Ectomycorrhizal fungi - Molecular tools to study species and functional diversity

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Propositions / Stellingen

1. Molecular methods provide essential tools for the identification and relative quantification of ectomycorrhizal mycelium.

This thesis.

2. The claim that molecular quantification of ectomycorrhizal fungi is generally unnecessary as ectomycorrhizas are countable macroscopic packages *(Horton and Bruns, Molecular Ecology 10, 2001)* underestimates the ecological importance of the extramatrical mycelium.

This thesis.

3. Establishing species richness - functional diversity relationships remains one of the most intractable challenges in ecological research.

J.R. Leake, New Phytologist 152, 2001.

4. Nothing ages as rapidly as the newest molecular techniques.

5. Het succes van promovendi in hun (toekomstige) loopbaan kan worden geoptimaliseerd door professionele coaching en loopbaanbegeleiding.

VSNU, 2002.

6. Het zou de gezondheid van de veeteelt- en pluimveesector ten goede komen indien in de toekomst het welzijn van dieren niet wordt gedefinieerd als de afwezigheid van stress, maar als de aanwezigheid van geluk.

Vrij naar Koos van Zomeren, 2002.

7. Eeyore's gezegde 'brains first, then hard work' geldt niet alleen voor ezels, maar ook voor wetenschappers.

Pooh's little instruction book, A.A. Milne.

8. Slechts het voorbijgaande is van blijvende waarde.

Eugène Ionesco.

Stellingen behorende bij het proefschrift:
'Ectomycorrhizal fungi - Molecular tools to study species and functional diversity'
Renske Landeweert, Wageningen, 6 juni 2003

To see a world in a grain of sand
and a heaven in a wild flower
hold infinity in the palm of your hand
and eternity in an hour.

— William Blake, Auguries of Innocence

Abstract

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The extramatrical mycelium of ectomycorrhizal (EM) fungi actively mobilizes nutrients from organic sources through excretion of enzymes and from mineral sources through excretion of organic acids. An observation of tunnel-like structures in weatherable mineral grains gave rise to the hypothesis that these were formed by the hyphae of EM fungal species. Tunneled minerals have been found in the weathered E horizon of podzols, a typical soil type for boreal forests. In these forests the EM fungal community is highly species-rich and this thesis describes the vertical distribution of fungal species throughout the podzol profile, with special emphasis on the weathered E horizon. Identification of EM fungal species from podzol horizons through morphological and molecular identification of EM root tips revealed a significant relationship between the EM fungal species composition and soil horizons. The vertical distribution of the EM extramatrical mycelium in podzol horizons was studied with molecular identification techniques based on total soil DNA extracts and a cloning method. The root tip study as well as the mycelial study showed that many EM fungal species exist in the mineral soil that do not occur in the organic layer of a podzol. A high correspondence was furthermore found between the basidiomycete diversity detected by the molecular analysis of root tips and of hyphae.

This thesis further describes the use of molecular techniques for the quantification of EM fungal species in soil. By comparing molecular techniques with conventional quantification techniques, it was shown that molecular methods provide tools to determine the biomass of individual fungal species in soil. The molecular methods enabled identification and relative quantification of two EM fungal species separately in a more-species environment and showed consistent results.

In conclusion, this thesis demonstrates that molecular techniques facilitate identification and relative quantification of individual soil fungi and in addition highlights the pitfalls of the molecular methodology. As differences in amount and organization of the extramatrical mycelium produced by different EM fungal species may reflect the different ecological roles that the fungi may have, the detection of hyphae initiates possibilities for *in situ* studies on substrate specificity, niche partitioning and succession of EM fungal species below ground.

Keywords: Ectomycorrhizal fungi, community, diversity, hyphae, podzol, vertical distribution, mineral weathering, molecular identification, molecular quantification, ITS.

Contents

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General introduction

General introduction

Ectomycorrhizal (EM) fungi are a group of mutualistic soil fungi, which live in symbiosis with trees, woody perennials and some herbaceous plants. About 6000 species of EM fungi are thought to exist (4) and although the EM symbiosis is restricted to less than 5% of the terrestrial plant species, EM fungi present the major part of the soil fungal biomass in boreal forest systems (4). EM fungi colonize the roots of trees, thereby forming a hyphal mantle around root tips and a Hartig net of hyphae that penetrate between the cortical root cells. From these EM root tips (ectomycorrhizas) the EM fungus explores the surrounding soil by the formation of extensive extramatrical mycelium. Because of its high surface to volume ratio, the mycelium has a much higher absorbing surface than roots and is therefore of primary importance to the tree for the uptake of nutrients and water. In return, the fungus obtains photosynthates from the host tree. EM fungi may take up nutrients dissolved in the bulk soil solution. In addition, the fungi can mobilize N and P from organic material through enzyme production and the fungi can also mobilize base cations and phosphorus from minerals through production of low molecular weight (LMW) organic acids.

Boreal forest systems typically occur on one dominant podzol soil type. The podzols studied in this thesis are classified as haplocryods (1), are highly stratified and are characterized by four distinct horizons (3). Typically, the upper organic (O) horizon is underlain by a mineral eluvial (E) horizon, an illuvial (B) horizon and a parental C horizon. The formation of these horizons is induced by complexation and mobilization of iron and aluminum from the weathered E horizon and subsequent precipitation of the complexes in the underlying B horizon. In 1997, weatherable feldspar grains from the E horizon of a Swedish podzol were found to contain microscopic tunnel-like structures that sometimes contained fungal hyphae. Feldspars contain plant nutrients and can be weathered by fungal exudates like LMW organic acids. Representing a major part of the soil fungal community in boreal forest systems and being known for exuding LMW organic acids, it was hypothesized that EM fungi would form these tunnels when exploring the soil (2). The EM fungi would exude the complexing LMW organic acids at their hyphal tips, bore themselves into minerals and would thereby obtain a unique source of nutrients. In doing so, EM fungi would no longer depend on or need to compete with other microbes for dissolved nutrients available from the soil solution. In fact, EM fungi were hypothesized to have found a way to by-pass the soil solution rich in toxic aluminum, and would via this by-pass deliver mineral nutrients to the trees.

Outline of this thesis

In 1998, a program titled 'Rock-eating mycorrhizas: Where, why, how?' was started at Wageningen University that focused on the formation of the observed microscopic tunnels in mineral grains and the role that EM fungi might play in this mineral weathering process. The work presented in this thesis has been carried out as part of that program. The project was aimed at providing insight into the distribution of EM fungi in a podzol profile in relation to their possible functional role as weathering agents.

Some of the first questions that needed to be addressed after the start of the project were: Which species of EM fungi may be involved in mineral weathering and does fungal weathering capacity depend on the EM fungal species considered (Chapter 2). As detection of a single fungal species inside a tunnel would not prove that its hyphae actually formed the tunnel, no attempts were made to follow this approach. Instead it was decided to focus on the composition of the EM fungal community in the soil horizon where tunneled minerals were found to determine whether specific fungal species occur in this particular horizon. This would enable us to select for fungi that would probably be able to dissolve minerals and these could then be isolated and used for mineral weathering experiments.

EM community studies are often conducted through a combination of morphological and molecular identification of EM fungi on colonized root tips (Chapter 3). When considering the root tip diameters however, it is clear that the 4 to 10 μm-wide tunnels in minerals can not be formed by root tip penetration. To understand which fungi might possibly form tunnels in mineral grains, EM fungi needed to be identified that had a major part of their extramatrical mycelium in the soil horizon where tunneled minerals were found. Molecular methods were used to identify EM mycelium from four podzol horizons (Chapter 4), including fungal species from the E horizon with tunneled minerals. Yet, whether the obtained fungal species composition also represented the abundance of fungal species in the community remained unknown, as a natural system had been sampled containing unknown quantities of unknown fungi.

To fully exploit the molecular potential to answer ecological questions, it is essential that molecular methods enable identification as well as quantification of fungi in soil. To verify the significance of molecular data for quantitative analysis of fungal DNA extracted from soil, a pot experiment was performed (Chapter 5). The use of clones as a quantification tool was assessed and conventional fungal biomass measures were compared with molecular methods measuring DNA amounts in soil.

Although for fungal biomass quantification molecular methods are useful tools to consider (see Chapter 7), their compatibility with root tip identification methods needs to be considered as well. In Chapter 6, the fungal diversity in a natural forest

system was studied by two different molecular approaches. Here, the root tip identification approach was compared with the mycelial (total soil DNA) identification approach in order to find out whether the fungal diversity outcome would be different.

Finally, in Chapter 7, the root tip and mycelial approach are put in perspective and their use for fungal community analysis is discussed. The use of molecular methods is compared to other methods currently available to quantify fungal biomass in soil. Some methodological aspects regarding the use of DNA for fungal identification and quantification are discussed and possible methodological approaches for future studies suggested. This final chapter summarizes the results presented in this thesis and describes molecular methods as tools in future studies on the spatial distribution of EM fungal species and on their possible functional role as mineral weathering agents.

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2

Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals

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Traditional theories about the role of ectomycorrhizal fungi in plant nutrition have emphasized quantitative effects on uptake and transport of dissolved nutrients. Qualitative effects of the symbiosis on the ability of plants to access organic N and phosphorus (P) sources have also become increasingly apparent. Recent research suggests that ectomycorrhizal fungi mobilize other essential plant nutrients directly from minerals through excretion of organic acids. This enables ectomycorrhizal plants to utilize essential nutrients from insoluble mineral sources and affects nutrient cycling in forest systems.

Physical and chemical weathering of the earth's primary rock results in a release of dissolved mineral elements and residues into the biological environment. The rise of vascular land plants about 400 M ya ago presumably led to vastly increased mineral weathering (6,11). Some of the photosynthetic products formed in plant leaves end up as organic acids that are exuded by plant roots into the surrounding soil. Together with CO_2, which is pumped into the soil via root respiration and heterotrophic respiration of soil microorganisms, these organic acids greatly enhance the dissolution of primary silicate minerals (Box 1), thereby mobilizing essential lithophilic plant nutrients. Silicate minerals like feldspars, micas, hornblende and pyroxene provide calcium (Ca), magnesium (Mg) and potassium (K) and apatite is the main primary mineral source of phosphorus (P). The carbon-rich root exudates also support large communities of root-associated microorganisms (rhizosphere bacteria and fungi) that further accelerate weathering of minerals by excreting organic acids, phenolic compounds, protons and siderophores (12,18). Such plant-induced mineral weathering must have facilitated the subsequent spread of land plants by increasing the availability of essential plant nutrients and by producing secondary minerals, such as clays and iron (Fe) and aluminium (Al) oxides, providing soil material for anchorage and water holding. Continuing today, essential plant nutrients enter ecosystems through biogeochemical weathering of primary minerals. In order to understand soil ecological processes, knowledge of the potential influence of plant roots and their associated microbiota on weathering processes is essential.

Organic acids as weathering agents

Soluble organic acids affecting mineral weathering in soils originate from various sources. Medium- to high molecular weight organic acids, such as humic substances, are less effective in promoting mineral dissolution than low molecular weight (LMW) organic acids produced by plant roots and soil microorganisms (33).

Box 1. Mineral weathering

Chemical weathering is the transformation of rock-forming primary minerals into dissolved substances and secondary mineral residues under the influence of water, acids, complexing agents and oxygen. Biological weathering or biochemical weathering is mediated by organisms. Secondary minerals include clay minerals such as kaolinite, and oxides of Fe and Al. These, together with resistant primary minerals such as quartz, make up the bulk of highly weathered soils.

Examples of weathering reactions under the influence of H^+ are the transformation of an alkali feldspar ($KAlSi_3O_8$) into kaolinite ($Al_2Si_2O_5(OH)_4$, a secondary mineral):

$$2KAlSi_3O_8 + 2H^+ + 9H_2O \rightarrow Al_2Si_2O_5(OH)_4 + 4H_4SiO_4 + 2K^+ \quad [1]$$

and the complete dissolution of this alkali feldspar:

$$KAlSi_3O_8 + 4H^+ + 4H_2O \rightarrow Al^{3+} + 3H_4SiO_4 + K^+ \quad [2]$$

The low solubility of Al at pH values of >4.5 is a major reason for the slow dissolution rates of Al silicates such as feldspars (between 10-13 to 10-18 $mol.m^{-2}.s^{-1}$), which form the bulk of weatherable primary minerals in most soils. Certain water-soluble organic anions [L^{n-} (L, ligand)], for instance oxalate or citrate, form strong complexes with cations such as Al^{3+}:

$$Al^{3+} + L^{n-} \rightarrow AlL^{3-n} \quad [3]$$

This complexation decreases the concentration of aqueous Al^{3+}, thereby increasing the solubility and weathering rate of minerals containing Al. Complexed Al is less toxic to plants than Al^{3+}.

Although constituting only a minor fraction of the total organic acids in the soil solution, LMW organic acids are generally considered to be the most important biological weathering agents in soils, owing to their acidifying and complexing capacities (5,33). Depending on the number and configuration of carboxylic and phenolic groups and the related acid strength, organic acids provide H^+ for protonation of the mineral surface and chelate cations(46) (Box 1). Concentrations of LMW organic acids in the bulk soil solution are generally too low to accelerate mineral weathering, but concentrations in the microenvironments around microbes, hyphae and roots are often high enough to do so(3-5,13). Of several LMW organic acids released by plant roots, bacteria and fungi, oxalate, citrate and malate are the

strongest chelators of trivalent metals such as Al^{3+} and Fe^{3+} (21). Oxalic acid has the highest acid strength and also forms complexes with K, Ca, Mg, manganese (Mn), zinc (Zn), copper (Cu), Al and Fe (16). It is commonly produced in large quantities by many different fungal species (14). Oxalate is a by-product of the hydrolysis of oxaloacetate originating from the citric acid and glyoxylate cycles, and is also formed by oxidation of glyoxilate (14). The citric acid cycle is upregulated by growth-limiting concentrations of Mn, Fe and Zn (16). Although probably toxic to fungi (16), oxalic acid is functionally significant to different groups of fungi. Functional roles of oxalate vary from detoxification of metals, increasing the susceptibility of plant tissue to pathogenic fungal penetration by decreasing host tissue pH(14) or being an electron donor in lignocellulose degradation (16).

Nutrient mobilization by symbiotic fungi

Lichenous fungi

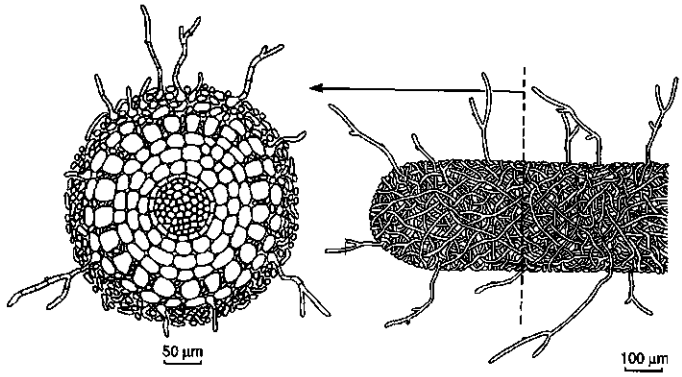
In common with free-living fungi, symbiotic fungi also produce and excrete organic anions and protons. Well known examples include lichens, which are formed by the association of lichenous fungi and photosynthetic algae or cyanobacteria, often growing on solid rock. The mycobiont mobilizes elements from the rock by excreting a range of acids, including oxalic acid, derived from carbohydrates provided by the photobiont (4). The hyphae penetrate cracks in the rock surface (25), form trench-like features and etch pits in the mineral surface(48) and enhance biotite dissolution underneath lichen thalli (47).

Ectomycorrhizal fungi

Over 80% of all land plants form some type of symbiotic association with mycorrhizal fungi. By colonizing plant roots, mycorrhizal fungi absorb nutrients and water from the soil and usually obtain plant photosynthates in return. The transfer of carbon from plant to fungus is analogous to the transfer within lichen thalli (4). The ectomycorrhizal symbiosis is the dominant type of association between roots of most long-living woody perennials and trees with members of the Basidiomycota and Ascomycota (40) (Box 2). Ectomycorrhizal symbioses are restricted to <5% of terrestrial plant species, but members of the Pinaceae and Fagaceae, which dominate temperate forests, together with members of the Myrtaceae and Dipterocarpaceae from (sub)tropical regions, are predominantly ectomycorrhizal. As many as 6000 fungal species may form ectomycorrhizal associations (40) in which the fungus forms a mantle of fungal material around the root tip (Box 2). The hyphae that grow outward from the mantle into the surrounding soil are very efficient nutrient scavengers, owing to their high surface area:mass ratio and their ability to penetrate

Box 2. The structure of ectomycorrhizas

Ectomycorrhizas typically consist of an intimate association between a living root tip and a basidiomycete or ascomycete fungus[a]. The fungus forms a mantle of fungal material around the root tip and penetrates the intercellular space between cortical root cells, forming a 'Hartig net'.



Transfer of materials between fungus and plant takes place in the Hartig net. External hyphae grow outwards from the fungal mantle into the soil and the fungal mycelium might differentiate into rhizomorphs, which are hyphal aggregates that can transport water and nutrients over several dm to the root. The ectomycorrhizal fungus produces hormones that suppress root-hair growth and might induce typical dichotomous branching of the mycorrhizal root tip. Single mycorrhizal root tips can transform into clusters of root tips colonized by a single fungal species.

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microsites that are inaccessible to plant roots. The expanding mycorrhizal mycelium exploits a larger soil volume than roots alone. The positive effects of ectomycorrhizal fungi on plant nutrition have traditionally been attributed to the quantitative effects of the extraradical mycelium on uptake of dissolved nutrients from the soil solution and subsequent transport to the plant roots. More recently, attention has been paid to qualitative effects of the symbiosis on plant nutrition and the ability of ectomycorrhizal fungi to use organic N and P sources (Box 3), which would otherwise be largely unavailable to plants (40). Ectomycorrhizal fungi might also be able to access pools of P within mycelia of saprotrophic fungi (29). These

types of nutrient uptake effectively represent 'short cuts' to traditionally described pathways of decomposition and mineralization and highlight the important function of ectomycorrhizal fungi in forest nutrient recycling.

Mineral weathering by ectomycorrhizal fungi

Experimental evidence

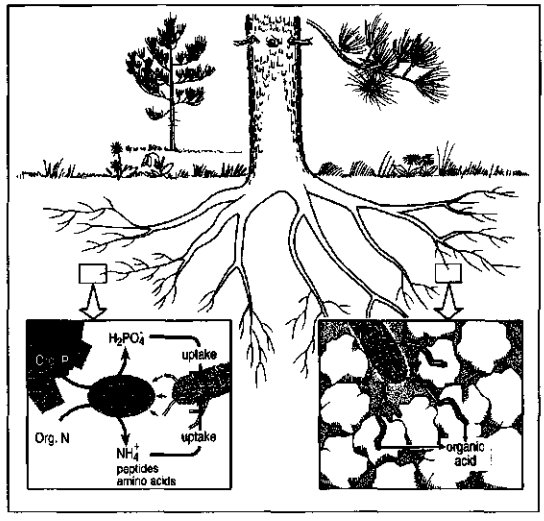
Recently, the potential ability of ectomycorrhizal fungi to actively mobilize and translocate essential plant nutrients from minerals has become a focus of attention (Box 3). As in lichenous fungi, ectomycorrhizal fungi receive carbon from their host and solubilize surrounding weatherable minerals through excretion of organic acids. When grown in vitro on agar plates, different ectomycorrhizal fungal species produce oxalic acid, solubilize Ca-phosphates deposited on agar (23,27) and mobilize K^+, NH_4^+ and Ca^{2+} trapped inside mineral interlayer spaces (35-37). Simultaneous depletion of K^+ and Mg^+ in the growing medium increases mineral weathering (37). Weathering performance in vitro can vary greatly between different ectomycorrhizal fungal species (23), between different strains belonging to the same fungal species (23) and between monokaryotic and dikaryotic mycelia of the same ectomycorrhizal fungal species (32).

Weathering of soil minerals *in vivo* has been observed for ectomycorrhizal fungi growing in symbiosis with host tree seedlings (28,34,43-45). Phosphorus is mobilized from apatite (43) and K is mobilized from biotite (44,45) by ectomycorrhizal pine seedlings in long-term pot experiments. In these experiments only traces of organic acids are detected in the rhizosphere of mycorrhizal pines and the release of essential plant nutrients is seldom positively correlated to the oxalic acid concentration in the soil solution (44). High micro spatial variability in concentrations and rapid microbial consumption of carbon-rich plant and fungal exudates obscure the relationships between organic acid concentrations and ectomycorrhizal effects on weathering and nutrient uptake under more natural conditions. Dual inoculation with rhizobacteria and an ectomycorrhizal fungus shows that exudation of organic acids by roots is indeed modified by the presence of ectomycorrhizal fungi as well as rhizobacteria (28). Furthermore, ectomycorrhizal fungal exudations, bacterial numbers and bacterial activity change with the kind of mineral applied (34), thereby probably influencing mineral weathering effects. When grown in axenic conditions, ectomycorrhizal pine seedlings generally produce more oxalic acid than do nonmycorrhizal seedlings (1).

Box 3. Nutrient mobilization by ectomycorrhizal fungi

As well as the quantitative effects on plant nutrient uptake (increase in uptake surface and exploited soil volume) the ectomycorrhizal fungus influences the uptake of plant nutrients in two qualitative ways:

(a) Via enzyme production the ectomycorrhizal fungus can utilize organic N and P forms, which would otherwise remain largely unavailable to roots. Nutrient mobilization from amino acids, peptides, proteins, amino sugars, chitin and nucleic acids has been shown[a], together with transfer of N and P into the host plant[a,b]. Direct hyphal absorption of amino acids and simple peptides can also occur[a].



(a) (b)

(b) Through organic acid excretion the ectomycorrhizal fungus can mobilize P, K, Ca and Mg from solid mineral substrates[c,d,e]. In addition, tunnels in weatherable minerals enable ectomycorrhizal hyphae to reach the interior of the minerals and access P from apatite inclusions. Essential nutrients become available to the host plant via the ectomycorrhizal mycelium[c]. Analogous to their organic nutrient mobilizing capabilities, the abilities of different ectomycorrhizal fungi to mobilize inorganic nutrients might be species specific[f].

(for references see next page)

References box 3:

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e) Wallander, H. (2000) Use of strontium isotopes and foliar K content to estimate weathering of biotite induced by pine seedlings colonised by ectomycorrhizal fungi from two different soils. *Plant Soil* 222, 215-229

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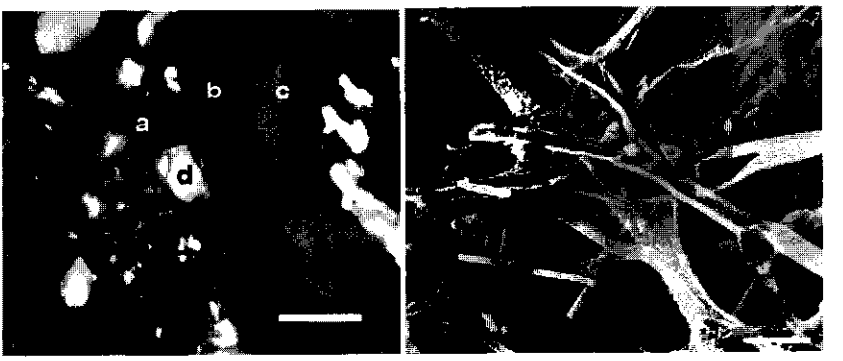
Ectomycorrhizal fungi and weathering of forest soils

A clear example of ectomycorrhizal mineral weathering is provided by mat-forming fungi. In forest soil, certain species of ectomycorrhizal fungi (*Hysterangium* spp, *Hydnellum* spp and *Gautieria* spp) form mat-like structures at the interface of the surface humus layer and upper mineral soil. These fungal mats can cover several m^2 of forest floor (15). The mineral soil within this concentrated mass of hyphae is often weathered more strongly than is the surrounding soil and this has been attributed to the excretion of oxalic acid by the mat-forming ectomycorrhizal fungus (15). Within the mats, calcium oxalate crystals are abundant and decomposition rates and nutrient availability are increased relative to the adjacent soil (15). Concentrations of essential plant nutrients in fungal mat tissue might fluctuate throughout the year, depending on the activity of the host trees(15).

Ectomycorrhizal root tips can also change the mineralogy of surrounding soil. In the immediate vicinity of roots, root-induced weathering takes place and ectomycorrhizal fungi can enhance this process by excretion of organic acids(3). Lower pH in ectomycorrhizosphere soil contributes to increased weathering of micas and K feldspars around the colonized root tips, increasing cation exchange capacity and concentrations of soluble and exchangeable K^+, Ca^{2+} and Mg^{2+} (2,3).



(A) (B)

Figure 1. A thin-section of (A) a cross-sectioned ectomycorrhizal root tip illustrates how (a) ectomycorrhizal hyphae, emanating from (b) the fungal mantle around (c) a root, enclose (d) mineral particles from the adjacent soil. The direct contact between the hyphae and the mineral surface is revealed on (B) a SEM picture of branching hyphae that cover and penetrate a mineral particle. Scale bars are 50 μm and 10 μm respectively.

Mineral weathering by individual ectomycorrhizal hyphae

Weathering of soil minerals is not only brought about by ectomycorrhizal root tips, but can also be enhanced by ectomycorrhizal hyphae that radiate outwards from the colonized root tips. In soil, hyphae tightly enclose mineral particles (38,42) (Fig. 1) and penetrate mineral interlayer spaces (38). In two-compartment experimental pot systems ectomycorrhizal hyphae exploit root-free soil compartments amended with apatite and free P from them(43).

Even the interior of weatherable minerals might become exploited by ectomycorhizal hyphae. Thin section micrographs of feldspars and hornblendes from coniferous forest soil show open tunnels with rounded ends, curved tracks and a diameter of 3-10 μm (22,42). The tunnels with a constant diameter differ morphologically from the crystallographically oriented etch pits and saw-toothed cracks that would result from chemical weathering (24) (Box 4). The presence of hyphae inside the tunnels indicates that single hyphae reach the interior of the tunnelled weatherable minerals (7,22,42). Exudation of organic acids at the hyphal tips would free essential cations such as K^+, Ca^{2+} and Mg^{2+} from the mineral interior (42) and could be responsible for formation of the tunnels (7,22,42). Subsequent transport of dissolved cations through the ectomycorrhizal mycelium towards the host tree roots could benefit tree growth (20). It has been shown that isotopically labelled Mg can be translocated over a distance of several centimeters to ectomycorrhizal roots, improving the Mg nutrition of the host plant (20). Some

feldspars contain apatite inclusions (39,42) and ectomycorrhizal hyphae might access these enclosed P sources through 'tunnel growth', thereby exploiting a mineral P source unavailable to plant roots. Selective dissolution of Ca-rich inclusions in volcanic glass has been ascribed to acid excretion by invading plant symbiotic fungal hyphae (7) and bacteria in groundwater systems selectively colonize and weather P-rich feldspars when P is in short supply (39).

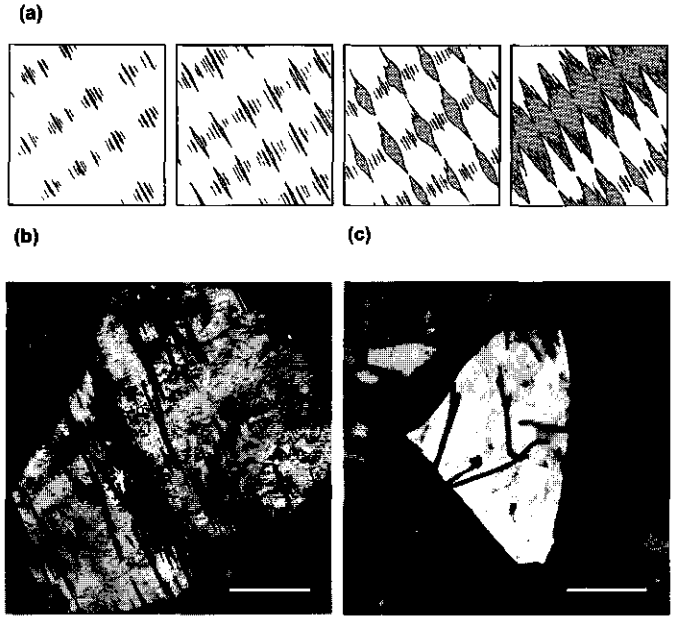
Implications of ectomycorrhizal weathering.

In summary, ectomycorrhizal fungi commonly produce organic acids, enabling them to actively mobilize plant nutrients from minerals. The implications of ectomycorrhizal fungal mineral weathering are manifold. First, it redefines our traditional view on the role of ectomycorrhizal fungi in root nutrient uptake. The effect of mineral nutrient mobilization by ectomycorrhizal fungi adds to the established quantitative effects on uptake and translocation of dissolved elements from the soil solution and the more recently studied qualitative effects of enzymatic release of N and P from organic sources (40) (Box 3). In addition to producing a large surface area in contact with the soil, ectomycorrhizal hyphae can actively dissolve their mineral surroundings and release essential plant nutrients (Box 3). The selective colonization and weathering by ectomycorrhizal hyphae of K-, Mg- or Ca-rich feldspars could specifically improve K, Mg or Ca nutrition of trees.

Second, ectomycorrhizal hyphae can access enclosed nutrient sources in minerals that are unavailable to roots. Apatite inclusions in feldspars (2) are unavailable to roots but could be exploited by hyphae. Organic acid excretion in the tunnels will accelerate further disintegration of the minerals and result in enlarged weatherable surface areas, accessible to roots as well as fungi. Roots will promote mineral weathering, but just as their effectivity in nutrient uptake is restricted by their limited distribution compared to fungal mycelium, so will their effectivity as weathering agents. Third, excretion of oxalate by ectomycorrhizal fungi could reduce effects of soil acidification on forest productivity. At present, acidification of coniferous forest soils because of anthropogenic atmospheric inputs is widespread (8). This acidification has been accompanied by changes in the ratio of base cations:Al,resulting in high concentrations of Al^{3+} in the bulk soil solution, which could hamper root uptake of Ca^{2+} and Mg^{2+} and inhibit root elongation. In response to Al stress, plant roots might secrete oxalic acid and detoxify Al^{3+} by formation of Al-oxalate complexes (30).

Box 4. Formation of etch pits

(a) Chemical weathering of minerals leads to formation of regularly arranged, and regularly shaped, angular cavities, so-called etch pits. Etch pits form because minerals dissolve preferentially at and along crystallographically determined dislocations and planes. Saw-tooth cracks eventually form when side-by-side aligned etch pits coalesce[a] (Figure a, from left to right).



Thin section micrographs in cross-polarized light showing (b) a chemically weathered feldspar with partly coalesced etch pits. In contrast, a thin section micrograph of (c) a tunnelled feldspar shows a more or less irregular pattern of open, tubular pores, 3-10 μm in width that criss-cross the interior of the mineral.

Reference:

a) Berner, R.A. and Holdren, G.R. (1979) Mechanism of feldspars weathering. II. Observations of feldspars from soils. *Geochim. Cosmochim. Acta* 43: 1173-1185

When exposed to elevated Al, mycorrhizal pine seedlings, as well as nonmycorrhizal pine seedlings, show enhanced oxalic acid production (1). In addition to formation of Al-oxalate complexes, ectomycorrhizal fungi could alleviate Al stress to the trees by mobilizing base cations (Ca^{2+} and Mg^{2+}) from microsites inaccessible to the tree roots (42).

A fourth implication involves soil forming processes and their biogeochemical consequences. Taking into account that ectomycorrhizal associations are widespread and were established at least 50 M ya ago (26), mobilization of mineral elements by ectomycorrhizal hyphae and ectomycorrhizal root tips has probably contributed to soil formation and global element cycling via effects on mineral weathering and podzolization (42).

Prospects

In most boreal forest systems N is the growth-limiting nutrient for the standing trees (8,9,41). Nitrogen limitation impairs the synthesis of amino acids or other N compounds in plant tissues and leads to a relatively increased diversion of assimilates to plant roots (31). An increased below-ground carbon input might also result from elevated levels of atmospheric CO_2. With more carbon available, the activity and growth of ectomycorrhizal fungi is stimulated (10,17), resulting in increased organic N use and uptake of ammonium (9). In addition, a high carbon input and low N, P and trace metal availability upregulates the fungal citric acid cycle (16,19), stimulating oxalic acid production. Increased oxalate production enhances the availability of essential cations from soil minerals. The exact regulation of the ectomycorrhizal weathering process remains to be determined, but plant growth-limiting N concentrations and elevated atmospheric CO_2 concentrations are likely to increase mobilization of nutrients from minerals.

Biogeochemical weathering of minerals is brought about by many different groups of soil organisms. Some microorganisms might be associated with plant roots or ectomycorrhizal mycelium and the relative contribution of each group to mineral weathering is difficult to determine in situ. Potential ectomycorrhizal fungal weathering rates can be calculated (42) but quantification of its effects and the relative contribution of ectomycorrhizal inorganic nutrient mobilization to nutrient cycling have yet to be studied.

At present, increased biomass harvesting in intensive forestry practices leads to increased nutrient losses from boreal forest systems. In order to maintain long-term forest productivity, nutrients leached or removed from the forest system need to be replaced. Coinciding with elevated CO_2 levels, the ecological relevance of active

mobilization of inorganic nutrients by ectomycorrhizal fungal species could become more apparent in the near future.

The impact of plant-, lichen- and microbe-induced weathering on long-term geological processes is still under debate (4,6,11) and quantifying the relative contribution and ecological significance of ectomycorrhizal weathering provides a new research challenge.

Glossary

Apatite: group of calcium phospate minerals.
Axenic: *in vitro* cultures of microorganisms in absence of any other organism.
Biotite: mica type of mineral containing K, Fe and Mg.
Ectomycorrhizal fungus: fungus able to form ectomycorrhizas.
Etch pit: lens-shaped hollow inside a mineral, caused by the action of water and its solutes.
Extraradical mycelium: network of fungal hyphae growing away from the root tip into the surrounding soil.
Feldspar: primary aluminium silicate group of minerals. The major primary source of Ca and K in most ecosystems.
Hornblende: primary silicate group of minerals, the major primary source of Mg and Fe in ecosystems.
Hypha: fungal thread.
Lichenous fungus: fungus that grow in symbiosis with a green alga or cyanobacterium to form a lichen.
Lithophilic: derived from a rock.
Mica: group of aluminium silicate minerals containing K and possibly Mg and Fe that readily separate into thin leaves.
Mobilization: release by solubilization or mineralization.
Mycobiont: fungal component of a lichen.
Photobiont: photosynthesizing component of a lichen.
Primary mineral: unchanged rock mineral.
Rhizomorph: root-like structure formed by fungal hyphae.
Saprotrophic: able to feed on dead organic matter.
Secondary mineral: altered mineral, formed by weathering of a primary mineral.
Septate: divided by partitions.
Siderophore: compound that chelates iron.
Thallus: simple, undifferentiated body of a lichen.
Tunnel: hollow inside mineral characterized by a constant diameter and a rounded end.
Volcanic glass: amorphous mixture of silicates produced by the cooling of lava too rapidly to permit crystallization.
Weathering: chemical or physical transformation.

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3

Vertical distribution of ectomycorrhizal fungal taxa in a podzol profile

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Summary

- Podzols are highly stratified soils that often underlie boreal forests. Within these boreal forest soils, the species richness of the ectomycorrhizal (EM) fungal community is high, but the vertical distribution of EM fungi is largely uninvestigated.

- EM root tips were sampled from seven horizons in three columns of a 52 cm deep podzol profile. Root tips were sorted into morphotypes and the EM fungi identified by sequencing of the ITS-rDNA region. The distribution of EM fungal taxa was examined using correspondence analysis.

- A significant relationship between EM fungal species composition and soil horizon was found. *Tomentellopsis submollis,* three *Piloderma* species and *Dermocybe* spp. were found predominantly in the upper horizons while other species within *Cortinarius spp.* and *Suillus luteus* were associated with the lower mineral horizons.

- Two thirds of the root tips were found in the mineral soil and half of the distinguished EM fungal species were restricted to the mineral horizons. This highlights the need to include the mineral soil when assessing EM fungal species diversity in order to gain a more accurate representation of the whole fungal community.

Introduction

Boreal forests characteristically develop podzol soils. Slow decomposition rates in these ecosystems lead to the development of a surface layer of organic matter, where partial decomposition results in formation of organic acids, which percolate with rainwater through the soil. In the underlying, upper mineral soil, soluble complexes are formed between organic acids and Fe and Al, creating a weathered, eluvial E horizon. The organic matter-metal complexes percolate further through the profile and precipitate below the E horizon, creating a characteristic rust coloured illuvial B horizon overlying the parental C horizon. As few burrowing animals thrive in these soils, mixing is limited, leading to the conservation of visible horizons in the soil profile (22,31). Podzol soils are poor in easily accessible nutrients and plants and microorganisms compete for the scarce resources (21). Symbiotic ectomycorrhizal (EM) fungi colonise the fine roots of boreal forest trees and play an essential role in tree nutrient uptake (27).

Although the highest fine root density in boreal forest soils is found in the organic and upper mineral soil horizons (23,26,28), tree roots can be found at great depths (18). At all depths, fine roots are colonised by EM fungi (6), yet most EM fungal community studies restrict sampling to the upper, organic part of the soil profile (16) and thus ignore the EM root tips in the deeper mineral soil layers.

Chemical and mineralogical properties of soils change with depth, creating a number of different habitats for microorganisms and the EM fungal community is likely to change throughout the soil profile. Results from studies that have examined the distribution of morphologically defined EM taxa in soil, either directly in soil samples (6,8,12) or on bait seedlings in organic and mineral substrates (8,12,13), suggest that there may be large differences in species composition between the organic layer and mineral soil. Molecular techniques and the use of sequence databases enable identification of taxa with high resolution (16). Recently, Dickie *et al.* (5), using T-RFLP analysis of DNA extracted from soil mycelium, found differences in EM fungal species composition between different components of the forest floor (L, F and H layers) and the B horizon of the mineral soil in a North American *Pinus resinosa* stand. Zhou & Hogetsu (35) also used T-RFLP to map the three-dimensional distribution of EM root tips in a Japanese *Larix kaempferi* stand but found no clear vertical distribution patterns.

In the present study, root samples were collected from a Swedish podzol and the EM fungal species distribution on the root tips was investigated in relation to the location of the roots in different soil horizons. Samples were collected to a depth of 52 cm and included the different components of the organic and mineral soil (the O, E, B and C horizons). Fungal species were identified from EM root tips

to genus or species level using a combination of morphological identification and sequencing of the ITS-rDNA region. The aim of this study was to investigate whether the species composition of an EM fungal community in a podzol would differ between soil horizons. This information is an important prerequisite when attempting to assign ecological niches to individual species.

Materials and methods

Study site

Soil samples were collected in August 1999 from three columns dug in a podzol profile at a mixed coniferous forest site in the north of Sweden (Nyänget, 64°15'N, 19°45'E). The soil has developed from basal glacial till. The dominant tree species were 60-80 year old Norway spruce (*Picea abies* [L.] Karst.) and Scots pine (*Pinus sylvestris* L.) with undergrowth consisting mainly of *Vaccinium myrtillus* L., *V. vitis-idaea* L. and *Deschampsia flexuosa* [L.] Trin. (17).

Soil samples

Three 20x20 cm vertical soil columns were collected from locations 6m apart. Each column consisted of four distinct soil horizons: an upper organic horizon (O), a strongly weathered eluvial horizon (E), an enriched illuvial horizon (B) and the parent material (C). The soil horizons were distinguished by their colour and found at the following average depths: O, 0-3 cm; E, 3-18 cm; B, 18-35 cm, and C, >40 cm. The E horizon was further divided into an upper E1 with visible organic matter and a lower E2 horizon with visibly less organic matter. The B horizon was also further divided into a upper strongly illuvial B1 and a lower partially illuvial B2. An intermediate EB layer was distinguished in two of the columns where there was no sharp transition between the E and the B horizons. The soil samples from the O horizon down to the B2 horizon were contiguous in each column. To ensure pure parental material, the C-horizon samples were taken close to the bottom of the column, resulting in a gap between the B2 and the C samples. The soil was sealed in plastic bags, transported back to the laboratory and stored at +5 °C. Soil and stone (> 1cm) volume from each sample was recorded. Soil samples were coded according to column number followed by the seven horizon codes O, E1, E2, EB, B1, B2 and C.

Extraction and morphological identification of root tips

From each of the three intact O horizon samples, three sub-samples were taken using a 3 cm diameter corer. In mineral soil all root tips were examined except where root tip density was high, sub-samples were taken after careful mixing. Each

soil sample was soaked in water for 15 minutes before roots were extracted by wet sieving using a combination of 2, 1 and 0.5 mm sieves. Root tips were collected from the sieves using a dissection microscope and forceps.

Living root tips from each sample were grouped into morphotypes according to macro- and microscopic criteria (1). For each sample the number of root tips in each morphotype group and the number of non-mycorrhizal root tips were recorded. Root tip numbers from the sub-samples were bulked. The first time a morphotype was encountered, five representative root tips from that group were taken for molecular identification and individually frozen (–70°C) in micro centrifuge tubes. When fewer than five tips of a new morphotype were found, all tips were selected. On the subsequent occasions that a morphotype was found, one or two representative root tips were sampled and frozen.

DNA extraction, amplification and sequencing

DNA was extracted from root tips (9) excluding the initial freeze-thawing step. Following a modification of the protocol described by Henrion *et al.* (14) the ITS region of the rDNA was amplified by PCR (25). The universal primers ITS1 and ITS4 (White *et al.*, 1990) or the fungal specific primer ITS1F and the basidiomycete specific primer ITS4B (9,33) were used, depending on which primer pair gave the best PCR products. PCR was performed using 0.26 u High Fidelity DNA polymerase per µl reaction volume in a kit buffer with $MgCl_2$. An additional 0.065 µl of 25 mM $MgCl_2$ was added per µl reaction volume. In the reaction mix the final concentrations of all four nucleotides and each primer were 2 µM and 0.3 µM respectively. DNA template was added as 25% of the final reaction volume. PCR was performed in 50 µl with a program of denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 30s, 50°C for 45s and 72°C for 60s. From 15 samples, where no species identity was obtained using ITS sequencing, the large subunit was also sequenced using the primers ITS3 and Lr21 (15). The PCR protocol was modified for this reaction by increasing the annealing temperature to 52°C and running only 30 cycles. The quantity and quality of PCR products were examined by gel electrophoresis (9) and visualised using GelDoc and Quantity One 4.1.0 by BioRad. Double-banded PCR products were separated on 1% agarose gel at 90V for 3-4 hours. Gel plugs were cut out from the bands with a Pasteur pipette and dissolved in 200 µl of deionised water overnight. Separated bands were re-amplified using the same PCR protocol as used in the initial amplification.

PCR products were purified using the Quiagen PCR purification kit (250) and sequenced using ABI PRISM™ BigDye™. Sequencing was performed in 10µl reaction volume with a final primer concentration of 0.32µM. Each sample was separately sequenced with the two primers that had previously given the best PCR

product for the sample. For problematic samples, additional sequencing was performed using the internal primers ITS2 and/or ITS3 (33). The sequence reaction was performed with 25 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4 minutes. The sequence products were purified by ethanol precipitation, resuspended in TRRM at 96°C for 2 minutes and analysed by ABI PRISM™ Genetic Analyzer. Consensus sequences for each sample were produced using Sequence Navigator 1.0.1., Applied Biosystems Inc.

To determine the degree of homology within each morphotype, all sequences from each morphotype were aligned using ClustalW at EMBL (30). One representative sequence from each sequence homology group was then selected. Alignment was repeated with the selected sequences from all morphotypes to determine homology between morphotypes. The obtained clusters were considered to represent individual taxa. The identity of these taxa was investigated by comparing representative sequences from each cluster with sequences in the GenBank database at NCBI using the BLAST program (2). Names were assigned to each taxa according to the obtained BLAST matches (Table 1). The identification of several *Piloderma* species was made possible by comparing the obtained sequences to unpublished sequences obtained from sporocarps (K-H. Larsson, unpublished).

Statistical analysis

The relatedness in species composition of the EM community between different soil samples was examined using correspondence analysis (ADE-4 version 2001, CNRS, Lyon, France). The analysis was performed on the relative abundance of mycorrhizal tips of each of the distinguished taxa. This was done to correct for the high variation in root tip density between horizons. ANOVA was used to test for a relationship between the position of the samples on the ordination axes (sample scores) and the location of the samples in the three different soil columns. The soil horizons were assigned numbers 1 to 7 from the organic horizon (O) down to the parent material (C) and a linear regression was performed with ordination axis values against horizon number.

Results

Root distribution and mycorrhizal colonisation

Root tip density was highest in the organic horizon, declining to 3-15% of the O horizon density in the eluvial horizon (E2), and increasing again in the illuvial horizon (B) to 10-30% of the O horizon value. The lowest density was found in the C horizon (Fig. 1). However, due to the greater thickness of the mineral horizons, almost two thirds (65%) of the total root tips from all three columns were found in the mineral soil. The degree of mycorrhizal colonisation varied between 60% and 98% and no clear patterns could be seen with respect to depth or soil column.

Classification of mycorrhizal groups

Using morphological characters, 39 morphotypes were distinguished from a total of 8275 root tips examined. Of 247 root tips selected for genetic identification, 75% were successfully sequenced. Alignment with GenBank sequences, as well as with unpublished sporocarp sequences, enabled identification of 95% of the sequenced mycorrhizal tips. Of the initial 39 morphotype groups, no sequences were obtained from six, and the remaining 33 could be rearranged into 22 taxa (Table 2) on the basis of clustering of sequence data. Eleven of the taxa could be identified to species level and eight to genus level. Three taxa remained unidentified.

Table 1. Total numbers of root tips and percentages of mycorrhizal root tips per dm^3 soil for each horizon of three soil columns.

	Column1		Column2		Column3	
Horizon	Number of roots/dm^3	% mycorrhizal	Number of roots/dm^3	% mycorrhizal	Number of roots/dm^3	% mycorrhizal
O	1976	74	1792	96	3307	96
E1	259	94	542	92	1252	95
E2	277	96	104	90	109	89
EB	191	94	973	93		
B1	466	93	375	93	365	66
B2	530	80	160	98	705	79
C	99	60	66	95	62	86

Table 2. Grouped (un)identified EM fungal taxa with NCBI accession numbers. Similarity percentages with best Blast matches are shown (%). Genetic subgroups of taxa are listed A – D in the column 'Sub'.

Taxon	Sub.	Best Blast match	%	bp	NCBI Acc.no.
Cortinarius spp.	A	AF037224	94	545	AF481373-74
	B	U56032	95	569	AF481375-76
	C	AF335446	95	657	AF476972
					AF476990
	D	AF335446	96	542	AF481377
Dermocybe spp.	A	AF323113	90	560	AF476989
	B	AF325563	96	609	AF481380
	C	U56038	100	635	AF481378-79
Helotiales spp.	A	AF486119	99	447	AF476977
					AF481385
	B	AJ534704	98	513	AF476973
					AF481386
	C	AF149082	99	414	AF476981
					AF481383-84
	D	AY078134	100	402	AF481371
	E	AJ430403	97	454	AF481372
	F	Z81441	93	327	AF481370
Lactarius utilis		AJ534936	95	688	AF476975
Piloderma byssinum		AY010279	100	541	AF477003
Piloderma fallax	A	AY010281	99	569	AF476982
	B	AY010281	95	595	AF481388
Piloderma reticulatum					AF476978-79
					AF481387
					AF494442
Piloderma sp. 1					AF476998-99
Piloderma sp. 2					AF476971
Piloderma sp. 3					AF476986
Piloderma sp. JS15686					AF476983
Russula decolorans		AF418637	100	650	AF476996
Russula adusta		AY067652	99	965	AF476997
		AF218544	99	965	AF476997
Suillus luteus		AJ272413	99	657	AF476994
Tomentellopsis submollis		AJ410766	100	577	AF476974
Tricholoma portentosum		AB036896	97	795	AF477002
Tylospora spp.	A	AF052563	100	546	AF476969
	B	AF052560	99	885	AF476970
		AF325323	99	885	AF476970
Unidentified no.12				747	AF477004
Unidentified no.15				495	AF477000
Wilcoxina rehmii		AF266708	99	509	AF476993
Black roots	A	AY112935	99	468	AF476968
	B	AF274772	97	503	AF476967
	C	AJ308340	99	400	AF481389
Unidentified doubles				472	AF476985
					AF481369

Table 3. Root tips per 100 ml soil for each horizon of three soil columns.

Column	Taxon	Horizon O	E1	E2	EB	B1	B2	C
Column1	*Inocybe sp.*	5						
	T.submollis	9						
	P.fallax	24		<1				
	Dermocybe spp.	1	4	2	4			
	Black roots	21	<1	1	3	5	3	
	P.bicolorata	2	<1	<1	<1	<1	<1	
	Non mycorrhizal	51	2	1	1	3	11	4
	Cortinarius spp.	2				10	11	<1
	P.reticulatum	67	17	11	1		4	1
	Tylospora spp.	16	1	2	1		1	4
	Other	<1	<1	<1	<1			<1
	Lactarius utilis			8	5	13		
	Piloderma sp. 2			1	2	8	15	<1
	Piloderma sp. 3				2	7	8	
Column2	Black roots	9						
	R.decolorans	161	45	1	<1			
	Tylospora spp.	2			2			
	Non mycorrhizal	6	4	1	6	3	<1	<1
	T.submollis		5					
	Cortinarius spp.			8	15			
	P.bicolorata			<1		<1		
	Piloderma sp. 2				3	7		
	S.luteus				67	21	14	
	Piloderma sp. 1				2	6	<1	
	Hebeloma sp.					<1		
	Unidentified no.15						<1	
	Russula adusta							5
	Wilcoxina sp.							<1
Column3	*P.byssinum*	20						
	T.submollis	10						
	Dermocybe spp.	2	1					
	P.fallax	15	7	<1				
	Hygrophoprus sp.	5	3	1				
	P.atheleum	<1	<1			<1	<1	
	Black roots	15	2				<1	
	Non mycorrhizal	12	6	<1		12	14	<1
	P.reticulatum	239	104	2			31	
	Cortinarius spp.	10		<1		8	12	1
	Tomentellopsis sp.		<1	1				
	Other		<1	<1			1	
	Tylospora spp.		<1	<1				<1
	Piloderma sp. 2			1				
	Lactarius utilis			<1				
	P.bicolorata			3		9	<1	
	S.luteus			<1		7	11	1
	Tricoloma sp.							4

Three taxa (*Helotiales* spp., 'Black roots' and 'Unidentified doubles', see Table 2) were excluded from statistical analyses because of weak correlation between morphotype and genetically identified taxa and due to their scattered occurrence in a number of other taxa as secondary colonisers (i.e. double bands). Apart from an unidentified double colonizer, the removed groups consisted of morphotypes characterized by their black mantles, like the morphotype "Black roots", including *Cenococcum geophilum*, *Pseudotomentella tristis* and *Helotiales* sp., as well as the taxa *Helotiales* spp. encompassing roots from the morphotype Piceirhiza bicolorata.

Of the six unsequenced morphotypes, three were represented by few mycorrhizal tips (< 8) and were removed from further analyses. The remaining three unsequenced morphotypes (*Inocybe* sp., *Hygrophorus olivaceoalbus* and an unidentified taxon belonging to the Thelephorales), together with the 19 identified taxa, were used in the statistical analysis.

Description of some of the identified taxa

Seven taxa belonging to the genus *Piloderma* were found in this study. Four of the seven taxa, *P. reticulatum, P. byssinum, P. fallax* and *Piloderma* sp. JS15686, were identified to species level by alignment with sporocarp sequences. The other three, *Piloderma* spp. 1, 2 & 3, were assigned to the genus based on their location within a *Piloderma* phylogenetic tree (K.-H. Larsson, unpublished). The genus *Piloderma*

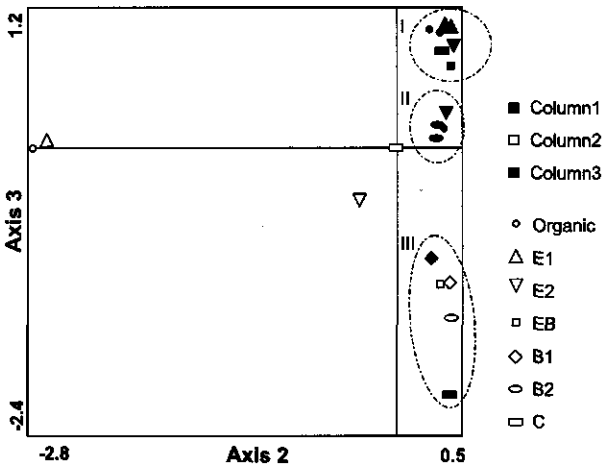

Figure 1. Sample scores of correspondence analysis, axis 2 and 3. The three columns are represented by different colours and the horizons are represented by different shapes.

colonised 52% of all root tips in this study. *P. reticulatum* was by far the most common taxon colonising 41% of all sampled root tips. The second most commonly sampled species was *Suillus luteus*, sampled from horizon EB to B2 in column 2 where it colonised 76% of all sampled root tips and in horizon E2 to B2 in column 3 where it colonised 21%.

The taxon *Tylospora* spp. encompassed two different morphotypes, identified as *T. fibrillosa* and *T. asterophora*. Using morphotyping it was not possible to discriminate consistently between the mycorrhizas formed by the two species within the genus and they were therefore merged into a single taxon with genus identity. Morphotypic separation within the genus *Cortinarius* was also low. The subgroup *Dermocybe* could be distinguished, and the taxon *Dermocybe* spp. was defined. This contained at least three species. It was apparent from the molecular data that, on some occasions, superficial morphological similarities caused erroneous identification of *Dermocybe* root tips as *Piloderma fallax* and *vice versa*. Morphotyping was unable to satisfactorily separate different *Cortinarius* species and these were thus grouped into the taxon *Cortinarius* spp., containing four minor subgroups.

Dual colonisation of mycorrhizal root tips

Distinct double PCR bands were observed as a result of amplification with ITS1 and ITS4 from 48 samples and weak double PCR bands were detected from another 45 samples. From most double-banded samples a single PCR product was obtained

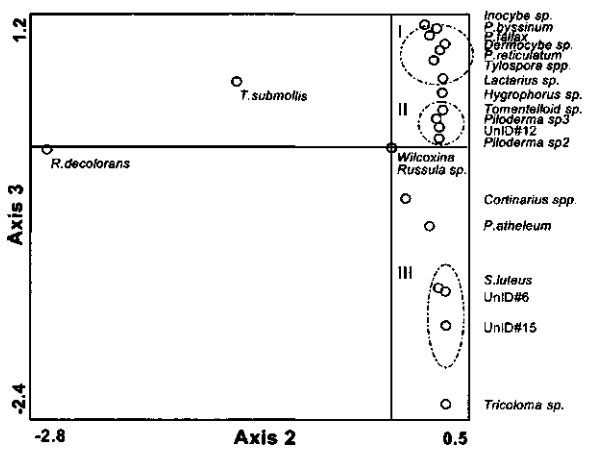

Figure 2. Species scores of correspondence analysis, axis 2 and 3. Symbols represent the EM taxa. The clusters I, II and III are superimposed on the species scores.

when amplifying the samples with the basidiomycete specific primer ITS4B, instead of the universal primer ITS4. Successful gel separation of double PCR products from 19 individual roots resulted in two sequences from each sample. Of these, each root tip generally yielded one sequence homologous to the representative sequence of the morphotype. Apart from the sequence associated with the visible EM fungal coloniser, the majority of double bands yielded different sequences within the monophyletic group of *Helotiales* spp., clustering with sequences of root tips that were morphologically identified as Piceirhiza bicolorata. The remaining six double band sequences formed a separate cluster of unidentified double colonizers. The frequency of double bands varied among the different EM fungal taxa and their occurrence did not appear to be entirely random. Most remarkable was the occurrence of double-banded PCR products in almost all of the analysed root tips colonised by *S. luteus* (data not shown).

Vertical distribution of mycorrhizal taxa

The distribution of mycorrhizal taxa throughout the soil profile is illustrated in Table 3. Of 22 distinguished groups, two were restricted to the organic horizon while 11 were found only in the mineral horizons. Of the latter group, three were found only in the C horizon (Table 3). The vertical distribution of mycorrhizal taxa was analysed using correspondence analysis (Fig. 1-3). The first four axes in the

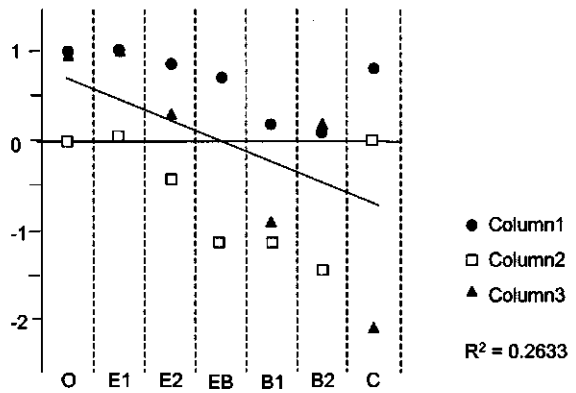

Figure 3. Regression analysis shows a statistical significant relation between the CA sample scores on axis 3 and the horizon definitions. The three columns are represented by different shapes and colours.

correspondence analysis together accounted for 60% of the total variation (inertia) of the data set (18%, 16%, 15% and 11% respectively). While the sample scores on the first two ordination axes could not be attributed to the location of the samples in the soil profile, the variation along the third ordination axis could clearly be related to differences in community composition between the soil horizons and between the soil columns (Fig. 1, 2). There was a statistically significant ($p = 0.02$) relationship between the sample scores on axis 3 and the horizons from which the samples were taken (Fig. 3). There was also a statistically significant association between the sample scores on axis 3 and the soil column from which the samples were taken ($p = 0.03$). The sample scores on the remaining ordination axes correlated weakly or not at all with the location of the samples in the soil profile or soil columns.

All variation along the first ordination axis was due to the separation of sample 2C from the rest of the samples. The EM fungal community in this sample consisted of only two taxa, *Russula adusta* and *Wilcoxina rehmii*, neither of which was found in any other sample. The variation along the second ordination axis could be largely attributed to the occurrence of *Russula decolorans*, that colonised more than 90% of the root tips in the two uppermost horizons of soil column 2 (samples 2O and 2E1) but was absent in the other soil columns (Fig. 1).

As the deviating species compositions of the samples 2O, 2E1 and 2C is explained by the first two ordination axes, these samples obtained sample scores close to zero on the third ordination axis. The difference in community composition between samples was largely reflected in their distribution along the third ordination axis, with O and E samples obtaining higher values and samples from deeper horizons obtaining lower or negative values (Fig. 1). The distribution of column 1 sample scores on axis 3 were less spread out than the samples from the other two columns. This followed the rather homogeneous species composition of column 1, where *P. reticulatum* and *Tylospora* spp. occurred throughout the whole profile. From the species scores along axis 3 (Fig. 2), the major taxa - those taxa that were found in more than one of the samples - that were associated the O and E samples could be identified as: *P. fallax*, *Dermocybe* spp., *P. reticulatum*, and *Tylospora* spp. The major taxa associated with the lower horizons were *S. luteus* and *Cortinarius* spp.

No statistical relationship could be found between the species composition and the chemical properties of the mineral horizons in which they occurred (data not shown).

Discussion

The high root tip density of the organic horizon found in this study is in line with earlier findings (18). Most studies of below ground EM fungal diversity have focused on the upper organic horizon where root tip density is high. In the present study, although root tip density was highest in the organic horizon, two thirds of all root tips in the 53 cm deep soil columns were recovered from the mineral soil.

Almost all taxa typically occurred in only part of the soil profile (Table 3). When occurring in several horizons, most taxa had a continuous distribution over adjacent horizons rather than a discontinuous distribution. This suggests that the growth of some mycorrhizal fungi is favoured in specific parts of the soil profile. The non-even distribution of species throughout the columns could also be a result of single mycelial individuals colonising a large number of adjacent root tips (29,36). As evident from the correspondence analysis, there was a significant relationship between the species composition and the soil horizon from which the roots were collected (Fig. 3). This suggests that the observed vertical species distribution patterns are not just products of stochastic variation.

Most taxa that were found in the organic layer were also found in the upper eluvial soil horizon (E1). The major separation in species composition was thus found between the organic and eluvial horizons on one hand and the deeper mineral soil on the other (Fig. 1). The similar species composition of the eluvial soil and the organic horizon could possibly be explained by the relatively large amount of organic material present in the upper mineral horizons, particularly in E1. On the other hand Dickie *et al.* (5) found differentiation in species composition even between different parts of the forest floor (the O horizon). By pooling all components of the forest floor (L, F and H), the present study may have missed parts of the vertical variation. However, by including deeper parts of the mineral soil and separating the different mineral soil components this study may have included variation that was not covered by Dickie *et al.* (5). In the present study one half of the EM fungal taxa were found exclusively in the mineral soil horizons.

The dominance of the genus *Piloderma* in this study is consistent with the suggested ecological importance of this genus in many boreal forest ecosystems (7). *P. fallax*, a species commonly identified in other studies (often referred to as *P. croceum*), was restricted to the organic and eluvial horizons. Goodman and Trofymow (12) found *P. fallax* exclusively in the organic horizon. In a study by Heinonsalo *et al.* (13), *P. fallax* was found on bait seedlings planted in humus material (O horizon) but not on those planted in mineral soil (B horizon). *P. byssinum* was restricted to the O horizon, although it was found in one column only. The most abundant species in this study, *P. reticulatum,* occurred predominantly in

the O and E horizons. The three other *Piloderma* taxa that were found in this study appear to be hitherto undescribed species. They were all found in the mineral soil only. *Piloderma* species do not form conspicuous fruiting bodies and the restriction of these new species to the deeper mineral soil may explain why they have not been found on roots in earlier investigations.

Tomentellopsis submollis appears to be restricted to the upper part of the profile. Pink morphotypes, most likely *T. submollis,* are regularly identified on roots from the organic horizon of Scots pine in Fennoscandia (19). *Suillus luteus* occurred in two columns in which it was only found in the mineral soil, occurring from the lower eluvial (E2) horizon and downwards. Both Danielsson & Visser (4) and Heinonsalo *et al.* (13) suggest that *Suillus* species constitute a higher relative proportion of the total mycorrhizal community on bait seedlings planted in mineral soil than on seedlings planted in organic material. The greater abundance of the pine specific *Suillus* species in the mineral soil could partially be explained by the greater rooting depth of pine compared to spruce (24). This may also be a factor of relevance for *Lactarius utilis,* which also occurs in the lower soil profiles. *Dermocybe* spp. were almost exclusively found in the E-horizon whereas other *Cortinarius* spp. were predominantly found from the E2 horizon downwards. Species within the genera *Suillus* and *Cortinarius* have been highlighted among those forming numerous fruitbodies although being weakly represented in communities of fungi colonising root tips in the organic soil horizon (3,10). The common practice of excluding the mineral soil from community studies undoubtedly contributes to the discrepancy frequently observed between perceived above- and below-ground mycorrhizal community structures (16).

In this study three different sequence groups were obtained from roots with black mantles. Sequences homologous to *Cenococcum geophilum* amplified only from root tips found in the organic horizons. These data correspond with those of Goodman & Trofymow (12) and Fransson *et al.* (8) who both found that *C. geophilum* was more common in the organic layer than in the mineral soil. In the present study *Phialophora finlandia* sequences were obtained from root tips in other horizons. This is in agreement with the findings of Heinonsalo *et al.* (13) where a black morphotype was found to be *C. geophilum* when colonising roots in the organic soil and *P. finlandia* when colonising roots in the mineral soil. *Phialophora* forms an EM fungal morphotype, commonly referred to as Piceirhiza bicolorata, that can be mistaken for a roughly defined *Cenococcum* morphotype (32). Consequently the abundance of P. bicolorata has been suggested to be greatly underestimated (32).

In addition to the present study, a parallel study was conducted (Chapter 4,(20)) in which amplification and cloning of soil DNA was used to investigate the distribution of extraradical mycelium in soil samples collected concomitantly to the

root samples examined in this study. In general, the same species were detected when comparing the EM fungal species composition on root tips with the species composition of extraradical mycelia. Of 16 EM fungal species identified in DNA extracts from root free soil, all but one were also found on root tips. In several cases species that were abundant on root tips were not detected in soil extracts (e.g. *S. luteus* and *P. reticulatum*), and others that were abundant in soil extracts were rarely detected on roots (e.g. *Dermocybe* spp. and *Piloderma* sp. 3).

The high occurrence of amplification of DNA from fungi other than the main visible coloniser may partly be explained by the occurrence of ascomycetous double colonisers that coexist with the mycorrhizal fungi on the roots. In other cases, the additionally amplified DNA could be ascribed to other EM fungal fungi that were abundant in the same soil sample. In laboratory microcosms, the secondary colonisation of mycorrhizal root tips already colonised by mycorrhizal fungi has been observed (34). During replacement of one fungus by another, root tips were simultaneously colonised by both fungi. Sometimes conflicting results of morphological and molecular identification may thus be a consequence of the dynamic character of the mycorrhizal community. A potential source of error, not explicitly considered in many studies, is the possibility that there is systematic variation in the efficiency of extraction and amplification of DNA between different fungi (11). Secondary colonisers may be present without changing the morphology of the mycorrhiza and still amplify more strongly than the primary coloniser.

In conclusion, the results of the present study indicate that there may be significant variation in EM fungal species composition between soil horizons of boreal forest podzols. In some of these soils a high proportion of the total number of root tips is present in the deeper mineral horizons and some EM fungal taxa are restricted to these deeper horizons. At present there is still little available information about the processes determining this distribution but it is clear that further studies of EM fungal diversity and function should include the roots sampled from deeper mineral horizons.

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4

Molecular identification of ectomycorrhizal mycelium in soil horizons

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Abstract

Molecular identification techniques based on total DNA extraction provide a unique tool for identification of mycelium in soil. Using molecular identification techniques, the ectomycorrhizal (EM) fungal community under coniferous vegetation was analysed. Soil samples were taken at different depths from four horizons of a podzol profile. A basidiomycete specific primer pair (ITS1F - ITS4B) was used to amplify fungal ITS sequences from total DNA extracts of the soil horizons. Amplified basidiomycete DNA was cloned and sequenced and a selection of the obtained clones was analysed phylogenetically. Based on sequence similarity, the fungal clone sequences were sorted into 25 different fungal groups or operational taxonomic units (OTUs). Out of 25 basidiomycete OTUs, 7 OTUs showed a high nucleotide homology (≥ 99%) with known EM fungal sequences and 16 were exclusively found in the mineral soil. The taxonomic position of 6 OTUs remained unclear. OTU sequences were compared to sequences from morphotyped EM root tips collected from the same sites. Of the 25 OTUs, 10 OTUs had a ≥ 98% sequence similarity with these EM root tip sequences. The present study demonstrates the use of molecular techniques to identify EM hyphae in various soil types. This approach differs from the conventional method of EM root tip identification and provides a novel approach to examine EM fungal communities in soil.

Introduction

Ectomycorrhizal (EM) fungi are major components of the soil fungal community in most boreal and temperate forests and several (sub) tropical forests. The EM fungi efficiently take up water and organic as well as inorganic nutrients from the soil and translocate these to colonised tree roots, receiving host carbohydrates in return. The extraradical mycelium is the part of the EM association actively involved in uptake of nutrients and water. Species specific variations in EM extraradical mycelial structure and activity enable single EM fungal species to exploit specific resources (30). The concept of ecological specialisation among EM fungi is generally accepted now (14) and underlines the proposed importance of EM fungal species diversity in soil ecosystems (2,7,21). To gain more insights on the functional roles of different EM fungal species and understand their functional diversity it is essential to obtain information about ecologically relevant features of the symbiosis, in particular the spatial distribution of EM extraradical mycelium.

Until the advent of molecular techniques it has been impossible to acquire data on mycelial distribution of individual fungal species in soil. Most EM diversity studies are currently based on EM root tip inventories. Morphotype techniques combined with molecular techniques enable identification of EM fungi on root tips extracted from soil and provide information on EM fungal species diversity on a fraction of the tree root system. When using molecular techniques for identification of fungal material on roots, the distribution of extraradical mycelium in the bulk soil remains unknown. Yet, molecular identification techniques can also be used to detect fungal hyphae in substrates other than roots and therefore enable identification of mycelium in soil.

Molecular identification based on the extraction of total environmental DNA enables identification of fungi directly from the environment, irrespective of morphology or growth stage. Fungal DNA can selectively be amplified from total environmental DNA extracts, using the polymerase chain reaction (PCR) and a pair of primers that allow specific amplification of the fungal ribosomal DNA (rDNA) gene cluster. With temperature gradient gel electrophoresis (TGGE) (36), denaturing gradient gel electrophoresis (DGGE) (25,29,37) or single-strand conformation polymorphism (SSCP) (23), fungal communities can subsequently be analysed. The 18S-rDNA and ITS regions have been used for construction of fungal clone libraries, enabling identification of non-mycorrhizal fungal species from church window glass (34), soil (3) and decaying plant material (6) through selective clone sequencing.

These molecular approaches have recently also been applied to the investigation of EM fungal communities in soil, using restriction fragment length polymorphism

(RFLP) (9) and terminal restriction fragment length polymorphism (T-RFLP) (11). EM fungi are not a monophyletic group and EM specific primers therefore do not exist. Primers generally used for identification of EM fungi have enhanced specificity for basidiomycetes (13) and target the ITS regions of the rDNA gene cluster, including the 5.8S rDNA gene. The rDNA gene cluster is presented by one to several hundred copies per genome within the fungal nucleus and therefore provides a good target region (4, 38). Until now, the basidiomycete specific ITS1F-ITS4B primer pair (13) has been used to amplify DNA extracted from fruitbodies (7,8,31), fungal cultures (13,33), infected plant material (13) and from EM root tips (7).

This study presents the use of the ITS1F-ITS4B primer pair to amplify basidiomycete DNA from soil DNA extracts, followed by a cloning and sequencing procedure, in order to identify the EM fungi present. Through identification of basidiomycete mycelium in soil, the EM fungal community structure can be analysed in a novel way, excluding EM root tips from the analysis. The soil samples that were analysed originated from four distinct horizons of a podzol profile, under coniferous vegetation, situated in the northern part of Sweden. The clone sequences obtained were grouped and compared to sequences from a selection of morphotyped EM root tips collected concomitantly from the same podzol profiles.

The aim of the underlying study was threefold: First, to use molecular methods for identification of EM mycelium in soil. Second, to investigate the distribution of EM mycelium in a vertical soil profile and finally to compare the obtained clone sequence data to EM root tip sequence data.

Materials and methods

Soil samples

The investigated site is located in the northern part of Sweden within the area of the Svartberget Forest Research station (Nyänget, 64°15'N, 19°45'E). The soil is a basal glacial till and the soil type is classified as a Typic Haplocryod or a Haplic Arenosol (19), characterised by four distinct podzolic horizons. The organic mor layer (O, 0-3 cm) is underlain by a strongly weathered eluvial horizon (E, 3-18 cm) and an enriched illuvial horizon (B, 18-35 cm). The transition of the B horizon into parent material (C, >40 cm) at the bottom of the soil profile is gradual. The coniferous forest is dominated by Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) and the main undergrowth consists of Blueberry (*Vaccinium myrtillus*), Lingonberry (*Vaccinium vitis-idaea*) and Wavy hair-grass (*Deschampsia flexuosa*).

In August 1999 three pits were dug about 6 m apart from each other. Pit 1 and 3 were situated 1 m from a Norway spruce tree, while pit 2 was closest (1 m) to a Scots pine tree. From one side of each pit the soil was sampled along three vertical columns. Working downward from the organic (O) layer, a small block of soil (5x5 cm) was carefully removed from the centre of each horizon (E, B and C horizon) using fine stopping knifes. The soil of each horizon was sealed separately in a plastic bag, transported back to the lab and frozen at -70 °C. The E horizon was divided in two parts when sampled, viz. an upper E1 and a lower E2. In doing so, a distinction could be made between fungi found in the upper E1, close to the organic layer, and fungi found more close to the B horizon. Table 1 gives average pH values and average total C contents (g/kg soil) of the pooled soil samples from each horizon of the three pits. Total C content of the soil samples was determined according to a wet oxidation procedure (KurmiesC procedure) (15). There are large variations in carbon percentages (30-62%) of organic matter with different soil types and therefore total C content cannot be converted to organic matter content of the different soil horizons.

DNA extractions from soil samples

From the three pits, DNA was extracted from three soil samples taken from each horizon. Mineral soil was gently sieved (1 mm) to discard EM root tips. Visual checks were performed to make sure no root tips had passed the sieve. Organic horizon samples that could not be sieved were checked visually under the dissecting microscope and root tips were removed manually. DNA was extracted from 5 g wet weight of mineral soil and 1 g wet weight of the organic samples, using a bead-beater as described by Smalla *et al.* (35). DNA was purified twice with the Wizard® DNA Clean-Up System (Promega) according to the manufacturer's purification protocol before amplification

Table 1. pH and total C contents of soil samples taken from five horizons of three pits

Horizon	pH (CaCl)[a]	Total C, g/kg (KurmiesC)[a]
E1	3.7 ± 0.1	6.5 ± 1.7
E2	3.8 ± 0.1	5.6 ± 0.3
B1	4.9 ± 0.6	21.7 ± 8.1
B2	5.7 ± 0.2	7.6 ± 1.1
C	5.3 ± 0.1	3.9 ± 0.5

[a] Results are averages and standard deviations for three pits

PCR amplification

For each pit separately, the three DNA extracts from each horizon were pooled prior to PCR, to reduce sample numbers for the cloning procedure. DNA extracts of the B1 and B2 horizon were also pooled. DNA extracts from the C horizons could not be amplified due to low template concentrations and were left out of any further analyses. The remaining 12 samples (four soil horizons O, E1, E2 and B, from three different pits) were used for PCR and for the cloning procedure. DNA amplification of the ITS regions was performed on a Hybaid PCR Express thermocycler with the primers ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS4B (5'- CAG GAG ACT TGT ACA CGG TCC AG -3') (13). The PCR reaction mix consisted of 5 µl 10x PCR buffer 2 (Roche), 200 µM concentrations of each deoxynucleoside triphosphate, 200 nM concentrations of each primer, 0.5 µl Expand enzyme mix (Roche), 38 µl sterile Ultrapure water and 1 µl of 1:50 diluted template DNA (see Table 2 for template DNA concentrations). The following thermocycling pattern was used: 94°C for 3 min (1 cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 3 min (30 cycles); and 72°C for 10 min (1 cycle). Amplified DNA fragments (0.7-kb) were visualised on 1% agarose gels.

Cloning of ITS1F-ITS4B PCR products

The successfully amplified PCR products (0.7-kb) were ligated into the pGem-T vector which has 3'-T overhangs to facilitate cloning of PCR products (Promega, Madison, WI, USA). Ligation mixtures were transformed into Ultracompetent E.coli XL1-Blue (Stratagene, Cambridge, UK) according to the manufacturer's instructions. The bacteria were plated out in three dilutions. From each sample 100 white colonies were picked, cultured overnight in 2 ml Luria broth and then frozen at -70 °C.

Table 2. Amounts of total double-stranded DNA extracted from soil samples of five horizons of three pits

Horizon	Double-stranded DNA (ug/g soil)[a]
O	23.7 ± 7.5
E1	7.2 ± 2.9
E2	5.8 ± 1.1
B	4.4 ± 3.2
C	0.42 ± 0.2

[a] Results are averages and standard deviations for three pits.

Identification of the cloned fungal ITS fragments

Thirty clones from each sample were amplified and analysed on a 1% agarose gel. Clones with bands of the right size (0.7-kb) were purified with the QIAquick Purification Kit (Qiagen) according to the manufacturer's protocol. The 0.7-kb fragments were sequenced in one direction on an ABI377 DNA sequencer by cycle sequencing using the dye terminator system (Eurogentec, Seraing, Belgium) and the ITS1F primer. In order to identify the obtained clone sequences, all clone sequences were subjected to multiple alignments using ClustalW (DNA Star Program) and used to construct phylogenetic trees by neighbor-joining using the Treecon program (version 1.36, Yves van der Peer). Based on the clusters that were formed clones were sorted into groups or operational taxonomic units (OTUs). A representative of each OTU was compared phylogenetically to sequences of known species in the Genbank database of the National Center for Biotechnology Information (NCBI) using the Blast program. A phylogenetic tree was then constructed with all clones from each OTU and the most homologous sequences from the database. Database sequences yielding the greatest percent similarity to the clone sequences were chosen as best match for each OTU. The purpose of this study was to assign a taxon name to each OTU rather than to provide a phylogenetic analysis of each obtained OTU. Assignment to taxon categories was done as follows: Sequence similarity $\geq$ 99%, identification to species level; sequence similarity 95 - 99%, identification to genus level; sequence similarity $\leq$ 95%, identification to family or ordinal level. Neighbor-joining trees were consequently constructed with *Amanita muscaria* as outgroup and 100 bootstrap replicates.

Statistical analysis of clone distribution

A permutation test was performed to determine whether the occurrence of OTUs was related to soil horizons. A statistic (summore) was calculated on the actual data measuring the association between OTU and soil horizons. This summore was compared to the null permutation distribution (i.e. under the hypothesis of no association) of a statistic calculated from random permutations of the data. The following three assumptions have been used: 1) the number of OTUs per pit and per horizon was held fixed, 2) the quantitative aspect of the number of clones representing each OTU was ignored (in each soil horizon an OTU was considered either to be present or not) and 3) the presence of an OTU in a horizon was considered dependent on the presence of the same OTU in adjoining, contiguous, layers, while the presence of an OTU was considered to be independent of the presence of other OTUs.

The data sets of the three separate soil pits were combined for the permutation test. The test was performed exclusively with 11 OTUs represented by clones

originating from more than one soil pit (OTUs 4, 5, 7, 8, 9, 10,12, 15, 18, 21 and 24). The data set shows that over all three pits, 18 out of 44 fields in the 11x4 matrix (11 OTUs x 4 horizons) are empty and contain no OTU. This variable 18 is called the 'summore'. Subsequently a test was performed to determine the probability under the null permutation distribution of finding a value of summore of 18 or more, i.e. to test whether the value of summore = 18 is coincidental. Designed for this purpose, a statistical program was used to take random samples from all possible OTU - horizon distributions having total values equal to the actual total values found. In total 24000 permutations were performed (12). For 24000 permutations, 1200 values of summore $\geq$ 18 are required to reject the null-hypothesis of a random OTU distribution, at $P \leq .05$.

Comparison of clone and root tip sequences

Using the Blast program (NCBI) clone sequence data were compared to sequence data obtained from root tips extracted from soil samples taken concomitantly with the soil samples used in the presented study (32). Nucleotide sequences of the root tip data are accessible under NCBI numbers AF476965-477005, AF481369-481389 and AF494441-494447.

Nucleotide sequence accession numbers

The nucleotide sequences of the clones representing each OTU have been submitted to NCBI and are accessible under numbers AY097035-097059.

Results

DNA extractions and PCR reactions.

Total DNA was successfully extracted from all soil samples (Table 2). Due to too low template concentrations however, DNA extracts from the C horizons could not be amplified at template dilutions equal to the other samples and therefore C samples were left out of any further analyses. DNA extracts from the O and B horizons had a dark brown colour probably due to co-extracted organic matter. Double purification of extracted DNA allowed successful PCR amplification of 1:50 diluted template DNA, using the ITS1F - ITS4B primer pair.

Phylogenetic grouping of clone sequences.

Sequencing of 30 clones from all 12 soil samples resulted in 318 successful sequencing results. To determine whether analysis of 30 clones from each sample was sufficient to detect the most common fungal species, 20 extra clones from one

sample (E1 horizon sample from pit 1) were sequenced and rarefaction analysis was applied. Rarefaction is a statistical method to estimate the number of species expected in a random sample of individuals taken from a collection (20). From the collection of 50 sequences, representing 6 fungal species (OTUs), rarefaction analysis shows that the expected number of species in a sub-sample of 30 clones is 5.1 ± 0.7. Therefore, detection of the most common species seems likely when analysing 30 clones/sample.

After multiple alignments and constructions of phylogenetic trees, the obtained clone sequences were sorted in 29 different OTUs. Four OTUs that were represented by one single clone sequence (OTU 14, 16, 26 and 29) were not used any further, leaving 25 different OTUs. Figure 1 presents a phylogenetic tree with the 25 OTUs and sequences from the GenBank database with highest sequence similarity to clone sequences.

Four main fungal orders were distinguished, based on the classification of Hibbet and Thorn (17). Within these four orders most terminal clades are well supported with high bootstrap values. The Boletales order includes OTU 20 (*Suillus*-like sp.). The Polyporales order includes the *Piloderma*-like OTUs 5, 10, 18 and 21 and the *Tylospora*-like OTU 9 (Fig. 1). Strong bootstrap support is found for the Russulales order, including OTU 8 (*Lactarius*-like sp.), OTU 19 (*Russula*-like sp.) and OTU 28 (unknown). The order Agaricales includes OTUs 1-3, 6, 11-13 and 22-24. Within the Agaricales order, the Cortinariaceae clade is strongly supported. However, another presumed member of this clade (OTU 4) does not group within this well-supported Cortinariaceae clade.

For 14 out of the 25 OTUs a Blast search sequence similarity ≥ 95% was found (Table 3). Out of the total 25 OTUs, 19 OTUs represented basidiomycetes with known affinity, while the taxonomic position of 6 other basidiomycete OTUs remained unclear. Four unidentified OTUs were represented by clones from the mineral E and B horizons only. Of the 19 basidiomycete OTUs, 7 OTUs showed a high (≥ 99%) nucleotide similarity to known EM fungal sequences and could be assigned species names. Five basidiomycete OTUs showed a 95 - 99% sequence similarity to known EM fungal species and could be assigned to genus level. Four basidiomycete OTUs with a low sequence similarity (≤ 95%) to known EM fungal sequences were assigned to family level. For 3 OTUs a highest sequence similarity was found with non-mycorrhizal fungal species (*Ceraceomyces eludens*, *Galerina pseudomycenopsis* and *Cryptococcus terricola*).

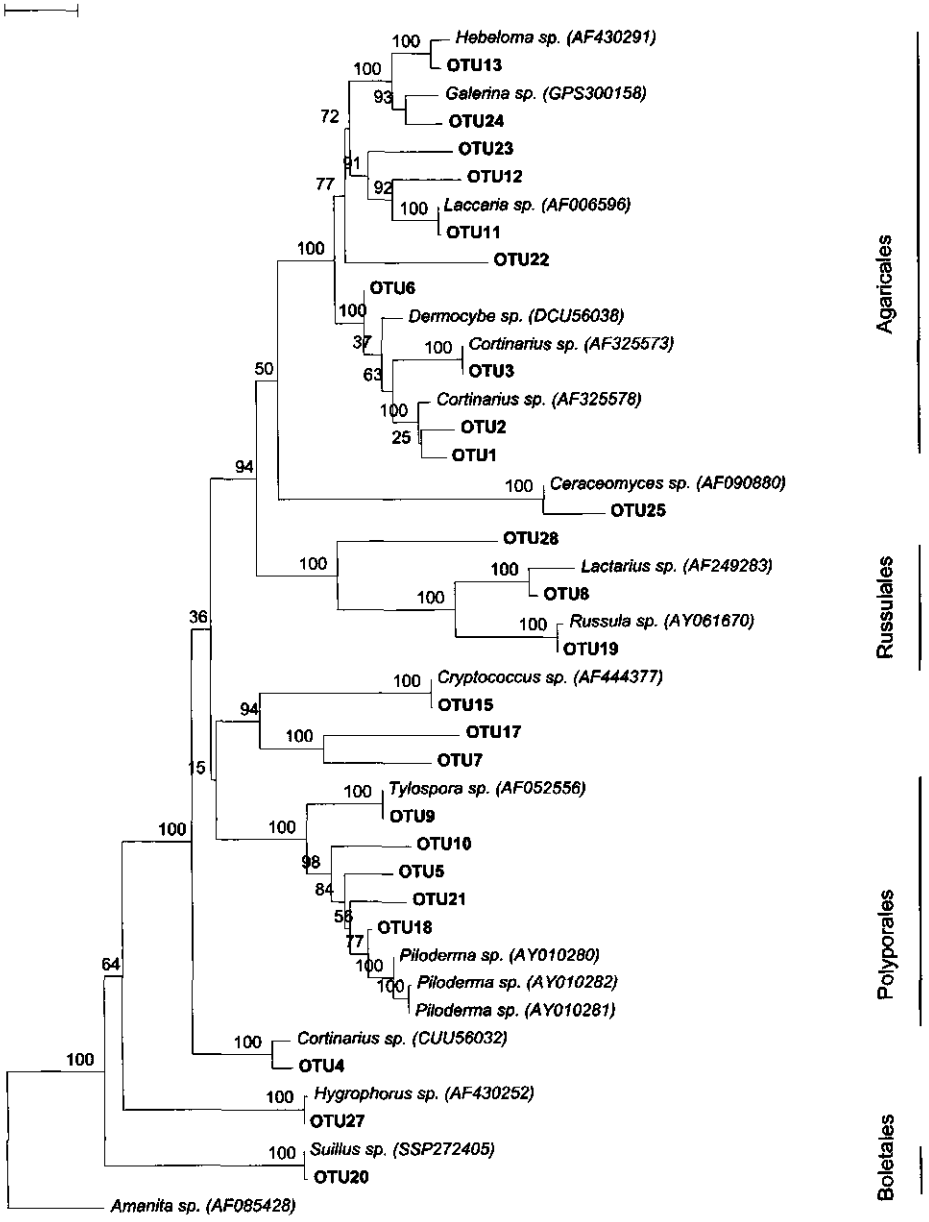

Figure 1. Neighbor-joining tree representing the phylogenetic relationships of 25 OTU sequences obtained from three soil pits to the most closely related sequences obtained from Blast searches. The scale bar indicates 0.1 substitutions per site.

Table 3. Fungal species from the GenBank database with highest sequence similarity to each OTU and distribution of OTUs over four horizons[a].

OTU	Blast match[b]	Accession no.	Similarity (%)	No. of clones in soil horizon:				Total no. of clones
				O	E1	E2	B	
22	None			3				3
23	None			3				3
25	*Ceraceomyces eludens*	AF090880	99.0	9				9
24	*Galerina atkinsoniana*	GPS300158	92.1	3	1			4
1	***Cortinarius acutus***	AF325578	95.3	27	2	1		30
18	***Piloderma fallax***	AY010281	94.9	5	4	4		13
19	***Russula decolorans***	AY0061670	99.2	23	20	2		45
21	***Piloderma fallax***	AY010281	97.6	8	1	2		11
15	*Cryptococcus terricola*	AF444377	99.5	7	1	1	1	10
3	***Cortinarius collinitus***	AF325573	99.8		19	3		22
6	***Dermocybe crocea***	DCU56038	99.6		5	2		7
11	***Laccaria laccata***	AF006596	99.6		1	5		6
27	***Hygrophorus olivaceoalbus***	AF430252	99.3		8	7		15
10	***Piloderma fallax***	AY010280	93.2		7	6	19	32
4	***Cortinarius umbilicatus***	CUU56032	96.0		1	14	8	23
2	***Cortinarius acutus***	AF325578	92.7			6		6
8	***Lactarius deliciosus***	AF249283	93.5			4		4
9	***Tylospora asterophora***	AF052556	99.8			2		2
5	***Piloderma fallax***	AY010282	92.0			9	25	34
7	None					2	13	15
12	None					1	2	3
13	***Hebeloma incarnatulum***	AF430291	96.1				4	4
17	None						2	2
20	***Suillus sp.***	SSP272405	99.5				5	5
28	None						6	6

[a] Data for the three pits are pooled

[b] Boldface indicates EM fungal species

Distribution pattern of OTUs in soil profile

The 25 OTUs consisted of variable numbers of clone sequences, often originating from different soil horizons. Ten OTUs were represented by clones from just one horizon, while the other 15 OTUs were represented by clones from two or more adjoining horizons. Table 3 gives the combined results of three soil pits. Three OTUs were found exclusively in the organic horizon, while sixteen OTUs were found exclusively in the mineral soil. Six OTUs (1, 15, 18, 19, 21 and 24) were represented by clones from the organic as well as the mineral E soil

Table 4. OTUs with a high sequence similarity to sequences from EM root tips extracted from the same soil pits, ranked according to nucleotide similarity percentage.

Clone sequence data				Blast match	Root tip sequence data	
OTU	Genus	Acc. no.[b]	Horizon[a]	(%)	Acc. no.[b]	Horizon[a]
6	*Dermocybe sp.*	AY097040	E1,E2	100	AF494447	O
19	*Russula sp.*	AY097051	O,E1,E2	100	AF476996	O
3	*Cortinarius sp.*	AY097037	E1,E2	99.8	AF476991	E1
8	*Lactarius sp.*	AY097042		99.8	AF476975	B1
10	*Piloderma sp.*	AY097044	E1,E2,B	99.7	AF476986	B1
18	*Piloderma sp.*	AY097050	O,E1,E2	99.5	AF481388	E1
5	*Piloderma sp.*	AY097039	E2,B	99.4	AF494446	E2
21	*Piloderma sp.*	AY097053	O,E1,E2	98.8	AF494442	O
22	none	AY097054	O	98.6	AF476965	O
9	*Tylospora sp.*	AY097043	E2	98.3	AF476970	B2
1	*Cortinarius sp.*	AY097035	O,E1,E2	97.1	AF476989	O
4	*Cortinarius sp.*	AY097038	E1,E2,B	95.9	AF481373	B1
20	*Suillus sp.*	AY097052	B	92.1	AF476994	B1

[a] Horizon(s) from which the clone or root tip was extracted

[b] GenBank accession numbers

horizons. Seven OTUs were exclusively found in the E horizons and of these, three (OTUs 2, 8 and 9) were exclusively found in the deeper E2 horizon. OTUs 4 and 10 were found in E1, E2 and B horizons and were not seen in the organic layer. Three OTUs (5, 7 and 12) were found in the E2 as well as B horizon and four OTUs occurred in the B horizon only. Clones representing OTU 15 were found in all four soil horizons.

Seven OTUs with a high sequence similarity (≥ 99%) to EM fungal sequences from the GenBank database were distributed as follows: In the O and E horizons OTU 19 (*Russula decolorans*) was found; in the E horizons OTUs 3 (*Cortinarius collinitus*), 6 (*Dermocybe crocea*), 9 (*Tylospora asterophora*), 11 (*Laccaria laccata*) and 27 (*Hygrophorus olivaceoalbus*) were found and in the B horizon OTU 20 (*Suillus* sp.) was found.

Statistical analysis of OTU distribution

A permutation test was performed to determine whether the OTU distribution is related to soil horizons. This test was performed with 11 OTUs represented by clones from more than one soil pit. The fact that one pit was dominated by a different tree species probably increased the variation in fungal species composition

between the three pits. As a result, 14 OTUs did not appear in more than one soil pit and were not included in the statistical analysis. In total 24000 permutations were carried out, resulting in 1489 values of summore $\geq$ 18, leading to a calculated statistic of .06. At a probability level of 6% the test indicates that the distribution of the OTUs over the four horizons is not random.

Clone sequences versus root tip sequences

An EM root tip inventory of the same soil pits carried out concomitantly with the presented study revealed the presence of 25 different EM fungal types on a selection of the root tips sampled (32). Of the 19 basidiomycete OTUs, 2 OTU had 100% sequence similarity to root tip sequences (OTUs 6 and 19). Similarity percentages of 98 - 99 % were found for OTUs 3, 5, 8, 9, 10, 18, 21 and 22. While OTUs 5, 10, 18 and 21 matched poorly (92 - 97%) with three Piloderma fallax sequences from the GenBank database, they matched well ($\geq$ 98%) with four different root tip sequences, representing four potentially different Piloderma species. Although the identity of OTU 22 remained unresolved after the Blast search, a good match (98.6%) was found with a root tip sequence. This strongly indicates that this OTU groups within an EM fungal genus. Of 13 OTUs with $\geq$ 92% sequence similarity to root tip sequences, 10 OTUs occur in the same horizons as from where the matching root tips were extracted.

Discussion

This paper demonstrates the use of molecular identification techniques to study the occurrence of EM hyphal material in various soil horizons. For this study the use of ITS sequences was chosen to enable comparison with ITS sequences available from EM root tips sampled concomitantly from the same pits. In most cases, the resolving power of the ITS proved to be enough to retrieve closely related taxa from the GenBank database. Rarefaction analysis showed that the amount of clones analysed was sufficient to detect the most common fungal species. Construction of neighbor-joining phylogenetic trees with 25 OTUs and related GenBank sequences placed most OTUs in four main fungal orders. For 10 OTUs a Blast search sequence similarity of $\geq$ 99% was found with Genbank basidiomycete sequences, enabling identification to species level. Four OTUs with 95 - 99 % sequence similarity were assigned to genus level, while five OTUs with $\leq$ 95% sequence similarity were assigned to family level (Table 3). Other studies considered nucleotide homologies of 87% (8) and 94% (24) sufficient for the placing of unknown sequences in known fungal taxa.

In some cases however, the resolving power of the ITS sequence proved too weak for correct taxonomic placement of an OTU, as illustrated in the Cortinariaceae clade (Figure 1). Within the Cortinariaceae clade, up to 30% base pair variation within the ITS regions and 5.8S gene sequences is possible (22), likely affecting the taxonomic placement of the Cortinarius-like OTU 4 in this study. In addition, the taxonomic position of six basidiomycete OTUs remained unresolved, as ITS sequence data from closely related species were not available in the database. While the resolving power of the fungal ITS regions would often enable identification of distantly related species (7), unknown ITS sequences remain uninformative if they belong to groups that are under-represented in sequence databases (18). Sequence databases tend to be biased towards sequences from fungal species that either produce distinctive sporocarps or grow well in culture and the continuing addition of sequences from more fungal species is needed to get beyond the current 'unknowns' (18). Four unresolved OTUs came from the mineral E1, E2 and B horizons. These OTUs might represent fungal species that have been missed in earlier studies as they might only occur in deeper soil layers or do not form sporocarps.

Most current inventories concerning EM fungal communities are based on EM root samples taken from organic horizons. The present study reveals that 16 out of 25 OTUs were exclusively detected in the deeper mineral soil. This demonstrates that the general concept of fungal species from deeper soil profiles not accounting for much of the fungal species diversity (5) might no longer be valid. In a podzol profile, apart from being situated at different depths, each soil horizon has distinct chemical and physical properties. Several factors could influence fungal species distribution along a vertical soil profile (27) as species differ in sensitivity to environmental factors like O_2 and CO_2 (5), pH (10, 27), soluble Al^{3+} (26), soil moisture and organic matter content (1) or predation (28). This study was not set up to determine factors influencing fungal species distribution along a vertical gradient, yet the results indicate that the detected species are not equally distributed over four podzol horizons. Higher pH values and total C amounts (Table 1) in the B horizon compared to the E horizon could influence OTU distribution. In this study, EM fungal species like *Hebeloma* or *Suillus* were found in the mineral B soil high in total C contents, whereas *Cortinarius*, *Dermocybe*, *Tylospora* and *Laccaria* were found in the strongly weathered mineral E soil with low total C contents. Organic and mineral nutrient availability vary in each horizon and fungal species presence in a certain horizon could depend on its ability to take up nutrients from organic or inorganic sources. Considerable amounts of extramatrical mycelium of *Suillus* spp. in the mineral E and B horizons have been observed in microcosm systems and have been related to the suggested role of these species in mineral weathering processes (16).

The permutation test performed indicated a non-random vertical distribution of OTUs along the podzol profile at a probability level of 6%. The distribution of the 11 analysed OTUs appears to have some relation to the soil horizons, taken into account that the test performed relies on some important assumptions that influence its outcome. Based on the fact that OTUs from two or more horizons always occur in bordering horizons (Table 3), it was assumed that the occurrence of an OTU in one horizon might depend on its occurrence in one or two adjoining horizons. Quantitative clone aspects were not taken into account in this study and the finding of one clone in a horizon was considered to represent the presence of an OTU in that particular horizon. Due to the continuity of mycelium in the soil, occurrence of (small amounts of) mycelium in more than one soil horizon is likely. These detected, however ecologically insignificant, amounts of mycelium in bordering horizons might have been the cause of the test's outcome being only marginally significant at $P \leq .06$. This fact illustrates the difficulty of accurately detecting spatial structure when sampling in natural systems. In fact, for many spatial analysis methods it is difficult to detect significant spatial patterns for small sample sizes ($n \leq 10$) (12) and analysis of 11 OTUs proved not ideal.

Clone sequence data were compared to sequence data obtained from root tips to find out whether the distribution of mycelium was related to root tip distribution. EM fungal identification through root tip identification presents a very dissimilar approach to EM fungal community analysis when compared to molecular identification of fungi in soil DNA extracts. In this study, although sampled from the same pits and soil horizons, DNA was extracted from soil samples other than the samples that the root tips originated from. Nevertheless, the results (Table 4) show that for 7 OTUs, clone sequence data matched well ($\geq$ 99 %) with the root tip data, indicating that the demonstrated molecular approach is technically robust. One unidentified OTU (OTU 22) matched with a root tip sequence, indicating that the fungus belongs to an EM fungal group that is not yet present in the GenBank database. Four Piloderma-like OTUs had a high sequence similarity with 4 different *Piloderma*-like root tip sequences, indicating that several *Piloderma* species await identification. In addition, the OTU - root tip comparison shows that the EM mycelium primarily colonises the horizon in which the EM root tip is found.

This study shows that the use of molecular methods enables identification of EM mycelium in soil and provides a complementary approach to conventional EM root tip identification in EM community studies. Both approaches, however, are subject to bias. While the sampling of clustered root tips often inadequately reflects the full species richness below ground (18), each step involved in the molecular analyses of an environment could be a source of bias, which will lead to a distorted view of the

real world (39). How these molecular uncertainties affect the outcome of molecular fungal community analyses needs to be studied in greater detail.

In conclusion, the present study demonstrates that molecular identification methods provide a novel tool for future studies on EM fungal community dynamics. These molecular methods allow identification of EM mycelium in soil and therefore permit a unique view on the EM fungal community below ground, independent of EM root tip occurrence. The active soil mycelium has clear functional significance (18) and compared to data on colonised root tip numbers, spatial distribution of EM mycelium better reflects substrate preference and potential nutrient mobilising and uptake capacities of EM fungal species. EM mycelial distribution data will provide significant information on potential ecological roles of different EM fungal species or functional groups in soil ecosystems. Although the accuracy of molecular methods for quantitative fungal community analysis and fungal activity measures needs to be assessed, the use of molecular identification methods will provide valuable new insights into EM fungal community dynamics.

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5

Quantification of ectomycorrhizal mycelium in soil by Real-Time PCR

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Abstract

Mycelial biomass estimates in soils are usually obtained by measuring total hyphal length or by measuring the amount of fungal specific biomarkers like ergosterol and phospholipid fatty acids (PLFAs). These methods determine the biomass of the fungal community as a whole and do not allow species-specific identification. Molecular methods based on the extraction of total soil DNA and the use of a gene as biomarker enable identification of mycelia directly from the environment. To test whether also the biomass of individual fungal species in soil can be determined with molecular methods, three molecular techniques were compared. The growth of extramatrical mycelium of two ectomycorrhizal (EM) fungal species (*Suillus bovinus* and *Paxillus involutus*) in soil was monitored by denaturing gel gradient electrophoresis (DGGE), a cloning technique and Real-Time Quantitative PCR and compared to results obtained with hyphal length determination and PLFA analysis. The molecular methods enabled identification and relative quantification of both species separately in a more-species environment and showed consistent results. Amounts of target DNA per gram soil were used to quantitatively compare soil samples. Over time in a more-species environment, increasing amounts of *S. bovinus* DNA and decreasing amounts of *P. involutus* DNA were found. This work demonstrates that molecular methods provide tools to determine the biomass of individual fungal species in soil.

Introduction

Many different ectomycorrhizal (EM) fungal species exist in natural ecosystems and form symbioses with woody perennials and trees. The EM symbiosis is characterised by the presence of colonised root tips and the development of extramatrical mycelia radiating into the soil. EM fungi on root tips can be identified with morphological and molecular methods and for that reason EM root tips are frequently treated as countable units (19) that can conveniently be used to quantify the presence of individual EM fungal species on tree root systems. The ecological importance of specific EM fungi in ecosystems is thereby often regarded proportional to the number of EM root tips. Besides the extent of root tip colonisation however, variations in exploration and exploitation structure of the extramatrical mycelia largely determine the water and nutrient uptake, transport and transfer capacities of individual EM fungi. Although the characteristics of the extramatrical mycelium largely establish the functional role of individual EM fungal species (1), identification and quantification of mycelia in soil has been more problematical than identification and quantification of EM root tips. Only recently have molecular methods enabled identification and quantification of EM extramatrical mycelia in soil (6,10,16,21,22). Commonly, mycelial biomass estimates in soils are obtained by measuring total hyphal length or by measuring fungal specific biomarkers like ergosterol and phospholipid fatty acids (PLFAs) (12,24,29), but these methods do not distinguish EM fungal species from other fungal species. Inclusion of a non-mycorrhizal control treatment and subtracting the amount of fungal biomass in the control treatment from the fungal biomass in the mycorrhizal treatment is sometimes used to solve this problem (2,11,30). This practice can only be applied in artificial systems and besides, fungal growth is generally influenced by several factors, including competition with other fungi. The hyphal growth rate of a non-mycorrhizal fungus in a control treatment is therefore not necessarily equal to its growth rate in a treatment with mycorrhizal fungi.

At present, molecular techniques enable the use of a gene as biomarker and facilitate identification of hyphae directly from the environment. Based on the extraction of total soil DNA, molecular techniques like denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), competitive PCR and cloning-sequencing procedures have been used to identify EM mycelia from soil (6,10,16,21,22). Nonetheless, identification in itself only reveals the presence of a fungal species in a fungal community. In order to study species dominance, succession and interaction with other mycelia, the biomass of individual fungal species in soil needs to be measured. For quantification of specific target DNA in environmental samples, Real-Time Quantitative PCR can be used. Very

small quantities of DNA can be detected by Quantitative PCR, making it an important tool for disease risk assessments (9,18). Still, a certain quantity of fungal DNA does not necessarily correlate with a certain quantity of fungal biomass. Filamentous fungi differ in cell lengths, cell volumes and amounts of DNA per cell and it is therefore not possible to obtain a universal conversion factor to convert DNA quantities to fungal biomass (16). Yet, as stated by Guidot et al. (16), for a relative comparison of fungal abundance in soil samples, expressing the results in terms of the amount of DNA per gram soil may be sufficient. So far, only a few studies have used molecular data obtained from soil DNA extracts in a quantitative way. Mycelia of Hebeloma cylindrosporum have been quantified by competitive PCR in natural soil (16) and clone numbers obtained after a cloning-sequencing procedure have been used to quantify fungal propagule numbers in forest soil (6). Both applications show that relative quantification of individual fungal species in complex and mixed-species environments is feasible with molecular techniques. Still, how data obtained with these molecular methods relate to data obtained from conventional fungal biomass measures has not been studied.

In this study, a comparison was made between conventional and molecular methods to quantify the presence of EM hyphae in soil. Pinus sylvestris seedlings were inoculated with none, one or two EM fungal species and grown in pots. Fungal presence in the potting soil was determined over time by conventional methods (direct hyphal counts and PLFA analysis) and by three different molecular methods. Samples were analysed by 1) DGGE, 2) a clone library was screened, and 3) Real-Time Quantitative PCR was applied. The aim of this study was to compare different hyphal quantification techniques and to test whether molecular methods like cloning and Real-Time Quantitative PCR can be used to obtain relative measures of hyphal abundance in a mixed-species environment.

Materials and methods

Plant and fungal material

Two EM fungi (*Suillus bovinus* 'BL97-14', fruitbody isolate from Uppsala, Sweden; and *Paxillus involutus* 'nr.17', fruitbody isolate from Sheffield, UK) were grown on MMN medium (23) and sub-cultured twice before transplanting them to autoclaved (1 min at 120 °C) cellophane placed on MMN medium, resp. three and five weeks before inoculation. The two fungi were grown separately on the cellophane as well as jointly on one piece of cellophane. Scots pine seeds (Pinus sylvestris) were soaked in distilled water for 16 hours and surface sterilised with H_2O_2 (30%) for 30 min. Sterilised seeds were sown in a mixture of perlite:vermiculite (1:1, v/v), moistened with a modified Ingestad solution (100 N / 9 P / 54 K / 6 Ca / 6 Mg / 9 S) (8,20)

and placed in a climate chamber (16 h photoperiod at 70 Watts/m2, day/night temperatures of 20/16°C and 80% humidity). Six weeks after sowing the seedlings were inoculated using a sandwich technique according to the protocol of Colpaert et al. (7). Seedlings were inoculated with *S. bovinus* or *P. involutus* (hereafter named single-species treatment [SS], either 'Suillus' or 'Paxillus') or with both fungi (hereafter named two-species treatment [TS]). A control group of seedlings was not inoculated but was treated the same as the inoculated seedlings. Inoculated and control seedlings were transferred to plant containers (60 ml syringes) filled with acid-washed, sieved perlite, as described by Colpaert et al. (8). For one month, the plant containers were flushed daily with 10 ml modified Ingestad solution. One month after inoculation, inoculated and control seedlings were transplanted to 1 L pots, filled with a mixture of sand:peat:vermiculite (5:4:2, v/v) and immediately after transplantation watered with the modified Ingestad solution (20 ml). Two seedlings were placed about 5 cm apart in each pot. Pots were placed in a climate chamber (16 h photoperiod at 70 Watts/m2, day/night temperatures of 20/16°C and 80% humidity) and circulated weekly. Pots were watered daily with dH_2O and were given 10 ml modified Ingestad solution weekly. The seedlings were grown for three months and every month three pots of each treatment were taken randomly for harvest.

Harvest

Upon harvest, the top 2cm of the potting mixture was removed and discarded. Roots were separated from the remaining potting mixture and carefully shaken to remove the excess of adherent soil. The potting mixture was homogenised by hand and sub-samples of 100 g were frozen immediately (-15 °C). These were used for PLFA and DNA extractions.

Hyphal staining

Hyphae were stained with fluorescent brightener F3397 (Sigma) which binds to beta 1-3 and 1-4 polysaccharides, i.e. cellulose and chitin. Soil samples were diluted in water (30 g FW soil/190 ml dH_2O), mixed in a food processor for 1 min. and 9 ml of this solution was fixed by adding 1 ml of formaldehyde (37%). Fixed solutions were shaken and allowed to settle for 2 minutes. Then 12µl was taken out, smeared on a coated microscope slide (one 12 µl drop covering a 200 mm2 hole in the slide coating) and air-dried. Subsequently a drop (50 µl) of freshly prepared fluorescent brightener F3397 solution (1mg/ml) was added and samples were stained in the dark for 2 hours. After removing the stain solution the slides were rinsed three times 20 minutes with dH_2O and finally dried. Stained and dried samples were covered with a drop of immersion oil and a cover slide and stored in the dark at 6

°C. Hyphal lengths were estimated by epifluorescence microscopy using a gridline intersection method over 100 randomly selected microscopy fields (3). Total hyphal lengths (m/g DW soil) were calculated according to the procedures described by Bloem (3) and Paul et al. (25).

PLFA determination

Extraction and analysis of PLFAs was done according to the method modified from Frostegård et al. (1993) (26). Two gram (FW) of the potting mixture was extracted with chloroform:methanol:citrate buffer mixture (1:2:0.8) and the lipids were separated into neutral lipids, glycolipids and phospholipids on a silicic acid column. From the phospholipid fraction 3 ml was used to extract PLFAs. The quantity of PLFA 18:2 ω6,9 was determined for the DW of each sample and used as an indicator of fungal biomass, as it is suggested to be mainly of fungal origin in soils (26).

DNA extraction and amplification

DNA was extracted from 7 g (FW) of the well-mixed potting mixture, using a Bead-Beater (27). DNA was purified twice with the Wizard® DNA Clean-Up System (Promega) according to the manufacturer's purification protocol before amplification.

For the cloning procedure, DNA amplificaion of the ITS regions was performed on a PCR Express (Hybaid) with the basidiomycete specific primer pair ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS4B (5'- CAG GAG ACT TGT ACA CGG TCC AG -3') (15). The PCR reaction mix consisted of 1 µl 50x diluted template DNA, 38 µl sterile Ultrapure water, 5 µl 10x PCR buffer 2 (Roche), 200 µM of each deoxynucleoside triphosphate, 200 nM of each primer and 0.5 µl Expand enzyme mix (Roche). The following thermocycling pattern was used: 94 °C for 3 min (1 cycle); 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min (30 cycles); and 72 °C for 10 min (1 cycle).

For the DGGE procedure, ITS sequences were amplified on a PCR Express (Hybaid) with the primers ITS1F and ITS4B-GC. ITS4B-GC is similar to ITS4B and includes a GC clamp on the 5' end to stabilise the melting behaviour of the DNA fragments. The PCR reaction mix consisted of 1 µl 50x diluted template DNA, 39 µl sterile Ultrapure water, 5 µl 10x PCR buffer 2 (Roche), 200 µM of each deoxynucleoside triphosphate, 200 nM of each primer and 0.5 µl Expand enzyme mix (Roche). The following thermocycling pattern was used: 94 °C for 3 min (1 cycle); 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min (35 cycles); and 72 °C for 10 min (1 cycle).

DGGE analysis

The presence of successfully amplified PCR products (ITS1F-ITS4B primer pair) was confirmed by analysing 2 µl of PCR product on a 1% agarose gel, stained with SYBRGold (Molecular probes) and visualised using a CCD camera system (Syngene). Ten microliters of the obtained PCR products were subsequently analysed by DGGE. Gel electrophoresis was performed on an 8% acrylamide gel containing a linear denaturant gradient from 20% to 60% of urea and formamide. The 100% solution contained 4,0 ml acrylamide, 0.3 ml 50x TAE buffer, 6 ml formamide and 6.3 g ureum, supplemented with distilled water to a final volume of 15 ml. Gels were run overnight at a constant temperature of 60 °C, with 80 V for 18 h. After completion of the electrophoresis, gels were stained with SYBRGold and documented on a Syngene CCD camera system (Genesnap 4.00.00, copyright Synoptics Ltd. 1993-2000).

Cloning procedure

Successfully amplified PCR products (ITS1F-ITS4B primer pair) of the two-species treatment were cloned into the pGem-T vector (Promega, Madison, WI, USA). Ligation mixtures were transformed into Ultracompetent E.coli XL1-Blue (Stratagene, Cambridge, UK) according to the manufacturer's instructions. The bacteria were plated out in three dilutions. From each sample 20 white colonies were picked with a toothpick, resuspended in the PCR mixture and amplified directly with the ITS1F and ITS4B-GC primer pair. The amplified products were identified by DGGE analysis, using the same DGGE conditions as described under 2.6. A *S. bovinus* - *P. involutus* marker was used to identify the Suillus or Paxillus band in each sample.

Development of specific primers and probes

Design of the two Paxillus and Suillus primer and probe sets was based upon sequences obtained from DNA extracts of the *P. involutus* and *S. bovinus* fungal cultures. A 5.8S primer (5'-TGA ATC ATC GAA TCT TTG AAC G-3', 22bp, Tm=60 °C, 36% GC) was used for Paxillus as well as Suillus amplification. A ITS2 primer was used to detect Suillus sequences (5'- CCC GAA CAC GAA TAT TCA GG -3', 20bp, Tm=60 °C, 50% GC) and another ITS2 primer was used to detect Paxillus sequences (5'- CCA TCG CTA ATG CTT TTA AGG -3', 21bp, Tm=60 °C, 43% GC). Specificity of the primers was tested by amplification of eight cultured EM fungal species, including *S. variegatus* and another isolate of *S. bovinus* and 18 EM fungal species from basidiocarps collected in The Netherlands, including *S. bovinus* and *P. involutus*. Amplification of *S. bovinus* from the original culture, the basidiocarp as well as slight amplification of the second *S. bovinus* culture was

observed with the Suillus primer pair. Besides amplification of *P. involutus* from the original culture and the basidiocarp, slight amplification was observed for *Laccaria bicolor*, *L. proxima* and *Gymnopilus sapineus* with the Paxillus primer pair. Hybridisation probes were used for the sequence-specific detection of *S. bovinus* and *P. involutus* target DNA with the LightCycler. Two oligonucleotide probes were developed for each species. Each probe is labelled with a different marker dye (fluorecein or LCRed640) and fluorescence occurs when both probes are bound to adjacent sequences on the target DNA. Two probes were developed for detection of Suillus sequences (5'- GAG CAT GCC TGT TTG AGC GTC AGT AAA -3', 27bp, Tm=65.5 °C, 48.1% GC; and 5'- LCRed640-CTC AAC TCC TCT CGA TTG ACT TCG -3', 29bp, Tm=66.5 °C, 51.7% GC). Two other probes were developed for detection of Paxillus sequences (5'- CAA CCA TCC CTC GAT TCG TTT CGA G -3', 25bp, Tm=65.8 °C, 52.0% GC; and 5'- LCRed640-GTT TGG CTT GGA TTT TGG GGG C -3', 22bp, Tm=67.3 °C, 54.5% GC).

Quantitative PCR

DNA extracts from the two single-species treatments and from the two-species treatment were amplified by Quantitative PCR. Quantitative PCR was performed on the LightCycler (Roche), using LightCycler Capillaries and LightCycler Software version 3.5.3. (Roche, 2001). Twenty µl reaction solutions were pipetted in each capillary. The Suillus reaction mix contained 2µl 10x diluted template DNA, 6.5 µl water, 2 µl LightCycler FastStart reaction mix buffer, 3 mM MgCl2, 0.5µM of each primer, 0.2 µM of probe 1 (fluorecein) and 0.2 µM of probe 2 (LC-Red640). The Paxillus reaction mix contained 2 µl 10x diluted template DNA, 7.8 µl water, 2 µl LightCycler FastStart reaction mix buffer, 3mM MgCl2, 0.5µM of each primer, 0.2 µM of probe 1 (fluorecein) and 0.4 µM of probe 2 (LC-Red640). All chemicals originated from the LightCycler-FastStart DNA kit (Master Hybridization Probes, Roche). The following thermocycling pattern was used for the Suillus PCR: 95 °C for 10 min (1 cycle), 95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 10 sec (45 cycles), followed by 1 cycle at 55 °C for 15 sec. The following thermocycling pattern was used for the Paxillus PCR: 95 °C for 10 min (1 cycle), 95°C for 10 sec, 60 °C for 15 sec and 72 °C for 10 sec (45 cycles); and 72 °C for 10 min (1 cycle), followed by 1 cycle at 55 °C for 15 sec. Dilutions of known amounts of ITS plasmid inserts were used as standard curve under the assumption that amplification efficiency of plasmid target DNA was equal to the amplification efficiency of sample target DNA. LightCycler results (µg target DNA/20 µl PCR mixture) were converted to pmol target DNA/g soil (DW). LightCycler product formation was confirmed by melting curve analysis and by analysing PCR products on a 1% agarose gel, stained with SYBRGold.

Statistics

Data analysis was performed using SAS programs (SAS 8, copyright 1999 by SAS Institute Inc.) and SPSS (SPSS 8.0, copyright 1997 by SPSS Inc.). All analyses were performed on log transformed data. A normality test (SPSS, Non-Parametric test, Sample K-S) and a test of Homogeneity of Variances (SPSS, One-Way ANOVA) showed that the log transformed data sets were normally distributed and that their variances were spread homogeneous. A correlation analysis (SPSS, Pearson correlation, 2-tailed) was performed to check for correlations between PLFA and hyphal data for four treatments. GLM analysis (SAS) was used to test for significant differences between treatments at each sampling time and to test for significant differences for each treatment over time (Tukey's Studentized Range Test). For each treatment at each month, three replicates (n = 3) were used.

Results

PLFA and hyphal length results for all treatments

In all four treatments, over three months, the total hyphal lengths (Fig.1A) showed the same trend as the amounts of PLFA 18:2ω6,9 (Fig.1B). The Suillus treatment showed a significant increase in hyphal length as well as amounts of PLFAs, while the Paxillus treatment and the two-species treatment remained constant over time. The controls showed a significant increase in hyphal lengths as well as PLFAs over time. Correlations between PLFA concentration and the hyphal lengths of the Suillus treatment, the two-species treatment and the controls (resp. R_S = .828, R_{TS} = .937 and R_C = .867) were significant at the 0.01 level (2-tailed). The correlation for the Paxillus treatment (R_P = .283) was not significant.

LightCycler results for single species treatments

LightCycler PCR product formation was confirmed by melting curve and agarose gel analysis, with the melting curves showing one distinct peak and the agarose gel showing one distinct band of the right size per sample. The standard curves for both the Paxillus and Suillus LightCycler analyses were highly reproducible (data not shown). In general, the amounts of DNA measured in the Suillus treatment (Fig.2) were higher than the amounts of DNA in the Paxillus treatment. Both treatments showed no significant changes over time. Standard errors were high for both treatments. No significant correlations between PCR data and PLFAs or hyphal lengths for the single-species treatments were found.

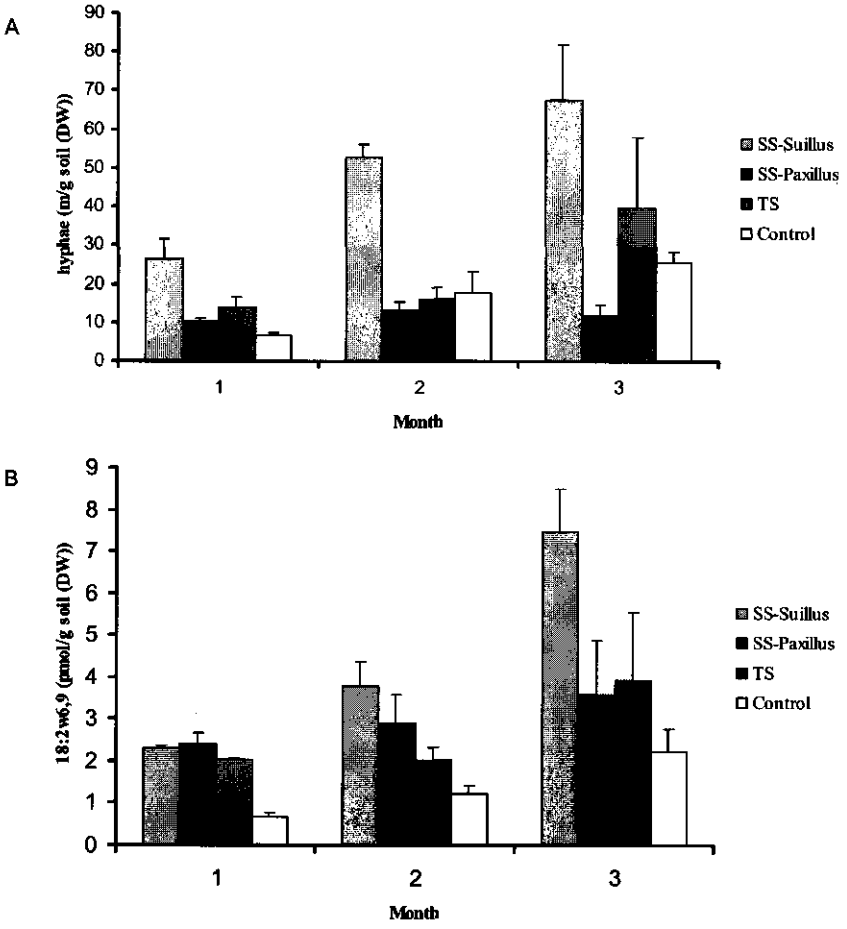

Figure 1. Fungal colonisation of the potting-mixture over three months determined by (A) hyphal lengths and (B) phospholipid fatty acid (PLFA) 18:2ω6,9 analysis. SS indicates the single-species treatments and TS indicates the two-species treatment. Error bars indicate ± SE, n = 3. Different letters within each month indicate statistically different values (GLM/Tukey's Studentized Range).

DGGE results for two-species treatment

DGGE analysis confirmed the presence of Suillusand Paxillus DNA in the two-species treatment soil (Fig.3). Over time, DGGE band intensities varied as the average band intensity of Paxillus DNA decreased while the average band intensity of Suillus DNA increased. DGGE analysis also revealed the presence of bands that did not belong to either Paxillus or Suillus, indicating the presence of other fungi in the treatment and control soil (data not shown). No attempts were made to identify these fungi.

Clone counts of two-species treatment

Suillus as well as Paxillus clones were detected after amplification, cloning and DGGE analysis of extracted DNA from the two-species treatment (Fig.4A). Fungal clones other than Suillus or Paxillus were also found but these were excluded from the analysis. The majority of clones found in the first month were Paxillus clones (> 97%) but their frequency decreased during the following two months to 0%. After the second month the frequency of the Suillus clones increased from 55% to 100% at the end of the experiment.

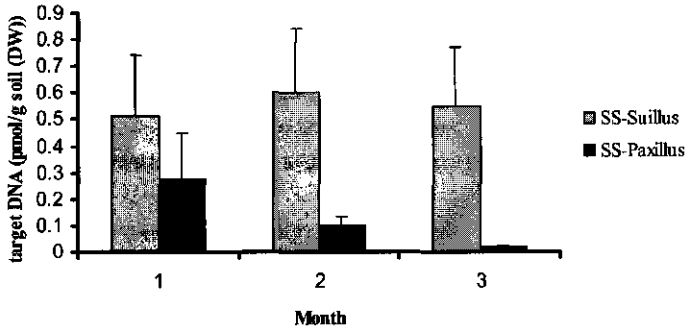

Figure 2. Concentrations of target DNA in the potting-mixture over three months for the two single-species treatments (SS), as determined by LightCycler analysis. Error bars indicate ± SE, n = 3.

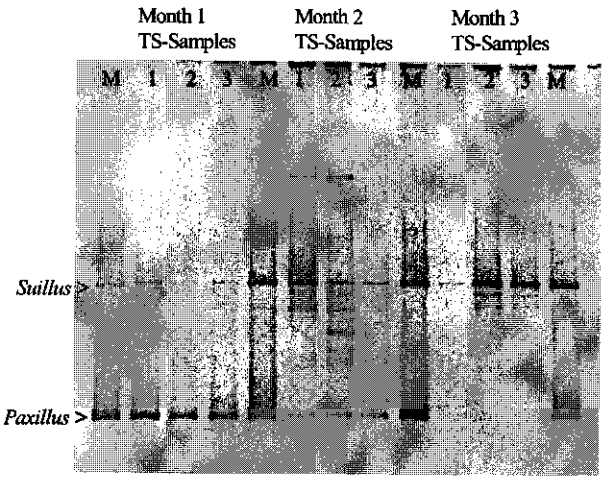

Figure 3. DGGE of amplified ITS rDNA fragments from the two-species treatment (TS), representing the fungal community in the potting-mixture over three months. Marker lanes (M) show the *Suillus-Paxillus* marker. For each month, TS-samples 1,2 and 3 represent the results of three pots that were destructively harvested.

LightCycler results for two-species treatment

DNA of Suillus as well as Paxillus was detected and quantified in the potting mixture of the two-species treatment (Fig.4B). Over time, the amount of Suillus DNA increased from 0.01 to 0.34 pmol/g soil DW, while the amount of Paxillus DNA significantly decreased from 0.12 to 0.01 pmol/g soil DW. Standard errors were high for both fungi.

Discussion

Using molecular tools to obtain fungal biomass estimates

This study has shown that molecular methods enable identification and quantification of hyphal material in an artificial mixed-species environment. Like the use of hyphal lengths and PLFAs, DNA soil extracts proved a useful source for fungal biomass estimations. The three molecular methods tested showed consistent results and provided tools to analyse species-specific fungal abundance in soil.

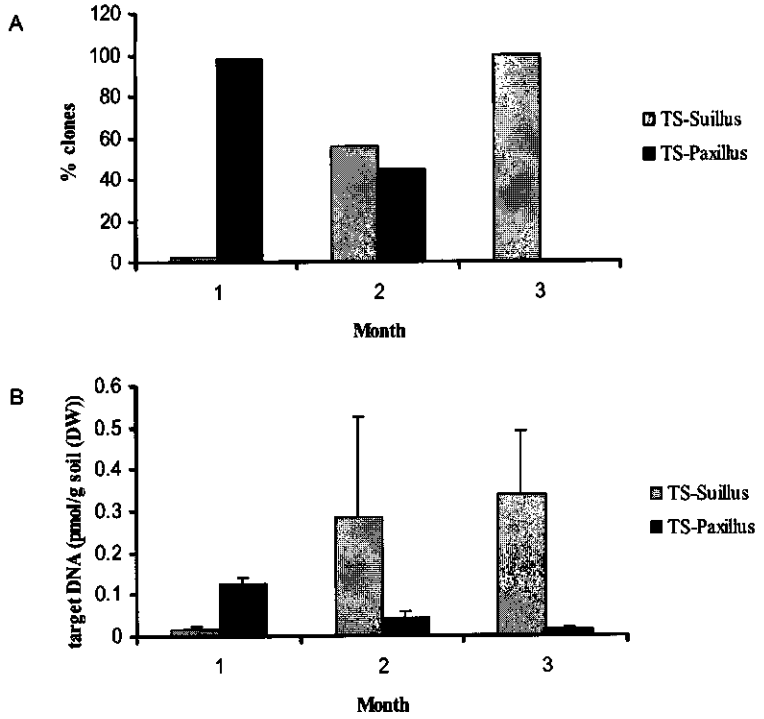

Figure 4. Concentrations of target *Suillus* and *Paxillus* DNA in the potting-mixture over three months for the two-species treatment (TS), as determined by (A) clone counts and (B) LightCycler analysis. Error bars indicate ± SE, n = 3.

First of all, DGGE fingerprinting provides a powerful tool in microbial ecology to assess the microbial diversity in environmental samples (14). In this study, DGGE analysis allowed detection of the two inoculated EM fungi and allowed furthermore detection of fungi that had spontaneously invaded the soil. In addition, DGGE band intensities provided an indication of fungal abundance. The average band intensities of Suillus in the two-species treatment were found to increase, while the average intensities of Paxillus were found to decrease. This trend was confirmed with the other two molecular methods. Nonetheless, quantitative use of DGGE implies plateau phase quantification of PCR products, as band intensities do not increase anymore after a certain number of PCR cycli (5). Only with the use of an

internal standard can DGGE band intensities accurately quantify the abundance of the corresponding organism in the template DNA mixture (5).

The second molecular method being applied was the screening of a clone library. Cloning in *E.coli* is a method to separate PCR-amplified DNA fragments from a mixture (28). Cloned DNA fragments can be sequenced to identify them to species level, but this is a costly procedure. In this study, a number of cloned fragments was analysed by DGGE, allowing rapid and relatively cheap identification. Cloning techniques have been applied to identify fungi in different soil fungal communities (4,22) but the influence of cloning systems on quantitative detection of fungi is poorly investigated (28). Nevertheless, ITS clone numbers have been used to quantitatively compare sites for different functional groups of fungi (6). In this study, determination of the percentage of clones originating from Suillus or Paxillus showed a trend equal to the DGGE results over time and revealed the presence of other fungi besides Suillus and Paxillus.

The third method used to quantify amounts of DNA was Real-Time Quantitative PCR. Specific DNA quantification is obtained with two fluorogenic probes that hybridise to a region of the target DNA defined by two PCR primers. To standardise the PCR quantifications in this study, dilutions of cloned Suillus and Paxillus DNA were used under the assumption that the amplification efficiencies of plasmid target DNA and sample target DNA were equal. Analysing all samples in duplicate however showed that LightCycler soil sample results fluctuated, while analysis of the standard plasmid samples did not reveal these fluctuations. The standard samples produced highly reproducible standard curves and variances due to pipetting inaccuracies can therefore be excluded as a source for fluctuating results. In contrast to the purified plasmid DNA extracts, co-extracted components may have contaminated the soil DNA extracts. Co-extracted contaminants can inhibit PCR amplification (28) and even low concentrations may affect the amplification efficiency of the highly sensitive LightCycler. Differences in homogeneity of samples analysed in duplicate may, however small, have differentially affected the LightCycler efficiency. Due to the exponential nature of the PCR reaction, small changes in amplification efficiency will have a large impact on its outcome and may have caused the fluctuations observed in this study. Still, in correspondence to the DGGE and clone data, Quantitative PCR showed increasing amounts of Suillus DNA and decreasing amounts of Paxillus DNA in the two-species treatment.

All three molecular methods revealed a similar trend in the two-species treatment, with increasing amounts of Suillus DNA and decreasing amounts of Paxillus DNA over time. In contrast, PCR analysis of the single-species treatment revealed no significant changes in Suillus and Paxillus DNA amounts over time.

This suggests that interaction between the two EM fungi influenced Paxillus growth negatively in the two-species treatment.

Using hyphal lengths and PLFAs to obtain fungal biomass estimates

Three molecular techniques were compared in this study to hyphal length measurements and PLFAs analyses. Amounts of PLFA 18:2ω6,9 are generally being used as indicators for the presence and abundance of fungi in soils (13). It is thereby assumed that each fungal species in a community contributes to the PLFA profile in proportion to its biomass (17). In this study, the amounts of PLFA 18:2 ω6,9 correlated well with the hyphal lengths of three treatments. For the Paxillus single-species treatment however, a relative large amount of PLFA 18:2ω6,9 was found compared to the hyphal length data. Contrary to other reports (24), there were no indications from the hyphal length and PLFA results of the single-species treatments that Suillus produces relatively more PLFAs than Paxillus. Hyphal lengths and PLFAs in the non-inoculated control treatment showed a gradual increase in time as the potting mixture became colonised by invading fungi. DGGE banding pattern analysis allowed for detection of fungal species, other than*S. bovinus* and *P. involutus*, which had indeed spontaneously invaded the potting mixtures of the controls as well as the treatments. The fungi that spontaneously invaded the treatment soils also contributed to the measured hyphal lengths and PLFAs profiles of these treatments, but not necessarily to the same extent as indicated by their presence in the control soils. The growth rate of a non-mycorrhizal fungus in a control treatment does not necessarily equal its growth rate in a more-species treatment, where competition for resources occurs. This study indeed showed that the sum of the hyphal lengths and PLFAs amounts from Suillus and Paxillus in the two single-species treatments did not represent the total amounts of hyphae and PLFAs in the two-species treatment. The practice of subtracting the amount of fungal biomass in the control treatment from the total fungal biomass (2,11,30) in order to estimate mycorrhizal fungal biomass is therefore problematic.

Concluding remarks

Where the choice for a (vital) stain determines whether living and/or dead fungal hyphae are quantified, this distinction is less clear when analysing fungal DNA. The changes in species abundance found with molecular methods can not directly be translated to changes in fungal biomass. This is illustrated by the fact that even in the single-species treatments no significant correlation was found between the DNA data and the hyphal and PLFA data. Copy numbers of ITS genes on the genome can vary between fungal species. For quantification purposes it would therefore be most reliable to use a biomarker gene with a known copy number. Copy numbers of

the ITS genes and their extraction efficiency were assumed equal for the two fungal species tested in this study, although this likely caused some bias. Still, PLFAs are used extensively to quantify fungal biomass, despite the fact that their quantity depends on the fungal species considered and its active membrane area (24). The use of DNA adds much-needed specificity to hyphal quantification measures and the present uncertainties might not be much different from those of PLFA use.

In conclusion, the molecular methods used in this study have enabled identification and (relative) quantification of mycelia of two EM fungi in a mixed-species environment. The results of this study demonstrate the high potential of molecular methods to assess relative changes in fungal biomass over time, thereby providing a species-specific measure to quantify mycelia in soil.

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6

Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach

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Abstract

Molecular methods based on soil DNA extracts are increasingly being used to study the fungal diversity of ectomycorrhizal (EM) fungal communities in soil. Contrary to EM root tip identification, the use of molecular methods enables identification of extramatrical mycelia in soil. To compare fungal diversity as determined by root tip identification and mycelial identification, six soil samples were analyzed. Root tips were extracted from the six samples and after amplification, the basidiomycete diversity on the root tips was analyzed by denaturing gel gradient electrophoresis (DGGE). The soil from the six samples was sieved, total soil DNA was extracted and after amplification, the basidiomycete diversity in the soil fractions was analyzed by DGGE. Fourteen different bands were excised from the DGGE gel and sequenced and to eight bands fungal taxon names could be assigned. Out of a total of 14 fungal taxa detected in soil, 11 fungal taxa were found on root tips, of which seven were EM fungal taxa. To examine whether the sieving treatment would affect EM species diversity, two different sieve mesh sizes were used and in addition, the organic soil fraction was analyzed separately. DGGE analysis showed no differences in banding pattern for the different soil fractions. The organic fraction gave the highest DGGE band intensities. This work demonstrates that there is a high correspondence between basidiomycete diversity detected by molecular analysis of root tips and soil samples, irrespective of the soil fraction being analyzed.

Introduction

Ectomycorrhizal (EM) fungi live in symbiosis with mostly trees and woody perennials. About 6000 species of EM fungi are known to exist, forming species-rich, highly dynamic and complex communities below ground (15). The high species richness and the apparent non-random distribution of species complicate sampling of EM communities to assess species richness. Many EM fungal species can be classified by morphological identification of colonized EM root tips and often the number or percentage of colonized root tips is used as a measure of species abundance (15). For identification of EM fungi, root tips are typically extracted from soil and sorted by morphological criteria. A carefully selected, small portion of these sorted root tips is subsequently used for molecular identification to species level. Often, not more than 2-5% of the total number of sampled root tips is used for identification by molecular methods (e.g., (11,13)) and conspicuous types are sometimes considered characteristic enough to be identified without molecular methods (6). However, visual sorting of fungal morphotypes can lead to bias. Without species verification through molecular analysis, root tip sorting can lead to underrepresentation of species, as different fungal species with a uniform morphology may be regarded as one morphotype. Molecular analysis of a relatively small root tip selection of that morphotype can be insufficient to detect the different species it contains and these species will be underrepresented in the molecular species lists. Rare but distinct types in contrast will be overrepresented in the molecular species lists, as atypical root tips are always grouped into distinct morphotypes and therefore always included in the molecular analyses. This root tip sample bias would be overcome when all sampled EM root tips are included in the molecular analyses. Single root tip DNA extractions are however not realistic when analyzing thousands of root tips and therefore extracted root tips are sometimes pooled prior to DNA extraction (16). Detection of a fungal species then depends on its initial quantity and on its DNA extraction and amplification efficiency.

Studies on fungal species diversity are increasingly conducted with molecular techniques and based on the mycelial presence of fungi in soil or other substrates. Recently, molecular methods based on total soil DNA extracts were used to detect and identify EM mycelium in natural soil (4,7,12). These molecular methods enable the identification of fungi in soil without the extraction or cultivation of the organisms themselves. The use of soil DNA extracts to identify EM fungi does not involve morphological sorting of EM root tips and sample bias related to root tip sorting does therefore not occur.

Now that studies of the diversity of EM mycelia are becoming more common, it would be useful to compare fungal diversity obtained from molecular mycelia

identification to fungal diversity obtained from EM root tip identification. To make sure that detected differences in species diversity are not induced by the methods used, the identification methods should be kept as similar as possible in both approaches and root tip sorting should not be applied, as it might induce sampling bias. In addition, the method used to extract root tips from the soil that will be used to detect mycelium should be considered carefully. Other studies have removed root tips from soil samples by visually checking for the presence of root tips under the dissecting microscope or by sieving the soil before extracting total soil DNA (4,7,12). Several sieve mesh sizes have been used to separate soil from EM root tips but it is unknown whether and how these different sieve mesh sizes influence the outcome of the study. EM root tips or even fragments of hyphal mantles that end up in the soil fraction will co-amplify and likely increase species diversity or abundance as determined from soil DNA extracts.

In the present study the species diversity of an EM fungal community in a *Pinus sylvestris* stand in The Netherlands was studied by molecular EM root tip identification as well as by molecular EM mycelia identification. To avoid methodological differences between the two approaches, the same molecular identification techniques were applied to the root tip samples and the mycelial soil samples and the EM root tips were not grouped in morphotypes. DNA was extracted from pooled root tips and soil and ITS fragments were amplified with basidiomycete specific primers. Denaturing gradient gel electrophoresis (DGGE) was used to analyze band diversity from the root tip and soil samples. A selection of the DGGE bands was excised from the gel and sequenced in order to identify the fungal taxa present. Furthermore, DGGE band diversity was analyzed in two different fractions of sieved soil and, separately, in the organic soil fraction. The aim of this study was to determine whether the detected basidiomycete diversity would differ according to the root tip or soil DNA approach used and whether the diversity would depend on the soil fraction analyzed.

Materials and Methods

Field site and soil sampling

In June 2002 soil cores were taken in a forest dominated by Scots pine (*Pinus sylvestris* L.) in a drift sand area in the central part of The Netherlands (Hulshorsterzand, 52° 21'N, 5° 44'E). In 1994 the litter layer had been removed from this stand and sporocarp production of EM fungi was monitored in 1998 (14). In 2002, 8 years after the sod-cutting, a very thin litter layer had re-developed. Six 2 to 3-year old pine seedlings were selected and soil cores (Ø 6 cm) were taken that included the seedling. The soil was sampled to a depth of 10 cm and after transport

to the lab samples were frozen at -70°C until processing. After defrosting, roots and soil were separated on a 2 mm sieve. Root tips were removed from the sieve and were pooled per sample in a micro-centrifuge tube, cleaned by several dH_2O washes and ground with a tube mortar in sodium-phosphate buffer. From the six sieved soil samples (2 mm fraction), sub samples of 50 g were air dried and sieved again over a 1 mm and 0.3 mm sieve. From three sieved soil samples, the 1mm soil fraction was further separated into a light organic fraction and a heavy sand fraction by careful shaking. Of these three soil samples, DNA was extracted from the 1mm total soil fraction, 1mm organic fraction, 1 mm sand fraction and the 0.3 mm total fraction. A selection of shriveled, dry and clearly dead root tips was collected from the 1 mm fraction of one soil sample, cleaned by several dH_2O washes and ground with a tube mortar in sodium-phosphate buffer.

DNA extraction and amplification

DNA was extracted from 0.5 g of all soil fractions and 50 µl of the root tip suspensions, using a Fast DNA Spin kit for soil (BIO101 Systems) according to the manufacturer's instructions.

For the DGGE procedure, ITS sequences were amplified on a PCR Express (Hybaid) with the primers ITS1F and ITS4B-GC (8). ITS4B-GC is similar to ITS4B and includes a GC clamp on the 5' end to stabilise the melting behaviour of the DNA fragments. The PCR reaction mix consisted of 1 µl 50x diluted template DNA, 39 µl sterile Ultrapure water, 5 µl 10x PCR buffer 2 (Roche), 200 µM of each deoxynucleoside triphosphate, 200 nM of each primer and 0.5 µl Expand enzyme mix (Roche). The following thermocycling pattern was used: 94°C for 3 min (1 cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 3 min (35 cycles); and 72°C for 10 min (1 cycle).

DGGE

The presence of successfully amplified PCR products (ITS1F-ITS4B primer pair) was confirmed by analyzing 2 µl of PCR product on a 1% agarose gel, stained with SYBRGold (Molecular probes) and visualized using a Syngene CCD camera system (Genesnap 4.00.00, copyright Synoptics Ltd. 1993-2000). Ten µl of the obtained PCR products of the root tip and 1 mm soil samples and 5 µl of the obtained PCR products of the different soil fractions were subsequently analyzed by DGGE. Gel electrophoresis was performed on an 8% acrylamide gel containing a linear denaturant gradient from 20% to 60% of urea and formamide. The 100% solution contained 4 ml acrylamide, 0.3 ml 50x TAE buffer, 6 ml formamide and 6.3 g ureum, supplemented with distilled water to a final volume of 15 ml. Gels were run overnight at a constant temperature of 60 °C, with 80 V for 18 h. After completion

of the electrophoresis, gels were stained with SYBRGold and documented on a Syngene CCD camera system.

Sequencing of DGGE bands

Bands of interest were excised from the DGGE gel and allowed to stand in 50 µl distilled water for 30 min. After mixing, 1 µl of this solution was added to the PCR mix, containing 39 µl sterile Ultrapure water, 5 µl 10x PCR buffer 2 (Roche), 200 µM of each deoxynucleoside triphosphate, 200 nM of each primer (ITS1F-ITS4B primer pair) and 0.5 µl Expand enzyme mix (Roche). DNA from the DGGE bands was re-amplified whereby the following thermocycling pattern was used: 94°C for 3 min (1 cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 3 min (35 cycles); and 72°C for 10 min (1 cycle). Obtained PCR products were analysed on a 1% agarose gel. Bands of the right size (0.7-kb) were purified with the QIAquick Purification Kit (Qiagen) according to the manufacturer's protocol. The 0.7-kb fragments were sequenced in one direction on an ABI377 DNA sequencer by cycle sequencing using the dye terminator system (Eurogentec, Seraing, Belgium) and the ITS1F primer. To identify the obtained sequences, all sequences were compared to sequences from the GenBank database, making use of the Blast program from the National Center for Biotechnology Information (NCBI). Subsequently, alignments were made with all obtained sequences and a selection of the best matching sequences obtained from GenBank, using ClustalW (DNA Star Program). Finally, phylogenetic trees were constructed by neighbor-joining using the Treecon program (version 1.36, Yves van der Peer). Based on the clusters that were formed sequences were sorted into groups and assigned taxon names. The obtained nucleotide sequences are to be submitted to NCBI and are currently available on request.

Results

Soil samples versus root tip samples

DNA extracted from dry, dead root tips could not sufficiently be amplified and produced a hardly visible product on a 1% agarose gel (data not shown). DNA was successfully extracted from the six root tip and soil samples (1 mm fraction) and fungal DNA was amplified with the ITS1F-ITS4B primer pair. The DGGE banding pattern revealed a total of 14 clear, different bands (Fig. 1). Out of this total of 14 bands, 11 bands were detected in the root tip as well as the soil samples, whereas three bands were exclusively found in the soil. Band no.11 was found in every soil sample, but in none of the root tip samples. For five out of six samples (root tips as well as soil) the banding patterns were quite similar, whereas one sample (sample 6) revealed another, different banding pattern. Two bands (band no. 8 and 9) were

exclusively found in sample 6. Sequencing of re-amplified bands was not in all cases successful and out of 39 sequencing reactions, 19 sequences were obtained that could be used for sequence analysis (Table 1). Sequence analysis through Blast matching and phylogenetic tree construction revealed several *Rhizopogon* spp., a *Cortinarius* sp., a *Lactarius* sp. and an 'uncultured ectomycorrhiza' on the root tips as well as in the soil (Table 1). Sequences obtained from six bands did not satisfactorily match with anything in the database and remained unknown. Two of these unknowns were exclusively detected in the soil.

Different soil fractions

DNA was successfully extracted from three soil samples that had passed a 1 mm and 0.3 mm sieve and also from the separated 0.3 mm organic and sand fraction. DNA was successfully amplified with the ITS1F-ITS4B primer pair. DGGE analysis revealed that within each sample, all fractions showed a similar banding

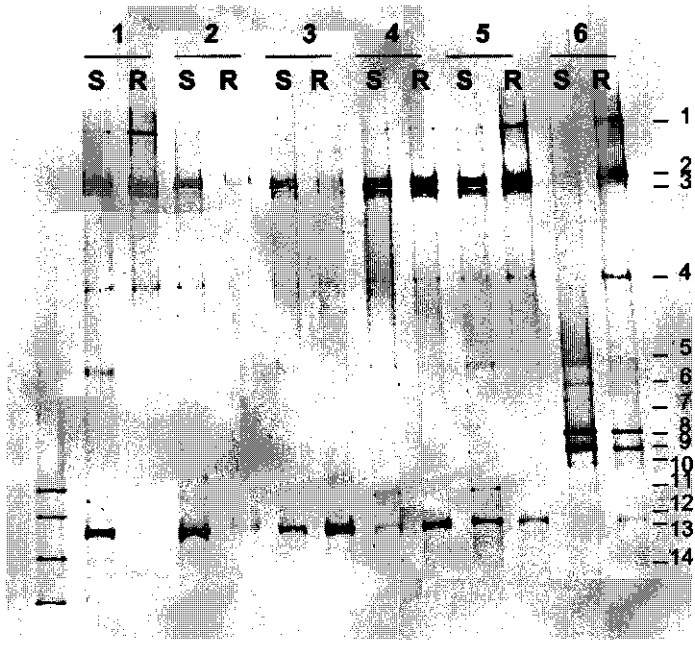

Figure 1. DGGE of amplified ITS-rDNA fragments representing the basidiomycete community in soil (S) and on root tips (R) of six samples (numbered 1 to 6). Bands numbered 1, 2, 4, 5, 8, 9 and 13 could be matched to database sequences and were identified as EM fungal taxa. Band no. 11 was identified as a non-mycorrhizal fungus.

pattern with varying band intensities between soil fractions (Fig. 2). In all three samples, the 1 mm organic fraction had the highest band intensity.

Conclusions

Fungal community analysis by DGGE showed that fungal diversity detected in soil was a little higher (14 bands) than the fungal diversity detected on root tips (11 bands). Out of a total of 14 bands that were distinguished, eight were assigned a taxon name, including seven taxa that were identified as EM fungal taxa. Sequence analysis revealed three different *Rhizopogon* sp., while the official species list based on fruitbody occurrence reports of one species only, namely *Rhizopogon luteolus* (14). Due to partial sequences present in the GenBank database, the *Rhizopogon* sp. could not be identified with higher resolution. One band was identified as *Cortinarius* sp. and this finding corresponds with the report of high numbers of *C.fusisporus*

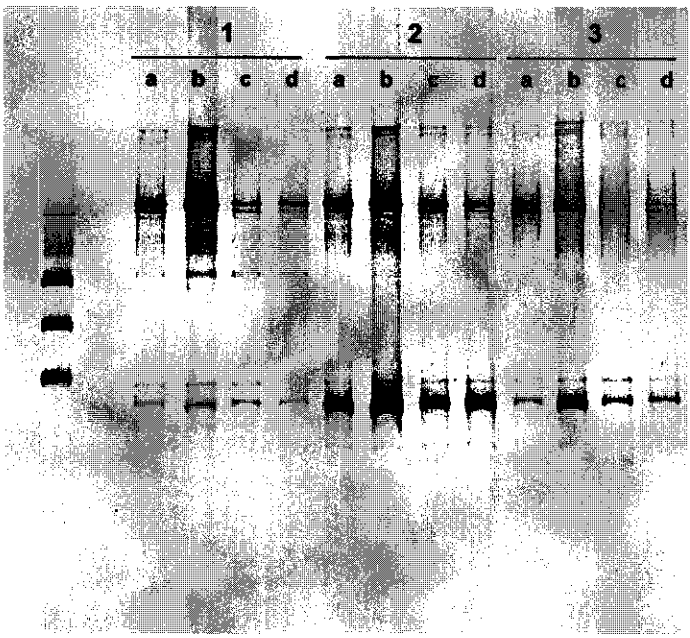

Figure 2. DGGE of amplified ITS-rDNA fragments representing the basidiomycete community in four different soil fractions of three soil samples (numbered 1 to 3). Lanes a) represent the 1 mm total soil fraction; lanes b) represent the 1 mm organic soil fraction; lanes c) represent the 1 mm sand fraction and lanes d) represent the 0.3 mm total soil fraction.

Table 1. Ectomycorrhizal fungal taxa identified from root tips and/or soil after DGGE analysis, named after their best Blast match, indicated in the column 'Blast match'.

Band	Identity	Blast match	Soil	Root	#bands sequenced[A]	#sequences[B]
1	*Rhizopogon sp. 1*	AF062939 AF115834	x	x	5	3
2	*Rhizopogon sp. 2*	AF115834	x	x	5	4
3	Unknown1		x	x	0	0
4	*Rhizopogon sp. 3*	AF062936	x	x	6	1
5	*Cortinarius sp.*	AF268894	x	x	4	1
6	Unknown2		x	x	1	0
7	Unknown3		x	x	0	0
8	Uncultured ecto 1	AF440673	x	x	2	2
9	Uncultured ecto 2	AF440673	x	x	2	2
10	Unknown4		x	x	0	0
11	*Cryptococcus sp.*	AF444321	x		5	3
12	Unknown5		x		0	0
13	*Lactarius sp.*	AF096989	x	x	9	3
14	Unknown6		x		0	0
				Total:	*39*	*19*

[A] Number of excised bands (from different lanes) that was sequenced.
[B] Number of obtained sequences that could be used for sequence analyses.

fruitbodies in 1998 (14). The finding of *Lactarius* sp. corresponds with the report of *L. hepaticus* fruitbodies and the 'uncultured ectomycorrhiza' that was found as best database match probably belongs to the Cantharellales, a taxon represented by *Cantharellus cibarius* at the study site. In general, all detected EM fungal taxa had been reported earlier as fruitbodies and were now, in all cases, detected in soil as well as on root tips. The only identified non-mycorrhizal fungus (*Cryptococcus sp.*) was exclusively found in the soil. Other studies on soil DNA extracts using fungal specific primers have found several non-mycorrhizal fungal species in soil (4,12) and also detected *Cryptococcus* sp. (12).

No fungal species were exclusively detected on root tips, indicating that the mycelia of most species occur in high enough quantities in soil to be detected by the molecular methods. When root tips are not analyzed, EM fungal species that do not form an extensive amount of extramatrical hyphae may remain undetected in the soil DNA approach. However, *Russula* sp. and *Lactarius* sp., both having smooth hyphal mantles and lacking large amounts of emanating hyphae (1), have been

detected in molecular studies on soil fungal diversity (4,7,12). The present study also demonstrates that *Lactarius* sp. can be detected with molecular methods in soil as well as on root tips (Table 1).

The identity of six fungal taxa in this study remained unknown. Two of these were exclusively found in soil, indicating that these might have been non-mycorrhizal fungal species, or EM fungal species that do not form, or did not form at the time of sampling, a symbiosis with tree seedlings. Except for differences in hyphal presence, the differences in detected fungal species in the soil and root tip samples could also have been caused by the fact that the soil samples contained other fungal material than just hyphae. The use of total DNA extracts to identify fungi does not distinguish between DNA extracted from hyphae, spores or sclerotia. Not many fungi belonging to the Basidiomycota form sclerotia and co-extraction of DNA from sclerotia should therefore be no problem when analyzing an EM fungal community. Spores however may persist on and in the soil, especially in autumn. The samples analyzed in this study originated from a sod-cut forest plot, where fruitbodies of more than 20 EM fungal species had been collected earlier (14). Fourteen basidiomycete species were detected in the soil DNA extracts from the topsoil of this plot. If extensive co-extraction of DNA from spores had occurred in this particular case, it would be reasonable to assume that more than 14 fungal species would have been detected. Although co-extraction of DNA originating from spores does not seem to be of importance in this study, it might have been if, for example, soil cores would have been taken in autumn when many species sporulate. Since spores are expected to be found in the upper layers of the soil, co-extraction of DNA from spores would be of no major concern when sampling deeper soil layers, a practice increasingly being applied (7,12).

Detection of single fungal species in soil through total soil DNA and PCR application has proven a useful method to rapidly and accurately identify fungal pathogens in soils and pre-symptomatic crops (5). In contrast to the detection of fungal pathogens, accurate detection of EM mycelium in soil involves total removal of (fragments of) EM root tips. Separation of root tips from soil samples is often done by sieving the samples (9,12) or by the removal of root tips with a pair of forceps under a dissecting microscope (4,7,12). In some cases complete hyphal strands and mycelium have been extracted from soil (17) (H. Wallander, pers. comm.).

To determine whether species richness depends on the soil fraction used, DNA was extracted from several soil fractions in this study. Small root tips passed the 2 mm sieve and further sieving (1 and 0.3 mm) was applied to separate these from the soil. The 1 and the 0.3 mm sand fraction gave equal DGGE banding patterns, indicating that these two sieve mesh sizes were equally effective in separating root

tips from sand. Still, successful removal of all root tips from a soil sample largely depends on the quality of the sample, as samples containing large organic matter fragments can not be sieved and should be checked by hand. Although sieving with a 1 mm sieve proved sufficient in this study, careful examination is needed in each individual case to guarantee that no root tips or mantle fragments remain behind in the soil being extracted. While remaining root tip fragments may not be a huge concern when using mycelial data for identification purposes, it could lead to a distorted view when using molecular data to quantify fungal presence in soil.

Analysis of the 1 mm and 0.3 mm soil fractions showed that equal DGGE banding patterns were obtained for the two sand fractions, but that the highest band intensities were obtained for the 1 mm organic fraction. This 1 mm organic fraction consisted of fragments of dry and dark organic matter, including fragments of shriveled EM root tips. Extracted DNA from a carefully rinsed selection of these shriveled, dry root tips could not be amplified. It has been shown before that DGGE band intensities can provide an indication of fungal abundance in the template DNA mixture (3) but in this case it is unlikely that DNA from the dead root tips caused high band intensities. Instead, providing a nutrient-rich substrate, the dead root tips might have been covered by fungal hyphae. It has been shown before that patches of organic matter are preferentially colonized by hyphae (2) and it can therefore be assumed that the intense DGGE band intensities from the organic matter fraction reflected DNA extracted from active mycelia.

The present study demonstrated that species diversities determined by the root tip or total soil DNA approach show high correspondence. After analyzing the root tip and soil fraction from the same soil samples with the same molecular methods, a correspondence between both methods of 11 out of 14 fungal taxa was obtained. In this case, the diversity obtained with the total soil DNA approach was even more comprehensive than the diversity obtained with the root tip analysis, although the two additional fungal taxa in the soil remained unidentified. In this study, total soil DNA analysis was therefore as robust as root tip analysis, indicating its potential for future diversity studies. Sampling of EM root tips is typically inadequate to get a true picture of species richness (10) and the use of soil DNA extracts can reduce several sample biases related to root tip sampling (see Chapter 7). The use of molecular identification methods to study EM hyphae in soil will become more common in the near future, as these techniques permit *in situ* testing of hypotheses on resource partitioning and niche differentiation of individual fungi (7). Methods based on soil DNA extracts might be a good alternative when analyzing species diversity of an EM fungal community, but it depends on the nature of the study whether the answers needed are best provided by a root tip or mycelial approach.

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7

General discussion

Contents

1. Introduction

In the following chapter I will summarize and discuss some ecological and methodological aspects regarding the study of ectomycorrhizal fungal communities by molecular techniques. After introducing the use of molecular methods and giving the background of this thesis (§ 1), the root tip and hyphal approach to fungal community studies will be put in perspective (§ 2). To explore the potential and possible pitfalls of the molecular techniques, I will present an overview of some methodological considerations regarding molecular community analyses (§ 3 and 4). Finally, the potential use of molecular methods to study particular fungal roles in ecosystems will be discussed (§ 5) and final conclusions are given in § 6.

1.2. Molecular tools and the taxonomy of microorganisms

The development of molecular techniques over the past 15 years has greatly enhanced our ability to study the composition of microbial communities in soil and other substrates. Detection and identification of a microbe now no longer depend on the culturability of the species. These methods and techniques have revolutionized prokaryote taxonomy and ecology, by discovering new clades of eubacteria and archaea with unique metabolic functions and by making abundantly clear that the cultivable species are a biased subset of total species richness.

Albeit at a somewhat slower pace, molecular methods and techniques are now about to change fungal taxonomy and ecology. Fungi are a highly diverse group of organisms, ranging from single cell to multicellular species. Some individual multicellular fungi are among the largest and heaviest organisms in the world (53). Estimates of fungal diversity have suggested that the group is much more species-rich than previously thought. Hawksworth (29) reviewed various estimates of global fungal diversity and concluded that a 1,5 million species estimate is likely. Of the 80000 to 100000 fungal species described so far, only 17% can be grown in culture (8), and culture methods therefore yield biased results of total fungal diversity. Only in a small number of cases species identification of the fungal mycelium is possible by morphological means. Traditionally, identification of fungi is based on fruit bodies, infection structures or resting spores. The development of molecular techniques has greatly improved our ability to identify fungi from a wide range of substrates, independent of their growth morphology or fruiting stage. Many fungal species will occur in the soil environment at some stage in their life cycles, but relatively few species have been identified from soil (8). Soil fungi are an important part of the soil microbial community and play fundamental roles in nutrient cycling within the soil. It is in particular within this complex multi-species environment that

molecular methods have greatly increased (and will continue to increase at a high rate) our ability to detect and identify individual fungal species.

1.3. Identification of 'rock-eating fungi'

The work presented in this thesis focused on ectomycorrhizal (EM) fungal species, a major group of mutualistic soil fungi. It was aimed at providing insight into the distribution of EM fungi in a podzol profile in relation to their possible functional role as weathering agents ('rock-eating mycorrhizal fungi'). In 1997, microscopic tunnel-like structures were observed in weatherable mineral grains, which were sometimes occupied by hyphae, and it was hypothesized that these tunnels were formed by hyphae of EM fungi (37). The tunneled minerals were found in the weathered, eluvial (E) horizon of a podzol profile. At the start of my project, preliminary work suggested that both mycorrhizal and saprotrophic fungi colonize rock substrate (51), but it was unknown which fungal species are present in the weathered E horizon. Therefore, the work presented in this thesis has been a direct result of asking a relatively simple question: 'Which species of EM fungi have their extramatrical hyphae in the weathered horizon of a podzol profile?'. Answering that question seemed a prerequisite for addressing the various issues of mineral weathering by EM fungi, as reviewed in Chapter 2.

Traditionally, EM fungal community studies have been based on identification of EM root tips (ectomycorrhizas) by morphological and / or molecular methods (Chapter 3). As the methods to identify EM fungi from soil were not sufficient to detect individual hyphae in soil, our simple research question initiated a wide range of analyses related to molecular identification of EM fungi and has finally brought us to where we stand now. At present, we can identify EM fungi in a soil profile by identifying their extramatrical mycelium (Chapter 4) and we can make relative quantifications of individual hyphae by the use of molecular techniques (Chapter 5). Yet, a comparison of the strengths and weaknesses of both identification methods is important. Does the new '*mycelial view*' (33) yield the same fungal diversity picture as the old '*root tip view*'? The answer to this question must necessarily remain provisional. Less than two years ago Horton and Bruns (33) reviewed the molecular revolution in EM ecology based on root tip studies and highlighted complications of molecular quantification of mycelium. The work of Dickie *et al.* (17), Guidot *et al.* (27) and my thesis are a clear indication of how rapidly the molecular field has been progressing and highlight some methodological considerations (discussed in § 3 and 4) and questions on functional diversity (discussed in § 5): Compared to root tips, how well do mycelia represent the fungal community below ground? What are possible sources of bias inherent in the mycelial view? How should (further)

validation of the mycelial approach proceed? Can we answer ecological questions when detecting not the organism itself, but its DNA? Is all DNA from a fungal community extracted and detected in representative amounts? How well does detected DNA represent a living fungal community? Should we focus on RNA instead of DNA to assess fungal activity? Should we detect specific functional genes if we are interested in particular functional roles that fungi can have in ecosystems? For answers to these questions we need to consider several aspects related to EM root tip and mycelial diversity and evaluate the methodologies currently used to detect EM fungal species diversity.

2. Species diversity of EM fungal communities

2.1. A root tip view

EM root tips with a hyphal mantle and Hartig net represent the component of the EM symbiosis where nutrients and photosynthates are exchanged between the two symbionts. The hyphal mantle has furthermore a storage function, which enables the symbiosis to respond to pulses of nutrient release, a highly relevant factor in seasonal climates. The high concentration of fungal hyphae on the roots allows identification of the fungal symbiont and EM root tips are therefore used to study EM fungal species diversity in soil. The sampling of EM root tips is in general however inadequate to get a complete picture of species richness (33). This problem is both caused by the spatial and temporal patterns of EM root tip occurrence, and by the sampling methodologies used. EM fungal communities are species-rich, dynamic and patchily distributed at a fine scale below ground (33). Varying root tip densities and the fact that most EM fungal communities consist of a few common and a large number of rare species complicate the assessment of EM fungal diversity (56). Identification of EM fungal species on root tips is often achieved by a combination of morphological and molecular methods. Morphotype sorting often precedes molecular identification and a selection of root tips from each morphotype group is molecularly analyzed to identify the fungal species. Sometimes specific EM fungal morphotypes are regarded characteristic enough to be recognized with certainty and molecular analyses are not performed. Dahlberg *et al.* (16) claimed that mycorrhizas of *Piloderma croceum* and *Cenococcum geophilum* were characteristic enough to be recognized with certainty. However, within the well recognizable *Piloderma* morphotype, cryptic species seem to exist (Chapter 3 and 4) and it has also been shown that different fungal species have been included in the *Cenococcum* morphotype (Chapter 3, (30)). Accurate morphotype sorting is crucial as, in general,

only 2 - 5% of the total number of sampled root tips is used for molecular identification (38,39). Depending on morphological characteristics, fungal species might become overrepresented or underrepresented in samples and morphotype grouping may therefore induce sample bias in EM fungal diversity studies.

2.2. A mycelial view

The extramatrical mycelium represents the part of the EM symbiosis that is actively involved in mobilization, uptake and translocation of nutrients and presents an ecologically significant component of the EM symbiosis. The fungal biomass of EM mycelia and EM root tips has been estimated at 700 - 900 kg ha^{-1} in the humus layer of a temperate forest soil and it has been suggested that approximately 80% of this biomass is derived from EM extramatrical mycelia (60). EM fungal species differ in amount and organization of extramatrical hyphae and these differences in exploration and exploitation structures may represent predictive features relevant to the ecological classification of EM fungi (2,27).

Where the spatial distribution of mycelia in soil reveals substrate selectivity of individual EM fungi, root tip numbers will reflect numbers of nutrient exchange sites between the symbionts. A root tip and mycelial view on fungal species diversity might therefore represent different aspects of the EM fungal function. The extent to which both methods are complementary and how both views are affected by the methodological approaches taken, still needs to be studied in detail. The mycelial approach can make the identification of fungal species from soil DNA extracts objective and reproducible, as sample bias induced by root tip sorting does not occur when analyzing the soil mycelium. Still, each physical, chemical and biological step involved in the molecular analysis of an environment is a source of bias, which will lead to a partial and incomplete view of the real world (57).

To fully explore the potential of the soil DNA based techniques I will consider possible sources of bias encountered in this thesis.

3. Methodological aspects related to total soil DNA analysis

3.1. Total DNA extraction

For molecular assessment of fungal diversity in soil, DNA from all fungal species present in the soil needs to be extracted and amplified in representative amounts. Representative extraction requires that cells from all fungi are lysed with equal efficiency. All fungi have chitin-glucan cell walls, but cell wall structure and chemical

composition can vary according to the taxonomic fungal group or imposed stress (34). Although recovery rates are difficult to determine (57), now that commercial soil DNA extraction kits are available and cells are disrupted by strong mechanical bead-beating methods, we can assume that most hyphal cells will be lysed efficiently (13).

Lysis efficiency of cells and DNA recovery rates will also be affected by the organic matter and clay content of the soil (3,23,57). In our study, different amounts of total soil DNA were extracted from four horizons with different mineralogical and chemical compositions (Chapter 4). The extracted amounts were highest in the organic layer and decreased with depth. This extracted DNA reflects total environmental DNA, including DNA of prokaryotes, fungi and micro- and mesofauna of which biomass will decrease with depth in a soil profile. PLFA analysis has shown that bacterial and fungal biomass decreases with depth in a podzol profile (22) and total incidence of fungi was found to be greatest in the litter layer and to decrease with depth in another stratified profile (17). EM root tip numbers (Chapter 3) were found to decrease with depth in the podzol profile studied and furthermore, the biomass of soil fauna may be orders of magnitudes smaller than that of bacteria and fungi combined (6). These findings demonstrate that the decreasing amounts of total soil DNA reflect the expected patterns of fungal and bacterial biomass in a podzol profile. The differences in obtained DNA amounts were not an extraction artefact caused by the differences in chemical composition of the horizons, indicating that the DNA extraction protocol was technically robust. In each individual case however, the extraction protocol needs to be adjusted to the soil type under investigation, to reduce DNA losses (23).

3.2. DNA amplification

PCR amplification of the extracted DNA is the next step involved in the molecular analysis of a fungal community. Several problems can arise when amplifying DNA from a complicated matrix like soil: 1) PCR inhibition may occur due to co-extracted contaminants, 2) differential amplification may occur or 3) artificial PCR products can be formed (57).

- To minimize PCR inhibition, DNA purification protocols have to be adjusted to the soil type used (23). In our study, PCR amplification was possible after purification and without strong dilution of the template DNA or the use of additives to reduce inhibition effects of contaminants (Chapter 4).
- Differential amplification of DNA fragments might occur with variable length or GC content of the target region. Target length can vary between and sometimes within species, depending on the target region used. Differences in GC content

determine the melting temperature to separate DNA strands and may lead to differential amplification. Differences in GC content on the genome of the Ascomycota (38-54%) compared to the Basidiomycota (50-63%) (26) should be taken into account when using universal primers to assess fungal species diversity. Effects of bias induced by differences in GC content for lower taxonomic groups is very difficult, if not impossible, to assess in a fungal community with unknown species composition.

- The formation of artificial PCR products like chimeras can be a problem in PCR analysis, as it can suggest the presence of organisms that do not actually exist in the sample investigated (57). Chimeric molecules may be formed when an ITS1 and ITS2 region that do not belong together join and form an artificial molecule. In our study (Chapter 4) we found no evidence for the presence of such artificial PCR products after verifying whether the ITS1 and ITS2 regions would separately align with the same organism.

The exponential nature of the PCR amplification process and a lack of preferential amplification can cause relatively small amounts of target DNA in the template to remain below the molecular detection threshold. Specific primers can detect tiny quantities of fungal material, as was shown for the detection of fungal pathogens in soils (7,15) and of EM fungi in a potting mixture (Chapter 5). With the use of a more general primer set, however, in a multiple-competitive PCR approach, such tiny amounts of specific DNA can remain undetected. The extent of this problem could be investigated by adding small amounts of DNA of a foreign fungal species to a soil mixture, and determine the amplification threshold. In general, creating artificial DNA template mixtures including DNA from different fungal species could establish the significance of DNA detection thresholds.

3.3. Primer choice and species richness analysis

Representative amplification of DNA from all fungal species from a fungal community requires primers that amplify all members of the fungal community without bias, while excluding the co-amplification of other eukaryote sequences (4). Although it was recently suggested that primer bias may be less significant than previously thought, there are contrasting reports on the specificity of primers amplifying the 18S-rDNA (4). The 18S-rDNA region in fungi is highly conserved and the use of primers that amplify the relatively variable ITS regions on the rDNA results in higher taxonomic resolution.

In our study we chose to use the ITS primer pair ITS1F and ITS4B (25) with high specificity for fungi belonging to the Basidiomycota, as it allowed us to

compare ITS sequences obtained from our clone library (Chapter 4), with ITS sequences obtained from root tips (Chapter 3). A drawback of choosing a primer pair specific for fungi from the Basidiomycota is that fungi from the other major phyla (Ascomycota, Zygomycota, Glomomycota) remain undetected. This could be a problem for EM fungal studies, as several clades within the Ascomycota also contain EM-forming species. The use of ITS primers would in theory allow identification of fungi to species level when ITS fragments are sequenced, but this may in practice be problematical due to a scarcity of available ITS sequences in databases (Chapter 4). However, numbers of submitted fungal sequences in public databases increase daily. When the manuscript for Chapter 4 was first submitted, the sequence of OTU 27 could not be matched satisfactorily – but when the paper was revised and resubmitted 6 months later, meanwhile a high (99.3%) homology with a sequence of *Hygrophorus olivaceoalbus* was obtained. Filling the database with further fungal sequences therefore remains a top priority, a point also made by Horton and Bruns (33) and by Anderson *et al.* (4).

Other identification methods that do not require cloning and sequencing of fungal sequences can also detect fungal species richness in samples. Although these methods enable identification at a lower level of resolution, they require less financial input than the cloning and sequencing procedure. When processing a large number of samples or when a high level of taxonomic accuracy is not needed, techniques like ARISA (50), RFLP (1,13,36), T-RFLP (17,40,44), DGGE (49) (Chapter 5 and 6) or TGGE (52) can be very useful to describe fungal species richness.

In conclusion, it is clear from the above overview, that the molecular analysis of an EM fungal community based on soil DNA extracts may include methodological bias. Some of these biases will be related to the environment studied, others to the practical methods being applied. A proper assessment of these methodological biases is needed before we can start reducing them and in the overview above I have given some suggestions on where to start. In addition, to assess laboratory-induced biases and to enable comparisons of molecular fungal diversity estimates by different laboratories, reference samples should be made, sent to and analyzed in a variety of laboratories worldwide. The analysis of reference samples could be an essential (first) step to validate the accuracy of fungal species diversity estimates.

4. Application of molecular tools to detect the active fungal community

4.1. Identification of DNA to detect living fungi

While root tips might be selected for their living status, the major limitation of molecular soil DNA based techniques for EM community studies is the lack of discrimination between living and dead material, or between active and dormant forms (8). Root tip turnover is estimated at 2 to 4 months and living root tips are by appearance relatively easily distinguished from dead and decaying ones (54). These fresh-looking root tips can be sorted and selected for molecular analyses, assuring that the fungal diversity analysis is based on living material.

Total soil DNA extracts are expected to contain all DNA from the sample, including DNA from living and perhaps dead organisms and spores. Considering the high microbial activity in soils, dead fungal material will degrade fast, except perhaps for its more resistant cell-wall compounds like chitin. The ability to utilize DNA as a source of phosphorus and carbon is widespread among saprotrophic fungi, and many EM fungi also show DNA degrading activities (35). In fact, successful extraction and amplification of fungal DNA from root tips largely depends on the freshness of the root tip sample, as the DNA in the samples degrades rapidly (33). In further support of that claim our results show that a collection of shriveled, dry root tips could hardly be amplified (Chapter 6). To establish how quick fungal DNA degrades in soil, it would be useful to analyse soil samples to which tissue from a foreign fungal species is added, and test for successful amplification of that specific DNA over time. Whether DNA from fungal structures like spores is co-extracted would in the first place depend on whether large amounts of spores are present in the sample, which is not in all cases very likely (17). When sampling deeper soil layers, a high occurrence of spores is not to be expected, as dispersal through soil is limited compared to dispersal through the air and some soil fauna feed on fungal spores (21). The turnover of mycelia in soil has been estimated at weeks rather than months (55) and it therefore seems reasonable to expect that the major part of extracted DNA originates from living tissues.

4.2. Measures of DNA to quantify fungal biomass

Generally, biomarkers like ergosterol (58,59) or phospholipid fatty acids (PLFAs) (Chapter 5, (47)) are used to detect and quantify fungal biomass. Ergosterol and phospholipids are correlated to active membrane area and are thought to reflect the occurrence and abundance of living organisms, despite the fact that their amounts

per unit biomass are not constant for all organisms at all physiological activities (47). The largest drawback of these methods, however, is that they do not allow for quantification of individual fungal species.

Contrary to the use of ergosterol or PLFAs, the use of a gene as biomarker could permit detection of individual fungal species in soil. For identification purposes the variable ITS region of the ribosomal DNA gene cluster is often used as it allows for identification of fungal species at high taxonomic resolution. Genes occur on the genome as single-copy genes or as multi-copy genes and the ribosomal genes occur in large clusters spread over the genome, their numbers varying from 60 to 120, depending on the fungal species considered (27). This variation in ITS copy number constrains interpretation of ITS gene quantities as a measure for fungal biomass. Single-copy genes may offer a solution to that, but the detection limit of such genes is then a much more critical issue. Besides variable gene copy numbers, the nuclear genome size can also vary widely between fungal species. For fungi belonging to the Basidiomycota, sizes ranging from 8 to 46-Mb have been reported (43). A first report on the genome size of an EM fungal species indicates that the *Paxillus involutus* genome is 23-Mb, including 11% repetitive DNA that probably to a large extent represents rDNA sequences (43). With species-specific genome sizes, unknown copy numbers and variable fungal cell lengths and cell volumes it is not possible to obtain a universal conversion factor to convert DNA quantities to fungal biomass (27). In the absence of such universal conversion factor, it may be sufficient to express the quantification results in amounts of DNA per gram of soil when comparing spatial or temporal variations of single species biomass (Chapter 5, (27)).

4.3. Methods to quantify DNA

Several molecular methods can be used to measure relative abundance of DNA. In Chapter 4 a cloning procedure was applied and the combined results from Chapters 4 and 5 provide strong circumstantial evidence that clone numbers can be treated as abundance data, as was also shown by Chen *et al.* (13). Another molecular approach to EM fungal community analysis was introduced by Dickie *et al.* (17), whose data suggest that the height of the T-RFLP peaks can be treated as abundance data. Band intensities obtained by TGGE or DGGE can also be used to quantify differences in species abundance if an internal quantitative standard is used, as was shown for PCR-DGGE analyses of bacterial mixtures (9). The data from Chapter 5 indicate that DGGE band intensities from an EM fungal mixture show a pattern consistent with clone numbers and Quantitative PCR results. Guidot *et al.* (27) described an elegant cPCR method to quantify an EM fungal species in soil. By

including a known amount of a plasmid-cloned competitor sequence in the PCR mix, quantities of 3.10^{-4} µg µl^{-1} of genomic DNA could be detected. Besides the design of specific primers, this method also needs the design of a competitor. This competitor should be homologous to the genomic sequence to be quantified, except for an insertion or a deletion, so that both PCR-sequences can easily be distinguished in gel electrophoresis (27). This makes the cPCR method only applicable for the quantification of individual species in a sample. An alternative to cPCR is Quantitative PCR (or real-time PCR) which calculates the starting copy numbers of target genes in the template mixture through on-line monitoring of the exponential phase of the amplification process. Quantitative PCR has successfully been used to quantify the presence of fungi in soils and other environments (Chapter 5, (7,15,28)). In our study we could detect $1,2.10^{-4}$ µg µl^{-1} target gene DNA (Chapter 5) and detection of 2.10^{-11} µg µl^{-1} target gene DNA has been reported (7), indicating the high sensitivity of the Quantitative PCR method. Like cPCR, the Quantitative PCR method quantifies DNA from individual species in a sample and needs the design of specific primers and probes. Once the method is running, many samples can be analyzed at once within one hour and there is no need to run electrophoresis gels, minimizing sample handling. This advantage is however traded off against the high costs of the specialized equipment and chemicals needed for Quantitative PCR analysis, making it an unavailable tool for many laboratories at present.

The recent methodological developments and results obtained show that the issue of molecular quantification is currently being addressed by various means. As methods in this area develop very rapidly, an acceptable solution for several of the issues mentioned above may be available soon. For the time being, the uncertainties regarding species-dependence of the DNA quantification methods might be balanced by the advantage of quantifying individual mycelia.
Finally, the uncertainties regarding DNA quantification might not be much different from the uncertainties regarding species and activity-dependence of the often-used ergosterol or PLFA quantification (Chapter 5).

4.4. Extraction of RNA to detect fungal activity

Detection of a fungal species by means of its DNA indicates its presence and, possibly, biomass. DNA detection does not necessarily indicate that a fungal species is physiologically active at the moment of detection, as inactive hyphae or spores will also contain DNA. To detect activity, RNA analysis would be a solution. Extraction of RNA from soil however is complicated, as RNAs are highly susceptible to degradation by RNases during the extraction procedure (57). For

bacteria, the amount of ribosomal RNA per cell is roughly proportional to their metabolic activity and RNA extracted from ribosomes isolated from soil has been used to detect active bacteria (18). For fungi, however, this approach has not yet been reported. A loss of approximately half of all ribosomes during extraction from soil has been reported earlier for bacteria (19). In such cases the advantage of using RNA to detect metabolically active cells is traded off against the fact that the data cannot be used quantitatively.

5. Application of molecular tools to detect functional diversity

5.1. Detection of functional genes

DNA analysis of a fungal community reveals the potential activity of a fungal community when specific functional genes are detected. This was shown in a study by Luis *et al.* (45), where in a forest soil the occurrence of laccase genes was used to detect fungi with oxidative potential. Although the organisms themselves were not detected, the use of basidiomycete specific primers assured that the genes detected indeed originated from fungi belonging to the Basidiomycota.

Particularly in the case of EM fungi, the step from demonstrating genes to demonstrating the activity of genes is large, because it cannot be assumed that genes are always functional. EM fungi form a polyphyletic group of fungi, and it has been suggested by Hibbett *et al.* (31) that the symbiotic behavior was repeatedly gained and lost from saprotrophic ancestors. In a study that compared 48 species of EM fungi, more than 50% of the tested species contained peroxidase genes, indicating retained saprotrophic potential (14). The possibility for lignin degradation enables the EM fungus to use nitrogen and phosphorus from lignin-rich organic materials (63). Although it seems unlikely that these genes are functionally expressed when an EM fungus grows in symbiosis with a tree, differential gene expression within basidiomycete mycelia has been found. In the absence of morphogenetic differences, physiological heterogeneity may occur in time and place within individual hyphae (11). For hexose-regulated genes differential expression has been shown for hyphal mantle material and hyphal material from the Hartig net (46). It would be interesting to see whether the expression of peroxidase genes will similarly be different for hyphae that form the mantle and Hartig net and extramatrical hyphae that explore the forest floor for organic nutrients.

Not all fungal genes are as well described and characterized as some of the laccase, peroxidase or hexose-regulated genes and therefore, not all potential functions of fungal species can be detected in a similar way. In addition, not all

functions that fungi perform are directly regulated by specific genes and some functional roles might be the result of a cascade of physiological events involving many different genes. Besides the enzymes that enable many EM fungal species to utilize organic nitrogen and phosphorus forms, many EM fungal species produce Low Molecular Weight (LMW) organic acids that enable them to dissolve solid mineral substrates (Chapter 2). LMW organic acids like oxalic acid are commonly produced by many different fungal species. Oxalate is a by-product of the hydrolysis of oxaloacetate originating from the citric acid and glyoxylate cycles and it is therefore not to be expected that a specific oxalate gene exist on the fungal genome. Analyzing the expression of genes related to organic acid metabolism, as is done for plants, could however be an important step ahead, especially if the expression of these genes in specific tissues and in response to nutrient deficiency will be examined. Regulation of oxalate production is complex and different carbon sources, nitrogen sources (ammonium or nitrate) and phosphorus availability affect oxalate and citrate production in fungi. Up-regulation of the citric acid cycle furthermore occurs with growth-limiting concentrations of manganese, zinc and iron (24), indicating that the production of oxalate can only to some extent be related to nutrient availability. At present, when trying to identify fungi in soil that actively weather soil minerals, a functional gene approach would be difficult, if not impossible.

5.2. Mineral weathering activities of EM fungi

My project started with the hypothesis that tunnel-like structures found in weatherable minerals (feldspars) in the E-horizon of boreal podzols are produced by EM fungi. A functional gene approach to detect mineral weathering activities of specific fungal species in the E horizon could verify this hypothesis, but is unachievable, at least for the time being. Instead, now that fungi from strongly weathered soil horizons can be identified (Chapter 3 and 4), it would be more useful to see whether they can be isolated, grown in culture and used in weathering experiments. The work presented in Chapter 4 has revealed a number of fungal species that occur in the top part of the weathered E horizon (E1), where the highest numbers of mineral tunnels were detected (32). If we combine these findings with the findings from Chapter 5 showing that fungal clones from soil DNA extracts can give a measure of relative biomass quantity, we can select two fungal species that have a high occurrence in this particular part of the E horizon. Many clones resembling *Russula decolorans* were found in the organic as well as the E1 horizon, while most clones resembling *Cortinarius collinitus* were found in the E1 horizon. So far, most *in situ* studies of the mineral weathering capacities of EM fungi

have been performed with a relatively small selection of fungal species, often including *Paxillus* sp. or *Suillus* sp. (61,62). Criteria for species selection have often, and most likely in this case too, not been based on putative importance in that specific function, but on the ease with which experiments can be executed. Fitter (20) has eloquently expressed his feelings that ease of experimentation favors weedy species, thereby giving a limited or even distorted view of functional diversity. Earlier *in vitro* studies have shown that several EM fungal species can dissolve mineral nutrients by excretion of soluble fungal exudates (eg. (42,48)). Now that EM fungi have been identified in the mineral horizons of forest soil, it would be interesting to see how these fungi would perform in mineral weathering experiments. Although organic acid exudation of fungal species determined in experimental studies under optimal growth conditions does not reflect the actual situation in the field, it will reflect the potential capacity of fungal species to weather minerals.

Besides the focus on LMW organic acid production, the potential production of extracellular polymeric substances by fungi could be another significant weathering factor to focus on. Mucogenic substances surround hyphae that penetrate cracks in stones and rock and increase fungal mediated weathering (A. Gorbushina, pers.com.; E. Burford, pers.com.). The mucilage may possibly contain extracellular polysaccharides, which influence the precipitation of calcium oxalate (12) and could prevent crystal formation in the observed tunnels in feldspar grains.

The work presented in this thesis has not taken into account the aspects of mineral weathering induced by bacteria. It remains difficult to a priori discount the possibility that bacteria associated with the mycelium of EM fungi are also involved in, or even primarily responsible for, the mineral weathering activity. Bacteria colonize mineral substrates, excrete extracellular polysaccharides and LMW organic acids and etch mineral surfaces (5) and it has been found that specific bacterial communities may co-occur with EM fungi in mineral soil (30). Molecular studies based on total DNA extracts can be very useful in this research field as they allow for detection of the EM fungi as well as co-occurring microorganisms.

5.3. Vertical distribution of EM fungal species

The contents of this thesis focused on the occurrence of EM fungi in different podzol horizons, with emphasis on the weathered E horizon. The dominating general assumption that most EM root tips are found in the organic layer of the forest floor (10) has caused that most EM diversity studies have only sampled the top 10 cm of the soil profile. A recent study and this thesis have shown that a significant part of the fungal community is found in the deeper soil layers and that

Table 1. Identified and unidentified OTUs and root tips from four horizons of a podzol profile, named after their closest Blast match[a]. 'Hyphae' indicates ectomycorrhizal (EM) and unidentified fungal species detected by the total soil DNA approach (OTUs), while 'Root tips' indicates EM and unidentified fungal species detected by root tip identification (root tips). Data from Chapter 3 and 4, adapted.

Hyphae	Soil horizons				Root tips	Soil horizons			
	O	E1	E2	B		O	E1	E2	B
Unknown	x				*Inocybe sp.*	x			
Unknown	x				*P.byssinum*	x			
Cortinarius acutus	x	x	x		*T.submollis*	x	x		
Piloderma fallax	x	x	x		***P.fallax***	x	x	x	
Russula decolorans	x	x	x		***R.decolorans***	x	x	x	
Piloderma fallax	x	x	x		*Dermocybe spp.*	x	x	x	
Cortinarius collinitus		x	x		***Hygrophorus sp.***	x	x	x	
Dermocybe crocea		x	x		*P.bicolorata*	x	x	x	x
Laccaria laccata		x	x		*Tylospora spp.*	x	x	x	x
H.olivaceoalbus		x	x		*P.reticulatum*	x	x	x	x
Piloderma fallax		x	x	x	*P.atheleum*	x	x		x
Cortinarius umbilicatus		x	x	x	*Cortinarius spp.*	x		x	x
Cortinarius acutus			x		*Tomentellopsis sp.*		x	x	
Lactarius deliciosus			x		*Lacterius utilis*			x	x
Tylospora asterophora			x		*S.luteus*			x	x
Piloderma fallax			x	x	***Piloderma sp. 2***			x	x
Unknown			x	x	*Piloderma sp. 1*				x
Unknown			x	x	*Piloderma sp. 3*				x
Hebeloma incarnatulum				x	***Hebeloma sp.***				x
Unknown				x	*Unidentified no.15*				x
Suillus sp.				x					
unknown				x					

[a] Data represent pooled findings for three pits.

[b] Boldface indicates species identified as hyphae and root tip with the same vertical pattern.

its composition may be different from the community found in the topsoil (Chapter 3 and 4, (17)). Results obtained from root tips as well as from hyphae demonstrate that a vertical distribution pattern exists for EM fungal species in a podzol profile (Table 1). The data in Table 1 have been adapted from Chapter 3 and 4 and reveal that some species are found in horizons at the bottom of the pit, while others are found more close to, or in the organic layer. For some individual species (boldface, Table 1), the same vertical pattern was revealed by both the root tip and mycelial data. The finding indicates that root tips and mycelium of some fungal species may

co-occur in the same soil horizon(s). If the root tips had been extracted from the soil that was used for the total DNA analysis, this correspondence would probably have been better, as was shown in Chapter 6. The root tip as well as the mycelial data show that the occurrence of most species is not restricted to one horizon but is mostly spread over two or more adjoining horizons. For soil mycelia, this rather broad species distribution over adjoining horizons in a stratified soil profile has also been found by Dickie *et al.* (17). The fact that occurrence of mycelia of single fungal species seems not to be restricted by horizon borders raises interesting questions on ecological factors that define niche differentiation (41). In Chapter 3, the vertical distribution of fungal species could not be related to the chemical composition of the soil horizons. The root tip sampling effort might not have been extensive enough to show consistent patterns in relation to the soil chemistry and explanations for spatial variation on a vertical scale remain in the realm of speculation. Yet, niche differentiation by horizontal as well as vertical spatial partitioning of the forest floor and functional differentiation related to the vertical spatial axis may contribute to the high species richness of EM fungal communities (17).

Conclusions

The work presented demonstrates that the application of molecular techniques facilitates identification and relative quantification of individual fungi in soil. The use of molecular techniques as described in this thesis will probably result in an increasing number of fungal species being detected in soil and will thereby largely influence fungal taxonomy.

Yet, adding new species names to fungal species lists is arguably not the most promising aspect of the new molecular potential. For EM fungal community studies for instance, the use of these techniques will probably not significantly change our concept of the high species richness of EM fungal communities, since comparisons in this thesis between the root tip view and mycelial view yielded more or less the same species richness (but not necessarily an equal species composition). Yet for EM fungal ecologists, having spent years of looking at EM root tips, these techniques finally enable the detection of the extramatrical mycelium of individual EM fungal species. The extramatrical mycelium is the part of the EM symbiosis that actively takes up nutrients and the differences in amount and organization of the extramatrical mycelium produced by individual species may reflect the different ecological roles that species have in ecosystems. The possibility of detecting EM mycelia in soil now facilitates detailed *in situ* studies on substrate specificity, niche

partitioning, succession and longevity of hyphae. Furthermore, the identification of mycelia of specific fungal species colonizing certain substrates in the field will possibly help to select for the most ecologically relevant fungal species for specific experimental work.

In the near future, the information on the mycelial distribution of species might complement the information obtained from root tip distributions. Whereas the identification of root tips provides information on the number of fungus - tree exchange sites, the identification of mycelium permits detection of the exploration potential of a particular fungus. Considering the methodological constraints of both the root tip and the mycelial identification method, the nature of the research question will largely determine the value of both methods. For detection of *EM species diversity*, it can be argued that root tip identification, although prone to morphotype bias, can effectively identify dominant fungal species in a forest plot and relatively easily detect scarce, but characteristic fungal species as well. When hyphae are identified these scarce types may be underrepresented in the species list, yet morphotype bias will not influence the overall diversity outcome. If *EM functional diversity* is studied, the spatial distribution of mycelium of different fungal species will provide insight on potential roles of individual fungi. In such a case, the distribution and identity of the mycelium will provide more detailed functional information than the distribution of root tips.

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Summary / Samenvatting

Summary

The extramatrical mycelium of ectomycorrhizal (EM) fungi represents a significant component of the EM symbiosis, as the mycelium is primarily involved in the uptake of water and nutrients from the soil. The extramatrical mycelium may also actively mobilize nutrients from organic and mineral sources through excretion of enzymes and organic acids respectively. The capacities of EM fungi to actively dissolve minerals got new attention in 1998, when microscopic tunnel-like structures were found in weatherable mineral grains. It was hypothesized that these were formed by the hyphae of EM fungal species and this hypothesis formed the start of the work presented in this thesis. In Chapter 2 of this thesis, an overview is given of the mineral weathering capacities of EM fungi. Past research shows that free-living and symbiotic fungi can weather minerals, but there are no documented observations on EM fungi that form tunnels in minerals. Despite circumstantial evidence, it remains unknown whether EM fungi form tunnels in weatherable minerals. The relative contribution of the EM fungal weathering potential to the total forest nutrient budget still needs to be quantified, in order to determine the ecological relevance of EM fungal weathering. Yet, no matter whether the EM fungi obtain their nutrients from the inside of the mineral grains through tunnel formation, or from the outside through surface weathering, it is clear from past research that EM fungi can actively dissolve minerals.

Tunneled minerals had only been found in the weathered E horizon of podzols, which are typical soil types for boreal forests. It was also known that EM fungal communities in boreal forests are highly species-rich, but it was unknown whether species composition and diversity would differ between the organic and mineral horizons of podzols. Many different EM fungal species can excrete organic acids, but it was unknown which fungal species weather minerals *in situ*.

Traditionally, EM fungi are identified from soil through morphological and molecular identification of EM root tips, and this approach was used in Chapter 3 to identify fungal species in the weathered E horizon of a podzol profile. EM root tips were sampled from four horizons of a podzol profile, in order to determine whether the species composition of the strongly weathered E horizon would differ from the other horizons. Root tips were sorted into morphotypes and the EM fungi were identified by sequencing of the ITS-rDNA region. Using correspondence analysis, a significant relationship was found between EM fungal species composition and soil horizon. *Tomentellopsis submollis,* three *Piloderma* species and *Dermocybe* spp. were found predominantly in the upper horizons while other species within *Cortinarius* spp. and *Suillus luteus* were associated with the lower mineral horizons. Half of the number of identified taxa were exclusively found in the

mineral horizons, highlighting the need to include the mineral soil when assessing EM fungal species richness of a study site. No fungal taxa were exclusively identified in the E horizon. In Chapter 3, the vertical distribution of EM fungal taxa over the different podzol horizons was obtained by root tip sampling, whereas the organic acid excretion of fungi takes place at the hyphal tips. In addition, the high species richness of EM fungal communities, the fact that EM root tips often occur in clusters and morphotyping difficulties complicate root tip sampling.

In Chapter 4 therefore, the distribution of EM mycelium was studied in a podzol profile, making use of molecular identification techniques based on total soil DNA extracts. Similar to the root tip sampling form Chapter 3, soil samples were taken at different depths from four horizons of a podzol profile. A primer pair with high specificity for fungi belonging to the Basidiomycota was used to amplify fungal ITS sequences from the total DNA extracts. Amplified basidiomycete DNA was cloned and sequenced and a selection of the obtained clones was analyzed phylogenetically. Based on sequence similarity, the fungal clone sequences were sorted into 25 different fungal groups or operational taxonomic units (OTUs). Out of 25 basidiomycete OTUs, 7 OTUs showed a high nucleotide homology ($\geq$ 99%) with known EM fungal sequences and 16 were exclusively found in the mineral soil. The taxonomic position of 6 OTUs remained unclear. The obtained OTU sequences were compared to the sequences from the EM root tips analyzed in Chapter 3. Of the 25 OTUs, 10 OTUs had a $\geq$ 98% sequence similarity with specific EM root tip sequences, which often were obtained from the same soil horizon. Besides the fact that this finding demonstrates that the total soil DNA approach is technically robust, it also shows that the root tips and mycelium of one fungal species co-occur in the same soil horizon.

Root tips are in general being seen as entities that can be counted, and root tip numbers are used to indicate the biomass quantity and ecological importance of fungal species in ecosystems. The quantitative analysis of the soil mycelium determined by molecular methods is less straightforward (Chapter 5 and 7). The molecular techniques enable identification of fungal species directly from soil, but it is unknown whether DNA quantities do relate to mycelial biomass quantities. Mycelial biomass estimates in soils are usually obtained by measuring total hyphal length or by measuring the amount of fungal specific biomarkers like ergosterol and phospholipid fatty acids (PLFAs). These methods determine the biomass of the fungal community as a whole and do not allow species-specific identification.

To investigate whether the biomass of individual fungal species in soil can be determined with molecular methods, three molecular techniques were compared with conventional quantification techniques in Chapter 5. The growth of extramatrical mycelium of two EM fungal species (*Suillus bovinus* and *Paxillus*

involutus) in soil was monitored by denaturing gel gradient electrophoresis (DGGE), a cloning technique and Real-Time Quantitative PCR and compared to results obtained with hyphal length determination and PLFA analysis. The molecular methods enabled identification and relative quantification of both species separately in a more-species environment and showed consistent results. As it was not possible to directly relate quantities of DNA to fungal biomass, the quantity of target DNA per gram soil was used to compare soil samples. The work presented in Chapter 5 demonstrated that molecular methods provide tools to relatively quantify fungal presence in soil. Combining the results from Chapter 4 and 5 (as was done in Chapter 7) indicated that the clone numbers obtained in Chapter 4 could be interpreted as quantitative data. In doing so, two fungal species could be identified that show a high hyphal occurrence in the weathered E horizon, namely *Cortinarius collinitus* and *Russula decolorans*.

Now that root tips as well as hyphae could sufficiently be detected and used to study the EM fungal diversity in soil, it would be useful to compare both approaches. To compare fungal richness as determined by root tip identification and mycelial identification, six soil samples were analyzed in Chapter 6. Root tips were extracted from the six samples and after amplification, the basidiomycete diversity on the root tips was analyzed by DGGE. The soil from the six samples was sieved, total soil DNA was extracted and after amplification, the basidiomycete diversity in the soil fractions was analyzed by DGGE. Fourteen different bands were excised from the DGGE gel and sequenced and eight bands were assigned fungal taxon names. Out of a total of 14 fungal taxa detected in soil, 11 fungal taxa were found on root tips, of which seven were known EM fungal taxa. To examine whether the sieving treatment would affect EM species diversity, two different sieve mesh sizes were used and in addition, the organic soil fraction was analyzed separately. DGGE analysis showed no differences in banding pattern for the different soil fractions. The organic fraction gave the highest DGGE band intensities. Chapter 6 demonstrates that there is a high correspondence between basidiomycete diversity detected by molecular analysis of root tips and soil samples, irrespective of the soil fraction being analyzed.

In general, the work presented in this thesis demonstrates that molecular techniques facilitate identification and relative quantification of individual soil fungi and in addition highlights the pitfalls of the molecular methodology in the final Chapter 7. Although the use of molecular techniques may lead to the detection of new fungal species in soil, several aspects of the molecular techniques and the properties of the soil being analyzed may influence the detected fungal diversity. Nevertheless, sampling bias related to morphotype sorting does not occur when analyzing the soil mycelium. The work presented in this thesis has shown that

detection of mycelium in soil may reveal a species richness comparable to the species richness detected with root tip analysis. As differences in amount and organization of the extramatrical mycelium produced by individual species may reflect the different ecological roles that fungi have in ecosystems, the detection of hyphae initiates possibilities for *in situ* studies on substrate specificity, niche partitioning and succession of EM fungal species below ground. Future studies should determine what the significance of the molecular pitfalls is when compared to the sampling and sorting issues related to root tip identification. For now, it will depend on the research question being addressed whether EM fungal species diversity is best studied by a root tip or mycelial approach.

Samenvatting

Het in de bodem aanwezige (extramatrikale) mycelium van ectomycorrhizaschimmels (EM-schimmels) is, naast het mycelium op de wortel, een belangrijk bestanddeel van de EM-symbiose omdat het de opname van water en plantenvoedende stoffen uit de bodem verzorgt. Naast passieve opname kan het mycelium door de uitscheiding van enzymen en organische zuren ook actief nutriënten beschikbaar maken uit respectievelijk organische stof en mineralen. Het vermogen van EM-schimmels om mineralen te verweren kwam opnieuw in de belangstelling te staan in 1998, toen microscopische (kleiner dan 1/50 mm), tunnelachtige structuren in verweerbare mineralen werden gevonden. Er werd aangenomen dat deze tunnels waren gevormd door de hyfen van EM-schimmels en deze aanname vormde de start van het werk dat in dit proefschrift wordt gepresenteerd. In Hoofdstuk 2 van dit proefschrift wordt een overzicht gegeven van het vermogen van schimmels om mineralen te verweren. Onderzoek uit het verleden laat zien dat zowel vrij-levende als symbiotische schimmels mineralen kunnen verweren, maar er zijn geen waarnemingen bekend van tunnelvormende EM-schimmels. Momenteel zijn er slechts indirecte bewijzen dat EM-schimmels tunnelachtige structuren in mineralen kunnen vormen. Uit Hoofdstuk 2 blijkt verder dat de relatieve bijdrage van verwering door EM-schimmels op het totale nutriëntenbudget van een bos zal moeten worden gekwantificeerd om de ecologische betekenis van verwering door schimmels vast te stellen. Los van het feit echter of EM-schimmels nutriënten opnemen vanuit het binnenste van mineralen door de vorming van tunnelachtige structuren of van de buitenkant van mineralen door verwering van de oppervlakte, onderzoek uit het verleden heeft onomstotelijk aangetoond dat EM-schimmels mineralen kunnen verweren.

Mineralen met tunnelachtige structuren zijn tot op heden alleen gevonden in de verweerde E horizont van podzolbodems, een kenmerkend, gelaagd bodemtype voor boreale bossen. De EM-schimmelgemeenschap is in deze boreale bossen zeer soortenrijk, maar eventuele verschillen in de soortensamenstelling van de organische en minerale horizonten van een podzolprofiel waren niet eerder bestudeerd. Ook was het onbekend welke EM-schimmelsoorten ter plaatse onder natuurlijke omstandigheden mineralen verweren, terwijl wel van veel soorten bekend was dat zij organische zuren uitscheiden.

Traditioneel worden EM-schimmels in bodems op naam gebracht door een combinatie van morfologische en moleculaire identificatie van gemycorrhizeerde worteltoppen. Deze aanpak is gebruikt in Hoofdstuk 3 om schimmels op naam te brengen in de verweerde E horizont van een podzolprofiel. Uit vier horizonten van

een podzolprofiel werden de EM-worteltoppen bemonsterd om te bepalen of de soortensamenstelling van de schimmels in de E horizont zou verschillen van de andere horizonten. De worteltoppen werden naar uiterlijke kenmerken gegroepeerd in verschillende morfotypen en de schimmels werden op naam gebracht door het bepalen van de basenvolgorde van het ITS-rDNA. Met behulp van een ordinatietechniek, correspondentieanalyse, werd vervolgens een significant verband gevonden tussen de soortensamenstelling en de horizonten. *Tomentellopsis submollis*, drie *Piloderma* soorten en één *Dermocybe* soort werden voornamelijk boven in het bodemprofiel gevonden, terwijl *Suillus luteus* en soorten binnen *Cortinarius* spp. in de minerale horizonten onder in het profiel werden gevonden. De helft van het aantal gevonden soorten werd alleen gevonden in de minerale horizonten, aantonende dat het belangrijk is ook de dieperliggende minerale horizonten te bemonsteren wanneer de soortenrijkdom van een gebied wordt bepaald. Er werden geen soorten gevonden die uitsluitend in de E horizont voorkwamen.

De verdeling van soorten over het bodemprofiel werd in Hoofdstuk 3 bestudeerd door middel van identificatie van gemycorrhizeerde worteltoppen, terwijl organische zuren door schimmels worden uitgescheiden aan de hyfentoppen. De grote soortenrijkdom van EM-schimmelgemeenschappen, het feit dat worteltoppen met dezelfde schimmelsoort vaak groepsgewijs voorkomen en afwijkingen die ontstaan door onjuiste morfologische typeringen bemoeilijken het analyseren van worteltoppen. Daarom werd in Hoofdstuk 4 de verticale verdeling van mycelium over de horizonten geanalyseerd met behulp van moleculaire technieken, gebaseerd op extracten van al het DNA uit de bodem. Net als in Hoofdstuk 3 werden bodemmonsters genomen uit vier horizonten van een podzolprofiel. Met behulp van een primerpaar dat een hoge specificiteit heeft voor schimmels die tot de basidiomyceten behoren, werden specifiek de ITS-rDNA-fragmenten van deze schimmels uit de bodemextracten vermeerderd. Het aldus vermeerderde DNA-materiaal werd gekloneerd, van een aantal van deze kloons werd de basenvolgorde bepaald en deze sequenties werden in een evolutionaire stamboom geanalyseerd. Gebaseerd op de overeenkomstige samenstelling van dat stukje DNA werden de kloons in 25 groepen of 'operational taxonomic units' (OTUs) ondergebracht. Van die 25 OTUs bleken 7 OTUs een hoge overeenkomst (≥ 99%) te vertonen met bekende schimmelsequenties en van 6 OTUs kon niet nauwkeurig bepaald worden tot welke groep binnen de basidiomyceten ze behoorden. Zestien OTUs werden alleen in de minerale horizonten gevonden. De verkregen sequenties van deze OTUs werden vergeleken met sequenties van worteltoppen uit Hoofdstuk 3. Van de 25 OTUs bleken 10 OTUs een overeenkomst in basenvolgorde van ≥ 98% te vertonen met sequenties die van gemycorrhizeerde worteltoppen afkomstig waren. Vaak waren beide DNA-sequenties uit de dezelfde horizont afkomstig. Behalve het

feit dat deze vergelijking aantoont dat deze aanpak om al het DNA uit de bodem te isoleren, het ITS-rDNA van basidiomyceten specifiek te vermeerderen en op naam te brengen, technisch gezien voldoet, laat deze vondst zien dat worteltoppen en mycelium van éénzelfde soort voornamelijk in dezelfde horizont voorkomen.

In het algemeen worden worteltoppen gezien als telbare eenheden en daarom worden aantallen gemycorrhizeerde worteltoppen vaak gebruikt om de biomassa en ecologische betekenis van een schimmelsoort in een bodem aan te geven. De kwantitatieve bepaling van mycelium met moleculaire methoden lijkt minder eenvoudig (Hoofdstuk 5 en 7). Met behulp van moleculaire technieken kunnen schimmels direct in bodems op naam worden gebracht, maar het is nog onbekend of er een rechtstreeks verband bestaat tussen hoeveelheden DNA en de biomassa van het mycelium. Bepalingen van mycelium biomassa worden meestal verkregen via hyfenlengte bepalingen of door de kwantitatieve bepaling van schimmelspecifieke stoffen zoals ergosterol en bepaalde vetzuren. Deze methoden geven echter de biomassa van de gehele schimmelgemeenschap en laten geen soortspecifieke bepalingen toe. Om te onderzoeken of de biomassa van afzonderlijke schimmelsoorten in de bodem kan worden bepaald met moleculaire technieken werden in Hoofdstuk 5 drie moleculaire technieken vergeleken met conventionele technieken ter bepaling van biomassa. De groei van het extramatrikale mycelium van twee EM-schimmelsoorten (*Suillus bovinus* en *Paxillus involutus*) in grond werd gedurende drie maanden gevolgd met behulp van DGGE, een kloneringtechniek en Real-time kwantitatieve PCR. Deze resultaten werden vergeleken met resultaten verkregen uit bepalingen van hyfenlengtes en specifieke vetzuren. Met behulp van moleculaire methodes kon het mycelium van beide schimmels apart geïdentificeerd worden en konden relatieve hoeveelheden mycelium per soort worden vastgesteld. De verschillende methoden gaven vergelijkbare resultaten. Omdat het niet mogelijk bleek om de hoeveelheden DNA direct te koppelen aan biomassa werd de hoeveelheid DNA uitgedrukt in ng DNA per gram grond en konden de verschillende monsters met elkaar vergeleken worden. Het werk dat in Hoofdstuk 5 wordt gepresenteerd laat zien dat moleculaire methoden kunnen worden gebruikt voor de relatieve bepaling van biomassa of aantallen van schimmels in de bodem. De combinatie van de gegevens uit Hoofdstuk 4 en 5 (zoals beschreven in Hoofdstuk 7) toont aan dat de kloonaantallen zoals verkregen in Hoofdstuk 4 kwantitatief kunnen worden geïnterpreteerd. Op deze manier werd zichtbaar dat *Cortinarius collinitus* en *Russula decolorans* relatief veel voorkomen in de verweerde E horizont.

Nu zowel worteltoppen als mycelium kunnen worden gebruikt om schimmels in bodems op te sporen en aan te tonen is het noodzakelijk beide manieren van aanpak met elkaar te vergelijken. Om soortenrijkdom zoals bepaald met moleculaire identificatie van worteltoppen en hyfen met elkaar te vergelijken werden 6 grondmonsters geanalyseerd in Hoofdstuk 6. Worteltoppen werden uit deze grondmonsters gehaald en na DNA-vermeerdering werd de diversiteit van basidiomyceten op de worteltoppen geanalyseerd met DGGE. De grond van de 6 monsters werd gezeefd, al het DNA werd geëxtraheerd en na DNA-vermeerdering werd ook hier de diversiteit van basidiomyceten in verschillende grondfracties geanalyseerd met DGGE. Veertien banden werden uit de DGGE gel gesneden voor bepalingen van de basenvolgorde en 8 banden konden tot op soortsniveau worden geïdentificeerd. Van 14 soorten die werden aangetroffen in de bodem werden er 11 gevonden op de worteltoppen, waarvan 7 werden herkend als EM-vormende schimmels. Om te onderzoeken of de zeefgrootte invloed zou hebben op het aantal soorten dat aangetoond kon worden, werden twee verschillende zeefgroottes gebruikt en werd ook de organische fractie apart geanalyseerd. DGGE analyse liet geen verschillen zien voor de verschillende bodemfracties. De organische fractie gaf de hoogste bandintensiteiten, hetgeen een aanwijzing is voor de grootste schimmelbiomassa in deze fractie. Hoofdstuk 6 laat zien dat er een grote overeenkomst is tussen de diversiteit van basidiomyceten zoals bepaald via analyses van gemycorrhizeerde worteltoppen en hyfen, onafhankelijk van de bodemfractie die bekeken wordt.

Het werk dat in dit proefschrift wordt gepresenteerd laat zien dat moleculaire technieken kunnen worden gebruikt om bodemschimmels op naam te brengen en relatief te kwantificeren. Daarnaast beschrijft het de moeilijkheden van moleculaire technieken (Hoofdstuk 7) en laat zien dat bepaalde aspecten die samenhangen met moleculaire procedures of de geanalyseerde bodems de gedetecteerde rijkdom aan schimmelsoorten kunnen beïnvloeden. De soortenrijkdom zal echter niet vertekend worden door aspecten die wel kunnen optreden bij morfologische typeringen van worteltoppen.

Dit proefschrift laat verder zien dat de soortenrijkdom zoals bepaald door moleculaire analyses van mycelium vergelijkbaar is met die bepaald door analyse van worteltoppen. EM-schimmels produceren soortsafhankelijke hoeveelheden mycelium en verschillen ook in de ruimtelijke verdeling daarvan. Deze soortsspecifieke verschillen hangen mogelijk samen met de ecologische functies van schimmels in ecosystemen. Nu het mycelium aangetoond, op naam gebracht en gekwantificeerd kan worden, is het mogelijk om in het veld onderzoek te doen naar substraatspecificiteit, nisdifferentiatie en de successie van schimmelsoorten in de bodem. Toekomstig onderzoek zal moeten aantonen hoe belangrijk de beperkingen

van de moleculaire methoden zijn wanneer deze worden vergeleken met de beperkingen die samenhangen met worteltopbemonstering en indeling in morfotypen. Voorlopig zal het afhangen van de onderzoeksvraag of soortenijkdom van EM-schimmels het best wordt bestudeerd met een benadering via gemycorrhizeerde worteltoppen of via mycelium.

Dankwoord

Dankwoord

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Levensloop

Renske Landeweert werd op 23 augustus 1971 geboren te Deventer. Op de Vrije School De IJssel te Zutphen behaalde zij in 1989 haar MAVO-diploma. Na het behalen van haar HAVO diploma aan Scholengemeenschap Midden-IJssel te Deventer vertrok zij in 1990 naar Australië en Indonesië, waar zij gedurende 8 maanden woonde, werkte en rondreisde. Na thuiskomst begon zij aan de opleiding Milieukunde aan het Van Hall Instituut te Groningen. Om specifieker te kunnen studeren stapte zij na het afronden van de HBO-propedeuse over naar de Rijksuniversiteit Groningen, waar zij in 1992 begon aan de opleiding Biologie. Hier werd haar interesse gewekt voor de plantenwereld en in het bijzonder voor symbiotische samenlevingsvormen daarin. Naast de afstudeerrichting Milieubiologie besloot zij haar studie te richten op de afstudeerrichting Plantenfysiologie. In 1995 volgde zij gedurende één trimester een aantal colleges aan de Wageningen Universiteit. Tijdens twee stageperiodes in Mozambique en in Canada maakte zij vervolgens kennis met de praktische kant van het uitvoeren van wetenschappelijk onderzoek en bestudeerde zij de effecten van arbusculaire mycorrhiza-schimmels op pinda's, ginseng en gemuteerde erwten. Gedurende deze stageperiodes werd duidelijk dat zij in de toekomst graag zou werken als wetenschappelijk onderzoeker. Na afronding van haar studie Biologie in mei 1998 kwam zij in september 1998 dienst van de NWO en werd zij aangesteld als onderzoeker in opleiding aan de Wageningen Universiteit. Hier maakte zij voor het eerst kennis met ectomycorrhizaschimmels en leerde deze te identificeren tijdens een studiebezoek van drie maanden aan de SLU in Zweden in 1999. In samenwerking met het RIVM te Bilthoven onderzocht zij vervolgens de (on)mogelijkheden van het gebruik van moleculaire technieken om ectomycorrhizaschimmels in de bodem te identificeren. In maart 2003 werd haar oio-aanstelling afgerond met het gereedkomen van het proefschrift dat voor u ligt. In mei 2003 treedt zij in dienst bij Blgg Oosterbeek voor de oprichting van een moleculair, microbiologisch laboratorium te Wageningen.

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