*Sebacina* aff. *epigaea* AF490393	100.0					
Sebacina sp1	*Sebacina* sp DQ974768	96.2	4.1	12.5	0.147	0.208	0.113
Sebacina sp3	*Sebacina* aff. *epigaea* AF490393	91.0					
Sebacina sp4	*Sebacina* sp DQ974770	92.6	2.7				
Sebacina sp5	*Sebacina epigaea* AF490397	98.6		3.1			
Sebacina sp6	*Sebacina vermifera* AF202728	80.1					
Sebacina sp7	n.s.	n.a.					
Thelephora sp1	*Tomentella sublilacina* AJ889976	88.5					
Thelephora terrestris	*Thelephora terrestris* UDB000971	100.0	2.7				
Tomentella aff. *subclavigera*	*Tomentella subclavigera* UDB000259	93.9					
*Tomentella badia*1	*Tomentella badia* UDB000961	96.5	1.4	6.2			
*Tomentella badia*2	*Tomentella badia* UDB000961	98.0		3.1			
*Tomentella badia*3	*Tomentella badia* UDB000961	97.6	1.4	9.4	**0.002**	0.710	0.786
*Tomentella bryophila*1	*Tomentella bryophila* UDB000253	100.0	4.1				0.042
*Tomentella bryophila*2	*Tomentella bryophila* UDB000035	99.8	4.1	12.5			
*Tomentella coerulea*1	*Tomentella coerulea* UDB000266	99.6		3.1			
*Tomentella coerulea*2	*Tomentella coerulea* UDB000266	94.2		3.1			
*Tomentella ellisii*1	*Tomentella ellisii* UDB000231	99.6	1.4				

*Tomentella ellisii*2	*Tomentella ellisii* UDB000219	98.5			0.785		
*Tomentella lapida*1	*Tomentella lapida* UDB000270	100.0					
*Tomentella lapida*2	*Tomentella lapidum* AF272941	95.6					
Tomentella sp1	*Tomentella subtestacea* UDB000034	91.3		3.1			
*Tomentella stuposa*1	*Tomentella stuposa* UDB000248	99.8		3.1			
*Tomentella stuposa*2	*Tomentella stuposa* UDB000245	99.8	8.1	28.1	0.725	0.365	**<0.001**
*Tomentella stuposa*3	*Tomentella stuposa* AY010277	94.3	1.4	3.1	0.131		
*Tomentella stuposa*4	*Tomentella stuposa* UDB000967	99.6	1.4	3.1	**0.026**		**0.003**
Tomentella sublilacina	*Tomentella sublilacina* UDB000777	99.8	40.5	25.0	0.059	**0.008**	0.029
*Tomentella terrestris*1	*Tomentella terrestris* UDB000222	96.5	1.4	3.1			
*Tomentella terrestris*2	*Tomentella terrestris* UDB000222	99.6					
*Tomentella terrestris*3	*Tomentella terrestris* UDB000201	99.3		3.1			
*Tomentella terrestris*4	*Tomentella terrestris* UDB000222	93.6					
Trichophaea aff. *hybrida*	*Trichophaea* cf. *Hybrida* DQ200834	95.3	2.7	9.4	0.079	**0.003**	0.053
Tuber sp1	*Tuber* sp. DQ069050	90.0	1.4				
Tuber sp2	*Tuber* sp AJ534705	99.8					
Tuber sp3	n.s.	n.a.					
Tylospora asterophora	*Tylospora asterophora* AF052558	100.0	8.1	15.6	0.858	0.274	0.415
*Tylospora fibrillosa*1	*Tylospora fibrillosa* AF052563	100.0	35.1	43.8	0.133	**0.012**	0.454
*Tylospora fibrillosa*2	*Tylospora fibrillosa* AF052562	100.0	1.4				
Unidentified sp2	n.s.	n.a.	1.4				
Unknown sp1	*Astraeus hygrometricus* DQ682996[¶]	79.6	1.4		0.083		

*n.s., not sequenced; n.a., not applicable;
[¶]based on nLSU sequence.

VIII

Tedersoo L, Jairus T, Horton B, Glen M, Kõljalg U.
Ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest. Unpublished.

ECTOMYCORRHIZAL FUNGI IN A TASMANIAN WET SCLEROPHYLL FOREST

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ABSTRACT

Ectomycorrhizal symbiosis is a widespread plant nutrition strategy in Australia, especially in semiarid regions. This study was undertaken to study the diversity, community structure, host and soil preference of ectomycorrhizal fungi in a Tasmanian mixed wet sclerophyll forest. Using anatomotyping and rDNA sequencing, more than 110 species of ectomycorrhizal fungi were recovered from root tips of *Eucalyptus regnans* (Myrtaceae), *Pomaderris apetala* (Rhamnaceae) and *Nothofagus cunninghamii* (Nothofagaceae). Most of the frequent species from several ectomycorrhizal lineages displayed substantial host preference. The community of ectomycorrhizal fungi was dominated by the *Cortinarius* and *Tomentella-Thelephora* lineages, followed by *Russula-Lactarius*, *Clavulina*, *Descolea* and *Laccaria*. Apart from the presence of *Descolea*, the phylogenetic community structure is similar to that in boreal and temperate forests of the Northern Hemisphere.

INTRODUCTION

Ectomycorrhizal (EcM) symbiosis plays an important role in nutrient cycling in many Australian ecosystems (Ashford & Allaway 1982; Reddell & Milnes 1992; Reddell *et al.* 1999; Tommerup & Bougher 1999). Semiarid Australian flora includes a surprising diversity of EcM host plants, including Myrtaceae, Mimosoideae, Papilionoideae, Rhamnaceae, Goodeniaceae, Asteraceae, Casuarinaceae, Euphorbiaceae, *etc.* (Pryor 1956; Warcup 1980; Kope & Warcup 1986; Bellgard 1991; Brundrett & Abbott 1991; Reddell & Milnes

1992). The closest relatives of these plants often form exclusively arbuscular mycorrhiza in other continents (Ducousso & Thoen 1991; Reddell & Milnes 1992). Many of the Australian EcM hosts are able to form both arbuscular mycorrhiza and EcM, depending on species, age, soil nutrient status and availability of inoculum (Lapeyrie & Chilvers 1985; Reddell *et al.* 1986; Warcup 1990; Brundrett & Abbott 1991).

Semiarid sclerophyll habitats are the most widespread ecosystems in Australia and thus, much of the mycorrhizal research has focused on these habitats. The ecology of EcM fungi in moist coastal and submontane forests has been little studied (Reddell *et al.* 1999), although rain forest habitats prevailed before 30–25 Mya. Climate changed due to progressive global cooling and drying, which in turn resulted from the opening of Tasman sea and rapid northward movement of Australian continent (Crisp *et al.* 2004; Hill 2004). Subsequently, members of sclerophyll communities evolved from rain forest-inhabiting ancestors and radiated rapidly since 25 Mya (Ladiges *et al.* 2003, 2005; Steane *et al.* 2003; Crisp *et al.* 2004; Lavin *et al.* 2005). During climate change, wet forests dominated by gymnosperms and *Nothofagus* were successively replaced by sclerophyll and scrub vegetation dominated by Myrtaceae, Casuarinaceae and Mimosaceae (Crisp *et al.* 2004; Hill 2004). Therefore, it was hypothesized that certain *Nothofagus*-associated EcM fungi switched to eucalypts and other EcM plants following major changes in vegetation (Bougher & Malajczuk 1985; Bougher *et al.* 1994).

Accumulating information from fungal fruit-body surveys suggests that Australian EcM fungi are highly diverse (May & Simpson 1997; Bougher & Lebel 2001), with an estimated number of *ca*. 6500 species (Bougher 1995). Recent studies on soil mycelia support these findings in New South Wales and Queensland (Bastias *et al.* 2006; Midgley *et al.* 2007). Extensive fruit-body surveys involving epigeous or hypogeous fruiting taxa reveal that the EcM lineages of *Cortinarius, Descolea, and Russula-Lactarius* are the most species rich (Claridge *et al.* 1999; Lu *et al.* 1999; Bougher & Lebel 2001; Gates *et al.* 2005; Ratkowsky & Gates 2005). The *Russula-Lactarius* and *Tomentella-Thelephora* lineages, however, dominate soil EcM fungal communities, followed by *Cortinarius* and *Inocybe* (Bastias *et al.* 2006; Midgley *et al.* 2007).

Many stipitate, 'agaricoid' genera comprise a large number of secotioid or hypogeous-fruiting members in Australia (Bougher & Lebel 2001). Most of these hypogeous taxa have been described as entirely new genera or families (e.g. Trappe *et al.* 1996). Their high abundance in Australian semiarid woodlands has been attributed to seasonal climate and co-evolution with small marsupials that consume and distribute these taxa (Johnson 1996; Trappe & Claridge 2005). Surprisingly, most non-hypogeous Australian EcM fungal genera are shared with the Holarctic region (May & Simpson 1997). Watling (2001) hypothesized that many EcM boletes may have followed the migrating vegetation from Indo-Malay and New Guinea to Australia *via* Pleistocene land

bridges (and possibly earlier) that explains their wide distribution in SE Asia and Australia. Conversely, other taxa are shared with *Nothofagus* forests in New Zealand and southern South America, suggesting ancient vicariant distribution or more recent dispersal (Bougher *et al.* 1994; Watling 2001; Moyersoen *et al.* 2003). Certain taxa such as Mesophelliaceae (Trappe *et al.* 1996), *Descolea* (Bougher & Malajczuk 1985) and *Rozites* (Bougher *et al.* 1994) are far more diverse in Australia compared to other continents, suggesting their Australian origin.

Plant species differ in litter quality, especially lignin and calcium content that contribute to soil patch development in mixed forests (Ashton 1975; Hobbie *et al.* 2006). In particular, soil humus and nutrient concentration are considered among the most important determinants of EcM colonization in Australian trees (Chilvers & Pryor 1965; Reddell & Malajczuk 1984; Reddell *et al.* 1986). Soils with different fire history, vegetation and humus type develop distinct EcM fungal communities (Reddell & Malajczuk 1984; Launonen *et al.* 1999; Bastias *et al.* 2006) in Australia. Similarly, differential soil quality influences the community composition of EcM fungi in the Holarctic region (Lilleskov *et al.* 2002; Toljander *et al.* 2006). Because vegetation drives soil quality and *vice versa*, the preference for host *per se* and host-mediated soil effects on EcM fungi are poorly understood. The most common EcM fungi are usually associated with multiple host plants in the Holarctic region (Horton & Bruns 1998; Kennedy *et al.* 2003; Richard *et al.* 2005; Ishida *et al.* 2007). Exceptions include the closely related genera *Suillus*, *Rhizopogon* and *Chroogomphus* (the Boletaceae-Sclerodermataceae lineage) that are specific to certain Pinaceae (Molina & Trappe 1982), and EcM symbionts of *Alnus* (Betulaceae; Molina 1979) that are all absent from the Australian indigenous flora. Most *in vitro* synthesis experiments suggest that both Australian plants and fungi associate with multiple symbiotic partners (Chilvers 1973; Warcup 1980, 1990; Kope & Warcup 1986, Reddell *et al.* 1999; but see Malajczuk *et al.* 1982). In contrast, Chambers *et al.* (2005) argued that *Pisonia grandis* R. Br. (Nyctaginaceae) forms host-specific associations with two *Thelephora-Tomentella* spp. in Great Barrier Reef islands. Australian native fungi are usually incompatible with the introduced pines (Chilvers 1973; Malajczuk *et al.* 1982), but colonize European hardwoods (Diez 2005). Similarly, several fungal taxa native to African hardwoods or American conifers, form EcM with eucalypts (Malajczuk *et al.* 1982; Tedersoo *et al.* 2007b; but see Chen *et al.* 2007).

Australian EcM host plants are suggested to have obtained their EcM symbionts from *Nothofagus*, probably the oldest extant EcM taxon in Australia (Horak 1983; Hill 2004). Therefore, we hypothesize that EcM fungal communities in a Tasmanian wet sclerophyll forest are highly diverse and lack host specificity. This study further aims at uncovering the relative importance of host root and host-mediated soil preference on EcM fungal community

structure. Combining anatomotyping and sequencing, we demonstrate that the EcM fungal community in a Tasmanian wet sclerophyll forest is species-rich and phylogenetically diverse, but substantially influenced by host trees.

MATERIALS AND METHODS

Study site

Sampling was performed in a wet sclerophyll forest at Tall Forest Walk, Mt. Field National Park, Tasmania (geocode 42º40.9'S, 146º42.2'E; altitude 250 m a.s.l.) in August 2006. Mt Field National Park has a long history of conservation and recreational management, being first reserved in 1885 and proclaimed a national park in 1917. The vegetation of the study site forms a tall open forest. *Eucalyptus regnans* F. Müll. (EcM host) forms a canopy at approx. 60 m. The subdominant canopy layer consists of *Pomaderris apetala* Labill. (EcM host), *Acacia verniciflua* Cunn. (confirmed as non-EcM at this site), *Nothofagus cunninghamii* (Hook.) Oerst (EcM host), *Atherosperma moschatum* Labill., *Olearia argophylla* (Labill.) F. Müll. and a few *Acacia melanoxylon* R. Br. (putatively EcM). The understorey is covered by tree ferns (*Dicksonia antarctica* Labill.), *Pittosporum bicolor* Hook. and *Coprosma quadrifida* (Labill.) Rob. Forest floor is covered by ferns, including *Histiopteris incisa* (Thunb.) J. Sm., *Hypolepis rugulosa* (Labill.) J. Smith and *Blechnum* spp, and bryophytes. Decaying boles and branches of all decomposition stages are abundant, indicating primary conditions. Soils are derived from Permian mudstone and siltstone parent material and are deep gradational clay loam over light brown clay. The mean annual rainfall averages 1224 mm and mean daily minimum and maximum temperatures range between 5.3 °C and 16.2 °C (Maydena Post Office Station # 095063, 1992–2004).

Three plots (1 ha) were established 100–500 m apart in sites, where at least three EcM host trees – *E. regnans*, *N. cunninghamii* and *P. apetala* – co-occurred. Plots I and II were situated on a south-easterly aspect of a slope of approx. 1–5 degrees, whereas plot III was situated on steep (slope 10–25 degrees), eastern and western banks of a stream. From each plot, five root samples (15 x 15 cm to 5 cm depth) were collected from 0.2–1.5 m distance to trunks of each host tree, using a sharp knife. From each root sample, soil fraction was separated, dried at 70 °C and stored in paper bags for further chemical analyses. Roots were separated from remaining soil particles in tap water. After careful examination, EcM roots from each sample were sorted by plant species based on colour, ramification pattern, thickness and occurrence of nodules. Using a stereomicroscope, EcM root tips were assigned to morphotypes on each host species separately. Morphotypes were distinguished based on colour, roughness of mantle surface, occurrence of rhizomorphs,

emanating hyphae and cystidia. Several EcM clusters of each morphotype were mounted into 1% CTAB DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% cetyl trimethyl ammonium bromide) for storage and transportation. Soil and roots were processed within one and five days since collection, respectively. Usually several root tips from each morphotype per root species were further anatomotyped following Agerer (1991). Anatomotypes were kept separately for each plot. One or more root tips of each anatomotype per host and plot were subjected to molecular analyses.

Molecular analyses

DNA extraction was performed using a High Pure PCR Template Preparation Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, Indiana, USA) as outlined in Tedersoo (2007). The rDNA internal transcribed spacer (ITS) and nuclear large subunit (nLSU) were amplified as described in Tedersoo *et al.* (2006) using a primer ITS1F (5' cttggtcatttagaggaagtaa 3') in combination with a newly designed basidio-mycete-specific primer LB-W (5' cttttcatctttccctcacgg 3') or ascomycete-specific LA-W (5' cttttcatctttcgatcactc 3'). nLSU was amplified using a primer LR0R (5' acccgctgaacttaagc 3') in combination with newly designed basidio-mycete-specific primers LB-Y (5' tttgcacgtcagaatcgcta 3') or LB-Z (5' aaaaatggcccactagaaact 3'), ascomycete-specific primer LR3A (5' cacytactcaa-atccwagmg 3') or fungal-specific primer LR5F (5' cgatcgatttgcacgtcaga 3'). All newly designed primers are more profoundly described in Tedersoo (2007). PCR products were checked on 1% agarose gels under UV-light and purified using Exo-Sap enzymes (Sigma, St. Louis, Missouri, USA). Sequencing was performed using primers ITS4 (5' tcctccgcttattgatatgc 3') and/or ITS5 (5' ggaagtaaaagtcgtaacaagg 3') for the ITS region; ctb6 (5' gcatatcaataagcggagg 3') and/or LR5 (5' tcctgagggaaacttcg 3') for the nLSU. Contigs were assembled using Sequencher 4.7 (GeneCodes Corp., Ann Arbor, Michigan, USA). A value of 97.0% ITS region identity (excluding flanking 18S and 28S rDNA sequences) was used as a DNA barcoding treshold (molecular species criterion; Tedersoo *et al.* 2003). For *Cortinarius* and *Laccaria*, 98.0% treshold was used instead, because the ITS region is more conserved in these genera in Europe. All unique sequences were submitted to the UNITE database (Kõljalg *et al.* 2005). BLASTn and FASTA3 searches were performed against public sequence databases NCBI, EMBL and UNITE provide as precise identification for the EcM fungi as possible.

To confirm the identity of host tree, the plastid *trnL* region of root tip DNA was amplified using primers trnC (5' cgaaatcggtagacgctacg 3') and trnD (5' ggggatagagggacttgaac 3'). As revealed from agarose gels, *P. apetala*, *E. grandis* and *N. cunninghamii* differed in the size of the trnL region.

Statistical analyses

To compare the effects of host root and host-mediated soil on cumulative species richness of EcM fungi, rarefaction curves with 95% confidence intervals were computed using EstimateS ver. 8 (Colwell 2006). Sample-based minimal total species richness estimates Chao2 and Jackknife2 were calculated for each host species. For the whole community, ACE minimal richness estimate was additionally calculated. In these analyses, root samples were used as sampling units and fungal species were sampled randomly without replacement.

To study the effects of host root and host-modified soil on frequency of EcM fungal species, Fisher´s Exact tests were used at significance level $\alpha = 0.05$. To control false discovery rate and reduce familywise error rate associated with multiple testing, a sharpened procedure of Benjamini-Hochberg correction was used as implemented in Verhoeven *et al.* (2005). The combination of these procedures required at least three and four observations of each species to obtain statistically significant results for studying host root and host soil effects, respectively.

Using PC-Ord ver. 5 (McCune & Mefford 1999), Detrended Correspondence Analysis (DCA) was employed to unravel the effects of host species, host soil and plot on EcM fungal community structure. Root samples with binary-encoded, standardized species data were used in the analysis. All factors were transformed to dummy variables due to their unordered nature.

RESULTS

Combining sequencing and anatomotyping, 121 species of fungi were recovered from root tips of three host species (Appendix). Among these, 10 were strongly suspected to be putative saprobes or endophytes. Two anatomotypes consistently failed to amplify. The EcM fungal community comprised a few dominant and a large number of rare species. In particular, 61 (50%) of the taxa were found only once. The community was dominated by *Lactarius eucalypti* (in 47% of samples), followed by *Laccaria* sp1 (42%) and *Descolea* sp2 (31%). *Cortinarius* (incl. hypogeous members; 28 spp.), *Tomentella-Thelephora* (18 spp.), *Russula-Lactarius* (10 spp.), *Clavulina* (9 spp.), *Descolea* (including *Setchelligaster* and *Descomyces*; 7 spp.) and *Laccaria* (5 spp.) were the most species-rich lineages of EcM fungi. Of ascomycetes, both *Cenococcum geophilum* and members of Pezizales and Helotiales were detected as EcM symbionts.

All three host tree species were associated with multiple EcM fungi and displayed no particular specificity for any fungal lineage. Based on rarefaction curves, *Pomaderris apetala* roots and soil supported more species of fungi compared to *Eucalyptus regnans* and *Nothofagus cunninghamii*, although the

overlapping confidence intervals suggested that this effect was nonsignificant (Fig. 1a,b). Neither rarefaction curves nor minimal species richness estimates reached an asymptote when host species were analyzed individually or pooled. Of the estimators, Jackknife2 produced more stable and consistent estimates than Chao2. The total EcM root-associated fungal community at Tall Forest Walk was estimated to comprise between 180 (Chao2) and 210 (Jackknife2) species (Fig. 2). The slope of rarefaction curve exceeded that of estimators (except Jackknife2) when 40–45 samples were randomly sampled.

Most EcM-associated fungi (55%) that were observed more than once, colonized root tips of a single host tree species (Fig. 3). Thirty and 21 species could be statistically analyzed for host root and host soil preference, respectively. The frequency of 22 (73%) species was significantly affected by host roots, whereas 11 species (52%) were significantly more frequent in the soil of certain host trees. Notably, all species displaying host soil preference were significantly more frequent on the roots of this host. Host root effect was statistically more significant in 10 out of 12 cases where the *P*-values of host root and host soil substantially differed (Appendix). *Pomaderris apetala, E. regnans* and *N. cunninghamii* preferentially harboured 14, six and two of these host-biased taxa, respectively.

Both root and soil effects of *P. apetala* contributed to the main axis of DCA (eigenvalue 0.85) that explained 5.0 % and 19.0% of variation in species and species-environmental data, respectively (Fig. 4). DCA main axis effectively separated fungal communities of *P. apetala* and *E. regnans* roots, whereas samples comprising *N. cunninghamii* roots were situated at intermediate positions in the ordiantion diagram. As suggested by arrow length and direction, the two host effects were not distinguished by DCA, but appeared more important than plot effect in explaining the EcM fungal community structure.

DISCUSSION

EcM fungi formed a diverse community in a Tasmanian wet sclerophyll forest that compares well with Holarctic ecosystems (Horton & Bruns 2001; Richard *et al.* 2005; Tedersoo *et al.* 2006; Ishida *et al.* 2007). The Tasmanian EcM fungal community was dominated by the *Cortinarius*, *Tomentella-Thelephora*, *Russula-Lactarius*, *Clavulina*, *Descolea* and *Laccaria* lineages. Except for *Descolea*, these lineages form a substantial part of the Holarctic EcM fungal communities as well. In particular, the *Tomentella-Thelephora, Russula-Lactarius, Cortinarius, Inocybe* and *Sebacina* are among the most species-rich lineages in Holarctic ecosystems (Horton & Bruns 2001; Douglas *et al.* 2005; Izzo *et al.* 2005; Walker *et al.* 2005; Tedersoo *et al.* 2006; Ishida *et al.* 2007). The *Tomentella-Thelephora* and Boletaceae-Sclerodermataceae lineages dominate the belowground EcM fungal communities in tropical forests

(Sirikantaramas *et al.* 2003; Riviere *et al.* 2007; Tedersoo *et al.* 2007b), whereas *Cortinarius* is considered rare in the tropics (Peintner *et al.* 2003, but see Onguene 2000; Tedersoo *et al.* 2007b). In the Holarctic region, individual species of *Cortinarius* and *Laccaria* usually occur in low abundance and frequency belowground due to small genetic individuals and highly clumped distribution of EcM (Gherbi *et al.* 1999; Genney *et al.* 2006). In Tasmania, however, certain species of these lineages were among the most frequent EcM taxa. In addition, *Cortinarius* and *Laccaria* respectively colonized 47% and 15% of the EcM root tips in an old-growth *Nothofagus* forest in Victoria state, Australia (Tedersoo *et al.* 2007a), which suggests that these lineages may have different ecological roles and importance compared to Holarctic ecosystems. Some of the fungal lineages commonly observed in Tasmania are poorly represented in the Northern Hemisphere. These include *Descolea* (Bougher & Malajczuk 1985) and probably some lineages of Helotiales. As the EcM-forming species of Helotiales are closely related with many endophytic and/or ericoid mycorrhizal taxa (Vrålstad *et al.* 2002; Hambleton & Sigler 2005), additional morphological and physiological evidence for their true EcM lifestyle is required. Based on this unreplicated study site, it is impossible to conclude on the absence of certain EcM lineages in Tasmanian wet sclerophyll forests in general. Indeed, two lineages, Elaphomyces and Sordariales, were found from Victoria in spite of substantially lower sampling effort (Tedersoo *et al.* 2007a). Nevertheless, the *Suillus-Rhizopogon* group of the Boletaceae-Sclerodermataceae lineage and *Amphinema-Tylospora* (Atheliales) that are diverse and abundant in the Holarctic region (Taylor *et al.* 2000; Horton & Bruns 2001; Tedersoo *et al.* 2003), were not observed belowground in Tasmania or Victoria. In agreement with this, none of these taxa are reported as native to Australia (May & Simpson 1997).

Root morphology aided with length difference of chloroplast *trnL* region was successfully employed to distinguish the three host species belowground. Because most samples comprised roots of a single host species that grew the closest, separation of host root and host-mediated soil effects proved difficult using ordination methods. Nonetheless, statistical analyses of individual species revealed that host root preference was usually substantially more significant than preference for host-mediated soil effects. As suggested by DCA and the frequency of colonization of individual species, *Pomaderris apetala* and *Eucalyptus grandis* hosted distinct EcM fungal communities. Statistical analyses supported these results indicating that most of the common EcM fungal species preferred the roots of either *P. apetala* or *E. grandis*, although exclusive specificity was less common. *Nothofagus cunninghamii* usually shared its EcM fungi with *P. apetala* or *E. regnans*. Only Helotiales sp2, most likely a true EcM fungal species with a conspicuous mantle structure, was found exclusively on *N. cunninghamii*. Host-biased EcM fungal species were observed in many lineages, including *Cortinarius*, *Russula-Lactarius*, *Laccaria*,

Descolea, *Tomentella-Thelephora*, *Cenococcum*, *Clavulina, etc*. The lineages of *Laccaria, Tomentella-Thelephora, Cenococcum* and *Clavulina* comprise species with a broad host range in the Holarctic region, where host specificity is uncommon among the dominant fungal species (Horton & Bruns 1998; Kennedy *et al.* 2003; Richard *et al.* 2005; Nara 2006; Ishida *et al.* 2007) and usually restricted to certain taxonomic groups within some fungal lineages (e.g. *Leccinum, Rhizopogon, Suillus, Lactarius* sect. *Dapetes, Alnicola*). The substantial host preference as observed in this study may contribute to the significantly higher species richness at this site compared to a monodominant *Nothofagus* forest in Victoria state (Tedersoo *et al.* 2007a). In this study, *Cenococcum geophilum* was observed only on roots of *P. apetala* and *N. cunninghamii*, although Chilvers (1968) described typical *C. geophilum* mycorrhizas on eucalypts.

Usually, host generalist EcM fungi are considered important drivers of forest succession by facilitating seedling establishment of late-successional host trees (Horton *et al.* 1999; Kennedy *et al.* 2003; Dickie *et al.* 2004; Richard *et al.* 2005). Following this hypothesis, *P. apetala* and *E. regnans*, the pioneer, fire-dependent tree species may effectively exclude each other through priority effect and incompatible EcM symbionts. Late-successional *N. cunninghamii*, however, may be facilitated by their EcM symbionts that are shared with either *E. regnans* or *P. apetala*. This does not argue against the hypothesis that other Australian EcM hosts received their EcM symbionts from *Nothofagus*, although unambiguous evidence is still lacking. Due to differences in litter quality (Ashton 1975) and, possibly, historical habitat, eucalypts and Pomaderreae may have acquired the EcM fungi from *Nothofagus* spatially independently. Assuming that sclerophyll associations are relatively young, EcM fungi of Pomaderreae and eucalypts may have retained their host preference. If we consider the alternative possibility that all three (and many other Australian indigenous EcM plant lineages) host taxa gained EcM lifestyle and EcM associations from independent *sources*, these taxa should have evolved in spatial separation. However, except for the connection with New Guinea and Tasmania, Australian continent has been a compact land mass in the past. The extensive, moist areas covered by *Nothofagus* in the past and the evolution and radiation of *Eucalyptus*, *Acacia*, Casuarinaceae and Pomaderreae in similar moist climatic conditions render little chance of independent EcM acquisition in these plant taxa. Improved understanding of the development centre, dated evolution and biogeography of Australian EcM host plants could provide a better explanation for this hypothesis. In addition, DNA sequence data of *Nothofagus* EcM associates from other areas of endemism (e.g. South America) and resolved phylogenies of EcM fungi will probably uncover the primary *vs*. secondary nature of host preference among Australian EcM fungi. Experimental synthesis trials involving more species of *Eucalyptus* and Pomaderreae should be performed to rule out the *plant species* effects on mycorrhizal specificity.

In conclusion, this study provides strong evidence for host preference among the dominant EcM fungi in a Tasmanian wet sclerophyll forest. To substantiate these findings and eliminate the possibility of host-mediated habitat preference, soil chemical analyses are required. The underlying causes and mechanisms of host preference remain unknown, but deserve attention to learn the basic features of biogeography and host shifting in EcM symbiosis.

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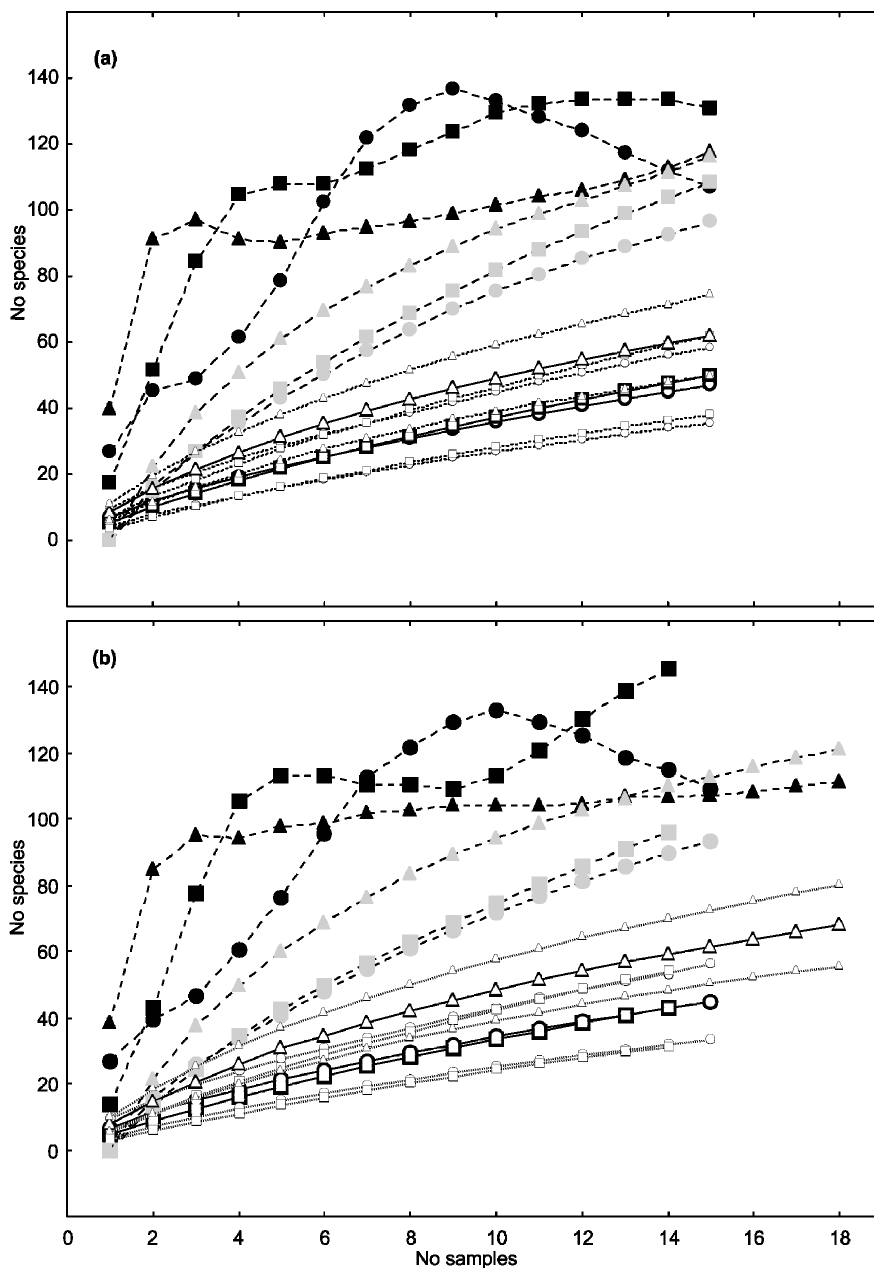

Figure 1. Rarefaction curves, their 95% confidence intervals (pointed lines with reduced symbols), Jackknife2 (shaded symbols) and Chao2 (closed symbols) minimal species richness estimates of ectomycorrhizal fungi in the study site. (a) among host neighbourhood; (b) among roots of host species. Triangles, *Pomaderris apetala*; squares, *Nothofagus cunninghamii*; circles, *Eucalyptus regnans*

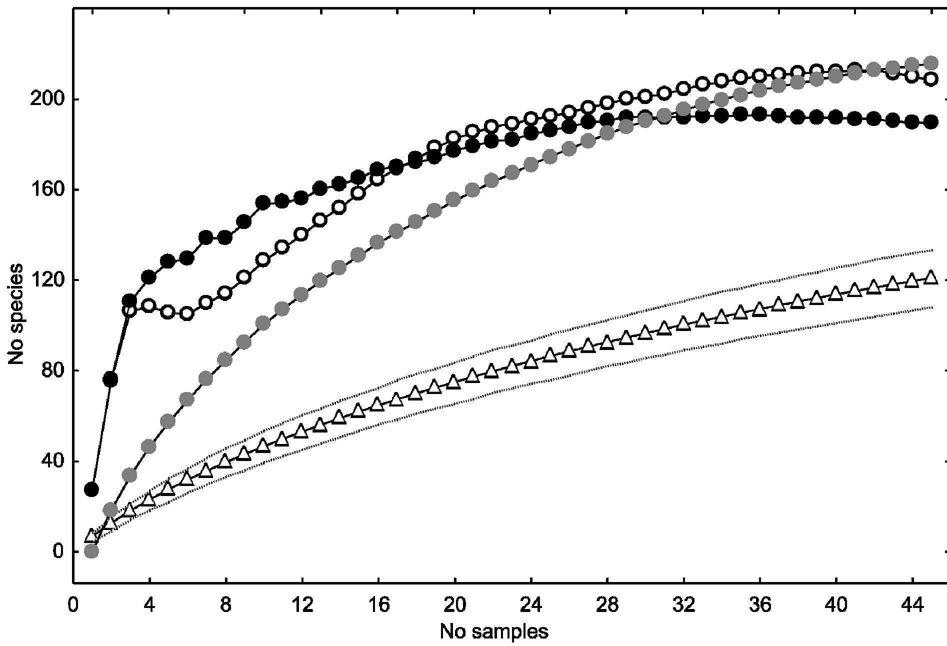

Figure 2. Rarefaction curve (open triangles), its 95% confidence intervals (pointed lines); Jackknife2 (shaded circles), Chao2 (closed circles) and ACE (open circles) minimal species richness estimates of ectomycorrhizal fungi in the study site.

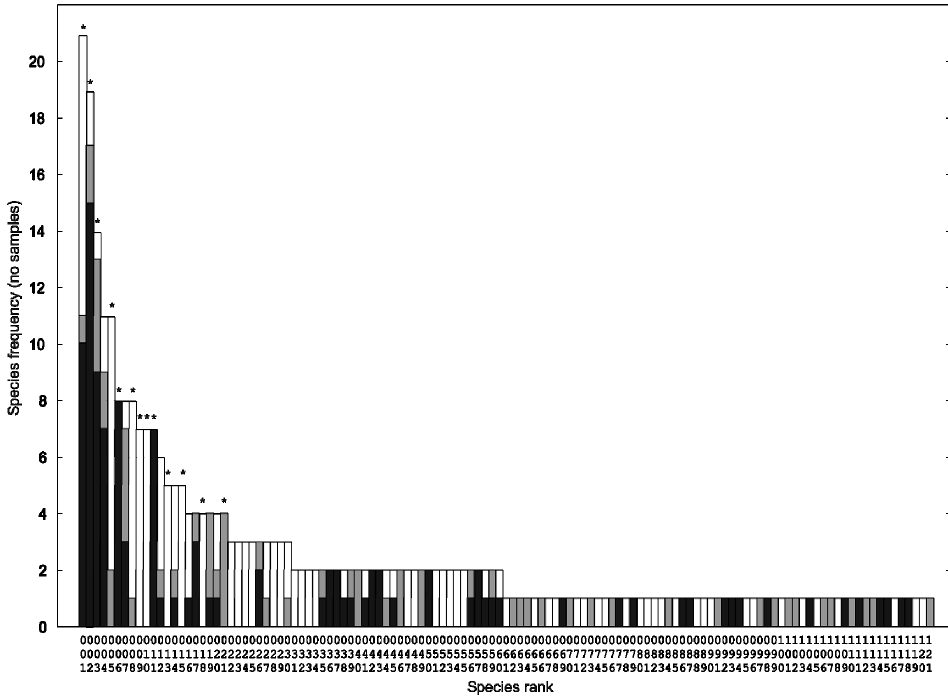

Figure 3. Frequency of ectomycorrhizal fungi on three host species in the study site. Columns represent the number of root samples (per host species), where the species were present. Differential shading demonstrates the weighted proportion of each host species. Closed symbols, *Eucalyptus regnans*; shaded symbols, *Nothofagus cunninghamii*; open symbols, *Pomaderris apetala*. Asterisks denote statistically significant host preference according to Fisher's Exact test, followed by sharpened Benjamini-Hochberg corrections according to Verhoeven *et al.* (2005). Species ranks are encoded in appendix.

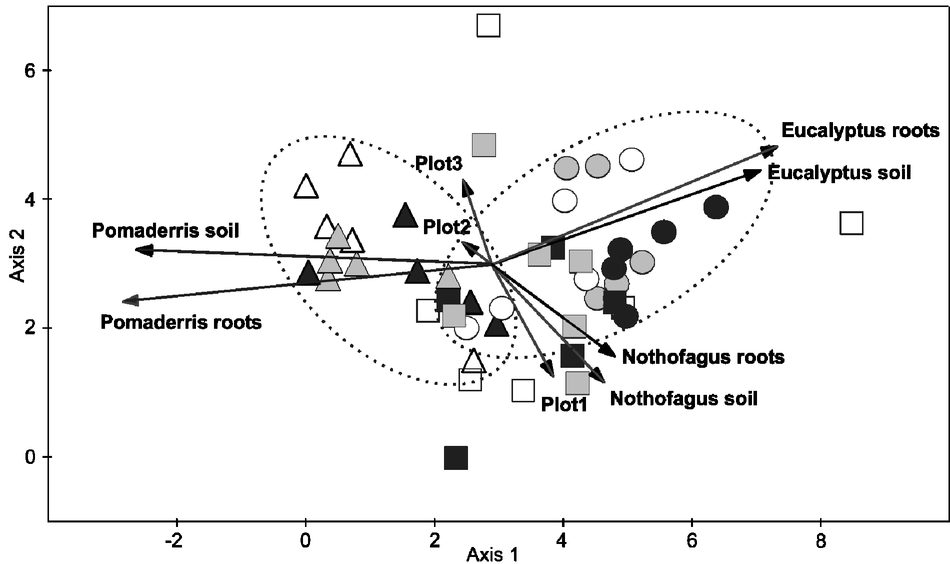

Figure 4. Detrended Correspondence Analysis (DCA) ordination demonstrating the relative importance of host root, host soil and plot effects (arrows) on community composition of ectomycorrhizal fungi. The first two axes have eigenvalues of 0.848 and 0.734, and explain 5.0% and 4.3% of the variance of species data, respectively. Triangles, *Pomaderris apetala*; squares, *Nothofagus cunninghamii*; circles, *Eucalyptus regnans*. Differential shading represents plots. Pointed ellipses indicate separation of root samples from *E. regnans* and *P. apetala.*

Appendix. Identification and host preference of ectomycorrhizal fungi. Best BLASTn/FASTA3 matches of the entire ITS region to database sequences are shown. *P*-values for host root and host soil preference are indicated. Statisticlly significant effects following Benjamini-Hochberg correction are shown in bold.

Species	Best sequence match		Rank	P-values			
		Identity		Host root		Host soil	
	Species and accession†	(%)‡		Observed‡	Treshold	Observed	Treshold
Lactarius eucalypti	*Lactarius eucalypti*	100.0	001	**0.002**	0.044	0.500	0.069
Laccaria sp1	*Laccaria laccata* AJ699075	96.1	002	**<0.001**	0.006	**0.001**	0.012
Descolea sp2	*Descolea recedens* AF325649	98.4	003	**0.002**	0.038	**0.027**	0.038
Russula sp1	*Russula cremoricolor* DQ974755	91.0	004	**0.054**	0.088	**0.014**	0.031
Laccaria sp3	*Laccaria laccata* AJ699074	99.0	005	**0.001**	0.025	**0.001**	0.015
Tomentella sp1	*Tomentella fuscocinerea* DQ974776	91.5	006	**<0.001**	0.013	**<0.001**	0.004
Unidentified sp1	n.s.	n.a.	007	0.199	0.156	0.488	0.065
Cenococcum geophilum	*Cenococcum geophilum* (Japan) AB251837	99.6	008	**0.004**	0.056	0.229	0.050
Tomentella sp9	*Tomentella ramosissima* U83480	98.1	009	**0.001**	0.031	0.179	0.046
Tomentella sp4	*Tomentella lateritia* UDB000268	94.5	010	**0.001**	0.038	**0.001**	0.019
Cortinarius sp2	*Cortinarius rotundisporus*	99.0	011	**<0.001**	0.019	**0.010**	0.027
Inocybe sp1	*Inocybe* cf. *glabripes* AJ889952	81.8	012	0.346	0.163	0.858	0.081
Tomentella sp8	*Tomentella badia* UDB000961	91.3	013	**0.009**	0.069	**0.007**	0.019
Tomentella sp7	*Tomentella stuposa* UDB000967	90.9	014	0.605	0.175	0.594	0.077
Clavulina sp4	*Clavulina* cf. *cristata* DQ974710	94.6	015	**0.009**	0.075	**0.007**	0.023
Tomentella sp2	*Tomentella stuposa* AY010277	91.6	016	0.366	0.169	0.302	0.054
Tomentella sp2	*Tomentella subclavigera* AY010275	93.8	017	**0.097**	0.100	0.302	0.058
Tomentella sp10	*Tomentella lateritia* UDB000963	92.6	018	**0.030**	0.081	**0.027**	0.034
Sebacina sp4	*Sebacina helvelloides* AJ966750	90.5	019	**0.061**	0.094	0.302	0.062
Clavulina sp6	*Clavulina* cf. *cristata* DQ974710	89.0	020	1.000	0.188	0.524	0.073
Helotiales sp2	*Leptodontidium elatius* AY781230	86.0	021	**0.006**	0.063	**0.027**	0.042

Hysterangium sp1	*Hysterangium cassirhachis* DQ365633	82.2	022	**0.101**	0.106	n.a.	n.a.
Laccaria sp6	*Laccaria laccata* AJ699075	96.0	023	**0.101**	0.113	n.a.	n.a.
Inocybe sp5	*Inocybe cf. glabripes* AJ889952	81.3	024	**0.101**	0.119	n.a.	n.a.
Unidentified sp2	n.s.	n.a.	025	**0.101**	0.125	n.a.	n.a.
Cortinarius sp20	*Cortinarius delibutus* AY669587	92.2	026	0.767	0.181	n.a.	n.a.
Helotiales sp7*	*Oidiodendron maius* AF062798	96.4	027	0.185	0.144	n.a.	n.a.
Helotiales sp6*	*Oidiodendron maius* AF062800	96.1	028	**0.101**	0.131	n.a.	n.a.
Helotiales sp10*	*Leptodontidium elatius* AY781230	80.4	029	**0.101**	0.138	n.a.	n.a.
Helotiales sp1	*Solenopezia solenia* U57991	84.0	030	0.185	0.150	n.a.	n.a.
Tomentellopsis larsenii	*Tomentellopsis larsenii* AF326980	99.2	031	n.a.	n.a.	n.a.	n.a.
Sebacina sp1	*Sebacina helvelloides* AJ966749	89.3	032	n.a.	n.a.	n.a.	n.a.
Russula sp8	*Russula chloroides* AF418604	86.4	033	n.a.	n.a.	n.a.	n.a.
Russula sp5	*Russula adusta* AY061652	89.2	034	n.a.	n.a.	n.a.	n.a.
Russula sp3	*Russula nigricans* AM113960	87.8	035	n.a.	n.a.	n.a.	n.a.
Russula sp2	*Russula nigricans* AY061695	86.3	036	n.a.	n.a.	n.a.	n.a.
Rhodocollybia sp1*	*Rhodocollybia dotae* AF505758	89.6	037	n.a.	n.a.	n.a.	n.a.
Pezizaceae sp1	*Terfezia arenaria* AF276674	76.7	038	n.a.	n.a.	n.a.	n.a.
Laccaria sp4	*Laccaria amethystina* UDB001492	94.3	039	n.a.	n.a.	n.a.	n.a.
Laccaria sp2	*Laccaria laccata* AJ699075	97.2	040	n.a.	n.a.	n.a.	n.a.
Inocybe sp2	*Inocybe lacera* AB211269	78.5	041	n.a.	n.a.	n.a.	n.a.
Endogonales sp1	*Endogone pisiformis* AF006511	partial	042	n.a.	n.a.	n.a.	n.a.
Descolea sp3	*Descolea maculata* DQ192181	99.8	043	n.a.	n.a.	n.a.	n.a.
Descolea sp1	*Descolea phlebophora* AF325656	100	044	n.a.	n.a.	n.a.	n.a.
Cortinarius sp9	*Cortinarius canthocephalus* UDB000674	95.0	045	n.a.	n.a.	n.a.	n.a.
Cortinarius sp8	*Cortinarius teraturgus* AF389151	96.0	046	n.a.	n.a.	n.a.	n.a.
Cortinarius sp6	*Cortinarius teraturgus* AF389151	94.3	047	n.a.	n.a.	n.a.	n.a.

Cortinarius sp19	*Cortinarius cephalixus* AY174784	92.2	048	n.a.	n.a.	n.a.	n.a.
Cortinarius sp13	*Cortinarius cystideocatenatus* AY669651	94.8	049	n.a.	n.a.	n.a.	n.a.
Cortinarius sp11	*Cortinarius obtusus* UDB000127	94.6	050	n.a.	n.a.	n.a.	n.a.
Cortinarius sp1	*Cortinarius firmus* AF389163	86.8	051	n.a.	n.a.	n.a.	n.a.
Cantharellus lineage sp2	*Hydnum albidum* AJ534709	70.2	052	n.a.	n.a.	n.a.	n.a.
Clavulina sp1	*Clavulina* cf. *cristata* DQ974710	82.5	053	n.a.	n.a.	n.a.	n.a.
Clavulina sp10	*Clavulina* cf. *cristata* DQ974710	75.9	054	n.a.	n.a.	n.a.	n.a.
Cantharellus lineage sp1	*Craterellus tubaeformis*	partial	055	n.a.	n.a.	n.a.	n.a.
Boletus sp1	*Boletus amygdalinius* DQ974705	78.2	056	n.a.	n.a.	n.a.	n.a.
Helotiales sp8*	*Dermea viburni* AF141163	84.8	057	n.a.	n.a.	n.a.	n.a.
Helotiales sp5	*Hymenoscyphus immutabilis* AY348584	85.3	058	n.a.	n.a.	n.a.	n.a.
Helotiales sp4	*Hyphodiscus hymenophilus* DQ227258	82.5	059	n.a.	n.a.	n.a.	n.a.
Helotiales sp13*	*Leptodontidium elatius* AY805569	91.5	060	n.a.	n.a.	n.a.	n.a.
Tricholoma sp1	*Tricholoma scalpturatum* AF377201	85.3	061	n.a.	n.a.	n.a.	n.a.
Tomentellopsis sp3	*Tomentellopsis bresadoliana* AJ410779	86.8	062	n.a.	n.a.	n.a.	n.a.
Tomentellopsis sp1	*Tomentellopsis larsenii* AF326980	92.7	063	n.a.	n.a.	n.a.	n.a.
Tomentella sp6	*Tomentella lateritia* UDB000963	90.3	064	n.a.	n.a.	n.a.	n.a.
Tomentella sp5	*Tomentella fuscocinerea* DQ974776	93.1	065	n.a.	n.a.	n.a.	n.a.
Tomentella sp15	*Tomentella fuscocinerea* DQ974776	91.4	066	n.a.	n.a.	n.a.	n.a.
Tomentella sp16	*Tomentella lilacinogrisea* UDB000953	89.8	067	n.a.	n.a.	n.a.	n.a.
Tomentella sp17	*Tomentella fuscocinerea* DQ974776	91.2	068	n.a.	n.a.	n.a.	n.a.
Tomentella sp14	*Tomentella atramentaria* DQ974722	89.8	069	n.a.	n.a.	n.a.	n.a.
Tomentella sp13	*Tomentella coerulea* UDB000266	91.4	070	n.a.	n.a.	n.a.	n.a.
Tomentella sp12	*Tomentella stuposa* UDB000965	94.8	071	n.a.	n.a.	n.a.	n.a.
Tomentella sp11	*Tomentella cinerascens* UDB000232	96.2	072	n.a.	n.a.	n.a.	n.a.
Descolea sp7	*Setchelliogaster* sp. DQ328214	99.6	073	n.a.	n.a.	n.a.	n.a.

Sebacina sp3	*Sebacina* sp. AF440664	88.3	074	n.a.	n.a.	n.a.	n.a.
Sebacina sp2	*Sebacina helvelloides* AJ966750	90.0	075	n.a.	n.a.	n.a.	n.a.
Russula sp7	*Russula littoralis* AY061702	83.5	076	n.a.	n.a.	n.a.	n.a.
Russula sp4	*Russula nauseosa* AY061733	91.7	077	n.a.	n.a.	n.a.	n.a.
Gauteria sp1	*Gauteria caudata* AF377057	partial	078	n.a.	n.a.	n.a.	n.a.
Ramaria sp1	*Ramaria ignicolor* AJ408386	72.3	079	n.a.	n.a.	n.a.	n.a.
Hysterangium sp2	*Hysterangium cassirhachis* DQ365632	80.8	080	n.a.	n.a.	n.a.	n.a.
Piloderma sp3	*Piloderma fallax* DQ179125	86.0	081	n.a.	n.a.	n.a.	n.a.
Piloderma sp2	*Piloderma byssinum* DQ365683	86.5	082	n.a.	n.a.	n.a.	n.a.
Piloderma sp1	*Piloderma fallax* AY010282	85.1	083	n.a.	n.a.	n.a.	n.a.
Pezizaceae sp2	*Terfezia arenaria* AF276674	76.8	084	n.a.	n.a.	n.a.	n.a.
Lactarius sp3	*Lactarius serifluus* AY332558	90.6	085	n.a.	n.a.	n.a.	n.a.
Lactarius sp2[¶]	*Lactarius subdulcis* AF218552	98.2	086	n.a.	n.a.	n.a.	n.a.
Inocybe sp4	*Inocybe fraudans* AJ889953	77.6	087	n.a.	n.a.	n.a.	n.a.
Inocybe sp3	*Inocybe lanuginosa* DQ367905	78.8	088	n.a.	n.a.	n.a.	n.a.
Descolea sp6	*Descolea* sp. AF325658	100.0	089	n.a.	n.a.	n.a.	n.a.
Descolea sp5	*Descomyces albus* DQ328209	98.1	090	n.a.	n.a.	n.a.	n.a.
Descolea sp4	*Descolea recedens* AF325649	91.5	091	n.a.	n.a.	n.a.	n.a.
Cortinarius sp7	*Dermocybe olivaceopicta* U56050	97.1	092	n.a.	n.a.	n.a.	n.a.
Cortinarius sp5	*Cortinarius teraturgus* AF389151	95.2	093	n.a.	n.a.	n.a.	n.a.
Cortinarius sp4	*Cortinarius badiovinaceus* UDB002221	92.7	094	n.a.	n.a.	n.a.	n.a.
Cortinarius sp35	*Cortinarius cannarius* AY669630	95.4	095	n.a.	n.a.	n.a.	n.a.
Cortinarius sp34	*Cortinarius walkeri* AY669632	93.8	096	n.a.	n.a.	n.a.	n.a.
Cortinarius sp33	*Thaxterogaster levisporus* DQ328105	96.8	097	n.a.	n.a.	n.a.	n.a.
Cortinarius sp30	*Dermocybe olivaceopicta* U56050	95.7	098	n.a.	n.a.	n.a.	n.a.
Cortinarius sp3	*Thaxterogaster albocanus* AF325599	92.9	099	n.a.	n.a.	n.a.	n.a.

Cortinarius sp24	*Cortinarius ombrophilus* AF389149	90.8	100	n.a.	n.a.	n.a.	n.a.
Cortinarius sp23	*Thaxterogaster albocanus* AF325599	93.0	101	n.a.	n.a.	n.a.	n.a.
Cortinarius sp22	*Cortinarius collariatus* AY033115	96.7	102	n.a.	n.a.	n.a.	n.a.
Cortinarius sp21	*Quadrispora tubercularis* DQ328113	95.1	103	n.a.	n.a.	n.a.	n.a.
Cortinarius sp18	*Dermocybe olivaceopicta* U56050	95.9	104	n.a.	n.a.	n.a.	n.a.
Cortinarius sp17	*Cortinarius olivaceobubalinus* AF539736	97.5	105	n.a.	n.a.	n.a.	n.a.
Cortinarius sp16	*Cortinarius cystideocatenatus* AY669651	96.8	106	n.a.	n.a.	n.a.	n.a.
Cortinarius sp15	*Thaxterogaster* sp. DQ328121	95.7	107	n.a.	n.a.	n.a.	n.a.
Cortinarius sp14	*Quadrispora tubercularis* DQ328113	92.7	108	n.a.	n.a.	n.a.	n.a.
Cortinarius sp12	*Dermocybe olivaceopicta* U56050	95.7	109	n.a.	n.a.	n.a.	n.a.
Cortinarius sp10	*Cortinarius teraturgus* AF389151	92.4	110	n.a.	n.a.	n.a.	n.a.
Coltriciella sp1	*Coltriciella dependens* AM412252	84.5	111	n.a.	n.a.	n.a.	n.a.
Clavulina sp9	*Clavulina* cf. *cristata* DQ974710	83.4	112	n.a.	n.a.	n.a.	n.a.
Clavulina sp8	*Clavulina* cf. *cristata* DQ974710	79.1	113	n.a.	n.a.	n.a.	n.a.
Clavulina sp7	*Clavulina* cf. *cristata* DQ974710	84.0	114	n.a.	n.a.	n.a.	n.a.
Clavulina sp3	*Clavulina* cf. *cristata* DQ974712	94.1	115	n.a.	n.a.	n.a.	n.a.
Clavulina sp2	*Clavulina* cf. *cristata* DQ974711	83.4	116	n.a.	n.a.	n.a.	n.a.
Boletus sp2	*Xerocomus chrysonemus* DQ066378	82.1	117	n.a.	n.a.	n.a.	n.a.
Helotiales sp9*	*Pezicula sporulosa* AF141172	96.2	118	n.a.	n.a.	n.a.	n.a.
Helotiales sp3	*Leohumicola minima* AY706329	89.9	119	n.a.	n.a.	n.a.	n.a.
Helotiales sp12*	*Cladophialophora* sp. EF016385	91.4	120	n.a.	n.a.	n.a.	n.a.
Helotiales sp11*	*Oidiodendron maius* AY624308	89.4	121	n.a.	n.a.	n.a.	n.a.

*Species that are probably saprotrophic or endophytic;
¶Based on nLSU sequence data;
†n.s., not sequenced;
‡n.a., not applied.

IX

Tedersoo L, Dunk C, Gates G, Jairus T, Lebel T, May T, Kõljalg U.
Establishment of ectomycorrhizal fungi on *Nothofagus cunninghamii* seedlings
on dead wood in Australian temperate wet sclerophyll forests. Unpublished.

ESTABLISHMENT OF ECTOMYCORRHIZAL FUNGI ON *NOTHOFAGUS CUNNINGHAMII* SEEDLINGS ON DEAD WOOD IN AUSTRALIAN TEMPERATE WET SCLEROPHYLL FORESTS

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ABSTRACT

Decaying wood provides an important habitat for animals and forms a seed bed for many shade-intolerant, small-seeded plant species, particularly *Nothofagus.* Using morphotyping and rDNA sequence analyses, we compared the ectomycorrhizal fungal communities of *N. cunninghamii* seedlings germinating in decayed wood with mature tree roots in the forest floor soil. Species of *Cortinarius*, *Russula* and *Laccaria* were the most abundant taxa in forest floor soil at Yarra, Victoria state. Of these species, only *Laccaria* dominated the seedlings in decayed wood. Similarly, *Laccaria* spp. dominated, followed by *Descolea* spp. and a Sordariales sp. in CWD at Warra, Tasmania. This study demonstrates that only a few species colonize isolated seedlings on decayed wood, but these species are taxonomically unrelated to the species dominating in similar habitats in Europe. We conclude that the formation of resupinate fruit body type on the underside of decayed wood is not related to seedling root colonization in decayed wood.

INTRODUCTION

Decaying trunks and larger branches (termed ‘coarse woody debris’, CWD) are important components in mature and old-growth forest ecosystems (Harmon *et al.* 1986). In addition to functioning as a carbon store and providing habitat and food source for many vertebrate and invertebrate species (Harmon *et al.* 1986;

Yee *et al.* 2001; MacNally *et al.* 2002), CWD forms an important microsite for seedling establishment in many mesic and humid ecosystems of the world (e.g. Howard 1973; Lawton & Putz 1988; Hofgaard 1993; McGee & Birmingham 1997; Narukawa & Yamamoto 2002). Seedling establishment and survival on CWD is particularly important in ectomycorrhizal (EcM) and/or small-seeded plants (Hofgaard 1993; Lusk & Kelly 2003) that is often ascribed to reduced litter accumulation, lower root and shoot competition, greater light availability and humidity (Harvey *et al.* 1978; Christy & Mack 1984; Harmon & Franklin 1989). Shade-intolerant *Nothofagus* species form EcM and produce small seeds (Veblen *et al.* 1996 and references therein). In most of its geographical range, *Nothofagus* spp. require severe disturbance or CWD for regeneration (Howard 1973; Read & Brown 1996; Lusk & Kelly 2003; Christie & Armesto 2003).

EcM symbiosis is considered an important nutritional strategy particularly from organic sources in nutrient-poor soils (Read *et al.* 2004). EcM plants may thus rely on their fungal symbionts in nutrient capture from strongly decayed CWD and/or its decomposer community (Lindahl *et al.* 2002; Tedersoo *et al.* 2003). In boreal forests, conifer and hardwood seedlings become EcM usually within a few months after seed germination in CWD (Christy *et al.* 1982; L. T. personal observation). Seedlings acquire their EcM symbionts either from spore-derived mycelium or from mycorrhizal mature tree roots that penetrate CWD from soil (Tedersoo *et al.* 2007b). In boreal forests, both species richness and frequency of individual fungal species on seedlings in CWD depends on the presence of root connections with mature host trees. Isolated seedlings harbour only a few EcM fungal species with broad successional range and belong to the orders of Atheliales and Thelephorales that form predominantly resupinate fruit bodies (Tedersoo *et al.* 2007b)

We hypothesized that EcM fungi that colonize isolated seedlings of *Nothofagus cunninghamii* (Hook.) Oerst in CWD are similarly species-poor and taxonomically closely related to these European members of Atheliales and Thelephorales. The EcM fungal community on seedlings at two sites and on mature *N. cunninghamii* roots at one site were examined, using anatomotyping and rDNA sequence analysis.

MATERIALS AND METHODS

Site description

Root tips of *N. cunninghamii* seedlings and mature trees were collected from an 1-ha site at Yarra National Park, Victoria state, Australia. The Yarra site harboured a wet sclerophyll forest dominated by *N. cunninghamii* that formed a monodominant stand in creek banks. A few *Acacia dealbata* (Link) Muell. trees were present, but other potential EcM hosts were virtually absent from the study

site. The understorey was dominated by a tree fern, *Dicksonia antarctica* Labill. The forest floor was strongly disturbed by lyrebird (*Menura novaehollandiae* Latham) activities. Small shrubs and grasses were sparse.

The Warra LTER near Tahune, Geeveston, Tasmania was chosen as a second site. The seedlings were collected from an area known as the Bird Track (43°05.6' S; 146°39.0'E), approximately 155 m a. s. l. The mean annual rainfall is 1080 mm. The bedrock type is quartzite with a dolerite talus (Alcorn *et al* 2001). The wet sclerophyll vegetation is dominated by *Eucalyptus obliqua* (L'Hérit.) with a subdominant layer comprising *Eucryphia lucida* (Labill.) Baill., *Atherosperma moschatum* Labill., *N. cunninghamii* and *Phyllocladus aspleniifolius* (Labill.) Hook. The understorey is made up of ferns *Anopterus glandulosus* Labill., *D. antarctica* and *Polystichum proliferum* (R.Br.) Presl. Large boles of *E. obliqua* are covered with bryophytes and support numerous seedlings and saplings of *N. cunninghamii* as well as various shrubs and ferns.

Sampling and DNA analysis

At Warra and Yarra, 1-5 year-old seedlings of *N. cunninghamii* were carefully pulled out from CWD and placed into plastic bags. It was ensured that no roots of mature EcM host trees occurred in the rooting zone of these seedlings. At Yarra, root samples of mature *N. cunninghamii* (15 x 15 cm diam. to 5 cm depth) were collected from the organic horizon in forest floor. Root systems were placed into Petri dishes with tap water, where root tips were separated into EcM morphotypes based on colour, surface texture, presence or absence of cystidia, emanating hyphae and rhizomorphs. Root tip clusters corresponding to each morphotype per root sample were inserted into 1% CTAB extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% cetyl trimethyl ammonium bromide) for further analyses. Subsequently, several root tips from each morphotype were subjected to anatomotyping as outlined in Agerer (1991). The DNA of one or two root tips of each anatomotype per site was extracted using a High Pure PCR Template Preparation Kit for Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, Indiana, USA). Using primer ITS1F (5' cttggtcatttagaggaagtaa 3') in combination with LB-W (5' cttttcatctttccctcacgg 3') or LA-W (5' cttttcatctttcgatcactc 3'), we selctively amplified the Internal Transcribed Spacer (ITS) region of basidiomycetes and ascomycetes, respectively, as described in Tedersoo (2007). In addition, the rDNA Large Subunit gene was amplified using primers LR0R (5' acccgctgaacttaagc 3') and LB-Z (5' aaaaatggcccactagaaact 3') or LR3A (5' cacytactcaaatccwagmg 3'). PCR products were checked on 1% agarose gels under UV light. Single products were purified using Exo-Sap enzymes (Sigma, St. Louis, Missouri, USA). For sequencing, primers ITS5, ITS4 and ctb6 were used. Contigs were assembled

using Sequencher ver. 4.7 (GeneCodes Corp., Ann Arbor, Michigan, USA). A value of 97.0% ITS region identity was used as a DNA barcoding criterion (Tedersoo *et al.* 2003). For *Cortinarius* and *Laccaria*, 98.0% criterion was used, because ITS region is relatively conserved in these genera. All unique sequences were submitted to EMBL sequence database. BlastN and fasta3 searches were performed against public sequence databases NCBI, EMBL and UNITE (Kõljalg *et al.* 2005) to provide as precise identification for the EcM fungi as possible.

Statistical analyses

To compare the species diversity between floor soil and CWD samples, species accumulation curves with 95% confidence intervals were computed using EstimateS ver. 8 (Colwell 2006). *Nothofagus cunninghamii* root samples from a mixed wet sclrophyll forest of Mt. Field National Park, Tasmania (Tedersoo *et al.* 2007a) were included to compare accumulating species richness between sites. To further compare species richness in CWD between different continents, samples from 1–2 year-old *Betula pendula* L. (Betulaceae) seedlings inhabiting CWD in Estonian boreal forests were also included. In these analyses, fungal species were sampled randomly without replacement and soil samples or individual seedlings were used as sampling units. To account for the effect of unequal size of root systems, the most abundant species from each sample of Australian study sites were randomly sampled using incidence-based rarefaction. T-tests, followed by Bonferroni corrections, were calculated to reveal differences in abundance of EcM fungal lineages among samples from forest floor soil and CWD at Yarra. Proportions of root tips from each root sample were arc sin-square root-transformed to meet the assumptions of parametric tests.

RESULTS

At Yarra, 24 and three species of EcM fungi were retrieved from forest floor soil and CWD, respectively. Eight species were found in the roots of *N. cunninghamii* seedlings on CWD at Warra. When rarefied to seven samples (samples where EcM root tips were present in CWD at Yarra), the number of species was reduced to 16.5 ± 4.5 (mean ± 95% CI) and 5.5 ± 2.2 species in forest floor soil at Yarra and in seedlings on CWD at Warra, respectively. Based on non-overlapping confidence intervals, seedlings on CWD had fewer species of EcM fungi compared to forest floor samples at both sites (Fig. 1a). When only the dominant species in each root sample were included in the analysis, the differences were no longer significant due to small sample size (Fig. 1b).

In terms of species richness, the EcM fungal community was dominated by the lineages of *Cortinarius* (including *Thaxterogaster*), *Laccaria*, *Descolea* (including *Setchelliogaster* and *Descomyces*) and *Tomentella-Thelephora* in forest floor soil at Yarra (Table 1). *Cortinarius* sp3, *Russula* sp9 and *Laccaria* sp7 were the most frequent species. *Laccaria* sp7 and *Tomentella* sp2; and *Laccaria* sp2 were the most common species on seedling roots in CWD at Warra and Yarra, respectively.

Members of the *Cortinarius* and *Russula-Lactarius lineages* that were the most frequent and abundant in forest floor soil at Yarra, were not observed in CWD neither at Yarra nor Warra. At Yarra, *Cortinarius* was significantly more abundant in forest floor soil compared to CWD (t_{20} = 20.8, $P < 0.001$), whereas *Laccaria* was significantly more abundant on roots of seedlings in CWD (t_{20} = 9.65, $P = 0.006$; Fig. 2).

DISCUSSION

The diversity of EcM fungi was surprisingly low in the pure, primeval stand of *Nothofagus cunninghamii* at Yarra. Further comparisons of rarefied species richness to *Nothofagus* roots at Mt. Field National Park, Tasmania revealed that EcM fungal accumulating species diversity was significantly lower at Yarra. This can be attributed to both monospecificity (DeBellis *et al.* 2006; Tedersoo *et al.* 2006, 2007a) or uniform overmaturity of the trees. Slowly growing and stressed plants are known to associate with less diverse communities of EcM fungi (Swaty *et al.* 2004; McHugh & Gehring 2006; Korkama *et al.* 2007). On the other hand, belowground species composition was similar at Yarra and Mt. Field (Tedersoo *et al.* 2007a). Namely, the EcM fungal lineages of *Cortinarius*, *Tomentella-Thelephora*, *Laccaria* and *Descolea* dominated the EcM fungal communities. Yarra and Mt. Field shared four of the observed species. As a major difference to Mt. Field, we detected neither *Cenococcum geophilum* nor *Clavulina* spp. on *Nothofagus* at Yarra and Warra. At Mt. Field, *C. geophilum* was significantly more frequent on *Pomaderris apetala* Labill. (Rhamnaceae), compared to *Eucalyptus regnans* F. Muell. and *N. cunninghamii*. In addition, most of the most common species displayed strong host preference, which was suggested to contribute to the high diversity at Mt. Field (Tedersoo *et al.* 2007a).

At least partly due to differences in root density and abundance, seedlings on CWD had fewer species of EcM fungi compared to mature tree roots in the forest floor. Low fungal species richness of seedlings in CWD has been reported from the Northern Hemisphere (Christy *et al.* 1982; Kropp 1982), where *Tomentella sublilacina* (Ellis & Holw.) Wakef., *Amphinema byssoides* complex and *Tylospora fibrillosa* Donk are the three most frequent species (Tedersoo *et al.* 2007b). These three species fruit on the underside of CWD and

are among the most abundant members of EcM fungal communities in boreal mixed forests (Tedersoo *et al.* 2007b). Closely related species are not known to associate with Australian indigenous host trees and were not detected belowground. In Australia, however, the EcM lineages of *Laccaria* and *Descolea* that form stipitate, agaricoid fruit-bodies, dominated on isolated seedlings in CWD. In contrast, *Laccaria* spp. rarely colonize seedlings on CWD in European boreal forests, although they inhabit root tips and fruit abundantly in other disturbed forest microsites (Tedersoo *et al.* 2007b; unpublished). In agreement with the European study, the fungi colonizing isolated seedlings on CWD are among the dominant or subdominant taxa in the forest floor soil (this study; Tedersoo *et al.* 2007a). However, the EcM fungal lineages of *Russula-Lactarius* and *Cortinarius* that codominated the forest floor soil, were lacking on seedlings in CWD. These taxa are considered late successional colonizers that may be excluded from isolated seedlings due to high resource requirements or poor infectivity from spores (Last *et al.* 1987; Gibson & Deacon 1990; Newton 1992; Hutchison & Piché 1995). Nevertheless, the results of this study further substantiate the hypothesis that resupinate fruiting habit on the underside of dead wood *per se* does not provide a competitive advantage on isolated seedlings in CWD (Tedersoo *et al.* 2007b).

In conclusion, seedlings of *Nothofagus* associate with EcM fungi during their establishment on CWD in Australian wet sclerophyll forests. The associated fungi are among the generalist dominants of the forest floor soil EcM fungal community, which is a similar phenomenon to boreal forests of the Northern Hemisphere. There is no evidence that preferential fruiting on dead wood is correlated with mycorrhizal colonization of seedlings on CWD. The direct contribution of EcM fungi to seedling establishment on CWD remains to be determined in future studies.

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Table 1. Identification and abundance of species of ectomycorrhizal fungi at Yarra and Warra. Best BLASTn or FASTA3 full-length matches are shown. Proportion of seedlings colonized by EcM fungal species are indicated.

Species	Best BLASTn/FASTA3 match	Identity (%)	Yarra forest floor (n = 16)	Yarra CWD (n = 7)	Warra CWD (n = 10)
Cortinarius sp1	*Thaxterogaster albocanus* AF325599	93.1	0.56	0	0
Russula sp1	*Russula mustelina* AY061727	87.4	0.50	0	0
Laccaria sp1	*Laccaria murina* AB211271	94.1	0.44	0.57	0.1
Peziza-Terfezia lin. sp1	*Terfezia arenaria* AF276674	76.8	0.38	0	0
Sordariales sp1	*Thielavia hyrcaniae* AJ271581	80.1	0.38	0	0
Descolea sp1	*Setchelliogaster* sp. DQ328087	99.5	0.31	0	0
Cortinarius sp2	*Cortinarius cystideocatenatus* AY669651	95.1	0.25	0	0
Tomentella sp1	*Tomentella subclavigera* AY010275	93.8	0.19	0.29	0.1
Cortinarius sp3	*Cortinarius amoenus* AF389160	93.7	0.19	0	0
Cortinarius sp4	*Cortinarius amoenus* AF389160	93.5	0.13	0	0
Cortinarius sp5	*Cortinarius amoenus* AF389160	91.9	0.13	0	0
Cortinarius sp6	*Thaxterogaster albocanus* AF325599	94.8	0.13	0	0
Cortinarius sp7	*Cortinarius walkeri* AY669632	96.5	0.13	0	0
Cortinarius sp8	*Cortinarius collariatus* AY033115	93.5	0.13	0	0
Tomentella sp2	*Tomentella botryoides* UDB000255	92.8	0.13	0	0
Tomentella sp3	*Tomentella lateritia* UDB000963	93.0	0.13	0	0
Cortinarius sp9	*Thaxterogaster levisporus* DQ328105	93.4	0.06	0	0
Descolea sp2	*Descomyces albus* DQ328209	93.2	0.06	0	0
Descolea sp3	*Descomyces albus* DQ328209	93.7	0.06	0	0
Descolea sp4	*Descomyces* sp. DQ328062	99.0	0.06	0	0
Elaphomyces sp1	*Elaphomyces muricatus* DQ974740	87.1	0.06	0	0
Laccaria sp2	*Laccaria laccata* AJ699075	95.1	0.06	0	0
Laccaria sp3	*Laccaria laccata* AJ699075	98.2	0.06	0	0
Russula sp2	*Russula clelandii* DQ328136	93.9	0.06	0	0
Inocybe sp1	*Inocybe relicina* AF325664	82.7	0	0.14	0
Laccaria sp4	*Laccaria laccata* AJ699075	97.2	0	0	0.3
Descolea sp5	*Descolea recedens* AF325649	98.4	0	0	0.2
Laccaria sp5	*Laccaria laccata* AJ699075	96.1	0	0	0.2
Sordariales sp2	*Lasiosphaeria glabrata* AY587914	80.5	0	0	0.2
Endogonales sp1	*Endogone pisiformis* AF006511	Partial match	0	0	0.1
Tomentella sp4	*Tomentella stuposa* UDB000967	90.9	0	0	0.1

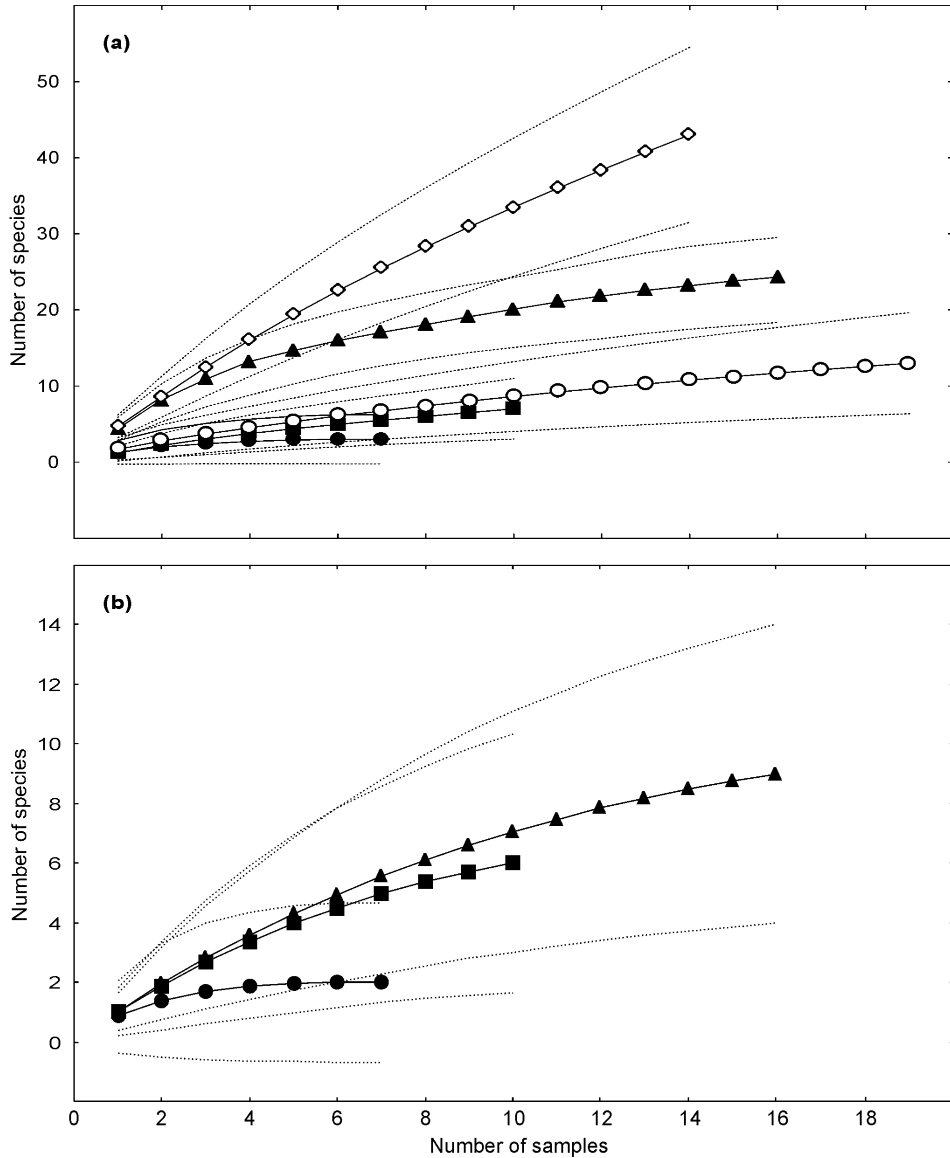

Figure 1. Rarefaction curves with 95% confidence intervals (pointed lines) demonstrating the accumulating ectomycorrhizal fungal species richness in different sites and habitats. (a) sample-based rarefaction with all species included; (b) incidence-based rarefaction with the most abundant species included. Open diamonds, *Nothofagus cunninghamii* root samples at Mt. Field National Park, Tasmania (Tedersoo *et al.* 2007a); closed triangles, root samples from forest floor soil at Yarra; open circles, 1–2 year-old *Betula pendula* seedlings on decayed logs in Estonia (Tedersoo *et al.* 2007b); closed circles, *N. cunninghamii* seedlings on decayed logs at Warra; closed squares, *Nothofagus cunninghamii* seedlings from decayed wood at Yarra.

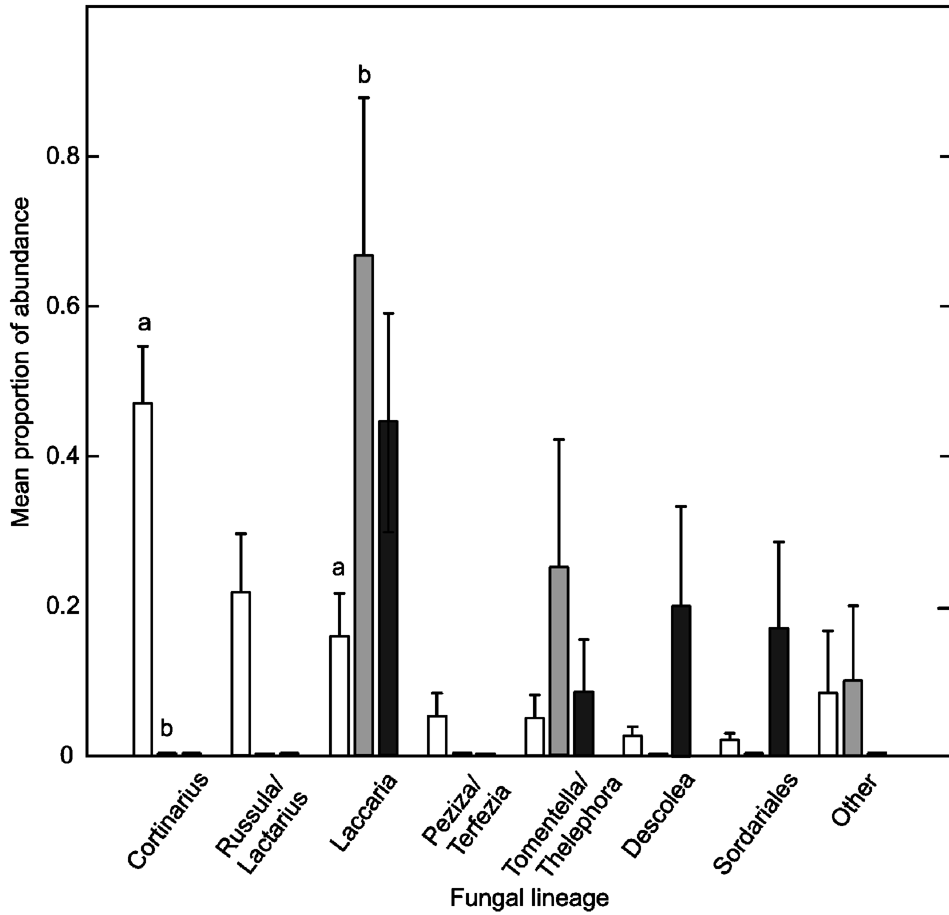

Figure 2. Mean (± S.E.) proportion of root tips colonized by members of different lineages of ectomycorrhizal fungi in forest floor soil at Yarra (open colums), decayed wood at Yarra (shaded columns) and decayed wood at Warra (filled colums). Letters denote statistically significant differences between decayed wood and forest floor at Yarra.

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Tedersoo L, Suvi T, Larsson E, Kõljalg U. Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. IMC8. August 2006. Cairns.

Tedersoo L, Hansen K, Perry BA, Kjøller R. Molecular and morphological diversity of pezizalean ectomycorrhiza. IMC8. August 2006. Cairns.

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Awards & Scholarships

British Mycological Society	Best student presentation. IMC8, Cairns, 2006 (2nd prize)
Doctorate School of Environmental Sciences	Graduent student scholarship, 2006
Doctorate School of Environmental Sciences	Graduent student scholarship, 2005
World Federation of Scientists	Graduate student scholarship, 2005
Ministry of Science and Education	Best student M.Sc. project, 2003 (1st prize)

Other activities and memberships

2002–	Member of the Estonian Naturalists' Society
2005–2006	President of the Estonian Mycological Society

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