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Georg-August-Universität Göttingen

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En Biologie Végétale et Forestière

Par

Marlis Reich

Application des techniques de génotypage à haut débit pour l'étude des communautés fongiques des sols

(Using high-throughput genotyping for monitoring communities of soil fungi)

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To a european spirit and an international exchange….

When I the starry courses know,
And Nature's wise instruction seek,
With light of power my soul shall glow…

On y voit le cours des étoiles ;
Ton âme, échappant à la nuit,
Pourra voguer à pleines voiles…

Erkennest dann der Sterne Lauf,
Und wenn Natur dich unterweist,
Dann geht die Seelenkraft dir auf…

(Faust I, Night, Johann Wolfgang Goethe)

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1. Preface

1.1.Summary

Forests are highly complex ecosystems and harbor a rich biodiversity above-ground and below-ground. In the forest soil a multilayer array of microorganisms can be found occupying various niches. They play essential roles in the mineralization of organic compounds and nutrient cycling (Fitter *et al.*, 2005). Mycorrhizal fungi are a highly abundant and functionally very important group of soil micro-organisms. They live in mutualistic symbiosis with the plants and deliver their plant hosts with nutrients and water and receive in return carbohydrates. Many environmental factors influence the richness and the composition of mycorrhizal communities. They can be grouped into biotic and abiotic factors. As mycorrhizal fungi are nearly wholly dependent on plant-derived carbohydrates it is not astonishing, that plant communities have a main impact on structure and function of mycorrhizal communities. This is achieved in a number of ways, e.g. by the host plant species, the age or successional status of the host tree/forest or physiological features such as litter fall, root turnover or root exudations of carbons (Johnson *et al.*, 2005). Recent research focused e.g. on the effect of host taxonomic distance on ectomycorrhizal (ECM) communities. It was reported that communities sharing host trees of similar taxonomic status showed similar structure compared to communities associated to more taxonomical distinct host trees (Ishida *et al.*, 2003). Anyhow, differences in ECM communities were observed when they were associated to two congeneric host trees with different leaf physiology (Morris *et al.*, 2008). An important abiotic factor in boreal and temperate forests is the climate change over seasons. Temporal patterns of ECM fungi occur during a year and can be explained by ecological preferences of fungal species and enzymatic adaptation to changing weather and changing resource conditions (Buée *et al.*, 2005; Courty *et al.*, 2008). All these studies reveal the diversity of factors and their impact on mycorrhizal communities. As mycorrhizal communities are playing an important role in forest ecosystems, the dynamics of mycorrhizal communities as response to environmental factors have to be studied to understand the global dynamic and biodiversity of forest ecosystems.

But how can we study and describe in detail such complex communities? In the last decades molecular biological detection techniques were developed and used alongside with classical morphological and anatomical-based methods. Especially the determination of ITS as DNA barcode for fungi and the adjustment of PCR conditions for the amplification of the total

fungal community opened the way for more detailed community studies (Horton & Bruns, 2001). Traditional molecular techniques such as ITS-fingerprinting or Sanger-sequencing were widely applied (reviewed in Anderson, 2006). However, these techniques are limited by the number of samples, which can be processed in a realistic time frame (Mitchell & Zuccharo, 2006). Identification of fungal taxa can nowadays be expanded to high-throughput molecular diagnostic tools, such as phylochips (a microarray to detect species) and 454 sequencing. The ongoing implementation of array technique led to its high-throughput capacity, as thousands of features can be fixed to the carrier glass. In the case of phylochips, features are oligonucleotides targeting barcode genes of the species of interest. So far, phylochips were used for the identification of bacterial species from complex environmental samples (Brodie *et al.*, 2006) or for few genera of pathogenic (Lievens *et al.*, 2003, 2005) and composting fungi (Hultman *et al.*, 2008). 454 sequencing is a newly developed sequencing technique combining the complete sequence process covering all subsequent steps from the barcode region of interest to the finished sequence (Margulies *et al.*, 2005). In first experiments 454 sequencing technique was used to sequence genomes (Andrie *et al.*, 2005) or transcriptomes (Bainbridge *et al.*, 2006). With the ongoing development metagenomic analysis were carried out. Bacterial community structures of different ecosystems were described with more than over 10,000 sequences (Huber *et al.*, 2007). So far, no studies were published on fungal communities by using 454 sequencing. Phylochip and 454 sequencing analysis started to revolutionize the understanding of bacterial community structure and have great potential to get new-insights into fungal community structure.

1.2.Objectives of my thesis

My thesis focused on the impact of host trees and seasonal changes on the fungal community composition. The main objectives were i) to describe the richness of ECM communities in beech and spruce plantations over a time-scale of one year and ii) to report the impact of the host tree species on the fungal community diversity. As described above high-throughput diagnostic tools have not been yet applied in studies focusing on fungi in forest ecosystems. Therefore, my goal was i) to develop and test a high-throughput phylochip to identify fungi on their ITS region, ii) to apply the developed phylochip in ecological studies, iii) to use 454 sequencing for exhaustive studies of fungal communities in a forest ecosystem, and iv) to report advantages and pitfalls of these two high-throughput approaches when used in fungal ecology studies.

1.3.Overview on the chapters

Chapter I: I give an overview on the research focusing on environmental factors, which influence mycorrhizal community composition and dynamics. Additionally, I discuss the pros and cons of detection techniques and their application in fungal community analysis.

Chapter II: We report the development of a small-scale phylochip to detect ECM fungi in mycorrhizal root tip samples of beech and spruce on our experimental site in Breuil, Burgundy, France. The phylochips were developed over two generations, first as a nylon, later as a glasslide array. The two generations of phylochips were evaluated by hybridizing artificial fungal community mixes. Results of environmental sample analysis were compared to results obtained by ECM root tip morphotyping and ITS-Sanger-sequencing on the same PCR product used for phylochip analysis.

Chapter III: We studied the impact of host trees, beech and spruce, and of seasonal changes on ECM communities in Breuil by using a large-scale phylochip. Design and development of the NimbleGen phylochip are described in detail. The NimbleGen phylochip differs to the phylochips described in chapter II in its size, as 23,393 fungal ITS-sequences were used to create 84,891 species-specific oligonucleotides for 9,678 fungal species. Oligonucleotides were spotted in four replicates on the phylochip. Results of phylochip analysis were validated with results of cloning/Sanger-sequencing.

Chapter IV: We describe the influence of tree species on total fungal community diversity in Breuil by using 454 sequencing. Soil samples were taken under two deciduous tree species (beech and oak) and under four conifers (spruce, fir, Douglas fir, pine). The ITS1-region was tagged for amplification. Between 26,000 and 36,000 sequences, depending of treatments, were generated, corresponding to 580-1,000 operational taxonomic units (OTU) (3% dissimilarity) for each treatment. Influence of tree species on fungal communities is discussed.

Chapter V: We compared the two high-throughput techniques, large-scale phylochip and 454 sequencing, against each other. Therefore fungal communities under plantations of spruce and beech of the experimental site of Breuil were analyzed on their ITS1-region. With this experiment, we tried i) to understand pros and cons of one technique over the other, ii) to explore favored possible fields of application of each technique and, iii) to discuss possible linkages of the two techniques in in-depth analysis of ecological studies.

Chapter VI: We tested the quantification of three different ECM fungal species associated with two different host tree species using automated ribosomal intergenic spacer analysis (ARISA). The use of this technique for semi-quantitative traceability of the ECM status of tree roots, based on the relative heights of the peaks in the electropherograms, is shown.

During my thesis, I participated in the *Laccaria*-Genome-project and was responsible for the annotation of the genes of the fatty acid metabolism. In the context of the consortium I contributed in the publication of two articles.

Chapter VII: We report the genome sequence of the ECM fungi *L. bicolor* and highlight gene sets involved in rhizosphere colonization and symbiosis. The 65-megabase genome assembly contains 20,000 predicted protein-encoding genes and a very large number of transposons and repeated sequences. The predicted gene inventory of the *L. bicolor* genome points to previously unknown mechanisms of symbiosis operating in biotrophic mycorrhizal fungi.

Chapter VIII: We explored the genome sequence of *L. bicolor* for genes involved in fatty acid metabolism. The pathways of fatty acid biosynthesis and degradation of *L. bicolor* were reconstructed using lipid composition, gene annotation and transcriptional analysis. Similarities and differences of theses pathways in comparison to other organisms and ecological strategies are discussed.

Chapter IX: I give some concluding remarks over the different detection techniques used during my thesis and discuss their pros and cons and possible fields of application. In some cases it might be interesting to link different techniques to get a complete view on fungal communities.

2. Chapter I: Introduction

2.1.Mycorrhiza in the focus of research

2.1.1. Mycorrhiza, a mutualistic symbiosis

A complex array of organisms have emerged since the genesis of life. Organisms which occupy the same niche are often forced to interact due to their close proximity. Interaction between organsisms occur in three different forms: (1) parasitism, where only one of the interaction partner benefits while the other one is harmed, (2) commensalism, where one partner benefits without harming the other, and (3) mutualism, where the fitness of both partners increases (Egger & Hibbett, 2004). Mutualistic symbiosis is the most prevalent interaction formed between the roots of land plants and fungi. Frank (1885) was the first one to recognize and describe the mutualistic aspects of what is now called “mycorrhiza” (Greek: roots of fungi). Nearly 95% of all land plants form mycorrhiza, including some non-vascular plants, ferns and other seedless vascular plants (Peterson *et al.*, 2004). It is assumed that fungi have played a crucial role in the colonization of land by plants (Remy *et al.*, 1994; Selosse & Le Tacon, 1998). The oldest fossil evidence of mycorrhiza is dated to the Ordovician period, 460 million years ago, which coincides with the first appearance of land plants (Redecker *et al.*, 2000).

In mycorrhiza nutrient exchange takes place between the symbiotic partners. The fungus delivers the plant with nutrients and water and receives in return glucose and in much lesser amount fructose, which are formed during the photosynthesis by the plant (Buscot *et al.*, 2000). Mycorrhiza enhances also the fitness of the plant by increasing their resistance against soil borne pathogens and toxic elements while also improving their drought tolerance (Smith & Read, 1997). These features make mycorrhiza, when associated with forest trees and crop plants, environmentally as economically interesting (Grove & Le Tacon, 1993; reviewed by Newsham *et al.*, 1995).

2.1.2. Different approaches to study mycorrhizal fungi

Considerable gaps still exist in our knowledge concerning the biology of mycorrhiza. To gain more insights into this relationship recent research has used ecological, physiological or genomic approaches to allow a broader understanding of the features and functionings of mycorrhiza. Ecologically, a comprehensive survey of the relationship between an organism to the environment is performed. “Environment” comprises of other organisms sharing the same niche or other biotic and abiotic factors (Agrawal *et al.*, 2007). In the context of mycorrhizal research, this means to study the influence of environmental factors on the community structure by looking at the distribution and abundance of different mycorrhizal species. Mechanical, biochemical and physical funtions of organisms are studied when using a physiological approach (Garland & Carter Jr, 1994). The plant-fungus interaction has been especially studied on the basis of nutrient exchange (Buscot *et al.*, 2000; Nehls *et al.*, 2007) or of pre-mycorrhizal signal exchange (Martin, 2006). Finally, the genomic approach considers the organization, structure and history of genomes (Mauricio, 2005) and is the basis of many other “omics” such as transcriptomics or proteomics (Ge *et al.*, 2003; Figure 1).

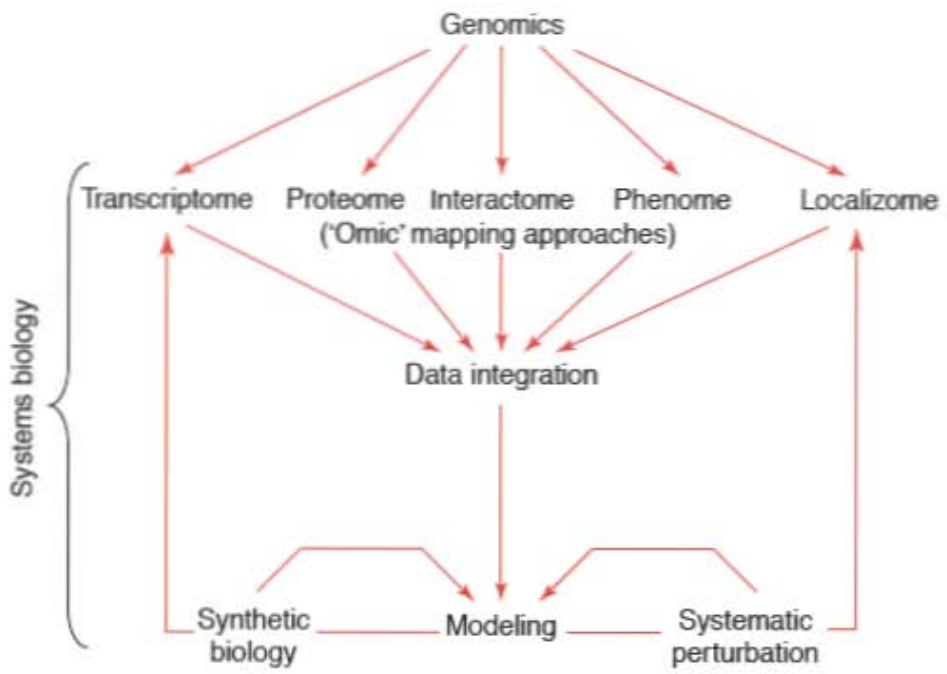


Fig. 1:Integrating “omic” information. With the availability of complete genome and transcriptome sequences (genomics), functional genomic and proteomic (or “omic”) approaches are used to map the trancriptome (comlete set of interactions), phenome (complete set of pheotypes) and localizome (localization of all transcripts and proteins) of a given organism. Integrating omic information should help to reduce the problems caused by false positives and false negatives obtained from single omic appporaches, lead to better functional annotations of gene products and the functional relationships between them, and allow for the formulation of increasingly relevant biological hypotheses (after Hui *et al.*, 2003).

Whitham *et al.* (2006; 2008) developed the idea that genomic studies help to understand community structure and ecosystem processes. They showed that the genotype of a species influences the fitness of another species in the same niche constituting an indirect genetic interaction. This interaction alters species composition and abundace in that niche causing a new community and phenotype ecosystem to develop (Figure 2). To understand the genomic components underlying these phenotypes the molecular mechanism regulating the interaction between the host plant and fungi should be studied in detail. Several genomes from fungi with different ecological background were recently sequenced and released (for an overview see Xu *et al.*, 2006; Martin & Selosse, 2008; Chapter VIII), allowing for comparative genome analysis to determine specific genes or gene structuring caused by different fungal life-styles (Chapter IX).

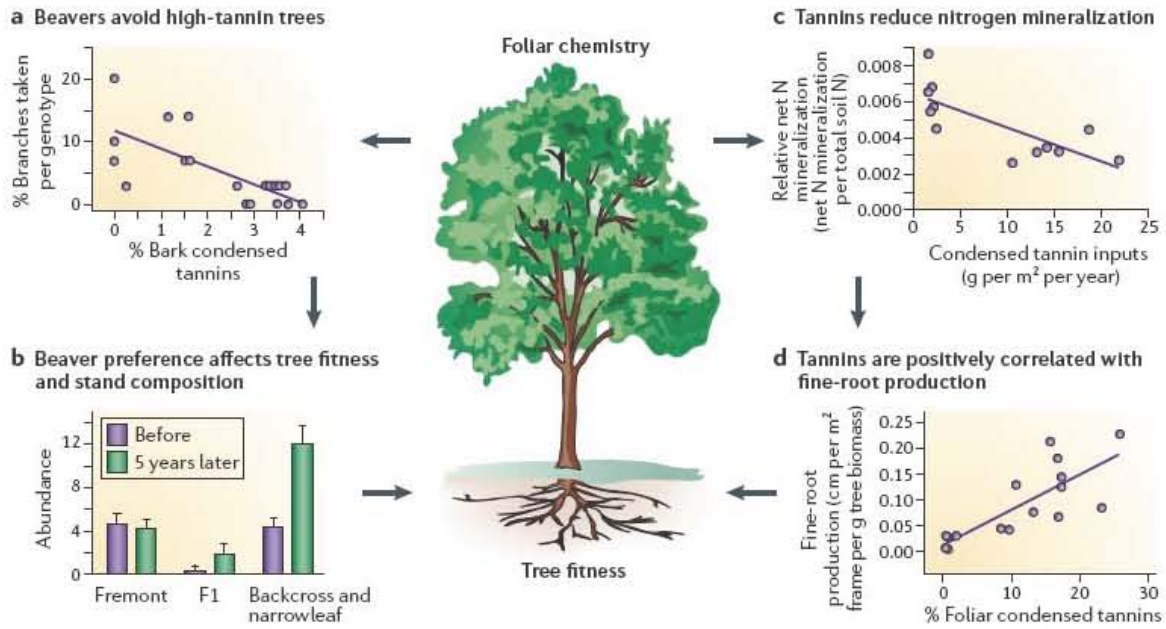


Fig. 2: Feedback relationships. Selection pressures that are exerted on foundation species can affect interactions with other species, which in turn might feed back to affect the fitness of the individual that produced the phenotype. Here we show how the condensed tannin phenotype in the poplar could affect the foraging of an important herbivore, nutrient cycling and nutrient acquisition. Panels (a) and (b) show that the beaver *Castor canadensis* is an important agent of natural selection in which interactions with a foundation tree species could affect many other species that depend on the tree for their survival. Beavers selectively fell trees low in condensed tannins, which in turn affects the fitness of different tree genotypes and cross types. After 5 years of selective tree felling, cross types that were high in condensed tannins nearly tripled in abundance, whereas the cross type lowest in condensed tannins had significantly declined in abundance and the cross type intermediate in condensed tannins (F1 hybrids) showed an intermediate increase in abundance. Panels (c) and (d) illustrate a potentially important feedback loop that presumably interacts through the microbial community to affect the tree's performance. Panel (c) suggests that an increased concentration of condensed tannins in leaves of individual trees can inhibit the microbially mediated process of nitrogen mineralization. In turn, variation in soil nutrients could feed back to affect the tree's investment into fine-root production to forage for limiting nutrients, which can affect tree performance. (after Whitham *et al.*, 2006)

«Mycorrhiza in the focus of research»

- mycorrhiza is a mutualistic symbiosis between plant roots and fungi
- the interacting partners exchange nutrients
- mycorrhizal research uses various scientific disciplines such as ecology, physiology and genomics
- ecological genomics can help to understand community structure and ecosystem processes

2.2.Variety of mycorrhizal types

2.2.1. Seven forms of mycorrhiza

Based on structural features seven different mycorrhizal types have been described which can be subdivised into two groups; the ectomycorrhiza (ECM) and the group of endomycorrhiza (Peterson *et al.*, 2004). The ectomycorrhiza is characterized by a hyphal mantle ensheating the roots and an intercellular hyphal net. Conversely, in the group of endomycorrhiza, fungal hyphae invade the root cells. Differences in the nature and the structure of the intracellular hyphal development can be described for the members of the endomycorrhizal group. The arbuscular mycorrhiza (AM), ericoid mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, orchid mycorrhiza and the ectendo mycorrhiza form the group of endomycorrhiza (Figure 3; Table 1). Only the arbuscular and the ectomycorrhiza will be discussed further, as they are the most commonly formed plant-fungal relationships formed in economical and ecological important plant species (Finlay, 2005).

2.2.2. Arbuscular mycorrhiza

The AM is the most ubiquitous type of mycorrhiza and is formed by 80% of all land plants (Smith & Read, 1997). It is especially formed by herbaceous plants and several tropical tree species, but it has also been reported to be formed by liverworts, ferns and by the geological "old" conifers (all but *Pinaceae* and *Gnetum*) (Peterson *et al.*, 2004). Beside this huge number of plant species (estimated 23,000) only ~160 fungal species were described to form AM, all belong to the phylum of *Glomeromycota* (Rosendahl & Stukenbrock, 2004; Table 1). It is assumed that much more fungal AM species exist as up to now only few biotopes and plant species were analysed in detail and nearly every new field study reveals undescribed species (Oehl *et al.*, 2003; Wubet *et al.*, 2003).

During the development of AM, a fungal hyphae attaches to the root surface and enters the root via an apressorium profilerating as intracellular hyphae over the root cells. Some hyphal ends branch dichotomously and form the so-called arbuscules (Figure 3, Figure 9). The host plasma membrane is elaborated over all branches of the arbuscules increasing the contact area between the plant and the fungus (Smith & Read, 2008). Here, nutrient exchange takes place. The fungus takes up glucose from the plant, while delivering the plant especially with water and phosphate, but also with amino acids and some cations such as e.g. Mg^{2+} and K^{+} (van der Heijden & Sanders, 2002).

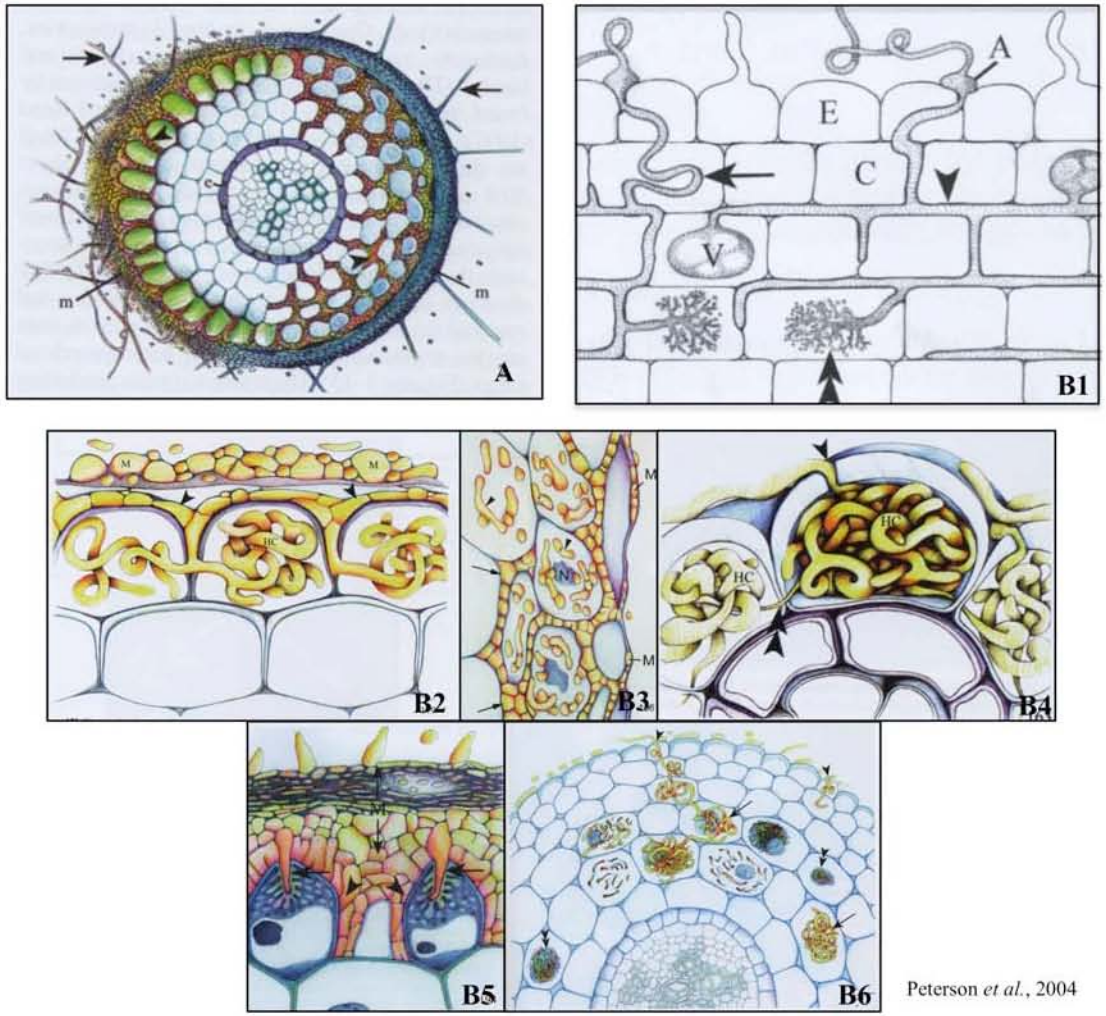


Fig. 3: Distinct mycorrhizal types.

Number	A	B1	B2	B4	B5	B6	B3
Form of mycorrhiza	**Ectomycorrhiza**	**Arbuscular mycorrhiza**	**Arbutoid mycorrhiza**	**Ericoid mycorrhiza**	**Monotropoid mycorrhiza**	**Orchid mycorrhiza**	**Ectendo mycorrhiza**
Photobiont	*Pinaceae*, *Betulaceae*, *Salicaceae*, *Myrtaceae*, *Dipterocarpaceae* *Fagaceae*	more than 80% of all land plants	*Arbutus*, *Arctostaphylos* and several genera of *Pyrolaceae*	*Ericaceae*, *Epacridaceae*, *Empetraceae*	*Monotropaceae*	*Orchidaceae*	*Pinus* and *Larix*
Mycobiontes	septed fungi, Asco-/Basidiomycetes and one *Endogone*	unsepted fungi, *Glomeromycota*,	some ectomycorrhiza forming fungi	*Hymenoscyphus ericae*, *Oidiodendron griseum*, some Asco-/Basidiomycetes	some ectomycorrhiza forming fungi	Basidiomycetes	a limited number of Ascomycetes
structure	• formation of a mantle (M) that covers considerable portions of lateral roots • Hartig net, hyphae between root cells (arrowheads) • extraradical mycelium (arrows) with hyphae or rhizomorphs that grow into surrounding soil	• appressorium (A) to penetrate epidermal cell (E) • frequently hyphal coils (arrow) • intercellular hyphae (arrowhead) in cortex (C) • arbuscules (double arrowhead) • - some fungi form vesicles (V)	• hyphal mantle (M) • paraepidermal Hartig net (arrowheads) • intracellular hyphal complex (HC)	• only in epidermal cells • hyphae enter cell through through thickened cell wall (arrowhead) • hyphal complexes (HC)	• hyphal mantle (M) • Hartig net (arrowheads) • fungal peds (arrows) • finger-like projections of host-derived wall material	• hyphae penetrate over epidermal cell (arrow head) • pelotons (hyphal coils) in cortical cells (arrows) • degradation of hyphal coils (double arrowheads)	• thin mantle (M) • Hartig net (arrows) • intracellular hyphae (arrow heads)

2.2.3. Ectomycorrhiza

In comparison to the AM, only approximately 8,000 plant species (3% of seed plants) form ECM (Smith & Read, 2008 t see a list of genera). Although this number is much smaller than that for plant species associated to AM, these host trees are disproportionately represented on a global scale. Vast areas in the Northern and Southern Hemispheres are populated by *Pinaceae, Abietaceae, Betulaceae, Salicaceae, Myrtaceae, Dipterocarpaceae* and *Fagaceae* (Finlay, 2005). While previous census placed the number of ECM fungal (EMF) species at approximately 5,500, ongoing research (especially in tropical forests) has detected many undescribed EMF species. Therefore, the number of EMF species is currently estimated between 7,000-10,000 species (Taylor & Alexander, 2005). Most of EMF are *Basidiomycota* and occur essentially in the order of *Agaricales*, but some *Ascomycota* are also EMF. Only one genus in the *Zygomycota*, *Endogone*, forms ECM (Table 1; extensive list of fungal species can be found in Smith & Read, 2008). Over the last years it has also become evident that non-*Agaricales* play an important role in ECM formation (Weiss *et al.*, 2004). The family of *Sebacinaceae* has especially attracted interest as their members very often form ECM and other mycorrhizal forms at the same time, building large networks between differently mycorrhized plants (Selosse *et al.*, 2007).

ECM is formed on the terminal feeder roots of plants and is comprised of three domains. 1) The mantle, a multi-layer hyphal structure, formed by the fungus on the external face of the root tip. 2) The fungal mycelium which extends out from the mantle surface into the soil, forming sometimes root-like structures, the rhizomorphs (Agerer, 2001) (Figure 9). The fungus degrades N- and P-compounds contained in soil organic matter by metabolic enzymes which are excreted from the extraradical hyphae. The excrition leads also to a dissolution of soil mineral particles and minerals (e.g. Ca^{2+}, Mg^{2+} or K^{+}) (Leake *et al.*, 2002). 3) The Hartig net, comprised of hyphae originating from the mantle, develops between root epidermal and cortical (in conifers) cells and forms a complex nutrient exchange interface over the surface of these cells (Smith & Read, 1997; Figure 3). Here, nutrient exchange takes place. The fungus takes up sugar from the plant, while delivering the plant with water and nutrients.

"Variety of mycorrhizal types"

- up to now seven different mycorrhizal types are described
- the most common mycorrhizal forms are the arbuscular- (AM) and the ectomycorrhiza (ECM)
- AM is formed by 80% of all land plants, but only a relative small fungal group, *Glomeromycota*, is involved
- ~ 7,000-10,000 fungal species form ECM
- both types of mycorrhiza form organs and cell structures, which are adapted to nutrient exchange between the host plant and the fungus and foraging in the soil

Box 1: Secrets of intraterrestial aliens

Only morphotyping was used in early mycorrhizal research to identify AMF (see section "Morphotyping"). For this reason the *Glomeromycota* were described as a group of only approx. 150 species (Smith & Read, 1997). It was assumed that they were host non-specific as in culture studies the few studied types showed promiscuous colonization (reviewed in detail by Helgason & Fitter, 2005). This picture changed with the use of molecular biological approaches. Up to 20 associated fungal species were found on the roots of one plant species containing primarily **new species unknown** from culture collections (Wubet *et al.*, 2003; Hijri *et al.*, 2006; Wu *et al.*, 2007). These new techniques enabled tracing and description of AMF in natural ecosystems, but ecological studies of AMF communities are hindered, as the **genome structure** of *Glomeromycota* causes difficulty to identify what constititutes an individual fungal strain (Rosendahl, 2008). Diversity studies very often use the ribosomal gene pool of a community to describe diversity. In the nucleus of common eukaryotes 75-100 identical copies of the ribosomal genes can be found. As *Glomeromycota* spores are coenocytic they contain between **2,000 – 20,000 nuclei** (Helgason & Fitter, 2005) and studies on the ribosomal genes have revealed **genetic variants** in a **single spore** (Sanders *et al.*, 1995). This has also been seen for other genes (Corradi *et al.*, 2004) leading to **two different hypotheses** concerning the genetic structure of AMF. The first hypothesis considers AMF as **homokaryon** with identical nuclei each coding for all genetic variations (e.g. Pawlowska & Taylor, 2004). In the counter-argument *Glomeromycota* are seen as **heterokaryon**, where several genetically distinct nuclei cohabit in one spore (e.g. Kuhn *et al.*, 2001; Hijri & Sanders, 2005) (discussed in more detail Young, 2008). Although a part of the *Glomus intraradices* genome has been sequenced (Martin *et al.*, 2008), the secret of *Glomeromycota* has not been resolved.

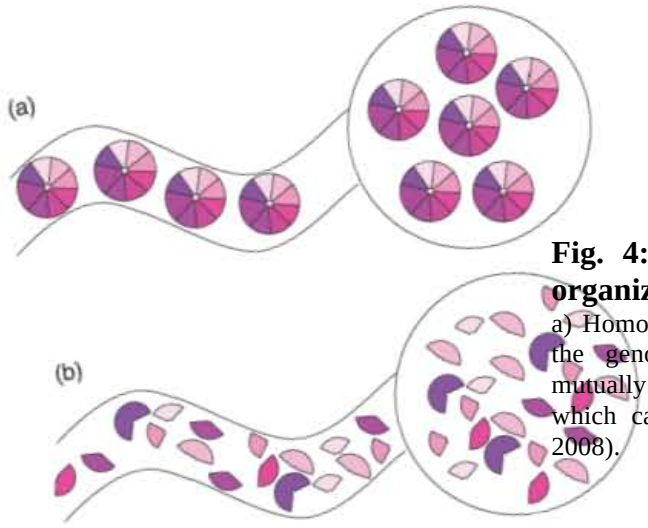


Fig. 4: The two hypotheses for genome organization in AMFs.
a) Homokaryon. Each nucleus has multiple copies of the genome. b) Heterokaryon. A consortium of mutually complementary nuclear lineages, none of which can survive without the other (after Young, 2008).

2.3.Ecology of mycorrhizal fungi

2.3.1. Environmental factors

Ecology studies the relationship of an organism/organism group to the environment. When studying the impact of environment on mycorrhizal communities, several main factors can be considered. Abiotic factors such as climate, natural conditions/ disasters or soil and soil features can be assesed. Biotic factors, that affect mycorrhizal groups, are host plants, organisms living in the same niche or more globally, all other organisms living on the host plants (e.g. insects). Human activities also have a high impact on mycorrhizal community structure. For example, land management restructures the soil or changes plant composition and density and thus effects symbiotic communities. While not every factor affecting the environment has a direct influence on mycorrhizal community, alterations to one of the main factors may change community structure indirectly (Figure 5). Although flow diagrams can help us to understand the connections between environmental factors and the studied organism, it should be kept in mind that ecological relationships are much more dense and variable and "factor research" can only give us a glimpse into this complex ecosystem.

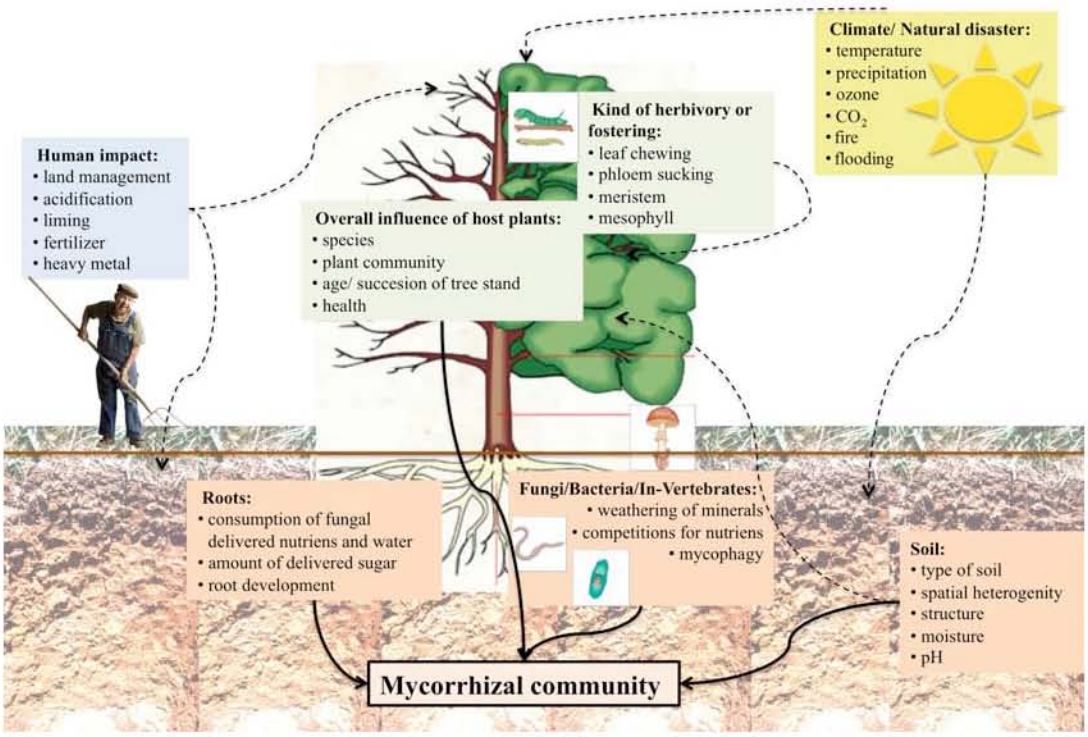


Fig. 5: Factors influencing mycorrhizal community structure. Some factors have an indirect influence on mycorrhizal communities (dotted lines) by changing active soil structure or plant health/stress. Constant lines represent direct influence.

2.3.2. Ecology of arbuscular mycorrhizal fungi

2.3.2.1. Biotic factors influencing arbuscular mycorrhizal communities

Host plant

Using molecular biological approaches, the hypothesis of non-host-specificity of AMF was rechecked. Vandenkoornhuyse *et al.* (2003) reported distinct AMF communities on three co-existing grass species. These results were supported by the work of Golotte *et al.* (2004), who demonstrated the selection pressure of plants on AMF communities by replacing indigenous vegetation with monocultures of grass species, and by Wubet *et al.* (2006) who studied the AMF communities of two coexisting conifers in Ethiopia. Pivato *et al.* (2007) demonstrated that plant genotypes can influence fungal community structure. While in this experiment strict host plant specificity was not shown, a preferential association of AMF was found with four annual *Medicago* species. The abundance of the fungi in the roots differed with the plant genotype. All obtained sequences belonged to the *Glomus* genus and mainly to the *Glomus* A group, which is in agreement with its wide distribution and its prevalence in roots of legumes (Pivato *et al.*, 2007). AMF genus diversity associated with one plant species has also been shown for *Prunus africana* in Ethiopia. Most abundant were *Glomeraceae* species, but *Diversisporaceae* and *Archaeosporaceae* were also found. Of the 22 species described, 20 were new to science (Wubet *et al.*, 2003).

The age of the host plant can also evoke a drastic shift in AM community composition. First and second year seedlings of two tropical plant species were used to demonstrate a strong shift of their associated AMF composition. Species that dominated in the first year were almost entirely replaced by previously rare species in the second year (Husband *et al.*, 2002a, 2002b). This suggests that the diversification of communities is based on the function of a number of plant-associated variables and that AMF composition change with the need of the plant.

Herbivory

Above-ground herbivory normally has a negative effect on mycorrhizal colonization and diversity (Gehring & Whitham, 2002). Additionally, the type of herbivore are a key to below-ground effect (Wearn & Gange, 2007). The influence of soil invertebrates on mycorrhiza is less studied (reviewed by Gange & Brown, 2002), but it is still unknown if these interactions result in a shift in AMF community composition.

"Biotic factors influencing arbuscular mycorrhizal communities"	
factor	**effect on AMF community**
plant species	• preferential association
plant age	• strong fungal community shift; diversification is based on function and is adopted to the need of the plant

2.3.2.2. Abiotic factors influencing arbuscular mycorrhizal communities

Site effect

Soil structure has a huge influence on mycorrhizal community composition. Rosendahl & Stuckenbrock (2004) described the community structure of AMF in undisturbed coastal grassland where fungi showed a spatial distribution. Dominant fungal species covered up to 10 m along a transect, while others formed small individual mycelia clusters. The authors assumed that species, which spread mainly by vegetative growth, are more abundant in undisturbed systems, while sporulating species favor disturbed systems. Oehl *et al.* (2003) compared the species diversity in arable soil on sites with low, moderate (with 7-year crop rotation) and high land use (monoculture). They found that species richness was lowered at the high-input monocroping site, with a preferential selection of species that colonized roots slowly but formed spores rapidly. While each site had its own specialistic species, some species were present on all three sites. A similar picture arises when looking on AMF communities of primary successional sites, e.g. those of a volcanic desert on Mont Fuji (Wu *et al.*, 2007). Here, fungal species diversity increased with decreasing altitude as did the diversity of plant species, an effect most likely due to less soil erosion. The dominant species found at higher altitudes remained dominant at lower altitudes (Wu *et al.*, 2007).

Edaphic factors

Physical soil features, such as moisture, have an ambigous influence on AMF species. AMF species richness in a *Populus – Salix* stand in a semiarid riparian ecosystem were positively related to gravimetric soil moisture and declined with distance from the closeby water channel (Beauchamp *et al.*, 2006). Conversely, standing water in soil has a negative affect on AMF species (Miller, 2000). Low soil pH negatively affects AMF species richness (Toljander *et al.*, 2008) and colonization rate (Göransson *et al.*, 2008). Increasing heavy metal concentrations generally decreases AMF richness (Zarei *et al.*, 2008). Among the few species colonizing plants on heavy metal polluted sites, distinctive abundance was reported. At a zinc waste in Poland the *Glomus* sp. HM-CL4 was the most effective colonizer, while four other species were found at moderate or low abundance (Turnau *et al.*, 2001).

N-deposition

As mycorrhizal fungi are involved in the nitrogen cycle, they are affected by anthropogenic N-fertilization/-deposition. Community shifts favoring *Glomus aggregatum, Gl. leptotichum* and *Gl. geosporum* were reported in coastal sage scrub or *Gigaspora gigantea* and *Glomus mosseae* in tallgrass prairie after N-fertilization (reviewed in Rilling *et al.*, 2002). It was hypothesized that due to increased N availability in the soil, P became the limiting factor instead of N thereby resulting in a community shift toward species adapted to low P (Rilling *et al.*, 2002) rather than accumulation of nitrophilic species (Toljander *et al.*, 2008).

Climate change

Among all the factors related to global environmental change, elevated CO_2 is the most studied with respect to mycorrhizal fungi. The response of AMF through increase of biomass or colonization rate is controversial (Drigo *et al.*, 2008). Wolf *et al.* (2003) reported an effect of CO_2 on AMF community composition, while in another study the speed of CO_2 increase influenced the AMF community: when CO_2 was elevated in a single high step composition changed sharply, while for gradual elevation no response was recognized (Drigo *et al.*, 2008).

"Abiotic factors influencing arbuscular mycorrhizal communities"	
factor	**effect on AMF community**
plant species	• host preference
plant age	• strong fungal community shift; diversification is based on function and is adapted to the need of the plant
soil structure	• undisturbed system: fungi spread mainly by vegetative growth • disturbed sites: sporulating species • specialists for specific sites • generalists
soil moisture	• depend on intensity • standing water negative effect
soil pH	• low pH has a negative effect
heavy metal	• negative effect
N-fertilization	• shift of community
elevated CO_2	• strenth of elevation step influences community

2.3.3. The ecology of ectomycorrhizal communities

2.3.2.3. Biotic factors influencing ectomycorrhizal communities

Host plant

Host plant composition is one of the major factors influencing EMF community structure (Anderson, 2006). Early research demonstrated that certain EMF show high host specificity while others have a broad host range (reviewed in Johnson *et al.*, 2005). Host-specific associations were described for *Pinaceae* with *Suillus* and *Rhizopogon* species (Molina & Trappe, 1982). Strong host specificity was found for the EMF genera of *Suillus, Rhizopogon, Alnicola* or *Leccinum*. *Leccinum versipelle, L. scabrum* and *L. holopus* were exclusively associated with *Betula* species. Other genera, such as *Cenococcum, Clavulina* or *Laccaria* are more promiscuous as they can associate with different plant species (Breitenbach & Kränzlin, 1984-2000). With the development of high-throughput molecular tools, preferences of EMF in a community can now be studied in detail (Horton & Bruns, 2001; Anderson, 2006). Ishida and colleagues (2007) studied EMF associated to eight different plant species of three different plant families in a mixed conifer-broadleaf forest. Host taxonomy influenced fungal composition and taxonomically close host species harbored the most similar EMF communities. In large part, the same host-specific EMF were shared between species of one genus (Figure 6). Beside host-specific fungi, EMF species with a broad host range on both angiosperms and gymnosperms hosts were also found. With a similar goal, Morris *et al.* (2008) studied the host-influence of congeneric trees on EMF communities. They sampled EMF under the deciduous *Quercus douglasii* and the evergreen *Q. wislizeni.* Their results showed that host species of the same genera can generate very different EMF communities, with only 40 shared of 140 fungal. The two oak species, though from the same genus, had a very different leaf physiology and structure, which might be also influencing EMF species composition.

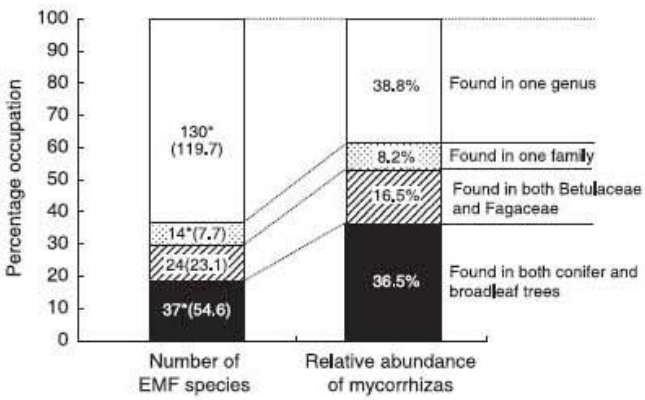


Fig. 6: Percentage occurrence of EMF species in relation to their host ranges. The relative abundance of mycorrhizas was calculated in each host range group. To test the bias of the observed proportion of each host range class, observed values were compared with values obtained after 1000 randomizations. In a randomization, each EMF species was randomly assigned to the number of host trees equal to its observed frequency. Asterisks indicate significantly biased values ($P < 0.05$). Mean numbers of EMF species obtained from randomizations were shown in the parentheses. (after Ishida *et al.*, 2007)

Ishida *et al.* (2007) assumed that host preference at any taxonomical level may provide new niches and support higher local species richness. The results of Tedersoo *et al.* (2008) support these hypothesis. They described EMF communities of three tree species of a Tasmanian wet sclerophyll forest and found striking differences between the two pioneer, fire-dependent tree species *Eucalyptus regnans* and *Pomaderris apetala*. The authors, following the hypothesis of Ishida and coworkers, concluded that these two tree species exclude each other through priority effect and hardly compatible EMF.

Sucessional state of forest

Mason *et al.* (1982) were the first group to consider the influence of forest age on EMF diversity. When they were looking at *Betula pendula* trees, which had recently colonized agricultural soil, they found two different groups of fungi over time: the “early-stage” fungi, which were associated to trees in their pioneer phase (young, first-generation trees on disturbed forest sites), and the “late-stage” fungi found in climax vegetation (after canopy closure of the forest site). Danielson (1984) added a third category, the “multi-stage” fungi: fungi that are present throughout the life of the stand. The main critic of the model proposed by Mason *et al.* was that their conclusions were based on an experiment conducted with a stand growing on agricultural soil. This is not a realistic circumstance for forests (Smith & Read, 2008). Another model had been developed earlier by Pugh (1980) who transferred the ecological strategy model from plants on fungi. The model distinguishs between fungi, which can be found on ruderal or pertubated sites (“R” fungi), and fungi, which are competitive and stress-tolerant, that can be found on mature forest sites (“C” and “S” fungi). The main difference of these two fungal groups is their type of reproduction. “R” fungi possess small, cordless basidiocarps, are short-living and reproduction occurs mainly through spores. “C” and “S” species produce larger and more resistant basidiocarps and build long, widely distributed mycorrhizal patches (Frankland, 1998; Figure 7). A study by Jonsson *et al*. (1999a), which looked at EMF associated with seedlings and old trees of Scots pine in an old virgin boreal forest, supports the concept of a mycelial network in older stands. They reported a very similar fungal species composition between seedling and old trees showing the continuity of EMF communities and fungal interconnections between different trees.

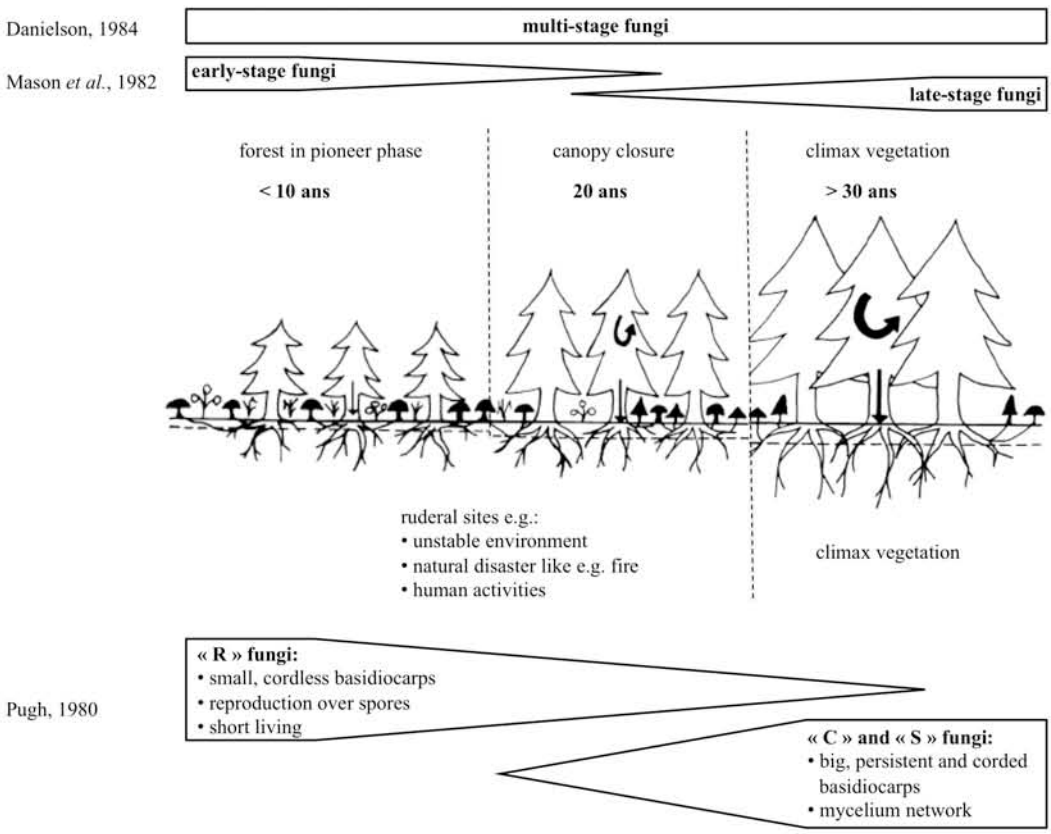


Fig. 7: Different concepts of the succession of mycorrhizal fungi during forest development/ ruderal sites and climax vegetation. R, ruderal; C, competitive; S, stress-tolerant (after Frankland, 1998).

Herbivory

Herbivores have an indirect influence on fungal community structure, as most plants reduce root growth when being attacked by herbivores. The carbon limitation caused by defoliation is the most likely reason for this mechanism: severe defoliation caused by the western spruce budworm reduced EMF occurrence more severly than moderate defoliation (Kolb *et al.*, 1999). In general, a negative effect from indirect herbivore interaction on EMF communities has been reported (Gehring & Whitham, 2002).

Saprotrophic fungi

Others than EMF, a huge number of saprotrophic fungi can be found in the soil, where a competition for the principle sources of nutrients occurs. Although it could be shown in a microcosm study that the saprotrophic fungus *Phaenerochaete velutina* reduced the growth rate and density of the mycelium of *Suillus bovinus* (Leake *et al.*, 2002), it is not clear what general influence the presence of saprotrophic fungi have on the overall EMF community structure.

"Biotic factors influencing ectomycorrhizal communities"	
Factor	**Effect on ectomycorrhizal community**
host plant	• host-specific associations or broad host range • taxonomically close plant species harbor more similar EMF communities than with other host plants • EMF community can be very distant between congeneric host plants, when physiology of the host plants differ a lot • host preferences at any given taxonomical level may provide new niches
host age/ succession of forest	• different concepts developed • early-stage, late-stage and multi-stage fungi dependend on canopy closure • ecological concept: "ruderal" fungi versus "competitive and stress-tolerant" fungi showing different reproduction strategies
herbivory	• depending on organism • above-ground herbivory often indirect negative effect over host plant
saprotrophic fungi	• competition for nutrients • unknown influence on community structure

2.3.3.1. Abiotic factors influencing ectomycorrhizal communities

Seasonal influence

Community structure of ECM is dependent on the seasons and fungi can be grouped by their response to temporal patterns (Koide *et al.*, 2007). Courty *et al.* (2008) described species, which were present only during a few months, while others were detected during the whole year, though abundance of certain species changed. They assumed that distribution and presence of a species depends on its ecological preference. Buée *et al.* (2005) showed contrasting seasonal patterns in metabolic activity of different ECM species. For example, ECM formed by *Clavulina cristata*, *Laccaria amethystina* and *Russula sp.*were significantly more abundant and active in winter than in summer.

Edaphic factors

Several studies have tried to determine the influence of edaphic factors upon EMF diversity. The vertical distribution of fungi has attracted special attention as fungal diversity may be explained from the niche partitioning due to the physiochemical features of each soil layer (Erland & Taylor, 2002). Mycelium distribution has been shown partition in a *Pinus resinosa* plantation (Dickie *et al.*, 2002). Species were divided into specialists to certain soil horizons or multilayer generalists, which shows that there is a wide range of substrate utilization patterns among different ECM species. The species richness was significantly lower in the deepest mineral layer than in the other layers (Dickie *et al.*, 2002). Rosling and coworkers (2003) documented the vertical distribution of ECM taxa over seven soil horizons in a podzol profile. They reported that two thirds of the root tips colonized were in the mineral soil, representing half of all ECM taxa found, as opposed to colonies in the highest fine root density in the organic horizons. The major separation of species composition was found between the organic and deeper mineral soil horizons. Taxa occuring in several horizons showed normally a continuous distribution over nearby soil layers.

When looking at the spatial distribution of saprotrophic and mycorrhizal fungi, mycorrhizal species predominate in the deeper soil profile while saprothropic fungi are mainly found in the litter layer (O'Brien *et al.*, 2005). Lindahl and coworkers (2007) compared this distribution pattern to patterns of bulk carbon:nitrogen ratios and ^{15}N contents in soil. High C:N ratios and an enrichment of ^{15}N in deeper soil layers showed a selective removal of N (which means that plant N is mobilized by root-associated mycorrhizal fungi) and root-derived C (Figure 8). Spatial distribution within a mycorrhizal community was also drawn by exploration type of mycelium (Agerer, 2001) and ecological function of the fungal species (Genney *et al.*, 2006).

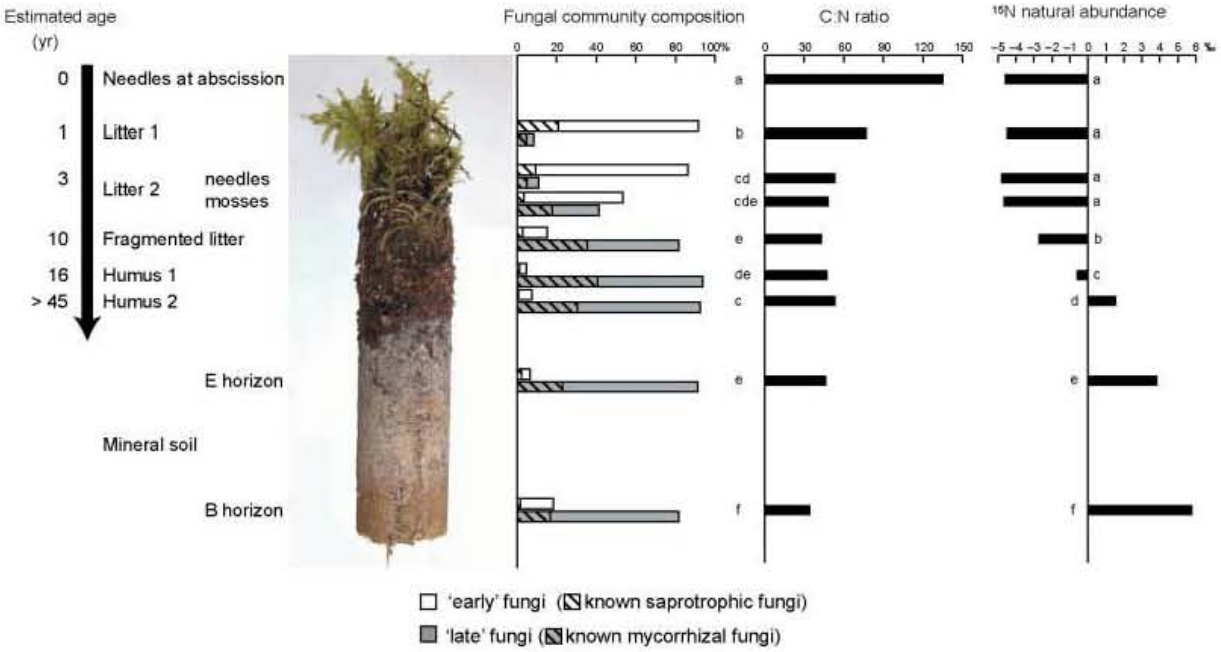


Fig. 8: Fungal community composition, carbon:nitrogen (C:N) ratio and ^{15}N natural abundance throughout the upper soil profile in a Scandinavian *Pinus sylvestris* forest. Different letters in the diagrams indicate statistically significant differences between horizons in C:N ratios and ^{15}N abundance, and the standard error of the mean was < 0.3‰ for ^{15}N natural abundance and < 3 for C:N ratio (n = 19–27, for recently abscised needles n = 3). The age of the organic matter is estimated from the average $\Delta^{14}C$ of three samples from each horizon (five samples of the litter 2 (needles) fraction) and needle abscission age (3 yr) is subtracted. Community composition data are expressed as the frequency of total observations. 'Early' fungi are defined as those occurring with a higher frequency in litter samples compared with older organic matter and mineral soil. 'Late' fungi are those occurring with a higher frequency in older organic matter (Lindahl *et al.*, 2007).

There are several other soil features or environmental conditions influencing soil structure leading to a different inventory of the mycorrhizal community composition. Most of these factors are due to anthropogenic activities. Decreasing soil moisture lowers ECM community, while the overall proportion of *Cenococcum geophilum* increases (Erland & Taylor, 2002). Soil pH is also an important parameter. Fungal species show different sensitivity to pH and respond with altered growth and colonization (Erland & Taylor, 2002). Some species have altered enzymatic capabilities as some enzymes have a narrow pH optima. The overall response of ECM community seems less affected by acidification than by liming as acid soils are more common in the native boreal forests of ECM's. Jonsson *et al.* (1999c) described changes of community structure caused by liming and discussed the difference of competitive balance between mycorrhizal and saprotrophic fungi. Rineau (2008) studied the mid-term effects of liming on the ECM community and showed that especially ubiquistic or competitive species replaced acidophilic and stress-tolerant species in the limed plots. The liming effect was found to have less effect in some plots after fewer than 20 years, which is why he considered liming as a short term, heterogenous effect.

Heavy metals also affect EMF. Fungi protect themselves against heavy metals by binding them into cell-wall components or storing them in their cytosol. The sensitivity of ECM communities to heavy metal stress is controversial. Hartley *et al.* (1997) found a negative effect on fungal diversity with an increase in dominant species. In contrast to the results of Hartley *et al.*, species richness of 54 fungal taxa was reported on European aspen on a heavy metal polluted site in Austria (Krpata *et al.*, 2008). In this stuy, species abundance followed community structure of undisturbed sites with a few abundant and a large number of rare ECM species. Only *Cenococcum geophilum* showed adaption to heavy metal in its distribution, as it was spread over several soil layers and normally appears preferentially in the organic layer (Krpata *et al.*, 2008).

N-deposition

ECM fungi are adapted to conditions of low mineral N availability and most of them are capable to extract N from organic sources. Sites with high N deposition affect EMF (Wallenda & Kottke, 1998) and species richness is reduced (Peter *et al.*, 2001). Lilleskov and coworker (2002) described the fungal community response to N polluted sites in more detail by identifying ECM root tips. They showed that the stress-tolerant species which occure

naturally, like *Cortinarius, Piloderma* and *Suillus*, where the mineralisation process is low, disappeared on N-rich sites and were replaced by generalistic species like *Laccaria, Lactarius* and *Paxillus*. Concordant with these data, strong taxonomic signature in biomass production was reported from 68 ECM species grown on nitrate as sole N source, although genes coding for a nitrate reductase were detected (Nygren *et al.*, 2008).

Fire

Wildfire is a major disturbance factor in forests and still occurs regurlarly in less fire controlled ecosystems. Two kinds of wildfire have to be distinguished, as fire intensity seems to play an important role: low intensity fires and intense stand replacing fires. Jonsson and coworkers (1999b) documented EMF communities in a burned versus unburned late-succesional stand in northern Sweden where fires have generally a low intensity. Most fungal species were shared between both sites. The evennes of species distribution was lower on the burned stand while species richness was not affected. In contrast, marked changes of mycorrhizal community structure were reported after intense stand replacing fires (e.g. Grogan *et al.*, 2000), as host plants were killed and the soil environment was altered. Horton *et al.* (1998) also reported a radical change of EMF community structure after intense fire, but the dominant species of the pre-burned community were also present in the post-burned community, although quantitatively reduced. Taylor & Bruns (1999) focused on the post-burned dominant species. They demonstrated that these fungi were present only as a small proportion of the mycorrhiza or only as propaguels before the fire, and when the fire removed other competitive fungi they became dominant. The idea of this “inoculum reservoir” is based on the position of these fungi within the soil before the fire. Propaguels can be either spores or mycelia fragments. The authors further reinforced through a seedling bioassay.

Climate change

Some research work focused on climate change and its influence on EMF. One of the factors caused by climate change is elevated CO_2. Increased atmospheric CO_2 enhances growth and productivity of several plant species including their root systems, which leads to increased ECM fungal colonisation (Rygiewicz *et al.*, 1997). Additionally, shift in EMF community structure was reported, where no ECM species of the community was dominant, but abundance of all species was increased (Godbold *et al.*, 1997). Gange *et al.* (2007) looked on recent changes in fungal fruiting patterns caused by climate change. They observed a delay in fruiting dates by 59% of the analyzed mycorrhizal species associated with broadleaf trees. No delay was reported for species associated to conifers. Whether this influences community structure is not yet clear.

"Abiotic factors influencing ectomycorrhizal communities"	
Factor	**Effect on ectomycorrhizal community**
season	• contrasting seasonal patterns and enzymatic activities
vertical soil distribution	• specialists for each horizon and multilayer generalists
soil moisture	• decreasing soil moisture lowers ECM community richness
soil pH	• fungal species show different sensitivity to pH
liming	• ubiquistic species or competitive species replace acidophilic and stress-tolerant species
N-deposition	• N-rich sites harbour generalistic ECM species
heavy metal	• influence on ECM community controversely discussed
wildfire	• dependent on fire intensity: low intensity = species distribution is lowered; high intensity = fungi with "inoculum reservoir" become dominant
elevated CO_2	• no dominance of one species

2.4.Techniques for identifying mycorrhizal fungal species

2.4.1. Morphotyping

The classical technique to determine fungal species is morphotyping. In this technique species are described according to their shape, colour and appearance of different fungal tissues or layers (Figure 9).

Arbusular mycorrhizal fungi

The taxonomy on morphology of the AMF is mainly based on the spores. Families and genera are mostly distinguished by hyphal attachement, while species are identified by their spore wall structure and substructures (Morton, 1988; Walker, 1992). Additionally, shape of arbuscules, vesicles and intraradical hyphae can be considered for determination. Until now there have been eight different arbuscular types described depending on host plant and fungus (Dickson, 2004). Walker (1992) noted the high percentage of misidentificaton and confusion over clear classification. The taxonomic concept of the AMF were developed over years and only some of the already described species were redescribed with the modern concept.

Ectomycorrhizal fungi

Below-ground studies on EMF communities are based on the presence of symbiotic ECM root tips, while above-ground studies are based on carpophore determination (Horton & Bruns, 2001). ECM can be easily collected, counted, weighed and analysed and analysis can be coupled with other techniques. Macroscopic determination relies on several features, such as the colour of the mantle, surface appearance, spatial organization, presence/absence of cystidia and sclerotia. For some species, rhizomorphs are attached to the ECM and are a helpful feature for species determination (Agerer, 1987-1998). If necessary the structure of the mantle and the Hartig net can be analysed microscopically.

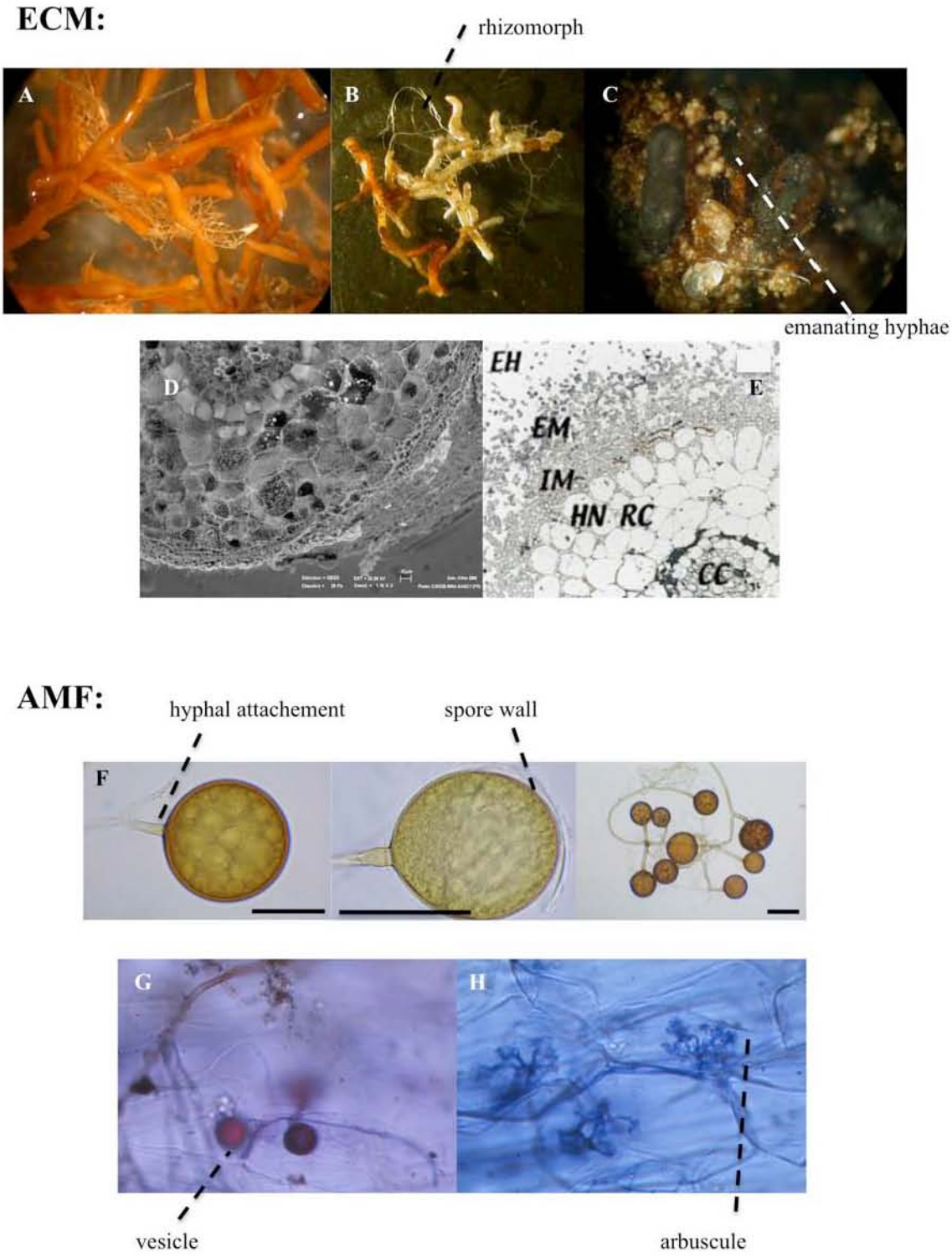


Fig. 9: Different fungal features used for morphotyping. (A) *Lactarius dulcis* ECM; (B) *Scleroderma citrinum* ECM with rhizomorphs (Reich); (C) emanating hyphae of *Cenococcum geophilum* ECMs; (D) *Lactarius subdulcis* ECM mantle transversal section (A,C,D; Rineau); (E) *Pisolithus tinctorius* ECM transverse section, external (EM) and internal mantles (IM), root cortex (RC), Hartig net (HN), extraradical hyphae (EH) (Martin *et al.*, 2001); (F) spores of *Glomus geosporum*, *G. mossae*, *G. intraradices*; bare size = 100 µm; (G) vesicles of *Glomus tenue*; (H) arbuscules of *Glomus tenue* (F,G,H Walker).

Pros and Cons

Morphotyping is a skill, which needs specialized training; furthermore it is a time-consuming technique and cannot be applied on large-scale studies. This is especially true in ecological studies where many morphotypes can stay unidentified.

Despite these deficits, morphotyping provides useful data: it is very important to describe new species and can also give insights into the functional role of fungi (Agerer, 2001). Genetically, the sequences of morphotypes could help to build a robust database with high-quality sequences and descrease the number of unidentified species in public databases.

"Morphotyping"

- morphotyping is the classical technique to determine fungal species
- for the determination of EMF the ectomycorrhizal root tips are used
- AMF are mainly determined by their spores; sometimes also the shape of arbuscules and vesicles contain some taxonomical informations
- very often morphotyping is not descriminating enough on species level; misidentification occurs
- morphotyping is especially important, when new species are described or voucher-specimens for sequence deposit are needed

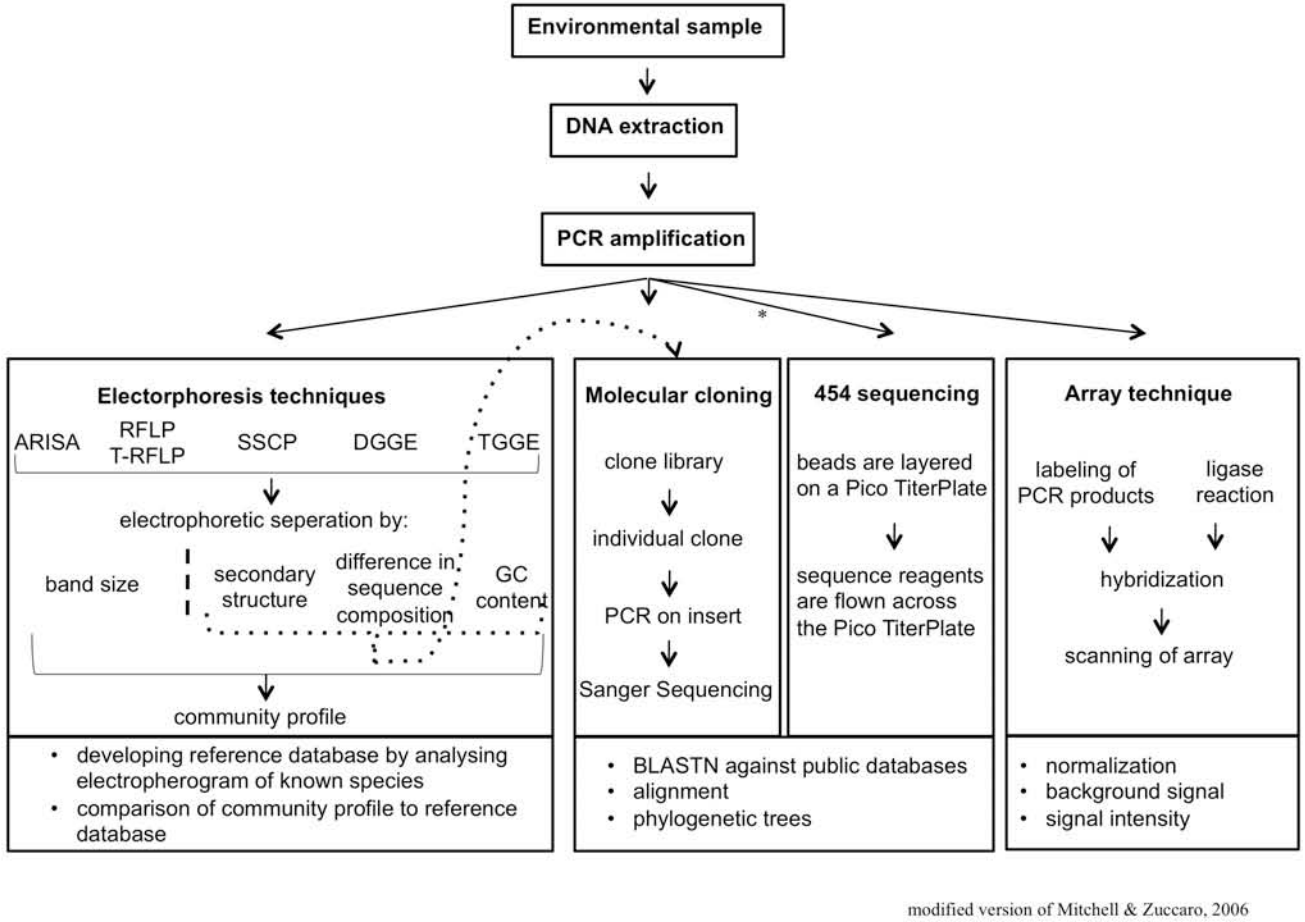


Fig. 10: PCR- based approaches to environmental nucleic acid analysis. DNA is extracted from the environmental source and is subjected to PCR amplification to produce a heterogeneous mixture of sequences. These are seperated into individual molecules by electrophoresis techniques (ARISA, amplified ribosomal intergenic spacer analysis; (T-) RFLP, (terminal-) restriction fragment length polymorphism; SSCP, single stranded conformational polymorhpism; DGGE, denaturant gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis), by cloning, by cleavage to beads or by hybridization. Results of the used techniques are viewed as banding patterns, sequences or signals on the array. Dotted file indicates the possibility after certain electrophoretic fingerprinting techniques to isolate single bands and to subject extracted PCR products to a cloning/Sanger sequencing step. *Single DNA fragments are bound to beads before running the PCR.

2.4.2. Molecular techniques to study fungal diversity

A variety of molecular techniques has been applied to assess the diversity of microorganism in environmental samples and has revolutionized our understanding of the dynamics of microbes in ecosystems. Most of these techniques have been adapted for ecological studies on fungal communities and will help us to understand fungal community diversity and functioning (Horton & Bruns, 2001). These molecular techniques have some advantages over the tradional morphotyping as they allow for high throughput studies. Beside this, molecular techniques also make it possible to track the diversity of communities in more depth, especially in detecting cryptic species or species, which are difficult to describe by morphotyping. Additionally, fungal mycelium abundance and distribution in soil can be analyzed.

Although there is a large set of molecular techniques, they all rely on polymerase chain reaction (PCR) (if immunological methods are excluded) (Figure 10). They rely on the enzymatic replication of a target sequence *in vitro* by using primers (a short strand of nucleic acid complement to the target sequence), which bind at the beginning and end of the choosen sequence to start the amplification reaction.

Markergenes

For the use of PCR in species identification, potential marker genes have to be carefully choosen. A marker gene has to show a high phylogenetic inference power to distinguish clearly between fungal families – genus – species, or even isolates, depending on the ecological question. Furthermore, marker genes must contain conserved sites for primer annealing and variable sequences inbetween primer sites. Most often high copy number genes are choosen as amplification is easiest (Mitchell & Zuccaro, 2006).

The most used gene regions for studying fungal ecology are the genes of the ribosomal RNA gene cluster (Anderson *et al.*, 2003). They exist in several copies in the genome organized in tandem repeats. These genes consist of variable and conserved regions (Figure 11).

The 18S gene (part of the small subunit (SSU) is the most conserved one of the rRNA genes. For most of the ECM species, the phylogenetical resolution goes very little beyond the family level due to their affiliation to the *Basidiomycota* and *Ascomycota*. However, some ECM groups (e.g. some *Pezizales* and *Cantharellaceae*) were determined on their 18S gene

sequence (Horton & Bruns, 2001). SSU sequences of the ancient fungal clade of the *Glomeromycota* show more variation than in the *Dikaryota*. Hence, this region is widely used to discriminate between AMF species or even below species level (Vandenkoornhuyse *et al.*, 2003). Schüßler *et al.* (2001) used also the SSU to separate the *Glomeromycota* from the *Zygomycota*.

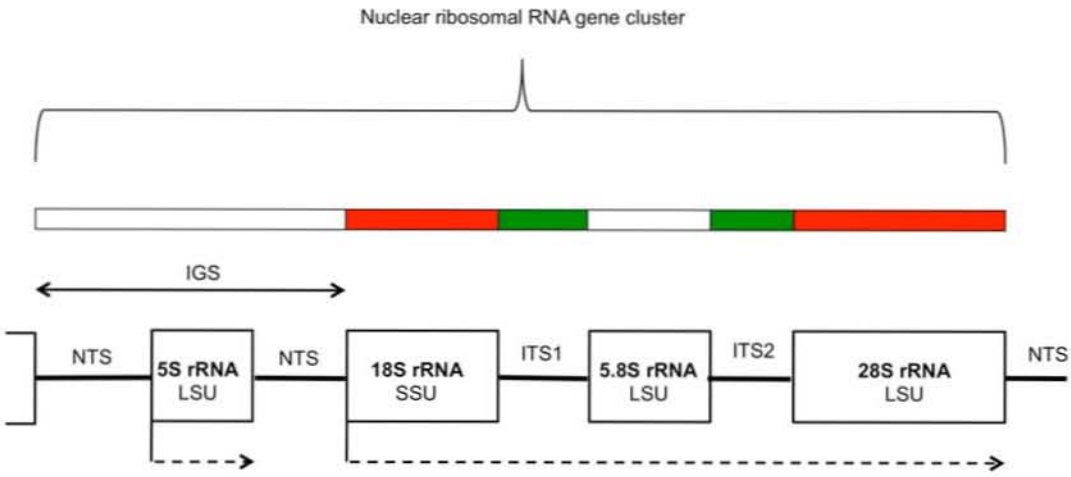


Fig. 11: The ribosomal RNA gene cluster. The cluster comprises four genes (5S, 5.8S, 28S, 18S), intergenic spacer (IGS with non transcribed spacer (NTS)) and internal transcribed spacer (ITS). Regions, which are transcribed, are indicated by dashed arrows. The degree of sequence conservation varies between these genetic regions and within the genes. Additionally, accumulation of mutations in these regions differ between EMF and AMF due to their different evolutional ages. Red marked regions are mostly used for AMF identification on species level, while green regions are mostly used for EMF.

The large subunit (LSU) genes are more variable, especially in the domains D2 and D8 in the 28S, which contain a lot of phylogenetic information. These variable regions occur in similar positions relative to the secondary structure within different organisms (Hopple & Vilgalys, 1999). The LSU is widely used in studies of AMF communities. In contrast, only few studies on EMF were carried out on the LSU genes due to the relative low number of sequences in public databases (Horton & Bruns, 2001; Moncalvo, 2000; Tedersoo *et al.*, 2009).

The internal transcribed spacer (ITS) regions are two spacer regions (ITS1 & ITS2), seperated by the 5.8S gene, and show a high sequence and size variation. Their size together can vary between 650 – 900 bp (including the 5.8S). The ITS regions are the most frequently used rRNA region to analyse phylogeny of EMF (Gardes & Bruns, 1991; Henrion *et al.*, 1992). Their resolution goes to species or beyond species level. The sequence variability is drawn by indels and repetitions, what can make alignment of ITS-sequences difficult. Recently evolved species have often a lack of species variability in the ITS. For community analysis of the *Glomeromycota*, the ITS is rarely chosen as a marker gene, because intraspecific sequence variability is very high.

In some studies, researchers used two rRNA genes to describe fungal communities (O'Brien, 2005). James *et al.* (2006) reconstructed the phylogeny of fungi with six different genes, using three of the rRNA genes, elongation factor 1-α and two RNA polymerase II subunits. Furthermore, genome scans and novel molecular insights have brought attention to other single-copy genes (Aguileta *et al.*, 2008). These strategies, however, are not transferable to large-scale fungal community detection approaches as there is a lack of sequence information in the public databases.

"Molecular techniques to study mycorrhizal fungi"

- molecular biological techniques allow high throughput studies
- they allow very often the detection of cryptic and unculturable species or species, which cannot be determined by morphotyping
- marker genes show high phylogenetic inference power
- rRNA genes, high copy genes, are widely used to determine fungi
- rRNA genes suit differently well to trace different fungal subgroups
- in some approaches, several genes or single-copy genes were used to trace fungi

Box 2: Primers & PCR

Some primers are **generic**, while others are **specific** to certain taxonomic groups. The specificity depends on the annealing site. During amplification some problems can occur, but by adjusting the PCR conditions most of them can be overcome:

Problem: **Co-amplification of non-target organisms** can lead to an inaccurate estimation of fungal diversity (Pang & Mitchell, 2005). *Solution:* The specificity of primers largely depends on the availability of enough suitable sequences to design and to compare the designed primer to other non-target groups. Designed primers have to be aligned against more species.

Problem: **Preferential annealing** of primers to certain templates more than to others influences molecular diversity assessments (Wubet *et al.*, 2003). *Solution:* It is necessary to use multiple primer sets. Using several group-specific primers enables better resolution and identification of species (Tedersoo *et al.*, 2006).

Problem: **Low target concentration** in the sample or **inhibitors** (e.g. polyphenols) present in many root samples hinder amplification (Redecker, 2002). *Solution:* A nested PCR can be applied (van Tuinen, 1998), which is a two-step PCR: a first PCR is run with a set of primers of broad host-template annealing range followed by a second PCR with more groupe-specific primers.

The most common primers for **fungal ITS amplification** are ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). ITS1F was designed with the intent to identify EMF, but it can also be used for **wide-scale fungal community** analysis (Lindahl, 2007; O'Brien, 2005). To reduce co-amplification bias, more specific primers have been designed such as ITS4B for the *Basidiomycota* (Gardes & Bruns, 1993) and ITS4A for the *Ascomycota* (Larena *et al.*, 1999) or NSI1 and NLB4 for *Dikaryomycota* (Martin & Rygiewicz, 2005).

ITS1F and ITS4 have also been used for identification of **AMF communities**, but they were coupled separately with five different *Glomales*-specific primers (Redecker, 2000).

Primers amplifying fungal rRNA regions are listed on:

- http://www.biology.duke.edu/fungi/mycolab/primers.htm
- http://aftol.biology.duke.edu/pub/primers/viewPrimers
- http://plantbio.berkeley.edu/~bruns/

2.4.2.1. DNA Fingerprinting techniques

Overview

In some molecular biological approaches PCR products are seperated into individual fragements by electrophoresis. The resulting fragment profiles, so-called DNA fingerprints, are used as information about the community diversity. These techniques can be classed into two subgroups. In the first one, the amplified fragments are separated by their different properties on a gel causing a different electrophoresis mobility of the molecules (denaturing-/ temperature- gradient gel electrophoresis (D-/T-GGE) and single strand conformation polymorphism (SSCP)). The second group comprises techniques where fragments are separated only by the size of the fragment (amplified ribosomal intergenic spacer analysis (ARISA) and restriction-/ terminal restriction- fragment length polymorphism (R-/ TR-FLP)) (Figure 11).

T-RFLP technique is the most popular fingerprinting technique and is widely used to describe species richness (Vandenkoornhuyse *et al.*, 2003) or to identify species in a community (Genney *et al.*, 2006; Lindahl *et al.*, 2007). PCR products are digested using restriction enzymes and only the terminal fragments are labelled. The fragment size of a band is compared to a T-RFLP database. Large databases are already developed, but very often they are build up on fungi with epigeous sporocarps or on morphotyped mycorrhiza. Thus, “hidden” fungi remain as “unknown” as they are very often missing from these databases. The most accurate determination can be achieved when the database is created from the same sample site. T-RFLP technique is discussed in depth in the review of Dickie & FitzJohn (2007).

Pros and Cons

All these techniques need relatively minimal technical equipment. Thus, quick sample processing and a relative high throuput of samples are possible. Furthermore, they are less expensive than sequencing (Dickie & FitzJohn, 2004) or array approaches. Most often these techniques are used to describe community structures. The presence of a band in the community profile is interpreted as presence of the corrsponding species. But the absence of a band does not necessarily stand for the absence of a species, as resolution for less abundant species is not high enough. Additionally, the separation of relatively small DNA fragments can be problematic (Muyzer, 1999). Sometimes a single band can consist of several fragments from different species (Mitchell & Zuccaro, 2006). Another problem are unknown species,

which cannot be even classified taxonomically as sequence information is missing. The band pattern of these “unknown species” can also derive from PCR artefacts or from nontarget species such as endophytes.

“Fingerprinting techniques”

- fingerprinting techniques separate PCR products into individual molecules by electrophoresis
- T-RFLP most popular fingerprinting technique
- minimal technical equipment, relative cheep in comparison to other techniques
- community profiles have to be compared to previously developed databases
- resolution is not high enough for detailed view on community

2.4.2.2. Sequencing techniques

Sanger-Sequencing

Another widely used technique for description of mycorrhizal fungi communities is sequencing of PCR-amplified loci (Ahulu *et al.*, 2006; Rosling *et al.*, 2003; van Tuinen *et al.*, 1998). The amplified sequences are compared to those available in public databases allowing an exact determination to species level or beyond. The first developed Sanger sequencing technology (Sanger & Coulson, 1975) is often combined with a cloning step, where the PCR products from a microbial community are separated by cloning individual molecules in a bacterial vector and constructing a gene library. The vector itself can easily be amplified and sequenced. Thus, sequencing can give a full record of what has been amplified if sequencing effort is high enough. Beside community description, sequencing is often used complementary to profiling fingerprinting techniques for subsequent species identification after e.g. T-RFLP-analysis (Figure 10; Burke *et al.*, 2005; Lindahl *et al.*, 2007).

The advantage of this combined approach is not only the description of the unknown or cryptic species at a taxonomic level or better, but also the identification of PCR artefacts like chimeric sequences, which influence the estimation of species richness (Anderson, 2006). Cloning/sequencing approach can become very costly and time-consuming with an increasing

number of samples and risks underestimation of species richness due to sample limitation. Until now the more complete view of the fungal diversity in soils realized by Sanger sequencing analyzed less than 1,000 sequences (O'Brien *et al.*, 2005). The bottlenecks for Sanger sequencing are library and template preparation and tedious sequencing procedures.

454 pyrosequencing

With the purpose to simplify this process, especially the *in vitro* sample preparation, Margulies *et al.* (2005) developed a modified pyrosequencing technique by combining several different technologies. This new 454 pyrosequencing technique comprises the complete sequence process covering all subsequent steps from the gene of interest to the finished sequence with a throughput of 10 megabases/hour (Rothberg & Leamon, 2008) (Figure 12). Also, sample preparation is much quicker than for Sanger sequencing as the preparation steps differ strikingly (Table 1). The 454 pyrosequencing technique developed very quickly and it became possible to sequence more complex genomes or species communities with time (Figure 13).

Table 1: Comparison of Sanger sequencing and 454 sequencing procedures for description of fungal communities

	Sanger sequencing	454 sequencing	time required
Isolation of DNA	x	x	~ 3 h
Amplification of marker gene	x	x	~ 3 h
Cloning	x		~ 3 h
Clone picking	x		~ 2 h
PCR on plasmids	x		~ 3 h
Purification of PCR products	x		~ 2 h
Running sequencer (capillary system)	x		~ 8 h
454 sequencing library		x	~ 5 h
Amplification in PCR microreactors		x	~ 6 h
Sequencing run (flowing system)		x	~ 4 h
Assembly of raw sequences	x	x	days to weeks

after Wicker *et al.*, 2006

It has already been applied for many different research aspects such as genomics (e.g. Andries *et al.* 2005), transcriptomics (Bainbridge *et al.*, 2006), metagenomics (e.g. Edward *et al.*, 2006) and functional metagenomics (Dinsdale *et al.*, 2008). In 2008, the 6 gigabase genome of J. D. Watson was sequenced in only two month. Overall, the results agreed with older results of Sanger sequencing of a human individual. Additionally, novel genes were discovered as Sanger sequencing can loose sequence information during the cloning step (Wheeler *et al.*, 2008). More complex genomes, such as the barley genome (Wicker *et al.*, 2006), or the transcriptome of *Medicago truncatula* (Cheung *et al.*, 2006) have also been partly sequenced using 454 pyrosequencing. Even 13 Mio base pairs of mammoth mitochondrial DNA were discovered in a metagenomic approach, revealing the high sequence identity of 98.55% with African elephants (Poinar *et al.* 2006).

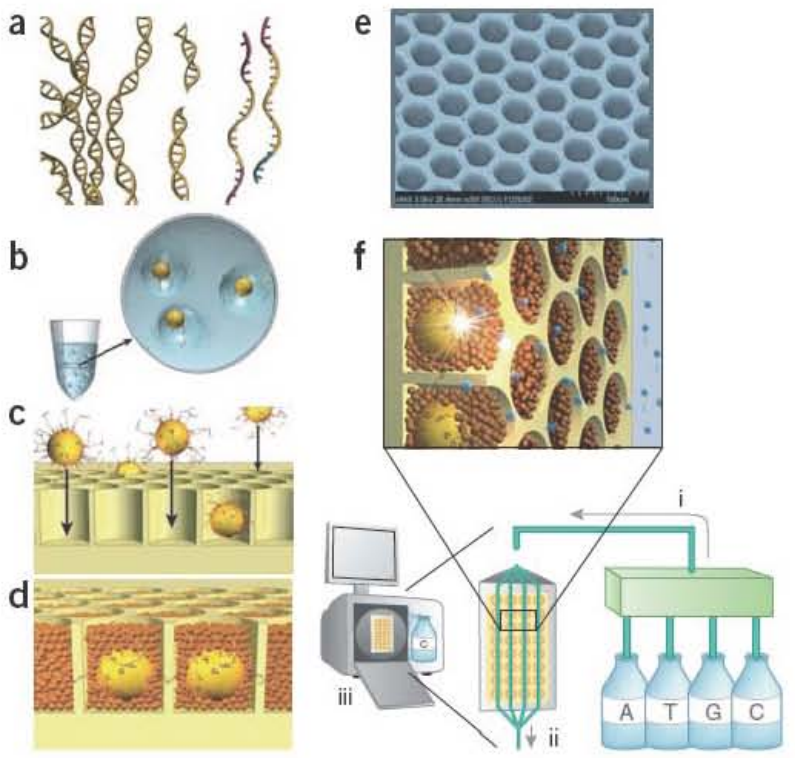


Fig. 12: Overview of the 454 sequencing technology. (a) Genomic DNA is isolated, fragmented, ligated to adapters and separated into single strands. (b) Fragments are bound to beads under conditions that favor one fragment per bead, the beads are isolated and compartmentalized in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template. (c) The emulsion is broken, the DNA strands are denatured and beads carrying single-stranded DNA templates are enriched (not shown) and deposited into wells of a fiber-optic slide. (d) Smaller beads carrying immobilised enzymes required for a solid phase pyrophosphate sequencing reaction are deposited into each well. (e) Scanning electron micrograph of a portion of a fiber-optic slide, showing fiber-optic cladding and wells before bead deposition. (f) The 454 sequencing instrument consists of the following major subsystems: a fluidic assembly (object i), a flow cell that includes the well-containing fiber-optic slide (object ii), a CCD camera-based imaging assembly with its own fiber-optic bundle used to image the fiber-optic slide (part of object iii), and a computer that provides the necessary user interface and instrument control (part of object iii) (Rothberg & Leamon, 2008).

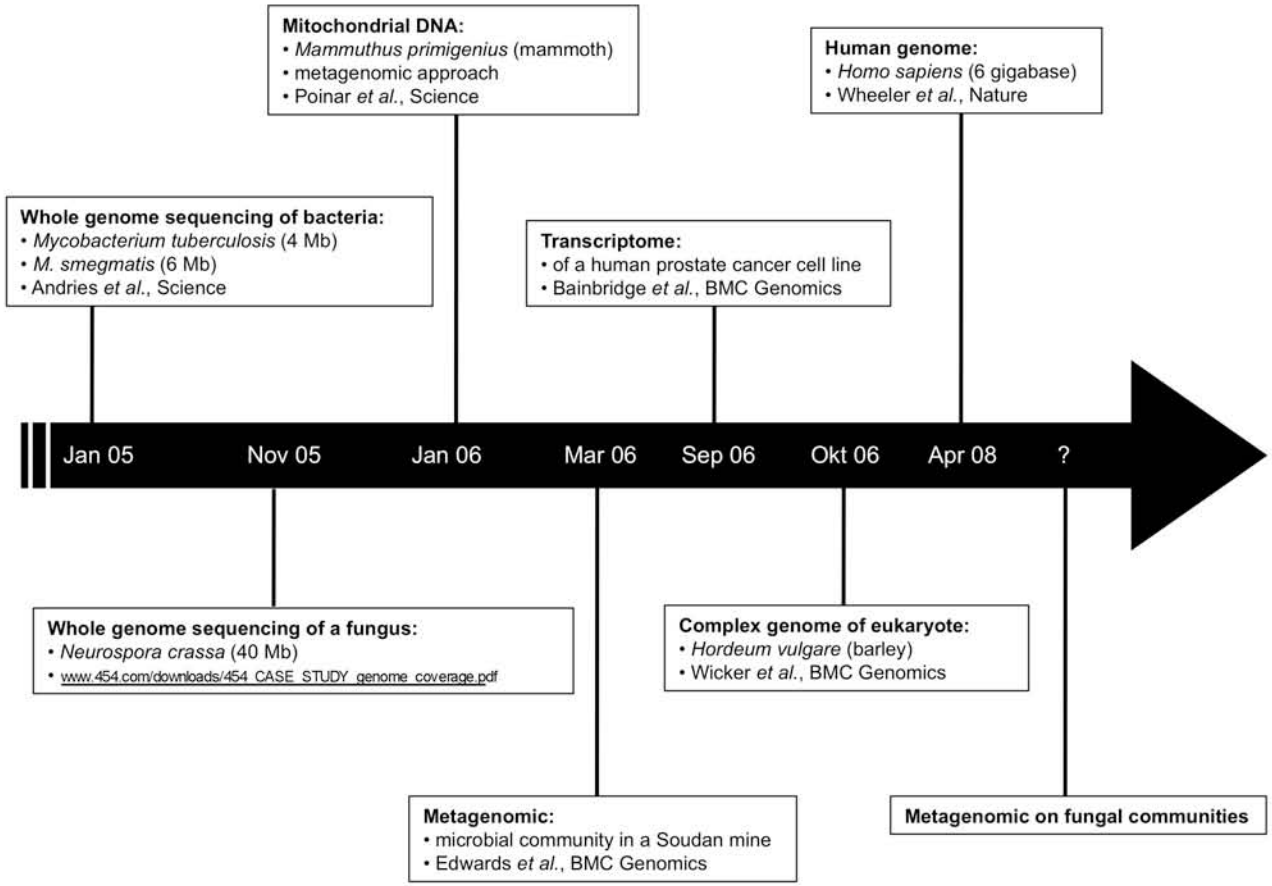


Fig. 13: Timeline of research projects using the novel 454 Sequencing technique, showing the use in a variety of different research purpose.

Metagenomic analysis using 454 pyrosequencing

Normally, metagenomic is the sampling of genome sequences of a community of organisms inhabiting a common niche. To date, metagenomic analyses have been largely applied to microbial communities. With the new sequencing techniques, metagenomic analysis will complete the in-depth understanding of species richness on earth. Initial 454 pyrosequencing studies on microbial population structures in a deep marine biosphere described more than over 10,000 bacterial operational taxonomic units (OTU) (threshold of 3% sequence identity) and the slope of the calculated rarefaction curve was still far from approaching the asymptote (Huber *et al.*, 2007). A comparison of four different sites in the ocean demonstrated striking differences of microbial communities. A relatively small number of different species always dominated the samples beside thousands of low-abundant species (Sogin *et al.*, 2006). Distinctness of microbial communities was also reported from two adjacent sites in a Soudan mine in Minnesota, USA where the divergent biochemistry of the available substrate separated the two communities. It was determined that species richness was much higher for the "oxidised" samples than for the "reduced" samples (Edwards *et al.*, 2006). 454 pyrosequencing was also used in functional metagenomics, which determines metabolic processes that are important for growth and survival of communitites in a given environment. Dinsdale and coworkers (2008) analyzed nine biomes from distinct sites. Strongly discriminating metabolic profiles across the environments were reported and the authors stated that different ecosystems cannot be distinguished by taxa but by their metabolic profiles. So far, no metagenomic or functional metagenomic studies have been published on fungal communities using 454 pyrosequencing.

Pitfalls of 454 pyrosequencing

The estimation of the number of sequences needed for in-depth description of communities remains crucial, as the number of needed sequences can vary with the chosen marker gene region. For example, Anderson *et al.* (2003) reported that more ITS sequences are needed than 18S sequences to achieve coverage of fungal diversity. Hence, programmes calculating rarefaction curves and running statistical tests on sequence number should be used (Weidler *et al.*, 2007). A problem linked to estimating the number of needed sequences is the determination of the cut-off value for taxonomic grouping, as sequence homology is influenced by inter- and intraspecific variation. Acosta-Martínez *et al.* (2008) proposed to calculate rarefaction curves with different cut-off levels starting with 0% sequence homology

up to 20%. 3% sequence homology seemes to be the most accurate estimation on species level and 5% on a genus level. The same authors described more than 2,000 OTUs in their study, with a 3% cut-off, but the rarefaction curve still did not reach the asymptote. Thus, also with 454 pyrosequencing an enormous sequencing effort has to be made and detailed characterization of community and diversity remains a challenge.

Although 454 pyrosequencing technique shows high-throughput potential, care should be taken in analysis and interpretation of the results. As large data sets are produced, analysis of sequences has to be automated. But automated BLAST can lead to misinterpretation, as the first hit of the BLAST may not be the best one. Analysis of 454 pyrosequencing data also needs higher amounts of storage capacity and CPU power than Sanger sequencing. Even more problematic for CPU power is the overall sequence comparison required in metagenomic analysis. Additionally, the outcome of metagenomic analysis is based on what we can infer from databases, and uncharacterized species or genes will hinder the in-depth understanding (Hugenholtz & Tyson, 2008).

The outcome of the sequences produced by 454 pyrosequencing of 100 to max. 300 bp bears a different error profile than the one produced by traditional Sanger sequencing and it can negatively influence accurate assembling of metagenomes in the absence of scaffolds (Edwards *et al.*, 2008). The assembling of highly repetitive sequence regions, such as found in the barley genome, also fails without a reference scaffold (Wicker *et al.*, 2006). In this case, it can be helpful to combine Sanger with 454 pyrosequencing technique. New data handling strategies have been developed to address these specific problems of 454 pyrosequencing (Trombetti *et al.*, 2007). Despite these technical problems, the ability of high-throughput techniques to determine subtle differences in community change or metabolic potential of communities will allow 1) to describe communities in-depth and 2) to detect environmental changes at early stages of perturbation.

"Sequencing techniques"

- 454 pyrosequencing has a higher throughput capacity and simplified sample preparation than Sanger sequencing
- 454 pyrosequencing enables massively parallel sequencing reaction and produces thousands of sequences in one run
- 454 pyrosequencing has been applied in genomics, transcriptomics and metagenomics
- metagenomic approach has been yet only applied on microbial communities and revealed new insights into their dynamics and structures
- new analysis programmes and higher CPU capacity is needed to overcome to the specific bias of 454 pyrosequencing

Box 3: Public sequence databases

The largest database is GenBank at the NCBI containing currently 4,228,658 fungal sequences of 24,364 fungal species (http://www.ncbi.nlm.nih.gov/Taxonomy/txstat.cgi; as of December 2008). Uneven representation of taxonomic groups and genomic regions is observed (Figure 14). This **deposit deficiency** makes identification of environmental sequences of some fungal groups more difficult than for others. Furthermore, accuracy of many sequences in databases is questionable and hinders identification. Bridge *et al.* (2003) showed that up to **20%** of the sequences of Genbank are **incorrectly named** or of **poor quality** for reliable comparison. Ideally, public-access databases should provide a working archive of available sequences, forming a **valuable resource**, analogous to herbarium and culture collections. In an initiative to provide high-quality ITS sequences of ECM fungi, the open-access database UNITE was founded by Kõljalg *et al.* (2005). The deposited sequences derive always from herbarium specimens and are linked to informations of the date, collector/source and ecological data. Only fungal specialist, accepted by UNITE, are allowed to add sequences. Another problem of the Genbank database is the large number (**27%**) of **unidentified** sequences (Nilsson *et al.* 2006). To monitor the taxonomic progress of the own deposited unidentified sequences over time, Nilsson *et al.* (2005) wrote the programme *emerencia*, which compares regularly the datasets of unidentified sequences with the identified from GenBank. Discussion of the **accuracy of GenBank** entries has become an important issue. Bidartondo wrote with 255 other researchers (2008) an open letter to NCBI asking for a cumulative annotation process of GenBank sequences, where third parties improve annotation. Until now, NCBI has rejected annotation. Only when changes can be backed by a publication third party annotation is allowed. It is likely, given the lack of good annotations at GenBank, that new more accurate databases will be built by networks such as UNITE or FESIN (http://www.bio.utk.edu/fesin/title.htm).

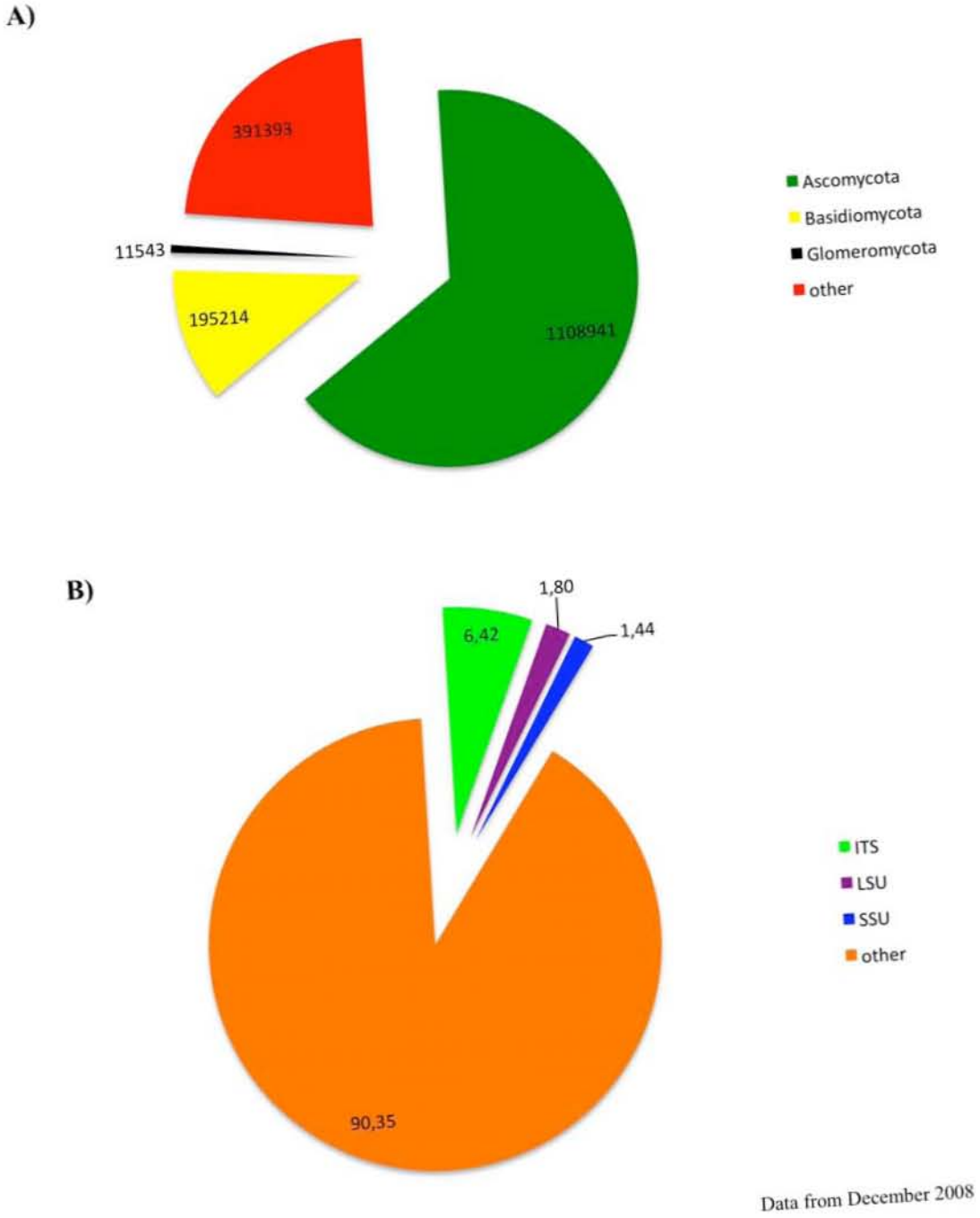


Fig. 14: Fungal sequences in GenBank. (A) Number of nucleotide sequences of *Ascomycota*, *Basidiomycota* and *Glomeromycota* and sequences from other fungi or undefined fungi. (B) Percentage of fungal rRNA nucleotide sequences.

2.4.2.3. Array technique

Microarrays consist of a solid surface, mostly a glass slide, onto which detection probes are chemically bonded. They allow a rapid parallel detection of several labelled and hybridized molecules of interest from a sample simultaneously (Figure 15). Arrays are versatile and flexible in their design and can be adjusted to different research features such as DNA, RNA or proteins. The first arrays are dot blot analysis and spotted nylon arrays (Saiki *et al.*, 1989). The first microarrays were developed with the purpose of monitoring gene expression in *Arabidopsis thaliana* (Schena *et al.*, 1995). The array technique has been further developed for complete genome analysis (complete genome array (CGA) or tilling array (Yamada *et al.*, 2003)), for tracking selected genes of key enzymes for certain metabolic pathways (functional gene array (FGA) (He *et al.*, 2007)) or for tracing and describing communities (phylochips, reviewed by Sessitsch *et al.*, 2006).

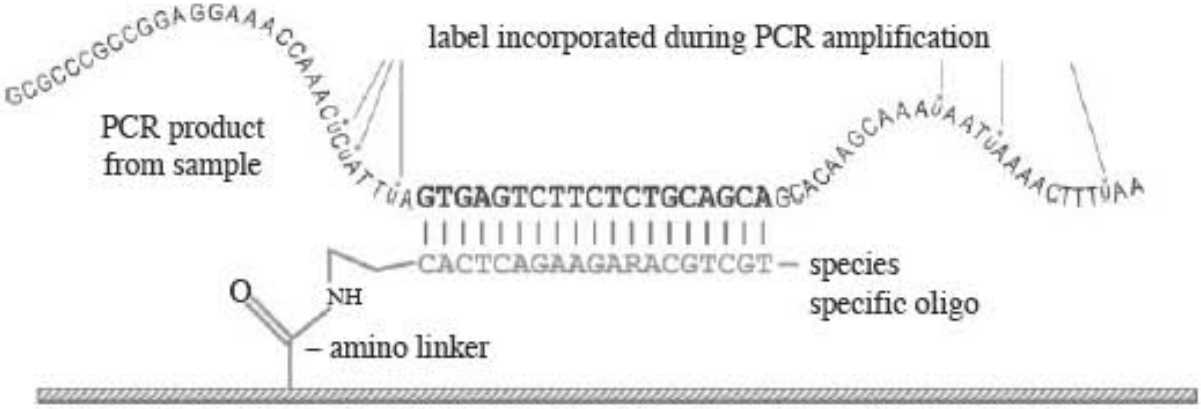


Fig. 15: Annealing of labelled amplicon with a specific oligonucleotide that is lined to a solid surface via an amino linker (Summberbell *et al.*, 2005).

Array approach used to study bacterial communities

Both, FGAs and phylochips are largely applied for bacterial community studies and can be used for large-scale detection. Brodie *et al.* (2006) developed a phylochip carrying 500,000 bacterial 16S probes to determine, if changes in microbial community composition were a factor in uranium reoxidation. Their analysis identified five clusters of bacterial subfamilies responding in different manner to the three studied reaction phases (Figure 16). They could even describe the reaction of individual members of different subfamilies in detail (e.g. the metal-reducing bacteria *Geobacteraceae*).

With a similar question in mind, He and coworkers (2007) developed the first array for studying biogeochemical processes and functional activities, the GeoChip, a type of FGA. The developed FGA carried 24,243 oligonucleotides representing more than > 10,000 genes

in greater than 150 functional groups such as nitrogen, carbon and sulfur cycling. The researchers monitored microbial community dynamics in groundwater undergoing an *in situ* biostimulation for uranium reduction and showed that the uranium concentration in groundwater was significantly correlated with the total abundance of c-type cytochrome genes. They showed that species can also be detected over functional genes using microarray approach (He *et al.*, 2007).

Another application of microarrays is in clinical diagnosis. Helpful here, as for the GeoChip, is the combination of probes used to identify individual bacterial species and probes used to identify virulence and antibiotic resistance genes on one array. Such a diagnostic tool has the potential to trace pathogens, but also to function as an early warning system for pathogenic bacteria that have been recently modified in their virulence or antibiotic resistance (Stabler *et al.*, 2008).

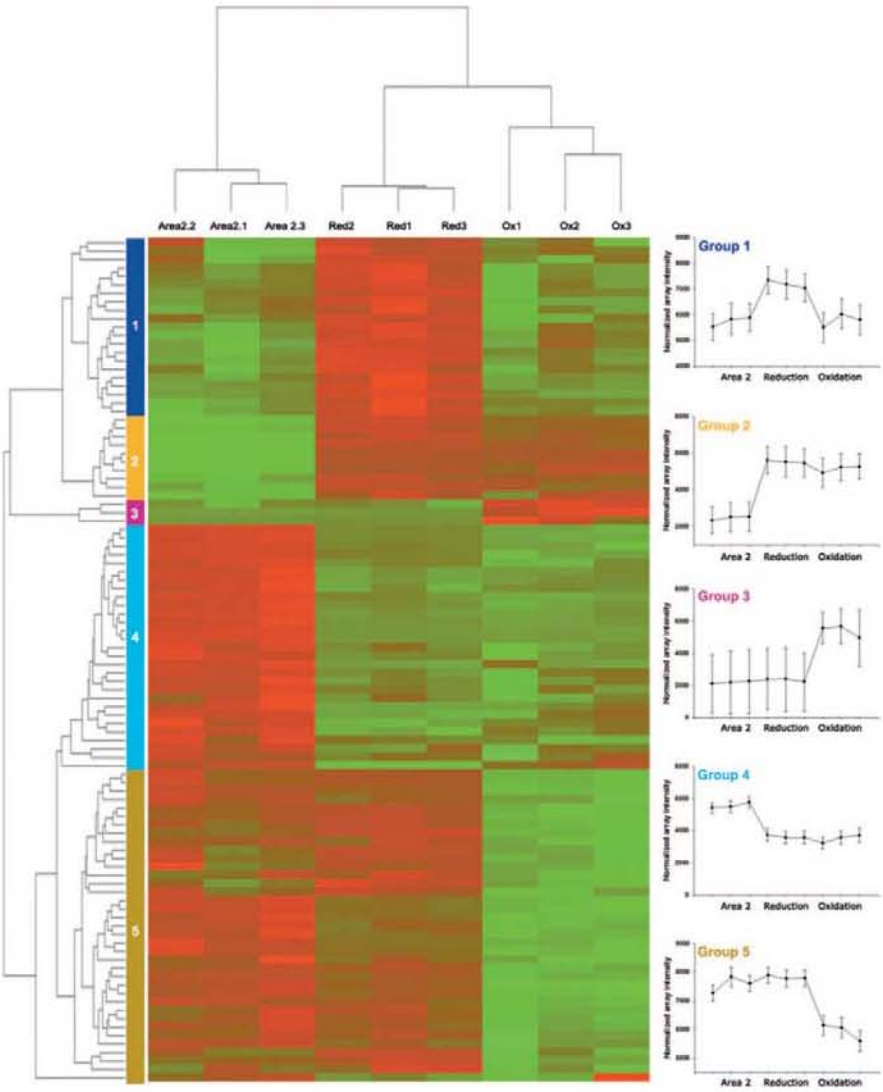


Fig. 16: Example of array analysis: heatmap and dendogram showing the response of 100 bacterial subfamilies under nine different conditions (Are2.2, 2.1, 2.3, Red2, 1, 3, Ox1, 2, 3). The colour gradient from green to red represents increasing array hybridization intensity. Five main response groups were detected, and the average intensity (HybScore) of the cluster group response is presented in line plots to the right of the heatmap (after Brodie *et al.*, 2006).

Table 2: Some operation steps, their problems and possible solutions in the development and application of DNA microarrays

Design step	Problems	Solutions/Improvements
In silico probe design	Identification of target group	• Creation of an aligned sequence database
	Assessing probe hybridization behavior	• Check probe specificity against up-to date sequence databases
		• Evaluate theoretical hybridization behavior by calculating thermodynamic properties for probe-target duplex and inter- and intra-molecular interactions of probe and target molecules
Specificity	Some probes show false-negative or false-positive signals	• Evaluate microarray by individual hybridization with reference nucleic acids; remove false-negative and highly cross-hybridizing probes
Uniform hybridization behavior	Different probes display different target-binding capacities	• Use probes with similar predicted Tm and GC
		• Uniform oligonucleotide probe length plus addition of tertiary amine salts to hybridization/ wash buffer
	Sensitivity thresholds differ among probes due to their different target binding capacities	• Determine range of sensitivity achievable with the microarray: hybridize concentration series of target organisms perfectly matching the probes with the lowest and highest duplex yield on the microarray

after Wagner *et al.*, 2007

Application of array technique in fungal research

Monitoring of fungal pathogens is also a keystone of pest management of plant diseases. Different phylochips were developed for the identification of *Fusarium* and *Verticillium* pathogens (Lievens *et al.*, 2003, 2005; Tambong *et al.*, 2006). The phylochip analysis showed the same results as plating analysis, but results were generally received within 24h. Therefore, the authors concluded that phylochip technique is rapid and efficient in the detection of pathogens because simultaneously identification of multiple species is possible (Lievens *et al.*, 2003).

Hultman and her coworkers (2008) focused on the fungal fraction in compost communities. Therefore, they constructed a microarray with fungi-specific oligonucleotides by aligning 11,881 fungal ITS sequences. They validated their phylochip by describing fungal species out of ten compost samples and confirmed the results with cloning/sequencing approach. As the phylochip showed a detection limit of 0.04% of the total DNA, it has the potential also to detect fungi on pathogenic levels.

ECM species were detected on a first-try small-scale phylochip developed by Bruns & Gardes in 1993. Five oligonucleotides designed for certain suilloid genera were used, but none of the probes exhibited their intended specificity. Using the probes collectively they worked well for identification of suilloid taxa of field collected mycorrhizae. El Karkouri *et al.* (2007) showed in dot blot analysis that identification of some *Tuber* species on their ITS motifs is possible. They traced *T. magnatum* and *T. melanosporum* in a blind test with 27 different fungal isolates showing that tracing of truffle species via DNA barcoding is possible.

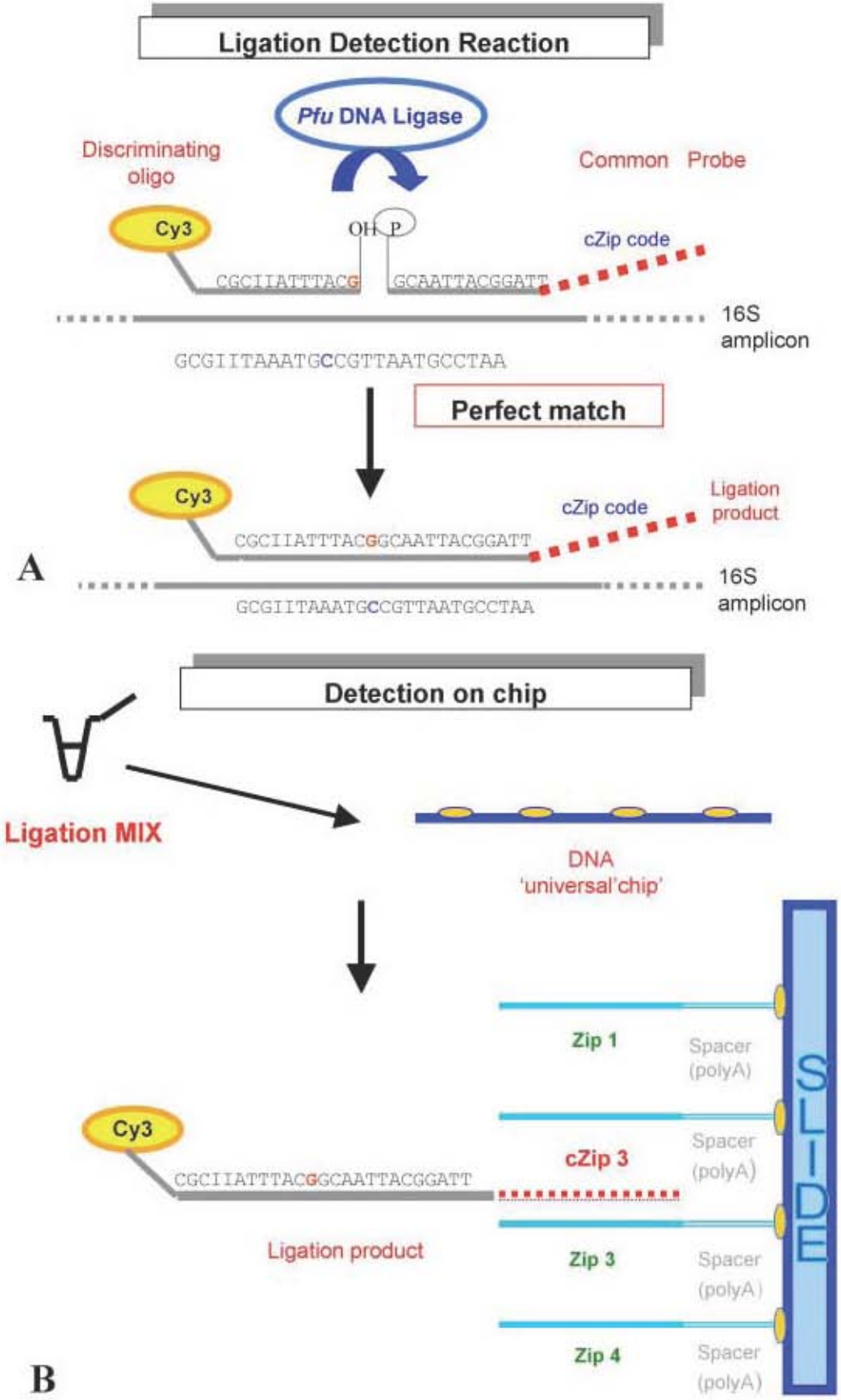
Array design/Array types

When a phylochip is designed, several steps have to be taken into account (Table 2). The first step in phylochip development is the selection of appropriate phylogenetic marker genes because good microcoding is highly dependent on the target region (see section Marker genes) and used sequences from databases (Wagner *et al.*, 2007). The specificity of the designed probes must also be tested by BLAST against public databases and against all designed probes. Furthermore, all probes must have nearly the same length, the same GC-content and should not form dimers or hairpins, which influence hybridization and signal intensity. One of the biggest problems of array technique is the detection of false positives (cross-hybridizations). Normally, the false positive signal drops out with decreasing sequence identity of probe and non-target template (Shiu & Borevitz, 2008), however, cross-hybridization is still widely reported (Sessitsch *et al.*, 2006). Therefore, it is important to

validate the designed probes *in silico* and then *in situ* and all bad probes should be deleted from array analysis. Additionally, hybridization and washing conditions should be optimized.

There are different kinds of array approaches developed to decrease the number of false interpretation. One of these approaches uses multiple probe detection, where only presence of a certain species is assumed when a certain number of the specific probes give a positive signal. Furthermore, the threshold of signal intensity, from which on a signal is considered as true positive, is carefully chosen. In another array approach a mismatch probe (MP), with an alteration at the 13th base position, is designed beside the real 25mer detection probe (DP) as found on Affimetrix arrays. After hybridization, signals are only considered as true positives, when 1) the intensity of the DP probe is "x" times greater than the intensity of the MP and 2) the difference in intensity, DP minus MP, is at least "x" times greater than the calculated value for the background (Brodie *et al.*, 2006). In another array approach, selective probes, ligation reactions and universal arrays are combined. Two probes are designed specific for one target sequence. One of the probes carries a fluorescent label and the other one a unique sequence, the so-called zipCode. In the presence of a proper template, both probes are ligated. The ligation mix is hybridized on a universal array, which is unrelated to a specific molecular analysis, but carries complementary oligonucleotides to the zipCodes. Hybridizing probes are detected via the fluorescent label (Busti *et al.*, 2002) (Figure 17). Using one of these approaches, very often the overall percentage of false positives becomes negligible (He *et al.*, 2007; Hultman *et al.*, 2008).

Fig. 17: Schematic representation of ligation detection reaction (LDR). (A) Each organism of intrest is identified by a Common Probe and a Discriminating Oligo. The common probe is phosphorylated on its 5' end and contains a unique cZip Code affixed to its 3' end. The discriminating oligo carries a fluorescent label (Cy3) on its 5' end, and a discriminating base at its 3' terminal position. The two probes hybridize adjacently to each other on the template DNA (PCR-amplified rDNA) and the nick between the two oligos is sealed by the ligase only if there is perfect complementarity at the junction. The reaction can be thermally cycled. (B) The presence of an organism is determined by hybridizing the content of a LDR to an addressable DNA Universal Array, where unique Zip Code sequences have been spotted (Busti *et al.*, 2002). see next page


Ligation Detection Reaction
Pfu DNA Ligase
Discriminating oligo
Common Probe
Cy3
OH P
cZip code
CGCIIATTTACG
GCAATTACGGATT
16S amplicon
GCGIITAAATGCCGTTAATGCCTAA
Perfect match
Cy3
cZip code
Ligation product
CGCIIATTTACGGCAATTACGGATT
A
16S amplicon
GCGIITAAATGCCGTTAATGCCTAA
Detection on chip
Ligation MIX
DNA 'universal'chip'
Zip 1
Spacer (polyA)
Cy3
cZip 3
Spacer (polyA)
CGCIIATTTACGGCAATTACGGATT
Ligation product
Zip 3
Spacer (polyA)
Zip 4
Spacer (polyA)
SLIDE
B

Pros and Cons

One problem using phylochips for community studies is that they can only detect taxa known to be present on the site. A nested approach by using probes specific on genus or family level can help to describe the cryptic species, at least taxonomically. Additionally, description of the community is not necessarily representing the active community. Nevertheless, array processing is easy to handle and results are received very quickly (within 24 h). Phylochip analysis can be used to investigate the biogeography of known species and to monitor intensively community dynamics of specific sites. Furthermore, they can be helpful in tracing specific species in clinical approaches.

"Array technique"

- four types of array exist: expression arrays, complete genome arrays, functional gene arrays (FGA), phylochips
- FGAs and phylochips have been especially developed to monitor bacterial communities and their dynamics and functions
- fungal phylochips were mostly developed to trace pathogenic fungi
- phylochips can be applied in pest management, clinical diagnosis and monitoring species
- different array approaches were developed to overcome cross-hybridization problems of probes

2.4.3. Closing words about detection techniques

I presented in the last sections different genotyping techniques. The technique used in a study should be chosen according to the focus on the studied fungi, as the techniques differ in their resolution and their specificity to detect various species. Following questions should be asked before taking a decision which detection technique will be applied in the study and how to interpret the data:

Questions for the design of the study

1. On which fungal group does my study focus?
2. How many species do I expect to find?
3. Do I want to make a detailed species inventory or do I want to get an overview on present taxonomical groups?
4. How many samples will be treated?
5. Do I want to have an exhaustive view on species richness?

Questions concerning the possibilities of a laboratory

1. Which technical facilities do I have in the laboratory?
2. How much money should be spent on the project?
3. Which skills have I, which ones my colleagues?
4. Do I have enough CPU power for evaluation of the data?
5. Do I need a bioinformatician?
6. Do I have appropriate databases to which my data can be compared?

Questions about data analysis and interpretation

1. How do I have to interpret the data?
2. Which statistical tests can I use?
3. How do I judge the results if I work on public databases?
4. Can I compare directly my results with the results of other publications?

Answering these questions may help to find the right detection technique for a research project. Coupling of different techniques can also be useful to obtain comprehensive analysis.

3. Chapter II: Development and validation of an oligonucleotide microarray to characterize ectomycorrhizal communities

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Development and validation of an oligonucleotide microarray to characterise ectomycorrhizal fungal communities

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Abstract

Background: In forest ecosystems, communities of ectomycorrhizal fungi (ECM) are influenced by several biotic and abiotic factors. To understand their underlying dynamics, ECM communities have been surveyed with ribosomal DNA-based sequencing methods. However, most identification methods are both time-consuming and limited by the number of samples that can be treated in a realistic time frame. As a result of ongoing implementation, the array technique has gained throughput capacity in terms of the number of samples and the capacity for parallel identification of several species. Thus far, although phylochips (microarrays that are used to detect species) have been mostly developed to trace bacterial communities or groups of specific fungi, no phylochip has been developed to carry oligonucleotides for several ectomycorrhizal species that belong to different genera.

Results: We have constructed a custom ribosomal DNA phylochip to identify ECM fungi. Specific oligonucleotide probes were targeted to the nuclear internal transcribed spacer (ITS) regions from 95 fungal species belonging to 21 ECM fungal genera. The phylochip was first validated using PCR amplicons of reference species. Ninety-nine percent of the tested oligonucleotides generated positive hybridisation signals with their corresponding amplicons. Cross-hybridisation was mainly restricted at the genus level, particularly for *Cortinarius* and *Lactarius* species. The phylochip was subsequently tested with environmental samples that were composed of ECM fungal DNA from spruce and beech plantation fungal communities. The results were in concordance with the ITS sequencing of morphotypes and the ITS clone library sequencing results that were obtained using the same PCR products.

Conclusion: For the first time, we developed a custom phylochip that is specific for several ectomycorrhizal fungi. To overcome cross-hybridisation problems, specific filter and evaluation strategies that used spot signal intensity were applied. Evaluation of the phylochip by hybridising environmental samples confirmed the possible application of this technology for detecting and monitoring ectomycorrhizal fungi at specific sites in a routine and reproducible manner.

Background

Ectomycorrhizal (ECM) fungi form a mutualistic symbiosis with tree roots and play key roles in forest ecosystems. In return for receiving nutrients and water from the soil via the roots, they receive carbohydrates as photosynthate from their host plants [1]. As is the case for other soil fungal species, the composition of the ECM community is affected by both biotic and abiotic factors; these include climate changes, seasons, soil micro-site heterogeneity, soil and litter quality, host tree species and forest management [2-6]. To describe in more detail the impact of environmental factors on community composition, long-term, year-round monitoring and a detailed spatial description of the community has to be carried out. However, analyses are very often hindered by a limited sample number and by the ephemeral or cryptic lifestyle of the fungi [7,8].

Over the last fifteen years, PCR-based molecular methods and DNA sequencing of nuclear and mitochondrial ribosomal DNA have been used routinely to identify mycorrhizal fungi [9]. However, these methods are time-consuming and are limited in the number of samples that can be treated in a realistic time frame [10]. With automated molecular genotyping techniques, appropriate DNA databases [11] and a better knowledge of ITS variability within fungal species [12], identification of fungal taxa in environmental samples can now be expanded from the aforementioned methods to high-throughput molecular diagnostic tools, such as phylochips [13]. So far, DNA arrays have been mainly used for genome-wide transcription profiling [14,15], but also for the identification of bacterial species from complex environmental samples [16] or for the identification of a few genera of pathogenic fungi and Oomycetes [17,18]. Phylochips may comprise up to several thousand probes that target phylogenetic marker genes, such as 16S rRNA in bacteria or the internal transcribed spacer (ITS) region in fungi [19]; indeed, the latter is one of the most widely used barcoding regions for fungi [20]. Phylochips have several advantages over traditional approaches, including higher specificity, cost efficiency, rapid identification and detection of target organisms, and the high numbers of samples throughput; therefore, they are increasingly used for the detection of bacterial and pathogenic fungi [21,22]. In the ECM fungal ecology field, the first application of ribosomal DNA arrays was reported by Bruns and Gardes [23]; they developed a specific phylochip (on nylon membranes) to detect Suilloid fungi. Recently, this approach has also been used for truffle identification [24]. To the best of our knowledge, no study has reported the construction and application of an ECM fungal phylochip to detect a large number of ECM fungal species that belong to various genera from environmental samples.

Here, we report the first application of a custom ribosomal ITS phylochip to describe the community composition of ECM fungi on roots. The phylochip carried specific oligonucleotides for 95 fungal species that belong to 25 ECM fungal genera. The specificity of the oligonucleotides was evaluated using ITS amplicons of known reference species. The method was then used to describe ECM fungal communities that were obtained from 30-year-old spruce and beech plantations. To validate the phylochip, morphotyping and ITS sequencing of the ECM root tips, together with sequencing of ITS clone libraries, were carried out. We discuss the pros and cons of the phylochip in comparison to conventional approaches, and outline its potential applications for environmental monitoring.

Results

Identification of ECM fungi from environmental samples by morphotyping/ITS sequencing and sequencing of ITS clone libraries

By combining morphotyping and ITS sequencing of individual ECM root tips, and sequencing of ITS clone libraries, 26 fungal species were identified on the roots of beech and spruce trees; these included 25 ECM fungi (Table 1). Rarefaction curves of clone library coverage nearly reached a plateau, which indicated a near complete sampling of the ECM species in the soil samples that were taken from under the beech and spruce. In order to detect only one more species from spruce samples and a further two species from beech samples, it would be necessary to increase the sequencing effort two-fold (Additional file 1). The species richness was very similar for the two plantations, with 13 and 16 species being associated with spruce and beech, respectively; however, the community compositions were clearly distinct. Only three ECM taxa were found on the root tips of both hosts: *Cenococcum geophilum*, *Xerocomus pruinatus* and *Tomentellopsis submollis* (Table 1). Sequencing of the ITS clone libraries or identification of individual ECM morphotypes revealed similar fungal ECM profiles. Most fungi that were detected on spruce roots by sequencing of the ITS library were also detected by morphotyping (Additional file 2). Of these morphotypes, nine were also supported by sequencing the ITS of individual morphotypes (Table 1). One taxon was only identified with morphotyping and ITS-sequencing of individual ECM morphotypes, and another was identified only by morphotyping. Overall, 9 of 13 taxa (69%) from the spruce roots were identified by both molecular methods. A total of 10 of 16 taxa (62.5%) from the beech roots were identified by both approaches. Sequencing of the ITS clone libraries resulted in the detection of an additional two taxa. One of these was related to an unidentified endophyte, which was difficult to identify by morphotyping alone as it is likely leaving inside the root tissues (Table 1). A single taxon was identified only by the morphotyping/ITS sequencing approach, and three

Table 1: Fungal taxa identified on root tip samples from spruce and beech by sequencing of the ITS clone libraries of the pooled ECM tips and morphotyping/ITS sequencing of the individual ECM root tips.

	Pooled ECM tips ITS cloning/ITS sequencing		Individual ECM tips Morphotyping/ITS sequencing	
Species name	**Acc. n°**	**Identities (%) (Unite◆/NCBI❍)**	**Acc. n°**	**Identities (%) (Unite◆/NCBI❍)**
ECM from *Picea abies*:				
Thelephora terrestris	EU427330.1	360/363 (100)❍	UDB000971	142/151 (94)◆
Cenococcum geophilum	UDB002297	375/379 (98)◆	UDB002297	211/216 (97)◆
Clavulina cristata	UDB001121	375/375 (100)◆	UDB 001121	281/289 (97)◆
Atheliaceae (Piloderma) sp	AY097053.1	343/362 (94)❍	EU597016.1	612/624 (98)❍
Cortinarius sp 1	AJ889974.1	361/367 (98)❍	UDB002224	232/242 (95)◆
Xerocomus pruinatus	UDB000018	348/351 (99)◆	UDB 000016	692/696 (99)◆
Tomentelopsis submollis	AM086447.1	319/324 (98)❍		morphotyping only
Inocybe sp	AY751555.1	249/266 (93)❍		morphotyping only
Xerocomus badius	UDB000080	375/379 (98)◆	UDB000080	400/417 (95)◆
Tylospora asterophora	UDB002469	353/354 (99)◆	UDB002469	591/594 (99)◆
Tylospora fibrillosa	AF052563.1	405/408 (99)❍	AJ0534922.1	561/578 (97)❍
Sebacina sp	not detected		UDB000975	162/168 (96)◆
Lactarius sp 1	not detected			morphotyping only
ECM from *Fagus sylvatica*:				
Pezizales sp	UDB002381	28/28 (100)◆	DQ990873.1	602/646 (93)❍
Sebacinaceae sp	EF619763.1	327/347 (94)❍	EF195570.1	495/497 (99)❍
Laccaria amethystina	UDB002418	356/360 (98)◆	UDB002418	276/277 (99)◆
Endophyte	AY268198.1	205/243 (84)❍	not detected	
Inocybe napipes	UDB000017	292/294 (99)◆	UDB000017	148/155 (95)◆
Xerocomus pruinatus	UDB000483	241/242 (99)◆	UDB000483	279/288 (96)◆
Cortinarius sp 2	UDB002410	416/437 (95)◆	UDB002410	227/239 (95)◆
Cortinarius sp 3	UDB002170	306/316 (96)◆	UDB002445	57/59 (96)◆
Cortinarius tortuosus	UDB002164	279/284 (98)◆	not detected	
Russula puellaris	UDB000010	313/315 (99)◆	UDB000010	246/247 (99)◆
Tomentellopsis submollis	UDB000198	272/273 (99)◆	UDB000198	224/228 (98)◆
Laccaria laccata	UDB000104	322/327 (98)◆	UDB000769	283/283 (100)◆
Cenococcum geophilum	not detected		UDB002297	216/222 (97)◆
Amanita rubescens	not detected			morphotyping only
Lactarius sp 2	not detected			morphotyping only
Tomentella sp	not detected			morphotyping only

For the sequence homology search, BLASTN was carried out with the NCBI (❍) and UNITE (◆) databases. Accession numbers (Acc. n°) and identities are given.

taxa were identified only by morphotyping. Using ITS1F and ITS4 primers [9] or NSI1/NLB4 [25], the ITS region from six ECM morphotypes (*Amanita rubescens, Inocybe sp 1, Lactarius sp 1 + 2, Tomentella sp 1, Tomentellopsis submollis*) were not amplified. The ITS regions from four fungi (*A. rubescens, Lactarius sp 1 + 2, Tomentella sp 1)* of those six morphotypes were also not amplified using the ITS clone library approach (Table 1). However, the use of the second primer pair, NSl1/NLB4, enabled the molecular biological characterisation of four morphotypes (*Piloderma sp., Sebacinaceae sp., Sebacina sp.* and *Pezizales sp.*) that were not amplified with ITS1f/ITS4.

Specificity of designed oligonucleotides

The specificity of the 95 designed oligonucleotides (Additional file 3) was evaluated using PCR amplicons that were generated from sporocarp tissues. PCR amplicons mainly hybridised to the phylochip oligonucleotides according to the expected patterns (Figure 1), and the patterns were highly reproducible in the replications conducted with each of the templates. The hybridisation signal intensities ranged from -22 (background value) to 44,835 units. Ninety-nine percent of the oligonucleotides tested generated positive hybridisation signals with their matching ITS. Cross-hybridisations were mainly observed within the *Cortinarius* and *Lactarius* species complex. Among the *Boletaceae* species, a few cross-hybridisations were observed between the species that belonged to the *Boletus* and *Xerocomus* genera. Within the *Amanita*, *Russula* or *Tricholoma* genus, rare cross-reactions occurred between single sequences from closely related species.

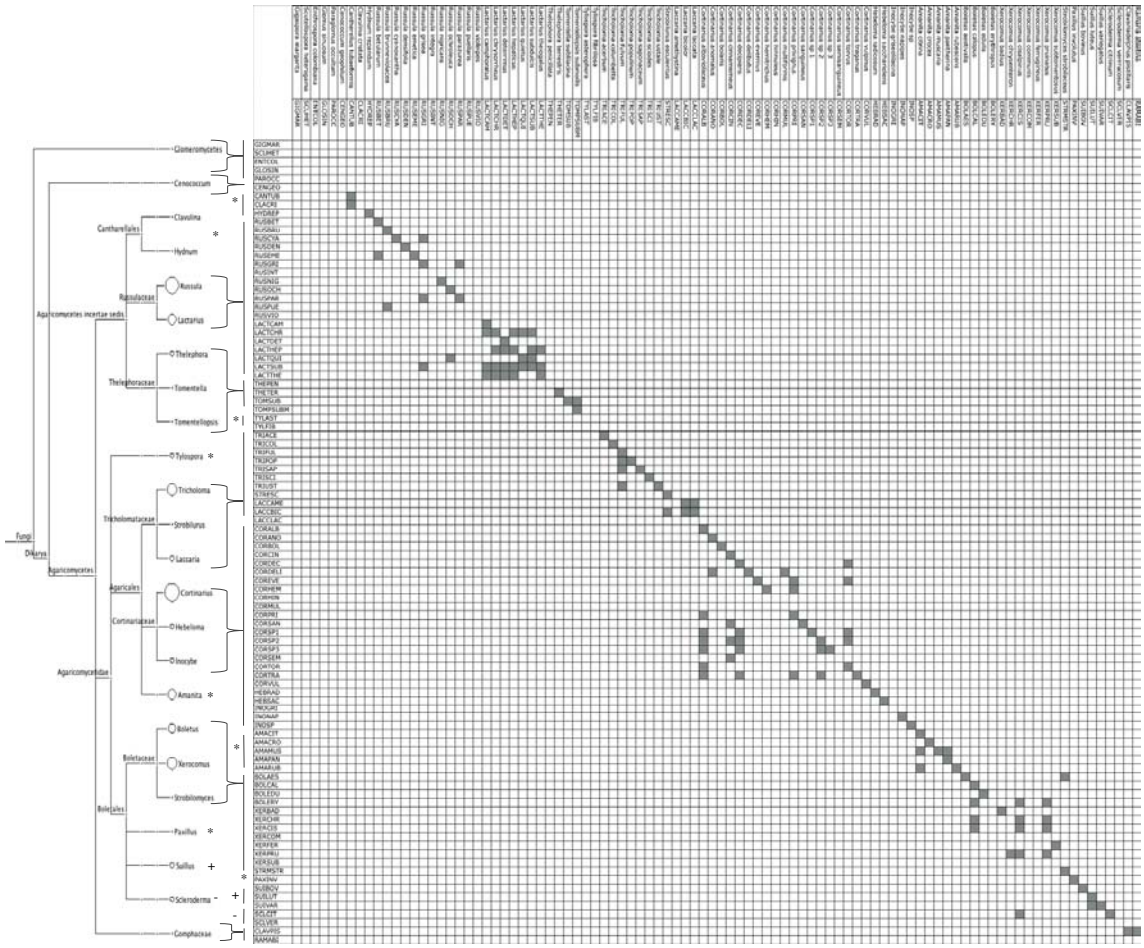

Figure 1

Hybridisation reactions of the species-specific fungal oligonucleotides. Reactions were tested by hybridising known fungal ITS pools to the phylochip. Vertical line indicates the fungal species used in the fungal ITS pools (hybridised probes), and the horizontal lines list the species-specific oligonucleotides. Grey boxes denote the positive hybridisation signals of an oligonucleotide obtained after threshold subtraction. The accompanying tree showing the phylogenetic relationship between tested fungal species was produced by the MEGAN programme. The size of the circle beside the genus name indicates the number of species of this genus used in the cross-hybridisation test.

Identification of ECM species in root samples using phylochip

The ITS amplicons that were obtained from the two different environmental root samples were labelled and hybridised to the phylochips. The phylochip analysis confirmed the presence of most of the ECM fungi that were detected with the morphotyping, with the ITS sequencing of individual ECM tips, and with the ITS clone library approaches that were obtained using the same PCR products (Table 2). The exceptions included the following fungal species for which corresponding oligonucleotides on the phylochips were lacking: *Pezizales* sp, *Atheliaceae (Piloderma)* sp, *Sebacina* sp, *Sebacinaceae* sp, and unknown endophytic species. The inability to detect these four morphotyped ECM fungal species by molecular typing suggests that these morphological identifications could be incorrect (Table 2).

Comparison of the abundance of sequences analysed by the cloning/sequencing approach and the species detection via the phylochip approach, indicated that the phylochip has the potential to detect taxa represented by approx. 2% of a DNA type in an environmental DNA sample. However, to assess the sensitivity of the current custom phylochip in more detail, further analyses will be carried out.

Discussion

Many different environmental factors influence the dynamics and the spatiotemporal structure of ECM com-

Table 2: Detection of fungal taxa from root tips of spruce and beech using different identification approaches.

Species name	Morphotyping/ITS sequencing of individual ECM tips	ITS cloning/sequencing of ECM tip pools	Phylochip
samples from *Picea abies*			
Thelephora terrestris	x	x	x
Cenococcum geophilum	x	x	x
Clavulina cristata	x	x	x
Atheliaceae (Piloderma) sp	x	x	no oligonucleotide
Cortinarius sp 1	x	x	x
Xerocomus pruinatus	x	x	x
Tomentellopsis submollis	morphotyping only	x	x
Inocybe sp	morphotyping only	x	x
Xerocomus badius	x	x	x
Tylospora asterophora	x	x	x
Tylospora fibrillosa	x	x	x
Sebacina sp	x		no oligonucleotide
Cortinarius sp 2			x
Russula integra			x
Cortinarius alboviolaceus			x
Cortinarius traganus			x
Amanita muscaria			x
Lactarius sp1	morphotyping only		
ECM from *Fagus sylvatica*			
Pezizales sp	x	x	no oligonucleotide
Sebacinaceae sp	x	x	no oligonucleotide
Laccaria amethystina	x	x	x
Endophyte sp.		x	no oligonucleotide
Inocybe napipes	x	x	x
Xerocomus pruinatus	x	x	x
Cortinarius sp 2	x	x	x
Cortinarius sp 3	x	x	x
Cortinarius tortuosus		x	x
Russula puellaris	x	x	x
Tomentellopsis submollis	x	x	x
Laccaria laccata	x	x	x
Cenococcum geophilum	x		x
Cortinarius sp 1			x
Cortinarius hinnuleus			x
Russula integra			x
Laccaria bicolor			x
Amanita rubescens	morphotyping only		
Lactarius sp2	morphotyping only		
Tomentella sp	morphotyping only		

munities [26,27,5,4]. A better understanding of the mechanisms underlying these dynamics will require year-round ECM monitoring at incrementally increased spatial resolutions. However, the limited number of samples that can currently be analysed hinders the use of molecular approaches for large-scale studies. With the ongoing development of high-throughput molecular diagnostic tools, such as DNA oligoarrays [19] and 454 pyrosequencing [28], larger scale surveys (in terms of both the frequency and depth of analysis) of soil fungi are now possible. Ecologically relevant sample throughput in the in the 100 to 1000 range is now accessible. So far, phylochips have been used for the identification of bacteria [29], viruses [30], and a few genera of closely related fungal species [18].

In the present study, we constructed a custom ribosomal DNA phylochip for the identification of ECM fungi that was based on the ITS1 and ITS2 regions. One of the great advantages of using ITS regions for oligonucleotide design is the high number of sequences that are available in public databases [12]. Furthermore, these regions are some of the most frequently used regions for the barcoding of ECM fungi [20], and compared to other possible barcoding regions, they show a high specificity at the species level [31]. We designed a total of 95 oligonucleotides,

from which 89 were species-specific for ECM fungal species. According to regular fruiting body surveys, these 89 ECM species are the most common species to be found in the long-term observatory of the Breuil-Chenue forest over the last ten years [32]. The ease with which high-quality species-specific oligonucleotides could be selected (mismatch in the middle of the designed oligonucleotide, without forming secondary structures), depended on the fungal genera. For example, the ITS sequences of *Laccaria* species showed only a few discriminative nucleotides that were spread as single nucleotide polymorphisms over the ITS1 and ITS2 regions. Consequently, prior to synthesis, oligonucleotide sequences were screened *in silico* for the presence of fortuitous similarities with fungal ITS sequences for which they were not designed.

The specificity of the spotted oligonucleotides was tested by hybridising ITS amplicons from reference species. Most of the oligonucleotides exhibited the expected hybridisation patterns (99% of the tested probes gave a positive signal with their corresponding ITS amplicon). However, cross-hybridisation was observed and it accumulated particularly in the genera *Cortinarius* or *Lactarius* that targeted other species in the same genus (Figure 1). With an estimated 2,000 spp. worldwide, *Cortinarius* is the most species-rich genus of mushroom-forming ECM fungi. Species delimitation within this genus is often controversial [33]. For these cryptic species, as for *Lactarius* or *Inocybe* species, the phylogenetic separation of species is ambiguous; indeed, most of these fungi have less than 3% intra-specific variability in the ITS region of their nuclear ribosomal DNA [34]. To keep cross-hybridisation low, we used a two-step data filtering process that involved: (i) accepting only spots with a significantly higher signal intensity value than the one obtained for the negative controls and, (ii) the requirement for a positive signal for at least four of the six replicates of one spot (see Methods). The hybridisation results were identical over the different replicates.

To test whether the current custom phylochip could be utilised in environmental studies that sought to describe the composition of an ECM community, ITS amplicons of root samples taken from beech and spruce plantations were hybridised to the array. As the focus of the current study was the validation of the phylochip, rather than an ecological study of the whole ECM fungal communities of the two plantations, a total of only six soil cores were used. The results of the phylochip were compared to the results that were obtained from the morphotyping/ITS-sequencing of individual ECM morphotypes and the sequencing of ITS clone libraries. Provided that the corresponding oligonucleotides were included on the array, all species that were detected by cloning-sequencing could also be identified with the phylochip. As the corresponding oligonucleotides were lacking on the phylochip, species belonging to the *Atheliaceae*, *Sebacinaceae* or *Pezizales* were not detected. Furthermore, the comparison of array signal intensity with ITS sequence frequency in the ITS clone library revealed the potential of the phylochip to detect taxa that were represented by approx. 2% of DNA types in the amplified DNA sample. However, the quantitative potential of this custom phylochip remains to be further accessed as bias linked to the PCR amplification could take place. The phylochip also detected species that were not expected according to the results obtained from the use of the other two approaches. This could be due to cross-hybridisations and/or to the fact that these under-represented species in the community could not be detected by the other approaches as the rarefaction curves of the ITS library sequencing method did not reach a plateau (Additional file 1).

When compared to each other, both of the other approaches provided similar, but not identical, profiles of the ECM communities. Approximately 70% of the species were detected using either method individually (Table 1). For the beech sample, three species were detected only by morphotyping as the PCR amplification of their DNA using ITS1F/ITS4 and/or NSI1/NLB4 primer pairs failed. Tedersoo et al. [35] showed that PCR of ITS from several ECM species failed using these universal fungal rDNA primers, and they stressed the need for additional taxon-specific PCR primers to be used for comprehensive genotyping of ECM communities. One of the morphotypes detected in the beech sample was a *Lactarius* species. In the same root sample, a *Pezizales* species was found by ITS-sequencing and cloning/sequencing; this suggests a possible co-colonisation of the ECM root tip [36]. ECM root tips can be colonised by more than one fungal taxon, by two different ECM species, or by one ECM species and an endophytic or parasitic species. Typically, these species are overlooked by the use of only morphotyping, but they can be detected by molecular biological approaches.

Conclusion

In this study, we demonstrated that identification of ECM fungi in environmental studies is possible using a custom phylochip. The detection of most of the species by the phylochip was confirmed by two other widely used detection methods. Although the possible application of the phylochip technique to other study areas is dependent on the fungal species to be analysed, high-quality sequence support for several temperate and boreal forest ecosystems is found in databases such as UNITE [11]. For the next generation of phylochips, we will add additional species-specific probes or use additional marker gene regions in the probe design to overcome the small number of observed cross-hybridisations. In addition, we will increase the number of specific oligonucleotides that are spotted onto the phylochip (up to 10,000) to adapt to the

taxonomic diversity found in soils at the study sites. Small-scale phylochips, so-called "boutique" arrays, such as the one designed in this study, are a time-saving and cheap approach for monitoring specific fungal species over years and/or in several hundred of samples. At the present time, the detection of a single species with our custom phylochip cost only one sixth of the price paid for the cloning/sequencing approach. The upscaling of detectable species on the phylochip (up to 10,000) will further lower the cost (by a factor of twenty). Thus, the phylochip approach should be an attractive method for routine, accurate and reproducible monitoring of fungal species on specific sites, in which a high sample throughput is required.

Methods

Site description and root sampling

The Breuil-Chenue experimental site is a temperate forest located in the Morvan Mountains (47°18'10"N, 4°4'44"E, France) at 650 m. The parent rock is granite and the soil is an alocrisol that is characterised by a pH ranging between 4 and 4.5, with moder type humus and micropodzolisation features in the upper mineral horizon. In 1976, a part of the original stand, composed mainly of beech (90% of the stems), oak and young birch on a homogeneous soil type, was clear-cut. Subsequently, beech (*Fagus sylvatica L.*) and spruce (*Picea abies (L.) H. Karsten*) were planted separately in 20 m by 20 m adjacent stands [37].

Sampling of the root tips was performed in each stand (beech and spruce) in October 2007. A drill was used to obtain three soil cores (4 cm diameter × 10 cm depth) from each of the two treatments, along 18 m transects in the middle of each of the two plantations. The distance between the soil cores was 6 m, and the samples were collected at distances of more than 0.5 m from the trees or the stumps. Soil cores were immediately transported to the laboratory in isotherm boxes and stored at 4°C. Within five days, the roots were manually separated from the adhering soil, gently washed, and then examined under a stereomicroscope at 40×. Morphological typing of all of the ECM tips (approximately 50-250 tips per sample) was performed according to Agerer [38].

ITS sequencing

An individual ECM root tip from each ECM morphotype was selected for molecular characterisation by ITS sequencing. The remainders of the ECM root tips in each sample were used for ITS amplification, cloning and sequencing, and phylochip analysis (Figure 2). The samples were conserved at -20°C. DNA was extracted from single ECM root tips, or from the pooled ECM tips, and it was subjected to PCR amplification to produce a specific ITS amplicon or a heterogeneous mixture of ITS sequences (Figure 2), respectively. ITS amplicons from single tips were directly sequenced. Heterogeneous mixtures of sequences were either used to construct ITS clone libraries or used directly for phylochip hybridisation.

The ECM roots (up to 100 mg fresh weight depending on the sample) were freeze-dried and ground in a ball mill MM200 (Retsch®, Haan, Germany). Ground tissue was resuspended in 400 µl AP1 buffer from the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France), and the DNA was extracted according to the manufacturer's instructions. Purified DNA was solubilised in dH_2O (~100 ng/µl) and stored at -80°C. The ITS was amplified as described in Buée et al. [5], using primers ITS1F and ITS4 [9] and/or NSI1 and NLB4 [25]. PCR products were purified using a 96-well filtration system (MultiScreen-PCR plates, Millipore Corporation, MA, USA) and sequenced with ITS1F and/or ITS4 primers and the Genome Lab DTCS Quick Start Kit (Beckman Coulter, Roissy CDG, France), using a CEQ 8000XL sequencer and the CEQ 8000 Genetic Analysis System. ITS sequences were assembled with the Sequencher program for Macintosh, version 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, USA), when sharing ≥ 97.0% identity. To identify the ECM fungi, BlastN was performed using ITS sequences that are available in the following public databases: NCBI http://www.ncbi.nlm.nih.gov/, UNITE http://unite.ut.ee/ and MycorWeb http://mycor.nancy.inra.fr/. ECM fungal morphotypes were considered to be identified at the species level when they shared ≥ 97% of their ITS region sequence identity with a sequence in these public databases [35].

Sporocarp collection and taxonomic identification

Three times per year, during the autumnal periods of 2004 to 2007, fungal sporocarps of all epigeous fungi were surveyed at the Breuil-Chenue experimental site, and mature fungal fruiting bodies that exhibited all the characteristics necessary for an unequivocal identification, were collected. An expert mycologist, Jean Paul Maurice (Groupe Mycologique Vosgien, 88300 Neufchâteau, France), used traditional mycological methods for taxonomic determination of the sporocarps [39]. They were named according to the new "French Reference of Mycology" http://www.mycofrance.org. Samples were taken from the inner cap tissue (50-100 mg) and ground using a ball mill MM 200 (Retsch). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. The ITS regions were amplified as described above, and they were used for hybridising the phylochips to assess the specificity of the designed oligonucleotides (see below).

Cloning and sequencing of ITS

Prior to cloning, the amplified ITS products that were obtained from the bulk ECM tips of all soil cores were

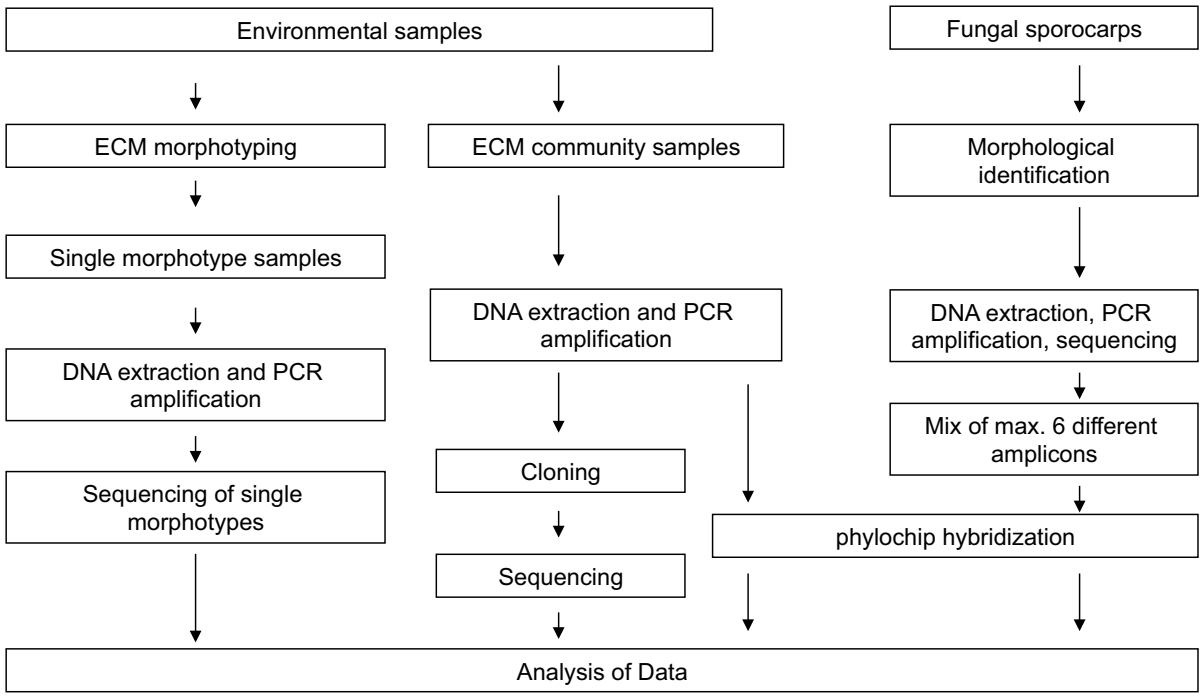

Figure 2
The different procedures used for molecular genotyping of ECM root tips and evaluation of the phylochip. DNA was extracted from individual ECM root tips or from pooled ECM root tips and subjected to PCR amplification to produce specific ECM ITS sequence or a heterogeneous mixture of ITS sequences, respectively. Individual ITS sequences were directly sequenced. The heterogeneous mixture of ITS sequences (ITS clone libraries) were either separated into individual molecules by cloning in bacterial plasmids or used directly for microarray hybridisation. The results of these three different technical approaches were analysed and compared. In addition, to test the specificity of the spotted oligonucleotides, the phylochips were hybridised with a heterogeneous mixture of ITS sequences from identified fungal sporocarps.

pooled to obtain only two samples: one sample each for the beech and spruce plantations. The amplified ITS fragments were cloned into *Escherichia coli* plasmids with the TOPO TA Cloning Kit, using the pCR®2.1-TOPO plasmid vector with a LacZα gene and One Shot DH5α chemically competent *Escherichia coli*, according to the manufacturer's instructions (Invitrogen, Cergy Pontoise Cedex, France). Seventy white recombinant colonies were selected; they were cultured overnight in LB medium and then frozen in glycerol at -80°C. Three microlitres of these bacterial suspensions were used directly for PCR, amplifying the inserts with M13-F (5'-GTAAAACGACGGCCAG-3') and M13-R (5'-CAGGAAACAGCTATGAC-3') primers. PCR was performed using the following protocol: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 30 s and 72°C for 3 min, with a final extension step at 72°C for 15 min. The PCR products were purified with MultiScreen HTS™ PCR filter plates (Millipore, Molsheim, France). Sequencing was performed with a CEQ 8000XL sequencer (as described above), in which the ITS1F and ITS4 primer pairs were used to obtain sequences with lengths of up to 600 bp that included the ITS1 region and part of the ITS2 region. Sequences were edited as described above. The sequences can be accessed in public databases using the accession number FN545289 - 545352. In addition, a rarefaction analysis was performed to measure the proportion of the estimated diversity that could be reached by sequence effort using the freeware software Analytic Rarefaction version 1.3 http://www.uga.edu/strata/software/Software.html.

Design of specific ITS oligonucleotide probes

To design specific ITS oligonucleotide probes for 89 ECM species, 368 ITS sequences of 171 ECM fungal species (around 600 bp) were aligned with the MultAlin program [40]. To take into account intraspecific ITS variability and sequencing errors, several ITS sequences from a number of

different species were used for the alignment. Single nucleotide polymorphisms and indels were identified by manual curation. The sequences, including the ITS1, 5.8S and ITS2 regions of the nuclear rRNA genes, were obtained from the public databases NCBI and UNITE. Perfectly matching oligonucleotides, 67 to 70 bases in length, were designed for each ITS sequence within the ITS1 or ITS2 regions. They were selected for optimal melting temperatures (Tm; 75°C ± 2.5°C) and GC content (45-55%) using the AmplifX 1.37 software http://ifrjr.nord.univ-mrs.fr/AmplifX. To enhance specificity, oligonucleotides that had selective nucleotides located in a central position were favoured. The specificity of the oligoprobes was first tested *in silico* by querying the oligonucleotide sequences against the UNITE and NCBI databases. An oligonucleotide was designed as a positive hybridisation control on the ITS region of *Arabidopsis thaliana*. Five additional 62- to 70-mer oligonucleotides that matched the LSU region of the *Glomeromycota* were used to measure the background signal resulting from unspecific hybridisation. To avoid cross-hybridisations with undescribed species or cryptic species, we did not use the ITS region of untargeted fungal groups as a negative control.

Spotting of glass slide microarray and hybridisation conditions

The 95 species-specific oligonucleotides (see above) were spotted; one well was spotted with only hybridisation buffer. Solutions of species-specific oligonucleotides were adjusted to a concentration of 600 pM and printed in triplicate by Eurofins, MWG/Operon (Cologne, Germany) on slide arrays with an activated epoxide surface. Oligonucleotides were bound via their 5' ends on the coating layer of the glass surface (for details, see http://www.operon.com). Arrays were prehybridised using the OpArray Pre-Hyb solution (Eurofins, MWG/Operon) according to the manufacturer's instructions. PCR-generated amplicons (maximal 30 ng/μl) were labelled with Alexa Fluor® 555 dye (Invitrogen, Cergy Pontoise, France) using the BioPrime® Plus Array CGH Indirect Genomic Labelling System Kit (Invitrogen) following the manufacturer's instructions. After the last purification step, labelled amplicons were concentrated with a vacuum concentrator centrifuge UNIVAPO 100 H (UNIEQUIP, Martinsried, Germany), and then dissolved in 7 μl sterile water. The sample hybridisation procedure followed Rinaldi *et al*. [41] and is fully described in sample series GSM162978 in the GEO at NCBI http://www.ncbi.nlm.nih.gov/geo/. Slide arrays were scanned using a GenePix 4000 B scanner (Axon-Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 532 nm for the Alexa Fluor 555 dye. Fluorescent images were captured as TIFF files and the signal intensity was quantified by GenePix Pro 5.0 software (Axon-Molecular Devices).

Specificity of oligonucleotides and validation of the phylochip

To validate the specificity of the designed oligonucleotides, PCR-amplified ITS fragments from the sporocarp tissues of known fungal species were hybridised (Figure 2). Prior to hybridisation, amplicons (5 ng/μl) from three to six different ITS amplicons were mixed in a 1:1 ratio. Species-specific ITS within a mix were chosen based on the *in silico* tested species phylogenetic distance (minimal 30% bp differences were observed between the oligonucleotides of one species and the ITS sequences of the other species in the mix). In total, 74 fungal species were probed via the fungal amplicon mixes. The PCR product that was amplified from the ITS region of *Arabidopsis thaliana* was added to all amplicon mixes (at a concentration of 5 ng/μl) as a positive hybridisation control. To test the possible use of this custom phylochip for describing ECM community composition in environmental samples, 10 μl of the PCR product that was amplified from the bulked ECM root tips of beech and spruce was used (spiked with the amplicon of *Arabidopsis thaliana)*. Six technical replicates were carried out for each sample (three block replications per slide × two slides per sample). The results of the cross-hybridisation test are outlined in Figure 1. The ITS-based cladogram was constructed for all tested fungal species using the default setting of the MEGAN software (version 3.0.2., [42]).

Array evaluation

Prior to further analyses, spots exhibiting poor quality (for example, as a result of the presence of dust) were flagged and excluded from the analyses. Hybridisation quality was surveyed using the positive (oligonucleotides of *Arabidopsis thaliana*) and negative controls (five oligonucleotides for the *Glomeromycota* (non-ECM species) and the one spot spotted with only hybridisation buffer) of each array. Data of the array were further used when (i) signal intensity values of the positive controls were within the group of oligonucleotides that showed the highest signal intensity values and (ii) the mean signal intensity value of the negative controls were a maximal 1.5% of the signal intensity with the highest value.

Individual spots were considered to be positive (species present in the sample) if their signal intensity showed a value that was five-fold higher than the averaged intensity value for all of the negative controls. Additionally, at least four of the six replicates per spot were required to generate a significant positive hybridisation. The threshold factor was fixed to five-fold after evaluation of the results of the arrays that were hybridised with the known amplicon mixes derived from sporocarp tissues (see "Sporocarp collection" and "Specificity of oligonucleotides"). Using a threshold factor of "5" defined the minimal 90% of all

species in the amplicon mixes as positive and filtered most false-positives (cross-hybridisation).

Authors' contributions

MR conceived and designed the array, set-up the clone library, acquired, analysed, and interpreted the data and drafted the manuscript. AK analysed and interpreted the array data. FM conceived and directed the project and drafted the manuscript. MB carried out the morphotyping and sequencing of the ECM root tips, drafted the manuscript and co-directed the project. All authors read and approved the final manuscript.

Additional material

Additional file 1

Rarefied species accumulation curve of fungal species detected in ECM root tip samples of (A) spruce and (B) beech. *Figures of the rarefaction curves of detected fungal species in ECM root tips of spruce and beech.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-241-S1.PDF]

Additional file 2

Species described by morphotyping with description of observed morphotypes according to Agerer (1987-2001). *List of all ECM species detected by morphotyping and detailed description of their morphotypes.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-241-S2.PDF]

Additional file 3

Sequences of the 95 species-specific oligonucleotides. *List of sequences of the 95 designed species-specific oligonucleotides.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-241-S3.PDF]

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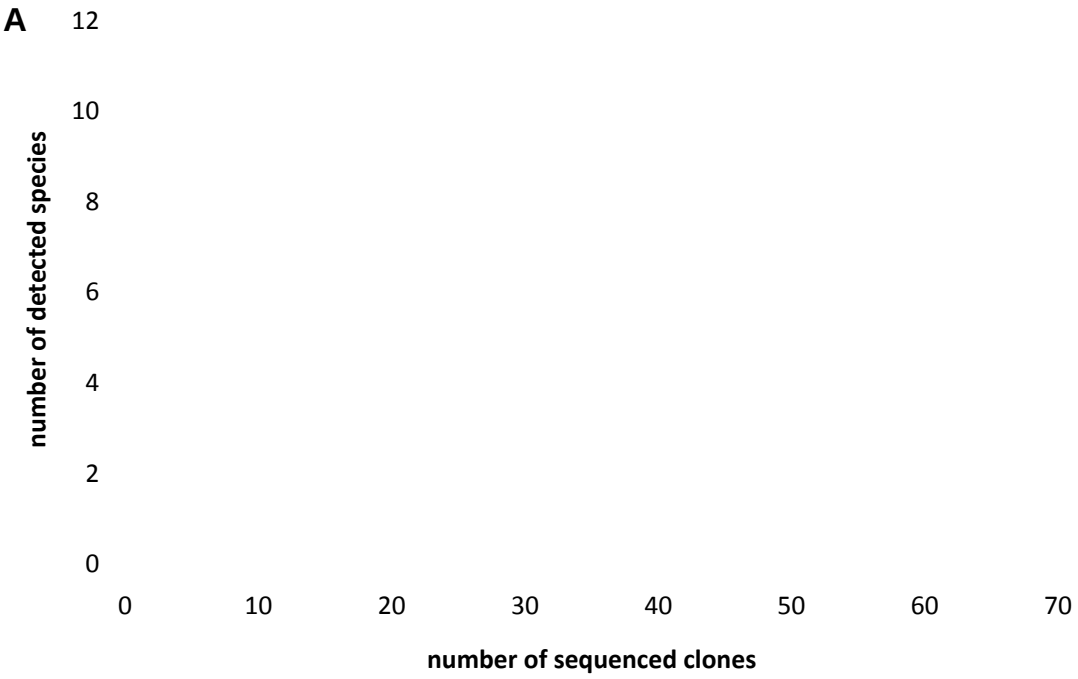
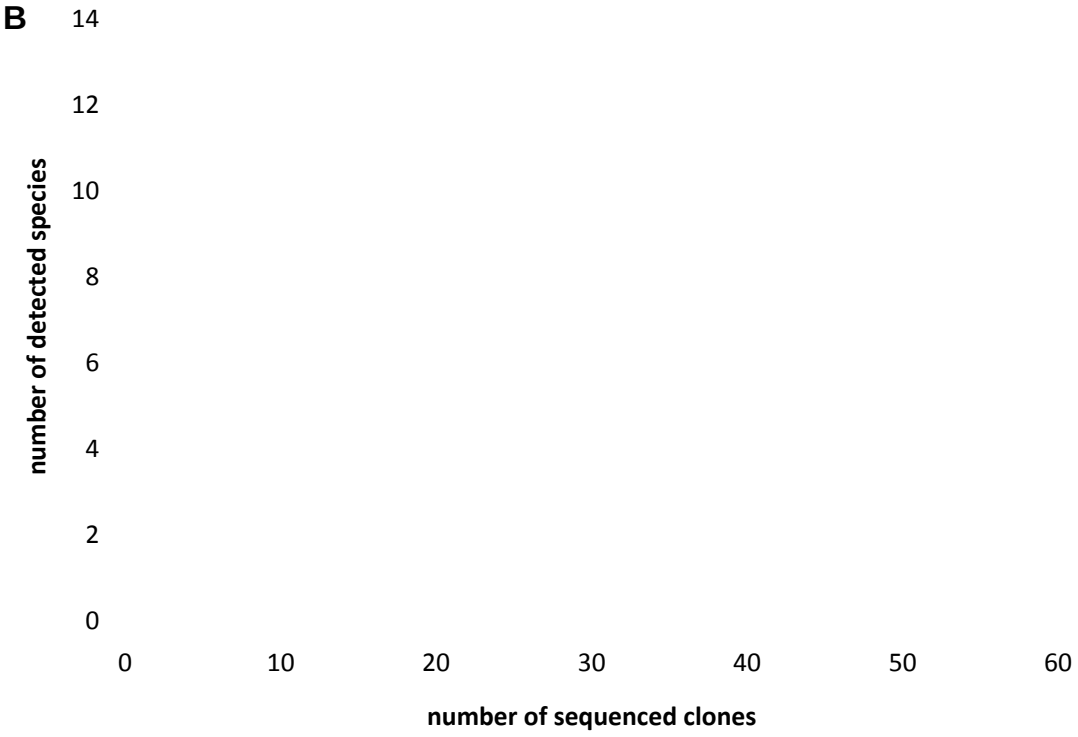
Additional file 1: Rarefied species accumulation curve of fungal species detected in ECM root tip samples of (A) spruce and (B) beech.





Additional file 2: Species described by morphotyping with description of observed morphotype according to Agerer (1987-2001).

ECM fungal species	Morphotyping
ECM from *Picea abies*	
Cenococcum geophilum	Dark and shiny mycorrhizal mantle with adhering soil debris within hyphae
Clavulina cristata	Monopodial mycorrhizae with velvet white mantle
Cortinarius sp1	Tortuous mycorrhizal tips with emanting hyphae and white rhizomorphs
Inocybe sp	Monopodial mycorrhizae, silvery and velvet mantle (cystidia) coated by soil particules
Lactarius sp 1	Pyramidal mycorrhizae, smooth mantle with laticifers
Piloderma sp	Branched mycorrhizae with cottony hyphae and few pale rhizomorphs on yellow-brown mantle
Sebacina sp	Monopodial mycorrhizae with pearly white mantle
Thelephora terrestris	Monopodial-pinnate mycorrhizal system, smooth yellow-brown mantle
Tomentellopsis submollis	Tortuous mycorrhizal tips with white emanting hyphae and pinkish rhizomorphs
Tylospora asterophora	Monopodial-pyramidal mycorrhizae, yellow-white smooth mantle and emanating hyphae
Tylospora fibrillosa	Pyramidal mycorrhizae, yellow-white smooth mantle and cottony hyphae
Xerocomus badius	Branched mycorrhizae with rhizomorphs, shiny-yellow mantle (presence of air)
Xerocomus pruinatus	Monopodial-pyramidal mycorrhizae with rhizomorphs, white mantle (presence of air)
ECM from *Fagus sylvatica*	
Amanita rubescens	Monopodial mycorrhizae with velvety claret-coloured mantle
Cenococcum geophilum	Dark and shiny mycorrhizal mantle with adhering soil debris within hyphae
Cortinarius sp 2	Tortuous mycorrhizal tips with cottony hyphae
Cortinarius sp 3	Tortuous mycorrhizal tips with emanting hyphae and white rhizomorphs
Inocybe napipes	Irregulary pinnate mycorrhiza, emanating hyphae enveloping mycorrhizal system
Laccaria amethystina	Monopodial mycorrhiza with purple tips and velvet surface mantle (emanating hyphae)
Laccaria laccata	Monopodial mycorrhiza with velvet honey-yellowish surface mantle
Lactarius sp 2	Pyramidal mycorrhiza, smooth honey-yellow mantle (laticiferous hyphae)
Pezizales sp	Dichotomous mycorrhizae with pale yellow mantle
Russula puellaris	Monopodial-pyramidal mycorrhizae, yellowish mantle with presence of air (light shiny)
Sebacinaceae sp	Dichotomous mycorrhiza, smooth greenish mantle
Tomentella sp	Monopodial-pinnate mycorrhiza with grainy brown mantle (few emanating hyphae)
Tomentellopsis submollis	Dichotomous mycorrhizae with several pink rhizomorphs on white mantle (with air)
Xerocomus pruinatus	Monopodial-pyramidal mycorrhizae with rhizomorphs, silvery-white mantle (presence of air)

Additional file 3: Sequences of the 95 species-specific oligonucleotides.

Species	Abbreviation	Oligonucleotide Sequence (5'-3')
Boletus aestivalis	BOLAES	GAGTGTCATGGAATTCTCAACCGTGTCCTCGATCTGATCTCGAGGCATGGCTTGGACTTGGGAGTTGCTG
Boletus calopus	BOLCAL	TTGAGTGTCATCGAATTCTCAACCATGTCTCGATCTATTTCAAGGCATGGCTTGGAGTTGGGGGTTTGCT
Boletus edulis	BOLEDU	CCTGAAATGCATTAGCGATGTTCAGCAAGCCTGAACGTGCACGGCCTTTTCGACGTGATAACGATCGTCG
Boletus erythropus	BOLERY	TTGAGTGTCATTTGAATTCTCAACCATGTCTTGATTGATTTCGAGGCATGGCTTGGACTTGGGGGTTGCT
Strobilomyces strobilaceus	STRMSTR	GCAAAGACGTCGGCTCTCCTCAAACGCATGAGCGGGACTAGCATGTCCGGACGTG
Xerocomus badius	XERBAD	GAGGATCTATGTATTTCATCATCACACCTATCGTATGTCTAGAATGTCATCGTCGACCACTGGGCGGCGA
Xerocomus chrysenteron	XERCHR	GTGCACGTCCACCTTTCTCTTACTCTCACACCTGTGCACACATTGTAGGTCCTCGAAAGAGGATCTATGT
Xerocomus cisalpinus	XERCIS	GAAAGCGGTCGGCTCTCCTGAAATGCATTAGCAAAGGACAGCAAGTCTGACGTGCACGGCCTTGACG
Xerocomus communis	XERCOM	GACGTGATAATGATCGTCGTGGGCTGAAGCGTCGGACATGCATCGATTGTCTTGTTTGCTTCCAAATCAC
Xerocomus ferrugineus	XERFER	CGTCCCCTCACCTTTTCTATCTACACACACCTGTGCACCTATTGTAGATCCCTCTCGAAAGAGAGGGAA
Xerocomus pruinatus	XERPRU	TTCGCTGTGCACGTCTTTCTTTTCGTCGACCTTTCTCTTACTCTCACACCTGTGCACACACTGTAGGT
Xerocomus subtomentosus	XERSUB	CTTTCTTCTTTCTTGGATGGAAAGTATGGCTTGGAGTTGGGAGTTGCTGGCAGAGACTGTCAGCTCTCC
Paxillus involutus	PAXINV	GCCTTTCCTTTGGAAGACCCTTTCTTCACACCCGTGCACACATTGTAGGTCTCCGCGAGGGGATCTATGT
Scleroderma citrinum	SCLCIT	GCATGTCTACAGAATGTCGTCCGTGGCGTCGGGCCACCGTAAACCATAATACAACTTTCAGCGATGGAT
Scleroderma verrucosum	SCLVER	GAGTGTCATCGAAACCTCAGACCGACCCTTCGACCCCGTCGGAGCTCGGTCTGGACTTGTGGGAGTCTGC
Suillus bovinus	SUIBOV	CAACTCCTCTCGATTGACTTCGAGTGGAGCTTGGATAGTGGGGGCTGCCGGAGACCTGAATATTCGTGTT
Suillus luteus	SUILUT	CGGAGACACTGGATTCGTCCAGGACTCGGGCTCCTCTTAAATGAATCGGCTCGCGGTCGACTTTCGACTT
Suillus variegatus	SUIVAR	GACCCGCGTCTTCATAAGCCCCTTCGTGTAGAAAGTCTATGAATGTTTTTACCATCATCGACTCGCGACT
Lactarius camphorata	LACTCAM	CGCTGGCTTTCAACGTTGTTGCACGCCGGAGCGTGTCCTCTCACATAACACAATCCATCTCACCCTTTGT
Lactarius chrysorrheus	LACTCHR	CCTCTCACATAATAATCCATCTCACCCTTTGTGCACCACCGCGTGGGCACCCTTTGGGATC
Lactarius deterrimus	LACTDET	CGCTGACTTTTTGAGACACAAAAGTCGTGCACGCCGGAGTGCGTCCTCTCACATAAAATCCATCTCACCC
Lactarius hepaticus	LACTHEP	GCAAGGGCTGTCGCTGACTCTATAAAGTCGTGCACGCCCGAGTGTGTCCTCTCACATAATAATCCATCTC
Lactarius quietus	LACTQUI	CTTCTAATCGTCTCAACCTTGCATCGAGACAAACGTCTGAGCGTGGCTCCCTTCCCTGGGAAACTCTCTC
Lactarius subdulcis	LACTSUB	GCTGTCGCTGACTCAAAGTCGTGCACGCCCGAGTGTGTCCTCTCACATAAATAATCCATCTCACCCTTTG
Lactarius theiogalus	LACTTHE	GTCGTGAAAACCTCAACCTCTTTGGTTTCTTCTGGGGACCAAAGCAGGCTTGGACTTTGGAGGCCTTTTG
Russula betularum	RUSBET	GGTCATTTTCGACCGCGGAAAGGATTTTGGACTTGGAGGCCTTTTGCTGGTTTCACCTTGAAGCGAGCTC
Russula brunneviolacea	RUSBRU	CTTTTTCTTTGACGAGAAAAGGAGTTTTGGACTTGGAGGTTCAATGCCCGCTTTCGGCATCGAAAGCGAG
Russula cyanoxantha	RUSCYA	CTTCAACCTTTCTTGGTTTCTTGACCGAGGAAGGCTTGGACTTTGGGGGTCTTTCATTGCTGGCCTCTTT
Russula densifolia	RUSDEN	GTCGTGAATTTCTCAAACCTTCTTGGTTTCTTGATCAAGAAGGCTTTGGACTTTGGAGGTCTTTGCCGGC
Russula emetica	RUSEME	CTCCTCCCAAATGTATTAGTGGGGTCTGCATTGTCGGTCCTTGGCGTGATAAGTTGTTTCTACGTCTTGG
Russula grisea	RUSGRI	GTGCATCACCGCGTGGGGCCCTTCTCTTTTCGGAGAGGGGGGTTCACGTTTTTACAAGAACGAACCATTA
Russula integra	RUSINT	CCCTTTTTGTTTGAAAAGGATTTTTGGACTTGGAGGTTCCATGCTCGCCTTTGCTTTTGAAGGTGAGCTC
Russula nigricans	RUSNIG	GTCGTGAAATTCTCAAACCTTCTTGGTTCCTTGACCAAGATGGCTTTGGACTTTGGAGGCGTTGTGCTGG
Russula ochroleuca	RUSOCH	CCTTTTTCTTTTTGGGAAAGGGTTTTTGGACTTGGAGGCTTTTTGCTGGCTTCACCTTGAAGTGAGCTCC
Russula parazurea	RUSPAR	CCTTGACGTGATAAGTTTGCTTCTACGTCTTGGGTTTCGCACTGTCGCACCGGAACCTGCTTCCAACCGT

Russula puellaris	RUSPUE	CCTTTTGTGCATCACCGCGTGGGTCCCCCTTTGCGGGAGGGCTCGCGTTTTCACATAAAACTTGATACAG
Russula violeipes	RUSVIO	GCGTGGGCCACCTTCTTTGGCTTGTTTCAAAGAGGTCGGTTCACGTTTTTACACACACACACCTTTATG
Amanita citrina	AMACIT	AGGTCCTTATGCAGCATGCAGGGAACTTTTGGACATTGGGAGTTGCTGGTCACTGATAAAGTGGCTGGCT
Amanita crocea	AMACRO	CCTGTGCACCGCCTGTAGACACTCTGTGTCTATGATATATGTCACACACACACACACAGTTGTTTTAGGC
Amanita muscaria	AMAMUS	GTCAAAACATGCACTTGAGTGTGTTTTGGATTGTGGGAGTGTCTGCTGGCTTTATGAGCCAGCTCTCCTG
Amanita pantherina	AMAPAN	CCTGAAAGACATTAGCTTTGGAGGGATGTGCCAAGTCGCTTCTGCCTTTCCATTGGTGTGATAGACG
Amanita rubescens	AMARUB	TGGGATTTTTGGACATTGGGAGTTGCCGGCTGCTGATAAAGTGGTGGGCTCTTCTGAAAAGCATTAGTTG
Laccaria amethystina	LACCAME	GGATACCTCTCGAGGCAACTCGGATTTTAGGGTCGCTGTGCTGTACAAGTCGGCTTTCCTTTCATTTCCA
Laccaria bicolor	LACCBIC	GCTTGGTTAGGCTTGGATGTGGGGGTTGCGGGCTTCATTAATGAGGTCGGCTCTCCTTAAATGCATTAGC
Laccaria laccata	LACCLAC	GGATACCTCTCGAGGCAACTCGGATTTTAGGATCGCCGTGCTGCACAAGTCGGCTTTCCTTTCATTTCC
Strobilurus esculentus	STRESC	CTTTGTACTCTTGTTGCTGTGTGCTGGCTTCTTCGGAAGTATGGTGCACGCTTGAGTGCAAGGGTCTTC
Tricholoma acerbum	TRIACE	AACCTTACTCAGCTTTCGCTAGTCGAGTTAGGCTTGGATATGGGAGTTTGTGGGCTTCTCGAAGTCGGCT
Tricholoma columbetta	TRICOL	CACTTTTATCGGTTGAATTAGGCTTGGATGTGGGAGTCTTTGCTGGCTTCGCAAGAGGTTGGCTCTCCTT
Tricholoma fulvum/ pseudonictitans	TRIFUL	CTACGCCATCATGTGAAGCAGCTTTAAATTGGGGTTGCTGCTCTCTAACAGTCTCTTTGGTGGGACAATT
Tricholoma populinum	TRIPOP	CCTAAAGTCGATCAGGCTTGGATGTGGGAGTTTGCGGGCTTTTCTAAAGTCGGCTCTCCTTAAATTT
Tricholoma saponaceum	TRISAP	CCTTTTCAGCATTTATGTTGATCAGGCTTGGATGTGGGAGTTTGCGGGCTTCTCAGAAGTCGGCTCTCCT
Tricholoma sciodes	TRISCI	GACTTGGAATATCTCTAGAGGCAACTCGGTTTTGAGGATTGCTGTGCGCAAGCCAACTTTCCTTACAC
Tricholoma ustale	TRIUST	CCTTTTCGGCTTTTTCTAAGTCGATTTAGGCTTGGATGTGGGAGTTTGCGGGCTTCTCTGAAGTCGGCTC
Cortinarius alboviolaceus	CORALB	CCTTCTCATTGCTGAGTGGTTTGGATGTGGGGGTTTGCTGGCCTCTTAAATGAGTTCAGCTCTCCTGAA
Cortinarius anomalus	CORANO	CTTCAGCTTTTGCTTGTTGAGTGTTGGATGTGGGGGGTCTTTTGCTGGCCTTTTTTTAGAGGTCAGCTTC
Cortinarius bolaris	CORBOL	CTCCACCTGTGCACCTTTTGTAGACCTGAATAGCTTTCTGAATGCTAAGCATTCAGGCTTGAGGATTGAC
Cortinarius cinnamomeus	CORCIN	CCAGGGTTTTTGACTTGTCGAGTGTTTGGATGTGGGGGTCTTTTGCTGGTCTCTTTTGAGGTCGGCTCCC
Cortinarius decipiens	CORDEC	GGGTTTGCTGGCCTTTTAAAAGGTTCAGCTCCTCTGAAATGCATTAGCAGAACAACCTTGCTCATTGGTG
Cortinarius delibutus	CORDELI	GAACAATTTGTTGACTGTTCATTGGTGTGATAATTATCTGCGCTATTGAACTGTGAGGCAAGTTCAGCTTC
Cortinarius evernius	COREVE	CTCCACCTGTGCACCCTTTGTAGACCTCCCAGGTCTATGTTGCTTCTTCATTTACCCCAATGTATGT
Cortinarius hemitrichus	CORHEM	CAAACCTTCTCTTTGTTGAGCGGTTTTGGATGTGGGGGTTTGCTGGCCTCTTAAAAAGGTTCAGCTCCTC
Cortinarius hinnuleus	CORHIN	CTAGGGAGCATGTGCACACCTTGTCATCTTTATATCTCCACCTGTGCACTTCTTGTAGGCCTTTCAGGT
Cortinarius multiformis	CORMUL	CTCCACCTGTGCACCTTTTGTAGACCTGGATATCTCTCTGAGTGCTTGCGCTCAGGTTTGAGGATTGATT
Cortinarius privignus	CORPRI	CTTCTCATTGCTGAGTGGTTTGGATGTGGTGGTTTGCTGGCCTCTTAAATGAGTTCAGCTCTCCTGAATG
Cortinarius sanguineus	CORSAN	CCTGTGCACCTTTTGTAGATCTGGATATCTTTCTGAATGCCTGGCATTCAGGTTTGGGGATTGACTTTGC
Cortinarius sp 1	CORSP1	GGGAGCATGTGCACGCCTTGTCATCTTTATATCTCCACCTGTGCACCTTTTGTAGACCCTTTCCAGGTCT
Cortinarius sp 2	CORSP2	CCTGATGGGTTGTTGCTGGTTCTCTGGGAGCATGTGCACACCTGTCATCTTTATATCTCCACCTGTGCAC
Cortinarius sp 2	CORSP3	GGGAGCATGTGCACGCCTGTCATCTTTATATCTCCACCTGTGCACTTTTGTAGACCTTCTGGGTCTATGT
Cortinarius semisanguineus	CORSEM	CTGGTCTCTTTTGAGATCGGCTCCCCTGAAATGCATTAGCGGAACAATTTGTTGACCCGTTCATTGGTG
Cortinarius tortuosus	CORTOR	GCATGTGCACACCTGTCATCTTTATATCTCCACCTGTGCACCCTTTGTAGACCTTCTCAGGTCTATGTTG
Cortinarius traganus	CORTRA	CAACCTTCTCTTGTTTGAGTGGTTTGGATGTGGGGGTTTGCTGGCTTCTTAAAAGGGTTCAGCTCCTCTG
Cortinarius vulpinus	CORVUL	CAACCTCTTCAGCTTTTGCTTGTTGAGCGTTGGATGTGGGGGGTCTGTTTTGCTGGTCTTCTCAGGTCAG
Hebeloma radicosum	HEBRAD	GCTTTTGTTGATACTGGCTTGGATATGGGGGTCTATTTTGCTGGCTTCTTTACAGATGGTCAGCTCCCC

Hebeloma sacchariolens	HEBSAC	CAGCTTTTGTTGATAACGGCTTGGATATGGGGGTTTTTTTTTGCTGGCTTCTTCACAGATGGTCAGCTCC
Inocybe griseolilacina	ISOGRI	GCTGTCCCTTCCTTTGGGTACGTGCACGCTTGTCATCTTTATTTCTACCCACTGTGCACATATTGTAGAC
Inocybe napipes	INONAP	CTGCTGGCTCTCCTCGGAGGGCATGTGCACGCTTGTTGTCCATTATTTCTCCCACTGTGCACAAATTGTA
Inocybe sp	INOSP	GGCACGTGCACGCCTGTTTTTATTTGCTTCTCCAACTGTGCACAAATATCGTAGACCTTAGCAAGGCCTA
Thelephora penicillata	THEPEN	AAATGAATCAGCTTGCCAGTCTTTGGTGGCATCACAGGTGTGATAACTATCTACGCTTGTGGTGGTC
Thelephora terrestris	THETER	CTCTGTAGTTCTATGGTCTGGGGGACCCTGTCTTCCTTCTGTGGTTCTACGTCTTTACACACACACTGTA
Tomentella sublilacina	TOMSUB	CTGGGGGACCCTGTCTTCCTGCCGTGGTTCTACGTCTTTACACACACTCTGTAATAAAGTCTTATGGAA
Tomentellopsis submollis	TOMPSUBM	GATCACGGAGCCCTGATGGGCAACGAATGCCCTCGTCTATGAATATTTTCACACACGCTCAAAGTATGAC
Cantharellus tubaeformis	CANTUB	CGGTCGCTTCCAATTGGGGGTTGACTCATAGGGGGTACATCTGTTTGAGGGTCATTTGTACCTTCTCAAA
Clavulina cristata	CLACRI	CACCTGTGCACATTTTTGAGGGAGTCTTGAGTTGGTTGCCGCTCTTGGGTGATTTTCTCACATTCCCTTA
Hydnum repandum	HYDREP	GGTATTCCGGGGAGCACACCTGTTCGAGTGTCATTGAAACTCTCAAATAAAGGTGGTTTTTGCAGACCAT
Clavariadelphus pistillaris	CLAVPIS	GAGGAGCATGCCTGTTTGAGTGTCGTGAATCTCTCTCAATCCCACCTCTGTGGGCTTGGATTTGGATG
Ramaria abietina	RAMABI	GCATTAGCGTTCCGCCGCGAGTTCGGTTTCGTAACGACGGTGTGATAAGTAACACTTTGACGCCGTCTGG
Tylospora asterophora	TYLAST	CCGAGCCCTTGAATCCCAAACACCACATGTGAACCCACCGTAGGCCTTCGGGCCTATGTCTTATCATATA
Tylospora fibrillosa	TYLFIB	CCCCCAACAAACACCGTGGGCCTTCGGGCCCGCGTATATTTACTCTGAATGTGTATAGAATGTAAACC
Cenococcum geophilum	CENGEO	GACGATTGACTCATGTTGCCTCGGCGGGCTCGCCCGCCAGAGGATACATCAAAAATCTTGTTTTAACGGT
Enthrospora colombiana	ENTCOL	TGATCACCTCGCCGTCGATAGCTTTGCTAACCTCGGTGGGATCTGATTAACTAGAGATTAGACTGATCGT
Gigaspora margarita	GIGMAR	CCTTGATAGATGTGATGTTTGGGGTCGAGGATTGCAACGGATACCCCTTCGGGGCTAGCCGCCTGATCT
Glomus sinuosum	GLOSIN	CGTGGTGTTGCTTTTGTGACGCTTCGGAATTGGGTCATCTTGATCCTTTGGGTTAAGAGACT
Paraglomus occultum	PAROCC	CACAAGTCCTCTGGAACGTGGCATCGTAGAGGGTGAGAATCCCGTCTCTGGTCGTTGTCTTGCAGGCA
Scutellospora heterogama	SCUHET	GTCAGCGTCGATTTTGGATATCATAAAATGATTGGGGGGAAGGTAGCTCCTTCGGGAGTGTTATAGCCCT
Arabidopsis thaliana	ARATHA	AGCTTTTATCTCGGTCTTGTCGTGCGCGTTGCTTCCGGATATCACAAAACCCCGGCACGAAAAGTGTCA

4. Chapter III: Diagnostic ribosomal ITS phylochip for identification of host influence on ectomycorrhizal communities

Marlis Reich, Marc Buée, Henrik Nilsson, Benoit Hilseberger, Annegret Kohler, Emilie Tisserant, Francis Martin

(in preparation for submission to New Phytologist)

5. Chapter IV: 454 pyrosequencing analyses of forest soil reveal an unexpected high fungal

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454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity

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Summary

- Soil fungi play a major role in ecological and biogeochemical processes in forests. Little is known, however, about the structure and richness of different fungal communities and the distribution of functional ecological groups (pathogens, saprobes and symbionts).
- Here, we assessed the fungal diversity in six different forest soils using tag-encoded 454 pyrosequencing of the nuclear ribosomal internal transcribed spacer-1 (ITS-1). No less than 166 350 ITS reads were obtained from all samples. In each forest soil sample (4 g), approximately 30 000 reads were recovered, corresponding to around 1000 molecular operational taxonomic units.
- Most operational taxonomic units (81%) belonged to the Dikarya subkingdom (Ascomycota and Basidiomycota). Richness, abundance and taxonomic analyses identified the Agaricomycetes as the dominant fungal class. The ITS-1 sequences (73%) analysed corresponded to only 26 taxa. The most abundant operational taxonomic units showed the highest sequence similarity to *Ceratobasidium* sp., *Cryptococcus podzolicus*, *Lactarius* sp. and *Scleroderma* sp.
- This study validates the effectiveness of high-throughput 454 sequencing technology for the survey of soil fungal diversity. The large proportion of unidentified sequences, however, calls for curated sequence databases. The use of pyrosequencing on soil samples will accelerate the study of the spatiotemporal dynamics of fungal communities in forest ecosystems.

Introduction

Fungi represent an essential functional component of terrestrial ecosystems as decomposers, mutualists and pathogens, and are one of the most diverse groups of the Eukarya (Mueller *et al.*, 2007). Studying the ecological factors that underlie the dynamics of fungal communities remains a challenge because of this high taxonomic and ecological diversity. PCR-based molecular methods and sequencing of ribosomal DNA have been used successfully to identify subsets of this species' richness (Vandenkoornhuyse *et al.*, 2002), and have provided insights into the ecological processes that affect the structure and diversity of fungal communities (Gomes *et al.*, 2003; Schadt *et al.*, 2003; Artz *et al.*, 2007). These advances are particularly noteworthy in below-ground studies of ectomycorrhizal (EM) fungi as a result of the combination of morphological and molecular identifications of EM root tips (Horton & Bruns, 2001; Martin & Slater, 2007). Spatial and temporal variations of fungal communities in forest soils are affected by numerous biotic and abiotic factors, including seasons, soil characteristics, stand age and host tree species (Nordén & Paltto, 2001; Peter *et al.*, 2001; Dickie *et al.*, 2002; Buée *et al.*, 2005; Genney *et al.*, 2005; Koide *et al.*, 2007; Tedersoo *et al.*, 2008).

The internal transcribed spacer (ITS) region is now widely used as a validated DNA barcode marker for the identification of many fungal species (Seifert, 2008). With improvements in sequencing techniques and dedicated DNA databases (Kõljalg *et al.*, 2005), recent studies have demonstrated the potential of large-scale Sanger sequencing of ITS for quantifying and characterizing

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soil fungal diversity (O'Brien *et al.*, 2005). To our knowledge, the species' richness of communities of soil fungi has not yet been assessed using high-throughput pyrosequencing.

In this article, we present the use of high-throughput tag-encoded FLX amplicon pyrosequencing (Acosta-Martinez *et al.*, 2008) to assess the fungal diversity in six soil samples from a French temperate forest site. We show that the abundance and diversity of fungi in the six soil samples were much higher than hypothesized previously. A few fungal taxa account for most of the species' abundance, whereas the majority of species are only rarely retrieved. There is reason to believe that the spatial diversity and difference in fungal richness among the six soil samples could be explained partly by forest management, that is, plantation tree species. The use of pyrosequencing on soil samples will accelerate the study of the spatiotemporal dynamics of fungal communities in forest ecosystems.

Materials and Methods

Study site and sampling

The experimental site of Breuil-Chenue forest is situated in the Morvan Mountains, Burgundy, France (latitude 47°18′10″, longitude 4°4′44″). The elevation is 640 m, the annual rainfall is 1280 mm and the mean annual temperature is 9°C. The parent rock is granite and the soil is an alocrisol, with a pH ranging between 4 and 4.5 (Ranger *et al.*, 2004). The native forest is an old coppice composed of beech (*Fagus sylvatica* L., 90% of the stems), Durmast oak (*Quercus sessiliflora* Smith), sporadic weeping birch (*Betula verrucosa* Ehrh) and hazel trees (*Corylus avellana* L.). In 1976, a part of the native forest was clear-cut and this area was planted with the following six species: beech (*Fagus sylvatica* L.), Durmast oak (*Quercus sessiliflora* Smith), Norway spruce (*Picea abies* Karst), Douglas fir (*Pseudotsuga menziesii* Franco), Corsican pine (*Pinus nigra* Arn. ssp. *laricio* Poiret var. *Corsicana*) and Nordmann fir (*Abies nordmanniana* Spach.). Six plots (1000 m^2 each), corresponding to these six plantations, were selected for the study. These plots were relatively contiguous, because the six plantations were distributed on a total area of *c.* 14 000 m^2. The site is surrounded mainly by native forest and Douglas fir plantations. In March 2008, eight soil cores (1 × 1 × 5 cm depth) were sampled independently along two 30 m transects in each of these six plots. After removal of the forest litter, the 48 soil cores were sampled in the organic horizon (depth, 0–5 cm) and transported to the laboratory in an ice chest (8°C). Soil cores from each plot were independently homogenized, and minor woody debris and roots (>2 mm) were eliminated. Finally, 500 mg of the remaining soil was subsampled for DNA extraction from each soil core.

DNA extraction, PCR and pyrosequencing

Genomic DNA was extracted from the 48 subsamples of soil using the 'FastDNA SPIN for Soil Kit' (MP Biomedicals, Illkirch, France), according to the manufacturer's instructions. Amplicon libraries were performed using a combination of tagged primers designed for the variable ITS-1 region, as recommended for the tag-encoded 454 GS-FLX amplicon pyrosequencing method (Acosta-Martinez *et al.*, 2008). The 48 genomic DNA samples were diluted to 1 : 5 and 1 : 100. These 96 diluted genomic DNA samples were amplified separately using the fungal primer pair ITS1F (5′-*Axxx*CTTGGTCATTTAGAGGA-AGTAA-3′) and ITS2 (5′-*B*GCTGCGTTCTTCATC-GATGC-3′) to generate PCR ITS rRNA fragments of *c.* 400 bp, where *A* and *B* represent the two pyrosequencing primers (GCCTCCCTCGCGCCATCAG and GCCTTG CCAGCCCGCTCAG) and *xxx* was designed for the sample identification barcoding key. The PCR conditions used were 94°C for 4 min, 30 cycles of 30 s at 94°C (denaturation), 50°C for 1 min (annealing) and 72°C for 90 s (extension), followed by 10 min at 72°C. The 96 PCR products were purified using the Multiscreen-PCR plate system (Millipore Corporation, Billerica, MA, USA), and then pooled to obtain six amplicon libraries corresponding to the six different forest soils. The amplicon length and concentration were estimated, and an equimolar mix of all six amplicon libraries was used for pyrosequencing. Pyrosequencing of the six amplicon libraries (from the ITS1F primer) on the Genome Sequencer FLX 454 System (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA) at Cogenics (Meylan, France) resulted in 180 200 reads that satisfied the sequence quality criteria employed (cf. Droege & Hill, 2008). Tags were extracted from the FLX-generated composite FASTA file into individual sample-specific files based on the tag sequence by the proprietary software COGENICS v.1.14 (Cogenics Genome Express FLX platform, Grenoble, France).

Sequence editing and analysis of the reads by operational taxonomic unit (OTU) clustering

The filtered sequences were trimmed using the *trimseq* script from the EMBOSS package (Rice *et al.*, 2000). Sequences shorter than 100 bp after quality trimming were not considered. The average length of the 166 350 edited reads was 252 bp. The resultant individual sample FASTA files were assembled in tentative consensus sequences using BLASTclust v.2.2.1.6 (Altschul *et al.*, 1997), with the requirement that at least 97% similarity be obtained over at least 90% of the sequence length (-S 97 -L 0.9). In order to identify OTUs, a random sequence was compared with the nonredundant GenBank database and a custom-curated database (C-DB, described below). The OTUs defined at

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97% sequence similarity (O'Brien *et al.*, 2005) were used to perform rarefaction analysis and to calculate the richness (Shannon) and diversity (Chao1) indices. The rarefaction analysis was performed using ANALYTIC RAREFACTION v.1.4 (Hunt Mountain Software, Department of Geology, University of Georgia, Athens, GA, USA). Calculation of the richness (Shannon) and diversity (Chao1) indices was performed using the ESTIMATES software package (version 8.00, R. K. Colwell; http://viceroy.eeb.uconn.edu/ EstimateSPages/Est-SUsersGuide/EstimateSUsersGuide.htm).

Phylogenetic assignment of the ITS-1 reads with MEGAN

In a second analysis, the individual sample FASTA files were evaluated using NCBI-BLASTn (Altschul *et al.*, 1997) against the nonredundant GenBank database (Benson *et al.*, 2008) and C-DB derived from the GenBank and UNITE (Kõljalg *et al.*, 2005; http://unite.ut.ee/index.php) databases. To construct C-DB, all fully identified fungal ITS sequences in GenBank and UNITE, as of November 2007, were screened for appropriate length (300–1500 bp), IUPAD DNA ambiguity content (less than five symbols) and taxonomic reliability, as established by Nilsson *et al.* (2006). A maximum of five sequences per species was selected at random, resulting in a total of 23 390 sequences representing 9678 Latin binomials. A post-processing Perl script generated best-hit files comprising the top 10 best BLAST hits with an *E*-value < $10e^{-3}$ for tentative species' identification.

According to their best matches, the rDNA ITS sequences were phylogenetically assigned using MEGAN v. 3.0.2 (MEtaGenome ANalyzer, Center for Bioinformatics, Tübingen, Germany) (Huson *et al.*, 2007), which provides unique names and IDs for over 350 000 taxa from the NCBI taxonomic database. The output files obtained from the nonredundant GenBank and C-DB databases were then processed. All parameters of MEGAN, including the lowest common ancestor (LCA) assignment, were kept at default values, except for the 'min support' option (regulating the minimum number of sequence reads that must be assigned to a taxon), which was set to either unity or five depending on the analysis (cf. Wu & Eisen, 2008).

Results

Analysis of the reads by OTU clustering

A total of 166 350 ITS-1 sequences passed the quality control, and the number of reads per sample (i.e. pools of eight soil cores per plantation) ranged from 25 700 to 35 600. A maximum of 1000 OTUs (including 594 singletons) was identified in the soil samples collected in the oak plantation of the experimental site, whereas only 590 OTUs (including 333 singletons) were identified in the same volume of soil collected in the beech plantation. From 4 g of forest soil and a mean of 30 000 reads, the number of OTUs obtained was *c.* 830 (± 73). The number of OTUs increased with the number of reads, and a plot of OTUs **vs** the number of ITS-1 sequences resulted in rarefaction curves that did not approach a plateau (Fig. 1), in spite of the large number of reads. At 97% similarity, the nonparametric Chao1 estimator (Chao *et al.*, 2005) predicted that the maximum number of OTUs probably ranges from 1350 to 3400 (data not shown) depending on soil sample, with a mean estimated OTU richness close to 2240 (± 360).

To identify the most frequent fungal taxa present in the organic soils from the Breuil-Chenue forest site, OTUs were clustered with all the 166 350 reads. The 26 most abundant OTUs represented 73% of the total reads (Table 1). The most frequent OTU was assigned to an 'uncultured fungus' in GenBank, but Menkis *et al.* (2006) suggested that it corresponds to the root plant pathogen *Ceratobasidium* sp. The six most abundant OTUs were distributed in three phyla and six distinct orders: Cantharellales, Mortierellales, Helotiales, Tremellales, Agaricales and Boletales (Table 1).

Analysis of reads with MEGAN

The set of individual DNA reads was also compared against the nonredundant GenBank database of known ITS sequences using BLASTn. MEGAN was used to compute the taxonomic content of the dataset, employing NCBI taxonomy to order and cluster the results (Huson *et al.*, 2007). Most of the sequences (71.5%) lack an explicit taxonomic annotation (Fig. 2a). To obtain a better assessment of the taxonomic diversity of the known species, sequences were queried against C-DB, containing only ITS sequences from known fungal species (see Materials and Methods section). After this curation, only 11% of the OTUs remained in the 'unclassified fungi' category, 81% in the Dikarya subkingdom and 8% in the Mortierellaceae family (Fig. 2b). With 43.7% of the remaining ITS sequences, the Basidiomycota represented the predominant fungal phylum in the pooled results from soils of the Breuil-Chenue plantations.

Comparative analysis of the six plantation soil samples revealed a distinct distribution of fungal phyla (Fig. 3). For instance, Basidiomycota accounted for 65% of OTUs in the soil cores collected in the oak plot, whereas this phylum accounted for only 28% of OTUs in the soil cores sampled in the spruce plot. Alternatively, soil samples from the spruce plot were characterized by a relatively high percentage (*c.* 17%) of species from the order Mortierellales, parasitic or saprobic fungi belonging to the Mucoromycotina (Hibbett *et al.*, 2007), with the number of ITS reads two- to five-fold higher than the five other forest soil samples (only 3% of Mortierellales in the soil cores from the oak plantation).

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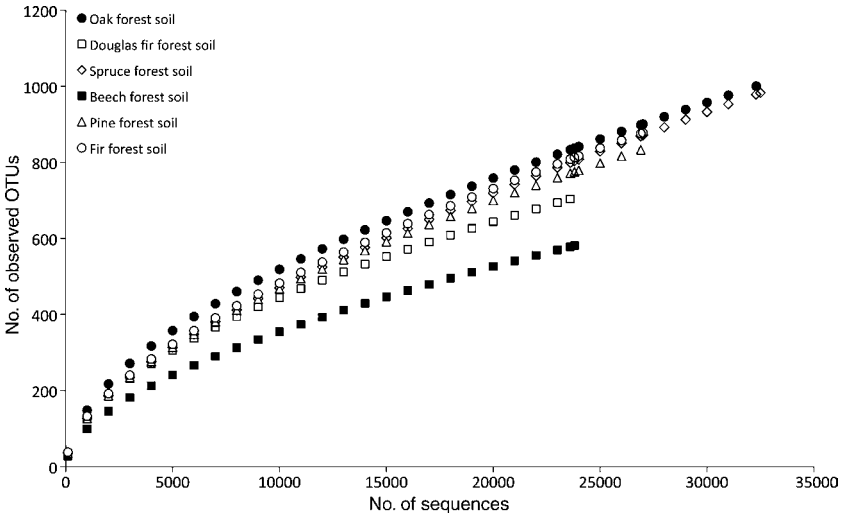

Fig. 1 Rarefaction curves depicting the effect of internal transcribed spacer (ITS) sequence number on the number of operational taxonomic units (OTUs) identified from the six soil samples. Between 25 680 and 35 600 sequences, depending on soil core, were generated, corresponding to 580–1000 OTUs (at 3% sequence dissimilarity). Forest soil (*x*, number of sequences; *y*, number of observed OTUs): oak forest soil (32330; 1001); Douglas fir forest soil (23624; 704); Norway spruce forest soil (32555; 983); Corsican pine forest soil (26908; 833); Nordmann fir forest soil (27054; 833); beech forest soil (23878; 581).

Table 1 List of the 26 most abundant fungal operational taxonomic units (OTUs) found in the forest soil of the Breuil-Chenue site

Closest NCBI database match	Closest accession number (NCBI)	Identities, length (%)	No. 454 reads
Uncultured fungus (*Ceratobasidium* sp.)[1]	DQ093748.1	189/190 (99)	20067
Uncultured *Cryptococcus*	FM866335.1	222/222 (100)	19452
Lactarius spp.[2] (*quietus*, *tabidus* and *Lactarius* spp.)	EF493299.1	212/214 (99)	11302
Scleroderma sp. (*citrinum*)	EU784414.1	274/285 (96)	8733
Uncultured Dermateaceae	FJ554441.1	258/268 (96)	5617
Uncultured Mortierellaceae	FJ475737.1	259/268 (96)	5253
Uncultured fungus sp. 2	EF521220.1	217/282 (76)	5099
Uncultured soil fungus sp. 1	EU806458.1	189/203 (93)	4562
Inocybe sp. (uncultured ectomycorrhiza)	FN393147.1	228/235 (97)	4476
Russula sp. (*parazurea*)	DQ422007.1	251/271 (92)	4390
Uncultured soil fungus sp. 2	DQ421207.1	219/227 (96)	4271
Uncultured *cryptococcus*	FJ554344.1	257/260 (98)	4041
Uncultured fungus (*Cyllamyces* sp.)	AM260910.1	82/85 (96)	3759
Uncultured soil fungus sp. 3	FJ553866.1	259/262 (98)	3403
Uncultured *Sebacinales*	DQ421200.1	245/297 (82)	2936
Uncultured basidiomycete	FJ475793.1	275/285 (96)	2703
Uncultured soil fungus sp. 4 (*Mortierellaceae*)	FJ554362.1	122/126 (96)	2087
Uncultured dothideomycete (*Cenococcum* sp.)	DQ273316.1	246/258 (95)	1443
Uncultured Helotiales	FJ552732.1	272/281 (96)	1370
Tylospora asterophora	AF052557.1	269/276 (97)	1220
Uncultured basidiomycete (*Cortinarius* sp.)	AM902090.1	231/248 (93)	1184
Amanita sp. (*spissa*)	AJ889924.1	237/242 (97)	1049
Pseudotomentella sp. (*tristis*)	AJ889968.1	232/237 (97)	1048
Uncultured Helotiales	FJ475783.1	239/264 (90)	1016
Uncultured soil fungus sp. 5 (*Mortierellaceae*)	EU807054.1	249/253 (98)	989
Boletus sp. (*pruinatus*)	AJ889931.1	239/244 (97)	974

The 166 350 reads were assembled into tentative consensus sequences with the requirement that at least 97% similarity be obtained over at least 90% of the sequence length (-S 97 -L 0.9). To identify OTUs, a random sequence was compared with the nonredundant GenBank database.

[1]This OTU was assigned to 'Uncultured fungus' in GenBank. It corresponds to *Ceratobasidium* sp. (Menkis *et al.*, 2006).

[2]The 11 302 reads of this OTU (*Lactarius* spp.) correspond to a complex of 6952 reads of *L. quietus*, 3489 reads of *L. tabidus* and 757 reads of other *Lactarius* species (as *L. theiogalus* and *L. rufus*) with ITS-1 sequences showing >97% homology.

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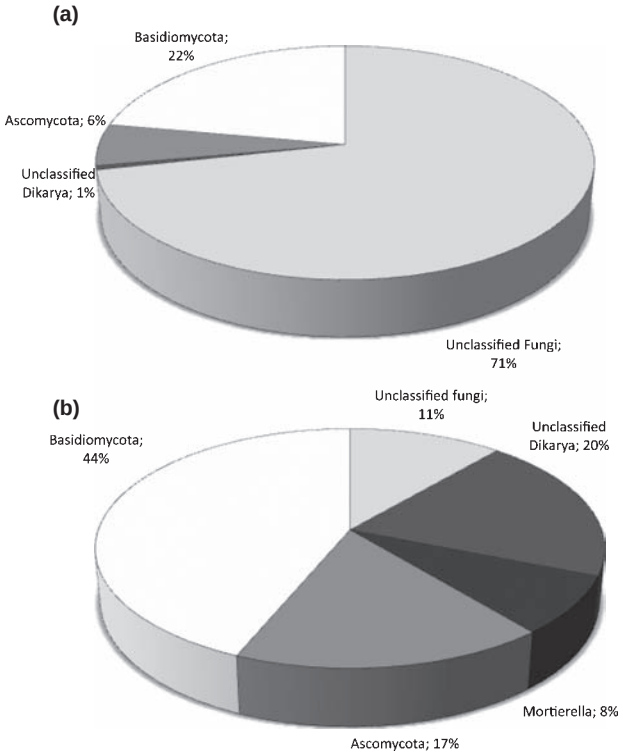

Fig. 2 Proportional distribution of different phyla and fungal groups in the sequenced internal transcribed spacer (ITS) clone libraries. The half-plate pyrosequencing produced 180 213 fungal sequences (166 350 after trimming procedures). Results obtained after BLASTn of sequences performed against GenBank and UNITE (a) or filtered database (b) (16 987 sequences, representing 9 678 fungal species), containing well-identified sequences and excluding all 'uncultured fungi' sequences and 'environmental sample' sequences (see Materials and Methods section for more details). Taxonomic clustering was performed in MEGAN (Metagenome Analysis) with the following lowest common ancestor (LCA) parameter values: 1, 35, 10 and 0 for Min support, Min score, Top percent and Win score, respectively.

At the family and genus levels, the fungal communities showed similar taxonomic distribution across all soil samples (Tables S1 and S2). Among saprotrophic, parasitic and mycorrhizal fungi, the genera *Ceratobasidium*, *Cryptococcus*, *Lactarius*, *Mortierella*, *Russula*, *Scleroderma*, *Neofabraea*, *Inocybe* and *Cenococcum* were the most prominent genera found in this study (Table 1). Moreover, numerous Agaromycotina families were common to all soil samples, with a strong representation of the EM species from the Boletales, Agaricales, Thelephorales, Russulales, Cantharellales and Sebacinales (according to Rinaldi *et al.*, 2008). Other EM genera, such as *Lactarius* and *Tylospora*, were mainly identified in the oak and spruce plots, respectively. EM fungi represented more than 50% of the 30 most abundant genera (Table S1).

At the species' level, the fungal community composition also revealed similar taxa between different soils (Table S2). The two yeast species, *Cryptococcus podzolicus* and *C. terricola*, occurring on the surface of roots and in the rhizosphere (Golubtsova *et al.*, 2006), were the most abundant Dikarya found in all organic forest soils of the Breuil-Chenue site. The plant pathogen fungus *Ceratobasidium* sp. was also dominant in all soil samples. EM species, such as *Cenococcum geophilum* and *Cortinarius* sp. (*saturninus* from C-DB), were also ubiquitous. Other species, such as *Scleroderma* sp. (*citrinum* or *bovista*), were very abundant in most plantations (between 1000 and 2000 reads), except under oak (*c.* 100 reads). By contrast, the oak-specific EM symbiont *Lactarius quietus* was restricted to the soil collected under the oak plantation. *Russula puellaris* was only identified in the Douglas fir forest soil samples and Corsican pine plantation soil samples, whereas *Russula vesca* was found in the soils from Corsican pine and Nordmann fir plots.

Discussion

This pilot study used 454 pyrosequencing to evaluate the fungal diversity in six distinct and spatially distant soil samples from a temperate forest. By sequencing a total of 166 350 PCR-amplified ITS-1 sequences, we identified

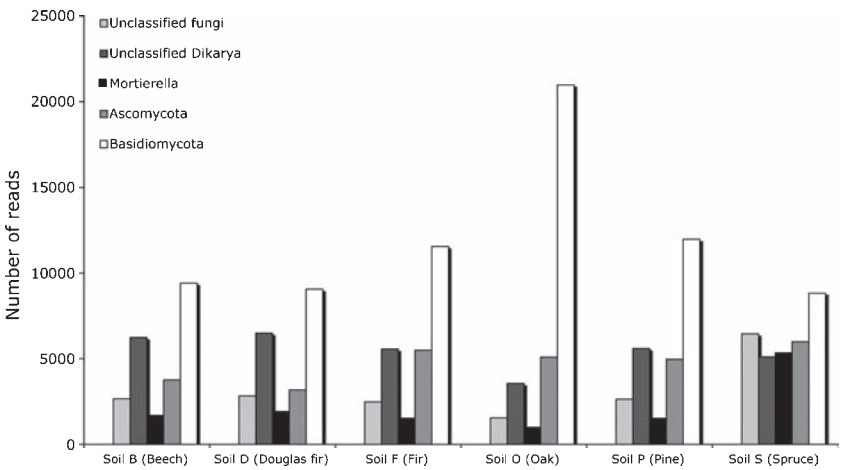

Fig. 3 Relative abundance of fungal phyla for each soil library. ITS-1 sequences were classified according to their BLASTn similarity by MEGAN (Metagenome Analysis). After using BLASTn against the curated database, taxonomic clustering was performed in MEGAN with the lowest common ancestor (LCA) parameter values set to 5, 35, 10 and 0 for Min support, Min score, Top percent and Win score, respectively.

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600–1000 OTUs in each of the forest soil samples. The nonparametric Chao1 estimator (Chao *et al.*, 2005) predicted that the mean number of OTUs in 4 g of forest soil was *c.* 2240 (± 360). Interestingly, 73% of the DNA reads corresponded to 26 taxa only, and a detailed analysis showed that the three most abundant OTUs were supported by 25–55% of reads whatever soil was considered. Using a cloning/Sanger sequencing approach, Fierer *et al.* (2007) have estimated a similar number of OTUs in rainforest soil samples (1 g), ranging between 1000 and 2000 OTUs in each community, depending on the parametric model used. Although we found 600–1000 OTUs in each of the forest soil samples, we highlighted between 249 and 408 taxonomic groups from these soil samples, supported by a minimum of two reads. Therefore, the number of singletons, which were close to only 1.8% of the total number of reads, corresponded to approximately 60% of the observed OTUs. This large proportion of OTUs, supported by unique reads, suggests that these sequences result from the sequencing of the numerous individuals isolated in the samples. This low abundance of numerous fungal taxa should be correlated with the inconspicuous nature of fungi and their dispersal ability. Hyphae and spores present in litter, leaves, pollen or needles, or the microscopic propagules, probably favour the spread of fungal species in diverse ecosystems. These species constitute a microbial reservoir (Finlay, 2002), which may play important functions in forest ecosystems facing environmental stresses.

At the present time, the ITS regions have been validated as the best DNA barcode marker for fungal species' identification (Seifert, 2008). In the present pyrosequencing experiment, and as reported in other studies (Liu *et al.*, 2008; Nilsson *et al.*, 2009), an average length of 252 bp for the ITS-1 sequences is long and sufficiently polymorphic to allow the identification of the majority of fungal OTUs at the species' or genus levels. A large part of the sequenced ITS regions belonged to unclassified fungi from incompletely annotated environmental samples. Lack of taxonomic annotation and errors in taxonomic assignments of ITS sequences deposited in the international DNA databases (Vilgalys, 2003) are major limitations to the survey of fungal species, and have hampered such efforts (Nilsson *et al.*, 2006; Bidartondo *et al.*, 2008; Horton *et al.*, 2009). For these reasons, the use of a curated ITS database (Nilsson *et al.*, 2005, 2006) should provide more pertinent taxonomic information. Using a curated database, we found that the majority of fungal sequences recovered belonged to the Dikarya (Ascomycota and Basidiomycota), which account for 81% of the OTUs. Basidiomycota was the most abundant phylum (43.7% of OTUs), whereas Ascomycota accounted for a much smaller percentage of the community (17.3%). These results are very similar to those of a large-scale survey of temperate forest soils carried out using Sanger sequencing (O'Brien *et al.*, 2005). By contrast, Schadt *et al.* (2003) found a large proportion of Ascomycota in 125 cloned fungal sequences from tundra soils. The Glomeromycota and Chytridiomycota were probably underestimated in our ITS-1 libraries as we have amplified ITS from soil DNA using primers designed for Dikarya (ITS-1F/ITS-2). In addition, these fungal taxa and several genera, including Glomeromycota, were underestimated in our survey, as poorly annotated ITS sequences from GenBank were excluded from C-DB (Vilgalys, 2003; Nilsson *et al.*, 2008; Ryberg *et al.*, 2009).

Lindahl *et al.* (2007) reported that saprotrophic fungi were confined to the surface of the boreal forest floor. This functional ecological group of fungi seems to be under-represented in our topsoil samples. Ryberg *et al.* (2009) reported that numerous saprotrophic species are also poorly represented in the sequence databases compared with mycorrhizal sequences, and this imbalance may explain the apparent bias. Moreover, the season of sampling can influence the pattern of fungal richness and the under-representation of some species in our current samples (Taylor, 2002; Koide *et al.*, 2007). However, several saprotrophic species were found in all six soil samples. For instance, the two ubiquitous anamorphic Basidiomycota yeast species (Fonseca *et al.*, 2000), *Cryptococcus podzolicus* and *C. terricola*, showed a large number of reads in the six forest soils, and three *Mortierella* species were also very abundant in the six soil samples (Table S2). Interestingly, the three functional ecological fungal groups (parasitic, saprotrophic and mutualistic) were represented by the three most abundant OTUs, belonging to *Ceratobasidium*, *Cryptococcus* and *Lactarius* genera, respectively.

Owing to the large proportion of unclassified fungi found in the present and other soil surveys, a collection of curated sequences for fungal identification is urgently needed. Nevertheless, several of these unclassified fungal sequences seem to correspond to a well-supported clade of Ascomycota, equivalent to a subphylum, and referred to as soil clone group I (Porter *et al.*, 2008).

Amongst the taxonomically assigned species, EM species from the Boletales, Agaricales, Thelephorales, Russulales, Cantharellales and Sebacinales were predominant in the six soil samples from different plantations (Tables S1 and S2), supporting recent results on EM community structure (Tedersoo *et al.*, 2008). These authors reported a host preference of EM fungi in wet sclerophyll forest, but revealed that the lineages of *Cortinarius*, *Tomentella–Thelephora*, *Russula–Lactarius*, *Clavulina*, *Descolea* and *Laccaria* prevailed in the total community studied. The wide distribution of these fungi is likely to favour their dissemination (Baker, 1966; Lockwood *et al.*, 2005), as are their resistance to environmental stresses and their capacity for invasiveness (Desprez-Loustau *et al.*, 2007).

The diversity and OTU richness between the six different forest soils suggest a strong spatial heterogeneity. Numerous

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factors could explain this diversity, including the influence of the host tree or the impact of the soil organic matter. Moreover, a difference in organic matter composition and functioning has been reported in previous topsoil analyses from three plantations of this site (Moukoumi *et al.*, 2006). The taxonomic information obtained in the present high-throughput survey shows an unexpectedly high richness of fungal species in forest soils. Additional 454 pyrosequencing-based surveys of fungal diversity will shed light on the factors that have the largest impact on the fungal communities.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Fungal community composition, at the genus level, in the six forest soils

Table S2 Fungal community composition, at various taxonomic levels, in the six forest soil samples

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Supporting Information

Table S1 Fungal community composition, at the genus level, in the six forest soils.

Analysis of the six datasets (using BLASTn against the curated database) was obtained after taxonomic clustering using MEGAN with the LCA parameter values set to 1; 35; 10 and 0 for Min support, Min score, Top percent and Win score, respectively. The values shown correspond to the number of reads in each taxonomic categories obtained from the 454-pyrosequencing. Grey background: the most abundant genera (>100 reads).

* Genera proposed as being ectomycorrhizal (according to Rinaldi *et al.* 2008); but the status of some species as mycorrhizal fungi is contested (EM?) and must be confirmed. Soils O, D, S, B, P and F were sampled in oak, Douglas fir, spruce, beech, pine and fir plantations respectively.

Genera	**Status (EM*)**	**Soil O**	**Soil D**	**Soil S**	**Soil B**	**Soil P**	**Soil F**
Acephala		1	0	10	0	0	0
Acremonium		0	1	0	0	0	0
Alatospora		8	0	1	0	0	0
Allantophomopsis		1	10	18	6	1	4
Amanita	EM	931	6	13	2	218	72
Amphisphaeriaceae		10	0	0	0	0	0
Aphanocladium		3	0	0	0	0	0
Arachnopeziza		4	0	1	3	1	0
Armillaria		3	2	0	0	0	0
Aspergillus		3	0	0	0	0	1
Athelia		0	3	5	7	4	4
Basidiobolus		1	0	0	0	0	1
Boletus	EM	9	3	10	0	56	10
Botryobasidium		12	14	25	61	27	20
Botryosphaeria		10	0	0	0	0	0
Bullera		0	1	1	0	0	0

Cadophora		5	0	0	0	0	0
Calcarisporium		3	0	0	0	0	6
Calocera		0	3	0	0	0	1
Capronia		5	2	3	0	0	3
Cenococcum	EM	177	158	366	194	184	405
Chaetosphaeria		75	16	13	28	30	23
Chaunopycnis		17	29	28	17	53	50
Chloridium	EM?	1	0	0	0	0	3
Cladophialophora		127	59	108	59	76	108
Cladosporium		2	1	2	2	2	1
Clavulina	EM	2	23	2	1	1225	144
Clitocybe		2	0	0	0	0	0
Clonostachys		2	35	1	0	0	0
Coprinellus		0	0	0	0	0	1
Cordyceps		11	0	3	0	4	0
Cortinarius	EM	238	157	259	156	131	1595
Cryptococcus		1096	4255	4213	5272	4505	4752
Cudoniella		0	0	0	2	0	0
Cylindrosympodium		0	16	0	0	0	0
Cystoderma		0	0	0	0	2	6
Dactylaria		19	4	10	3	7	4
Dermea		158	93	78	118	109	102
Diplogelasinospora		0	0	0	0	1	0
Discostroma		0	0	0	5	0	0
Doratomyces		1	0	0	0	0	0
Dwayaangam		3	0	0	0	0	0
Elaphomyces	EM	0	0	68	0	0	107
Eupenicillium		4	0	0	0	1	0
Exophiala		17	7	2	6	5	8
Fulvoflamma		1	4	3	0	3	17
Galerina		8	0	1	0	2	0
Ganoderma		0	0	2	0	5	1
Gelatinipulvinella		0	1	0	0	0	0

Geomyces		2	2	0	3	1	2
Gigasporaceae	AM	0	0	0	0	1	0
Gloeotinia		3	0	25	2	6	5
Grifola		0	1	0	0	0	0
Guehomyces		0	10	0	0	0	21
Gyoerffyella		0	1	0	0	0	0
Helicodendron		0	2	0	0	0	0
Hirsutella		1	1	0	0	0	0
Holwaya		54	4	17	1	6	4
Hyalodendriella		3	26	5	0	0	3
Hyaloscypha		0	0	3	0	7	0
Hydnotrya	EM	2	0	0	0	1	1
Hydnum	EM	0	0	0	0	1	274
Hymenoscyphus		0	0	0	0	1	1
Hypholoma		3	2	1	1	2	0
Hypocrea		4	2	0	0	0	0
Hypomyces		0	2	0	1	0	0
Inocybe	EM	7	769	875	1235	838	1123
Laccaria	EM	923	27	23	121	38	40
Lachnum		13	9	0	1	80	22
Lactarius	EM	10545	2	25	6	57	86
Lecythophora		22	11	11	2	6	3
Leohumicola		0	0	0	0	2	0
Lophodermium		0	0	1	0	1	0
Lycoperdaceae		0	0	0	0	1	0
Mariannaea		2	0	0	0	0	0
Mastigobasidium		0	0	0	0	0	1
Megacollybia		2	0	0	0	0	0
Meliniomyces	EM?	0	4	72	0	6	16
Metarhizium		8	1	18	0	4	0
Micarea		0	0	1	0	0	0
Microbotryomycetes		0	0	0	4	2	0
Microdochium		4	0	0	0	0	0

Microscypha		1	0	0	0	0	0
Mollisia		0	0	0	0	1	0
Monilinia		1	0	0	0	0	0
Mortierella		980	1925	5341	1677	1508	1517
Mycena		16	0	0	0	1	0
Nectriaceae		8	0	21	0	0	0
Nematoctonus		1	1	0	0	0	0
Neofabraea		160	951	972	1337	1120	1209
Neonectria		0	3	0	6	6	5
Nolanea		6	0	0	2	2	0
Oidiodendron	Ericoid M?	232	86	135	48	133	129
Oligoporus		3	0	0	0	0	0
Ombrophila		0	7	12	27	21	24
Ophiostoma		1	0	0	0	0	0
Parapleurotheciopsis		2	0	0	0	0	0
Paxillus	EM	7	0	0	0	0	1
Penicillium		136	90	23	7	37	36
Pezizaceae	EM?	12	26	13	15	81	12
Phallus		1	0	0	0	1	0
Phialemonium		3	0	0	0	12	0
Phialocephala		36	5	13	6	0	8
Phlebia		0	0	0	0	1	0
Phlogicylindrium		3	4	8	0	0	126
Piloderma	EM	0	0	0	0	0	1
Pleosporales		0	0	0	0	1	0
Pochonia		15	7	22	3	3	8
Polyporales		0	6	0	0	0	0
Preussia		0	1	0	0	0	2
Psathyrellaceae		4	0	1	0	0	0
Pseudaegerita		1	0	0	2	1	0
Pseudeurotiaceae		0	3	2	1	1	1
Pseudoclathrosphaerina		2	0	0	0	0	0
Pseudotomentella	EM	277	318	312	2	2	236

Psoraceae		0	1	2	5	4	2
Pucciniaceae		1	1	1	0	1	2
Ramularia		2	0	0	0	0	0
Readeriella		0	5	0	0	0	3
Rhizopogon	EM	0	1	0	0	0	0
Rhizoscyphus		25	5	1	1	6	2
Rhizosphaera		0	0	0	0	0	2
Rhodosporidium		6	0	2	0	0	0
Rhodotorula		4	1	2	0	0	0
Russula	EM	5174	1597	79	294	2755	769
Sarea		0	0	1	0	1	0
Scleroderma	EM	99	1110	1246	2077	1514	1851
Scleromitrula		2	0	0	0	1	0
Scleropezicula		39	2	13	1	15	8
Sclerotiniaceae		0	0	1	0	0	0
Scytalidium		8	3	2	2	5	4
Sebacina	EM	9	0	7	3	3	0
Sphaerobolus		0	0	2	0	0	0
Spirosphaera		35	0	0	0	0	0
Sporobolomyces		0	0	1	0	0	0
Sporothrix		1	0	0	0	0	0
Stachybotrys		11	4	4	6	3	4
Strobilurus		0	0	0	0	0	2
Talaromyces		5	1	0	3	2	0
Taphrina		4	0	0	0	0	0
Tephrocybe		2	0	0	0	0	0
Thelephora	EM	480	531	29	12	266	88
Thysanophora		1	1	1	0	0	3
Tomentella	EM	8	59	211	21	129	120
Tomentellopsis	EM	0	0	14	0	0	5
Trechispora	EM (?)	13	3	1	0	3	2
Tremella		1	2	7	0	2	0
Trichocladium		24	5	1	0	1	4

Trichoderma	EM?	5	4	1	0	4	3
Trichoglossum		6	1	0	2	0	1
Trichosporon		42	37	14	4	13	12
Tuber	EM	0	0	0	0	0	4
Tumularia		0	3	0	0	0	0
Tylopilus	EM	0	0	16	0	0	0
Tylospora	EM	3	0	1450	5	39	2
Umbelopsis		4	0	4	0	1	1
Umbilicariaceae		2	0	1	0	0	0
Vascellum		0	1	0	0	0	0
Venturia		0	1	31	0	0	0
Venturiaceae		8	0	0	0	0	0
Wilcoxina	EM	0	1	0	0	0	0
Xenasmatella		23	0	2	0	0	0
Xenochalara		0	0	0	0	0	1
Xerocomus	EM	870	36	47	39	60	161
Zalerion		0	0	0	0	0	7
Zignoella		149	0	1	0	6	0

Table S2 Fungal community composition, at various taxonomic levels, in the six forest soil samplings. Analysis of the six datasets was obtained after taxonomic clustering using MEGAN software with the LCA parameter values set to 5; 35; 10 and 0 for Min support, Min score, Top percent and Win score, respectively. Values correspond to the number of reads in each taxonomic categories obtained from the 454-pyrosequencing. **Amanita* sp. (*brunnescens*), previously only described in North America, was recorded in the soil samples from oak plot. The sequence of this OTU had 99% of identities (227/229) on GenBank with an uncultured soil fungus clone and the best hit with the first fully identified sequence was with *A. brunnescens*, but with 93% identities (189/203). ***Cortinarius* sp. (*saturninus*) is normally only associated with *Salix*, *Populus* or *Corylus*. Soils O, D, S, B, P and F were sampled in oak, Douglas fir, spruce, beech, pine and fir plantations, respectively.

	Soil O	**Soil D**	**Soil S**	**Soil B**	**Soil P**	**Soil F**
Acephala applanata	0	0	10	0	0	0
Agaricales	36	9	26	9	13	12
Agaricomycetes	0	5	11	0	6	5
Agaricomycetidae	109	5	13	0	6	51
Agaricomycotina	0	11	10	10	12	8
Alatospora acuminata	8	0	0	0	0	0
Allantophomopsis lycopodina	0	10	18	6	0	0
Amanita	0	0	0	0	0	5
Amanita sp. (*brunnescens*)*	8	0	0	0	0	0
Amanita fulva	22	0	0	0	0	0
Amanita rubescens	66	0	11	0	0	55
Amanita spissa	829	5	0	0	211	10
Amphisphaeriaceae	10	0	0	0	0	0
Ascomycota	1020	704	812	1174	1036	1146
Athelia epiphylla	0	0	5	6	0	0

Basidiomycota	17	28	22	15	16	23
Bionectriaceae	0	7	0	0	0	0
Boletales	0	37	34	94	50	66
Boletus	9	0	0	0	0	0
Boletus edulis	0	0	7	0	55	10
Botryobasidium subcoronatum	0	14	0	0	0	6
Botryobasidium subcoronatum	12	0	25	61	27	20
Botryosphaeria stevensii	10	0	0	0	0	0
Cadophora finlandica	5	0	0	0	0	0
Capnodiales	9	9	7	0	0	0
Capronia fungicola	5	0	0	0	0	0
Cenococcum geophilum	177	158	366	194	184	405
Chaetosphaeria chloroconia	53	13	13	21	23	19
Chaetosphaeriaceae	141	21	9	20	18	34
Chaunopycnis alba	17	29	28	17	53	50
Cladophialophora	0	0	7	0	0	0
Cladophialophora chaetospira	29	15	33	6	10	18
Cladophialophora minutissima	94	42	68	51	62	90
Clavulina cristata	0	23	0	0	1225	144
Clonostachys candelabrum	0	35	0	0	0	0
Cordyceps	7	0	0	0	0	0
Cortinariaceae	10	0	9	0	5	18
Cortinarius	14	6	6	0	0	1388
Cortinarius anomalus	81	0	0	0	0	0
Cortinarius obtusus	0	0	56	0	0	0
Cortinarius semisanguineus	0	0	0	0	0	51
Cortinarius tortuosus	0	0	6	0	0	0
Cortinarius delibutus	0	9	7	17	0	13
Cortinarius sp. (*saturninus*)**	143	142	184	138	122	141
Cryptococcus	7	0	0	0	0	0
Cryptococcus podzolicus	643	3592	3577	4407	3718	3991
Cryptococcus terricola	446	662	629	864	785	759
Cylindrosympodium lauri	0	16	0	0	0	0

Cystoderma amianthinum	0	0	0	0	0	6
Dactylaria appendiculata	19	0	9	0	7	0
Dermateaceae	235	0	23	16	19	11
Dermea	151	90	74	114	108	98
Dermea viburni	7	0	0	0	0	0
Dikarya	4540	8381	10411	7894	7080	7048
Discostroma tricellulare	0	0	0	5	0	0
Dothideomycetes	0	29	0	0	7	0
Elaphomyces muricatus	0	0	68	0	0	107
Exophiala	16	7	0	6	5	8
Fulvoflamma eucalypti	0	0	0	0	0	17
Fungi	560	903	1098	992	1124	960
Galerina pseudocamerina	8	0	0	0	0	0
Ganoderma applanatum	0	0	0	0	5	0
Gloeotinia temulenta	0	0	25	0	6	5
Guehomyces pullulans	0	10	0	0	0	21
Helotiales	46	0	0	28	0	0
Holwaya mucida	54	0	17	0	6	0
Hyalodendriella betulae	0	26	5	0	0	0
Hyaloscypha daedaleae	0	0	0	0	7	0
Hyaloscyphaceae	0	46	974	0	0	8
Hydnum albidum	0	0	0	0	0	274
Hypocreaceae	83	37	35	5	67	75
Hypocreaceae	108	8	20	0	24	20
Hypocreales	11	11	15	0	21	16
Inocybe	0	0	0	5	0	0
Inocybe napipes	0	0	0	0	0	100
Inocybe petiginosa	0	10	0	0	0	0
Inocybe umbrina	5	759	872	1230	837	1023
Laccaria	923	27	23	121	38	40
Lachnum	0	9	0	0	68	20
Lachnum sclerotii	9	0	0	0	11	0
Lactarius	101	0	9	6	0	48

Lactarius camphoratus	0	0	0	0	51	0
Lactarius glyciosmus	0	0	12	0	0	38
Lactarius quietus	6952	0	0	0	0	0
Lactarius tabidus	3489	0	587	0	95	305
Lecanorineae	0	0	0	5	0	0
Lecythophora mutabilis	22	11	11	0	6	0
Meliniomyces bicolor	0	0	0	0	0	8
Meliniomyces variabilis	0	0	72	0	0	8
Metarhizium	6	0	5	0	0	0
Metarhizium anisopliae	0	0	13	0	0	0
Microbotryomycetes	8	0	0	0	0	0
mitosporic Ascomycota	67	9	18	0	11	10
mitosporic Helotiales	16	0	57	0	11	0
mitosporic Orbiliaceae	8	10	0	0	9	0
Mortierella	251	349	760	469	412	438
Mortierella horticola	0	15	0	0	0	0
Mortierella gamsii	12	13	56	6	10	6
Mortierella humilis	196	454	2422	55	176	140
Mortierella hyalina	360	932	1817	1020	749	795
Mortierella macrocystis	159	162	284	127	158	134
Mycena	16	0	0	0	0	0
Nectriaceae	8	28	21	42	34	22
Neofabraea	125	885	917	1232	1029	1110
Neofabraea malicorticis	35	66	55	105	91	99
Nolanea sericea	6	0	0	0	6	0
Oidiodendron	68	43	77	22	100	89
Oidiodendron echinulatum	0	10	0	0	0	0
Oidiodendron pilicola	98	0	23	0	13	10
Oidiodendron scytaloides	7	10	11	0	0	5
Oidiodendron rhodogenum	59	19	19	21	18	21
Ombrophila violacea	0	7	12	27	21	24
Paxillus involutus	7	0	0	0	0	0
Penicillium	123	77	22	6	37	28

Penicillium coralligerum	6	0	0	0	0	0
Penicillium inflatum	5	8	0	0	0	0
Pezizaceae	12	26	13	15	81	12
Pezizomycotina	1224	587	1703	552	1646	1701
Phialocephala virens	5	5	12	0	7	8
Phialocephala xalapensis	31	0	0	0	0	0
Phialophora phaeophora	22	0	0	0	7	0
Phialophora phaeophora	0	0	0	7	0	0
Phlogicylindrium eucalyptorum	0	0	8	0	0	126
Pochonia	15	7	22	0	0	8
Polyporales	8	6	0	0	0	0
Pseudotomentella	0	0	85	0	0	0
Pseudotomentella tristis	277	318	227	0	0	236
Psoraceae	0	0	0	5	0	0
Readeriella gauchensis	0	5	0	0	0	0
Rhizoscyphus ericae	25	5	0	0	6	0
Rhodosporidium lusitaniae	6	0	0	0	0	0
Rhytismataceae	0	0	177	0	0	0
Russula	89	5	7	0	34	9
Russula cyanoxantha	275	0	0	0	0	0
Russula densifolia	0	0	0	0	0	436
Russula nigricans	372	118	0	0	0	0
Russula parazurea	4410	0	21	0	0	0
Russula grisea	0	0	0	139	0	0
Russula puellaris	0	1407	0	17	1183	0
Russula puellula	0	0	0	26	0	0
Russula rosea	0	0	0	13	0	0
Russula vesca	0	0	0	37	1510	276
Russula xerampelina	20	63	46	53	25	46
Russulaceae	11	0	0	0	10	0
Scleroderma sp.	98	1110	1246	2077	1514	1851
Sclerodermatineae	0	18	11	15	23	21
Scleropezicula alnicola	39	0	13	0	15	8

Scytalidium lignicola	8	0	0	0	5	0
Sebacina incrustans	9	0	5	0	0	0
Sordariomycetidae	438	18	23	28	30	25
Spirosphaera carici-graminis	35	0	0	0	0	0
Stachybotrys	11	0	0	6	0	0
Thelephora	7	14	0	0	0	0
Thelephora terrestris	0	429	5	0	5	86
Thelephora penicillata	471	88	23	9	261	0
Thelephoraceae	16	23	27	0	13	0
Tomentella	7	0	10	0	15	34
Tomentella sublilacina	0	55	201	0	110	82
Tomentella badia	0	0	0	17	0	0
Tomentellopsis submollis	0	0	14	0	0	5
Trechispora hymenocystis	13	0	0	0	0	0
Tremella	0	0	7	0	0	0
Trichocladium asperum	12	0	0	0	0	0
Trichocladium opacum	12	5	0	0	0	0
Trichocomaceae	41	9	7	15	22	14
Trichoderma	5	0	0	0	0	0
Trichoglossum hirsutum	6	0	0	0	0	0
Tricholomataceae	0	7	0	5	5	0
Trichosporon porosum	42	37	14	0	11	12
Tylopilus felleus	0	0	16	0	0	0
Tylospora fibrillosa	0	0	204	0	0	0
Tylospora asterophora	0	0	1246	5	37	0
Venturia	0	0	30	0	0	0
Venturiaceae	8	10	0	0	0	0
Xenasmatella vaga	23	0	0	0	0	0
Xerocomus badius	26	28	33	38	59	44
Xerocomus pruinatus	843	6	14	0	0	117
Zalerion arboricola	0	0	0	0	0	7
Zignoella pulviscula	149	0	0	0	6	0

6. Chapter V: Comparison of the capacity to describe fully identified fungal species using the two high-throughput techniques 454 pyrosequencing and NimbleGen phylochip

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(in preparation)

7. Chapter VI: Quantitative traceability of ectomycorrhizal samples using ARISA

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(in preparation for submitting to Forest Ecology and Management)

Abstract:

In the present context of growing interest for using ectomycorrhizal inoculation for practical purpose, a simple and semi-quantitative molecular technique is needed to trace and quantify the introduced fungi over time. Here, the quantification of three different ectomycorrhizal fungal species associated with two different host tree species was tested using automated ribosomal intergenic spacer analysis (ARISA). The results show that this technique can be used for semi-quantitative traceability of the ectomycorrhizal status of tree roots, based on the relative heights of the peaks in the electropherograms.

Keywords

Ectomycorrhiza, ARISA, semi-quantitative analysis, traceability, relative peak abundance

Introduction

In the present context of growing interest for using ectomycorrhizal inoculation for practical purpose (optimizing nursery techniques and forest stock production, improving the growth of plantation forests, producing edible mushrooms such as truffles, chanterelles or cepes, etc.), there is a need to quantify the ectomycorrhization rate in large-scale, field-based inoculation experiments, for instance when assessing the efficiency of inoculants or inoculation techniques, or when studying the competition between resident and introduced ectomycorrhizal fungi. As the heterogeneity of such field experiments and the number of replicate samples are high, the used technique must be simple and easy to handle.

Until now a wide range of molecular fingerprinting techniques have been used to trace and identify fungal species (Henrion et al. 1992, 1994; Erland et al. 1994; Selosse et al. 1998; Dickie et al. 2002; Pennanen et al. 2005), but the quantification of fungal species is most often poorly or not at all estimated. For research work concerning quantifications, very often the non-molecular based morphotyping followed by ectomycorrhizal root tip counting has been the method of choice. All the techniques used in the early works mentioned above relied on time-consuming and biased enzymatic digestions (Avis et al., 2007). Therefore, a more simple technique is needed for automated quantification or at least semi-quantification of mycorrhizal samples.

Automated Ribosomal Intergenic Spacer Analysis (ARISA) is one of the electrophoresis based fingerprinting techniques where the banding patterns are representing the different PCR-amplified species from the sample. In comparison to the widely used T-RFLP technique, multiple restriction digestion is not required. ARISA has mostly been used to describe bacterial communities (Hernandez-Rasuet et al. 2006; Ikeda et al. 2008), but also fungal communities (Torzilli et al. 2006) without linking the traceability of defined species with semi-quantitative capacity of this technique.

The aim of the present work was therefore to test whether the ARISA technique has the capacity to trace and semi-quantify inoculated fungal species in mycorrhizal associations. This hypothesis has been tested on ectomycorrhizal root tips formed by *Laccaria bicolor, Paxillus involutus* and *Scleroderma citrinum,* sampled from beech (*Fagus sylvatica*) and Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings of a tree nursery in North-Eastern France. Mycorrhizal tips formed by the three different fungi were mixed in defined quantities. The different species were identified by their specific ARISA electropherograms. Relative peak height, calculated for each fungal species, significantly reflected the ratio of the mixed root tips.

Materials and Methods

Biological material

Seeds of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) from provenance zone 412 (Washington State, USA) were stratified in moist peat at 4°C for 4 weeks to break dormancy. Seeds of beech (*Fagus sylvatica*) from provenance 201 Nord-East were sown directly.

The three ectomycorrhizal fungi *Laccaria bicolor* S238N (Di Battista *et al.*, 1996; short distance explotation type according to Agerer, 2001) (LC), *Paxillus involutus 01* (Px, long-distance exploration type with rhizomorphs) and *Scleroderma citrinum* Foug A (Sc, also long distance with rhizomorphs) were separately maintained on Pachlewski agar medium (Pachlewski & Pachlewska, 1974). Fungal inoculum was prepared by aseptically grown mycelium in a peat-vermiculite nutrient mixture (Duponnois and Garbaye 1991).

Nursery

The experiment was performed in a bare-root forest nursery in Champenoux (eastern France). Soil preparation, inoculation and growth conditions were carried out as described by Frey-Klett et al. (1999). Fungal inoculum of different fungal species was separately distributed in plots. Each plot had a size of 0.75m x 1.5 m and 0.6m distance to the next plot. Stratified seedlings of *Fagus* and *Pseudotsuga* were sown separately in the different plots with one seed every 2 cm. Seeds were covered by disinfected soil.

Sample processing and PCR

We harvested three individual seedlings of each host tree species, with their whole root systems, six month after sowing. After morphologically determining the ectomycorrhizal status of each seedling, ectomycorrhizal tips of the different morphotypes were pooled. For each host tree, four artificial, mixed samples were assembled, each one with different proportions of the three ectomycorrhizal types, but always with the total amount of 15 ectomycorrhizal root tips: (1) 5-5-5; (2) 13-1-1; (3) 1-13-1 and (4) 1-1-13 with *L. bicolor*, *P. involutus* and *S. citrinum*, respectively. Three biological replicates per mixed sample (15 ectomycorrhizal root tips, 20-30 mg fresh weight) were analysed. Furthermore, three biological replicates (mycelium samples, 100 mg fresh weight each) for each fungal species were harvested from fungal plate cultures. All samples were snap-freezed in liquid nitrogen and kept at -20°C, then ground in a ball mill. DNA was extracted from samples using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France).

Only the ITS region 1 from DNA extracts were amplified with WellRED fluorescent dye D4-PA labelled forward primer ITS1f (Sigma-Aldrich, Lyon, France) (5'-CTTGGTCATTTAGAGGAAGTAA-3') and unlabelled reverse primer ITS 2 (5'-GCTGCGTTCTTCATCGATGC-3'). The PCR reactions were carried out in a final volume of 25 µl reaction mixture containing 2.5 µl of 1x PCR buffer (MP Biomedicals, Illkirch, France), 1.5 mM $MgCl_2$ (MP Biomedicals), 1.4 µl of 16 mg/ml bovine serum albumine (Sigma), each deoxynucleoside triphosphate at a concentration of 0.05 mM, each primer at a concentration of 0.4 µM, 0.5 U of Taq DNA Polymerase (MP Biomedicals) and 2 µl of extracted DNA (corresponding to 6 to13 ng DNA in the final mix, depending on the sample). PCR included an initial denaturation step at 94°C for 1 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 50°C and 2 min at 72°C with a final extension for 10 min at 72°C. On each sample a technical replication was run.

Automated ribosomal intergenic spacer analysis (ARISA)

1 µl of 1:10 diluted PCR products were mixed with 0.5 µl DNA size standard 600 nt (Beckman Coulter®, Roissy, France) and 30 µl of sample loading solution (Beckman Coulter®). After covering the samples with a drop of oil, the intergenic spacer fragments were run on a Beckman Coulter CEQ 8000 Genetic Analysis System. Peaks with a peak height ≤ 5000 (dye signal, arbitrary unit) were excluded from the analysis. The threshold level for intraspecies variation was set to ± 1.5 bp, according to the variability observed from the analyses performed with the pure cultures of the three fungal species (results not shown). Relative peak height was calculated by dividing individual peak heights by the total peak heights per electropherogram.

Stastistical analysis

A correlation test was run using the Pearson's product-moment correlation function of the R software 2.7.2. Thereby the measured (ARISA-derived) relative abundance for each species in the mixed samples was compared to the theoretical (known from mixing the composite samples) relative abundance Correlation curves were drawn using the correlation curve function of the Excel software (2008, version 12.1.3).

Results

ARISA analysis of free-living mycelium (pure cultures) produced electropherograms with a large, specific rDNA ITS1 peak for each ectomycorrhizal species. The specific peak of *S. citrinum* was detected at a fragment length of 299 bp, the one of *L. bicolor* at 358 bp and of *P. involutus* at 397 bp (data not shown). The species specific peaks were used to correlate peak appearance to species presence in electropherograms of mixed samples. Comparison of electropherograms from technical and biological replicates indicated the conservation of peak height proportions and reflected the composition of mycorrhizal root tips from the three different fungi within mixed samples (Fig. 1). Fungal species present with only one ectomycorrhizal root tip in a mixed sample of fifteen, could still be detected.

However, as we wanted to know whether ARISA electropherograms could be used for semi-quantitative analysis, we compared the relative peak heights of mixed samples to theoretical relative peak heights using statistical tests (Fig. 2). Correlation analysis revealed the significance of linear regression of mixed sample ratios on theoretical ratios in both host trees with $r^2=0.76$; $p<0.0002$ and $r^2=0.98$; $p<0.00001$ for *Fagus* and *Pseudotsuga* samples respectively. Additionally, we calculated correlation coefficients and p-values for each ectomycorrhizal species separately: theoretical and experimental ratios were for all three tested species significantly correlated ($r^2>0.8$; p-values < 0.004, data not shown).

Discussion

Molecular fingerprinting techniques have been largely used to identify individual mycorrhizas (Gardes and Bruns 1993; Erland et al. 1994) or the influence of biotic and abiotic factors on fungal community structures (Dickie et al. 2002; Pennanen et al. 2005; Ishida et al. 2007). Nonetheless, identification in itself only reveals the presence of a fungal species in a fungal community. Quantification of ectomycorrhizas from environmental sample seems to be more problematic. One molecular approach allowing quantification is real-time quantitative PCR (qPCR). It is frequently used in clinical diagnosis (Manzin et al. 1995; Sedgley et al. 2004) or for growth assessment in mycological research (Cullen et al. 2001; Boyle et al. 2005). Regarding ECM, qPCR has been used for quantifying *Piloderma croceum* mycelium in pure cultures and rhizotron-grown *P. croceum* ectomycorrhizas (Schubert et al. 2003), or from soil (Landeweert et al. 2003). However, qPCR is a time consuming technique because the standardization process implies designing species-specific primers and a fastidious calibration procedure.

ARISA creates 'fingerprints' of fungal samples from profiles of the ITS region of fungi, based on the length of the amplified nucleotide sequence, which displays significant heterogeneity between species. It is frequently used to describe and identify bacterial (Hernandez-Rasuet et al. 2006; Ikeda et al. 2008; Lejon et al. 2005) as well as fungal communities (Torzilli et al. 2006) because it is very simple to operate. But anyhow the question is whether it can be used for quantitative traceability of defined fungal species. There are several factors influencing quantification. First, the fungal biomass of fungal species is likely to be variable because the structure and the proportion of extra-radical mycelium of ectomycorrhizas differ widely depending on the exploration type they belong to (Agerer 2001). Second, the amplification of the fungal DNA from an ectomycorrhiza is dependent on the presence/absence of inhibitors (e.g. polyphenolic compounds from the root tissues of the host-tree, or resulting from co-extraction of the DNA of the host tree) or on other species in the sample.

To study the possible use of ARISA as a quantitative tracing technique, we calculated the relative abundance of three ectomycorrhizal fungal species based on peak heights from known fungal mixes. The used ectomycorrhizas belonged to different exploration types (Agerer 2001) and were associated with two different host trees. DNA-extraction and PCR amplification were carried out on the complete ectomycorrhizal mix to analyse the above-described bias on quantitative use of ARISA.

Electropherograms of technical replicates showed similar results (data not shown), which indicates a minor influence of DNA-extraction and PCR bias on our experiment. Regression

lines of the mixed samples from the two different host trees revealed a significant host effect. However, the most important result is, that the significant correlation between the theoretical and experimental data supports the idea of using ARISA as a quantitative tracing technique.

The influence of the fungal species on the ARISA results has been evaluated by calculating regression for each species separately. A slight influence could be observed, but it can be disregarded for quantitative analysis because of the very highly significant overall correlation ($p<0.004$).

To conclude, ARISA can be used as an easy and reliable semi-quantitative technique to trace ectomycorrhizal development in the field. However, its present limitation is that it only determines the relative abundance of a given fungal species among the whole ectomycorrhizal community, but not the total ectomycorrhizal colonization of the root systems studied. Therefore, an interesting development of the method would be to design internal standards (e.g. rDNA from the root tissues) to assess this variables.

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Fig. 1: ARISA-electropectograms of four *Pseudotsuga menziesii* mycorrhizal root tip samples. Each sample contained in total 15 mycorrhizal root tips of three ECM species, but quantities of each fungal species differed between samples. Mycorrhizal root tips/sample: diagram A: 5-5-5; diagram B: 1-1-13; diagram C: 13-1-1; diagram D: 1-13-1 with *S. citrinum* (Sc)- *L. bicolor* (Lc)- *P. involutus* (Px).

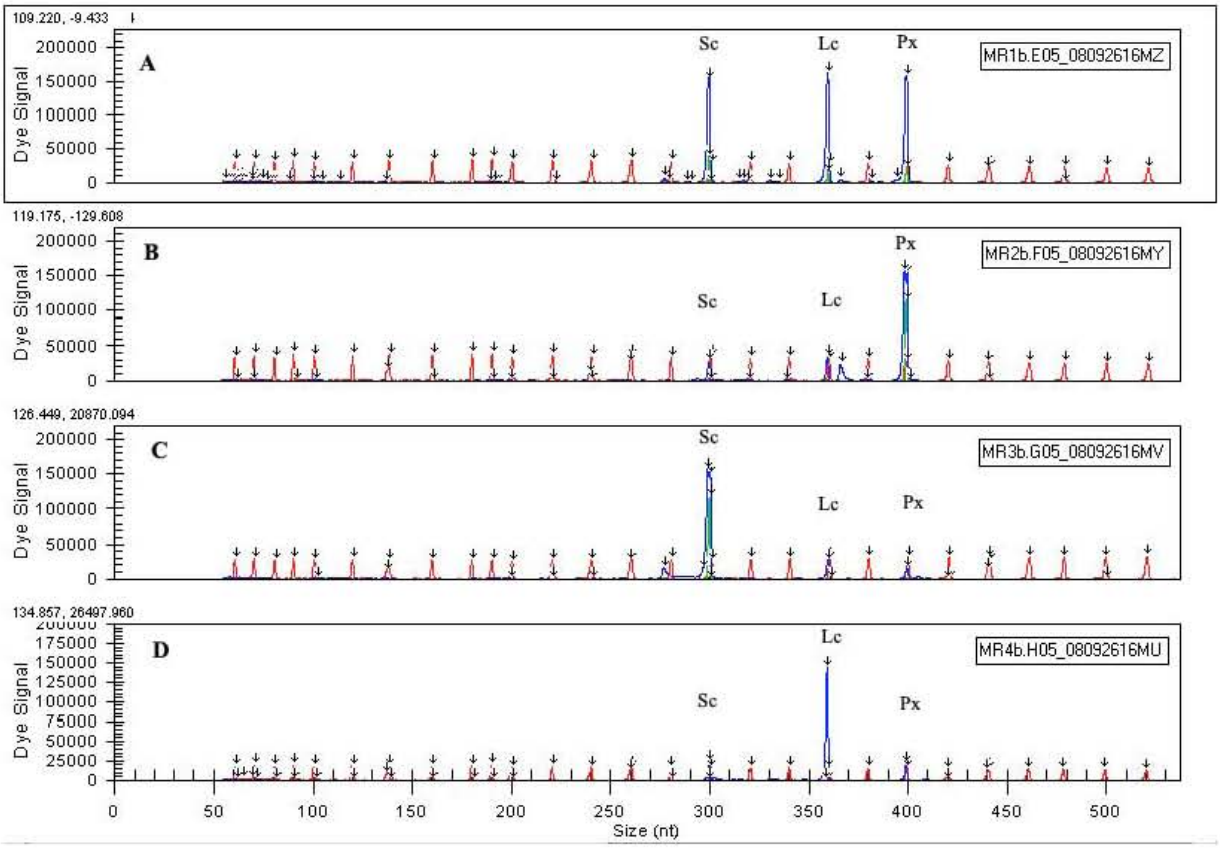
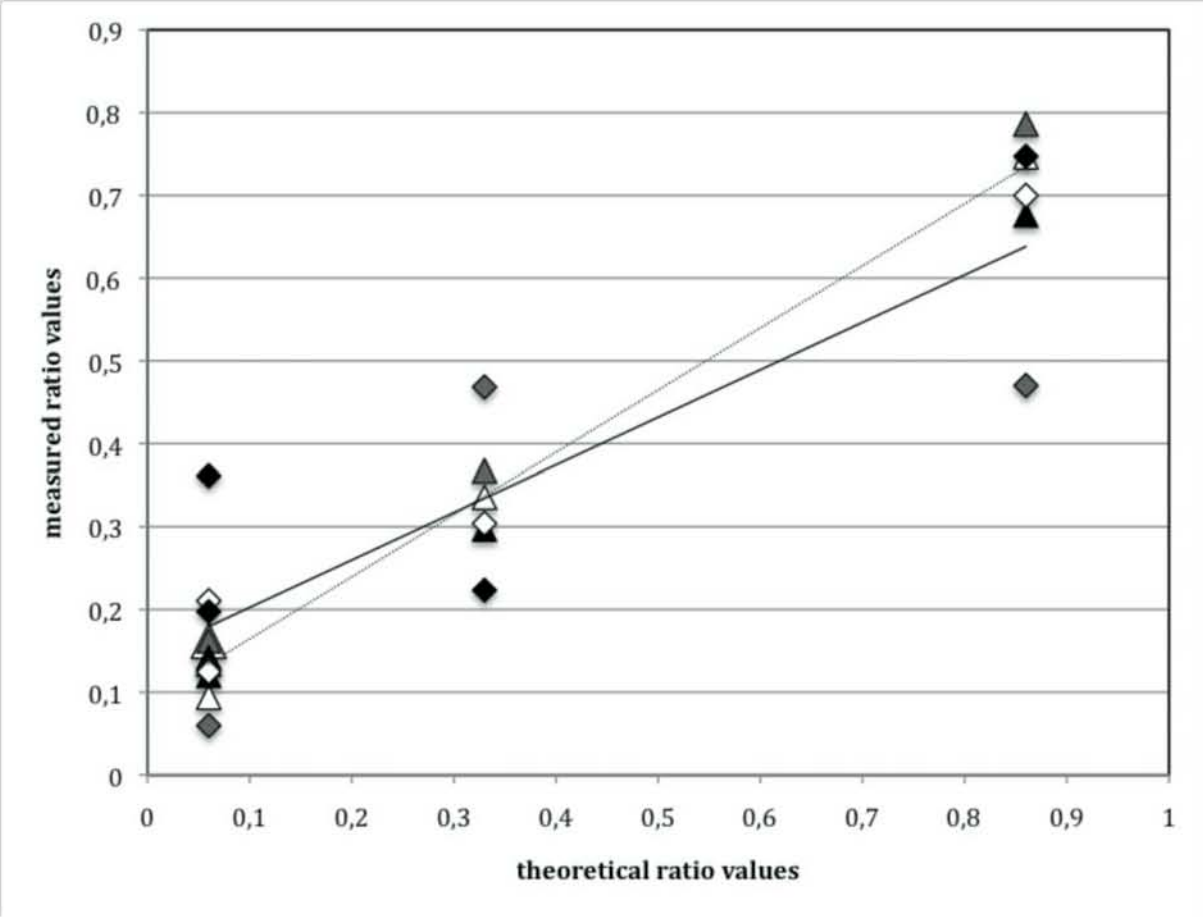


Fig. 2: Regression line between theoretical and measured ratio values of the four mycorrhizal root tip mixes, in which the mycorrhiza from three different fungi (black, *Laccaria bicolor;* white, *Scleroderma citrinum;* grey, *Paxillus involutus*) have been regrouped in following quantities: 5-5-5; 13-1-1; 1-13-1; 1-1-13. Two independent correlation curves were calculated for the mycorrhizal mixes dependent on host tree association: *Fagus sylvatica* (diamonds, constant line; R^2=0.764; p<0.001) and *Pseudotsuga menziesii* (triangles, dotted line; R^2=0.983; p<0.0001).



8. Chapter VII: Symbiosis insights from the genome of the mycorrhizal basidiomycete *Laccaria bicolor*.

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9. Chapter VIII: Fatty acid metabolism in the ectomycorrhizal fungus *Laccaria bicolor*

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Fatty acid metabolism in the ectomycorrhizal fungus *Laccaria bicolor*

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Key words: *Laccaria bicolor*, lipid metabolism, marker lipid, mycorrhiza, subcellular localization.

Summary

- Here, the genome sequence of the ectomycorrhizal basidiomycete *Laccaria bicolor* was explored with the aim of constructing a genome-wide inventory of genes involved in fatty acid metabolism.
- Sixty-three genes of the major pathways were annotated and validated by the detection of the corresponding transcripts. Seventy-one per cent belonged to multigene families of up to five members. In the mycelium of *L. bicolor*, 19 different fatty acids were detected, including at low concentrations palmitvaccenic acid (16:1(11Z)), which is known to be a marker for arbuscular mycorrhizal fungi.
- The pathways of fatty acid biosynthesis and degradation in *L. bicolor* were reconstructed using lipid composition, gene annotation and transcriptional analysis. Annotation results indicated that saturated fatty acids were degraded in mitochondria, whereas degradation of modified fatty acids was confined to peroxisomes.
- Fatty acid synthase (FAS) was the second largest protein annotated in *L. bicolor*. Phylogenetic analysis indicated that *L. bicolor*, *Ustilago maydis* and *Coprinopsis cinerea* have a vertebrate-like type I FAS encoded as a single protein, whereas in other basidiomycetes, including the human pathogenic basidiomycete *Cryptococcus neoformans*, and in most ascomycetes FAS is composed of the two structurally distinct subunits α and β.

Abbreviations: aa, amino acids; ACC, acetyl-CoA carboxylase; ACDH, acyl-CoA dehydrogenase; ACP, acyl carrier protein; ACT, acylcarnitine transferase; AcCT, acetylcarnitine transferase; ACX, acyl-CoA oxidase; ASG, acylated sterol glycoside; AT, acetyl-CoA-ACP transacetylase; CACT, carnitine/acylcarnitine translocase; DAG, diacylglycerol; DCI, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase; DCR, 2,4-dienoyl-CoA reductase; DGDG, diglycosyl diacylglycerol; DH, 3-hydroxyacyl-ACP dehydratase; ECH, enoyl-CoA hydratase; ER, β-enoyl reductase; EST, expressed sequence tag; FA, fatty acid; FAS, fatty acid synthase; FFA, free fatty acid; GC, glucosyl ceramide; HCDH, 3-hydroxyacyl-CoA dehydrogenase; IDH, isocitrate dehydrogenase; KCT, 3-ketoacyl-CoA thiolase; KR, 3-ketoacyl reductase; KS, 3-ketoacyl synthase; LACS: long chain FA-CoA ligase; MAG, monoacylglycerol; MFP, peroxisomal D-specific bifunctional protein harbouring hydratase-dehydrogenase activities; MT, malonyl-CoA-ACP transacylase; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; PPT, phosphopantetheinyl transferase; PXA: peroxisomal long-chain FA import protein (ATP-binding cassette (ABC) transporter); SE, sterol ester; SG, sterol glycoside; SGD, Saccharomyces Genome Database; SPE, solid phase extraction; TAG, triacylglycerol; TLC, thin-layer chromatography; WGEO, whole-genome expression oligoarray.

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Introduction

Fatty acids (FAs), the major building blocks of lipids, are common to all living cells. They are components of membranes and storage lipids. In addition to these central functions, FAs and their derivatives also play important roles as signalling molecules (Berg *et al.*, 2006) and as crucial compounds in establishing pathogenic plant–fungal interactions (Wilson *et al.*, 2004; Klose & Kronstad, 2006). Fungi generally accumulate significant amounts of lipids in hyphae as well as in spores, and these lipids serve as carbon and energy sources during starvation and spore germination (Martin *et al.*, 1984; Laczko *et al.*, 2003; Trépanier *et al.*, 2005).

In mutualistic interactions, such as mycorrhizas, the fungal partner is dependent on carbon supply by the host. Arbuscular mycorrhizal fungi metabolize plant-derived carbohydrates into lipids, yielding lipid bodies which can then be transported from the intra- to the extraradical hyphae; during their journey to the hyphal tips, lipids are partly consumed but re-shuttling also takes place (Sancholle *et al.*, 2001; Bago *et al.*, 2002a,b). During the formation of ectomycorrhizas, the composition of FAs also shows drastic changes, probably also involving lipid translocation (Martin *et al.*, 1987; Laczko *et al.*, 2003).

In addition, FAs have been used as markers to characterize microbial communities (Olsson 1999; van Aarle & Olsson, 2003; Bååth, 2003) and soil food webs (Ruess *et al.*, 2002). The unsaturated FA palmitvaccenic acid, 16:1(11*Z*) (x:y(z) denotes an FA with *x* carbons and *y* double bonds in position *z* counting from the carboxyl end), which corresponds to the denomination 16:1(ω5) or 16:1(n-5), has been widely applied to estimate arbuscular mycorrhizal fungi in soil samples and quantify fungal colonization (van Aarle & Olsson, 2003; Nilsson *et al.*, 2005; Stumpe *et al.*, 2005). The phospholipid FA 18:2(9*Z*,12*Z*) (=18:2(ω6,9)) was used to assess the amount of ectomycorrhizal fungi (Bååth *et al.*, 2004) but occurs also in saprophytes (Olsson, 1999). Despite the widespread utilization of FAs as biological markers and their importance for many life functions, little is known on lipid biosynthesis and degradation in ectomycorrhizal fungi.

The objective of this study was to identify and characterize the set of genes involved in FA biosynthesis, modification, and degradation in the recently sequenced *Laccaria bicolor* genome (Martin *et al.*, 2008). Starting with the analysis of all FAs and many lipid classes our analysis included cataloguing predicted FA metabolism proteins, predicting their subcellular localisation and validation of their expression by transcriptional analysis on whole genome arrays. Phylogenetic analyses of fatty acid synthase (FAS) revealed striking differences in FA biosynthesis between *L. bicolor* and other sequenced fungi.

Materials and Methods

Fungal culture, ectomycorrhiza synthesis and harvest

Free-living mycelium of *Laccaria bicolor* (monocaryotic strain S238N-H82 (INRA Nancy), phylum: Basidiomycota, Agaricales, Hydnangiaceae) was grown on 10 cellophane-covered agar plates containing Pachlewski medium (Di Battista *et al.*, 1996) for 3 wk in darkness at 25°C. Only the three most healthy looking mycelia, which were evenly grown over the agar plate, were used for futher analysis. Ectomycorrhizas of *L. bicolor* S238N/*Pseudotsuga menziesii* were synthesized by growing Douglas fir seedlings for 9 months in polyethylene containers filled with a peat-vermiculite mix (1:1, v/v) and mixed with 2.5% (v/v) *L. bicolor S238N* inoculum as described previously (Frey-Klett *et al.*, 1997). Inoculated Douglas fir plantlets were grown in a glasshouse with 25°C : 15°C (day : night) temperature and fertilised once a week with 80 mg l^{-1} KNO_3 and 0.2 ml l^{-1} Kanieltra (COFAZ, Paris, France). Additionally, plants without fungal inoculum were grown under the same conditions and further Douglas fir seedlings were raised under axenic conditions for 2 months (Dučić *et al.*, 2008). Ectomycorrhizal root tips of *L. bicolor* and noninfected fine roots of control plants were identified under the dissection microscope and stored frozen in liquid nitrogen for further analyses.

Microarray analysis

RNA extraction of mycelium and ectomycorrhizas was carried out using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA preparations (three biological replicates) were amplified using the SMART PCR cDNA Synthesis Kit (Clontech, Saint Quentin Yvelines, France) according to the manufacturer's instructions. Single dye labelling of samples, hybridization procedures, data acquisition, background correction and normalization were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland) following their standard protocol. The *L. bicolor* 4-plex (4 × 72k) whole-genome expression oligoarray v 2.0 (WGEO), manufactured by NimbleGen (Madison, WI, USA), contains three independent, nonidentical, 60-mer probes per whole gene model. For 4702 gene models the array contains technical replicates. To estimate a cut-off level for expression, the mean signal intensity of 2032 random probes present on the microarray was calculated. Gene models with a signal intensity 3-fold higher than the calculated cut-off value of 78 were considered as transcribed. In the present study, signal intensities for transcripts of genes related to FA metabolism are shown. Relative expression levels were calculated as transcript signal in ectomycorrhiza/transcript signal in free-living mycelium. Student's *t*-test, in combination with false discovery rate multiple testing corrections included in the ArrayStar 2 analysis software (DNASTAR Inc., Madison, Wisconsin, USA), was used to identify genes whose transcripts changed significantly with *P*-values ≤ 0.05.

Quantitative RT-PCR

To validate array analyses, quantitative RT-PCR was performed for selected genes (whose protein identification numbers (IDs)

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can be found at http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html), namely acetyl-CoA carboxylase (ACC; ID: 187554), Δ^9-fatty acid desaturase (scaffold 8; ID 189797), Δ^{12}-fatty acid desaturase (scaffold 3; ID 292603), 3-ketoacyl-CoA synthase (subunit of fatty acid-elongase of ELO type; ID 186873) and fatty acid synthase (FAS; scaffold 11; ID 296983), and for three house-keeping genes, namely elongation factor 3 (ID 186873), GTPase β-subunit (ID 190157) and metalloprotease (ID 245383). Primers were designed using the software AmplifX 1.37 (http://ifrjr.nord.univ-mrs.fr/AmplifX) and Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) and are shown in Supporting Information Table S1.

Quantitative PCR assays were performed in Low Profile Thermo-Strips (ABgene, Surrey, UK) using a Chromo 4 Detector (MJ Research, Waltham, MA, USA). The reaction mixture contained 2 × iQ SYBR Green Supermix (Bio-Rad), 300 nM of each primer and 18.75 ng of cDNA from *L. bicolor* mycelium or ectomycorrhizas. Three biological and three technical replicates were analysed for each tissue. Data were analysed using the relative quantification method of Pfaffl (2001). Quantitative real-time PCR supported the WGEO analyses as the two methods gave similar results, although different extracts were used (Table S2).

Extraction and detection of lipids by gas and thin-layer chromatography

Fungal and plant tissues were freeze-dried (Dura-Top™/FTS Systems™; BioBlock Scientific, Illkirch Cedex, France). For gas chromatography/flame ionization detection (GC/FID), FAs from 10 mg of lyophilised fungal or plant material was converted to their methyl esters (Miquel & Browse, 1992). For quantification of the FAs, 20 µg of triheptadecanoate was added and the sample was re-dissolved in 10 µl of acetonitrile for GC analysis performed with an Agilent (Waldbronn, Germany) 6890 gas chromatograph fitted with a capillary DB-23 column (30 m × 0.25 mm; 0.25 µm coating thickness; J&W Scientific, Agilent, Waldbronn, Germany). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. Two different temperature gradients were used for the fatty acid methyl ester (FAME) analysis. The temperature gradient was either 150°C for 1 min, 150–200°C at 8 K min^{-1}, 200–250°C at 25 K min^{-1} and 250°C for 6 min or 150°C for 1 min, 150–200°C at 4 K min^{-1}, 200–250°C at 5 K min^{-1} and 250°C for 6 min (Stumpe *et al.*, 2005; Przybyla *et al.*, 2008).

For thin-layer chromatography (TLC) analysis of different lipid classes, total lipids were extracted from 50 mg of lyophilized fungal material with chloroform:methanol (1 : 2, v/v) for 4 h at 4°C. After centrifugation (3000 ***g*** for 5 min at 4°C), the supernatant was collected and the pellet was re-extracted with chloroform:methanol (2 : 1, v/v) for 16 h at 4°C. The resulting lipid extracts were combined and filtrated with cotton wool soaked with $NaSO_4$ and dried under streaming nitrogen. The total lipids were separated into neutral lipids, glycolipids and phospholipids on a solid phase extraction column (Strata SI-1 Silica, 100 mg/1 ml; Phenomenex, Aschaffenburg, Germany). Neutral lipids were eluted with 10 ml of chloroform, glycolipids with 10 ml of acetone:2-propanol (9 : 1, v/v) and finally phospholipids with 10 ml of methanol:acetic acid (9 : 1, v/v). These three fractions were further resolved on 10 × 20 cm silica gel 60 TLC plates (Merck, Darmstadt, Germany): Neutral lipids were developed with petroleum ether:diethyl ether:acetic acid (70 : 30 : 0.5, v/v/v), glycolipids with chloroform:methanol (85 : 15, v/v) and phospholipids with chloroform:methanol:acetic acid (65 : 25 : 8, v/v/v). The individual lipid classes were identified after incubation in $CuSO_4$ solution (0.4 M $CuSO_4$ in 6.8 % (v/v) H_3PO_4) and heating at 180°C.

Gene annotation of FA metabolism in *Laccaria bicolor* S238N-H82

Putative genes that encode FA metabolism enzymes initially were identified based on the automatic annotation in the publicly accessible *Laccaria* genome database (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/) at the Joint Genome Institute (JGI). Additionally, searches were performed using a range of sequences of FA metabolism proteins and genes (Mekhedov *et al.*, 2000) available from fungi (*Cryptococcus neoformans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) at the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/) and the Universal Protein Resource (UNIPROT) (http://expasy.org/) to probe the *Laccaria* genome database using the BLASTN, TBLASTN, and BLASTP algorithms as incorporated in the JGI accession page and the INRA *Laccaria* DB (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/). The putative homologues that were detected were characterized based on conserved domains (CDD of NCBI; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), identities, and E-values. *Laccaria bicolor* gene models were corrected when necessary. Manual annotation was carried out using the artemis software (http://www.sanger.ac.uk/Software/Artemis/). The manually annotated gene sequences were aligned and verified using the programs ClustalX (http://www.embl.de/~chenna/clustal/darwin/) and genedoc (http://www.psc.edu/biomed/genedoc/). Each curated homologue was further used for a BLAST search at the JGI, YeastDB (http://www.yeastgenome.org/) and Broad-MIT Institute (http://www.broad.mit.edu/) databases to check for similar genes in other fungi, including *Aspergillus nidulans*, *Coprinopsis cinerea*, *C. neoformans*, *N. crassa*, *Ustilago maydis*, *Phanerochaete chrysosporum* and *S. cerevisiae*.

Subcellular localization of putative proteins was predicted using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/), mitoprot server (http://ihg.gsf.de/ihg/mitoprot.html), predotar server (http://urgi.versailles.inra.fr/predotar/predotar.html), 'The PTS1 predictor' server (http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp) and wolf psort (http://

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Table 1 Fatty acid composition of free-living mycelium, ectomycorrhizas of *Laccaria bicolor* (EM) and *Pseudotsuga menziesii* (Douglas-fir) roots

FA	Mycelium (%)	EM (%)	Uninoculated roots (%)
14:0	0.19 ± 0.01	nd	nd
15:0	0.97 ± 0.03	nd	nd
16:0	14.33 ± 0.22b	16.09 ± 5.01b	9.60 ± 1.42a
16:1 (9*Z*)	0.27 ± 0.01c	0.23 ± 0.02b	0.15 ± 0.01a
16:1 (11*Z*)	0.09 ± 0.03a	0.26 ± 0.08b	0.05 ± 0.04a
14-Me-16:0	nd	2.76 ± 0.35a	5.99 ± 0.88b
18:0	0.82 ± 0.06a	2.59 ± 1.91a	2.55 ± 0.66a
18:1 (9*Z*)	27.19 ± 1.59b	4.06 ± 1.60a	5.32 ± 1.67a
18:1 (11*Z*)	0.05 ± 0.01a	0.51 ± 0.21b	0.50 ± 0.25b
18:2 (5*Z*.9*Z*)	nd	1.52 ± 1.65a	0.90 ± 0.31a
18:2 (9*Z*.12*Z*)	49.51 ± 2.49a	39.73 ± 8.01b	20.40 ± 7.03b
18:3 (5*Z*.9*Z*.12*Z*)	nd	2.53 ± 0.76a	3.84 ± 1.63a
18:3 (9*Z*.12*Z*.15*Z*)	0.15 ± 0.02a	2.96 ± 0.93b	3.98 ± 1.36b
20:0	0.30 ± 0.04a	3.78 ± 0.78b	7.62 ± 2.30c
20:1 (11*Z*)	0.25 ± 0.01a	nd	0.22 ± 0.10a
20:2 (11*Z*.14*Z*)	0.09 ± 0.01a	0.34 ± 0.42a	0.24 ± 0.11a
20:3 (5*Z*.11*Z*.14*Z*)	nd	1.89 ± 0.23a	4.33 ± 1.25b
21:0	0.03 ± 0.01a	nd	1.15 ± 1.07b
22:0	0.50 ± 0.11a	7.40 ± 1.84b	14.18 ± 4.02c
23:0	0.23 ± 0.05a	1.99 ± 0.70a	0.71 ± 0.16a
23:1 (14*Z*)	0.13 ± 0.01a	0.34 ± 0.22a	0.38 ± 0.34a
24:0	4.20 ± 0.71a	3.05 ± 1.07a	3.40 ± 1.07a
24:1 (15*Z*)	0.64 ± 0.04a	6.70 ± 1.99b	3.28 ± 2.03b
18:0 DiCOOH	nd	1.35 ± 0.41a	2.74 ± 1.43b
20:0 DiCOOH	nd	2.35 ± 0.65a	4.31 ± 1.96b
22:0 DiCOOH	nd	1.79 ± 0.57a	2.35 ± 1.17a
	(μmol g^{-1} DW)	(μmol g^{-1} DW)	(μmol g^{-1} DW)
Total amount of fatty acids	119.51 ± 4.18b	37.33 ± 8.06a	26.94 ± 2.93a

nd, not detected.
Mycelium was cultivated as described in Materials and Methods and harvested after 22 d. Douglas-fir seedlings were inoculated with *L. bicolor.* Ectomycorrhizas and roots of uninoculated seedlings were harvested after 8 months. Roots of axenically grown Douglas-fir seedlings showed the same lipid composition. Data indicate means (n = 4– 6 biological replicates) ± SE. The relative abundance of individual compounds is given as weight %. The total amount of fatty acids is given in μmol g^{-1} dry weight. Different letters in rows indicate significant differences at $P \leq 0.05$.

wolfpsort.seq.cbrc.jp/) prediction algorithms. *Laccaria bicolor* S238N-H82-derived sequences were used for BLAST analysis of the expressed sequence tag (EST) database available at INRA *Laccaria* DB (A. Kohler, unpublished).

Phylogenetic analysis

The protein sequences of fungal FASs were phylogenetically analysed using the ARB program (Ludwig *et al.*, 2004). Alignments were constructed using ClustalX (Thompson *et al.*, 1997) and manually edited over the ARB alignment interface. Ambiguous alignment positions were excluded from the phylogenetic analysis and a 50% similarity filter was set. To estimate phylogenetic relationships, alignments were analysed using the PROTEIN MAXIMUM LIKELIHOOD program including a hidden Markov model (Felsenstein & Churchill, 1996). No outgroup sequence was used.

Results

An inventory of FAs in *L. bicolor* and its host *P. menziesii*

In *L. bicolor*, 19 different FAs were detected (Table 1; see also Fig. S1 for GC/FID profiles). Their chain lengths varied from 14 to 24 carbon (C) atoms. The FAs 14:0 and 15:0 were only found in free-living mycelium, whereas all other FAs also occurred in sporocarps (not shown). The most abundant FAs were palmitic acid 16:0, oleic acid 18:1(9*Z*) and linoleic acid 18:2(9*Z*,12*Z*) contributing 14, 27 and 50%, respectively, to the total amount of FAs present in the mycelium (Table 1).

With the exception of 14:0 and 15:0, all FAs detected in the fungus were also present in roots of uninoculated as well as aseptically grown Douglas-fir (not shown). However, overall FA concentrations in roots from uninoculated plants were significantly lower than those in fungal tissues (Table 1). In comparison with roots, fungal tissues contained higher concentrations

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of the unsaturated FAs 18:1(9*Z*) and 18:2(9*Z*,12*Z*), respectively. Roots contained seven additional FAs not present in fungal tissues. Among these, 14-Me-16:0 was the most abundant (6%). Three root-specific unsaturated FAs (taxoleic acid (18:2(5*Z*,9*Z*)), pinolenic acid (18:3(5*Z*,9*Z*,12*Z*)) and sciadonic acid (20:3(5*Z*,11*Z*,14*Z*))) contributed 0.9, 3.8 and 4.3%, respectively, to the total amount of FAs. All four of these FAs are typical components of gymnosperm lipids (Wolff *et al.*, 1997a,b). Di-carboxylic acids of 18:0, 20:0 and 22:0 FAs have been found in tree species before (Wolff *et al.*, 1997a) and contributed together about 9.3% to root FA content (Table 1).

Ectomycorrhizas showed a mixed FA pattern containing all root-specific FAs but lacking 14:0, 15:0 and 20:1(11*Z*) and 21:0; that is, compounds that were only present at very low concentrations in fungal tissues and just above the detection limit in roots (Table 1). This suggests that these FAs were diluted in ectomycorrhizas to below the detection limit.

Based on the decreases in six of the root-specific FAs (see above) in mycorrhizas (Table 1), one can estimate that the contributions of root and fungal tissues in the harvested ectomycorrhizas were about 66 and 34%, respectively. A notable exception was the root-specific taxoleic acid, which was actually slightly increased in ectomycorrhizas (Table 1).

In our search for ectomycorrhiza-specific changes in FAs, we compared the relative abundance of FAs in the different tissues. Ectomycorrhizas were significantly enriched in palmitvaccenic acid (16:1(11*Z*); 5.4-fold) and linoleic acid (18:2(9*Z*,12*Z*); 2.2-fold). Linoleic acid (18:2(9*Z*,12*Z*)) was the most abundant FA in both Douglas-fir and *L. bicolor* and showed a strong decline with increasing age in the latter organism (Fig. S2). By contrast, palmitvaccenic acid (16:1(11*Z*)) was among the rarest of the root FAs (0.05%), displayed the highest absolute and relative enrichment factors in ectomycorrhizas and showed no age-dependent changes in mycelium (Fig. S2).

To analyse the lipid classes that constituted the membranes of *L. bicolor*, lipid extracts from 14-d-old mycelium were fractionated into neutral lipids, glycolipids and phospholipids, respectively, and separated by TLC (Fig. 1). The fraction of neutral lipids consisted of free sterols, sterol esters and free FAs as well as mono-, di- and triacylglycerols. The fraction of glycolipids consisted of acylated and nonacylated sterol glycosides and another glycolipid with chromatographic properties similar to either a diglycosyl diacylglycerol or a diglycosyl sterol (Fig. 1; DGDG). The fraction of phospholipids consisted of phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. Taken together, these results show that lipids harbouring esterified FAs constituted a major proportion of the lipid fraction of *L. bicolor*.

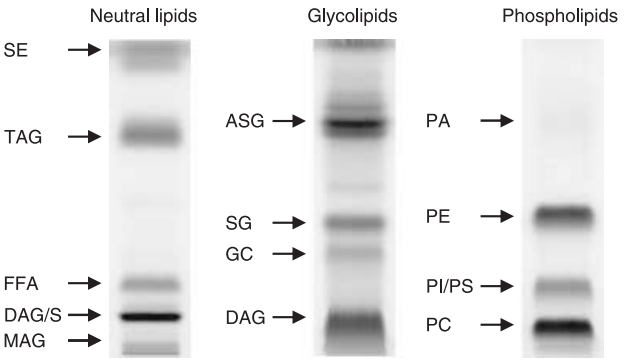

Fig. 1 Thin-layer chromatography (TLC) analysis of different lipid classes isolated from the lipid fraction of *Laccaria bicolor*. The lipids were extracted from lyophilized fungal material (50 mg) fractionated by solid phase extraction (SPE) into different lipid classes and analysed by TLC as described in the Materials and Methods. DAG, diacylglycerol; FFA, free fatty acids; MAG, monoacylglycerol; S, free sterol; SE, sterol ester; TAG, triacylglycerol; ASG, acylated sterol glycoside; GC, glucosyl ceramide; SG, sterol glycoside; DGDG, diglycosyl diacylglycerol (tentative); PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine.

The gene catalogue of FA metabolism

We annotated 63 genes of lipid metabolism belonging to 33 gene families. Among the annotated genes, 71% belonged to multigene families consisting of up to five members, which were, however, not clustered (Table 2). All enzymes needed for FA biosynthesis and seven gene families for FA modification processes, such as desaturation, were found. Sixteen gene families were found for β-oxidation (Table 2). Furthermore, five gene families were annotated for reactions involved in the glyoxylate and citrate cycle (for detailed annotation and information on carbohydrate metabolism, see Deveau *et al.* 2008). Sixty-six per cent of all annotated genes showed EST evidence (Table 2). For five genes alternative splicing was detected.

To determine whether annotated functional genes of FA metabolism were expressed, transcript profiling was conducted in mycelium and ectomycorrhizas of *L. bicolor* using WGEO arrays (Tables 3, 4). Predicted functional genes showed signal intensities well above the cut-off level, whereas gene models considered as pseudogenes showed zero or weak signal intensity within the range of the background signal. Four gene models for FA biosynthesis and one for FA degradation were not represented on the array (Tables 3, 4). The transcription of several genes for FA biosynthesis was decreased or remained unaffected in ectomycorrhizas compared with mycelium (Table 3). A putative 3-ketoacyl reductase that may be involved in the interconversion of acetoacetate and 3-hydroxy butyrate was the only gene whose relative transcript abundance increased in ectomycorrhizas compared with mycelium (Table 3). Among 33 genes related to FA degradation, the expression of carnitine/acylcarnitine translocase (CACT) and 3-ketoacyl-CoA thiolase (KCT) was suppressed and that of acylcarnitine transferase I (ACT I) was increased in ectomycorrhizas compared with mycelium (Table 4).

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Table 2 The annotated gene families of lipid metabolism in *Laccaria bicolor* S238N

Putative function	EC number	Localization	Protein ID	EST	PG	AS	Localization
Fatty acid biosynthesis							
Acetyl-CoA carboxylase	EC 6.4.1.2	Scaffold 2	187554	y	n	n	C
Acetoacetyl-CoA thiolase	EC 23.1.9	Scaffold 9	166928	y	n	n	C
FA-synthase	EC 2.3.1.86	Scaffold 11	296983	y	n	n	C
FA-synthase	EC 2.3.1.86	Scaffold 5	314211	n	y	n	–
FA-synthase	EC 2.3.1.86	Scaffold 57	313054	n	y	n	–
FA-synthase	EC 2.3.1.86	Scaffold 25	330158	n	y	n	–
FA-synthase	EC 2.3.1.86	Scaffold 7	399323	n	y	n	–
3-ketoacyl reductase	EC 1.1.1.100	Scaffold 57	255231	y	n	n	C
Acyl carrier protein		Scaffold 1	164630	y	n	y	C
Fatty acid desaturation and modification							
3-ketoacyl-CoA synthase (FA-elongation enzyme; ELO type)		Scaffold 1	186873	y	n	n	ER
Long-chain FA-CoA ligase	EC 6.2.1.3	Scaffold 9	319094	y	n	n	C
Δ^9-FA-desaturase	EC 1.14	Scaffold 1	301202	n	n	n	ER
Δ^9-FA-desaturase	EC 1.14	Scaffold 8	189797	y	n	n	ER
Δ^9-FA-desaturase	EC 1.14	Scaffold 27	295043	y	n	n	ER
Δ^9-FA-desaturase	EC 1.14	Scaffold 31	399315	n	y	n	ER
Δ^9-FA-desaturase	EC 1.14	Scaffold 79	399316	n	y	n	ER
Δ^{12}-FA-desaturase	EC 1.14.19	Scaffold 3	292603	y	n	n	ER
Δ^{12}-FA-desaturase	EC 1.14.19	Scaffold 41	309734	n	n	n	ER
Δ^{12}-FA-desaturase	EC 1.14.19	Scaffold 51	254799	n	n	n	ER
Δ^{12}-FA-desaturase	EC 1.14.19	Scaffold 23	303237	n	y	n	ER
Δ^{12}-FA-desaturase	EC 1.14.19	Scaffold 47	399320	n	y	n	ER
Δ^{15}-FA-desaturase	EC 1.14.19	Scaffold 3	245369	y	n	n	ER
Cytochrome b5 protein		Scaffold 4	183113	y	n	n	C
Cytochrome b5 reductase	EC 1.6.2.2	Scaffold 1	300832	y	n	n	C
β-oxidation (mitochondria)							
Acylcarnitine transferase	EC 2.3.1.7	Scaffold 6	189447	y	n	n	M
Acylcarnitine transferase	EC 2.3.1.7	Scaffold 11	184843	y	n	n	M
Acyl-CoA dehydrogenase	EC 1.3.99.13	Scaffold 25	191220	y	n	n	M
Carnitine/acylcarnitine translocase		Scaffold 1	301012	y	n	n	M
Carnitine/acylcarnitine translocase		Scaffold 5	183526	y	n	n	M
Enoyl-CoA hydratase	EC 4.2.1.17	Scaffold 10	234621	y	n	n	M
Enoyl-CoA hydratase	EC 4.2.1.17	Scaffold 4	245955	y	n	n	M
Long-chain FA-CoA ligase	EC 6.2.1.3	Scaffold 9	319094	y	n	n	C
3-ketoacyl-CoA thiolase	EC 2.3.1.16	Scaffold 38	185981	y	n	y	M
3-ketoacyl-CoA thiolase	EC 2.3.1.16	Scaffold 1	300690	y	n	n	M
3-hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	Scaffold 2	187873	y	n	n	M
3-hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	Scaffold 4	168519	n	y	n	M
β-oxidation (peroxisomes)							
Acetylcarnitine transferase 1	EC 2.3.1.7	Scaffold 3	188234	y	n	y	P (PTS1)
Acetylcarnitine transferase 2	EC 2.3.1.7	Scaffold 2	244106	y	n	y	C
Acyl-CoA oxidase	EC 1.3.3.6	Scaffold 9	319093	n	n	n	P (PTS1)
Acyl-CoA oxidase*	EC 1.3.3.6	Scaffold 11	190411	y	n	n	P (PTS1)
Acyl-CoA oxidase*	EC 1.3.3.6	Scaffold 11	190400	y	n	n	P (PTS1)
Acyl-CoA oxidase*	EC 1.3.3.6	Scaffold 25	320108	y	n	n	P (PTS1)
Long-chain FA-CoA ligase	EC 6.2.1.3	Scaffold 6	189537	y	n	n	P (PTS1)
D-multifunctional β-oxidation protein	EC 4.2.1 + EC 1.1.1.35	Scaffold 11	234865	y	n	n	P (PTS1)
Peroxisomal long-chain FA import protein 1		Scaffold 1	242594	y	n	n	P –
Peroxisomal long-chain FA import protein 2		Scaffold 4	141760	n	n	n	P –
$\Delta^{2,4}$-dienoyl-CoA reductase	EC 1.3.1.34	Scaffold 19	251259	n	n	n	P (PTS1)
3-ketoacyl-CoA thiolase	EC 2.3.1.16	Scaffold 1	301021	n	n	y	P (PTS2)
3-ketoacyl-CoA thiolase	EC 2.3.1.16	Scaffold 4	311721	y	n	n	P (PTS1)
3-hydroxy-2-methylbutyryl-CoA dehydrogenase	EC 1.1.1.178	Scaffold 24	320095	n	n	n	P (PTS1)
Δ^3,Δ^2-enoyl-CoA isomerase	EC 4.2.1.17	Scaffold 8	325594	n	n	n	P (PTS1)
$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase	EC 5.3.3	Scaffold 19	300535	n	n	n	P (PTS1)
$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase	EC 5.3.3	Scaffold 52	312422	y	n	n	P (PTS1)

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Table 2 *continued*

Putative function	EC number	Localization	Protein ID	EST	PG	AS	Localization
Catalase	EC 1.11.1.6	Scaffold 68	123238	y	n	n	P (PTS1)
NADP-dependent isocitrate dehydrogenase	EC 1.1.1.42	Scaffold 7	317084	y	n	n	P (PTS1)
NAD-dependent isocitrate dehydrogenase	EC 1.1.1.41	Scaffold 4	311861	y	n	n	P (PTS1)
Glyoxylate cycle							
Aconitase	EC 4.2.1.3	Scaffold 6	189452	y	n	n	C
Aconitase	EC 4.2.1.3	Scaffold 8	291064	y	n	n	C
Aconitase	EC 4.2.1.3	Scaffold 83	397786	n	n	n	C
Citrate synthase	EC 2.3.3.1	Scaffold 11	297019	y	n	n	P (PTS2)
Isocitrate lyase	EC 4.1.3.1	Scaffold 3	171643	y	n	n	P (PTS1)
Malate dehydrogenase	EC 1.1.1.37	Scaffold 9	326114	y	n	n	P (PTS1)
Malate synthase	EC 2.3.3.9	Scaffold 5	314107	y	n	n	P (PTS2)

*Contains an N terminal cytochrome b5 domain.
Localization of enzymes was predicted as described in the Materials and Methods (C, cytosol; ER, endoplasmatic reticulum; M, mitochondrion; PM, plasma membrane; P, peroxisome; PTS1/2, sequence contains peroxisomal target sequence 1/2; –, truncated sequence or not predictable). FA, fatty acid; EST, expressed sequence tag (y, yes; n, no); PG, pseudogene; AS, alternative splicing.

Table 3 Expression analysis of annotated genes of the fatty acid (FA) biosynthesis pathway in ectomycorrhizas (EM) and free-living mycelium of *Laccaria bicolor* strain S238N

Putative function	Scaffold	Protein ID	Signal intensity of transcript	Log_2 transcript ratio EM/mycelium	*P*-value
Acetyl-CoA carboxylase	2	187554	22288	**–2.03**	**0.03**
Acetoacetyl-CoA thiolase	9	166928	3533	–0.07	1
FA-synthase	11	296983	41552	–2.32	0.07
FA-synthase	25	330158	0		
FA-synthase	5	314211	0		
FA-synthase	57	313054	0		
FA-synthase	7	399323	No#		
Δ^9-FA-desaturase	1	301202	2720	+0.92	0.15
Δ^9-FA-desaturase	27	295043	44026	–0.23	0.20
Δ^9-FA-desaturase	8	189797	14616	**–3.47**	**0.02**
Δ^9-FA-desaturase	31	399315	No#		
Δ^9-FA-desaturase	79	399316	No#		
Δ^{12}-FA-desaturase	51	254799	2330	+0.29	0.78
Δ^{12}-FA-desaturase	23	303237	0		
Δ^{12}-FA-desaturase	3	292603	57915	–0.32	0.50
Δ^{12}-FA-desaturase	41	309734	3202	**+1.17**	**0.05**
Δ^{12}-FA-desaturase	47	399320	No#		
Δ^{15}-FA-desaturase	3	245369	7246	–1.05	0.12
3-ketoacyl-CoA synthase	1	186873	39429	–0.13	0.66
FA-elongation enzyme (ELO type)					
3-ketoacyl reductase	57	255231	2193	**+2.82**	**0.03**
Acyl carrier protein	1	164630	46644	–0.67	0.20
Long-chain FA-CoA ligase	9	319094	15403	–1.16	0.10
Cytochrome b5 protein	4	183113	23363	–0.10	0.62
Cytochrome b5 reductase	1	300832	21125	**–1.04**	**0.01**

Ectomycorrhizas were collected from *L. bicolor*-inoculated Douglas-fir seedlings. Mycelium of *L. bicolor* was cultured axenically. For each gene, its putative function, the scaffold containing the gene model sequence, the protein ID, and the mean signal intensity of mRNA from mycelium of *L. bicolor* are indicated. Relative changes in transcript signal intensities of ectomycorrhizas/mycelium are expressed as log_2-transformed data. Data are the means of three biological replicates. *P*-values ≤ 0.05 indicate significant changes (bold typeface) in the relative transcript abundance in ectomycorrhizas relative to free-living mycelium. For details, see the Materials and Methods.
No#, no oligonucleotides present on whole-genome expression oligoarray (WGEO).

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Table 4 Expression analysis of annotated genes of the fatty acid (FA) catabolism in ectomycorrhizas (EM) and free-living mycelium of *Laccaria bicolor* strain S238N

Putative function	Scaffold	Protein ID	Signal intensity of transcript	Log_2 transcript ratio EM/mycelium	*P*-value
Mitochondria					
Acylcarnitine transferase	6	189447	7756	+0.67	0.31
Acylcarnitine transferase	11	184843	522	–0.64	0.51
Acyl-CoA dehydrogenase	25	191220	10974	–0.74	0.33
Carnitine/acylcarnitine translocase	1	301012	16594	–0.55	0.26
Carnitine/acylcarnitine translocase	5	183526	16972	**–0.57**	**0.02**
Enoyl-CoA hydratase	10	234621	42118	–0.53	0.18
Enoyl-CoA hydratase	4	245955	2269	–0.68	0.22
Long-chain FA-CoA ligase	9	319094	15403	–1.16	0.10
3-ketoacyl-CoA thiolase	38	185981	4331	+2.24	0.11
3-ketoacyl-CoA thiolase	1	300690	1490	+0.53	0.35
3-hydroxyacyl-CoA dehydrogenase	2	187873	17086	+0.62	0.19
3-hydroxyacyl-CoA dehydrogenase	4	168519	No#		
Peroxisomes					
Acetylcarnitine transferase 1	3	188234	6486	**+2.44**	**0.03**
Acetylcarnitine transferase 2	2	244106	25232	+0.04	1.00
Acyl-CoA oxidase	9	319093	12203	–1.56	0.20
Acyl-CoA oxidase	11	190411	16777	–0.55	0.66
Acyl-CoA oxidase	11	190400	17307	–0.13	1.00
Acyl-CoA oxidase	25	320108	9386	+0.13	1.00
Long-chain FA-CoA ligase	6	189537	8455	+0.11	1.00
D-multifunctional β-oxidation protein	11	234865	27842	–0.01	1.00
Peroxisomal long-chain FA Import protein 1	1	242594	25413	–0.40	0.47
Peroxisomal long-chain FA Import protein 2	4	141760	0		
$\Delta^{2,4}$-dienoyl-CoA reductase	19	251259	8447	+1.31	0.23
3-ketoacyl-CoA thiolase	1	301021	16954	–0.55	0.38
3-ketoacyl-CoA thiolase	4	311721	22397	**–0.54**	**0.05**
3-hydroxy-2-methylbutyryl -CoA-dehydrogenase	24	320095	16327	+0.20	0.89
Δ^{3},Δ^{2}-enoyl-CoA isomerase	8	325594	706	–0.26	0.69
$\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA-isomerase	19	300535	12697	+1.22	0.16
$\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA-isomerase	52	312422	6600	+0.72	0.27
Catalase	68	123238	14515	–2.00	0.55
NADP-dependent isocitrate dehydrogenase	7	317084	22310	–0.70	0.36
NAD-dependent isocitrate dehydrogenase	4	311861	34574	–0.23	0.40

Ectomycorrhizas were collected from *L. bicolor*-inoculated Douglas-fir seedlings. Mycelium of *L. bicolor* was cultured axenically. For each gene, its putative function, the scaffold containing the gene model sequence, the protein ID, and the mean signal intensity of mRNA from mycelium of *L. bicolor* are indicated. Relative changes in transcript signal intensities of ectomycorrhizas/mycelium are expressed as log_2-transformed data. Data are the means of three biological replicates. *P*-values ≤ 0.05 indicate significant changes (bold typeface) in the relative transcript abundance in ectomycorrhizas relative to free-living mycelium. For details, see the Materials and Methods.
No#, no oligonucleotides present on whole-genome expression oligoarray (WGEO).

Reconstruction of the pathway of FA biosynthesis in *L. bicolor*

Using the FA composition, gene annotation and expression analysis of FA biosynthetic genes, we constructed a pathway for FA biosynthesis and modification in *L. bicolor* (Fig. 2, Table 3). This pathway starts with the production of palmitic acid (16:0) or perhaps to a small extent with myristic acid (14:0) because traces of this compound were present in the mycelium (Table 1, Fig. S1). FA biosynthesis takes place in the cytosol, as is the case for vertebrate and fungal FA biosynthesis (Lomakin *et al.*, 2007). Palmitic acid is synthesized in a stepwise fashion by two enzymatic complexes: the 'activating' ACC and the FAS, which adds in each internal cycle two carbon atoms to the growing carbon chain from malonyl-CoA. For ACC, only a single gene model was identified and curated in the draft genome. WGEO indicated that transcripts for both genes were expressed in free-living mycelium and ectomycorrhizas (Table 3). However, ACC was strongly suppressed in ectomycorrhizas relative to mycelium (Table 3). In contrast to mammalian FAS,

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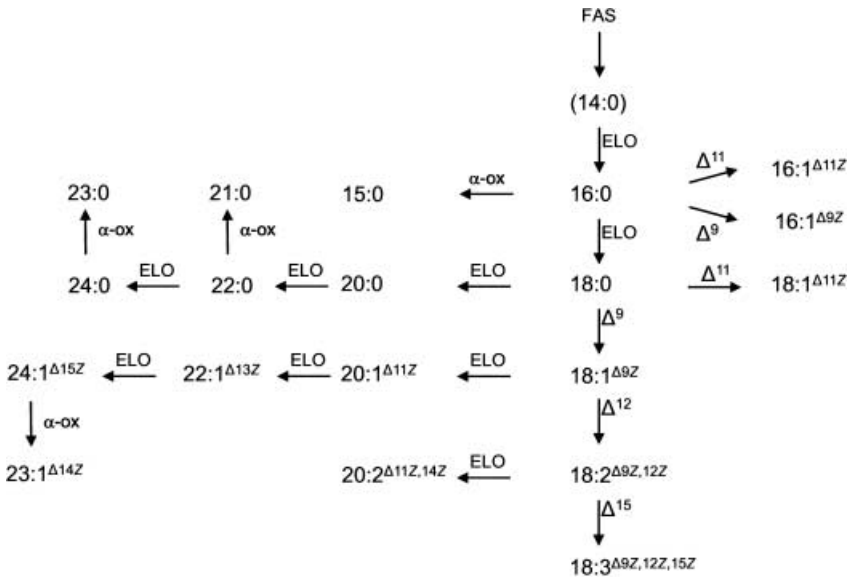

Fig. 2 Fatty acid (FA) biosynthesis pathway in *Laccaria bicolor*. All FAs detected by gas chromatography/flame ionization detection (GC/FID) analysis are included. In total, 10 different protein families are needed for the complete modification process. α-ox., unknown α-oxidation enzyme; Δ^9, Δ^9-FA-desaturase; Δ^{11}, Δ^{11}-FA-desaturase (tentative); Δ^{12}, Δ^{12}-FA-desaturase; Δ^{13}, Δ^{13}-FA-desaturase (tentative); Δ^{14}, Δ^{14}-FA-desaturase (tentative); Δ^{15}, Δ^{15}-FA-desaturase; ELO, FA-elongase.

the FAS from *L. bicolor* does not contain a thioesterase domain (Fig. 3). Therefore, the products released from this fungal FAS are probably acyl-CoAs. In addition, an acetoacetyl-CoA thiolase was identified that may be involved in sterol metabolism, but the function of the cytosolic acyl carrier protein (ACP) remains to be clarified.

The FAS gene family consists of five members. However, only one member of this family encoded a protein that contained all catalytic activities necessary for the internal cycle adding two C atoms to the growing fatty acid chain. This FAS gene was the only one for which EST supports existed (Table 2) and it was the only one with transcript levels significantly above the detection limit (Table 3). It is not yet clear whether the other genes are truncated sequences or code only for certain subunits or activities that may be part of an additional mitochondrial FAS. As their transcripts were below the detection limit under normal metabolic conditions, it is unlikely that they have functions in FA biosynthesis, although we cannot exclude their involvement as the metabolic rate of mitochondrial FAS may be low.

The product of FAS, palmitic acid, is modified by adding two C-atoms by an elongation step (ELO-type; Leonard *et al.*, 2004), by introduction of double bonds in the carbon C-chain by desaturases (Shanklin & Cahoon, 1998), and by shortening of the chain by one C-unit to yield uneven-numbered C chains. To synthesize the entire set of FAs, three different enzyme families are needed: desaturases, elongases and an α-oxidation system. For the first group of enzymes, in total 11 genes were annotated: five FA-Δ^9-desaturases, five FA-Δ^{12}-desaturases and one FA-Δ^{15}-desaturase. However, among these genes, two Δ^9- and two Δ^{12}-desaturases were annotated as pseudogenes; they showed no expression on WGEO and lacked EST support (Tables 2, 3). Furthermore, one 3-ketoacyl-CoA synthase (KS) as a subunit of an ELO-type FA elongase was annotated (Tables 2, 3). However, we failed to identify additional components of this complex. While an elongase complex is required for stepwise extension of the 16-C chain to 24-C, we were not able to identify the enzymes involved in α-oxidation that are necessary to yield the uneven-numbered FA C chains with 15-C, 21-C and 23-C atoms, respectively. From these results the pathway of FA biosynthesis shown in Fig. 2 was constructed.

From sequence data it was not possible to identify putative Δ^{11}-desaturases, which are required for formation of 16:1(11*Z*) and 18:1(11*Z*). As we found three Δ^9-desaturases, it is possible that one of them acted as a Δ^{11}-desaturase or that desaturation at Δ^{11} occurs as a side activity of Δ^9-desaturases (Fig. 2).

Phylogenetic analysis of FAS

FAS is encoded by one remarkably large gene, which is translated into the second-largest protein so far recognized in *L. bicolor* (3935 aa). In fungi, the FAS complex usually consists of eight enzymatic activities, including phosphopantetheinyl transferase (PPT) (Jenni *et al.*, 2006). However, the structures of FAS differ in different kingdoms. In bacteria and plants, all activities are encoded by distinct genes building a multienzyme complex of seven subunits (without PPT). In vertebrates and humans, all required FAS domains are found in one gene (Schweizer & Hofmann, 2004), as in *L. bicolor*. However, our analysis of several other basidiomycetes and ascomycetes whose sequences have been published (http://www.jgi.doe.gov/, http://www.yeastgenome.org/ and http://www.broad.mit.edu/) showed that fungi have no common FAS structure and that there are differences even in the group of basidiomycetes. A monomeric FAS protein such as that in *L. bicolor* was also encoded in *C. cinerea* (TS.ccin_1.191.21), *P. chrysosporium* (136804) and *U. maydis* (UM0297.1), whereas the basidiomycete *C. neoformans* (CNAG_02100.1, CNAG_02099.1) and the ascomycetes *Neurospora crassa* (NCU07307.3, NCU07308.3), *Saccharomyces*

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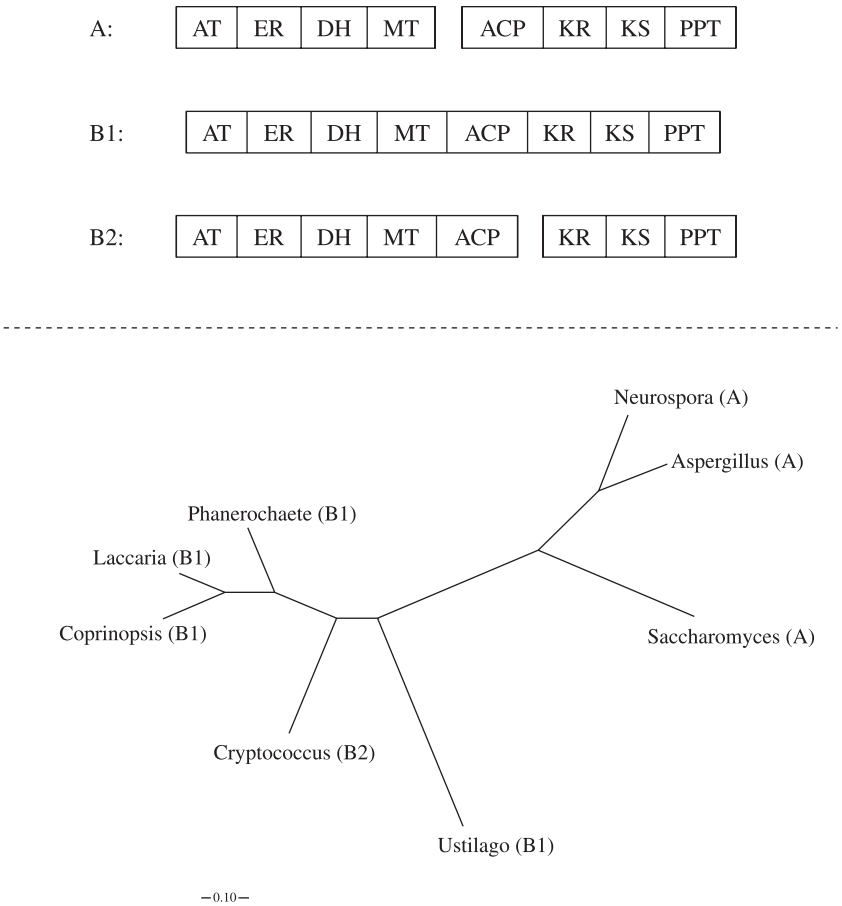

Fig. 3 Linear arrangement of functional domains of the fatty acid synthase (FAS) polypeptide of seven related fungi (top) and their phylogenetic relations based on the β-subunit of FAS (bottom). A, ascomycetes; B1 and B2, basidiomycetes with differences in the coding of the subunits of FAS. The following fungi (accession number) were analysed: *Aspergillus nidulans* (Broad Institute: AN9408.3); *Coprinopsis cinerea* (Broad Institute: TS.ccin_1.191.21); *Cryptococcus neoformans* (Broad Institute: CNAG_02099.1); *Laccaria bicolor* (Joint Genome Institute (JGI): 296983); *Neurospora crassa* (Broad Institute: NCU07307.3); *Phanerochaete chrysosporum* (JGI: 136804); *Saccharomyces cerevisiae* (SGD: YKL182W); *Ustilago maydis* (Broad Institute: UM0297.1). AT, acetyl-CoA-ACP transacetylase; ER, β-enoyl reductase; DH, 3-hydroxyacyl-ACP dehydratase; MT, malonyl-CoA-ACP transacylase; ACP, acyl carrier protein; KR, 3-ketoacyl reductase; KS, 3-ketoacyl synthase; PPT, phosphopantetheinyl transferase.

cerevisiae (YKL182W, YPL231W) and *Aspergillus nidulans* (AN9407.3, AN9408.3) encoded the eight enzyme domains in two genes, building a multienzyme complex composed of α- and β-subunits.

As the gene structure of FAS differs among fungi, three different phylogenetic analyses were conducted employing: (1) sequences of the β-subunit (including acetyl-CoA-ACP transacetylase, β-enoyl reductase, 3-ketoacyl-ACP dehydratase and malonyl-CoA-ACP transacylase), (2) sequences of the α-subunit (including 3-ketoacyl-ACP reductase, 3-ketoacyl-ACP synthase, phosphopantetheinyl transferase and in some organisms acyl carrier protein) and (3) merged sequences of the α- and β-subunits. The three calculated trees were similar (data not shown) and confirmed the typical phylogenetic positions of basidiomycetes and ascomycetes (Fig. 3). *Laccaria bicolor* and *C. cinerea* formed a subgroup relatively close to *P. chrysosporium* (Fig. 3). Phylogenetically, the β-subunit sequences of these three fungi were more closely related to that of *C. neoformans* (in which FAS is encoded by two genes) than to that of *U. maydis* (in which FAS is encoded by one gene). The ascomycetes formed their own subgroup, with *N. crassa* and *A. nidulans* more closely related to each other than to *S. cerevisiae*.

Furthermore, the linear arrangement of the functional domains of the FAS polypeptide differed among the fungi encoding this complex in two proteins. In *C. neoformans* the ACP is encoded by the gene of the β-subunit, whereas in the ascomycetes ACP is encoded by the gene of the α-subunit (Fig. 3).

FA degradation

Based on predicted target sequences, FA degradation by the β-oxidation cycle probably took place in two different organelles in *L. bicolor* (Tables 2, 4): (i) in mitochondria, as in animals and some algae as well as in their evolutionary ancestors the Gram-positive bacteria, and (ii) in peroxisomes, as in animals, plants and most eukaryotic micro-organisms (Fig. 4).

The annotation of the β-oxidation enzymes and expression analyses indicated that FA degradation involved the same steps as reported previously for mitochondria of eukaryotic systems and Gram-positive bacteria (Wanders & Waterham, 2006; Graham, 2008). After being activated in the cytosol, FAs are imported via the carnitine cycle (Fig. 4a). The degradation cycle consists of separate enzymes, all of which were annotated and contained predicted mitochondrial target sequences: one acyl-CoA-dehydrogenase, two enoyl-CoA hydratases, two 3-hydroxyacyl-CoA dehydrogenases, and two 3-ketoacyl-CoA thiolases. The electrons that derive from β-oxidation can be transferred to the respiratory chain and acetyl-CoA can be

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metabolized via the Krebs cycle. In contrast to peroxisomes (see next paragraph), no additional enzymes related to the β-oxidation cycle were identified, suggesting that only saturated FAs can be degraded in the mitochondria of *L. bicolor*. Expression analysis of mitochondrial genes showed no significant changes between mycelium and ectomycorrhizas, with the exception of one gene for carnitine/acylcarnitine translocase (scaffold 5) whose relative transcript levels were approximately 30% decreased in ectomycorrhizas compared with mycelium (Table 4).

In the case of peroxisomes, FAs are probably first imported via an ABC transporter and then activated in the matrix, as we identified a peroxisomal long-chain FA-CoA ligase (Table 2).

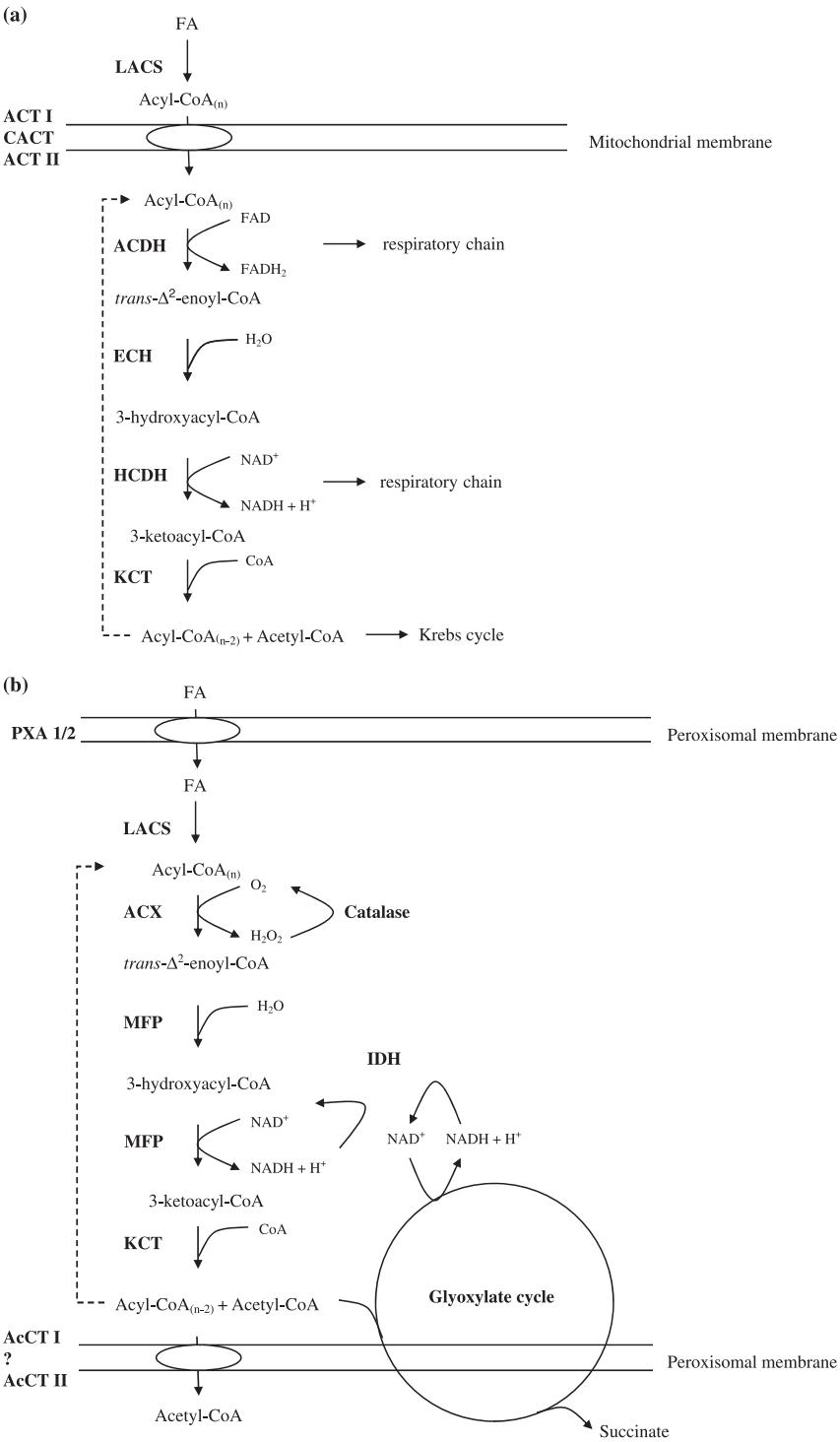

Fig. 4 Schematic representation of possible enzymatic reactions of the β-oxidation cycle in peroxisomes and mitochondria of *Laccaria bicolor* based on annotation and lipid profile data. Enzymes are presented in bold typeface. (a) In mitochondria: fatty acids (FAs) are activated in the cytosol and transported into the organelle via the carnitine cycle. A four-step oxidation cycle oxidizes only saturated FAs. The dashed array indicates that the molecule undergoes more than one round of the cycle for complete oxidation. (b, c) In peroxisomes: FAs are imported into the organelle via an ABC transporter and then activated. Then two possible oxidation cycles may occur, as represented in (b) and (c), respectively. (b) A four-step cycle oxidizes saturated FAs. Only the first enzyme differs from the scheme in the mitochondria. The resulting acetyl-CoA may be directly exported either via a putative acetyl-carnitine transport system or via the glyoxylate cycle. Regeneration of the cofactor NAD occurs via NAD-dependent isocitrate dehydrogenase. (c) Two out of three alternative pathways are shown, which include auxiliary enzymes to oxidize unsaturated FAs (B and C). ACDH, acyl-CoA dehydrogenase; ACT, acylcarnitine transferase; AcCT, acetylcarnitine transferase; ACX, acyl-CoA oxidase; CACT, carnitine/acylcarnitine translocase; DCI, $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase; DCR, 2,4-dienoyl-CoA reductase; ECH, enoyl-CoA hydratase; IDH, isocitrate dehydrogenase; HCDH, 3-hydroxyacyl-CoA dehydrogenase; KCT, 3-ketoacyl-CoA thiolase; LACS, long-chain FA-CoA ligase; MFP, peroxisomal D-specific bifunctional protein harbouring hydratase-dehydrogenase activities; PXA, peroxisomal long-chain FA import protein (ABC transporter).

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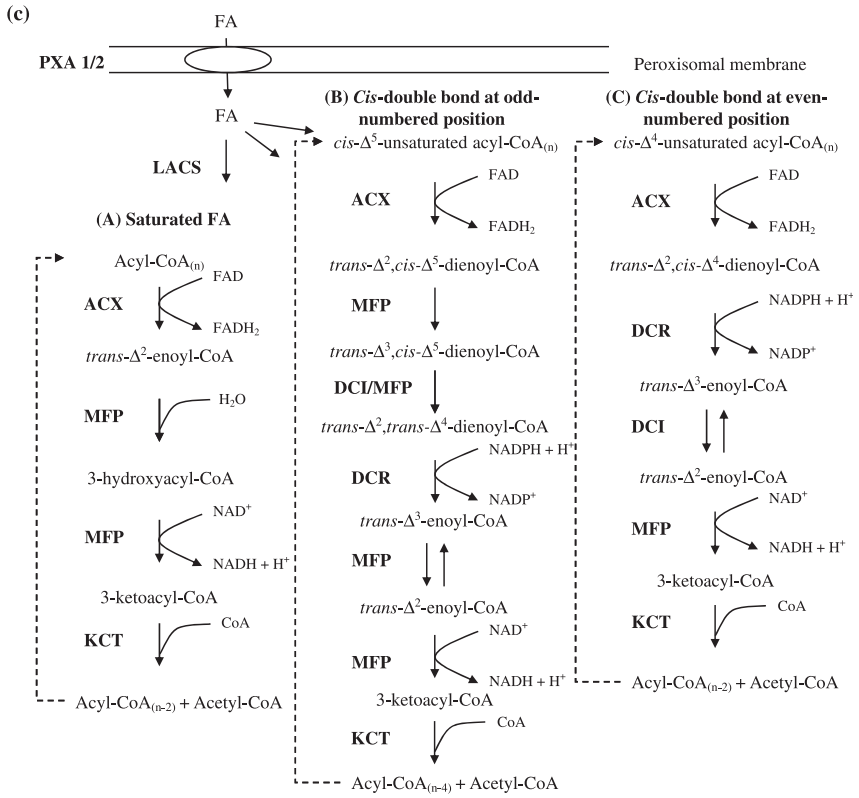

Fig. 4 *continued*

The following genes encoding enzymes for β-oxidation contained putative peroxisomal target sequences: four acyl-CoA oxidases, one multifunctional β-oxidation protein (harbouring enoyl-CoA hydratase and 3D-hydroxyacyl-CoA dehydrogenase) and three 3-ketoacyl-CoA thiolases. One catalase was annotated. In addition, four further enzyme families were identified, two 3,5-2,6-dienoyl-CoA isomerases, one 2,4-dienoyl-CoA reductase, one 3-hydroxy-2-methylbutyryl-CoA dehydrogenase and one 3,2-trans-enoyl-CoA isomerase, allowing *L. bicolor* to degrade not only saturated FAs (Fig. 4b), but also mono- and polyunsaturated FAs (Fig. 4c). For double bonds at odd numbers, two degradation pathways exist; one involves only the Δ^3,Δ^2-trans-enoyl-CoA isomerase in addition to the four standard activities (Fig. 4c, pathway B) and the other involves three additional auxiliary enzymes: $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, $\Delta^{2,4}$-dienoyl-CoA reductase and Δ^3,Δ^2-trans-enoyl-CoA isomerase (pathway not shown). In the case of double bonds at even numbers, one pathway exists. It involves $\Delta^{2,4}$-dienoyl-CoA reductase and Δ^3,Δ^2-trans-enoyl-CoA isomerase (Fig. 4c, pathway C). The electrons that derive from β-oxidation are being transmitted (1) to oxygen, in the case of acyl-CoA oxidase, or (2) to the NAD^+ that is being recycled by a peroxisomal NAD^+-dependent isocitrate dehydrogenase, in the case of the dehydrogenase activity of the multifunctional protein (van Roermund *et al.*, 1998). The acetyl-CoA formed is either metabolized via the glyoxylate cycle or directly exported to the cytosol via a putative carnitine cycle (Fig. 4b). Acetylcarnitine transferase, which mediates cytosolic export, was the only gene involved in FA degradation whose expression was significantly increased in ectomycorrhizas compared with mycelium (Table 4). With the exception of 3-ketoacyl-CoA thiolase (scaffold 4), whose relative transcript level decreased, the expression of all other genes of FA degradation in peroxisomes remained unaffected in ectomycorrhizas compared with mycelium (Table 4).

Discussion

Distribution of lipids in *L. bicolor*

The FA pattern of *L. bicolor* was composed of 19 components, most of which were also detected in ectomycorrhizas (Table 1, Fig. S2). The FA patterns appear to be species-specific, as different fungal species were distinguished by their lipid composition (Martin *et al.*, 1984; Ruess *et al.*, 2002). *Laccaria bicolor*/*P. menziesii* ectomycorrhizas (this study), as well as *Pisolithus tinctorius*/*Pinus sylvestris* ectomycorrhizas (Laczko *et al.*, 2003), showed a more diverse FA pattern than free-living mycelium. However, this was caused by the presence of additional FAs of plant origin. In both *P. tinctorius* and *L. bicolor*, the most abundant FAs were palmitic acid (16:0), oleic acid (18:1(9*Z*)) and linoleic acid (18:2(9*Z*,12*Z*)). As FA composition follows taxonomy, this is characteristic not only of other ectomycorrhizal

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fungi (Martin *et al.*, 1984; Pedneault *et al.*, 2006), but also of many other fungi (van der Westhuizen *et al.*, 1994).

Interestingly, *P. tinctorius* (Laczko *et al.*, 2003), *L. bicolor* (this study) and *Paxillus involutus* (C. Göbel,unpublished data) were found to contain palmitvaccenic acid 16:1(11*Z*), an FA that is enriched in arbuscular mycorrhiza, and has been used as a maker for mycorrhizal colonization (Bååth, 2003; van Aarle & Olsson, 2003; Stumpe *et al.*, 2005; Trépanier *et al.*, 2005). This FA was absent in the lipid fraction of the host plant of *P. tinctorius*, *Pinus sylvestris* (Laczko *et al.*, 2003), whereas we detected small amounts of this FA in Douglas-fir roots, even in seedlings raised under sterile conditions (not shown). However, it should be noted that the occurrence of 16:1(11*Z*) seems to be restricted to the genus *Pseudotsuga*, as revealed by a screening of 100 different tree species (C. Göbel *et al.*, unpublished results).

The formation of Δ^{11}-FA in *L. bicolor* remains unclear. 16:1(11*Z*) could be an unspecific by-product of 16:1(9*Z*) formation. In this case, we would expect a constant ratio of 16:1(9*Z*) and 16:1(11*Z*), assuming the same turnover of both FAs. However, the ratio changed because the concentration of (9*Z*) decreased in older tissues while the amount of (11*Z*) remained almost constant (Fig. S2). It is unlikely that this FA derives from elongation of 14:1(9*Z*), as we did not find any 14:1(9*Z*). It is, therefore, likely that one of the three genes annotated as Δ^9-desaturases may act as Δ^{11}-desaturase. This will require further analysis.

FA biosynthesis

We annotated five gene families that are involved in FA biosynthesis. In most organisms, FAs are synthesized from acetyl- and malonyl-CoA by a reaction sequence consisting of seven catalytic steps (Berg *et al.*, 2006). In most bacteria and plants, these enzyme activities are encoded by distinct genes, but in fungi and vertebrates this biosynthetic pathway is catalysed by a large multifunctional protein (Jenni *et al.*, 2007). Previously, it was assumed that this protein forms a homodimer (type I) in mammals and consists of a heterododecameric complex in fungi (type II; Schweizer & Hofmann, 2004). In contrast to this model, we found that several species of the Basidiomycotina harbour the classical yeast-like type II FAS, but *L. bicolor*, *U. maydis* and *C. cinerea* have a vertebrate-like type I FAS (Fig. 3). The three fungal species represent symbiotic, biotrophic, and saprophytic life styles, respectively. Some fungal species show a niche-dependent adaptation of FA biosynthesis. For example, *Malassezia globosa* is strictly dependent on host lipids because there is no FAS gene in its genome (Xu *et al.*, 2007). Although *L. bicolor* – at least in symbiosis – mainly relies on its host for its carbon resource, a reduction in the number of genes coding for enzymes of FA biosynthesis was not observed, as all genes required at least for cytosolic FA biosynthesis were present (Tables 2, 3). However, we observed a suppression of ACC transcription in ectomycorrhizas compared with free-living mycelium (Table 3). As ACC activity is required at the start of FA synthesis, suppression of this activity may be a reason for the decreased concentrations of FAs in ectomycorrhizas compared with mycelium (Table 1). This theory is also supported by the fact that there was no evidence for increased lipid degradation in ectomycorrhizas (Table 4). Johansson *et al.* (2004) also reported a decreased expression of some fungal genes related to lipid metabolism in the *Paxillus involutus*/*Betula pendula* symbiosis. Whether the life style of *L. bicolor* – free-living or in symbosis – is an important determinant for the activation of FA biosynthesis will require further experiments.

In addition to the basic set of enzymes necessary for FA production, we found modifying enzymes whose activities resulted in a substantial pool of different FAs (Fig. 2). These genes were expressed in ectomycorrhizas as well as in mycelium. This indicates that, in contrast to *Glomus* species, whose FAS appears to be active only in the intraradical and not in the extraradical mycelium (Trépanier *et al.*, 2005), *L. bicolor* can synthesize palmitic acid (16:0) and derived FAs in all tissues. Such differences in FA metabolism may partly explain the strict obligate biotrophism of arbuscular mycorrhizal fungi compared with the more flexible life style of ectomycorrhizal species.

An important difference between mycelium and ectomycorrhizas was an approximately 7-fold reduction in the abundance of 18:1(9*Z*) in the latter tissue. This cannot be explained by a 'dilution' effect attributable to plant tissues but suggests either a decrease in Δ^9-FA desaturase activity or an increased modification of 18:1(9*Z*). Our data seem to support the first hypothesis. As the expression of Δ^9-FA desaturase with protein ID 189797 was strongly suppressed, this enzyme may be the major one required for 18:1(9*Z*) formation.

FA degradation

The organization of the β-oxidation cycle differs among plants, animals and fungi (Berg *et al.*, 2006). In plants and yeast it is only found in peroxisomes or glyoxysomes. In animals the classical β-oxidation cycle takes place in the mitochondria, whereas peroxisomes harbour only a special β-oxidation system for the degradation of either very long chain or branched chain FAs. Here we provide evidence that, in *L. bicolor*, β-oxidation cycle enzymes are targeted to mitochondria as well as to peroxisomes. Both organelles are involved in FA catabolism in filamentous ascomycetes, basidiomycetes, zygomycetes and oomycetes, with some exceptions (Cornell *et al.*, 2007). In these microorganisms, the mitochondrial cycle is responsible for the breakdown of short-chain FAs while the peroxisomal cycle is used for the degradation of long-chain FAs (Maggio-Hall & Keller, 2004; Maggio-Hall *et al.*, 2008). We found a similar situation in *L. bicolor*, as auxiliary enzymes for the degradation of unsaturated FAs are located exclusively in the peroxisomes. For the two 'starter' enzymes of the two differently located cycles, the mitochondrial acyl-CoA dehydrogenases and the peroxisomal acyl-CoA oxidase, one and four gene family members were annotated, respectively. This implies that *L. bicolor*

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has different enzymatic specificities and can handle a broad range of substrates of lipids. The latter function may be especially important when *L. bicolor* assumes a saprophytic life style.

In addition to the β-oxidation cycle, we found all the genes that constitute a glyoxylate cycle in *L. bicolor*. Therefore, the fungus harbours peroxisomes of a more specialized type, called glyoxysomes, that have been described in seedlings (Graham, 2008) and other fungi before. In fungi, this cycle is important not only for carbon source utilization, but in addition for pathogenesis, development, and secondary metabolism (Idnurm & Howlett, 2002; Asakura *et al.*, 2006; Hynes *et al.*, 2008).

Overall, our analysis shows that *L. bicolor* contains all the genes required for FA synthesis, modification and degradation. Comparison of transcript analysis, lipid concentrations and composition suggested that the amount of FAs may be regulated via ACC and the abundance of oleic acid by Δ^9-FA-desaturase on scaffold 8.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Gas chromatography/flame ionization detection (GC/FID) analysis of fatty acid methyl esters isolated from the lipid fraction of different tissues of *Laccaria bicolour*.

Fig. S2 Changes in fatty acid concentration in *Laccaria bicolor* grown for 30 d in liquid culture media.

Table S1 List of primers used for quantitative RT-PCR

Table S2 Comparison of the relative expression of selected genes using whole-genome expression oligoarray (WGEO) and quantitative RT-PCR

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10. Chapter IX: Conclusions in French

Contexte et résumé général

Les forêts sont des écosystèmes extrêmement complexes et l'anthropisation relativement faible à laquelle elles sont soumises favorise leur hétérogénéité, source de biodiversité et de niches biologiques. Dans les sols forestiers, la vie microbienne est particulièrement exubérante et les communautés fongiques y tiennent une place majeure, dominées par ltrois grands groupes fonctionnels, les saprophytes, décomposeurs de bois et de litière, les champignons mutualistes, biotrophes ectomycorhiziens (ECM), et les espèces pathogènes. Ces organismes jouent un rôle fondamental dans la minéralisation des composés organiques, l'alimentation hydrominérale des arbres et dans la régulation des cycles majeurs, en particulier les cycles du carbone et de l'azote (Boddy and Watkinson, 1995 ; Fitter *et al.*, 2005). Si les champignons saprophytes utilisent le C de la litière consécutivement à des processus cellulolytiques et ligninolytiques, les champignons ECM reçoivent du C, par la symbiose qu'ils établissent avec les racines des arbres. Mais de nombreuses espèces ectomycorhiziennes présentent des comportements variant du semi-saprophytisme à la symbiose stricte (Högberg *et al*., 1999).

Dans les écosystèmes forestiers, la richesse et la diversité des communautés fongiques contrastent fortement avec le faible nombre d'espèces ligneuses. Les facteurs qui influencent le développement de ces communautés et le maintien d'une telle diversité sont encore mal connus. Les modifications environnementales, parfois consécutives aux activités anthropiques, peuvent avoir des conséquences variables sur ces communautés fongiques (Wallenda and Kottke, 1998; Erland and Taylor, 2000). Dans ce cas, les études concernant une gestion productiviste ont mis en évidence une simplification de la strate ligneuse par plantation ou substitution d'essence, une baisse de la fertilité des sols et une modification de la diversité fongique (Le Tacon *et al*., 2001). Les changements d'essences et les plantations d'espèces productives, lié parfois à des enrésinements massifs, sont au cœur des stratégies de gestion sylvicole. Des recherches récentes ont portées l'accent sur la spécificité d'hôte et l'influence des arbres sur la structure des communautés fongiques. L'influence de l'hôte a été bien illustrée dans de récentes études portant sur les communautés ectomycorhiziennes et indiquant une spécificité d'hôte importante chez espèces fongiques, en particulier vis-à-vis

des conifères et des feuillus (Ishida *et al.*, 2003), mais aussi un impact fort de d'espèces ligneuses différentes mais issues du même genre (Morris *et al.*, 2008). Par ailleurs, de nombreux éléments structurant évoluent en fonction du temps et des saisons, intimement liés aux facteurs pédo-climatiques. Ainsi, il a été démontré que ces communautés fongiques, en particulier chez les espèces ECM, évoluées au cours du temps, selon leur potentiel d'adaptation aux changements climatiques ou aux variations phénologiques des plantes hôtes auxquelles ces microorganismes sont associés (Buée *et al.*, 2005; Courty *et al.*, 2008). C'est la raison pour laquelle, une meilleure compréhension de la structuration de ces communautés microbiennes est nécessaire pour que l'aménagement des forêts s'inscrive dans une gestion durable de l'écosystème et respectueuse de la biodiversité.

Cependant, pour investir une telle diversité microbienne et mieux appréhender les moteurs de structuration de ces communautés, il est nécessaire de développer et d'affiner les outils de diagnostiques taxonomiques. Plusieurs dizaines d'espèces fongiques sont couramment associées à un même arbre et d'un arbre à l'autre, l'hétérogénéité du cortège fongique porte cette estimation à plusieurs centaines d'espèces à l'échelle d'un peuplement. Cette diversité de champignons s'illustre très bien en période automnale, lors de l'émergence des fructifications, mais aussi tout au long de l'année par des études de diversité axées sur l'analyse de la morphologie des apex ectomycorhiziens et de leur mycélium. Ce mycélium externe peut être, par exemple, hydrophile ou hydrophobe, plus ou moins ramifié, diffus ou agrégé en rhizomorphes… (Agerer, 1987-1998). Les approches écologiques concernant ces champignons symbiotiques reposent traditionnellement sur l'inventaire des carpophores visibles au sein l'écosystème forestier étudié. Beaucoup de travaux reposent encore sur cette démarche, non destructive mais saisonnière (Garbaye et Le Tacon 1982, Peter *et al*. 2001, Egli *et al*. 2006). Cependant, nombres de champignons ectomycorhiziens ne génèrent que des fructifications discrètes, comme les *Tylospora* spp. ou *Tomentella* spp., ou des carpophores épigés, comme les truffes, ou ne fructifient même pas, comme *Cenococcum geophilum* (Jany et al. 2002). Diverses études comparatives ont donc montré que les carpophores ne donnaient qu'une image tronquée de la diversité ectomycorhizienne (Peter *et al.* 2001, Buée *et al.* données non publiées), qui pouvait être décrite plus précisément via l'analyse morphologique ou moléculaire des apex racinaires ectomycorhizées (Agerer 1987-1998, Buée *et al.* 2005, Courty *et al.* 2006). Au cours de ces 15 dernières années, la généralisation de la PCR et le développement de nombreuses techniques d'empreintes moléculaires qui en dérivent (T-RFLP, SSCP, TGGE, etc.) ont permis une meilleure description de la diversité de ces

champignons symbiotiques (pour revue, voir Anderson, 2006). Ces approches PCR permettent de comparer des régions de différents génomes fongiques, et grâce à certaines d'entre elles, de discriminer les espèces sur la base du polymorphisme de ces séquences, après les avoir comparées à des bases de données (NCBI[1], UNITE[2]). Les régions les plus exploitées pour ces études de diversité moléculaire des champignons sont situées au sein d'un locus répété de l'ADN ribosomal nucléaire : les Internal Transcribed Spacer ou ITS (Horton & Bruns, 2001). Cependant, malgré l'automatisation de ces approches moléculaires, il semble indispensable d'intégrer des techniques d'analyse de la diversité à très haut débit, à l'heure où l'estimation de la richesse fongique se porte à plus de 1,5 millions d'espèces (Hawksworth, 2001). Ainsi, les perspectives technologiques, à court terme, reposent sur le développement de puces à ADN taxonomiques (« phyloarrays ») spécifiquement conçues pour réaliser des études de diversité à haut débit à l'image des microréseaux d'ADN développés pour l'étude de certaines communautés bactériennes (Brodie *et al.*, 2006). Jusqu'à présent, au phyloarray n'a été développé pour des études d'écologie moléculaire des champignons à large spectre, c'est-à-dire incluant l'ensemble d'une communauté. En effet, seuls quelques travaux se sont focalisés sur des études diagnostiques de pathogènes fongique par cette stratégie de microréseaux d'ADN (Lievens *et al*., 2003, 2005). Parallèlement à au développement de cette technologie, les sciences du vivant sont ébranlées par l'émergence des nouvelles techniques de séquençage massif, ou pyroséquençage, (Rothberg and Leamon, 2008) qui permettent d'envisager le décodage de plus d'un million de séquences en un seul « run ». Les premières application du pyroséquençage en écologie microbienne ont été éprouvés ces dernières années sur les communautés procaryotiques (Sogin et al. 2006; Roesch et al. 2007), mais aucun travail n'a encore été publié sur les communautés fongiques.

Ma thèse avait donc pour objectifs d'évaluer l'impact de l'arbre hôte, en particulier dans le cadre de la gestion forestière, sur la composition de la communauté fongique. L'approche s'inscrivant dans le temps, l'étude de la variation saisonnière des espèces et de la structuration temporelle a également été abordée. Comme décrit précédemment, le pré requis initial de ce travail a été le développement d'outils à haut débit pour l'analyse de cette diversité, en particulier la génération et la validation de la première puce taxonomique fongique, mais aussi l'exploitation des technologies de pyroséquençage en écologie microbienne (**Chapitre 1**).

Dans un dispositif expérimental de la forêt domaniale de Breuil-Chenu (Nièvre), au nord-est du Massif Central dans le Morvan, la diversité des champignons forestiers a été étudiée par ces différentes approches et les résultats obtenus ont été comparés aux données acquises par

des techniques plus traditionnelles, allant du morphotypage des apex mycorhiziens au clonage séquençage (Sanger). Le peuplement initial est un taillis sous futaie (TSF) constitué principalement par du hêtre et du chêne. En 1976, des plantations de six essences différentes (hêtre, chêne, épicéa commun, douglas, sapin de Nordmann et pin laricio) ont été réalisées après coupe à blanc du TSF afin d'évaluer, entre autre, l'influence de l'enrésinement sur les sols et la diversité biologique. Dans deux études distinctes, nous avons détaillé le développement successif de deux générations de puces taxonomiques (phyloarrays), respectivement spécifiques (1) des champignons ectomycorhiziens puis (2) de l'ensemble des espèces rattachées au royaume fongique. Ce dernier travail repose sur l'analyse bioinformatique de 23 393 séquences qui ont permis de générée 84 891 oligonucléotides spécifiques de 9 678 espèces fongiques actuellement décrites sur la base du typage moléculaire de la région ITS (**Chapitre 2 et 3**). Ces outils ont permis de conforter l'effet particulièrement structurant de la plante hôte sur les espèces fongiques associées, en comparant deux essences distinctes : le hêtre et l'épicéa.

Profitant de l'émergence des nouvelles technologies de séquençage au cours de ma thèse, nous avons éprouvé l'approche 454 pyroséquençage en écologie microbienne. Ainsi, la diversité fongique de l'ensemble de la communauté a été décrite à partir d'échantillons du sols collectés sous 6 essences distinctes sur le site de Breuil (chêne, hêtre, épicéa, pin Corse, sapin de Douglas et sapin de Nordmann). Cette technologie nous a permis de générer plus de 185 000 « amplicons » et d'obtenir pour chaque traitement entre 26 000 et 35 000 séquences, correspondant, après analyse bioinformatique et alignement sur les bases de données, à 600 – 1000 OTUs, selon les plantations (**chapitre 4**). La comparaison de cette technique de séquençage à haut débit avec les approches de phyloarrays (micro-réseaux d'ADN) met en évidence une complémentarité forte des deux techniques, mais aussi illustre les contraintes des approches moléculaires à haut débit, intimement liées à la qualité d'incrémentation des bases de données (**Conclusion**). De manière intéressante, les résultats acquis par affiliation à des bases de données épurées (n'intégrant que les séquences taxonomiquement bien identifiées) montre une distribution homogène des genres fongiques sous les différentes espèces d'hôtes. Mais, l'analyse comparative des données, à l'échelle de l'espèce, conforte encore un effet structurant de l'hôte, en particulier opposant les feuillus entre eux (chêne et hêtre) et les feuillus versus conifères.

Malgré toute la richesse de ces techniques moléculaires et l'immense volume de données qu'elles génèrent, la mycologie et la taxonomie morphologique des champignons restent des sciences fondamentales et essentielles, puisque c'est le couplage des analyses morphologiques

et moléculaires, à partir des fructifications ou des différentes structures fongiques, qui permet l'enrichissement et l'incrémentation des bases de données, indispensables au développement de l'écologie moléculaire et à l'exploitation des nouvelles technologies à haut débit. Le maintien de la diversité des approches taxonomiques et des compétences en écologie microbienne reste un réel challenge pour la communauté scientifique.

Ainsi, que la forêt soit vierge, aménagée ou même très artificialisée (plantation d'essences exotiques à croissance rapide), l'interdépendance entre les arbres et les champignons est particulièrement étroite. Par conséquent, toute perturbation d'origine naturelle ou anthropique (pollution, sylviculture) retentit sur ces interactions en équilibre plus ou moins instable. Les connaissances actuelles sur les relations forêt-champignons, acquises grâce à un effort de recherche récent mais croissant, montrent donc clairement que tout projet à long terme de gestion durable des ressources forestières doit se préoccuper de la stabilité des équilibres biologiques interactifs, dans lesquels les champignons ectomycorhiziens sont des partenaires déterminants. Il existe aussi désormais des outils de diagnostic et, dans certains cas, des moyens d'intervention pour contrôler et maîtriser les relations arbre-champignon. Leur maîtrise pourrait se révéler essentielle à la mise en œuvre d'une sylviculture plus durable.

Note sur l'annotation du génome de Laccaria bicolor

Au cours de ma thèse, mon laboratoire d'accueil a coordonné le séquençage et l'annotation du génome du champignon ectomycorhizien modèle Laccaria bicolor. Bénéficiant d'un contexte scientifique particulier, j'ai pu m'impliquer fortement dans le consortium d'annotation de ce génome et participer à la publication d'un article dans la revue Nature. Plus précisément, j'ai été impliqué dans l'annotation des gènes liés au métabolisme lipidique, en particulier 63 gènes majeurs de la voie de biosynthèse des acides gras. L'analyse de ces différents gènes a été réalisées à l'échelle génomique, mais également au niveau transcriptomique. Ce travail a également fait l'objet d'une publication dans la revue New Phytologist.

11. Chapter X: Conclusions

Where we are, what we know…

Forests are highly complex ecosystems and are important resources in ecological, social and economic dimensions. In the course of quickly increasing deforestation, discussion about sustainable forestation and forest conservation becomes more and more urgent. For an optimal protection of forests, the global status of biomes within forests and their response to environmental factors have to be understood (Sukumar, 2008). A high diversity of biomes can be found above-ground and below-ground. Fungi are important components of soil biomes and act as decomposers, pathogens or mycorrhizal mutualists.

Fungal communities show a high diversity in richness and structure as several environmental factors influence their composition. To understand functioning of soil biomes the impact of single factors such as edaphic factors, climate, fire and anthropogenic activities on fungal communities has been studied in the last fifteen years (Dahlberg, 2001). It was shown that plant composition has a major impact on the structure and functioning of fungal communities. The presence of dominant and rare fungal species differs between forest types influenced by the different availability of water and soil nutrients and microclimate conditions. Additionally, indirect influence over tree debris, such as needle, dead leaves or cones has been reported (Takumasu et al., 1994; Zhou & Hyde, 2001).

Recent research focused on the effect of host taxonomic affiliation on ectomycorrhizal (ECM) communities. It was reported that communities sharing host trees of similar taxonomic status showed similar structure compared to communities associated to more taxonomical distinct host trees (Ishida *et al.*, 2007). The influence of the replacement of a native forest by mono-specific plantations on fungal diversity is controversially discussed. In temperate coniferous plantations high fungal diversity was observed (Newton & Haigh, 1998, Humphrey *et al.*, 2000), while in exotic conifer plantations lower fungal diversity than in native hardwood stands was reported (Villeneuve *et al.* 1989, Ferris et al., 2000).

Detection techniques

In the last 15 years, ecological studies on fungal communities have been carried out using different molecular biological tools very often combined with classical techniques such as morphotyping. Especially the determination of ITS as DNA barcode for fungi and the adjustment of PCR conditions for the amplification of the total fungal community allowed first insights into fungal community dynamic (Horton & Bruns, 2001). However, detailed ecological studies of fungal communities stayed challenging as the used techniques limited the number of samples, which can be processed in a realistic time frame.

Identification of fungal taxa can nowadays be expanded to high-throughput molecular diagnostic tools, such as microarrays. First microarrays were developed with the aim to monitor gene expression and to analyze polymorphisms. But ongoing development allowed the use of array technique to describe community structures (phylochips) as thousands of species-specific probes can be fixed to the carrier glasslides. Successful application of phylochips in ecological studies was demonstrated by the identification of bacterial species from diverse ecosystems and of few genera of pathogenic and composting fungi (Sessitsch *et al.*, 2006). First application of a small-scale phylochip (southern dot blot) to trace ectomycorrhizal fungi (EMF) was reported by Bruns and Gardes (1993), who developed a specific Nylon-phylochip to detect Suilloid fungi. Recently, this approach has been also used for truffle identification (El Karkouri et al., 2007); but no study has reported the construction and application of a large-scale fungal phylochip to detect fungal species from environmental samples.

During the course of my PhD-thesis another high-throughput technique was developed and became the method of the year 2008: 454 pyrosequencing. This is a sequencing technique combining the complete sequence process covering all subsequent steps from the gene of interest to the finished sequence (Margulies *et al.*, 2005). In first experiments, 454 pyrosequencing technique was used to sequence bacterial genomes, but with its ongoing development it could also be applied in metagenomic analysis. Bacterial community structures of different ecosystems have already been described with more than over 10,000 generated sequences (Huber *et al.*, 2007). So far, no studies have been published on fungal communities by using 454 pyrosequencing.

The aims of my thesis

The project of my thesis was to describe the richness and the diversity of fungal communities under different mono-specific plantations of a temperate forest in Breuil-Chenue (Burgundy, France; Ranger *et al.*, 2004). Thirty-five years ago the native deciduous forest (oak, beech, birch and hazel tree) was substituted by mono-plantations of six different tree species (Norway spruce, Nordmann fir, Douglas fir, Corsican pine, oak and beech). First inventories of fungal communities have already been made by carpophore observations. Therefore, the aims of my thesis were (i) to describe the impact of host tree species on ECM communities under beech and spruce over the time-scale of one year, and (ii) to give an exhaustive description of the composition and richness of fungal communities under the six different tree species.

To overcome the above described problems of classical detection techniques, we (i) developed a large-scale phylochip to trace several fungal species simultaneously, (ii) validated the developed phylochip to test its capacity to describe fungal communities from environmental samples, and (iii) applied the developed phylochip and 454 pyrosequencing in our ecological studies. Additionally, we compared results of fungal genotyping using these two novel high-throughput techniques, 454 pyrosequencing and phylochip, against each other to describe inherent bias of each technology.

Genotyping using phylochips

Small-scale phylochips (nylon- and OPERON-glasslide-phylochips)

In a pilot experiment, we developed in a single oligonucleotide probe approach a small-scale ribosomal ITS phylochip carrying probes for 89 ECM species. The sensitivity and specificity of the oligonucleotides were evaluated using mixed known fungal ITS sequences. The phylochip was then used for characterizing ECM fungal communities sampled from 35-year-old spruce and beech plantations on the Breuil-Chenue experimental site. Morphotyping and ITS sequencing of ECM root tips, together with sequencing of ITS clone libraries of these environmental samples, validated the application of the phylochip for fungal ecological studies.

93% of the oligonucleotides generated positive hybridization signals with their corresponding ITS. 52% of the probes gave a signal with only species for which they were designed. Cross-hybridization was reported mainly within the genera of *Cortinarius* and was restricted to the genus showing a low intragenerus ITS sequence divergence (< 3%). Detection limit of the phylochip was highly dependent on the amount of spotted oligonucleotides. Weak signal

intensities were already detected with an amount of 0.0001 pmol of spotted oligonucleotides. The phylochip confirmed the presence of most ECM fungi detected with the two other approaches except for the fungal species for which corresponding oligonucleotides were not present on the phylochip. 13 and 15 ECM fungal species associated to spruce or beech were identified. Thus, we concluded that fungal ITS phylochips enable the detection and monitoring of ECM fungi in a routine, accurate and reproducible manner and facilitate the study of community dynamics, but also information about taxonomic affiliation of species.

Large-scale phylochip (NimblGen array)

Gaining from this pilot experiment, we designed in a second step the first generation of phylochip reporting all fungal ITS sequences deposited at the NCBI GenBank and UNITE database for large-scale analysis of soil fungal communities. For the oligonucleotide design, we downloaded all available fungal ITS sequences (~41,000) from public databases. Several ITS sequences of the public databases showed a low quality and were often truncated. In addition, numerous deposited ITS sequences corresponded to unidentified species or were poorly taxonomically annotated. Thus, we subjected all downloaded sequences to an automated quality control based on a series of scripts written by Henrik Nilsson (University of Göteborg, Sweden). Thus, only fully identified, high-quality fungal ITS-sequences were used for the design of our phylochip. To overcome the observed cross-hybridization within the analysis of the nylon- and OPERON-phylochips we opted for five oligonucleotides per sequence. The design of the oligonucleotide-probes was carried out by NimbleGen Systems (Mason, WI, USA) using their appropriate software. On this database, we applied a serious of post-design filtering scripts. Hereby, we excluded all oligonucleotides (i) with sequence overlap elsewhere than the ITS1 and ITS2 i.e., coding RNA sequences, and (ii) those which showed less than 3 bp difference to sequence of other species than they were designed for. Only high-quality oligonucleotides for 7,151 fungal species remained after these filtering steps on the NimbleGen phylochip. This represents 74% of the fully identified, trustful sequences of Genbank.

We used the NimbleGen-phylochip to access the impact of the host tree species on ECM communities in beech and spruce plantations (Breuil-Chenue site) over the time scale of one year. Comparison of the ECM community accessed by ITS cloning/sequencing and the NimbleGen phylochip approach confirmed the feasibility of this “All Fungal ITS” phylochip in ecological studies. The phylochip genotyping showed a higher sensitivity than the sequencing approach as additional ECM species, endophytic, saprophytic and pathogenic

fungi were detected in tree roots. However, cross-hybridization between probes was observed for several taxa having very similar ITS sequences (e.g. *Russulaceae*). A mutiple probe approach on ITS regions cannot overcome these cross-hybridization problems. The low number of phylogenetically informative base pairs that are found in some fungal taxa (e.g. *Russulaceae*) hindered oligonucleotide design on ITS regions. Difficulties in distinguishing certain ECM species based solely on their ITS-region have been reported elsewhere (Edwards & Turko, 2005), which is consistent with our observation that cross-hybridizations accumulated in certain fungal taxa. Owing to this limitation, certain fungal taxa can only be determined to the genera level using the "All Fungal ITS" phylochip. For an identification of all species to the species level, an oligonucleotide-design including multiple probes and multiple genes is likely needed. Furthermore, phylochips can only detect species for which oligonucleotides were designed, but a nested approach by using probes specific on genus or family level can help to describe the cryptic species.

The advantage of the current phylochip analysis, however, is the accurate description of hundred to thousand species to a taxonomical level. Additionally, ITS phylochip approach enables a reproducible detection in a routine manner prossessing a high number of samples in a relative short time. Thus, we proposed to use phylochip genotyping in studies monitoring the spatial and temporal diversities of well-described fungal communities.

The NimbleGen phylochip was used to access the influence of host tree species on ECM community diversity in the beech and spruce plantation at Breuil-Chenue. In total 59 fungal species were detected among them 53 EMF. Thirty-one species were only identified in one of the samples and in all but one sample endophytic, saprotrophic and pathogenic fungi were present. Probable cross-hybridization was observed in the genera of *Cortinarius*, *Laccaria* and *Lactarius*.

Nineteen fungal species were exclusively associated to beech. Three of them, *Russula emetica, Mycena galopus* and *Entoloma conferendum,* were present during all seasons while *Lactarius tabidus* was present in the samples taken in October and March. In contrast, 26 fungal species were specific to spruce. Seven species were present in the samples of October and March while all other species were only present in samples of one sampling time point.

Correspondence analysis separated the samples of the beech and spruce along the first axe while samples of October were separated from samples taken in March and May on the second axe. The described host preference is in consistence with known host preferences like for *Lactarius subdulcis, L. theiogalus* and *Russula parazurea* to deciduous forest trees, which we found only under beech. In spruce samples we identified also EMF with known spruce

preference like *Cortinarius rubrovioleipes, C. sanguineus* and *C.traganus* and *Rhizopogon* species. *Xerocomus badius* and *X. pruinatus*, two widely described ubiquistic fungi (Courtecuisse, 2000, 2008), were present in all samples. Most of the partitioning of the found species corresponded to carpophore observations carried out at the same experimental site over seven years (Buée *et al.*, in preparation). Thus, we showed that host preference of EMF reported for mixed forest in Japan (Ishida *et al.*, 2007), California woodlands (Morris *et al.*, 2008) or Tasmanian wet sclerophyll forest (Tedersoo *et al.*, 2008) can also be found in mono-plantations of temperate forests.

Beside the host preference we observed additionally temporal partitioning of ECM communities. Seasonal influence on ECM communities has already been reported by other authors, which assumed an indirect influence of seasonal changes on host physiology such as root elongation after leaf senescence or weathering effects like changing soil moisture (Buée *et al.*, 2005; Courty *et al.*, 2008; Koide *et al.*, 2007). However, since we have taken samples solely one year long we cannot show with evidence which of the seasonal dependent factors structured mostly ECM communities on our experimental site.

Comparison of the high-throughput genotyping techniques

To compare the two high-throughput technologies, NimbleGen phylochip and 454 pyrosequencing, we described the fungal communities in soil under beech and spruce of the Breuil-Chenue site. Differences in the repertoire of soil fungal species were observed in the term of species richness, taxonomical diversity and taxonomic affiliation.

454 pyrosequencing

454 pyrosequencing generated for each soil DNA sample over 2,500 ITS sequences corresponding to ~600 operational taxonomic units (OTU's) present in the beech soil sample and to ~1,000 OTU's found in the spruce soil sample (OTU was defined as species with ITS sharing 97% identity). Resampling curves of both samples were still far from reaching an asymptote meaning that the full species richness was not detected. Only 15 species were fully identified for the spruce sample and six for the beech sample using the GenBank database for BLAST analysis. This shows, that the automated analyses of generated sequences bear an inherent bias of 454 pyrosequencing. The relative large proportion of low quality or wrongly annotated sequences found in public databases can cause misleading BLAST hits, which will be overseen by automated analyses. Additionally, the large number of undefined species (=unknown fungal species, no hit) can also hinder BLAST analysis to show always the best hit to fully identified species. To overcome this problem, we applied filtering steps to the database before blasting against it. In our dataset, before a filtering step, the number of undefined sequences was already 71%. When we blasted the sequences against a database containing only sequences of fully identified fungal species, 54 fungal species of the spruce sample and 33 of the beech sample were described as fully identified.

To minimize the number of questionable BLAST matches or technical artifacts a relative stringent E-value for BLAST analysis can be chosen. Nevertheless, using a too stringent E-value increases the number of undefined sequences showing that fine adjustment of E-value is critical. Therefore, we tested in a first step the default value of the NCBI BLAST server which is an E-value $\leq 10^{-03}$. Furthermore, an arbitrary setting of an overall threshold to 3% for interspecific ITS sequence divergence is questionable, as a wide range of intraspecific sequence divergence exists in fungal groups (from < 1% for *Russula spp* to > 15% for *Pisolithus spp*; Martin *et al.*, 2002).

As 454 pyrosequencing is a recently developed technique, it still needs a lot of improvement and optimization of various technical steps and analysis tools. Although novel programs, such as MEGAN (Huson *et al.*, 2007), AMPHORA (Wu & Eisen, 2008) and CAMERA (Seshadri

et al., 2007) have been developed to cope with deep sequencing data, fine-tuning of user's-friendly softwares capable to deal with a high number of sequences is necessary allowing the user under variable conditions to adjust filtering steps, threshold levels and E-values to the dataset and studied organism groups.

Now, 454 pyrosequencing is providing unexpected insights into fungal community richness and composition, but results still have to be handled cautiously. The comparison of species richness and diversity from different 454 pyrosequencing studies stays questionable until a standardized method to analyze and interpret data will be developed.

NimbleGen ITS phylochip

Amongst the 7,151 fungal species having reporter probes on the NimbleGen ITS phylochip, only ~70 species were detected on the beech and spruce plantation of Breuil-Chenue using the most stringent signal intensity threshold. This includes 25 fungal species of the beech DNA sample and 43 of the spruce DNA sample. With a less stringent signal threshold value, less-abundant species were detected increasing the number of taxa found to 147 and 104 in beech and spruce DNA samples, respectively. In the beech sample, however, the species number within certain genera increased enormously. This suggested that the results of the designed overall fungal kingdom phylochip can be used in two different approaches. The first option uses a stringent signal threshold to detect the most abundant known species with a high species-specificity. The other option relies on the use of the phylochip with a low stringent threshold signal detecting less abundant species, but accepting a significant level of cross-hybridization between closely related species. Then, the presence of certain taxa can be confirmed at the genera level, but not at the species level.

When taxonomic affiliation of the fully identified species detected by 454 pyrosequencing and NimbleGen phylochip were compared against each other, only few identical affiliations were found due to the inherent bias of each technique. However, none of the used high-throughput techniques described clearly more fungal taxa as fully identified species than the other one showing that multiple-approach stays crucial for accurate ecological studies.

Assessing soil fungal diversity using 454 pyrosequencing

With 454 pyrosequencing we described for the first time fungal community composition under six different tree species (*Abies nordmannia, Picea abies, Pinus nigra, Pseudotsuga menziesii, Fagus sylvatica, Quercus sessiflora*). Between 25,680 to 35,600 sequences were generated for the different soil DNA samples (180,213 fungal sequences in total) corresponding to 580 to 1,000 OTU's. However, for none of the samples the rarefaction curve reached an asymptote meaning that the number of occurring sequences (i.e., OTU) may be much larger. BLAST against databases with only fully identified fungal sequences defined 42% of sequences as *Basidiomycota*, 17% as *Ascomycota* and 8% as *Mortierella*. However, the largest part belonged to unclassified *Dikarya* (20%), unclassified fungi (11%) and unclassified fungi/Metazoa group (2%). These non identified OTU's could however be used to compare fungal diversity and OTU richness between communities in ecosystems.

Correspondence analysis separated oak soil DNA sample from the other soil samples on the first axe and beech sample from coniferous species on the second axe. The much larger number of *Basidiomycota* solely found in oak soil DNA samples can explain this. The majority of fungal families were represented in all six plantations, but when looking on species level strong host preference was found. The most abundant fungal species were *Cryptococcus podzolicus* (45,354 sequences in total) and *Scleroderma bovista* (19,928) present in all soil samples. *Inocybe umbrina* and *Mortierella hyaline* belonged to the five most abundant species in beech, Douglas fir, fir and spruce. *Lactarius quietus* was the third most abundant species (6,952), but was solely present in oak soil sample. However, the number of fully identified species was for each sample low. For example, only 33 known species were described for beech sample and 54 for spruce sample. This is a low % of the overall fungal community and this calls for DNA barcoding programs aiming to increase the number of fungal species characterized at the molecular level. Sequencing the ITS of herbarium is a way to increase this number of know species in DNA databases (Brock *et al.*, 2009). This shows that in-depth description on species level of fungal communities by 454 pyrosequencing stays challenging. Further analysis will especially focus on better exploitation of databases for the analysis of the huge amount of sequences produced.

Conclusion: high-throughput techniques

We could show that the "All Fungal ITS" phylochip and 454 pyrosequencing are promising techniques for in-depth analysis of fungal communities. However, both techniques show inherent bias influencing in different ways the results of ecological studies. This shows that multiple approach studies will give the most accurate and detailed results analysing fungal communities. Additionally, the high dependence of both high-throughput techniques on public databases strengthens the need of filling these gaps by continuing to work also with classical taxonomic approaches.

Phylochip		
-	**possible optimization**	**+**
• dependence on public databases	• filtering databases	• accurate description to a taxonomic level • reproducible detection • routine manner: high sample throughput in a relative short time • use for site-specific analysis
• cross-hybridization	• multi-gene/multi-probe approach • not all gene regions are feasible as barcode regions in phylochip approach	
• only species detected, for which oligonucleotide were designed	• nested probe approach	
• fixing threshold	• mathematical model	

454 pyrosequencing		
-	**possible optimization**	**+**
• high dependence on public databases	• filtering databases	• exhaustive studies, but need of enormous generation of sequences
• high number of undefined species	•	
• adjustment of E-value for BLAST analysis • different threshold of intraspecific sequence similarity between taxa	• development of analysis tools	

Conclusion: impact of host tree species on fungal community

The different genotyping surveys used in this project showed that different tree species strikingly impact the soil microbial communities including ectomycorrhizal fungi (EMF). Host preference of fungi was observed for EMF as *Lactarius quietus* interacting with oak or *Lactarius tabidus* and *Russula parazurea* with beech and certain *Cortinariaceae*, as *Cortinarius rubrovioleipes* and *C. sanguineus* with spruce. These observations supported and extended carpophore surveys (Buée *et al.*, in preparation). Fungal communities clearly differed between beech and oak plantations, and the other tree species plantations. This indicates the impact of forest management on fungal community structure, where monospecific plantations are substituted for mixed forests of beech and oak trees. The differences in fungal community composition and species distribution can be explained by the presence of specific fungal networks already formed before for oak and beech while for other tree species the specific fungal networks had to be developed. Additionally, differences in litter composition, understorey vegetation and canopy structure between the different tree species can also indirectly influence fungal community structure. Which of these indirect factors are mainly influencing fungal diversity and how fungal communities of plantations response to changing environmental factors will be focused in future studies.

12. References of Introduction and Conclusions

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