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Charakterizace společenstva hub, podílejícího se na rozkladu opadu v jehličnatých lesích Národního parku Šumava

Characterization of fungal community decomposing litter in the coniferous forests of the Šumava National Park

Diplomová práce

Školitel: Doc. Petr Baldrián, Ph.D.

Praha, 2012

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis Lucia Žifčáková, v. r.

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Abstrakt

Pochopenie cyklu uhlíku v ihličnatých lesoch, ktoré predstavujú veľké zásobárne uhlíku má zásadný význam pre naše chápanie prírodných procesov v rámci zmeny globálnej klímy. Rozpoznanie húb ako základných rozkladačov môže prispieť k tomuto pochopeniu. Huby sú schopné rozložiť množstvo substrátov a majú celý rad enzýmov, aby tak urobili.

V tejto štúdii prezentujem huby rozkladajúce opad v horských smrekových lesoch národného parku Šumava. Cieľom mojej práce bolo sledovať sukcesiu a zmeny spoločenstiev húb od skorých štádií rozkladu ihlíc *Picea abies* po rozklad organickej hmoty v humusovom horizonte pôdy. Tento cieľ bol čiastočne splnený skúmaním produkcie extracelulárnych enzýmov húb v rôznych fázach rozkladu ihličia a to ihličia pripevneného na vetvičkách spadnutých stromov až po ihličie z opadu na povrchu pôdy v neskorších fázach rozkladu. Okrem testovania húb na ich prirodzených substrátoch – opad, enzýmové aktivity boli tiež merané na agarových kultúrach, ktoré umožňujú porovnanie rôznych húb rôzneho pôvodu. Enzymatické aktivity boli merané na enzýmoch rozkladajúcich celulózu a látkynachadzajúce sa v opade. I keď ekológia endofitov a saprotrofov naznačuje rozdiely v produkcii enzýmov, tieto neboli zaznamenané. Enzýmove aktivity vrcholia ako sa spotrebováva málo úživný sladový agar a jediný zdroj živín – smrekové ihličie je ťažké rozložiť.

Ďalšia časť výskumu bola sústredena na hubové spoločenstvá izolované z pôdy, kde pokračuje rozklad ihličia. Hubové komunity v pôde boli sledovanéslovený 454–pyrosekvenáciu – metódou celého meatgenómu. Zameranie 454–pyrosekvenačnej štúdie bolo na úplnu a celulolytickú hubovú komunitu reprezentovanú *cbhI* génom. Aby som charakterizovala *cbhI* gén do detailu, naklonavala som *cbhI* sekvencie z niektorých húb izolovaných zo smrekového ihličia.

Cieľom bolo zistiť, do akej miery sa líšia celkové i celulolytické hubové spoločenstvo medzi pôdnymi horizontami a ročnými obdobiami. Potvrdila som, že horizont je výrazny diskriminačný faktor medzi ekologickými skupinami húb. Saprotrofné huby boli nájdené v L horizonte, zatiaľ čo väčšina mykoríznych v horizonte H. Množstvo bazidiomycétov bolo väčšie v organickom horizonte zatiaľ čo u askomycétov to bolo naopak. Zistila som, že signifikantne sa viac vyskytujúcich v jednom horizonte je 73% skúmanej časť celkového spoločenstva a na období závisí 37%. V skúmanej časti komunity zastúpenej *cbhI* génmi bolo 62% OTUs závislých na nejakom horizonte a 21% na určitom období. Výsledky ukazujú, že hubové komunity sú veľmi ovplyvniteľné faktormi životného prostredia.

Kľúčové slová (12): Hubová komunita, *Picea abies*, 454–pyrosekvenácia, *cbhI* – celobiohydroláza I, ITS – vnútorný prepisovaný medzerník, celulóza, Opadový horizont, Organický / humózny horizont, Enzýmy, Saprotrofické, mykorízne a parazitické huby, lesné pôdy

Abstract

Understanding of carbon cycling in coniferous forests that represent a large carbon sink is crucial for our understanding of natural processes under global climate change. Recognition of fungi as fundamental decomposers can contribute to this understanding. Fungi are able to decompose numbers of substrates and possess a variety of enzymes to do so

In this study I present litter decomposing fungi in mountain spruce forest from national park Šumava. The aim of my thesis was to follow succession and community changes of fungi from the early stages of decomposition of *Picea abies* needles until degradation of organic matter in the organic horizon of the soil. This aim was accomplished partly by recording the extracellular enzyme production of fungi in different stages of decomposition from needles attached to the twigs of a fallen tree to a litter material in later stages of decomposition on the soil surface. In addition to testing of fungi on their natural substrata – needle litter, enzyme activities were also measured in laboratory agar cultures, which allow comparison of diverse fungi with different origins. Enzyme activities were aimed at enzymes decomposing cellulose and compounds found in litter. Although ecology of endophytic and saprothrophic fungi suggest differences in enzyme production, these were not recorded. Enzyme activity peaks as poor malt extract agar becomes spent and the only nutritive source – spruce needles is difficult to decompose.

Another part of research was triggered on fungal communities isolated from the soil where decomposition of needles continues. Fungal communities in the soil were approached by 454–pyrosequencing method of the whole meatgenome. The focus of 454–pyrosequencing study was on a total and cellulolytic fungal community represented by *cbhI* gene as a proxy. To characterize *cbhI* gene in detail, its sequences from some of the fungi isolated from spruce needles were cloned. I have investigated to what extent does the abundance of fungi in general and cellulolytic fungi in particular, differ among soil horizons and seasons. I have confirmed that horizon strongly discriminates between fungal ecological groups. Saprotrophic fungi were found in L horizon while most of mycorhizzal in H horizon. The abundance of *Basidiomycota* in the organic horizon was higher than of *Ascomycota* and vice versa. I have found significant association with one of soil horizons for 73% of examined part of total fungal community and with a season for 37%. In examined part of community represented by *cbhI* gene pool, 62% OTUs depend on a soil horizon and 21% on a specific

season. The results show, that fungal communities are strongly influenceable by environmental factors.

Keywords (12): Fungal community**,** *Picea abies*, 454–pyrosequencing, *cbhI* – cellobiohydrolase I, ITS – internal transcribed spacer, Cellulose, Litter horizon, Organic/organic horizon, Enzymes, Saprotrophic, mycorrhizal and parasitic fungi, Forest soil

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Abbreviations

ABM arbuscular mycorrhizal fungi

ABTS 2,2´–azinobis–3–ethylbenzothiazoline–6–sulfonic acid

APS ammonium peroxydisulfate

BLAST basic local alignment search tool

BSA bovine serum albumin

CCA Canonical correspondence analysis

CTAB cetyl trimethylammonium bromide

DCA detrending canonical analyses

DMAB 3,3–dimethylaminobenzoic acid

DSE dark septate endophyte

EDTA 2,2',2",2'"–(ethane–1,2–diyldinitrilo)tetraacetic acid

ECM ectomycorrhizal fungi

ENDO endophytic fungi

ErM ericoid mycorrhizal fungi

GPS global positioning system

H horizon - organic horizon

HEPES 4–(2–hydroxyethyl)–1–piperazineethanesulfonic acid

ITS internal transcribed spacer

L horizon litter horizon

MBTH 3–methyl–2–benzothiazolinonehydrazone

ME malt extract

NCBI National Center for Biotechnology Information

OTU operational taxonomic unit

PCA principal component analysis

PCR polymerase chain reaction

CCA Canonical correspondence analysis

rDNA ribosomal deoxyribonucleic acid

SDS sodium dodecyl sufate

TAE tris–acetate–EDTA

UV ultraviolet

1. Introduction

Soil is the largest pool of carbon in the biosphere. The transformation of organic compounds by soil–inhabiting heterotrophic microorganisms affects global carbon and nutrient cycles, the climate and plant production. Decay of organic substances in soil is mediated by extracellular enzymes that degrade biopolymers embodied in plant and microbial cell walls and reduce them to soluble molecules for microbial consumption (Burns and Dick 2002).

Coniferous forest ecosystems can be found in the northern hemisphere in subpolar, moderate and partially in subtropical area. The widest continuous vegetation is the boreal forest biome – taiga, which covers 11% of Earths dryland (Sánchez *et al.* 2009). Coniferous forests play a prominent role in global carbon cycling as a carbon sinks (Myneni *et al.* 2001). Understanding of microbial involvement in decomposition in these ecosystems is thus required to estimate global C fluxes and their potential future changes (Buée *et al.* 2009). Flux of plant assimilates through tree roots into the biomass of ectomycorrhizal fungi and through plant litter to saprotrophic fungi and bacteria are the two key paths of carbon allocation into forest soils. Forests dominated by spruce (*Picea spp*.) constitute large ecosystems in boreal forest biomes and, due to climatic conditions, also in higher altitude forests in the temperate zones. Due to their economical importance, spruce forests are also abundant as plantation forests worldwide.

Coniferous forests subsoil is usually composed of podsols, with acidic pH around 4 and has low fertility, the acidity of the soils being often increased by acid rain (Bardgett 2005). As a consequence of the low pH and high content of complex compounds like lignin, cellulose, waxes and defensive compounds (e.g., polyphenols), decomposition of spruce litter is very slow. This material is hard to process for soil fauna, which further slows down the decomposition rate. Fungi are able to tolerate low pH and decompose recalcitrant compounds, unlike bacteria, which are limited by low pH, enzymatic equipment, higher requirements for certain nutrients and lower ability to tolerate environmental changes (Allison *et al.* 2008, Carpenter *et al.* 1987).

The present understanding of how ecosystem functions are influenced by soil biodiversity is far behind our understanding of how aboveground organisms contribute to these functions (Bowker *et al.* 2010). While several recent studies have used deep sequencing approaches to assess the diversity of soil bacterial components (Lauber *et al.* 2009, Roesch *et al.* 2007), the number of such studies addressing fungal diversity is still limited. Only a minor fraction of the estimated 1.5 million fungal species worldwide have been described (Hawksworth 2001).

The ecological roles of most fungal taxa are poorly understood since the complexity of fungal communities has so far limited our ability to estimate diversity and distinguish individual taxa (McGuire and Treseder 2010). The ability to assign functional trait values to species (or species groups) is a critical step in the interpretation of changes in community structure along environmental axes and would strengthen researchers' mechanistic understanding of fungal community assembly. This is an area where functional gene approaches may provide an important link to generalizing patterns across studies.

2. Literature outline

2.1 Characterization of fungal community decomposing organic matter in the forest soil

Studies using the isolation and analysis of individual microbial taxa together with recent molecular studies and observations of enzymatic activity in soils have revealed a typical pattern of litter decomposition.

When the newly shed litter reaches the forest floor, it is already colonized by endophytic fungi. In pine needle litter, for example, the well known endophytic fungus *Lophodermium pinastri* is frequently found. Some authors report beneficial effects of endophytes on their hosts (Carroll 1988, Clay1988), leading to the impression that the term 'endophyte' defines fungal symbionts with mutualtic properties. Some studies demonstrate, however, that potentially pathogenic fungi are able to live latently for a longer or shorter period in their hosts (Dayer and Sinclair 1991, Sieber *et al.* 1988). Thus Sieber–Canavesiu and Sieber (1993) divided endophytic fungi into two groups according to their ecology: endophytic fungi – isolated from green living needles, having only an endophytic phase and 'transition–fungi' – survivors in senescent tissues, recently dead tissues still attached to the plant and litter tissues, after endophytic life phase can switch to saprophytic. The ecological role of endophytes is not clear, but many stay in the dead litter and some have saprotrophic capabilities (Osono, 2006). In this early stage of decomposition, mainly soluble sugars and other low molecular weight compounds are lost from the litter (Berg *et al.* 1982). Enzymes degradating monosaccharides and disaccharides are also prominent but then rapidly decline (Sinsabaugh 2005). Some endophytic fungi in the early community also have cellulolytic

capacities and have been observed to cause significant mass loss in laboratory experiments (Korkama–Rajala *et al.* 2008).

According to the results of Sieber–Canavesiu and Sieber (1993) on *Abies alba* needles only *Exophiala sp.* and *Grovesiella abieticola* belonged to the first group of endophytes. Prominent representatives of the second group were *Cytospora pinastri, Hormonema dematioides, Rhizosphaera oudemansii, R. macrospora* and *Leptostroma sp*. The frequency of colonization by the members of the second group decreased quickly after needle fall. Transition–fungi were fully substituted within one year after needle fall by members of the third group, such as *Thysanophora penicillioides, Rhizoctonia sp., Gliocladium penicilliodes, Pseudomicrodochium sp., Cylindrocladium sp.* or *Coleophoma cylindrospora.* In the *Picea abies* (Scots pine) needles, unidentified ascomycetous species belonging to either the *Leotiomycetes,* primarily within *Helotiales,* and *Dothideomycetes* dominate the fungal community at early stages (Lindahl *et al.*2007). The current knowledge on the functional capacities of these fungi is, however, very limited. The first fungal community in the recently shed litter is later enriched with early basidiomycetous fungi. Species within the genera *Athelia* and *Sistotrema* are frequently found (Lindahl *et al.* 2007, O'Brien *et al.* 2005) as well as with another very common and widespread litter fungus, *Marasmius androsaceus* (Holmer and Stenlid 1991, Korkama–Rajala *et al.* 2008, Lindahl *et al.* 2007).

During the second phase of litter decomposition, cellulolytic enzymes are active and the main degradation of the polymer occurs. Laccase activity can also be observed relatively early in decomposition of litter with high contents of phenolic compounds (Sinsabaugh 2005). Typical litter basidiomycetes such as the species of the genera *Mycena, Clitocybe* and *Collybia* are prominent during this stage (Osono, 2007).

In final stages of organic matter succession, the abundance of the typical litter decomposers tends to decrease and mycorrhizal fungi start to dominate the fungal community in the humus–layer of both deciduous and coniferous forests (Lindahl *et al.* 2007, O'Brien *et al.* 2005).

It is presumed that if there is a shift in a fungal community during the succession, and if different organic compounds are available at different phases of decomposition, there should be differences between various succession stages of decomposing community and in terms of production of enzymes.

Previous study of Lindhal *et al.* (2007) shown, that fungal communities in the soil are spatially heterogeneous. Especially there is a separation of saprotrophic and ectomycorrhizal fungi along soil depth and continuing changes in C:N ratio, which reflects the changing

quality of decomposed organic matter. In the ‘late’ fungal community, defined as taxa occurring with a higher frequency in fragmented litter and humus than in the fresh litter, were described by Lindhal *et al.* (2007) ectomycorrhizal basidiomycetes, predominantly from the genera *Cortinarius* and *Piloderma. Ascomycota Capronia spp*. and *Rhizoscyphus ericae*, which both form mycorrhizal associations with ericaceous plants (Allen *et al.* 2003), were also common. Gadgil and Gadgil (1971) suggested that ectomycorrhizal and saprotrophic fungi compete with each other for N resources, and such competitive interactions could act to maintain the partitioning of the fungal community into two vertically separated and functionally distinct subcommunities. Saprotrophic fungi are more efficient than mycorrhizal fungi in colonizing and utilizing fresh, energy–rich litter (Colpaert and van Tichelen 1996) and may thus be able to outcompete mycorrhizal fungi in the upper part of the forest floor. However, as the C:N ratio decreases and the substrate becomes depleted in available energy, the saprotrophs become less competitive, which is consistent with the observed replacement of saprotrophs by mycorrhizal fungi that do not depend on litter–derived energy.

The above-mentioned spatial heterogeneity implies also heterogeneity in time because the organic substrate is changed over the years of decomposition, but also heterogeneity in decomposition during the year can be suspected; however, the current knowledge of the seasonal changes of fungal communities is limited. Such changes are expectable for several reasons. Carbon cycling in a forest changes during the year. In summer, trees photosynthesize and send simple forms of carbon to the roots, where mycorrhizal fungi can utilize them Saprothrophic fungi decomposing organic material in the upper forest soil thus cannot compete for niches with ectomycorrhizal fungi in summer. In winter situation changes, the photosynthate input into the mycorrhizal fungi from trees is significantly lower than in summer and some of them might even switch to saprothrophic lifestyle to be able to compete with saprothrophic fungi and to gain some nutrients. Several studies in temperate forests have supported this hypothesis, demonstrating that ectomycorrhizal root tips exhibit high extracellular enzyme activity during winter months when photosynthesis rates decline (Buée *et al.* 2005, 2007; Mosca *et al.* 2007).summer. The carbon availability for saprotrophic fungi is more–less the same over the year in coniferous forests. Since saprotrophes are better adapted for degradation of complex organic substances, they have advantage before ectomycorrhizal fungi and can compete better for nutrients in winter under the snow cover. During autumn in deciduous forests have saprotrophic fungi huge supplies of fresh litter after leaf fall and should dominate over mycorrhizal fungi. Courty *et al* (2007) also found that ectomycorrhizal root tips in an old–growth oak forest produce a suite of extracellular

enzymes in the early spring that show peak activity immediately before and following bud break. Within a season, litter transformation results in the changes of both the litter chemistry and fungal community composition with increasing abundance of the *Basidiomycota* (Osono, 2007). Seasonal changes of ECM fungi were observed in oak forest but the dominant taxa were present all time at considerable quantities (Courty *et al.* 2008, Walker *et al.* 2008). Both composition and metabolic activity (enzymes) of ECM show seasonal changes (Buée *et al.* 2005). Arbuscular mycorrhizal fungi exhibit seasonal changes in community composition (Dumbrell *et al.* 2011). High seasonal variation but low interannual variation was observed for total fungi in boreal forest (Izzo *et al.* 2005, Taylor *et al.* 2010).

Previous studies showed that (ecto–, arbuscular–, ericoid–) mycorrhizal fungi are able to decompose some simple organic substances and can transport amino acids even when associated with host plant (Abuzinadah and Read 1986, Bajwa and Read 1986, Finlay *et al* 1992, Hawkins *et al.* 2000). The capacity of ectomycorrhizal and ericoid mycorrhizal fungi to produce enzymes involved in degradation of organic matter and to mobilize organic forms of N is well documented from laboratory experiments (Abuzinadah *et al.* 1986, Read and Perez–Moreno 2003, Lindahl *et al.* 2005).

In the litter horizon where initial stages of litter decomposition occur, enzyme activities are typically substantially higher than in the deeper soil horizons (Šnajdr *et al.* 2008, Wittmann *et al.* 2004). Recent studies have shown that the activities of extracellular enzymes, especially those participating in lignocellulose degradation, are not only associated with litter decay but are also used for nutrient acquisition by microorganisms in the deeper soil horizons (Caldwell 2005, Moorhead and Sinsabaugh 2000). Soil horizons (L – litter horizon and H – organic horizon) exhibit profound differences with respect to enzyme activities (Baldrian *et al.* 2008, Šnajdr *et al.* 2008). Comparison of the effect of a site and season on enzyme activity showed that season played a principal role in the enzyme activity of the litter horizon measured in spoil heaps after brown coal mining. The highest activities of all enzymes were detected in October, which is most probably caused by the input of fresh litter, rich in easily available nutrients, into soil during the litter fall period (September–October). High ergosterol content points to fungi as the main litter decomposers. The importance of the litter compartment for plant biomass decomposition is also supported by the fact that the seasonality was more pronounced in the upper part of the topsoil (Baldrian *et al.* 2008).

Edwards and colleagues (2008) recently designed a set of PCR primers that target the catalytic region of the fungal glycosyl hydrolase family 7 cellobiohydrolase I gene (*cbhI*) in *Ascomycota* and *Basidiomycota*. Targeting this gene allows a representative group of

cellulolytic fungi to be detected and monitored in soil ecosystems and has been shown to be a useful comparative functional gene marker for soil fungal communities (Edwards *et al.* 2008). Cellulolytic ability is widespread in members of the *Ascomycota* (Eriksson and Wood 1985) and *Basidiomycota* (Baldrian and Valášková 2008, Lynd *et al.* 2002). However, cellobiohydrolases have so far been isolated from several white rot basidiomycetes and the plant pathogen *Sclerotium rolfsii*. They are apparently absent from most brown rot fungi (Kämper *et al.* 2006, Loftus *et al.* 2005).Cellobiohydrolase activity was also documented in litter–decomposing fungi (Steffen *et al.* 2007, Valášková *et al.* 2007) and some ectomycorrhizal fungi (Burke and Cairney 1998, Cao and Crawford 1993). Three forms of CBHI protein with different activities towards carboxymethyl cellulose were isolated from *Phanerochaete chrysosporium* (Garzillo *et al.* 1994, Uzcategui *et al.* 1991). Edwards *et al.* (2008) isolated three distinct *cbhI* genes from *Clitocybe nuda, Clitocybe gibba* and *Chlorophylum molybdites.* These sequences within the same fungus had a nucleotide mean pairwise similarity of 70 – 80%.

Edwards *et al.* (2008) was first who designed and used *cbhI* primers in environmental soil analysis. They succeed to identify into the groups sequences obtained from the soil covered with deciduous forest. Obtained *cbhI* were on the clades with *Trichoderma spp., Xylaria spp., Organicola grisea, Alternaria spp. Apergillus spp., Penicilium spp., Pleurotus osteratus, Irpex lacteus, Phanerochaete chrysosporium, Clictocybe spp., Volvariela volvacera* and there were also unidentified groups of forest floor–derived sequences. The most recent papers on fungal *cbhI* gene pools in soils show that several dominant fungal taxa possessing cellobiohydrolase belong to groups not yet recognized as cellulose degraders (Weber *et al.* 2010). Weber *et al.* (2010) showed that the *cbhI* gene pools are ecosystem–specific; *cbhI* sequences from the *Ascomycota* comprised the majority of sequences from loblolly pine plantation, while *Basidiomycota* sequences comprised the majority of the aspen plantation. Phylogenetic tree constructed by Weber *et al.* (2010) proved that many of the *cbhI* genes do not form taxon–specific monophyletic clades. Because the *cbhI* phylogeny does not always parallel that of the ribosomal gene sequences and many isolates possess multiple and different copies of *cbhI*, it is difficult to discern taxonomic identity of an environmental *cbhI* sequence from its nearest BLAST hit or position within the phylogeny unless the similarity to the known sequences is high.

For fungal succession it has been observed that the *Mucoromycotina* belong to the first colonizers followed by the *Ascomycota* (Torres *et al.* 2005). These fungi have limited ability to degrade lignin and are mainly regarded as cellulose decomposers or sugar fungi (Osono

2007). *Basidiomycota*, with their ability to degrade the recalcitrant lignin–containing litter material, appear only later in the decomposition process (Lundell *et al.* 2010, Osono 2007). Although mycorrhizal fungi may not be primary participants in cellulose degradation, higher exo-enzyme activity rates have been noted in mycorrhizal mats suggesting that they can enhance and alter saprotrophic activities, and perhaps richness, in the surrounding community (Entry *et al.* 1991, Kluber *et al.* 2010).

2.2 Specific aspects of fungal ecophysiology

Fungi combine the micro– and macroscopic lifestyles. Like microbial communities, fungal communities are highly diverse and poorly described. Their vegetative bodies are composed of microscopic filaments that interact directly with the environment at the micron scale. Fungal spores, often in the small–micrometer range (e.g., 10 to 20 micrometers), are produced in great numbers and are capable of long–distance dispersal. This microscopic aspect makes fungi nearly impossible to observe in their active, vegetative states and molecular tools are required for their identification and quantification.

On the other hand, fungi share many ecological similarities with macroorganisms. Like plants, fungi are sessile and compete for space in order to control access to resources. Although individual hyphae are microscopic, genets can occupy large spaces and can survive for many years (Smith *et al.* 1992).

Unlike bacteria, fungi do not seem to exhibit high frequency of horizontal gene transfer, so that their functional traits are relatively stable, and species concepts are useful and reasonably well developed (Taylor *et al.* 2000). The development of fungal–specific primers for amplification of the internal transcribed spacer (ITS) region of the ribosomal RNA genes (Gardes and Bruns 1993) opened the way for direct amplification of fungal DNA from complex substrates containing multiple sources of DNA, such as soil or plant tissue.

Despite their ubiquity and importance in terrestrial ecosystems, the ecological research concerning fungal communities has long been held back by the inability to identify species in their vegetative states. Although reproductive structures can be diagnostic, they are not ideal for ecological studies because they are produced infrequently in the field, often harbor cryptic species complexes, and do not accurately represent species abundances. However, the recent adoption and dissemination of DNA– and RNA–based molecular tools has greatly reduced the barriers to sampling and identifying fungi from vegetative material. At the same

time, improvements in techniques for measuring fungal biomass and nutrient uptake (e.g., the use of stable isotopes, phospholipids fatty acids and ergosterol) have confirmed the importance of fungi in key ecosystem functions, such as carbon and nutrient cycling (Hobbie and Hobbie 2006).

2.3 Different ecological strategies of fungi

Considerable diversity of fungal species can be present already in living leaves as endophytes (Arnold *et al.* 2007). Current results show that among fungi associated with dead litter before abscission fungal endophytes quantitatively dominate over their bacterial counterparts concerning biomass (Šnajdr *et al.* 2011). Although the ecological role of endophytes is not completely clear, the fact that these fungi can start to exploit dead leaves immediately after their senescence and before these come into direct contact with soil may point to their potential importance in the initial stages of decomposition. This is in agreement with observations that endophytic species can be found in the litter horizon of forest soils where they may continue with decomposition (Livsey and Barklund 1992, Mitchell *et al.* 1978) and cause significant needle decomposition (Korkama–Rajala *et al.* 2008). On the other hand, the decomposition ability of most endophytic and phyllosphere–associated fungi seems to be limited compared to species occurring later during succession (Osono 2006 and 2007, Korkama–Rajala *et al.* 2008), although exceptions may exist (Boberg *et al.* 2010). The lower decomposition ability of endophytes might, possibly, be explained by the fact that fresh litter contains more easily available substrates that do not require biopolymer–cleaving enzymes (Šantrůčková *et al.* 2006).

Mycorrhizal fungi are in contrary to endophytic fungi, widespread in the soil (Wang and Qiu 2006). The symbiosis between plant roots and fungi, referred to as mycorrhiza (literally, "fungus root"), is one of the most ubiquitous mutualisms in terrestrial ecosystems. These mycorrhizal associations enable plants to acquire mineral nutrients and water in exchange for photosyntheticaly derived sugars. It is likely that plant adaptation to life on land 400 million years ago was possible only with the help of mycorrhizal symbionts (Simon *et al.* 1993). Many plants depend heavily on mycorrhiza for mineral nutrition, and the absence of appropriate fungi can significantly alter plant community structure (Weber *et al.* 2005). Although most mycorrhizal interactions are thought to be mutualistic, there are examples of mycorrhizal symbioses in which plants are parasitized by fungi (Johnson *et al.* 1997) or fungi

are parasitized by plants, as in the case of certain non–photosynthetic plants that have become parasites on mycorrhizal fungi involved in mutualistic interactions with other photosynthetic plants (Bidartondo 2005). Although we know little about the saprotrophic capabilities of many ectomycorrhizal taxa, an intriguing possibility is that ectomycorrhizal fungi may have evolved good abilities to degrade the structural macromolecules (e.g. suberin) found in fine roots but not in above–ground litter. Because the fine roots colonized by ectomycorrhizal fungi are low in lignin (e.g. oak; Soukup *et al.* 2004) compared with above–ground litter, it is plausible that the enzymatic systems necessary to degrade fine roots are poorly adapted for degrading lignin–rich above–ground litter. Recent research has shown that ectomycorrhizal and ericoid mycorrhizal fungi may contribute to litter degradation (Talbot *et al.* 2008). Also the arbuscular mycorrhizal fungi are able to decompose organic substances independently of their host and thus must possess extracellular enzymes to do so (Hodge *et al.* 2001, Trojanowski *et al.* 1984).

Saprothrophic fungi live on organic substrates, which they are able to decompose and are not dependent on the living host plant. They typically express a wide set of enzymes involved in decomposition (Baldrian 2008). We can divide saprothrophic fungi into groups based on their ability to degrade lignin. Lignin modification and degradation has been most extensively studied in *basidiomycota*, in which a number of enzymes and mechanisms involved in lignin attack have been demonstrated (Kirk and Farrell 1987, Tuomela and Hatakka 2011,). White–rot basidiomycetes (notably *Phanerochaete chrysosporium*) are the most frequent wood–rotting organisms, because of their ability to degrade lignin, hemicelluloses, and cellulose, often giving rise to cellulose–enriched white material. Brown–rot fungi grow mainly on softwoods and represent only 7% of wood–rotting *basidiomycota*. They can also degrade wood polysaccharides after only a partial modification of lignin, which results in a brown material consisting of oxidized lignin, which represents a potential source of aromatic compounds for the stable organic matter fraction in forest soils (Martinez *et al.* 2005) Lignin is degraded to a lesser extent by brown–rot fungi, via a different mechanism to white–rot fungi (Dey *et al.* 1994). Soft–rot fungi secrete cellulases from their hyphae and this leads to the formation of microscopic cavities inside the wood, and sometimes to a discoloration and cracking pattern similar to brown rot (Duncan and Catherine 1960). Soft–rot are presented by ascomyctes and mitosporic species, such as *Chaetomium sp.* and *Ceratocystis sp.* Another group of saprotrophic fungi are litter decomposing fungi, amongst which the best reviewed group are litter decomposing basidiomycets. Litter decomposing basidiomycets produce ligninolytic oxidases and

peroxidases, which are thought to be responsible for the transformation of lignin (Martinez *et al.* 2005, Sinsabaugh 2010). In addition, they can also degrade and mineralize chemically related compounds including organic and fulvic acids as well as numerous xenobiotics (Baldrian 2008, Steffen *et al.* 2002, Tuomela *et al.* 2002). Since the ability of efficiently decomposing and mineralizing lignin and organic acids was demonstrated for several saprobic basidiomycets and their isolated extracellular Mn peroxidases (Hofrichter *et al.* 1998 and 1999; Hatakka 2001, Steffen *et al.* 2002), litter decomposing basidiomycets are regarded as the key players in microbial lignin and humus degradation in forest soil environments (Baldrian 2008, Osono 2007;. Mn–peroxidase, mannanase and xylanase were produced by *Mycena galopus* in *Picea sitchensis* litter (Gosh *et al.* 2003) and the litter–decomposer *Lepista nuda* produced laccase, endoglucanase, β – glucosidase and β – xylosidase on *Fagus sylvantica* buried leaves in soil (Colpaert and van Laere 1996). Recently, litter–decomposing fungi *Gymnopus sp., H. fasciculare, L. nuda, Marasmius quercophilus, Mycena inclinata* and *R. butyracea* were reported to produce laccase, Mn–peroxidase and a complete set of cellulose degrading enzymes on *Quercus sp.* litter (Staffen *et al.* 2007,Valášková *et al.* 2007).

2.4 Properties of coniferous forest soils

Soil properties include its physical, chemical and biological characteristics, which are represented by the bedrock composition, relief, time, vegetation, climate and representation of living organisms. All these characteristics affect the activity of microbial communities and vice versa (Šnajdr *et al.* 2008).

Forest soils are typical by their sharp vertical stratification. Stratification develops because litter material is accumulated on the soil surface and as subsequent mineralization continues, mineralized material is pressed deeper to the soil by new litter layers. Podzols in area of Plešné lake are stratified into layers of the organic litter layer consisting predominantly of decaying spruce needles, branches and bark (L horizon); the uppermost mineral horizon with accumulated humified organic matter (H horizon) and mineral soil. H horizon is typical by lower ratio of C:N than litter, lower amount of organic matter relative to L horizon, bigger resiliency of organic compound and the related smaller amount of microbial biomass and its activity (Šnajdr *et al.* 2008).

Litter decomposition is one of the main processes responsible for the formation of typical forest soils with developed organic horizons, rich in lignocellulose–derived organic compounds. To sum up, physical, chemical and biological properties along with the plant litter input, its microbial degradation along with environmental factors lead to the formation of profile typical for forest soils.

The two major sources of carbon in forest soils are plant litter and plant root exudates. While the root exudates usually contain soluble small–molecular–mass compounds, plant litter is mainly composed of plant cell wall polysaccharides (Baldrian 2009a). Forest plant litter decomposition is an important process in C, N and P cycles and soil formation. This process is controlled by abiotic factors, such as temperature, moisture, chemical composition of the litter, and microbial communities (Aber *et al.* 1990, Couteaux *et al.* 1995, Fassnacht and Gower 1999, Park and Matzner 2003, Pregitzer *et al.* 2004). Litter low in N and rich in lignin requires additional N to be decomposed and decomposition is not followed by N release into the soil (Meentemeyer 1978). *Picea abies* needles are exactly the case – they are rich in C and poor in N (**Chyba! Nenašiel sa žiaden zdroj odkazov.**), so their decomposition is incomplete unless extra N is supplied. Decomposition of spruce needles rich in lignin is slower than the decomposition of herbaceous understory litter containing more cellulose, nonstructural material and less lignin and related compounds. Deciduous forest litter has higher content of easily degradable substances like amino acids and simple sugars, which contributes to their faster decomposition (Bardgett 2005).

Chemical composition of *Picea abies* needles differ in various phases of their decomposition. Decayed needles have less C and more N than mature needles, because as mentioned above, during decomposition litter lose C while obtain N since it is need in lignocellulose decomposition process. Chemical composition of *Picea abies* needles is in **Chyba! Nenašiel sa žiaden zdroj odkazov.** (Šantrůčková *et al.* 2006); the senescent needles were sampled from trees invaded by bark beetle.

Table 1: Nutrient concentration in the mature, senescent and decayed spruce needles from Plešné Lake. Mean values from the material collected are given.

Species	Stage	C	N	P	DOC	DN	P_{OX}	Molar ratios			Mg	Mn	Ca	K	Al	Fe	Ca/Al
		mol kg^{-1}		mmol kg^{-1}	mol kg^{-1}	mmol kg^{-1}		C/N	C/P	N/P	mmol kg^{-1}						molar
Picea abies (needles)[A]	Mature	42.80	0.83	21.31				52.18	2040	39.05	15.25	5.15	77.1	29.5	4.32	1.28	17.83
	Senescent	40.75	0.87	32.60	2.87	38.3	1.01	46.95	1234	26.46	30.83	6.48	108.8	93.7	7.72	1.52	14.08
	Decayed	40.02	0.91	33.87	1.34	62.5	3.30	43.98	1182	26.87							

Explanation: A mean values from 1 to 3 year old needles.

Lignocellulose is the structural material of plant cell walls, and is therefore the main component of plant biomass. Lignocellulose consists of three main components: cellulose, hemicelluloses, and lignin (Lee 1997, Sjöström 1993).

Cellulose, which accounts for 30–50% dry weight of lignocellulose, is a polysaccharide composed of β–1,4–linked D–glucose units. Cellulose fibrils contain tightly structurally organized part – crystalline cellulose and loosely organized amorphic parts (Hon 1994). Amorphous domains are easy to decompose because they can be easily attacked by cellulolytic enzymes that ultimately convert it into glucose.

Hemicelluloses consist of various polysaccharides, mainly the xylans and mannans, which are closely associated with the cellulose filaments, and chemically linked with lignin. The major hemicellulose in hardwoods is xylan (15–30% dry weight), a polysaccharide composed of β–1,4–linked D–xylose units, which can be substituted with other monosaccharide units. Softwood hemicellulose contains mainly galacto-, gluco-mannan (15–20% dry weight), a polysaccharide composed of β–1,4–linked D–glucose, D–galactose and D–mannose units.

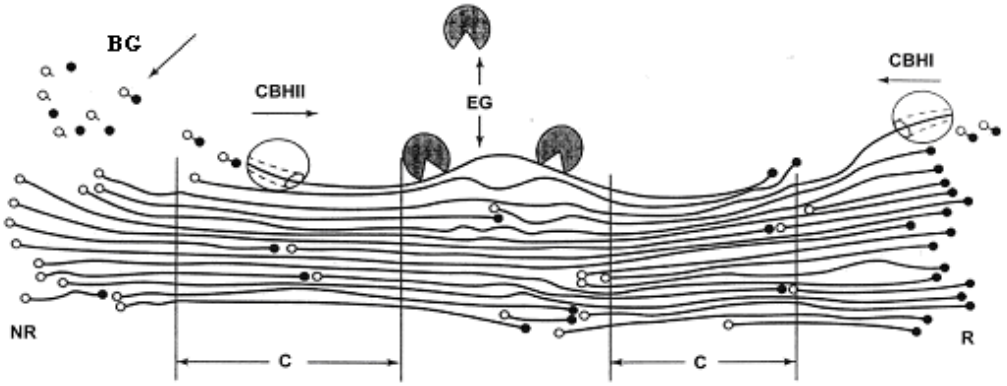
Lignin is a complex aromatic heteropolymer, composed of phenylpropanoid aryl–C_3 units, linked together via a variety of ether and C–C bonds. Lignin accounts for 15–30% dry weight of lignocellulose, in which it forms a matrix that is closely associated with the cellulose filaments, and is covalently attached to hemicelluloses. Lignin is formed by radical polymerization of guaiacyl (G) units from precursor coniferyl alcohol, syringyl (S) units from precursor sinapyl alcohol, and *p*–hydroxyphenyl (H) units from precursor *p*–coumaryl alcohol (Faix 1991). The ratio of G:S:H units varies from species to species, but softwoods usually contain G type lignins, containing mainly G units, while hardwoods are generally GS–type lignins, containing mixtures of G and S units and grass lignins are H type lignins containing a higher proportion of H units (Lee 1997). The ether and C–C linkages present in lignin are not susceptible to hydrolytic attack, and therefore, lignin is highly resistant to breakdown. The embedding of cellulose filaments in lignin provides a physical barrier to lignocellulose breakdown.

2.5 Enzymes degrading cellulose

Efficient solubilization of the heterogeneous and highly insoluble native cellulose requires the presence of multiple enzymes. Characteristic feature of most cellulases is a domain

structure composed of a catalytic domain linked by an extended linker region to one or more cellulose–binding domains (CBDs) (Tomme *et al.* 1995). It is well documented that CBDs are required for efficient hydrolysis of crystalline substrates. All cellulolytic enzymes share the same chemical specificity for β–1,4–glycosidic bonds, which are cleaved by a general acid–catalyzed hydrolysis,(McCarter *et al.* 1994). Different modes of action of cellulolytic enzymes on the polymeric substrate are commonly described as endo– and exo– types of attack (Wood *et al.* 1979) (Figure 1).

Figure 1: Decomposing of cellulose fibers by action of *CBHI*, *CBHI*I (cellobiohydrolase I and II), EG (endoglucanase) and BG (β–glucosidase), adapted from Teeri (1997)



Endoglucanases (EC 3.2.1.4) hydrolyze internal bonds at random positions of the less ordered (or amorphous) regions of cellulose. These enzymes generate chain ends for the processive action of another group of cellulases, the cellobiohydrolases (which are exoglucanases) (Himmel *et al.* 2007). A typical endoglucanase cleaves glycosidyc bonds along the length of the cellulose chains, resulting in a rapid decrease in the degree of polymerization (DP) of the substrate (Kleman–Leyer *et al.* 1994 and 1996). Because the glucan chains can remain associated with the rest of the crystal after a single bond cleavage at the surface, it takes a relatively long time before soluble products are observed after an endo– type of attack. Exoglucanases (also called cellobiohydrolases) are currently thought to be processive enzymes, initiating their action from the ends of the cellulose chains (Warren *et al.* 1996). These act in a unidirectional manner from either the non–reducing (EC 3.2.1.91) or the reducing (EC 3.2.1.176) ends of cellulose polysaccharide chains, liberating cellobiose as the major product. By sequence and structural comparison, cellulases and other glycoside hydrolases have been classified into families sharing similar structures and the same chemical reaction mechanisms. Cellobiohydrolases are classified into three glycoside

hydrolase families (GH6, GH7, and GH48) (Henrissat 1991). Of these families, only GH7 is thought to be exclusively fungal, and this family contains the *CBHI* cellobiohydrolases (mentioned above) and the EG1 endoglucanases from both *ascomycota* and *basidiomycota* fungi (Edward *et al.* 2008). β–D–glucosidases (EC 3.2.1.21) hydrolyze cellobiose into two glucose molecules (Himmel *et al.* 2007).

Cellulolytic fungi typically produce more different cellobiohydrolases (CBHs) belonging to different GH families. For example in *Trichoderma reesei*, *CBHI* makes up 60% and *CBHI*I 20% of the total cellulolytic proteins, accounting for most of its cellulolytic activity. These two enzymes can achieve complete, although slow, solubilization of cellulose crystals even without the help of endoglucanases (Chanzy *et al.* 1985).

CBHI and CBHII cellobiohydrolases liberate cellobiose from opposite glucan chains. Enzyme–kinetic data obtained using oligosaccharides labeled by ^{3}H at their reducing end or by ^{18}O at one glycosidic oxygen indicate that *T.reesei* CBHI prefers the reducing end while CBHII acts at the non-reducing ends (Barr *et al.* 1996).

The complementary activities of endo– and exoglucanases lead to synergy, demonstrated as the enhancement of activity of the enzyme pool over the summed–up activities of the individual enzymes. The endo–exo synergy is easy to understand as the endoglucanases provide free chain ends on the cellulose surface for the exoglucanases to act upon (Wood *et al.* 1979).

2.6 Other fungal enzymes involved in decomposition

As was stated above in section 2.4, spruce litter is composed mostly from hardly degradable compounds like lignocellulose, cellulose or hemicellulose, but also from easy decomposable substrates like pectins, mannans, simple sugars, proteins etc. Due to spruce needle litter chemical complexity, a wide array of enzymes is necessary for its decomposition.

Cellulases, in particular the complex consisting of endoglucanase, cellobiohydrolase (both discussed above in the section 2.5) and 1,4– α,β–glucosidase (EC 3.2.1.3/21), hydrolyze the long chains of cellulose, resulting in the liberation of cellobiose and finally glucose. 1,4–β–glucosidase (EC 3.2.1.21) is exocellulase hydrolyzing terminal non–reducing residues of a variety of β–D–glycoside substrates (including cellobiose, see above) releasing glucose units. It was found in many fungal taxa both saprothrophs and mycorrhizal (Baldrian 2008). Enzyme involved in degradation of starch is 1,4–α–glucosidase (EC 3.2.1.3.), which was

identified from several wood–rotting basidiomyctes and soil–inhabiting saprothrophs (Baldrian 2008).

Hemicellulases, such as endo–1,4–β–xylanase **(**EC 3.2.1.8), endo–1,4–β (EC 3.2.1.78) or exo–1,4–β mannanase (EC 3.2.1.25), exo–1,4–β–xylosidase (EC 3.2.1.37), are involved in the breakdown of different polysaccharide chains such as xylans and mannans. These are among the main components of hemicellulose, a much more heterogeneous polysaccharide than cellulose. Range of endoxylanases like1,4–β–xylosidase (EC 3.2.1.37), catalyzing random hydrolysis of β–1,4–glycosidic bonds in xylans was found in both white–rot and brown–rot fungi. β–glucuronidase (EC 3.2.1.131) is another enzymes involved in hemicellulose decomposition (Baldrian 2008) and was characterized from *Phanerochaete chrysosporium* (Castanares *et al.* 1995) and *Schizophyllum commune* (Johnson *et al.* 1989).

Between enzymes degradating lignin belongs laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), Mn–peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16). Laccases can oxidize a wide variety of substrates and play variety of roles, but mainly they degrade lignin by oxidative mechanism. Laccase is produced by many *Basidiomycota* and *Ascomycota*, both saprotrophic and mycorrhizal. Although there are some records of laccase in mycorrhizal basidiomyctes and brown–rotter *C.puteana* (Lee *et al.* 2004), their role in these fungi is not known in detail (Baldrian 2006). Ligninolytic peroxidases (lignin peroxidase, Mn–peroxidase, versatile peroxidase) participate in lignin decomposition. Their production is widespread among saprotrophic basidiomyctes although also some mycorrhizal fungi were demonstrated to possess genes encoding these enzymes (Baldrian 2008, Bödeker *et al.* 2009, Hofrichter 2002).

Lipolytic enzymes like esterase and lipase catalyze hydrolysis of lipidic backbone and function mainly in basal metabolism.

Hydrolytic enzymes accessing organic nitrogen to fungi are alanine aminopeptidase (EC 3.4.11.2) – release of an N–terminal amino acid, preferentially alanine from a peptide, amide or arylamide and leucine aminopeptidase (EC 3.4.11.1) releasing N–terminal amino acid, preferentially leucine. In the acquisition of phosphorus are applied acid phosphatase (EC 3.1.3.2) and phosphodiesterase (EC 3.1.4.1). Acid phosphatase together with alkaline phosphatase enables ectomycorrhizal and saprothrophic fungi to receive phosphorus from organic phosphates (Baldrian 2008). Sulfate containing phenol molecules are degraded by arylsulfatase (EC 3.1.6.1) and helps fungi to obtain sulfur from the nutrients.

The main source of chitin in the litter are fungal cell walls – those of death, but also of living fungi. Fungi overcome nitrogen deficiency experienced during litter colonization by

translocation of nitrogen from the older parts of mycelium. 1,4–β–N–acetylglucosaminidase (EC 3.2.1.52) is able to mobilize nitrogen supplies by hydrolysis of chitin. Compared to phytopathogenic fungi, the occurrence of chitinase in saprotrophic basidiomyctes did not attract much attention. However, production of extracellular chitinases was demonstrated in non-basidiomycetous microfungi isolated from *Quercus petraea* forest soil based (Baldrian *et al.* 2011). Production of extracellular chitinases was documented from root–infecting basidiomyctes and several ectomycorrhizal fungi (Hodge *et al.* 1995). Lindhal and Finlay (2006) have shown that secondary wood colonizers can use chitinases to degradate their own cell walls or primary colonizers' chitin walls.

2.7 Identification of fungi

While filamentous fungi are identified using mainly morphological characteristics, yeasts are identified using biochemical characteristics; such is their ability to utilize carbon and nitrogen compounds. However, these methods of identification are often problematic as there can be different morpho/biotypes within a single species. They are also time consuming and require a great deal of skills.

In the last two decades, the methods used to describe the diversity of microbial communities in soils have undergone a shift from cultivation–based approaches to more comprehensive culture–independent methods. This is of critical importance since only a minor fraction of a soil microbial community can be analyzed using cultivation–dependent techniques. Most recent molecular methods are based on the analysis of nucleic acids extracted from environmental samples.

A single locus, the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene, has become widely used for near–species–level identification (Horton and Bruns 2001). This region has four primary advantages over other regions: (1) it is multicopy gene, so the amount of starting material needed for successful amplification is low; (2) it has well–conserved fungal specific priming sites directly adjacent to multiple highly variable regions; (3) there are many sequences already available for comparison, which greatly facilitates the identification of unknown samples; and (4) it correlates well with morphologically defined species in many groups (Smith *et al.* 2007).

Although the consensus value for species discrimination using ITS is usually set at 97% (Amend *et al.* 2010, Buée *et al.* 2009, O'Brien *et al.* 2005, Tedersoo *et al.* 2010), it has been

shown that the ITS intra–specific similarity varied from 99% to 76% depending on the species in question (Nilsson *et al.* 2008). The fact that ITS is a multi–copy gene can be a hindrance, when one wants to quantify amount of fungi based on ITS qPCR.

In last decade, a new gene – RPB (RNA polymerase II) is used in phylogenetic studies besides ITS. RPB as fungal taxonomical marker was first used by Cheney *et al.* (2001) to identify fungal groups inside of *Microsporidia*. Though almost entirely coding, the studied region of RPB2 is more variable than ITS. The combined analyses of ITS and variable regions of RPB1 and RPB2 greatly increase the resolution and nodal support for phylogenies of *Cortinarius* group, which are closely related species that until now have proven very difficult to resolve with the ribosomal markers. We speculate that sequence information from RNA polymerase II genes have the potential for resolving phylogenetic problems at several levels of the diverse and taxonomically very challenging genus *Cortinarius* (Frøslev *et al.* 2005). The future prospects of fungal identification are that fungi will be molecularly identified based on multiple genes common to all fungi, but still able to distingue fungi into genera or species.

3. Aims

If there is a shift in a fungal community during the succession on decomposing organic matter reflecting the changing chemical composition of the material, there should be obvious disparity between various successional stages of the decomposer community in the production of extracellular enzymes. In addition to the differences in enzyme activities, the differences in organic matter quality among soil horizons may also cause the changes in the composition of the associated fungal community due to the differences in the availability of nutrients and the ability of fungal taxa to compete for nutrients and niches.

In addition to the spatial heterogeneity of soils which reflects the development of the soil horizon during long-term decomposition of dead plant biomass, seasonal changes in nutrient availability may also play a role in the shaping of the composition of fungal communities. One can assume that there can be some changes in decomposition rates and fungal ecology during the year, which can affect fungal communities. Saprothrophic fungi decomposing organic material in the upper forest soil are likely less successful in the competition with ectomycorrhizal fungi during summer, when ECM fungi receive carbon from the host trees. In winter situation changes, the photosynthate input into the mycorrhizal fungi from trees is

cedes and the switch to the saprothrophic processes in the soil can be expected. Ectomycorrhizal fungi should be thus spread in H horizon and proliferate in summer while in winter they should not be very abundant or extend their mycelia to L horizon for fresh nutrients. On the other hand litter saprotrophic fungi can thrive equally well in summer and winter due to the reserve of organic substances in the litter if they are not outcompeted by ectomycorrhizal fungi.

Sapling sites were chosen to be as most homogenous as possible with respect to the vegetation cover, moisture, pH etc., so that large differences in the distribution of fungi across sites were not expected.

4. Materials and methods

Methodology in this thesis is composed of two parts: isolation of litter decomposing fungi from spruce needles in different stage of fungal succession and examination of fungal communities and their decomposing abilities in the soil horizons. In the first part of experiment, spruce needles representing different phases of needle decomposition were collected and fungi were cultivated from them. Abilities of fungal strains to decompose different organic substances were examined by enzyme assays. The aim of this part was to show fungal succession on spruce needles from endophytes to litter saprotrophes and find out whether fungi from different successional phases have differ in their abilities to decay litter. The second part of research followed the first one in terms of monitoring succession of fungal communities in different layers of the soil representing variously old and decayed organic matter. I studied soil fungal communities by 454–pyrosequencing approach, which allows covering huge part of soil fungal diversity without disadvantages of cultivation. The goal was to identify fungal communities based on ITS sequences and also, which part of fungal community have cellulolytic abilities (established by sequencing of partial *cbhI* gene) and whether this two communities respond to distinct soil profiles (L – litter and H – organic horizon) or to sampling seasons (winter or summer).

4.1 Degradation of spruce litter by fungi

4.1.1 Study site and sample collection

Needle samples were obtained from Norway spruce (*Picea abies*) in the National Park Šumava, Trojmezná region 48°47′N 13°52′E at 1 300 m altitude. This area is covered with Norway spruce with patches of ash (*Fraxinus excelsior*). The mean annual temperature is 5.5 °C and mean annual precipitation 1 030 mm. The bedrock is granite and the prevailing soil types in this area are mainly podzols (Kopáček *et al.* 2002). Kopáček et al. (2002) and Veselý (1994) describe basic physico–chemical and biochemical properties of the soil.

Three types of needle samples were collected into sterile plastic bags: (1) dead green needles attached to twigs on recently dead trees (wind–fallen during the preceding 6 months); (2) freshly abscised brown needles exposed for 1–month in litterbags on the soil surface; and (3) litter needles collected from the L horizon. These three kinds of samples represent three different stages of decomposition:

(1) early decomposition of needles before colonization by soil fungi;

(2) initial stages of decomposition on the soil (litter) surface;

(3) later stages of decomposition.

4.1.2 Isolation of fungi

Needles were surface–sterilized by immersion into 30 % H_2O_2 and shaked for 90 s on a horizontal shaker. Surface sterilization was used to prevent the growth of surface–associated fungi. Ten needles from each sample were placed on the sterile MEA (20 g l^{-1} malt extract, 20 g l^{-1}agar) Petri dish. The plates were cultivated at a temperature 18°C /19 °C (day/night regime). After 4 – 5 weeks of incubation, colonies outgrowing from the needles were grouped according to morphology into morphotypes. Representatives of each morphotype were transferred onto fresh MEA plates. Sixteen strains representing the dominant morphotypes of stages 1 – 3 were used in subsequent analyses (Table 2). The strains were kept on MEA at 25 °C. The increase of a maximal colony diameter during the linear phase of the colony growth on MEA plates (the radial extension rate) was determined.

4.1.3 Identification of fungal strains obtained from spruce needles

Fungal strains were identified according to a combination of macro– and microphenotype characteristics with the molecular identification. DNA was isolated from pure agar cultures

using the Microbial DNA Kit (MoBio, USA). The manufacturer`s instructions were followed. Genomic DNA was used as a template in PCR reactions using primers for the ITS and 28S rDNA regions: ITS–1F (50–CTTGGTCATTTAGAGGAAGTAA–30) and NL4 (50–GGTCCGTGTTTCAAGACGG–30) (Gardes and Bruns 1993). PCR conditions and reaction mixture were the same as stated previously (Valášková and Baldrian 2009). Each 25–µl reaction mixture contained 16.75 µl H_2O, 2.5 µl 10x buffer for DyNAzyme II DNA polymerase, 1.5 µl BSA stock solution (10mg/ml), 1 µl ITS–1F primer (250 pM/µl), 1 µl NL4 primer (250 pM/µl), 1 µl fungal genomic DNA, and 0.75 µl DyNAzyme II DNA polymerase (final concentration 2 U/µl) and 1 µl PCR Nucleotide Mix (10 mM). Program for PCR amplification reaction consisted of initial denaturation at 94 ºC, 5 min., 35 cycles (94 ºC 1min., 50 ºC 1min., 72 ºC 1min.) and a 10–min final extension at 72 ºC. PCR products were sequenced as a single extension with primers ITS–1F or NL4 by Macrogen Inc. (Korea) using an ABI 3730 XL DNA Analyzer (Applied Biosystems). Sequences were manually edited in BioEdit before they were searched in BLAST (blastn) against the nucleotide database at NCBI (http://www.ncbi.nlm.nih.gov/blast). The isolates were assigned to species based on a combination of morphological characteristics and the best blastn match higher than 98 % similarity.

4.1.4 Extracellular enzymes assays measurements

4.1.4.1 Semi–quantitative assay of enzyme production in agar cultures

To measure activities of enzymes, where substrates for individual direct assays are not commercially available, namely the esterase, esterase lipase, α–galactosidase, β–galactosidase, β–glucuronidase, α–mannosidase, α–fucosidase was used API ZYM (Bio Merieux, France), semiquantitative analysis kit. Fungal strains were cultured on MEA at 25ºC for 14 days. After the incubation, 1 cm^2 of agar with about 7–days–old mycelium was homogenized with a mortar and pestle and supplemented with 2 ml of distilled H_2O. 65 µl of the resulting suspension were then delivered into the API ZYM cupules and incubated at 37 ºC for 4 hours as described in the manufacturer's instructions with slight modifications (Baldrian *et al.* 2011). One drop of each ZYM A and B were added to the cupules. The color reactions were read after 5 min of incubation and compared with the color code provided by the manufacturer. The results were recognized as 0 (0 nanomoles substrate hydrolyzed), 1 (5

nanomoles substrate hydrolyzed), 2 (10 nanomoles substrate hydrolyzed), 3 (20 nanomoles substrate hydrolyzed), 4 (30 nanomoles substrate hydrolyzed), 5 (40 nanomoles substrate hydrolyzed).

4.1.4.2 Production of extracellular enzymes in agar cultures

Enzymes were extracted from fungal cultures, which were growing on MEA medium at 10 ºC (the mean summer temperature at the site of isolation) in the dark, as described previously (Baldrian *et al.* 2011). Five 0.7 cm diameter plugs were cut off from 7–days–old mycelium plates, cut into small pieces and mixed with 50 mM sodium acetate buffer, pH 5.0 (3 ml cm^{-2}), and extracted for 2 hr at 4 ºC with constant mixing. Filtered extracts were used for analyses.

4.1.4.3 Production of extracellular enzymes on spruce needles

To measure enzyme activities in spruce needles colonized by fungi, isolates were cultured on MEA plates at 10 ºC in the dark. Two weeks after full colonization of a plate, when the most easily available nutrients were probably utilized, 10 spruce needles were added to each plate. Those freshly fallen needles were collected on soil surface in late autumn, air–dried and sterilized by gamma–irradiation. The needles were placed directly onto the fungal mycelia. After 14 and 35 days, 10 needles from 2 or 3 separate plates were removed and used for enzyme extraction. After 49 days, needles from plates of each strain were combined into three replicates (each from 2 or 3 separate plates) of 10 needles each, dried at 85 ºC until the constant mass and used for calculation of litter mass loss.

Needles for enzyme activity assays were homogenize with UltraTurrax (IKA Labortechnik, Germany) for 3 min at 8 000 rev min^{-1} in 10 ml of cold 50 mM sodium acetate buffer, pH 5.0. The homogenate was used as a sample in the enzyme assay.

4.1.4.4 Enzyme activity measurements

Activities of cellobiohydrolase (exoglucanase), β–glucosidase, β–xylosidase, α–glucosidase, N – acetylglucosaminidase, phosphatase, phosphodiesterase, arylsulphatase, alanine and leucine aminopeptidases were assayed by direct incubation of the needle homogenate with 4–

methylumbelliferone (MUF) or 7–amino–4–methylcoumarin (AMC)–based substrates based on (Vepsäläinen 2001) MUF–cellobioside, MUF–β–glucosidase, MUF–β–xylosidase, MUF–α–glucoside, MUF–N–acetylglucosaminide, MUF–phosphate, MUF–diphosphate, MUF–sulfate, L–alanine–AMC, and L–leucine–AMC, repectively. Fluorescence of the released reaction products was measured as previously described (Baldrian 2009b). The final concentration of fluorescence substrates in each of the 96 wells in 96 multi–well plate was 500 μM (100 μl of substrate in DMSO). Three technical replicates of 100 μl needle homogenate per well, were performed. For the background fluorescence measurement, 100 μl of 50 mM sodium acetate buffer, pH 5.0 were combined with 100 μl of 4–methylumbelliferone or 7–aminomethyl–4–coumarin standards to correct the results for fluorescence quenching. The multi–well plates were incubated at 40 °C, and fluorescence was recorded after 5 min and 125 min using the Infinite microplate reader (TECAN, Austria), using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The quantitative enzymatic activities after blank subtraction were calculated based on standard curves of MUF and AMC.

Laccase activity was measured by monitoring the oxidation of 2,2´–azinobis–3–ethylbenzothiazoline–6–sulfonic acid (ABTS) in 100 mM citrate – 200 mM phosphate buffer (pH 5.0) at 420 nm (Bourbonnais and Paice 1990). Manganese peroxidase (MnP) was assayed in succinate–lactate buffer (100 mM, pH 4.5). MBTH (3–methyl–2–benzothiazolinone hydrazone) and DMAB (3,3–dimethylaminobenzoic acid) were oxidatively coupled by the enzyme, and the resulting purple indamine dye was detected spectrophotometrically at 595 nm (Ngo and Lenhoff 1980). The results were corrected by the activities of the samples without manganese – the addition of manganese sulphate was substituted by an equimolar amount of ethylenediaminetetraacetate (EDTA). One unit of enzyme activity was defined as the amount of enzyme forming 1 nmol reaction product per min (1 U = 16.67×10^{-9} katal). Specifics of solutions composition and enzyme measurements are in section 8 Buffers and solutions.

4.1.5 Statistical analyses of enzymatic data

Statistical tests were conducted using the software package Statistica 7 (StatSoft, USA). Statistical significance of differences among groups of strains based on their occurrence during needle decomposition or their taxonomic placement (*Ascomycota* versus

Basidiomycota) was evaluated using one–way ANOVA. Pearson's correlations were calculated to find the relationships among enzyme activities and litter mass loss. Principal component analysis on enzyme production data was also used to discriminate these groups. Neither of these analyses showed differences between groups of strains at $p \leq 0.05$.

4.1.6 DNA isolation from fungal cultures

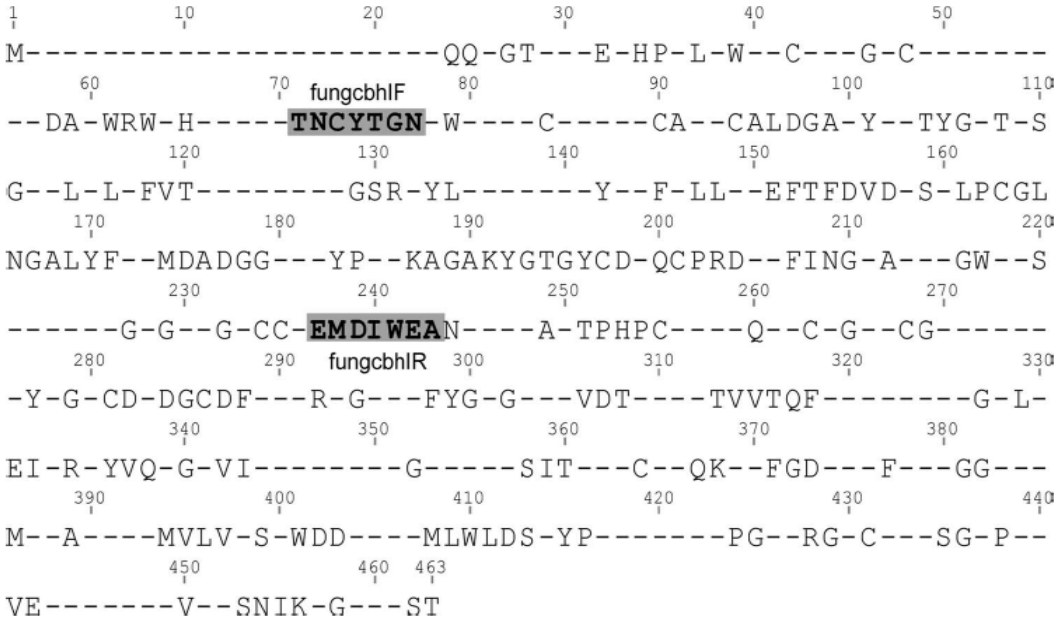
DNA was isolated from pure fungal cultures to obtain *cbhI* sequences by modified method from Sagova–Marečková *et al.* (2008). Mycelium from a MEA plate was carefully removed and placed into a sterile microtube with 400 µl of extraction buffer (50 mM NaH_2PO_4 (pH 8), 50 mM NaCl, 500mM Tris–HCl (pH 8), 5% SDS), 100 µl of pH 7.5 phenol and 100 µl of chloroform/isoamyl alcohol (24:1). Glass beads (0.25 g of 0.1 mm and 0.25g of 0.5 mm) were added and cell lysis was performed in Mini–BeadBeater–16 (Biospect product, USA) for 10 sec at speed 40. Homogenates were centrifuged for 3 minutes at 18 000 g and supernatants was transferred to sterile microtubes. The samples were extracted with phenol + chloroform/isoamylalcohol (1x the volume of supernatant). The mixtures were vortexed and centrifuged for 5 minutes at 6000 rpm. Supernatants were extracted with 1 volume of chloroform/isoamylalcohol (24:1). Again, the mixtures were vortexed and centrifuged for 5 minutes at 6000 rpm. 6M NaCl (1/3 volume of supernatant) and 10 % (w/v) CTAB in 0.7 M NaCl (1/10 volume of supernatant) were added to the supernatants. The mixtures were stirred and incubated at 65ºC for at least 30 min and then cooled down to laboratory temperature. Chloroform/isoamylalcohol (24:1) (1x the volume of mixture) was added to the mixtures, which were than shaken and centrifuged for 20 minutes at 4500 rpm. The supernatants were precipitated for 20 minutes with isopropanol (3/5 volume of supernatant) and Na–acetate (3M, pH 4.8, 1/10 volume of supernatant) at laboratory temperature. The mixtures were centrifuged for 20 minutes at 18 000 g. Liquid parts of the mixture was removed from the microtubes and sediments were washed with 200 µl of cold 70% ethanol. The sediments were centrifuged for 20 minutes at 18 000 g. Ethanol was discarded from tubes. The sediment (isolated DNA) was dried for 4 minutes using SpeedVac (Savant, USA) and dissolved in 50 µl of deionised water. Subsequently, DNA was purified by Geneclean Turbo Kit (Mpbio, USA) following manufacturer`s instructions. Concentration and quality of DNA was routinely measured using Nanodrop Spectrophotometer ND–100 (Microarray Core Facility) at 260 nm and 230 nm.

4.1.7 PCR amplification of fungal *cbhI* genes

To obtain the partial sequences of the fungal *cbhI* genes, PCR amplifications were performed on isolated and cleaned fungal DNA in the thermocyclers GenePro (Bioer, China). Degenerate primers fungcbhIF (5`–ACC AA[C,T] TGC TA[C,T] ACI [A,G]G[C,T] AA–3`) and fungcbhIR (5`– GC[C,T] TCC CAI AT[A,G] TCC ATC–3`) (Edwards *et al.* 2008, Figure 2) were used for amplification. Each 25–µl reaction mixture contained 16.75 µl H_2O, 2.5 µl 10x buffer for DyNAzyme II DNA polymerase, 1.5 µl BSA stock solution (10mg/ml), 1 µl fungcbhIF primer (250 pM/µl), 1 µl fungcbhIR primer (250 pM/µl), 1 µl fungal genomic DNA, and 0.75 µl DyNAzyme II DNA polymerase (final concentration 2 U/µl) and 1 µl PCR Nucleotide Mix (10 mM).

Program for PCR amplification reaction consisted of initial denaturation at 94 ºC, 3 min, 35 cycles (94 ºC 30 sec, 49 ºC 45 sec, 72 ºC 90 sec) and a 10–min final extension at 72 ºC.

Figure 2: Position of *cbhI* primers in the CBH amino acid sequence based on the consensus of 40 fungal amino acid sequences (adapted from Edwards *et al.*. 2008)



4.1.8 DNA electrophoresis

Products of PCR were visualized on agarose gel electrophoresis. Agarose gel (1.5%) was prepared from 0.75 g of agarose and 50 ml of 1 x TAE buffer. 50x TAE stock solution buffer was made of 100 ml of 0.5 EDTA (pH 8.0), 57.1 ml of ice acetic acid, 242 g Tris. Solid chemicals were dissolved in deionised water and pH was adjusted with acetic acid to 7.8. Water was added to final volume of 1 liter. Agarose with TAE buffer was boiled until agarose was completely dissolved and after cooling down it was poured into the electrophoretic device. 10 µl of 1% ethidium bromide was added before gel solidification. After placing a comb into tray with a gel, it was left to cool down. The solid gel was placed into electrophoretic tank with 1 x TAE buffer. 5 µl of PCR sample with added 1 µl of 6x DNA Loading Dye was loaded on gel. O'GeneRuler™ 100 bp Plus DNA Ladder (Fermentas, Estonia, 2 µl) was used as a size marker. Electrophoresis ran at 90 V for 40 min and was viewed under UV in transilluminator and photographed. The images were examined for DNA band size and quality.

4.1.9 Cloning of PCR products of *cbhI* gene amplification

CbhI gene clone libraries were constructed from PCR products. Two 50 µl PCR reactions were combined and purified using Wizard SV Gel and PCR Clean Up System (Promega, USA) and Mini Elute Purification Kit (Qiagen, USA)according to the manufacturer's instructions. The PCR products were subsequently ligated into pJET1.2/blunt cloning vector according to the manufacturer's instructions for CloneJET^{TM} PCR Cloning Kit (Fermentas, Estonia). The ligation product was purified with the equal amount of chloroform 24:1 isoamylacohol and transformed into electrocompetent cells – *Escherichia coli*, strain DH5α by electroporation. To 50 µl of thawed competent *E. coli* cells 1 µl of each ligation reaction was added and the tubes with mixtures of cells and ligation reaction products were left on ice. The mixtures were transferred into a cold electroporation cuvette. The cuvette was placed into the electroporator GenePulser XC (BioRad, USA) and pulsed once. After removing the cuvette from electroporator, 500 µl of 2x TY medium (1.6 g/l peptone, 1 g/l yeast extract, 0.5 g/l NaCl, pH 7) was added. The cells were resuspended with pipette and transferred to 1.5 ml tube and then shaken for 1 hour at 37°C. The cell suspension was poured out on 2x TY plates with ampicillin (0.5 g/l NaCl, 1 g/l yeast extract, 1.6 g/l peptone, pH was adjusted to 7 using NaOH, 0.1 g/l ampicillin, 2 g/l agar) and incubated at 37ºC overnight. The colonies containing an insert were able to grow on the above medium. When

the colonies grew, they were picked with a sterile toothpick and placed onto another agar plate with the selection medium. After incubation overnight at 37ºC, colonies were transferred into 1.5 ml tubes and resuspended in 300 µl of sterile deionized water. The suspensions were first incubated at 95ºC for 10 minutes and then cooled down on ice and for the second time incubated at 95ºC for 10 minutes. The cell lysate was centrifugated for 10 seconds and the supernatant was used as a template for colony PCR.

Alternatively, PCR products were ligated into the pGEM–T Easy Vector using pGEM®–T and pGEM®–T Easy Vector Systems (Promega), following the manufacturers' instructions. The ligation products were transformed into *E. coli* JM109 High Efficiency Competent Cells (Promega) by heat shock. 2 µl of each ligation reaction were added to 50 µl of thawed competent cells and tubes were left on ice for 20 minutes. Heat shock was performed for 45 seconds at 42ºC. Tubes were left on ice for 2 minutes. After that, 950 µl of SOC medium were added and cells in the medium were incubated and shaken for 1.5 hours at 37°C. The mixture was plated onto LB/ampicillin/IPTG/X–Gal plates (composition described below) and incubated overnight at 37°C. The successful transformants were crossed onto new LB/ampicillin/IPTG/X–Gal plates and incubated overnight at 37°C. After the incubation, colonies were transferred into 1.5 ml ependorf tubes and resuspended in 300 µl of sterile deionized water. Resuspended colonies were incubated at 95ºC for 10 minutes and then cooled down on ice and again incubated at 95ºC for 10 minutes. The cell lysate was centrifugated for 10 seconds and the supernatant was used as template for colony PCR for each sample. Colony PCR was performed for each sample as described below.

For both pGEM–T Easy Vector and pJET1.2/blunt cloning vectors, the used molar ratio of insert: vector was 3:1. Preparation of LB/ampicillin/IPTG/X–Gal plates and used buffers and solutions are listed in section 8 Buffers and solutions.

Colony PCR was performed using the primer pairs pJET2.1forward: 5' d(CGACTCACTATAGGGAGAGCGGC) 3' and pJET2.1reverse: 5' d(AAGAACATCGATTTTCCATGGCAG) 3' or 5′–d(TCACACAGGAAACAGCTATGAC)–3′ or, alternatively, using the pUC/M13 Forward Primer: 5′–d(GTTTTCCCAGTCACGAC)–3′ or the 5′–d(CGCCAGGGTTTTCCCAGTCACGAC)–3′ pUC/M13 Reverse Primer: 5′–d(CAGGAAACAGCTATGAC)–3′.

Each 50–µl reaction mixture contained 40 µl H_2O, 5 µl 10x buffer for DyNAzyme II DNA polymerase, 1 µl pJET2.1forward or pUC/M13 Forward primer (250 pM/µl), 1 µl pJET2.1reverse or pUC/M13 Reverse primer (250 pM/µl), 1 µl template DNA, and 1 µl

DyNAzyme II DNA polymerase (final concentration 2 U/µl) and 1 µl PCR Nucleotide Mix (10 mM).
Program for PCR amplification reaction consisted of initial denaturation at 94 ºC, 3 min, 35 cycles (94 ºC 30 sec, 60 ºC 30 sec, 72 ºC 105 sec) and a 10–min final extension at 72 ºC. All special chemicals used in methods are listed below in section 8.1.

4.1.10 Sequencing and sequence analysis of the *cbhI* genes

Approximately 40 µl of colony PCR amplified product was sent for sequencing to an external facility (Macrogen Inc., Korea). The *cbhI* fragments were sequenced by extension from pUC/M13 or pJET2.1 primers. DNA was sequenced under BigDyeTM terminator cycling conditions, using an automatic sequencer 3730xl (Macrogen Inc., Korea). *CbhI* sequences of individual clones were manually edited using the program BioEdit Version 7.0.0. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using the program Mafft online (Asimenos and Toh 2009). Sequences of clones from one organism showing similarity >98% were replaced in SeaView Version 4.3.2. (http://pbil.univ–lyon1.fr/software/seaview.html) with a consensus sequence representing one isoform of the enzyme.
Representative sequence of each isoenzyme was submitted to GenBank database. The sequences were late used to construct phylogenetic tree together with *cbhI* sequences obtained by 454–pyrosequencing.

4.2 Characterization of soil fungal community by 454 pyrosequencing

Aims here were to identify and characterize total and cellulolytic fungal community in terms of spatial and seasonal distribution. Soil samples were collected in winter and summer from three sampling sites and composite samples of L and H horizon separately were made. Metagenomes obtained were pyrosequenced and fungal species identified by PlutoF and Galaxy-454 pipeline. Cellulolytic OTUs were identified according to phylogenetic trees.

4.2.1 Study site and sample collection

Soil and litter samples were isolated from Šumava National Park near the summit of the Přilba (48°59.01 N, 13°35.05 E). Study area was located in the highest altitudes (1170–1200 m) of the Bohemian Forest mountain range (Central Europe) and was covered by an unmanaged spruce (*Picea abies*) forest. Soil and litter samples were taken on the 27.9.2010 (summer, S) and on the 23.3.2011 from three sites (winter, W), located 250 m from each other. Six topsoil samples located around the circumference of a 4–m–diameter circle were collected for each season. Litter horizon (L) and organic (organic) horizon (H) material were separately pooled. After removal of roots, L material was cut into 0.5 cm pieces and mixed; H material was passed through a 5 mm sterile mesh and mixed. Aliquots for nucleic acids extraction were immediately frozen and stored in liquid nitrogen. Dry mass content was measured after drying at 85 °C, organic matter content after burning at 650 °C and pH was measured in distilled water (1:10). Soil C and N content was measured using an elemental analyzer.

4.2.2 DNA isolation

4.2.2.1 Extraction and purification of DNA from soil samples

DNA was extracted in triplicate 0.300 g aliquots of each sample using the same method as was describe above in section 4.1.6. DNA was cleaned by Geneclean Turbo Kit (MPBio, USA). 1M HEPES/ 1M $CaCl_2$ solution (pH 7) was added prior to cleaning procedure, sample was left standing for 5 minutes and then Geneclean Turbo Kit manufacturer's instructions were followed.

4.2.2.2 PCR amplification of target gene sequences

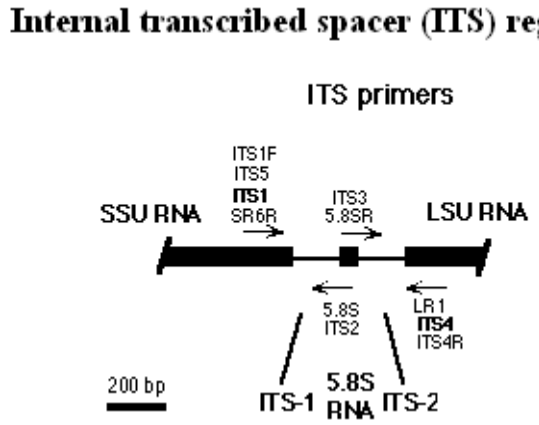
PCR reactions were performed independently for each extracted DNA sample. The volume of each PCR sample was 50 µl. PCR primers ITS1 5`(TCCGTAGGTGAACCTGCGG)3` / ITS4 5`(TCCTCCGCTTATTGATATGC)3` (White *et al.* 1990, Figure 3) were used to amplify the ITS region of fungal rDNA for fungal community analysis and fungcbhIF/ fungcbhIF (see 3.1.7) were used for the amplification of partial *cbhI* gene sequences.
Each 50–µl reaction mixture contained 45.5 µl H_2O, 5 µl 10x buffer for DyNAzyme II DNA polymerase, 3 µl of 10 mg/ml BSA, 2 µl forward primer (final concentration 10 pmol/µl), 2

µl reverse primer (final concentration 10 pmol/µl), 1 µl template DNA, and 1.5 µl 4% Pfu polymerase / DyNAzyme DNA Polymerase (final concentration 2 U/µl) and 1 µl PCR Nucleotide Mix (10 mM).

Program for PCR amplification reaction of fungal ITS consisted of initial denaturation at 94 ºC, 5 min, 40 cycles (94 ºC 30 sec, 51 ºC 45 sec, 72 ºC 90 sec) and a 15–min final extension at 72 ºC. For *cbhI* amplification, the program consisted of initial denaturation at 94 ºC, 5 min, 35 cycles (94 ºC 60 sec, 55 ºC 60 sec, 72 ºC 60 sec) and a 10–min final extension at 72 ºC.

PCR products were collected and cleaned by Wizard SV Gel and PCR Clean–Up System (Promega, USA).

Figure 3: Position of ITS1 and ITS4 primers in DNA coding for ribosomal genes (adapted from http://www.biology.duke.edu/fungi/mycolab/primers.htm)



4.2.2.3 Quantification of DNA by fluorescent dye binding

DNA concentration in purified PCR product was using the Quant–iT™ PicoGreen ds DNA kit (Invitrogen, USA). Each sample and standard was measured in triplicates on 96–well microplate. 100 µl of standard/sample and 100 µl of 200x diluted PicoGreen in 1x TE buffer were combined in each well. The concentrations of standards were 1 ng/µl, 0.001 ng/µl, 0.025 ng/µl, 0,00025 ng/µl and 0 ng/µl. Plate was incubated at room temperature for 2 minutes and fluorescently on Infinite microplate reader (TECAN, Austria). The excitation and emission wavelengths were 480 nm and 520 nm, respectively.

4.2.2.4 Ligation of tagged adaptors

The tagging of individual samples (PCR products obtained from the same sample) was performed using the Roche MID adaptors and GS Junior Rapid Library Preparation Kit, according to Rapid Library Preparation method Manual (Roche, USA). For each sample, 500 ng of PCR product was purified using Wizard SV Gel and PCR Clean–Up System (Promega, USA), eluted in 25 μl of water and the elute was cleaned again using the MinElute PCR Purification kit (Qiagen, USA) as recommended by the Rapid Library Preparation method Manual. First, ends of PCR products were repaired and blunted. Then, pyrosequencing adaptors carrying different MID adaptors (tags) were blunt end–ligated to each sample. Ligation products were treated with AMPure beads to remove small fragments, the resulting samples being eluted in 25 μl of TE buffer. 7 μl of the PCR product with ligated MID adaptor were analyzed electrophoreticaly (prepared as described above in section 4.1.8) to check the length and quantity of the product.

4.2.2.5 Emulsion PCR

In this approach, an oil–water emulsion is formed in which the aqueous phase contains the PCR reagents and the DNA template to be sequenced. Capture beads containing one of the oligonucleotide primers attached to them are also included. This oligonucleotide is complementary to one of the adaptor sequences used in library construction. The other PCR primer is present in the solution. After controlled and vigorous agitation of the oil–water system, emulsification takes place and millions of aqueous droplets are formed, within which PCR amplifications take place. Optimization of the concentration of DNA template, beads, and water droplets guarantees that only one bead carrying one template occurs in each droplet. Then, millions of copies of a unique DNA template are generated on each bead in a clonal PCR amplification (Siqueira *et al.*2012).

Prior to emulsion PCR copy number of designated gene was quantified by Kapa Library Quantification kit (KapaBiosystems, USA) following the manufacturers instruction. An emulsion PCR was prepared according manufacturer instructions (emPCR Amplification Method Manual–Lib–L) for GS Junior Titanium Series sequencing instrument. In the first step, AMP primer was ligated to a PCR product with MID adaptor, facilitating DNA capture bead attachment. Than emulsion PCR was performed to amplify attached molecule of DNA on the DNA capture bead. The PCR program consisted of initial denaturation at 94 ºC, 4 min and 50 amplification cycles (94 ºC 30 sec, 58 ºC 270 sec, 68 ºC 30 sec).

DNA capture beads were then transferred into 50ml Falcon tube and washed several times with the enhancing buffer, isopropanol, and ethanol. Sediment of beads was transferred into clean microtube and Melt solution was added. Melt solution contained NaOH and caused separation of DNA strands. Enrich primer was than ligated on free end of DNA attached to DNA capture bead. Through Enrich primer, Enrich magnetic bead was bound to the free end of DNA. Such modified beads were then washed several times with Enhancing buffer to remove poorly attached or unattached DNA capture beads. DNA capture beads were released from Enrich beads by Melt solution. Supernatant containing the enriched DNA capture beads was transferred to new tube and Seq primer was added.

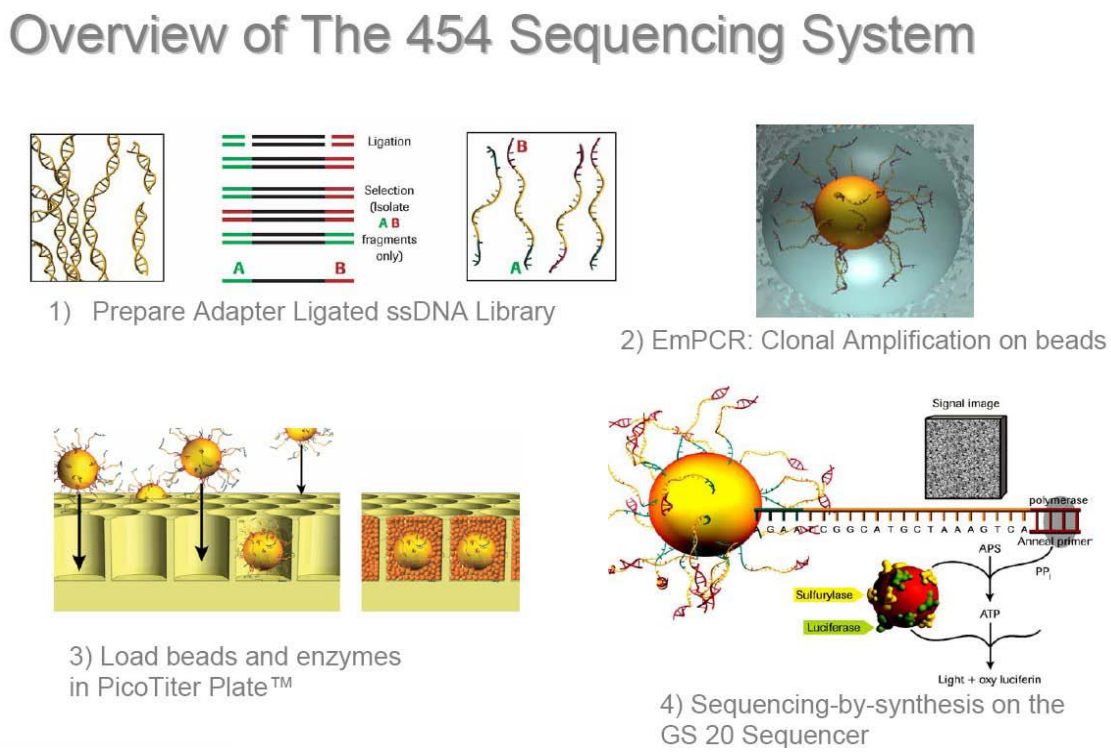
4.2.2.6 454 pyrosequencing – sequencing step

Prior to sequencing, the emulsion is broken, the DNA is denatured, and beads carrying single–stranded DNA are transferred to the wells of a picotiter plate in such a way that it permits a single bead to occur in each of the several hundred thousand wells. Because each bead has a fixed location in the plate, each sequencing reaction can be monitored. Beads containing the enzymes used in the pyrosequencing reaction steps are then deposited into each well. The pyrosequencing reaction takes place using a mixture of the single–stranded DNA template, the sequencing primer, and the enzymes DNA polymerase, ATP sulfurylase, luciferase, PPiase and apyrase (Figure 4). Two substrates are also included in the reaction – adenosine 5‘phosphosulfate (APS) and luciferin. The first one of the four deoxynucleotides (dNTPs) is added to the sequencing reaction, and the DNA polymerase catalyzes its incorporation into the DNA strand, in case there is a complementarity. During each incorporation event, a phosphodiester bond between the dNTPs is formed, releasing pyrophosphate (PPi) in a quantity equivalent to the amount of incorporated nucleotide. In sequence, the enzyme ATP sulfurylase converts PPi to ATP in the presence of APS. ATP is used in the conversion of luciferin to oxyluciferin mediated by the enzyme luciferase. This gives rise to light in intensity that is proportional to the amount of ATP used. Light is detected by a charge coupled device camera and detected as a peak in a pyrogram. The height of each peak is proportional to the number of nucleotides incorporated. The system is regenerated with apyrase that degrades ATP and unincorporated dNTPs and with PPiase enzyme in the buffer, which degradate pyrophosphate. Then, the next dNTP is added. Addition of dNTPs is performed one at a time. Generation of a signal indicates which nucleotide is the next one occurring in the sequence. As the process goes on, the

complementary DNA strand grows and the nucleotide sequence is determined according to the signal peaks in the program (Siqueira *et al.* 2012).

Sequencing itself was performed according to the manufacturer instructions (Sequencing Method Manual, Roche, USA) for GS Junior Titanium Series sequencing instrument. There are four layers of different kinds of beads loaded onto PicoTiterPlate. First layer is called Enzyme Beads Pre–layer and is the same as 3rd layer called Enzyme Beads Post–layer. The only difference is in the ratio of BB2 buffer and Enzyme Beads. I suppose that those two layers ensure proximity of enzymes like polymerase, bound on their surface, to DNA capture beads. The second layer between enzyme plies is DNA captured bead ply with Packing Beads and Control Beads XLTF added. The fourth layer is composed of PPiase Beads that contains beads with fixed PPiase enzyme. All plies are deposited on PicoTiterPlate centrifuging Bead Deposition Device with PicoTiterPlate inside.

Figure 4: An overview of the 454–pyroseqencing workflow (adapted from http://cage.unl.edu/454%20Introduction.pdf)



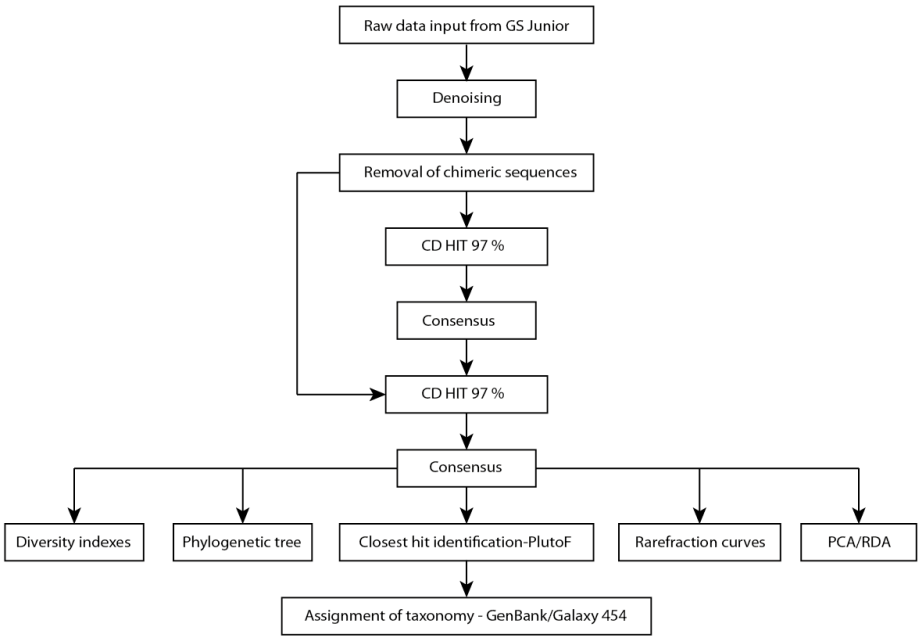
4.2.3 Analyses of 454–pyrosequencing data

ITS sequences obtained by pyrosequencing were processed as shown on the

Figure 5 below. Only the sequences beginning with the primer ITS1 were processed. After obtaining sequences from 454–pyrosequencing, ITS flowgrams were quality–filtered, separated into individual samples and pyrosequencing noise (method–dependent inaccuracies) were corrected using the Denoiser program (Reeder and Knight 2010). Chimeric sequences were detected using UCHIME (Edgar 2010) and deleted.

In the fungal community analyses, ITS sequences shorter than 380 bases were removed and the remaining sequences were truncated to 380 bases, which contained the ITS1 region, 5.8 S rDNA and a significant part of the ITS2 region. These sequences were clustered using CD–HIT (Li and Godzik 2006) at a 97% similarity threshold (O'Brien *et al.* 2005) to yield Operational Taxonomic Units (OTUs). Consensus sequences were constructed for each OTU using automated alignment tool. ITS sequences submitted to first CD HIT were combined into one file with consensus sequences, which served as seeds in the second CD HIT. Second CD HIT–est yielded final clusters, which were used for all subsequent analysis. PlutoF pipeline (Tedersoo *et al.* 2010) was used to identify best species hits in the GenBank (Benson *et al.* 2005) and UNITE databases (Abarenkov *et al.* 2010). Taxonomic identifications of the best species hits were retrieved from GenBank using the Galaxy 454 pipeline (https://galaxy.jgi–psf.org/).

Figure 5: Processing of ITS sequences

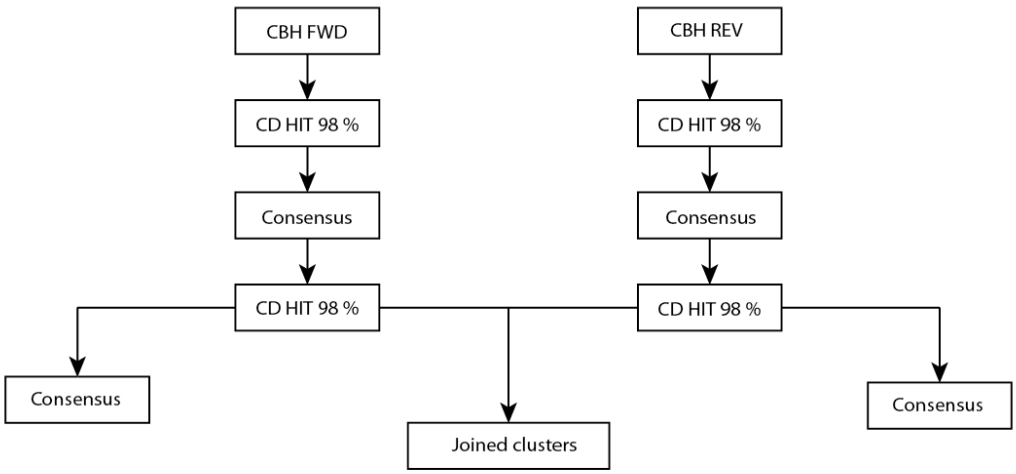


CbhI sequences were also quality–filtered, separated into individual samples and pyrosequencing noise was corrected using the Denoiser program (Reeder and Knight 2010). Chimeric sequences were detected using UCHIME (Edgar 2010) and deleted.

CbhI sequences shorter than 380 bases were removed and the remaining sequences were truncated to 380 bases (Figure 6). These sequences were clustered using CD–HIT (Li and Godzik 2006) at a 98% to yield OTUs. Consensus sequences were constructed for each OTU using automated alignment tool. *CbhI* sequences submitted to first CD HIT were combined into one file with consensus sequences, which served as seeds in the second CD HIT. This step yielded final clusters, which were used for all subsequent analyses (Figure 6).

Once consensus sequences were created for both reverse and forward sequences, the forward and reverse consensus sequences of all clusters with more than 3 members were merged based on the internal 200–bp overlap. From joined sequences consensus sequences were created and they were clustered by CD–HIT on 100% similarity level.

Figure 6: Processing of *cbhI* sequences



First 56 most abundant *cbhI* merged sequences were used for phylogenetic tree construction. Alignment was constructed using Mafft online (Asimenos and Toh 2009), intron positions were recorded, and introns were removed in BioEdit (Hall 1999). For identification, *cbhI* sequences were retrieved from GenBank Release 188.0 as well as from the fungal genomes available at the Joint Genome Institute. Additional sequences obtained from fungal isolates (see 4.1.3) were used as well. All nucleotide sequences except pseudogenes were translated into amino acid sequences.

Rarefaction and diversity analyses on OTUs/clusters were performed on 700 fungal and 267 *cbhI* sequences per sample, to eliminate the effect of sampling depth. Richness non–parametric Chao1 estimates were calculated using online tool Rarefaction Calculator (http://www.biology.ualberta.ca/jbrzusto/rarefact.php#ChaoEstimator).

Shannon–Wiener index and Evenness were counted using Shannon–Wiener Diversity Index / Shannon Entropy Calculator (http://www.changbioscience.com/genetics/shannon.html).

Statistical tests were performed using the software package Statistica 7 (StatSoft, USA). Statistical significance of differences in the abundance of fungal taxa among treatments was evaluated using one–way ANOVA with the Fisher LSD *post–hoc* test. Differences at $p \leq 0.05$ were regarded as statistically significant. The analysis of differences in the composition of total fungal communities, PCA on log transformed data was performed (Statistica 9.0, Statsoft, USA) on genera and subsequently on orders with relative abundance over 20 = relative abundance in percentage over 0.16%. To show differences between treatments (litter/humus and summer/winter) in the abundance of fungal taxa, CCA analysis in the program CANOCO (Braak 1990) was performed. CCA is a multiple regression for all species simultaneously with linear constraints on the regression coefficients. Principally, CCA is weighted PCA, where explanatory variables are linear combination of weighted environmental variables. T–value plots with the log data on genera, orders were created to visualize the effects of the season and the forest floor horizon. T–value biplots represent diagrams approximating a table of T–value statistics, each one corresponding to a simple regression model with one explanatory variable and one response (species). Species significantly associated with respective treatment in a T–value diagram are plotted inside the Van Dobben circles. Species with the positive correlation are in the red circle and those with negative one are plotted in the blue circle.

Non–metric multidimensional scaling (NMDS) on relative abundances of fungal genera was performed in the program Past (Hammer *et al.* 2001). For ITS, Euclidian distance measurement was chosen. STAMP (Statistical Analysis of Metagenomic Profiles) package (Parks and Beiko 2010) was used to analyze the significant differences in the ITS data among seasons and horizons within individual sampling sites. This approach was used to reduce the nonrandom effects of sites on the fungal community identified in the previous analyses (PCA, RDA). Differences among samples were examined using Fisher's exact two–sided test on datasets randomly resampled by the program.

Litter/humus abundance ratios for individual OTUs or fungal taxa were calculated as the sums of their relative abundances in the litter divided by the sum of relative abundances

among all sequences, and summer/winter ratio was calculated similarly. In the case of 2D graphs, the x coordinate was winter/summer ration, y coordinate was humus/litter ration and an area of circle is an mean relative abundance of the OTU.

Phylogenetic trees for 5.8S rDNA sequences of genera with abundance over 0.16% and for first 56 most abundant *cbhI* OTUs were constructed after aligning the sequences in the program Mafft online with default settings (Asimenos and Toh 2009). Alignments were treated by G-block (http://www.phylogeny.fr) to remove gaps from alignments. Aligned sequences were subjected to model testing using jModelTest 0.1.1 (Guindon and Gascuel 2003, Posada 2008). Evolution model for *CbhI* protein sequences was estimated using Prottest with default settings (http://darwin.uvigo.es/software/prottest.html). The best model for *cbhI* and 5.8S nucleotide sequences was K80+I and for *CbhI* protein sequences it was Dayhoff+G. BioNJ tree was constructed for *cbhI* nucleotide and protein sequences from 454–pyrosequencing data combined with the sequences available in the GenBank, in fungal genomes published on the Joint Genome Institute website and sequences obtained from fungal isolates in this study. Trees were bootstrapped 1000 times in the BioNJ tool on webpage http://www.phylogeny.fr with the Kimura 2–parameters substitution matrix (Dereeper *et al.* 2008) for *cbhI* nucleotide sequences and with Dayhoff matrix for *CbhI* protein sequences. The same procedure as was performed for *cbhI* nucleotide sequences was done for ITS sequences from metagenome, GenBank database sequences .

5. Results

5.1 Degradation of spruce litter by fungi

More than 50 morphotypes of microscopic fungi were isolated from *Picea abies* needles. Sixteen of the most frequently occurring ones were identified based on the rDNA region sequencing combined with macro- and micromorphology examinations (Table 2).

Table 2: Fungal strains isolated from *Picea abies* needles – identification. Abbreviations: (A) *Ascomycota,* (B) *Basidiomycota*.

Strain	GI No. of ITS	Identification	Division	Order	Source	Ecology *1	Reference
35	FR717226	*Fomitopsis pinicola (B)*	*Basidiomycota*	*Polyporales*	fallen tree	saprotroph, parasite	Sinclair and Lyon 2005
61	FR717228	*Peniophora sp. (B)*	*Basidiomycota*	*Russulales*	fallen tree	saprotroph, parasite	Dix and Webster 1995

17	FR715995	*Phacidiopycnis sp. (A)*	*Ascomycota*	*Helotiales*	fallen tree	saprotroph, parasite	DiCosmo *et al.* 1984
19	FR715996	*Phacidiopycnis sp. (A)*	*Ascomycota*	*Helotiales*	fallen tree	saprotroph, parasite	DiCosmo *et al.* 1984
26	FR715997	*Sirococcus sp. (A)*	*Ascomycota*	*Diaporthales*	fallen tree	parasite	Smith *et al.* 2003
148	FR667860	*Cistella acuum (A)*	*Ascomycota*	*Helotiales*	fallen tree, litterbag	saprotroph	Gremmen 1960
151	FR717232	*Cylindrocarpon magnusianum (A)*	*Ascomycota*	*Hypocreales*	fallen tree, litterbag	Saprotroph, parasite	Domsch *et al.* 2007
47	FR717230	*Chalara longipes agg. (A)*	*Ascomycota*	*Helotiales*	fallen tree, litterbag	saprotroph	Holubová-Jechová 1984
50	FR717231	*Chalara longipes agg. (A)*	*Ascomycota*	*Helotiales*	fallen tree, litterbag	saprotroph	Holubová-Jechová 1984
205	FR717225	*Ceuthospora pinastri (A)*	*Ascomycota*	*Helotiales*	litterbag	saprotroph	Gremmen 1960
194	FR717223	*Hormonema dematioides (A)*	*Ascomycota*	*Dothideales*	litterbag	Saprotroph, parasite	Gremmen 1960
190	FR717224	*Thysanophora penicillioides (A)*	*Ascomycota*	*Eurotiales*	litterbag	saprotroph	Iwamoto *et al.* 2005
200	FR822984	*Trichoderma sp. (A)*	*Ascomycota*	*Hypocreales*	litterbag	saprotroph, parasite	Domsch *et al.* 2007
24	FR717227	*Marasmius androsaceus (B)*	*Basidiomycota*	*Agaricales*	litter L horizon	saprotroph	Holmer and Stenlid 1991
85	FR717229	*Mycena galopus (B)*	*Basidiomycota*	*Agaricales*	litter L horizon	saprotroph	Frankland *et al.* 1995
22	FR822985	*Hormonema dematioides (A)*	*Ascomycota*	*Dothideales*	litter L horizon	saprotroph, parasite	Gremmen 1960

*1 Ecology of a particular species is derived from the current study and literature (see Reference).

Among the *Ascomycota*, the members of the order *Helotiales* were most frequently isolated with six morphotypes belonging to four genera *Ceuthospora, Chalara*, *Cistella* and *Phacidiopycnis*. By two morphologically distinct isolates were represented *Chalara longipes agg. (Helotiales), Phacidiopycnis sp. (Helotiales)* and *Hormonema dematioides (Dothideales)*, the remaining genera were represented by a single species. Among *basidiomycota*, two strains of the recognized wood decomposers *Fomitopsis pinicola* and *Peniophora sp*. were recorded from needles collected from a fallen tree while the typical litter-decomposers *Marasmius androsaceus* and *Mycena galopus* were found in needles collected from the litter horizon. The radial extension rate of all strains was relatively fast, at 50 - 508 μm hr^{-1} (Table 3).While the two *Chalara longipes agg*. isolates and the *Trichoderma sp.* were slow growers, the growth rate of *basidiomycota* was generally high,

especially that of *F. pinicola* and *Peniophora sp.* All but six strains (including all *basidiomycota*) were able to produce cellobiohydrolase (exocellulase) on MEA, a complex, nutrient-rich medium, although the production differed widely; highest activities were detected in *Sirococcus conigenus, M. androsaceus* and *Cylindrocarpon magnusianum* (

Figure 8). All isolates were able to produce β-glucosidase but the production by both strains of *H. dematioides* was very low. In general, strains with high cellobiohydrolase activity also exhibited high β-glucosidase activity ($p \leq 0.01$). Enzymes decomposing hemicelluloses were produced by all tested strains, but the production of individual enzymes differed widely (Table 3).

Table 3: Properties of isolated fungal strains. Oxidation of phenols was tested with ABTS as a substrate, activity of hydrolytic enzymes was assessed using the API ZYM test and radial growth rates were measured on MEA plates. The +/- indicates the presence or absence of enzyme activity, the values 0 – 5 are visual estimates of enzyme activity from zero (0) and low (1) to high (5). See 3.1.3.1 for detail, see section 3.1.3.1.

Identification	Fungal strain	Laccase (ABTS)	Alkaline phosphatase	Esterase	Esterase Lipase	Leucine arylaminidase	Valine arylaminidase	Acid phosphatase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	N-acetylglucosaminidase	α-Mannosidase	α-Fucosidase	Radial extension rate (µm h-1)
Fomitopsis pinicola (B)	35	+	1	1	0	0	0	5	1	2	4	0	4	4	0	0	508
Peniophora sp. (B)	61	+	1	2	1	3	2	5	1	3	0	3	4	4	4	5	469
Phacidiopycnis sp. (A)	17	+	2	2	1	2	0	5	1	2	0	4	5	0	1	0	294
Phacidiopycnis sp. (A)	19	+	1	1	0	1	0	5	1	1	0	4	3	0	1	0	286
Sirococcus sp. (A)	26	+	0	1	1	1	0	5	0	1	2	4	5	5	1	1	83
Cistella acuum (A)	148	+	5	1	2	3	0	5	2	1	0	2	5	1	4	0	58
Cylindrocarpon magnusianum (A)	151	+	1	2	2	0	0	3	0	0	0	1	4	5	0	0	253
Chalara longipes agg. (A)	47	+	2	1	3	3	0	5	0	0	0	4	4	0	2	0	50
Chalara longipes agg. (A)	50	+	3	1	1	4	0	5	1	2	0	0	5	5	3	0	53
Ceuthospora pinastri (A)	205	-	1	1	1	0	0	5	3	5	0	1	4	0	1	0	372
Hormonema dematioides (A)	194	-	1	2	1	3	0	5	0	1	0	2	3	1	0	0	132
Thysanophora penicillioides (A)	190	-	5	1	0	0	0	3	2	0	0	0	4	4	3	3	197
Trichoderma sp. (A)	200	+	1	1	1	2	0	5	0	1	0	5	5	4	5	0	55

Marasmius androsaceus (B)	24	+	0	2	1	1	0	5	3	2	1	4	4	5	0	1	153
Mycena galopus (B)	85	+	1	3	2	3	2	5	2	2	3	2	3	4	1	0	408
Hormonema dematioides (A)	22	+	3	2	1	3	0	5	0	0	0	0	5	0	1	0	283

When grown on MEA, all strains except *H. dematioides* 22 and *C. longipes agg*. 47 produced β-xylosidase (

Figure 8). The activities were usually substantially lower (5-10 x) than those of β-glucosidase but *F. pinicola, M. galopus* and *H. dematioides* 194 produced more
β-xylosidase than β-glucosidase. β-Galactosidase and α-amannosidase were produced by 12 strains each, α-galactosidase by 10 strains, α-fucosidase and β-glucuronidase by four strains (Table 3). 12 strains produced N-acetylglucosaminidase, often with high activities comparable to β-glucosidase (

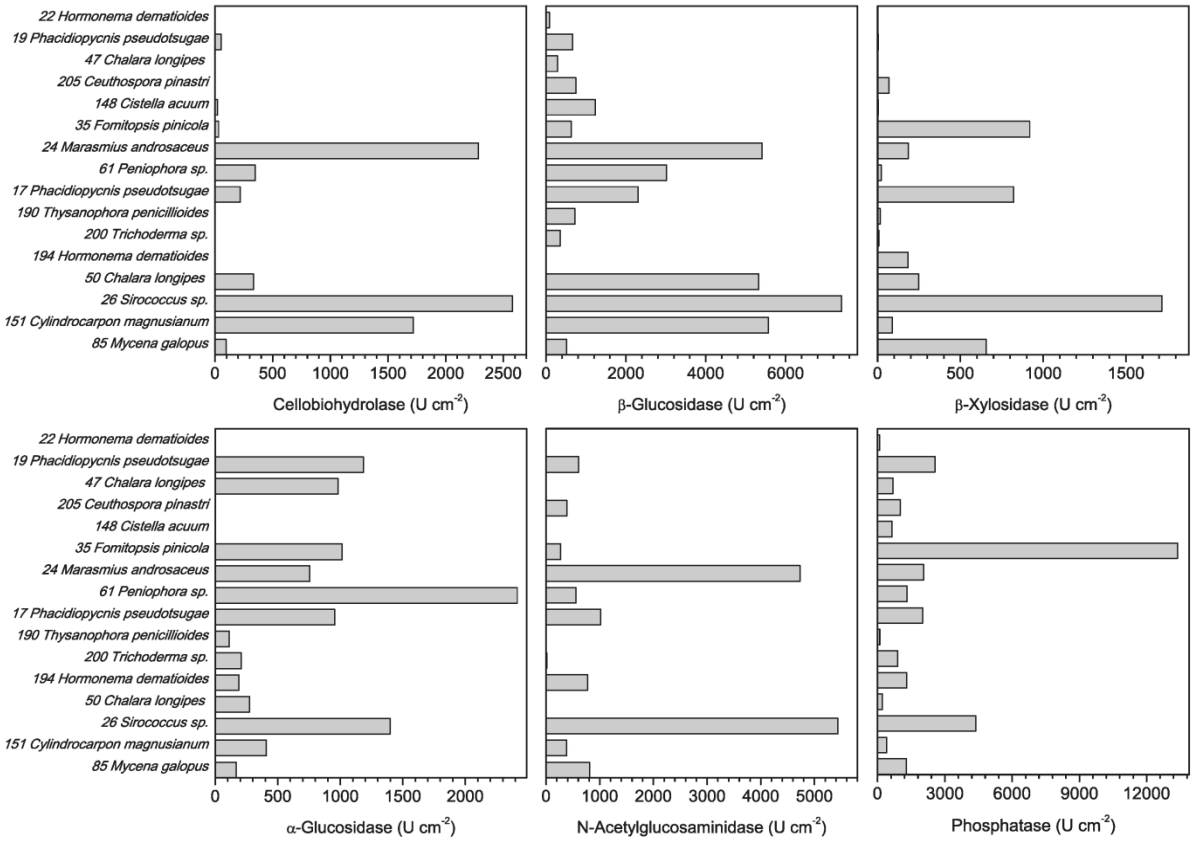
Figure 8). Among the other enzymes tested, phosphatase and esterase or lipase was produced by all 16 strains, phosphodiesterase by 13, leucine arylaminidase by 12, and α-glucosidase (amylase) by 11 strains. Only very low activities of arylsulfatase were produced by four strains, and valine arylaminidase by two strains; alanine and leucine aminopeptidases were not produced on MEA. Two species of *Basidiomycota* exhibited highly complex composition of their extracellular enzymatic systems: all enzymes except β-glucuronidase were produced by *Peniophora sp*. and all except α-fucosidase by *M. galopus*.

All fungal isolates showed rapid colonization of spruce needles with complete mycelial overgrowth before 14 d. Decomposition was estimated at 49 d since further mass loss was very slow for all strains (data not shown).

Significant decomposition of *P. abies* needles was recorded for all fungal isolates but the rates of dry mass loss varied greatly among fungi. Highest decomposition of 61 ± 9 % was recorded in the case of *H. dematioides* 22 and the

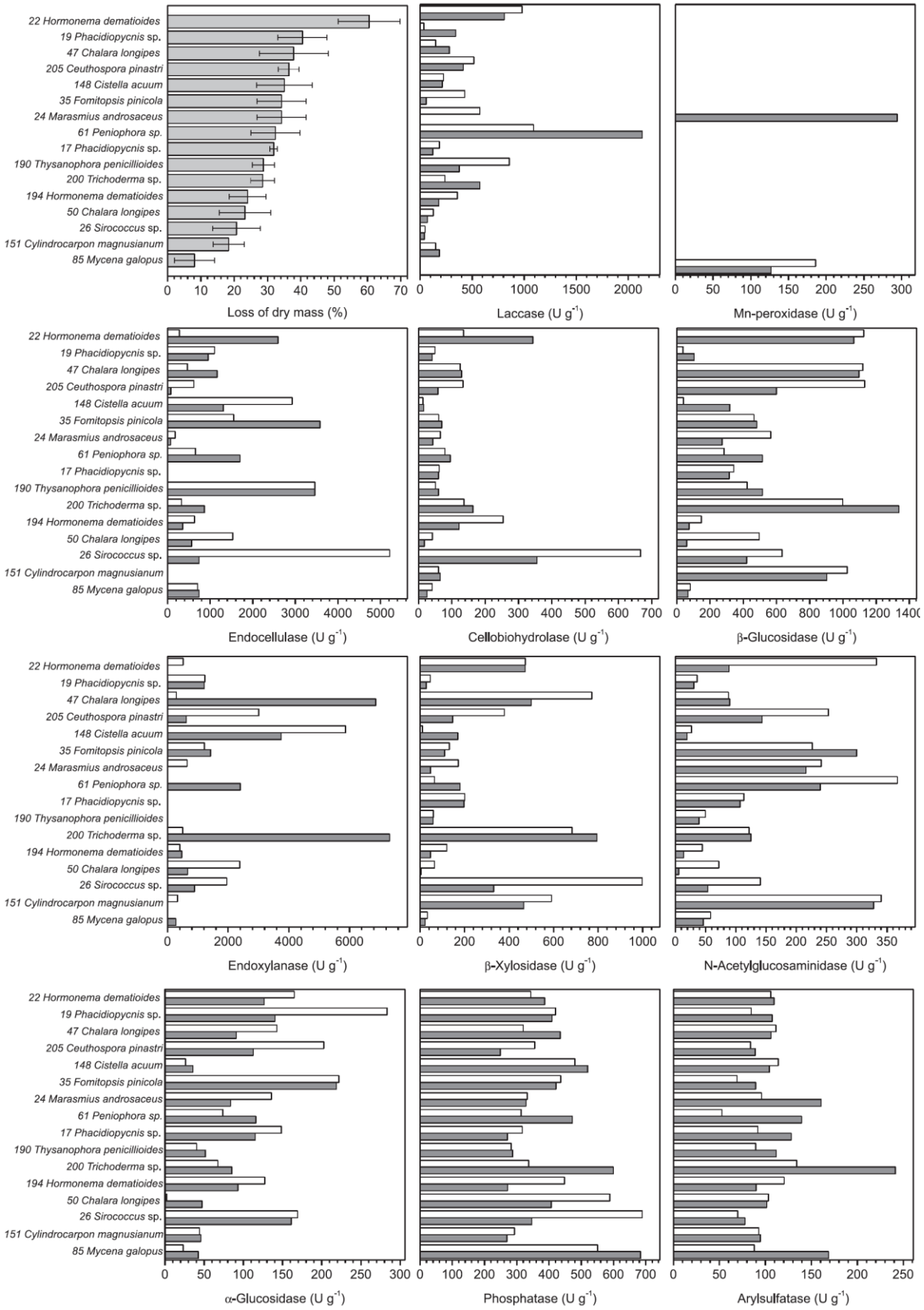
lowest one in *M. galopus* 8 ± 6 %. The rest of fungi exhibited mass losses between 18 % and 41 % (Figure 8).

Figure 7: Activity of extracellular enzymes in the cultures of fungal isolates from *Picea abies* needles on malt extract agar. Data represent means from three replicates; standard errors of the mean did not exceed 20 %.



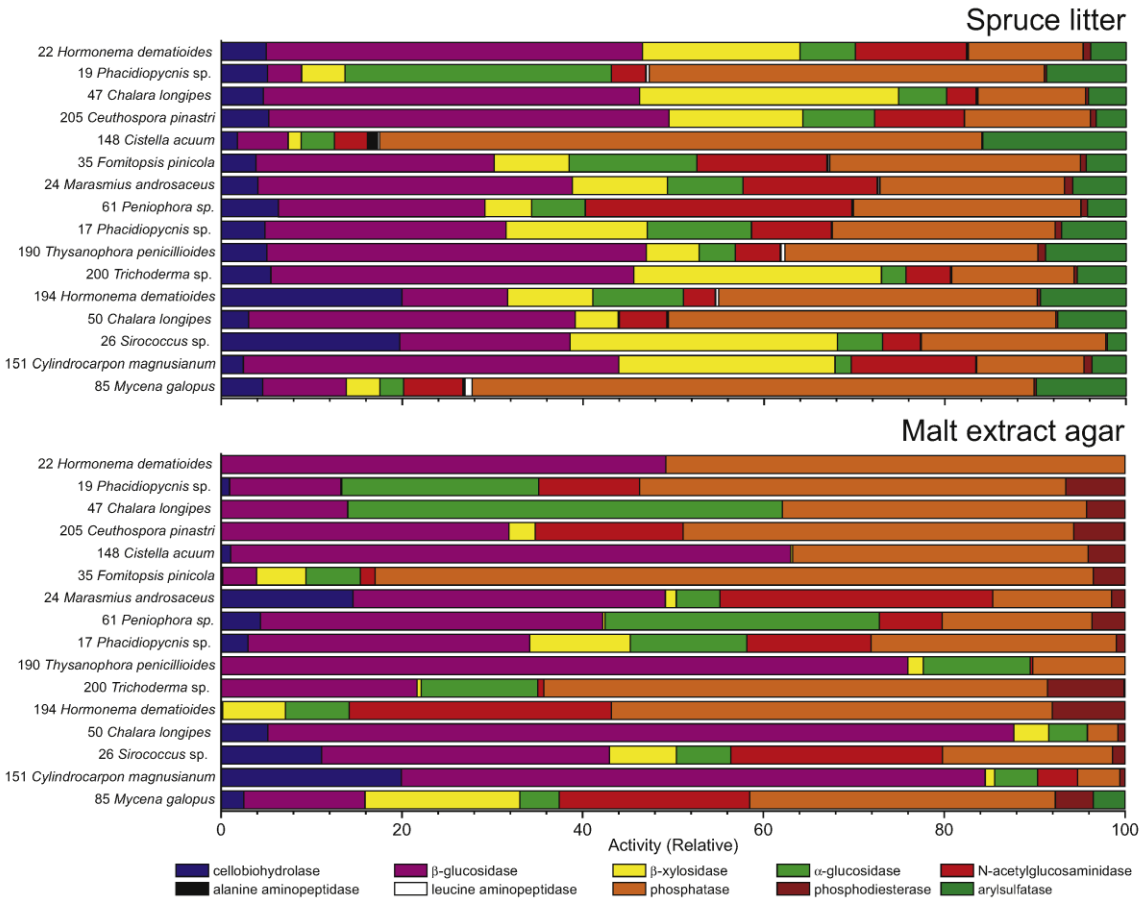
Enzyme production on the natural substratum of the studied fungi - sterile *P. abies* needles was substantially different from that on MEA (Figure 9). All fungi produced a more complete set of hydrolytic enzymes on needles. Enzyme production reflected the composition of the polysaccharidic substrate and thus the fungi produced on average higher relative activities of β-xylosidase and cellobiohydrolase on needles than on MEA; compared to β-glucosidase, relative production of both enzymes increased dramatically. On the other hand, activity of α-glucosidase was much higher on MEA. Alanine- and leucine aminopeptidases that were not detectable on MEA were both produced by 14 fungal strains on the needles, although at low and variable quantities; the activity of arylsulfatase was also highly increased on spruce needles compared to MEA (Figure 9).

Figure 8: Decomposition of sterile *Picea abies* litter after 49 d incubation with fungal isolates and activity of extracellular enzymes in the early stage (14 d, blue bars) and late stage (35 d, red bars) of decomposition. Bars represent standard errors of the mean (SE) from three replicates; for enzyme measurements, SE did not exceed 20 %.



Across the enzymes, activities measured on MEA showed significant ($p \leq 0.05$) correlation to those measured in needles only for cellobiohydrolase. In the agar plate test, all but three isolates exhibited phenol oxidation when assayed using ABTS (Table 3). In spruce needles, despite great variation, laccase activity was detected in all strains except *M. galopus*. This fungus and *M. androsaceus* also produced Mn-peroxidase. Among cellulolytic enzymes, cellobiohydrolase and β-glucosidase were produced by all strains while endocellulase was not detected in one of the two *Phacidiopycnis sp*. isolates and in *C. magnusianum*. Enzyme activities varied among the fungi with *Sirococcus sp.* showing the highest activities of both endocellulase and cellobiohydrolase. High activity of cellobiohydrolase, the processive enzyme decomposing cellulose, was also detected in both of the isolates of *H. dematioides*. Endoxylanase production was highly variable among fungi and also with time, but only *Phacidiopycnis sp*. 17 and *Thysanophora penicillioides* did not produce the enzyme at all. Activities of endocellulase, cellobiohydrolase, β-glucosidase and β-xylosidase, the enzymes acting on plant cell wall polysaccharides, showed significant correlations ($p \leq 0.05$) with each other across the fungal isolates.

Figure 9: Relative activity of hydrolytic enzymes of fungal isolates from *Picea abies* needles on needle litter (35 d) and on malt extract agar.



Activity of the other two tested polysaccharide hydrolases, N-acetylglucosaminidase and α-glucosidase varied among species but was relatively constant in time. All fungi showed relatively high and comparable production of phosphatase and arylsulfatase showing the importance of these enzymes for their growth on needles. When grouped according to their isolation source into colonizers of attached needles (present in green needles on fallen trees before contact with soil) and litter-decomposers (fungi isolated from litterbags or litter horizon material only), there was no significant difference in the production of any of the enzymes tested among these groups neither considering individual enzymes separately, nor using PCA analysis of data from all enzymes together. Also the differences in enzyme activities between *Ascomycota* and *Basidiomycota* were insignificant with the exception of Mn-peroxidase that was only found in *basidiomycota*. When the two strains causing the highest and lowest decomposition of needles were compared, *H. dematioides* 22 showed high activities of all cellulose and hemicellulose-decomposing enzymes except for endoxylanase while activities of all of these enzymes were relatively low in *M. galopus*, the slow decomposer.

5.2 Detection and sequencing of *cbhI* genes in fungal strains

In total 48 partial *cbhI* gene sequences were obtained from 3 *Basidiomycota*, 16 *Ascomycota* and 1 member of the *Mucoromycotina*. The fungi *Sirococcus conigenus*, *Phacidiopicnis sp.*, *Chalara longipes, Mycena galopus, Mucor hiemalis, Cylindrocarpon magnusianum, Thysanophora penicillioides, Hormonema dematioides, Trichoderma pleuroticola, Ceuthospora pinastri* were isolated from spruce needles in the catchment of the Plešné Lake, the other fungi have been obtained from culture collections as species occurring in the litter or soils from the Šumava National Park and identified using Stable Isotope Probing to incorporate cellulose-derived carbon from cellulose by Štrusová *et al.* (2012). Two *Russula* species were obtained and isolated from fungal sporocarps by T. Větrovský from NP Šumava. The overview of all species is in

Table 4. Some fungi have synonymous names used in the text below: *Phialophora malorum = Cadophora malorum, Phialophora luteo-olivacea = Cadophora luteo-olivacea, Sydowia polyspora = Hormonema dematioides.*

One to four genes were found in the studied fungi. Sequences with <98% similarity were considered genes isoforms of which most could be translated into amino acids without internal stop codons. Genes with stop codon in sequence after removing introns were considered pseudogenes. Pseudogenes were: gi 382929308 *Chloridium virescens*, gi 325152929 and gi 325152880, *Russula emetica*, gi 382929304 and gi382929305 *Mycena galopus*. The gi numbers of *cbhI* sequences deposited in GenBank are in the Table 4 **.**

Table 4: List of fungal species with *cbhI* genes and GenBank gi numbers of partial *cbhI* sequences.

Collection number*	**Fungal species cloned**	***cbhI* isoforms count**	**GI numbers of *CbhI* sequences in GenBank**			
	Basidiomycota					
Isolated by T.Větrovský	*Russula paludosa*	4	325152901	343129685	351735595	325152927
Isolated by T.Větrovský	*Russula emetica*	4	351735615	351735591	325152880	325152929
ZK85/08	*Mycena galopus*	3	351735593	382929304	382929305	
	Ascomycota					
CCF3784	*Cadophora malorum*	4	325152923	325152889	325152905	325152913
AK47/92	*Phialophora sp.*	4	325152878	325152917	310914183	310914176
ZK194/08	*Hormonema dematioides*	3	351735603	351735601	351735607	
ZK207/08	*Ceuthospora pinastri*	2	325152876	325152891		
CCF1782, CCF1787	*Chloridium virescencs*	3	325152870	351735613	382929308	

CCF3795	*Cadophora luteo-olivacea*	3	325152864	325152921	325152911	
AK289/05	*Hypocrea lixii*	2	351735619	325152887		
ZK50/08, ZK47/08	*Chalara longipes*	2	325152885	310914188		
CCF3045	*Leptodontium elatius*	1	325152868			
CCF3093	*Phialophora cinerescens*	2	325152925	325152903		
ZK26/08	*Sirococcus sp.*	2	351735621	351735605		
ZK151/08	*Neonectria ramulariae*	1	325152915			
CCF3410	*Oidiodendron cerealis*	1	325152866			
ZK19/08	*Phacidiopicnis sp.*	2	351735597	325152881		
ZK195/08	*Trichoderma pleuroticola*	1	351735599			
ZK190/08	*Tysanophora penicilloides*	1	325152897			
	Mucoromycotina					
ZK199/08	*Mucor hiemalis*	1	351735589			

* CCF - strains obtained from the Culture Collection of Fungi, Department of Botany, Charles University, Prague, Czech Republic, AK - fungal strains from personal collection of Alena Kubátová, ZK - fungal strains from personal collection of Zuzana Kolářová.

Sequences without introns, which were not pseudogenes, exhibited 50% to 90% similarity in the identity matrix produced in BioEdit 7.0.0. Some *cbhI* sequences were more similar between species than within the isoforms of the same species. *C. luteo-olivacea* had intra-species similarity of 60% while it was more similar to gi 325152905 of *Cadophora malorum* and gi 325152913 of *Cadophora malorum* (>90%) and to gi 325152876 of *Ceuthospora pinastri* (>80%). In addition, *C. malorum* had intra-species similarity of 60% and was similar to gi 325152868 of *Leptodontidium elatius* over 90% and to gi 325152876 of *Ceuthospora pinastri* and gi 351735619 of *Hypocrea lixii* over 80%. The two isoforms of *C. pinastri* were similar to each other 63.7% but gi 325152891 of *Ceuthospora pinastri* was similar over 90% to gi 325152889 of *Cadophora malorum*, gi 325152868 of *Leptodontidium elatius*, gi 351735589 of *Mucor hiemalis* and gi 325152897 of *Thysanophora penicillioides*. The second isoform gi 325152876 of *Ceuthospora pinastri* is identical on 100% with gi 351735619 of *Hypocrea lixii*. A sequence of gi 351735613 of *Chloridium virescens* is similar to other gi 325152870 of *Chloridium virescens* on 72% but is similar to gi 310914183 of *Phialophora* sp. on 99%. *CbhI* isoform of gi 351735593 of *Mycena galopus* was similar to gi 382929304 of *Mycena galopus* on 81% and to pseudogene gi 382929305 of *Mycena galopus* on 28 – 30% respectively, but showed 98% similarity with gi 325152929 of *Russula emetica*. Another example of inter-species similarity is isoform gi 325152903 *Phialophora cinerescens*, which is identical with gi 325152925 of *Phialophora cinerescens* on 64% but on 99% with gi 351735619 of *Hypocrea lixii* and gi 325152876 of *Ceuthospora pinastri*. Also two of three *cbhI* gene isoforms of gi 351735603 of *Sydowia polyspora* and gi 351735601 of

Sydowia polyspora were similar to gi 351735605 of *Sirococcus conigenus* over 90%. Other very similar genes were: gi 325152897 of *Thysanophora penicillioides* and gi 325152889 of *Cadophora malorum* and gi 325152868 of *Leptodontidium elatius* on 99%., gi 351735599 of *Trichoderma pleuroticola* exhibited 93% sequence similarity with gi 325152887 of *Hypocrea lixii* on 93%.

Product of *cbhI* PCR of requested size was obtained from *Acephala sp., Filobasidiella depauperata, Fomitopsis pinicola, Geomyces pannorum, Hymenoscyphus sp., Hypocrea viridescens, Marasmius androsaceus, Peniphora sp., Rhizoscyphus ericae, Scleroconidioma sphagnicola, Trichosporon pullulans* but I did not succeed to clone it. Neither of these species have any record of cellobiohydrolase I in Genbank, except *Geomyces pannorum*, which has a cellobiohydrolase-like gene.

5.3 Analysis of the *cbhI* gene pool in spruce forest floor

The main aim here was to describe distribution and richness of cellulolytic fungi in spruce forest soil. The questions asked here, were who are the members of cellulolytic community in spruce forest, whether some cellulolytic fungi have preferences for a one of examined soil horizons and whether their community is influenceable by seasonal changes.

5.3.1 Diversity of cbhI defined OTUs in spruce forest litter and soil

Pyrosequencing yielded the total of 8444 *cbhI* sequences of sufficient quality and length > 380 bp. There were 1276 sequences from LS, 3180 HS, 1900 LW and 2088 HW. Thus, diversity from L horizon in the summer can be underestimated. In one horizon and season were occurring 65% of clusters – mainly singletons: 205 in HS, 168 in HW, 213 in LW and 141 in LS. Sequences from 22 clusters were found ubiquitously across horizons and seasons.

Since the estimates of diversity depend on sampling effort, dataset was resampled at the same depth of 270 sequences per sample for the construction of rarefaction curves and calculation of diversity indices. Based on the rarefaction analysis (Figure 10), none of the samples reached plateau indicating insufficient sampling depth to obtain all sequences in the samples; the samples HW3 and HS3 appeared to be the less diverse in the dataset. This is in agreement with the Chao1-based prediction of diversity that was also smallest for samples HS3 and

HW3 (Table 5). Low number of singletons in HS3 further supports this conclusion. According to Fischer's exact test Chao1 estimated the highest number of OTUs in LW (p =

0.006), which contained high proportion of singletons. 39 ± 5.6 OTUs were needed to cover 80% of fungal diversity in LW, while only 26 ± 4.5 OTUs in LS. In average 33 ± 8 OTUs are needed to cover 80% of fungal diversity in the L horizon, but this value is only 19 ± 0.4 OTUs in the H horizon. There is an insignificantly higher estimated diversity in the L horizon than H because there are more singletons of sequences isolated from the litter.

Evenness index from the Table 5. indicates that species were distributed evenly through the population of cellulolytic fungi and there were no extremely dominant species. However, there were differences in the ratio of singletons to number of all clusters among samples.

Figure 10 Rarefaction curves of *cbhI* sequences resampled to the same sampling depth of 270 sequences.

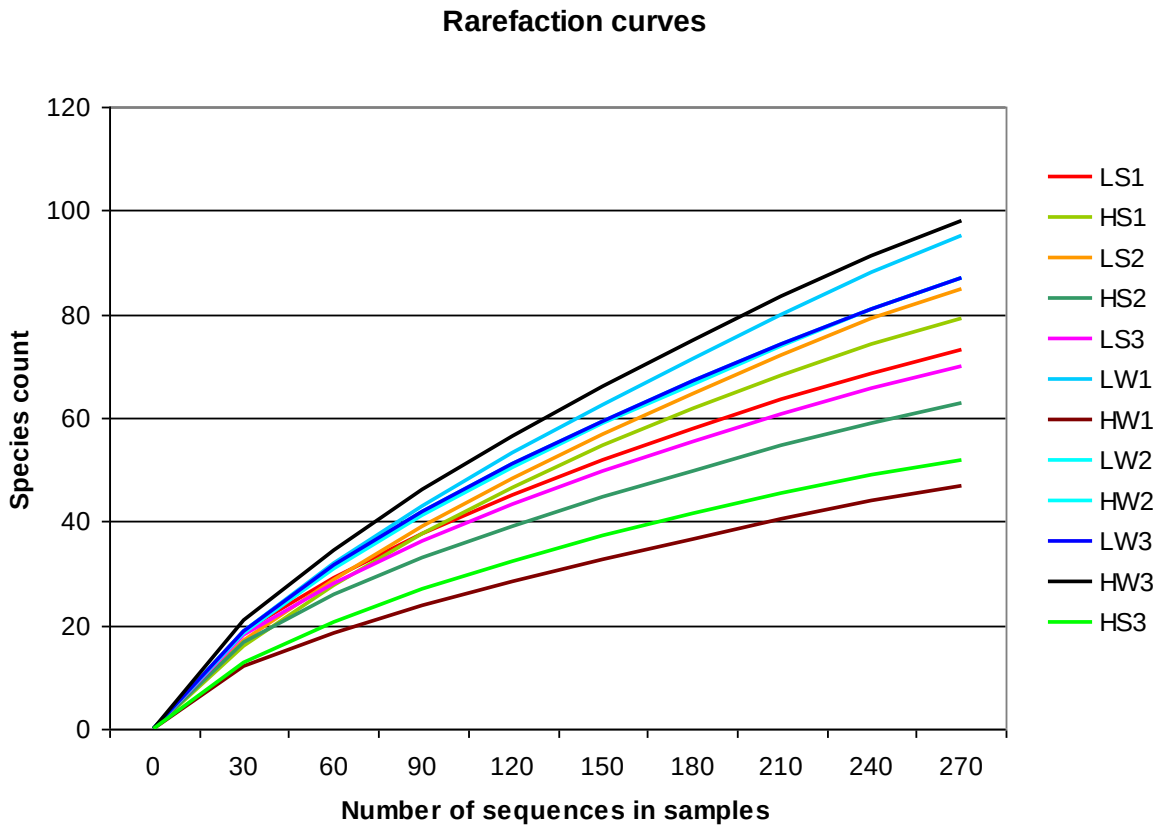


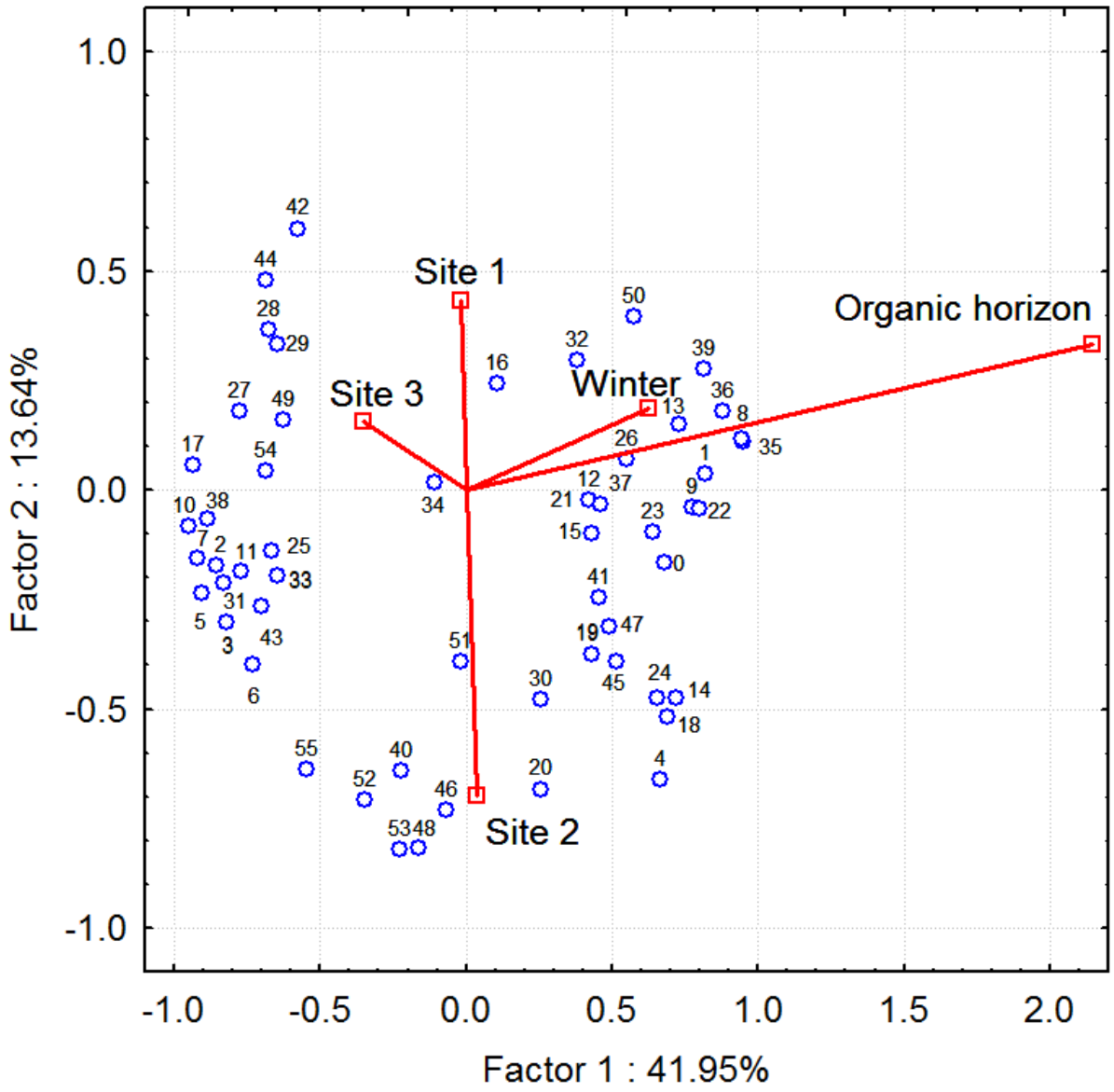
Table 5: Diversity estimates of *cbhI* gene pools in individual samples. The pyrosequencing-derived dataset of sequences was resampled at 270 sequences per sample.

Sample	Chao1	Chao1 SD	Shannon-Wiener	Evenness	Number of OTUs covering 80% of diversity	Number of observed OTUs
LS1	153	26	3.52	0.82	25	73
HS1	144	20	3.17	0.72	28	79
LS2	193	30	3.36	0.76	31	85
HS2	166	35	3.25	0.79	17	63
LS3	146	25	3.43	0.81	22	70
HS3	88	14	2.56	0.65	12	52
LW1	273	45	3.61	0.79	41	95
HW1	137	34	2.54	0.66	9	47
LW2	261	47	3.55	0.8	33	87
HW2	235	40	3.58	0.8	33	87
LW3	239	36	3.89	0.85	44	98
HW3	149	31	2.99	0.73	17	61

5.3.2 Statistical and multivariate analyses of cbhI defined OTUs distribution among environmental variables

Multivariate analyses were performed on the log transformed relative abundances of the 56 most abundant *cbhI* sequences that exhibited sufficient abundance in the dataset and thus non-random distribution among samples. ANOVA analysis with a Fischer's post-hoc test showed that 26 OTUs were significantly associated with particular horizon (0, 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 13, 14, 17, 22, 23, 26, 28, 31, 35, 36, 38, 39, 43, 54, 55), five with sampling site (16, 34, 41, 44, 53) and none with season. In the PCA analysis, the first two canonical axes explained 55.6% of total variability (Figure 11). OTUs were clearly divided by soil horizon into humus and litter horizons. Site 2 had the second biggest effect on dividing the cellulolytic community while the season had the smallest effect on the gene pool composition. In PCA asco – and basidiomycetes OTUs were not separated by soil horizon according their taxonomical classification but rather were mixed together.

Figure 11: PCA of 56 *cbhI* clusters showing dependence of OTUs on season, horizon and sampling sites.



When the same data were analyzed in statistical and imaging program Canoco, DCA analysis with detrending by segments on log-transformed sequence abundance data was performed first. Short gradients in data were observed, so linear analysis was used to describe cellulolytic community. On the second run CCA was performed. It is unimodal method, but when analyzing data with short gradient it behaves as linear method. When testing statistical significance of all canonical axes, they had p value ≤ 0.02. Under Monte-Carlo unrestricted permutations with variables Site, as supplementary variables, environmental variable Organic horizon was statistically significant at $p \leq 0.04$ and explained 0.43% of variability in data. The second tested variable Winter explained 0.087% of dataset variability and was not statistically significant at 5% level ($p \leq 0.24$). The total variance explained by horizon and

season together was 0.52% and the residual variance explained by supplementary variables was 0.22%.

Figure 12 represents the results of the CCA analysis as a triplot of species data, environmental variables and supplementary variables. If the red arrow marked Organic horizon is prolonged to other site of the same site, it divides species into to 2 sections in the graph in terms of dependency on the soil horizon. On the right side are genes which were more abundant in the H horizon, on the left side are those more abundant in the L horizon and in the middle are those distributed ubiquitously in both horizons. There was only a small effect of seasons on the cellulolytic community. Grey arrows represent supplementary factors, which were not included in the analysis. The direction of the arrows show the tendency of growing influence of factors if they were included in the analysis. If Sites will be included into analysis it would be impossible to separate effect of winter season and Site 2 since they have the same direction of influence. Figure 12 is divided into 4 quadrants, which correspond to sampling conditions. OTUs from different quadrants suppose to prefer different conditions. OTUs in the quadrant marked HS should prefer H horizon in summer, HW – H horizon in winter and analogically the same principle can be applied to LS and LW. Nevertheless, on the others Figure 13 and Figure 14 can be seen that this preference is no absolute and neither significant for all OTUs.

Figure 12: Triplot of environmental, supplementary and species data for 56 OTUs based on *cbhI* gene. Grey arrows represent supplementary variables, which are sampling sites – Site 1, 2, 3, which do not influence distribution of species in the graph. Triangles with names represent fungal species. Circles with marks HS (organic horizon summer), HW (organic horizon winter), LS (litter horizon summer), LW (litter horizon winter) represents sampling design.

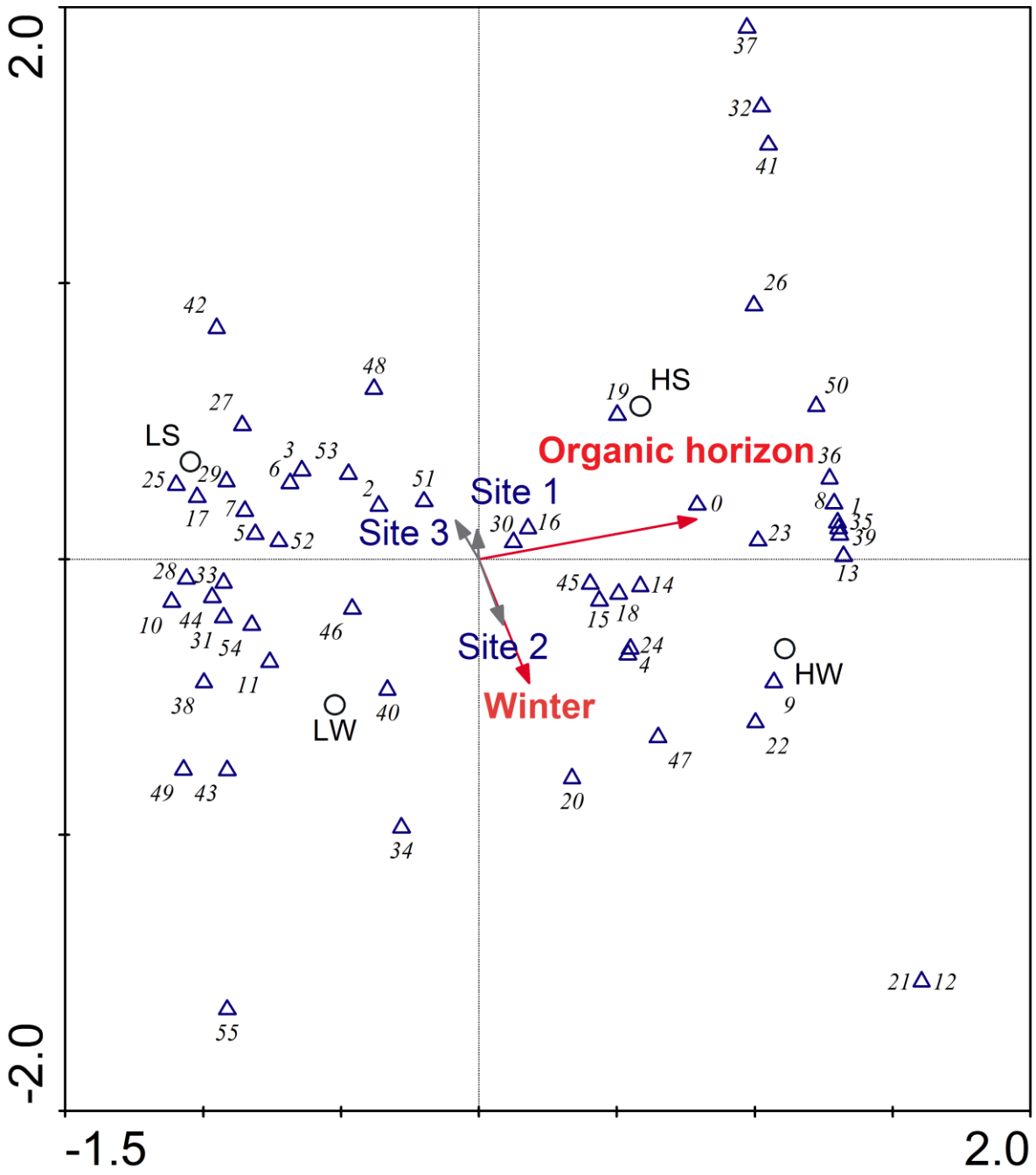
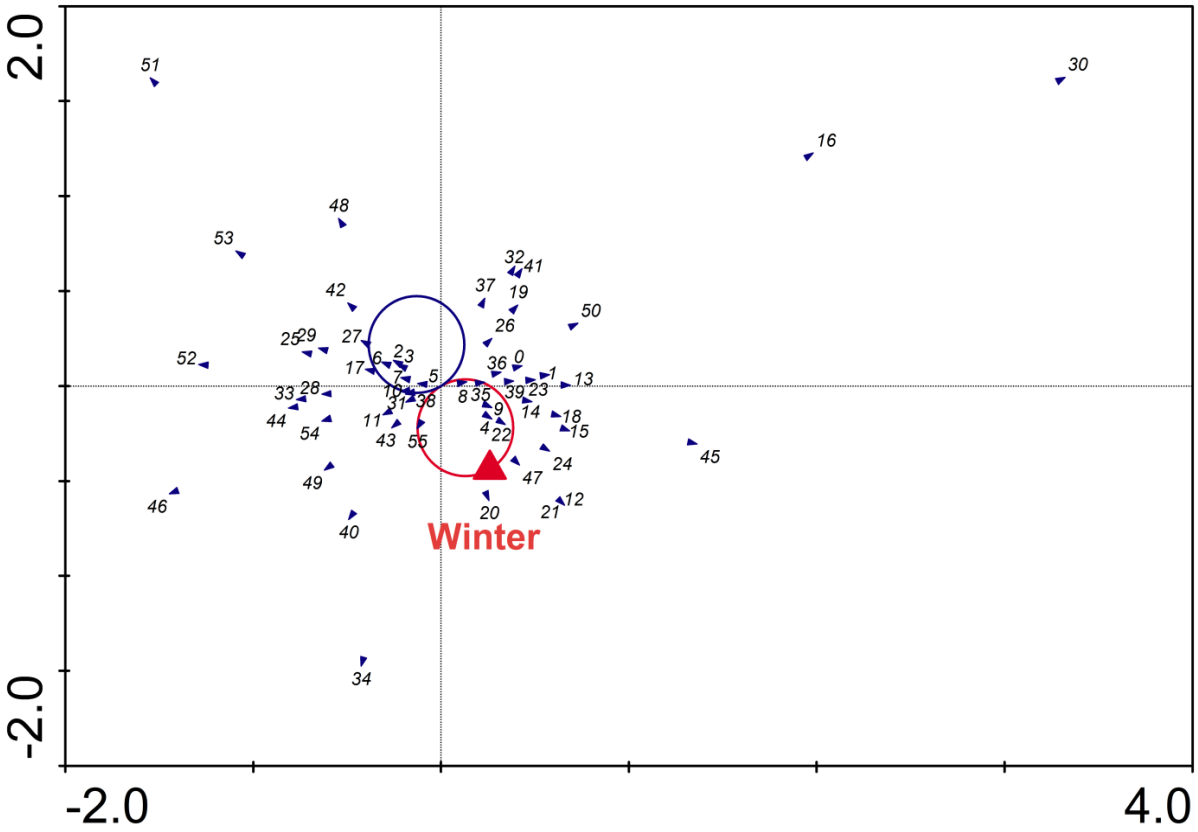


Figure 13 and Figure 14 present CCA t-value biplot analysis using Van Doben circles to detect the OTUs significantly associated with certain environmental factor (soil horizon and season). Six OTUs were significantly more abundant in the winter (4, 8 – *Ascomycota*, *Basidiomycota*: 9, 22, *Ascomycota*: 35, 55) and another six in the summer (*Ascomycota* – 2, 3, 5, 6, 7, 10).

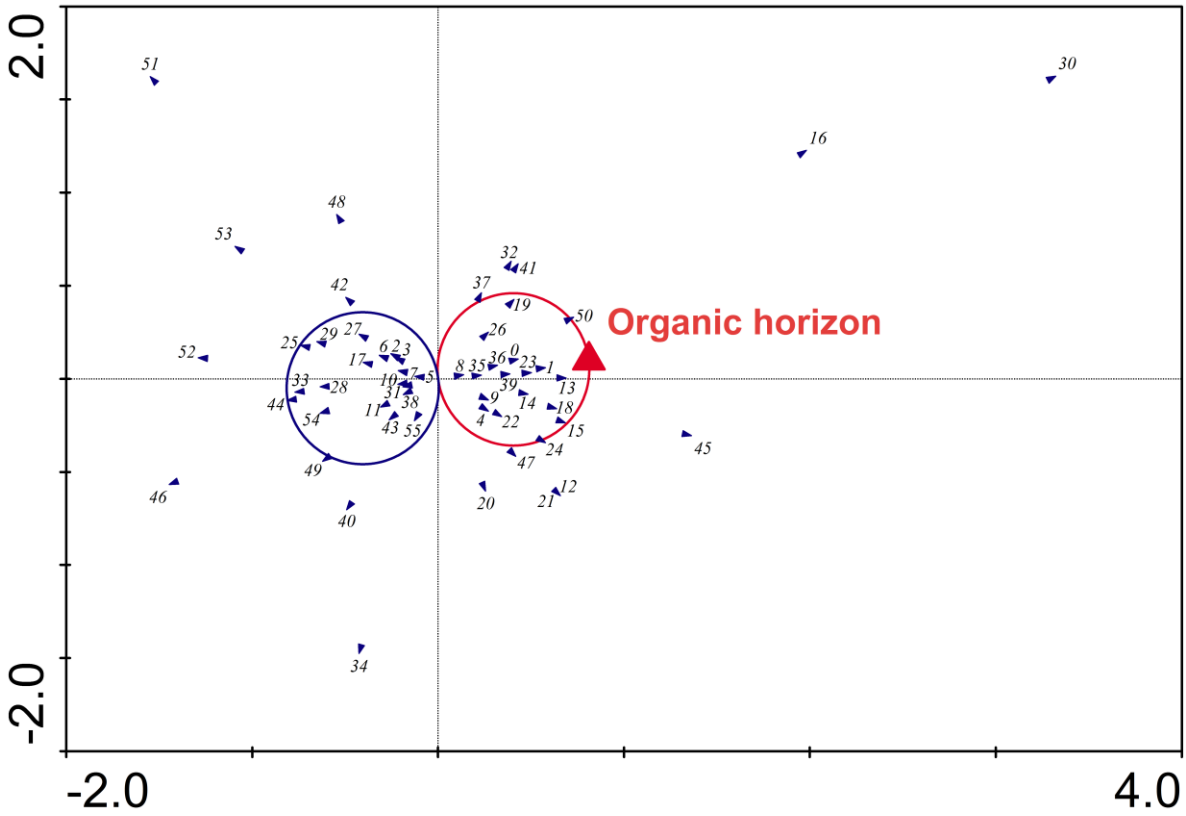
Figure 13: CCA t-values biplot with the indication of *cbhI* OTUs association with sampling seasons. OTUs inside the red circle are significantly more abundant in winter, these in the blue circle in summer.



T-plot analysis was used to identify OTUs associated with horizon. Eighteen OTUs were significantly associated with the H horizon (0, 1, 4, 8, 9, 13, 14, 15, 18, 19, 22, 23, 24, 26, 35, 36, 39, 50) of which 10 belonged to *Ascomycota*, 4 to *Basidiomycota* and the rest unidentified OTUs – identified based on BioNJ tree Supplementary Figure 15. Nineteen OTUs were significantly associated with the L horizon (2, 3, 5, 6, 7, 10, 11, 17, 25, 27, 28, 29, 31, 33, 38, 43, 44, 54, 55) of which 7 were *Ascomycota*, 6 *Basidiomycota* and the rest unidentified (

Figure 14). Nineteen OTUs did not show significant association with particular horizon. OTUs associated with winter and associated with H horizon were 4, 8 – identified from BioNJ tree as *Ascomycota*, 9 – *Basidiomycota*, 22 – *Basidiomycota*, 35 – *Ascomycota* and OTUs 2 – *Ascomycota*, 3 – *Ascomycota*, 5 – *Oidiodendron sp.*, 6 – *Pezizomycotina*, 7 – *Ascomycota* were associated with the summer and the L horizon. With respect to the distribution of cellulolytic community, there were both such clusters that occurred in both horizons and seasons and others, which were restricted to certain season or soil horizon.

Figure 14: CCA t-values biplot with the indication of *cbhI* OTUs association with soil horizon. OTUs inside the red circle are significantly more abundant in the soil, these in the black circle in the litter.



5.4 Analysis of total fungal community composition in spruce forest floor

5.4.1 Diversity of total fungal community in spruce forest litter and soil

Pyrosequencing yielded a total of 51550 raw ITS sequences of which 25037 started with the ITS1 (forward) primer and the rest with the ITS4 primer. Out of the 25037 forward sequences, 11866 were retained after the removal low quality sequences, sequences shorter than 380 bases and potentially chimeric sequences (Table 6). All these sequences were resampled at the same sampling depth of 700 sequences per sample for diversity analyses. Based on the rarefaction curves (

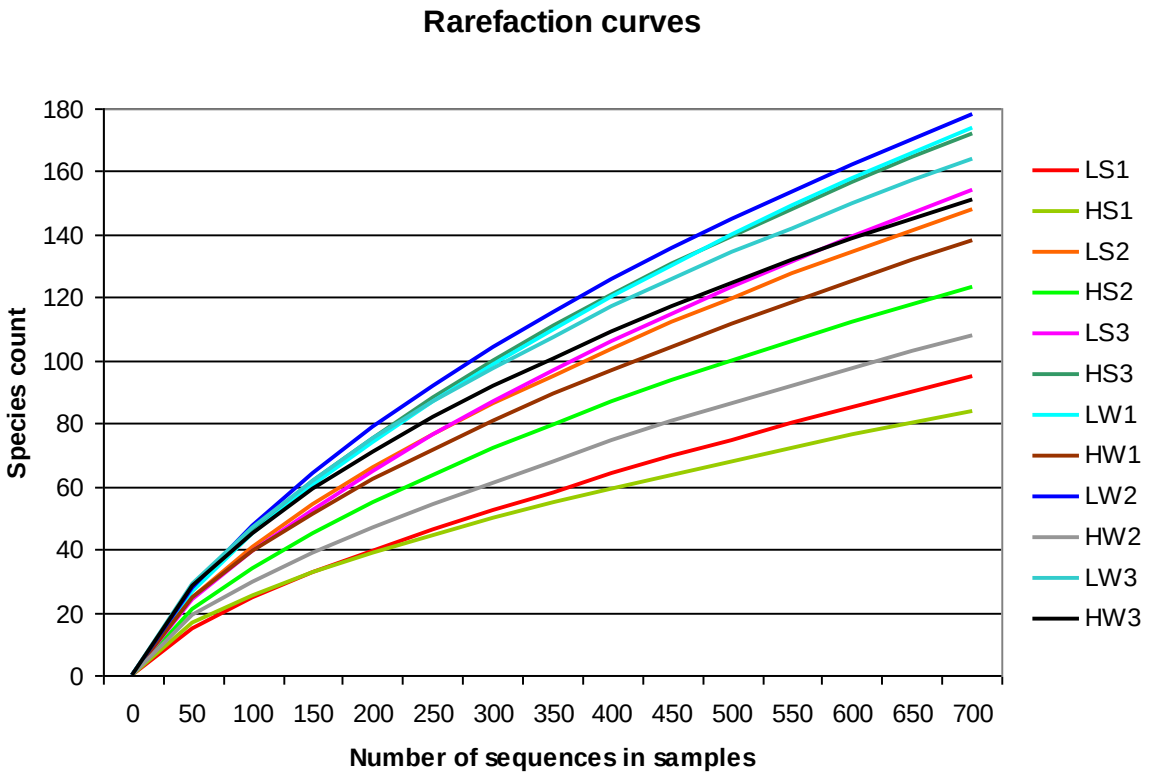
Figure 15), none of the samples reached plateau indicating insufficient sampling depth.

Table 6: Sequence counts of the forward fungal ITS sequences in the environmental samples from spruce forest.

Sample	Total sequences	Forward sequences surviving quality trimming
LS1	1439	733

HS1	3029	1490
LS2	1996	975
HS2	2017	1112
LS3	1864	706
HS3	1806	854
LW1	1838	754
HW1	2040	954
LW2	2548	1243
HW2	2298	1166
LW3	1787	793
HW3	2375	1086
Total	25037	11866

Figure 15: Rarefaction curves of fungal ITS sequences resampled to the same sampling depth of 700 sequences.



The L horizon samples were in average more richer in fungal species – 38 ± 17 OTUs were needed to cover 80% of fungal diversity comparing to 26 ± 15 OTUs in H horizon. This was similar to the *cbhI* sequences, where was insignificantly higher diversity in L horizon than H horizon. In total fungal community significantly more OTUs were needed to cover 80% o diversity in L horizon in winter when compared to H horizon or litter in summer ($p = 0.04$ and $p = 0.05$).

The total Evenness was higher in the winter than in the summer, but not significantly. There were higher differences among sites than among horizons in terms of fungal community evenness (Table 7), although none of them were significant. Fungal community had more rare species and was dominated by fewer abundant ones when comparing to the *cbhI* community. The estimated diversity for cellulolytic community than overall fungal community according Chao1 estimator is understandable since *cbhI* possessing fungi are just a part of the total fungal community. The total fungal community was less balanced in Evenness and Shannon index, which indicated that dominant fungi were more important in the total than *cbhI* community. Chao1 estimator predicted insignificantly higher diversity in the L than in the H horizon and in the winter than in the summer for both the total fungal community and the *cbhI* gene pool. In conformity with the *cbhI* Chao1 results, there was a higher estimated diversity in the L horizon than H but there were more rare species in the soil organic horizon. Estimated diversity was not significantly different according Fischer's exact test between horizons or seasons. In contrast with the *cbhI*, the highest estimated diversity according Chao1 was in the sample HS3, which had many singletons. This suggests that the cellulolytic community in the HS3 shows low diversity while the total fungal community is species-rich. The lowest Chao1 was found in HS1, which had the most sequences and an average number of singletons. Shanon-Wiener index was highest in LW3 and HW3, what indicates that there are quite many common species and species are distributed more-less equally, therefore is easier to predict the next species. From the Evenness we can tell, that ITS community on the site 1 in the summer was defined by dissimilar abundances of different species. There were probably unequally abundant species, with few dominant ones. At the site 1 only the 7-9 most abundant OTUs represented 80% of all sequences. PlutoF results suggest, that the highly abundant species found on this site in the summer was *Nolanea*, but according to GenBank it can be also *Mycena*, which has better maximal score when the same sequences is blasted. In all samples emerged *Nolanea* sp. as the dominant OTU, except for the HW3 and LW3, where the dominance of the first tree species was approximately the same.

Table 7: Diversity estimates of fungal community in individual samples. The pyrosequencing-derived dataset of sequences was resampled at 700 sequences per sample.

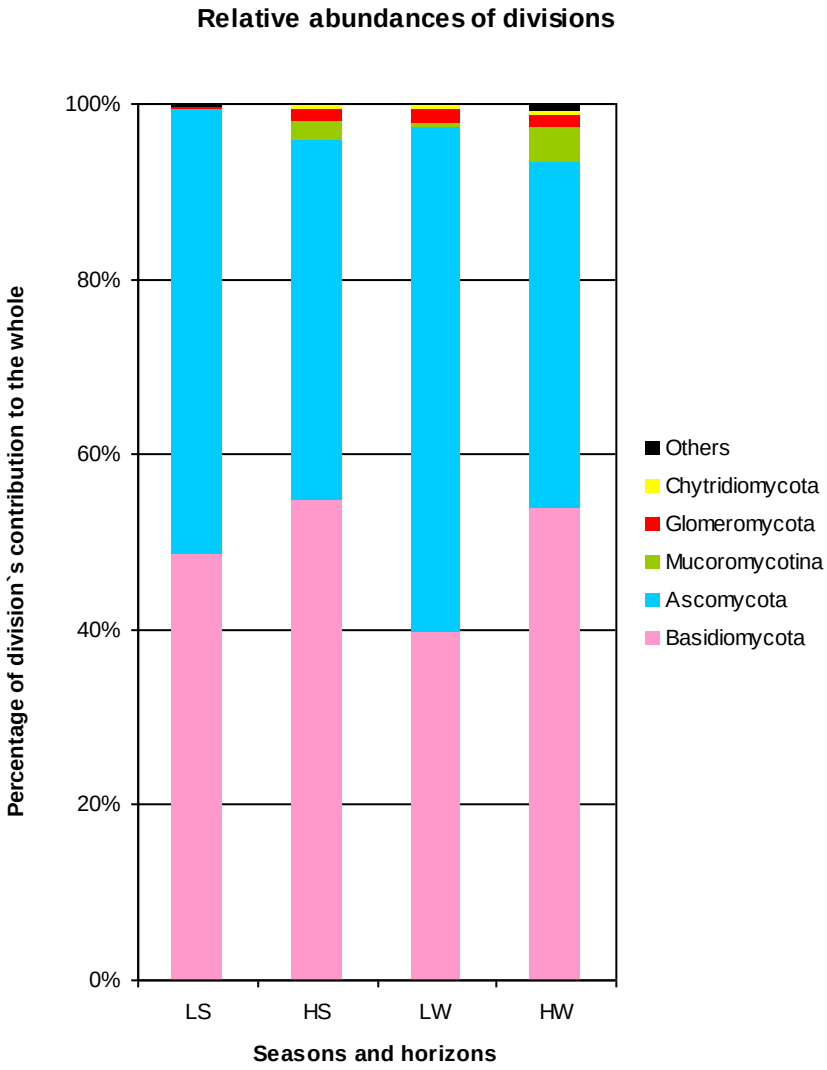
Sample	Chao1	Chao1 SD	Shannon-Wiener	Evenness	Number of OTUs covering 80% of diversity	Number of observed OTUs

LS1	299	51	2.05	0.45	7	95
HS1	196	32	2.50	0.56	9	84
LS2	320	37	3.47	0.69	33	148
HS2	244	29	3.21	0.67	20	123
LS3	350	40	3.52	0.70	35	154
HS3	401	46	3.79	0.74	48	172
LW1	348	34	3.62	0.70	50	174
HW1	374	52	3.56	0.72	28	138
LW2	359	36	3.76	0.73	55	178
HW2	265	38	3.00	0.64	13	108
LW3	328	35	3.99	0.78	46	164
HW3	265	26	3.89	0.77	40	151

5.4.2 Composition and ecology of total fungal community in spruce forest litter and soil

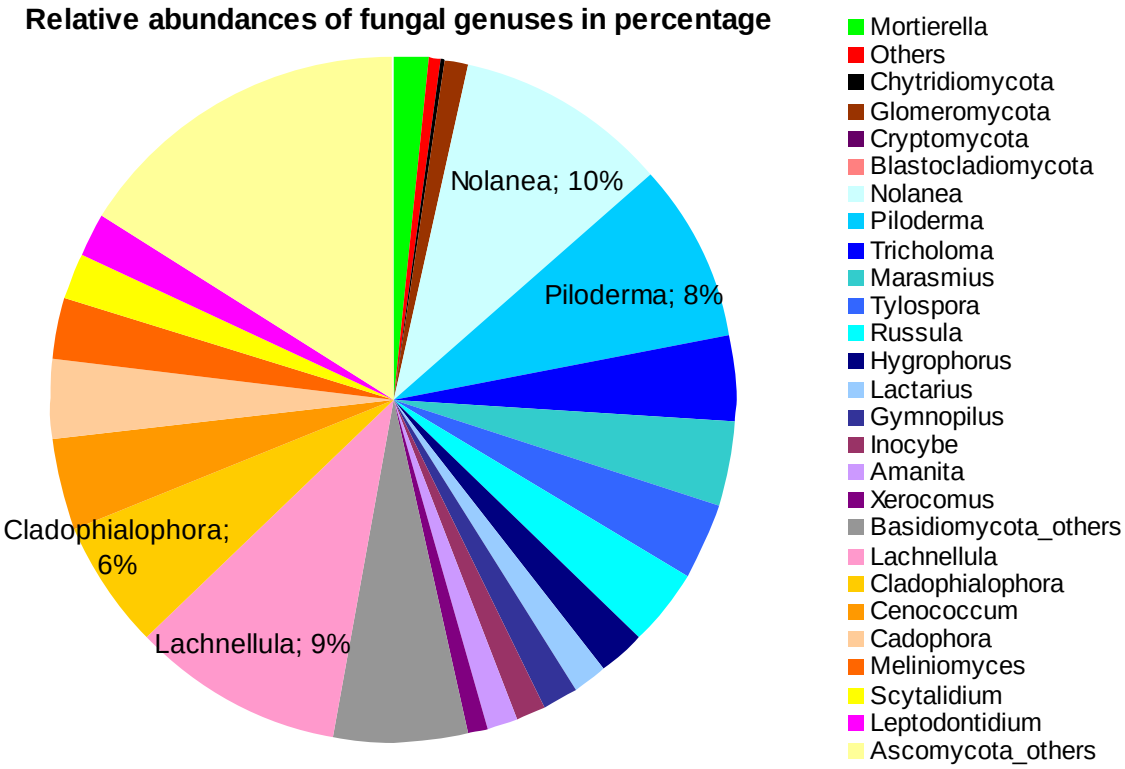
Fungi belonging to the *Ascomycota* were dominant in the litter horizon with 50 - 58% of sequences while the *Basidiomycota* dominated in the soil organic horizon with 55% of sequences in both seasons. The *Mucoromycotina* occurred mostly in the soil in both seasons. *Glomeromycota* and *Chytridiomycota* were present in both horizons and seasons but rather rare (Figure 16). Other sequences related to fungi in the dataset belonged to the *Entomophthoromycotina, Zoopagomycotina, Blastocladiomycota, Cryptomycota*, and others. Supplementary Figure 1 shows the abundance of fungal divisions in individual samples. We can notice that there were major differences between sites when it comes to the distribution of fungal divisions. Site 3 was the richest in the *Ascomycota* in both seasons and soil horizons. The *Ascomycota* were also very abundant on the second site in the litter horizon in both seasons. Site one, was, on the opposite, very rich in the *Basidiomycota*. On the site 3 in the organic horizon the abundance of the *Mucoromycotina* was also remarkable. These data show remarkable site-specific differences in the composition of the fungal communities.

Figure 16: Relative abundances of fungal divisions in the litter and soil of the spruce forest



The abundance of major fungal genera (> 1.6‰) in the whole dataset is summarized in the Figure 177. The *Basidiomycota* were represented by 49.3 %, the *Ascomycota* by 47.1 %, the *Mucoromycotina* by 1.68 %, the *Chytridiomycota* by 0.30 %, the *Glomeromycota* by 1.09 %, the *Blastocladiomycota* by 0.06 %, the *Cryptomycota* by 0.02%; other sequences (0.54 %) included the *Oomycota*, plants and other sequences. The most abundant genus of the *Basidiomycota* was *Nolanea* (10.0 %) and of the *Ascomycota* it was *Lachnellula* (9.8 %).

Figure 17: Relative abundances of major fungal genera in the whole dataset of spruce forest litter and soil.



Ecology was assigned to the genus identifications of individual fungal OTUs based on literature records. Significantly more ($p \leq 0.05$) symbiotic fungi included those with ECM, ABM, DSE, ErM, ENDO ecology were recorded in the organic horizon and, on the other hand, more saprotrophic fungi were found in the litter (Figure 18). The share of the ECM fungi in the community was by 20 – 30% higher in winter than in summer, but this difference was statistically insignificant. Endophytic fungi were more frequent in the L horizon than in the H horizon. Parasitic fungi were more abundant in the winter than in the summer due to the high amount of *Cadophora sp.* sequences in HW. Arbuscular-mycorrhizal fungi were almost absent in LS. Ericoid mycorrhizal fungi accounted for only 0.56% of all fungal ecological groups. Over 1% of sequences belonged to the lichenised fungi, where the most abundant genus, *Verrucaria*, was mainly present in the LW. Lichenised fungi were isolated more or less evenly from all sites and seasons. In the section Others were represented non-fungal sequences with abundance of 0.18%.

Figure 18: Relative average abundances of fungi according to their ecology showed on percentage scale (notes: SAP – saprothrophes, PAR – parasites, ORCH/SAP – orchideoid mycorrhiza or saprothrophe, LICH- lichenised fungi, ErM – ericoid mycorrhiza, ENDO – endophyte, ECM – ectomycorrhiza, DSE – dark septate endophytes, ABM – arbuscular mycorrhizae, Ambrosious – associated with bark beetle, Others – mostly plants)

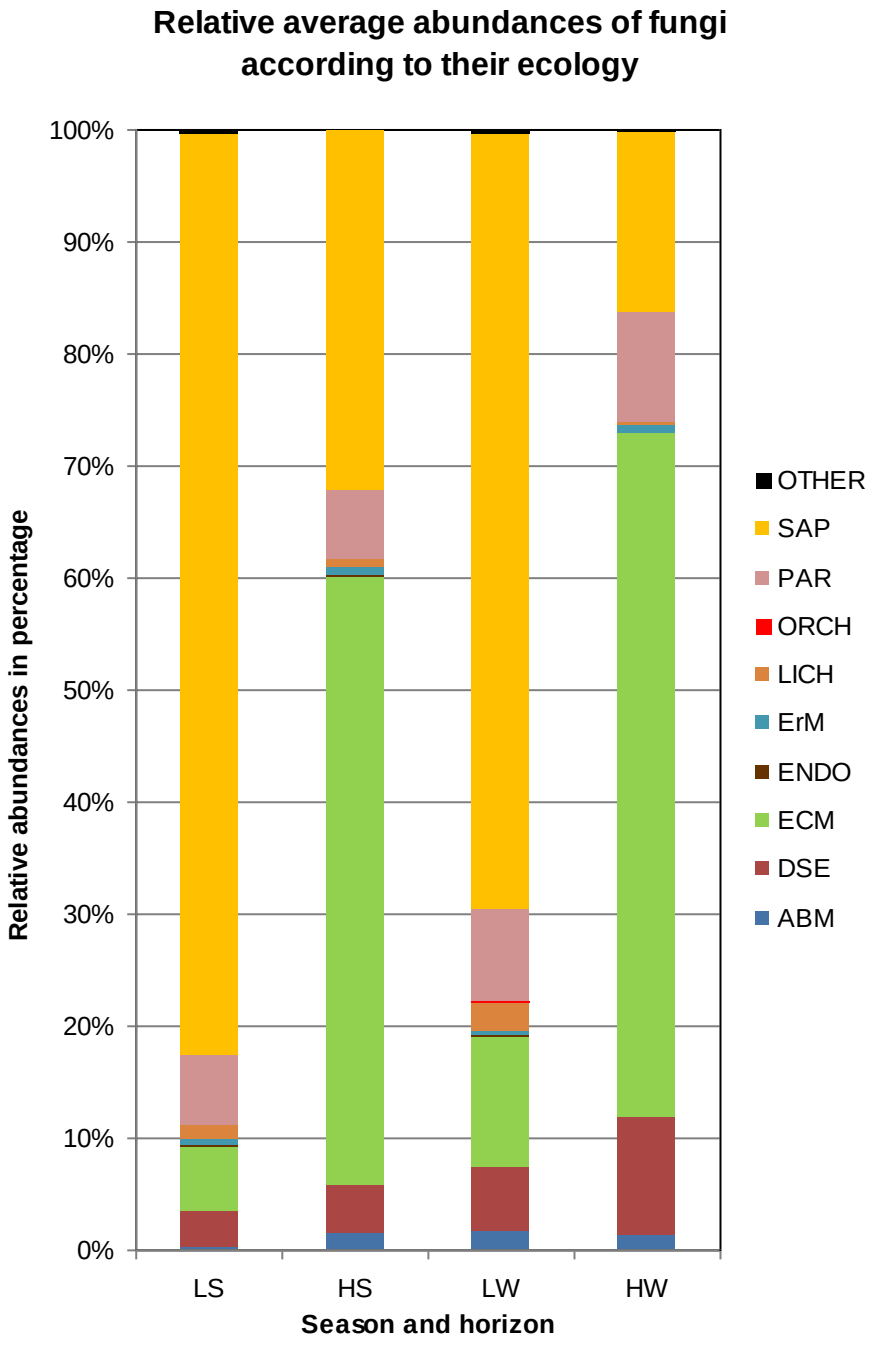
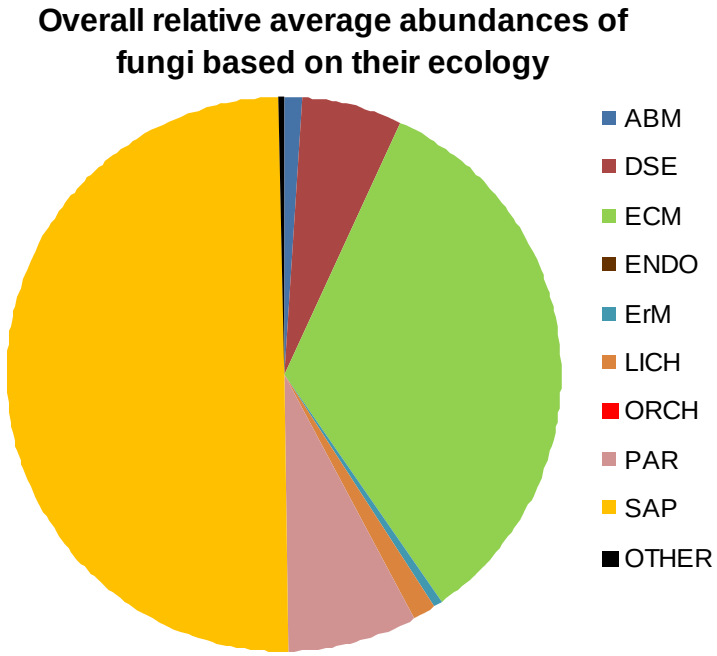


Figure 19 illustrates the overall composition of the fungal community by their ecology in the topsoil of the mountainous Norway spruce forest. There was approximately the same share of ectomycorrhizal (43%) and saprothrophic (39%) fungi, but much more species in saprothrophic fungi – 137 when compared to the ECM – 28 species. Other ecological groups of fungi had the following numbers of species: ErM 3, DSE and ENDO 5, ABM 7, LICH 24 to parasites with 61 species.

Figure 19: Composition of fungal community in the mountainous *Picea abies* forest by ecology (abbreviations are the same as in the Figure 25).



5.4.2.1 Statistical and multivariete analyses of distribution of fungal taxa among seasons and soil horizons

Graphs were constructed to show the preference of individual fungal genera for certain horizon or season. X axis , their relative occurrences were expressed by the litter / organic soil horizon (L/H) and summer / winter (S / W) ratios and depicted in Supplementary Figures 2-14.

On the Supplementary Figures 2 are depicted most abundant genera of total fungal community. It can be noticed that ECM fungi prefer HW, except *Piloderma.* On the other hand, most of saprotrophes occured mostly in LS.

The most abundant genus amongst *Basidiomycota* that were left after graphs fro *Agaricales* and *Agaricomycetes* were constructed, was *Cryptococcus* (0.9%), which was found in HW. Other genera occurring only in H horizon were *Auriculoscypha, Leucosporidiella, Puccinia, Pucciniastrum, Uromyces. Curvibasidium* depend on L horizon but not a season. None of genera in Supplementary Figures 3 depends on summer season.

Athelialen genus *Piloderma fallax* was the second most abundant among ECM fungi and had 100x more sequences from HS when comparing to other seasons and horizons

(Supplementary Figures 4). On the other hand, ECM genera *Russula, Xerocormus, Lactarius, Tomentella* were preffering HW. Saprotrophes like *Heterochaete, Clavulina, Ductifera, Xenasmantella* occurred in litter.

From the Supplementary figure 5 - *Agaricales* is evident that the members of the ectomycorrhizal genera *Amanita, Cortinarius, Hygrophorus, Tricholoma, Inocybe* are present only in the organic horizon while saprotrophes like *Mycena, Rhodocollybia, Galerina, Marasmius* occur in L horizon *Nolanea conferenda* (10%) was the most abundant fungus among ECM with more than 100x higher abundance in the L horizon than in the organic one and showed preference for summer.

In Supplementary Figures 6 are ascomycetes that were left after graphs for *Helotiales, Dothideomycetes, Eurotiomycetes, Lecarnomycetes, Sordariomycetes* were created. The most abundant genus from this group was endophyte/ saprotrophe/ parsite *Xenochalara* (0.88%). Abundant genera occurred in L horizon and vice versa. The ericoid mycorrhizal genus *Oidiodendron* was found only in the H horizon in both seasons. Orchideoid mycorrhiza was represented by *Gyoerffyella rotula* found in the litter horizon.

Most abundant genus amongst *Dothideomycetes* (Supplementary Figures 7) was ECM genus *Cenococcum* with hundreds of sequences in H horizon especially in the winter. *Dothideomycetes* species occurred mostly in L horizon.

Genera belonging to *Eurotiomycetes* (Supplementary Figures 8) were separated between two horizons with only two minor genera dependent on a season. *Cladophialophora* was the most abundant genus with 6.1% of relative abundance.

Cladonia (0.2%) was the most abundant among *Lecanoromycetes* (Supplementary Figures 9). There were genera showing clear preference for one of seasons or horizons but this can be due to insufficient amount of obtained sequences.

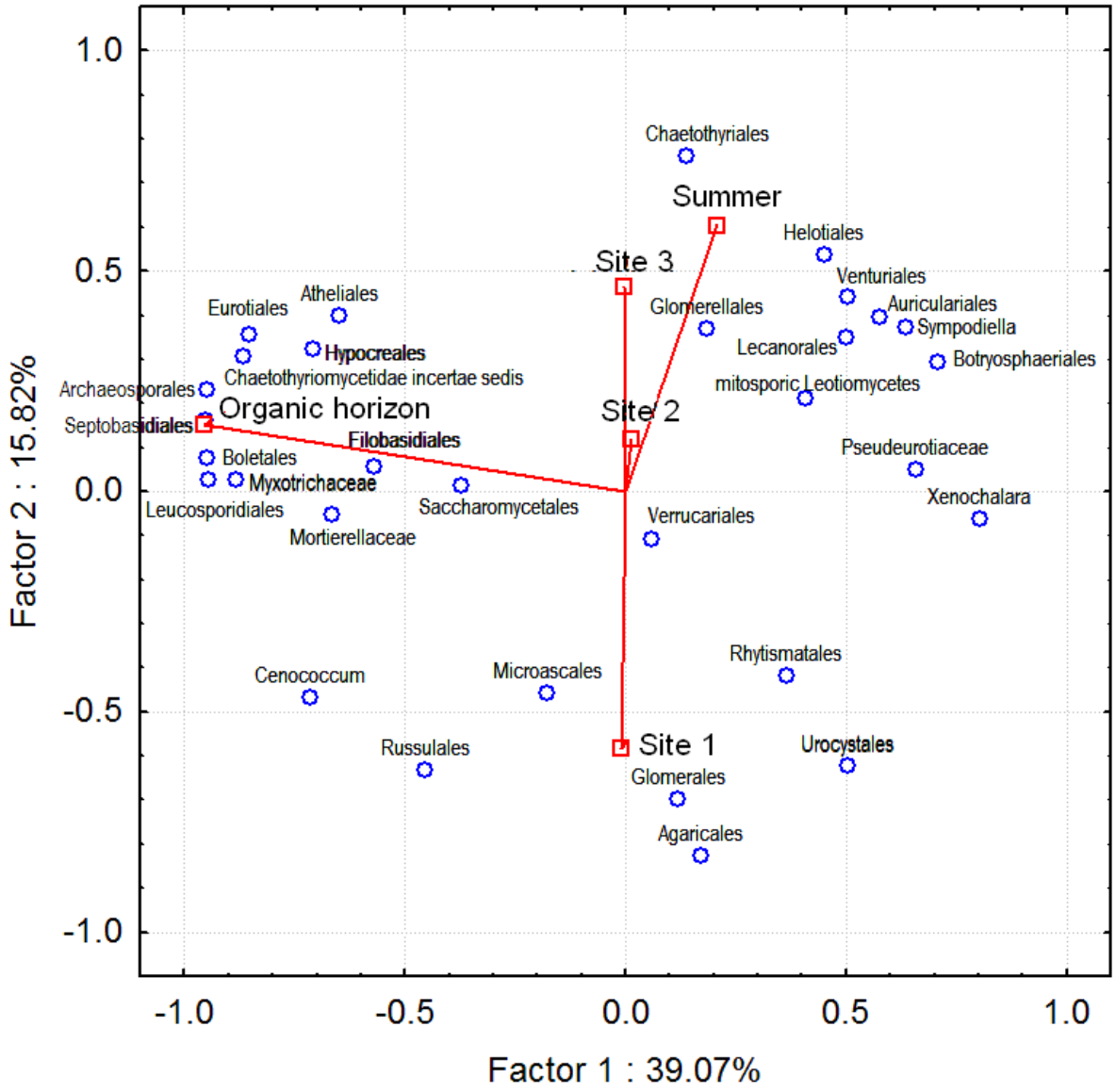
In *Sordariomycetes* graph (Supplementary Figures 10) parasites *Truncatella, Phlogicylindrium, Cordyceps* and saprotrophes *Acremonium, Polyscytalum, Pochonia, Ophiostoma, Zalerion* occurred only in l horizon. Some minor genera were found only in winter season: *Tolypocladium, Zalerion, Chaetosphaeria*. *Stachybotrys, Lecythophora, Thozetella, Ophiostoma* were found only in summer season.

Most of Helotialen (Supplementary Figures 11) genera prefered litter over organic horizon. In H horizon were found DSE *Acephala* and *Meliniomyces,* parasite *Neofabera* and saprotrophe *Scytalidium* in HS. Saprotrophe *Lachnellula* was the most abundant with 9.8% and depend on L horizon.

Glomeromycota were separated by soil horizons but not affected by season (Supplementary Figures 12). *Glomus, Gigaspora, Scutellospora, Entrophospora* were found only in litter while *Ambispora, Acaulospora, Paraglomus* only in H horizon.
There were only 6 members of *Chytridiomycota* on Supplementary Figures 13 and the most abundant saprotrophe Nowakowskiella (0.11%) was found only in H horizon.
8 genera from the taxonomical group, that used to be called *Zygomycota*, occurred only in H horizon and mostly in winter. *Mortierella* (1.6%) was the most abundant genus in this group (Supplementary Figures 14).

To show how total fungal community is influenced by environmental factors, principal component analyses was performed on non-transformed data of OTUs over 0.16% with relative abundances and it did not show any trends in orientation of species according to environmental variables (data not shown). The PCA on log transformed data of 61 OTUs and their orders showed clear spatial heterogeneity of fungal community (Figure 20 and Figure 21). On the left side of both PCA figures can be found species and orders belonging mostly to mycorrhizal fungi occurring in the H horizon and on the right side saprotrophic one found mostly in L horizon. Sampling sites divide PCA into lower and upper part, where in the lower half are species and orders abundant on Site 1 and not on other sites while fungi in the upper part were found on all sites. Dividing of species and orders according to season is not so clear, but OTUs in the upper half of the figure tend to be more abundant in summer and those on the opposite site in winter. The most of the variation was explained by horizons. The effect of seasons was low compared to the effects of horizons and sampling sites. The PCA of orders explained 54.89% variability in data and PCA of OTUs over 0.16% abundance explained 50.97%. The position of orders on the Figure 20 is the average from the position of OTUs in Figure 21.

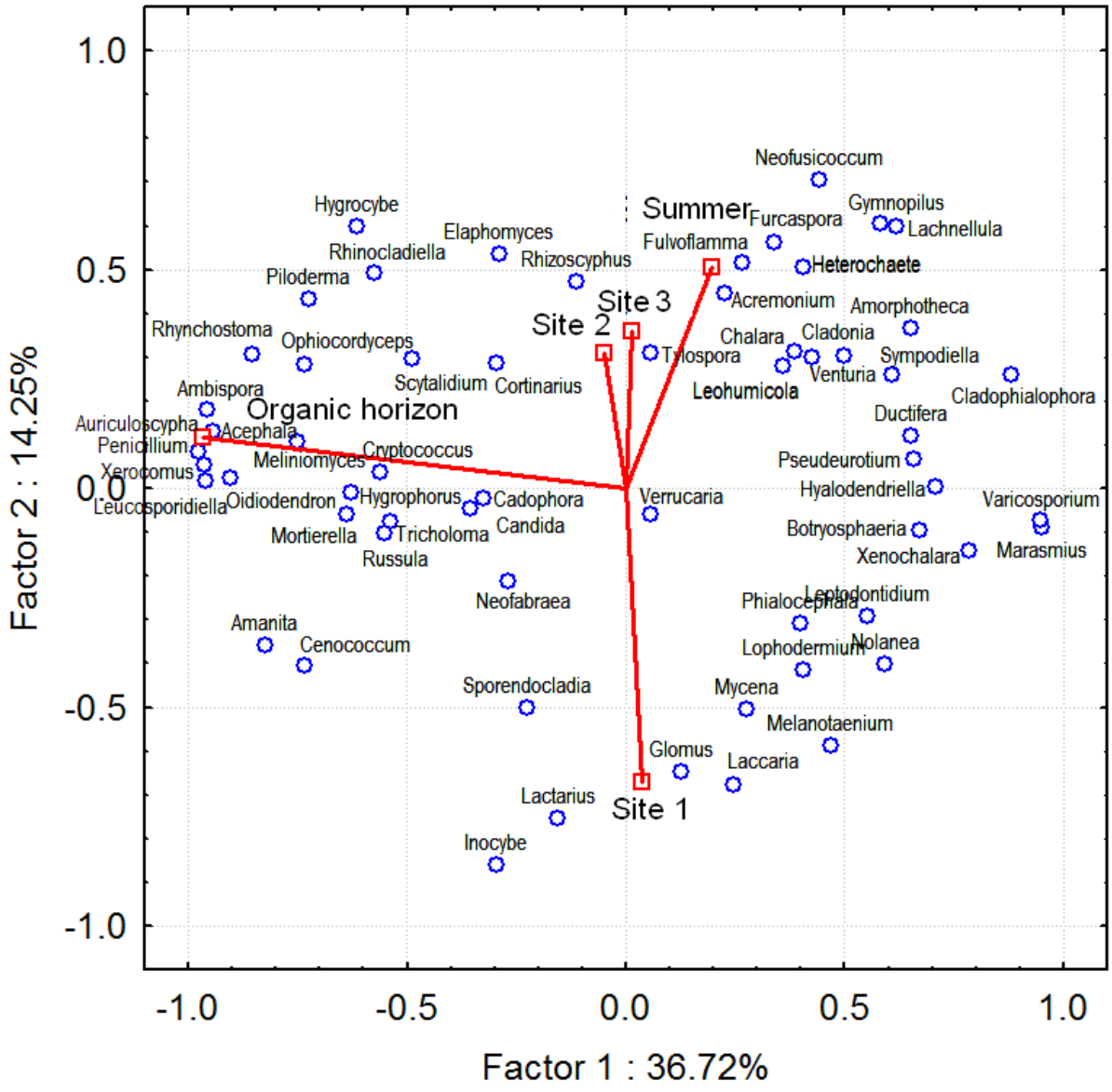
Figure 20: PCA analysis on the abundance of ITS orders and the effects of horizons, sites and seasons.



Species belonging to the order *Helotiales* assigned to the saprotrophs *Furcaspora eucalypti* and *Lachnellula calyciformis* and the saprotroph *Acremonium alcalophilum* of the *Glomerellales* order were more abundant in summer in litter horizon. Some OTUs from the *Eurotiales* were more abundant in the soil - like the saprotroph *Penicillium urticae* and the ectomycorrhizal fungus *Elaphomyces muricatus*. Also the OTUs from the *Hypocreales* had diverse ecology – the parasite *Ophiocordyceps sinensis* (taxon with low taxonomical support by PlutoF) was more abundant in the H horizon while the saprotroph *Sympodiella acicola* was mainly recovered from the litter. The whole order behaves as if it depends on organic horizon. From the order of *Chaetothyriales*, saprotrophic species *Cladophialophora minutissima* and *Amorphotheca resinae* were associated with the litter and summer season. The H horizon also contained more sequences of the ectomycorrhizal fungus *Cenococcum*

geophilum and the ericoid mycorrhizal fungus *Oidiodendron tenuissimum* and the L horizon the saprotrophs *Pseudeurotium bakeri* and *Marasmius androsaceus*. Majority of OTUs belonging to the *Agaricales* were distributed equally among sites although the whole order itself prevailed at Site 1. This is because half of order`s OTUs were found in litter like saprotroph *Marasmius androsaceus* and the parasite *Melanotaenium euphorbiae* (taxon with low % support by PlutoF) and the other half like ectomycorrhizal *Amanita sp.* or *Inocybe sp.* were abundant in organic horizon. Some species were strongly associated with H horizon like the parasite *Auriculoscypha anacardiicola* belonging to order *Septobasidiales*.

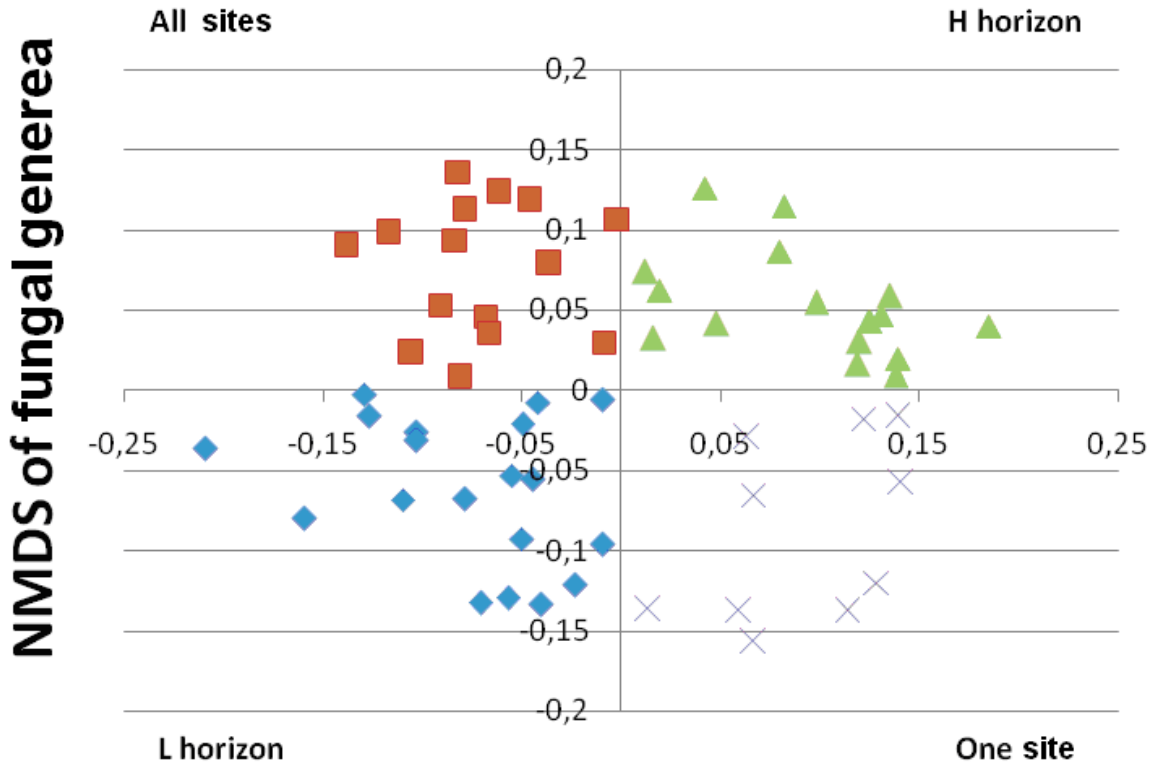
Figure 21: PCA analysis on the abundance of the 61 most abundant OTUs and the effects of horizons, sites and seasons.



The PC analyses explained only a small part of the total variability in fungal abundance. Non-metric multidimensional scaling (NMDS) was more efficient in exploring the relationships in the data. Furthermore, NMDS makes no assumption about data normality like PCA, and is thus better suited for data where normality is questionable. NMDS also allows the use of any distance measure of the samples, unlike other methods, which specify particular measures.

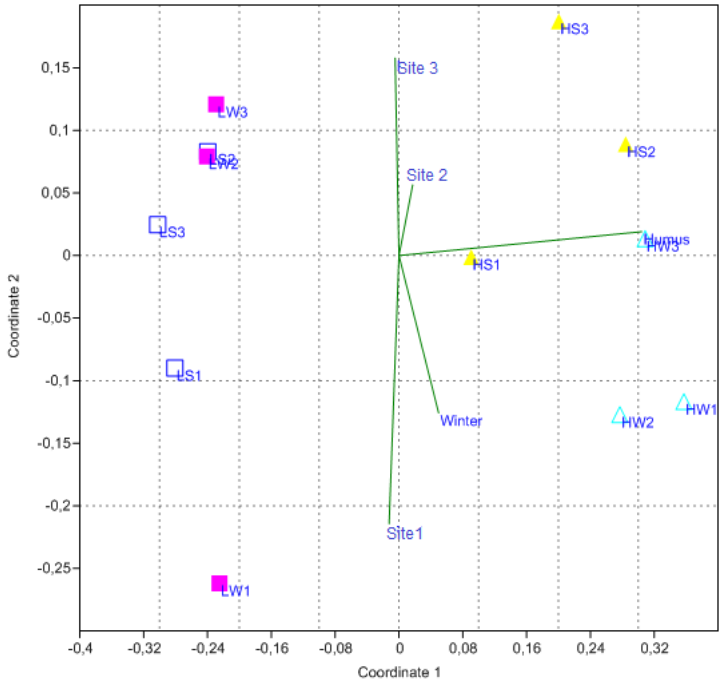
Non-metric multidimensional scaling (NMDS) analysis on the log-transformed abundances of 61 dominant OTUs showed that OTUs formed four groups depending on the site of occurence and soil horizon (Figure 22). The upper left quadrant of the plot contains fungi universally distributed among sampling sites: *Ductifera, Pseudeurotium, Phialocephala, Venturia, Rhizoscyphus, Lachnellula, Cladophialophora, Amorphotheca, Cadophora, Tylospora, Leptodontidium, Tricholoma, Meliniomyces, Cryptococcus, Mortierella*. The upper right quadrant harbors fungi occurring at all sites but constrained to the organic horizon occurred: *Elaphomyces, Neofabraea, Candida, Cortinarius, Leucosporidiella, Acephala, Amanita, Ambispora, Auriculoscypha, Scytalidium, Hygrocybe, Russula, Piloderma, Hygrophorus, Rhynchostoma, Penicillium, Xerocomus, Cenococcum.* Lower left quadrant of the graph is occupied by fungi occurring at all sites but mostly in the litter horizon (*Varicosporium, Sympodiella, Chalara, Hyalodendriella, Neofusicoccum, Botryosphaeria, Fulvoflamma, Melanotaenium, Cladonia, Leoorganicola, Furcaspora, Lophodermium, Verrucaria, Acremonium, Gymnopilus, Heterochaete, Marasmius, Nolanea, Xenochalara*) and finally the lower right quadrant contains fungi abundant at one single sites and mostly in the organic horizon (*Mycena, Glomus, Cortinarius, Sporendocladia, Laccaria, Rhinocladiella, Ophiocordyceps, Elaphomyces, Scytalidium, Lactarius, Inocybe*).

Figure 22: Non-metric MDS of fungal genera created with Euclidian similarity measurement



When the efects of horizons, seasons and sampling sites was explored by NMDS, the biggest effects were recorded for sites and horizons. Samples from both horizons were divided precisely but season effects in the litter horizon is hard to see (Figure 23). On the other hand, samples from the organic horizon were well separated by season. Samples from the litter horizon were more separated by sites – LS1 and LW1 were sampled from Site 1.

Figure 23: Non-metric MDS of 12 samples in dependences on environmental factors with Euclidian similarity. Squares are samples from litter horizon – full pink squares were sampled in winter and empty blue squares in summer. Triangles represents samples from organic horizon – full yellow triangles were sampled in summer and empty blue triangles in winter.



Three statistical programs were used to describe differencies in total fungal community between soil horizons in seasons: ANOVA, STAMP and Canoco. One-way Anova analysis did not reveal any significant differences among the abundances of fungal phyla amongst horizons, seasons or sampling sites. Generally, the effects of sampling sites tended to be greater than those of horizons and seasons for fungal orders and genera. ANOVA with a post-hoc test indicated significant differences among sampling sites in the following basidiomycetous genera: *Nolanea, Heterochaete,* and *Tylospora*. Significant differences amongst horizons were found for *Piloderma, Marasmius, Russula, Xerocomus, Auriculoscypha, Melanotaenium, Hygrocybe,* and *Leucosporidiella.* Fungi significantly affected by seasons were *Piloderma, Marasmius, Russula, Xerocomus, Auriculoscypha,* and *Leucosporidiella.* Ascomycetous genera that showed significant differences in abundance among sites were: *Lachnellula, Leptodontidium, Furcaspora*. The genera *Penicillium, Cladophialophora, Cenococcum, Meliniomyces, Amorphotheca, Rhynchostoma, Candida, Verrucaria, Sympodiella, Varicosporium,* and *Oidiodendron; Neofusicoccum* differed in abundance among seasons and *Cladophialophora, Cenococcum, Penicillium, Meliniomyces, Xenochalara, Rhynchostoma, Hyalodendriella, Varicosporium, Oidiodendron, Botryosphaeria* were preferentially associated with either the L or the H horizon.

Because the composition of fungal community differed widely among sampling sites, this seriously decreased the power of classical statistical tools to determine statistical significance of differences in abundance among seasons. For this reason, comparisons among horizons

and seasons were also performed for individual sites separately by random resampling corresponding pairs of datasets by the Fishers exact test. The trends in the abundance for certain treatments were regarded as statistically significant if they were found to be significant at all three sites separately at $p < 0.05$. To exclude positive results due to random recovery of sequences, only these 42 orders and 81 genera of fungi with the relative abundance over 1‰ in the whole dataset were tested and only 61 genera with relative abundance over 1.6‰ were discussed. The statistically significant effects in the dataset are shown in the Table 8 and Table 9. Significant differences among horizons or seasons were found for 9 orders of the *Basidiomycota*, 11 of the *Ascomycota*, 1 of the *Mortierellomycotina*, and 1 of the *Glomeromycota*. Ten orders were significantly more abundant in the L horizon, of which 7 were ascomycetes orders; 6 orders of the *Basidiomycota*, 3 of the *Ascomycota*, one of the *Mucoromycotina* and 1 of the *Glomeromycota* were more abundant in the H horizon. Two orders of the *Basidiomycota* and three of the *Ascomycota* were more abundant in the summer; another three orders of the *Basidiomycota*, three of the *Ascomycota* were more abundant in winter. Significant differences among horizons or seasons were further found for 16 genera of the *Basidiomycota*, 24 of the *Ascomycota*, 1 of the *Mucoromycotina* and 1 of the *Glomeromycota*. Fifteen ascomycetous genera were significantly more abundant in the L horizon, 10 in the H horizon, 5 in the winter 3 in the summer; ten basidiomycetous genera were more abundant in the H horizon, while only 5 in the L horizon, three genera were more abundant in summer and another three in winter. One genus of *Mucoromycotina* and one belonging to the *Glomeromycota* were abundant in the H horizon.

Table 8: Significant association of fungal orders with certain site, horizon or season based on random resampling of pairs of samples

Phylum	Order	site 1	site 2	site 3	STAMP horizons	STAMP seasons
Basidiomycota	*Agaricales*	+		+	L	S
Basidiomycota	*Ascomycota_others*		+	+	L	
Glomeromycota	*Archeosporales*		+	+	H	
Basidiomycota	*Atheliales*	+	+	+	H	W
Basidiomycota	*Auriculariales*		+	+	L	
Basidiomycota	*Boletales*	+	+	+	H	S
Ascomycota	*Botryosphaeriales*		+	+	L	
Ascomycota	*Dothideomycetes_others*	+	+	+	H	S
Ascomycota	*Eurotiales*	+	+	+	H	
Ascomycota	*Eurotiomycetes_others*	+	+	+	H	S
Basidiomycota	*Filobasidiales*		+	+	H	W
Ascomycota	*Helotiales*	+	+		L	W
Ascomycota	*Chaetothyriales*	+	+	+	L	S

Ascomycota	*Letiomycetes_others*	+		+	L	
Basidiomycota	*Leucosporidiales*	+	+		H	
Mucoromycotina	*Mortierellales*		+	+	H	
Basidiomycota	*Russulales*	+	+	+	H	W
Basidiomycota	*Septobasidiales*	+	+	+	H	
Ascomycota	*Venturiales*	+	+		L	
Ascomycota	*Verrucariales*	+	+		L	W

Table 9: Significant association of fungal genera with certain site, horizon or season based on random resampling of pairs of samples

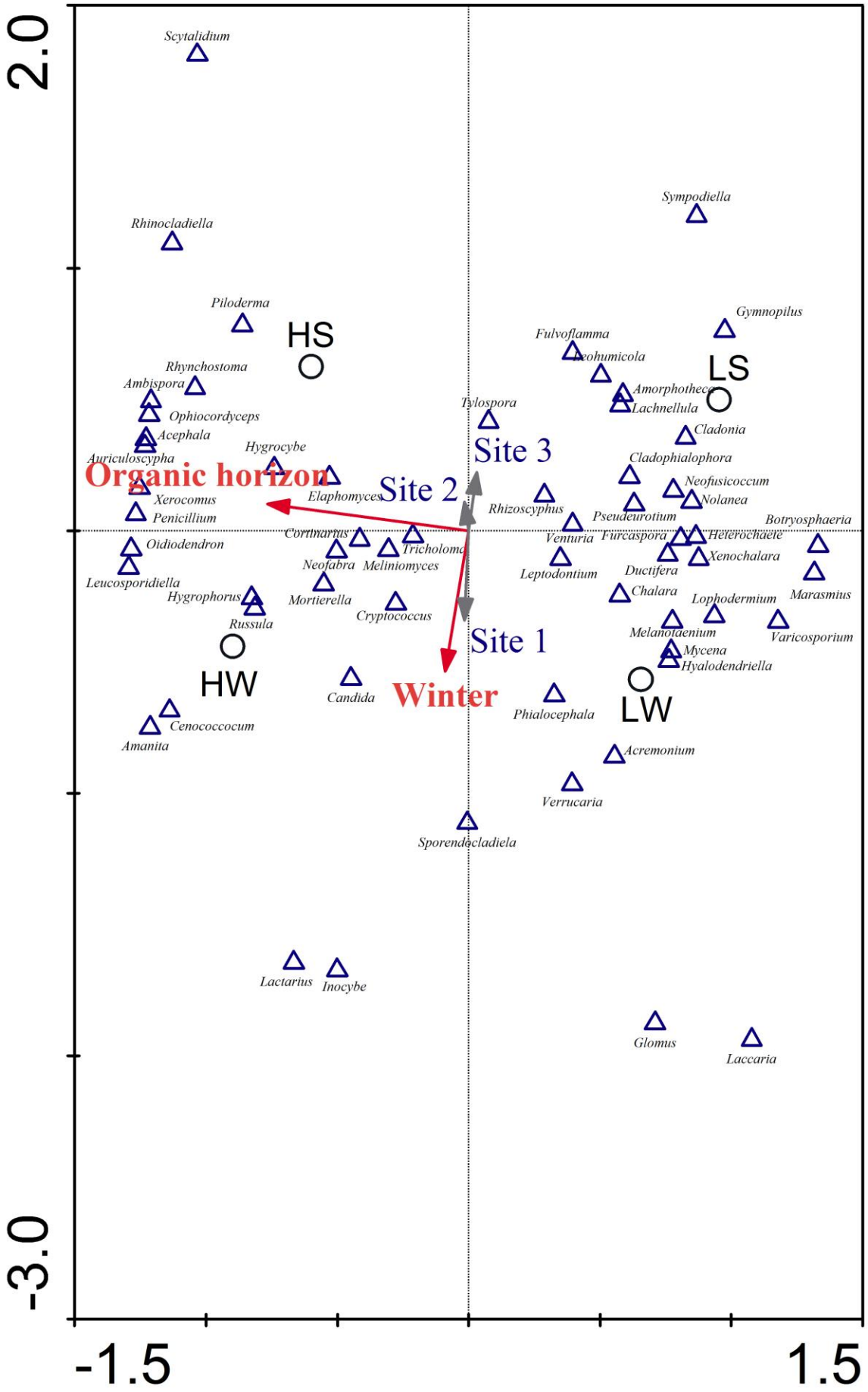
Phylum/subphylum	**Order**	**Genus**	**site 1**	**site 2**	**site 3**	**STAMP horizon**	**STAMP season**
Basidiomycota	*Agaricales*	*Amanita*	+	+		H	W
Basidiomycota	*Agaricales*	*Gymnopilus*	+		+	L	S
Basidiomycota	*Agaricales*	*Hygrocybe*	+		+	H	
Basidiomycota	*Agaricales*	*Hygrophorus*	+	+	+	H	
Basidiomycota	*Agaricales*	*Marasmius*	+	+	+	L	
Basidiomycota	*Agaricales*	*Nolanea*	+		+	L	
Basidiomycota	*Agaricales*	*Tricholoma*	+	+	+	H	
Glomeromycota	*Archaeosporales*	*Ambispora*		+	+	H	
Basidiomycota	*Atheliales*	*Piloderma*	+	+	+	H	S
Basidiomycota	*Atheliales*	*Tylospora*	+	+	+		S
Basidiomycota	*Auriculariales*	*Ductifera*	+	+	+	L	
Basidiomycota	*Auriculariales*	*Heterochaete*		+	+	L	
Basidiomycota	*Boletales*	*Xerocomus*	+	+	+	H	W
Ascomycota	*Botryosphaeriales*	*Botryosphaeria*	+	+	+	L	
Ascomycota	*Botryosphaeriales*	*Neofusicoccum*		+	+	L	
Ascomycota	*Dothideomycetes_others*	*Cenococcum*	+	+	+	H	W
Ascomycota	*Eurotiales*	*Elaphomyces*	+		+	H	
Ascomycota	*Eurotiales*	*Penicillium*	+	+	+	H	
Ascomycota	*Eurotiomycetes_others*	*Rhynchostoma*	+	+	+	H	S
Basidiomycota	*Filobasidiales*	*Cryptococcus*		+	+	H	W
Ascomycota	*Helotiales*	*Acephala*	+		+	H	
Ascomycota	*Helotiales*	*Cadophora*		+	+	H	W
Ascomycota	*Helotiales*	*Hyalodendriella*	+	+		L	
Ascomycota	*Helotiales*	*Lachnellula*	+	+	+	L	S
Ascomycota	*Helotiales*	*Meliniomyces*	+	+	+	H	W
Ascomycota	*Helotiales*	*Neofabraea*	+		+	H	
Ascomycota	*Helotiales*	*Phialocephala*	+	+		L	
Ascomycota	*Helotiales*	*Polydesmia*	+	+		L	
Ascomycota	*Helotiales*	*Varicosporium*	+	+	+	L	
Ascomycota	*Chaetothyriales*	*Amorphotheca*	+	+	+	L	S
Ascomycota	*Chaetothyriales*	*Cladophialophora*	+	+	+	L	
Ascomycota	*Chaetothyriales*	*Rhinocladiella*	+		+	H	
Ascomycota	*Leotiomycetes_others*	*Leptodontidium*	+	+	+	L	
Ascomycota	*Leotiomycetes_others*	*Oidiodendron*	+	+		H	
Ascomycota	*Leotiomycetes_other*	*Pseudeurotium*	+		+	L	

	s						
Basidiomycota	*Leucosporidiales*	*Leucosporidiella*	+	+		H	
Mucoromycotina	*Mortierellales*	*Mortierella*		+	+	H	
Ascomycota	*Pazizomycotina*	*Sympodiella*	+	+	+	L	S
Ascomycota	*Pazizomycotina*	*Xenochalara*	+	+	+	L	W
Basidiomycota	*Russulales*	*Russula*	+	+	+	H	
Basidiomycota	*Septobasidiales*	*Auriculoscypha*	+	+	+	H	
Ascomycota	*Venturiales*	*Venturia*	+	+		L	
Ascomycota	*Verrucariales*	*Verrucaria*	+	+		L	W

CCA analysis on log-transformed species data was performed to explore the relative contribution of the following factors: soil horizons and seasons on fungal community composition. Although CCA is a unimodal method it acts as a linear one when gradients are short and so it was suitable for the analysis of the dataset. All four canonical axes were statistically significant (p = 0.038);when the variable sites was made supplementary, than under Monte-Carlo unrestricted permutations, soil horizon effect was statistically significant at p = 0.02 and explained 0.35= of data varaibility. The season effect was marginally statistically significant (p = 0.052). The season explained 0.08% of dataset variablity. The total variance explained by horizon and season together was 0.38% and the residual variance explained by supplementary variables (sites) was 0.17%.

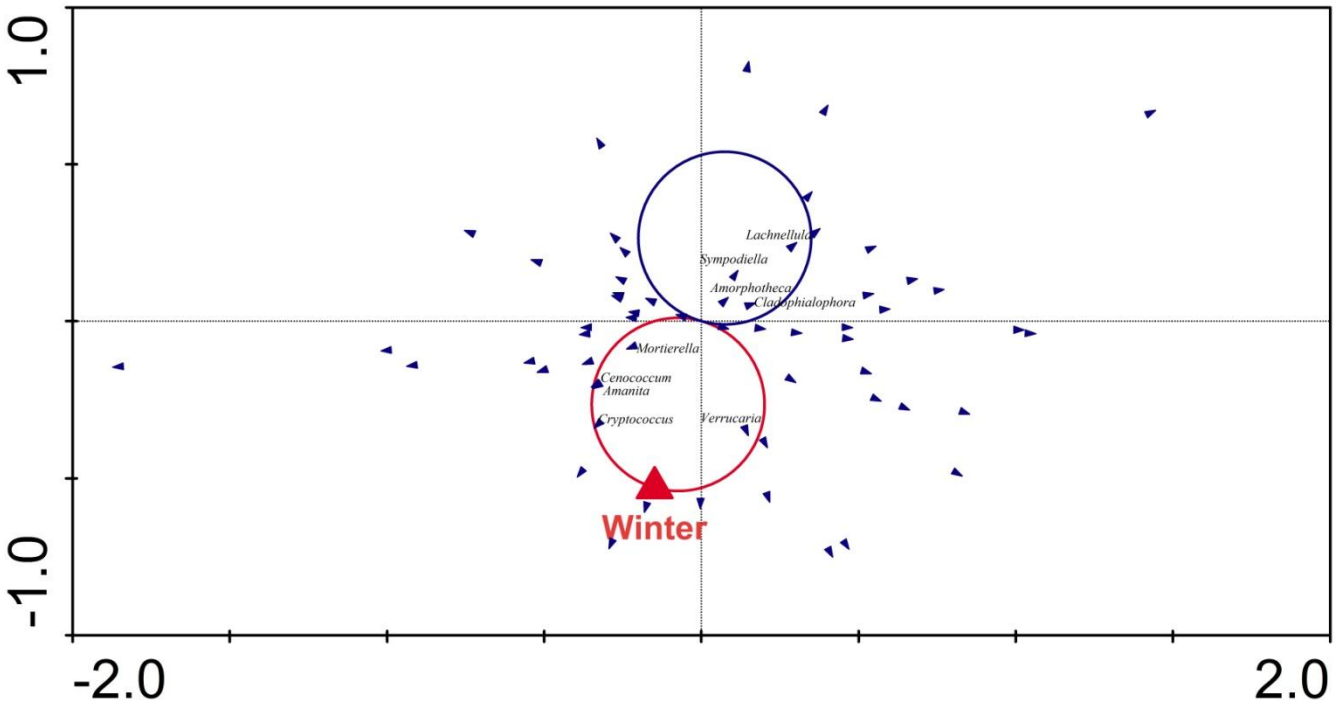
Triplot of genus abundance data, environmental variables and supplementary variables in the Figure 24 shows the association of individual genera with environmental factors. Grey arrows mark the supplementary factors (sites), which are not included in the analysis, but direction of the arrows shows the tendency of growing influence of factors if they were included in the analysis.

Figure 24: Grey arrows represent supplementary variables, which are sampling sites – Site 1, 2, 3, which do not influence distribution of species in the graph. Triangles with names represent fungal genera.


2.0
-3.0
-1.5
1.5
Scytalidium
Rhinocladiella
Piloderma
HS
Rhynchostoma
Ambispora
Ophiocordyceps
Acephala
Auriculoscypha
Hygrocybe
Organic horizon
Xerocomus
Penicillium
Elaphomyces
Site 2
Oidiodendron
Leucosporidiella
Hygrophorus
Russula
HW
Cenococcocum
Amanita
Cortinarius
Tricholoma
Neofabra
Meliniomyces
Mortierella
Cryptococcus
Candida
Site 1
Winter
Lactarius
Inocybe
Sporendocladiella
Sympodiella
Gymnopilus
Fulvoflamma
Acohumicola
LS
Amorphotheca
Lachnellula
Tylospora
Site 3
Cladonia
Cladophialophora
Neofusicoccum
Nolanea
Rhizoscyphus
Pseudeurotium
Botryosphaeria
Venturia
Furcaspora
Heterochaete
Xenochalara
Leptodontium
Ductifera
Chalara
Lophodermium
Marasmius
Melanotaenium
Varicosporium
Mycena
Hyalodendriella
LW
Phialocephala
Acremonium
Verrucaria
Glomus
Laccaria

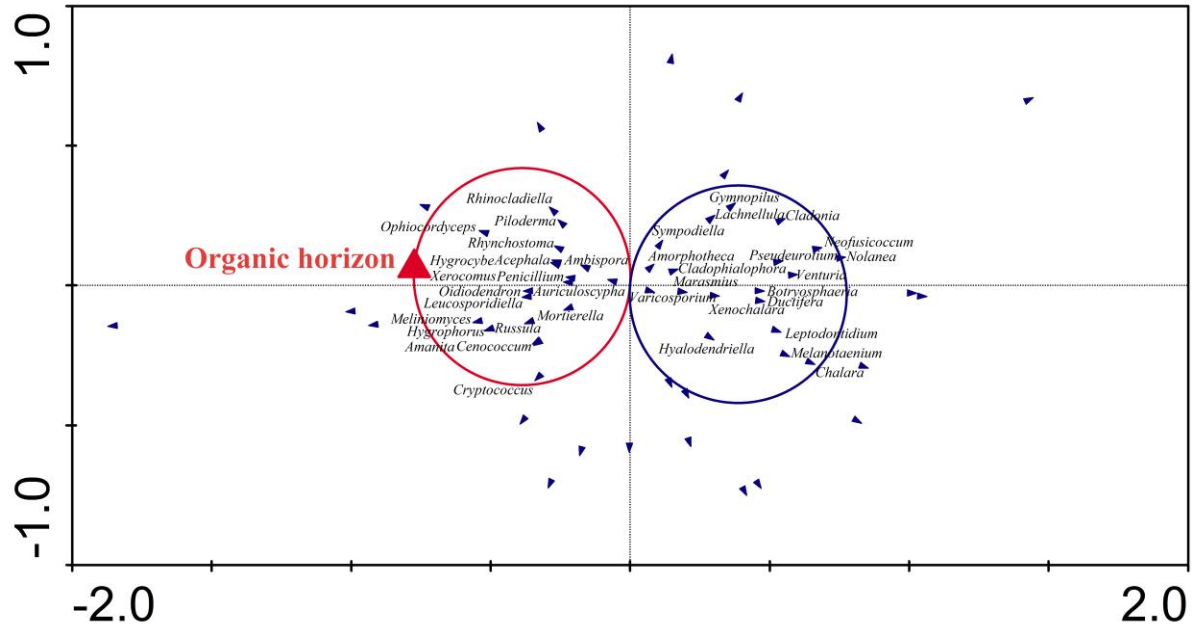
The Van Dobben circle analysis (Figure 25) identified the following saprotrophic genera to be significantly more abundant in summer: *Cladophialophora, Lachnellula, Amorphotheca, Sympodiella* and the fungi more abundant in winter: the ECM fungi *Amanita* and *Cenococcum,* the lichenised fungus *Verrucaria* and the saprotrophs *Cryptococcus* and *Mortierella.*

Figure 25: T-value plot of CCA analysis of 61 ITS based OTUs in dependence of season. Genera positively dependent on winter season have their arrow inside the red circle and species dependent on summer vice versa. For simplicity, only significantly affected species are named on the figure.



The saprotrophic genera *Pseudeurotium, Gymnopilus, Lachnellula, Sympodiella, Ductifera, Amorphotheca, Cladophialophora, Varicosporium, Chalara, Marasmius, Hyalodendriella,* mycorrhizal genera *Leptodontidium, Nolanea,* the parasites *Neofusicoccum, Venturia, Botryosphaeria, Melanotaenium,* the lichenised fungi of *Cladonia* and the endophytes *Xenochalara* were more abundant in the litter horizon (Figure 26). On the other hand, the ECM genera *Piloderma, Russula, Amanita, Cenococcum, Xerocomus, Hygrophorus,* the ericoid mycorrhizal genera *Oidiodendron,* the dark septate endophytes *Acephala, Meliniomyces,* the parasites *Ophiocordyceps, Auriculoscypha, Rynchostoma* and the saprotrophic fungi *Mortierella, Hygrocybe, Ambispora, Cryptococcus, Rhinocladiella, Leucosporidiella, Penicillium* were more abundant in the humus.

Figure 26: T-value plot of CCA analysis of 61 ITS based OTUs in dependence of horizon. Species positively dependent on organic horizon have their arrow inside the red circle and species dependent on litter horizon vice versa. For simplicity, only significantly affected species are named on the figure.



5.4.3 Phylogenetic analyses of cellulolytic and total fungal community

5.4.3.1 Phylogenetic analyses of *cbhI* nucleotide and protein sequences

A total of 1550 *cbhI* sequences from GenBank, and the fungal genomes (published by the Joint Genome Institute (JGI), USA, http://www.jgi.doe.org) were used identify environmental *cbhI* genes. The BioNJ tree was constructed for 128 sequences of which first 56 most abundant consensus sequences were the environmental ones, 46 were *cbhI* sequences obtained in this study from fungal isolates and the remaining sequences were from GenBank and JGI. Protein tree was constructed without pseudogene sequences of cloned fungi and environmental OTUs: 9, 12, 15, 14, 18,19, 30, 32, 34, 41, 44, 49, 53.

No identified sequences with high similarity could be assigned to the most of the environmental *cbhI* clusters and they thus remained unidentified at a species or genus level. The *cbhI* obtained from fungal isolates were highly helpful when trying to identify metagenomic *cbhI* isoforms in a tree.

Most of the *cbhI* clusters were specific for particular soil horizon and / or season as demonstrated for 56 dominant clusters in the Supplementary figure 15. Interestingly, related

clusters shared similar ecology in terms of the preference for particular horizon or season. This can be seen best in the Supplementary figure 16 where related clusters 29, 25, 42, 52, 33, 28 and 17 are all from the L horizon and are can be assigned to *Basidiomycota*. All unidentified *cbhI* clusters fall more-less to asco- or *basidiomycota* clade but one group of clades on the nucleotide tree Supplementary figure 15 cannot be assigned to any division due to the lack of bootstrap support. Clusters 1 and 51 are *basidiomycota* because they are on the same clade with *Jaapia argillacea, Phlebia brevispora* and *Mycena galopus* in both nucleotide and with *Phlebia brevispora* and *Volvariella volvacea* in protein tree. Cluster 34 is close to *Russula emetica / Mycena galopus* branch but in the protein tree it is within an unidentified group of sequences. Cluster 6 is identified as an ascomycota based both on DNA and protein sequence. Cluster 50 is ascomycete, because it clusters with *Ascomycota* in both trees, although only with low bootstrap values. Cluster 23 clusters together with clusters 8 and 35 found in H horizon in both trees and all these sequences are on the clade with the member of the *Mucoromycotina Mucor hiemalis* and with *Ascomycota Thysanophora penicillioides, Leptodontidium elatius, Cadophora malorum* and *Ceuthospora pinastri*; however, the position within this group is different in both trees. Clusters 30, 32 and 44 are assigned to the cluster of environmental sequences in both trees but are also on the same clade with *ascomycota* in the nucleotide tree, which has, however, only a small bootstrap value. Clusters 45 and 4 appear in protein tree as ascomycetes but in the nucleotide tree they cluster together with other environmental sequences. Cluster 2 clusters with a group of environmental sequences in the nucleotide tree but with *Ascomycota* in protein tree. Clusters 54 and 31 group together in the nucleotide tree but not in the protein tree, where they have lower branch support. Cluster 31 is on the same branch with *Chalara longipes* and cluster 54 is connected to the branch with asomycetous sequences in the protein tree although with low bootstrap values. Cluster 7 has low bootstrap supports in both trees, but clusters with ascomycetes in protein tree. Cluster 49 is close to *Cadophora malorum, Cadophora luteo-olivacea* and *Phialophora sp.* in the nucleotide tree with low bootstrap support while it groups with cluster 37 and *Russula emetica* in the protein tree with a 0.34 bootstrap value, thus the placement of this sequence remains unclear. Clusters 3, 20, 14, 19 are associated with the ascomycota *Glarea lozoyensis* in both trees. The clade of clusters 15, 18, 53 in the nucleotide tree had a bootstrap 1 while it was distributed among two remote clades in the protein tree with lower bootstrap support. Cluster 18 was on the branch with clusters 55, 15 in nucleotide tree and can be assigned to *Ascomycota*. Cluster 55 clustered in both trees with *Botryotinia fuckeliana* and *Sclerotinia sclerotiorum*, which suggests that it is an *ascomycete.*

Clusters 12, 38, 24 were on the same branch in both trees; in the nucleotide tree they were clearly associated with *Russula emetica*, while in the protein tree cluster 38 and 24 grouped with *Chalara longipes*. Clusters 43 and 5 were close to *Oidiodendron maius* in both trees with high bootstrap values. Cluster 37 was on the clade with *Russula emetica* with high bootstrap support in nucleotide tree but clustered with ascomycetes in protein tree, although its nucleotide sequence had similarity over 99% with *R.emetica*. Positions of clusters 10 and 13 are different among trees and show low bootstrap, but clusters with *Ascomycota* in both trees. In protein tree they were associated with *Ascomycetes*. Clusters 0 and 26 are on the clade with *Aspergillus sp*. in both trees. Cluster 36 clusters with different *Ascomycota* in both trees. Clusters 21 and 39 are on the same branch in both trees but do not group closely with any identified sequence. Cluster 46 is close to *Auricularia delicate* in nucleotide tree and to *Botryobasidium botryosum* but with higher support in the nucleotide tree. Clusters 33, 28, 17 are on the same branch in both trees with high support and cluster with *basidiomycota* sequences. Clusters 16, 48, 52 are on the same clade in the nucleotide tree and clusters 48, 52 are close to *Russula paludosa* thus can be assigned to *Basidiomycota* based on the protein sequence. Clusters 22 and 9 were associated with *Russula paludosa* in nucleotide tree and cluster 22 also in protein tree. Cluster 9 was on the clade with other environmental sequences and cluster 22 was on the clade with *Russula paludosa* and *Tramentes versicolor* and can thus be assigned to *basidiomycota*. Clusters 47 and 11 were on clade with *Russula paludosa* and *Tramentes versicolor* in nucleotide and protein tree, thus these clusters are probably *Basidiomycota*. Cluster 40 was close to *Tramentes versicolor* in both trees. Cluster 41 was on different clades in both trees and cannot be reliably assigned to a higher taxon. Cluster 27 in was on the clade with *Ascomycetes* in both trees. Clusters 29, 42, 25 were on the same clade with *R. paludosa* in both trees and can be assumed that they are *basidiomycota*. In the nucleotide tree, there is one clade and one branch of unidentified clusters, in the protein tree there are 5 unidentified clades. Some of sequences in these clades are identified in nucleotide tree – as cluster 12 being close to *R. emetica* with bootstrap value 1.

Interestingly some asco- and basidiomycota have very similar *cbhI* sequences. gi 325152901 *R.paludosa* was on the same clade with gi 361126979 *Glarea lozoyensis* in both trees. In addition, gi 351735591 *R.paludosa* was associated with ascomycetes in both trees. Also gi 351735589 *Mucor hiemalis* was associated with *Ascomycota* in both trees. Ascomycetous and basidiomycetous sequences were divided into phyla more precisely on the protein level than nucleotide ones, where pseudogens brought unnecessary noise.

5.4.3.2 Phylogenetic analysis of the ITS sequences

All consensus sequences of environmental OTUs with relative abundance over 1.6‰ were included into BioNJ tree. *Ascomycota* OTUs were found more often in the L horizon compared to the *Basidiomycota* (Supplementary figure 17). Consensus sequences of ECM fungi identified by PlutoF as *Russula ochroleuca* (OTU 5), *Lactarius rufus* (12), *Tylospora* (7) were found in the H and L horizon mostly in the winter. Other ECM fungi like *Xerocormus badius* (18), *Hygrophorus olivaceoalbus* (10), *Piloderma sp*. (2) were found in the H horizon only. Interestingly, sequences related to the *Mortierellales* were found in the H horizon even though these fungi are considered saprotrophs living on decomposing litter and organic matter.

In some cases, 165 bp of aligned 5.8S rDNA used to build a tree, were not accurate enough for an OTU to be assigned to the order or genus level. For example, consensus sequence of *Amanita sp.* (15) and one of *Tricholoma sp.* (8) did not cluster with asco – neither with basidiomyctes. In some cases, OTUs identifications by Plutof were flawed and these OTUs cluster in the tree somewhere else where they should. For example, consensus 43 clustered with *Ambispora callosa* and represented most distant sequence from the dataset. It was identified by PlutoF as *Candida sp.* with 97% of similarity but only 10% of coverage. Another misidentified fungus was consensus 58 identified by PlutoF as *Acremonium sp.* with 90% identity and 38% query coverage and it was clustering with *Glomus occultum,* although with low bootstrap support. In addition, other sequences from the bottom cluster of unidentified sequences confirmed flawed identification by PlutoF and clustered together because they were most distant from other sequences in alignment. Consensus sequence 68 identified by PlutoF as *Hygrocybe sp.,* which clustered with consensus sequence 55 (accurately assigned by Plutof to *Ambispora sp*.), showed similarity to uncultured *Basidomycota* (100% coverage and 95% similarity) and *Glomeromycota* (83% coverage and 97% similarity) from GenBank.

Most of environmental OTUs can be classified into orders or classes based on BioNJ tree. Some GenBank and environmental sequences were very closely related and thus created polytomies so OTUs can not be assigned to genera or order. This was case of some sequences in clusters *Agaricomycetes* and *Pezizomycotina*.

6. Discussion

Successional changes of dominant fungal taxa typically occur during the decomposition of plant litter (Frankland 1998). This also applies to needle litter that usually decompose more slowly *in situ* than other litter types (Osono *et al.* 2003; Osono and Takeda 2004; Korkama-Rajala *et al.* 2008). While the litter material in the initial stages of decomposition is often inhabited by opportunistic fungi, the proportion of efficient decomposers including ligninolytic basidiomycetes increases with time (Osono 2007).

In the present study, however, efficient decomposers of litter were isolated from all stages of needle litter decomposition and basidiomycetes were found to be associated with both the initial decay on trees (the wood associated species *F.pinicola* and *Peniophora sp*.) and the late stages, where litter-decomposers *M.androsaceus* and *M.galopus* were recovered. Though the former species are rather unexpected for litter needles, the presence of wood decomposing basidiomycetes does not seem accidental. Sokolski *et al.* (2007) recorded an unidentified member of *Peniophoraceae* and *Gloeophyllum sepiarium* as needle endophytes of *Picea mariana*. Neither *F.pinicola* nor *Peniophora sp.* were found in the soil metagenome. These wood-decaying fungi occurred only in the early stages of needles decomposition a probably were not able to compete with litter decaying fungi in later stages. In a previous study where direct isolation from *P.abies* needles after surface sterilization by ethanol and cupric chloride, the fungal isolate pool was dominated by opportunistic fungi from the genera *Penicillium, Cladosporium, Phomopsis* and *Mucor* (Przyby1 *et al.* 2008). In the present study, fast growing ubiquitous fungi (Table 1) were also isolated with low frequencies from needles in later stages of decay, but only *H.dematioides* and *T.penicillioides* were selected for further studies. Neither *H.dematioides* nor *T.penicillioides* were isolated from the soil horizons, but were isolated from the litterbag needles. This points out their restricted capacity for competition with fungi found in later stages of succession, because their enzymatic capabilities to decompose spruce needles were comparable (for *H.dematioides* isolate 194 from earlier stage even better than for the species isolate 22) to fungi found in later stages like *M.galopus* and *M.androsaceus*. In metagenome, *Mycena sp*. occurred mainly in L horizon, precisely in LW. In the cellulolytic community it was found in both horizons independently of the season. In total fungal community metageome, *Marasmius sp.* occurred only in L horizon with high abundance - 4% (when compared to most abundant species *Nolanea sp.* with 10%).

Changes in litter quality during decomposition are believed to be the most obvious cause of successional changes in the decomposer community. Indeed, the quality of litter changes substantially during decomposition. In both the *Q.petraea* and *P.abies* litters, cellulose decomposes more rapidly than hemicellulose, and lignin decomposition is very slow, resulting in its accumulation with time (Šnajdr *et al.* 2011). These chemical changes were well reflected in the relative activities of lignocellulose-decomposing enzymes during in situ decomposition of *Q. petraea litter*. Carbon mineralization during decomposition leads to a decrease of C/N and C/P ratio in litter (Osono and Takeda 2004, Voříšková *et al.* 2011). In *P. abies* needles, C/N ratio changes from 52 in mature needles over 47 in senescent needles to 44 in needles decayed in situ, and C/P decreases from 2 040 to 1 230 and 1 180 (Šantrůčková *et al.* 2006) and fungal succession on litter may partly also take place as a consequence of these changes.

Senescent spruce needles start to decompose while still attached to trees and these initial stages of decomposition may be performed by saprotrophic fungi already present in living needles as endophytes. Endophyte communities in *P.abies* needles are frequently dominated by *Lophodermium piceae* and other frequently recorded fungi include *Tiarosporella parca, T.penicillioides, Lirula macrospora* and *Rhizosphaera kalkhoffii* (Korkama-Rajala *et al.* 2008, Müller and Hallaksela 1998 and 2000). When spruce needles were left to decompose in laboratory microcosms only in the presence of endophytes, the same initial rate of decomposition was observed as in needles primed with nonsterile forest soil (Müller *et al.* 2001). On the other hand, litter containing exclusively endophytes decomposed more slowly than litterbags incubated in situ over a longer period of time (Korkama-Rajala *et al.* 2008). Only some endophytes continue decomposition when needles fall onto the forest floor. Within 2 years of in situ decomposition, *L.piceae* was still among the dominant needle litter-decomposers, but the abundance of basidiomycetes from the genera *Marasmius* and *Mycena* increased (Korkama-Rajala *et al.* 2008).

In our study, *L.piceae* was recorded only rarely and was not selected among the tested species. *L.piceae* was recorded in metagenome of the soil horizons. It was found in L horizon but also with low frequencies in H horizon. Lindhal *et al.* (2007) confirmed, that *Lophodermium spp.* belongs to the 'early' fungal community, defined as the taxa occurring with a higher frequency in litter samples compared to older organic matter and mineral soil.

C.longipes was previously shown to be involved in organic acid decomposition (Koukol *et al.* 2004). On the other hand, the same strain of *C.longipes* was not able to spread over sterile spruce needles in a microcosm experiment and caused no decomposition (Koukol *et al.*

2006). This species was also tested for decomposition ability on sterile *Abies* needles where it showed intermediate activity compared to other ascomycete strains (Osono and Takeda 2006) and on needles of *Pinus sylvestris*, where the result was similar (Boberg *et al.* 2010). The two strains used in our study belong to a complex of several species which are morphologically identical, but differ in their molecular characteristics and their phenotypical differences extend also to differences in their enzymatic capabilities.

Chalara sequences were also identified from the soil metagenome, where it occured in all horizons and season with the most of reads in LW, but it was not very abundant species. In cellulolytic communitiy it was found in LW.

The species *T.penicillioides* and *M.androsaceus* tested here previously showed similar decomposition rates on green and brown spruce needles (Koukol *et al.* 2008) but on *Abies* needles, *T.penicillioides* exhibited only slow decomposition (Osono and Takeda 2006). These differences may be attributed to intraspecific differences between strains.

In earlier decomposition tests, basidiomycetes were usually superior to ascomycetous species in litter (Osono and Takeda 2006, Osono 2007, Tanesaka *et al.* 1993) and basidiomycetes isolated from the same habitat as ascomycetes had significantly higher activities of ligninolytic enzymes and several hydrolases (Baldrian *et al.* 2011). These observations, however, might be biased by the testing of opportunistic fungi among ascomycetes that have limited capability of enzyme production. The comparison of enzyme activities in ascomycetes from *Q.petraea* topsoil obtained by the dilution plate method with isolates from surface-sterilized litter isolated in this study shows, that the former group produced significantly less esterase, lipase, acid phosphatase, β-glucosidase, N-acetylglucosaminidase, α-mannosidase and especially laccase (Baldrian *et al.* 2011), which indicates that interior colonizers may be superior in enzyme production.

My results show that the potential of fungi decomposing attached needles to produce extracellular hydrolytic enzymes is rich and essentially similar to that of later stage decomposers. Although the rate of production of individual enzymes differed among fungal isolates (and even between two strains of the same species), these differences did not correspond with the decomposition stage when the fungi were isolated.

If the fungi from different stages of decomposition have comparable abilities to decompose litter and to produce different extracellular enzymes, a question arises what is the reason for successional changes in the needle-associated decomposer community? One of the possible explanations is that while many fungi are able to decompose litter of different quality, their individual competitive abilities depend on litter composition and their communities change

as a consequence of the competition. Another explanation accepts occurrence of a rather stochastic group of species out of a set of species with preference for particular substrata. The dominance of a particular species can be due to a coincidence of several factors including geographical area, (micro)climatic conditions or biotic factors, such as the competitive/mutualistic interactions with other organisms present in the substratum.

The results of this study did not show a clear link between an individual enzyme or a group of enzymes, and overall needle decomposition. High decomposition rates compared to previous studies on fungal needle litter decomposition might be partly due to the pre-cultivation of fungal mycelia on a nutrient-rich medium before spruce needle addition. The methodology or strain differences may also be responsible for the observed difference between this study and that of Boberg *et al.* (2010) on *Pinus litter*. *Mycena sp*. was one of the most potent decomposers of *Pinus litter*, but the worst in this study, whereas the most potent decomposer in the present study was *Hormonema spp*., while a member of the same genus, *H.dematioides*

(Sydowia polyspora) barely caused any mass loss during 1 year on *Pinus* litter.

Malt extract agar represents a complex substratum theoretically suitable for the comparison of enzyme production in fungi of different origin but different nutritional requirements. Our results, however, indicate that enzyme assays based on ME agar cultures might overlook the activity of some enzymes expressed on litter and other natural substrata - natural substrata are, therefore, more suitable for the characterization of decomposition abilities of saprotrophic fungi. Our study shows that there are no major differences among fungi from different stages of *P.abies* needle succession in terms of decomposition rates and enzyme production, including between basidiomycete and ascomycetes. However, more studies on natural substrata accompanied by fungal competition studies are required to understand the ecology of successive enzymatic decomposition of litter.

Decomposition of organic matter by fungi, which begins in the needle litter, continues in the soil. Soil is a heterogeneous environment, which can be divided into soil horizons. Lindhal *et al.* (2007) and O'Brien *et al.* (2005) have already proved stratification of fungal OTUs across soil horizons. Fungal communities in studied *Picea abies* soil were diverse and vertically stratified due to different amount of organic matter, C and N content and decreasing nutrient availability with depth, creating two separated niches for fungi.

I have examined the effect of soil horizons and seasons on the soil fungal community. Courty *et al.* (2008) and Walker *et al.* (2008) observed seasonal changes in composition of

ectomycorrhizal fungal community in oak forest, but the dominant taxa were present all the time. Buée *et al.* (2005) also showed seasonal changes in community composition and metabolic activity of ectomycorrhizal fungi. In boreal forest, high seasonal but low interannual variation was observed for total fungal community (Izzo *et al.* 2005, Taylor *et al.* 2010).

In metagenome of total fungal community from the spruce forest topsoil at a sampling depth of 700 for ITS yielded 94-177 OTUs per sample. Most fungal sequences recovered from the L horizon belonged to saprothrophic fungi and these from the H horizon were mostly mycorrhizal fungi. The L horizon exhibited higher evenness – 38 ± 17 OTUs were needed to cover 80% of fungal diversity comparing to 26 ± 15 OTUs in H horizon, but significantly more species were needed to cover 80% diversity in L horizon in winter when compared to H horizon or litter in summer. This is in agreement with Baldrian *et al.* (2012), who studied the same area in the Bohemian Forest Natural Park, where 18 ± 8 OTUs were needed to cover 50% of diversity in L horizon and only 9 ± 3 OTUs in H horizon. Also in RNA community presented by Baldrian *et al.* (2012) was L horizon richer than H one. It seems that fungal community is more balanced in terms of species number in summer across horizons than in winter. The Chao1 estimates were significantly higher for the LW than in other samples. In Baldrian`s *et al.* (2012) paper, community Evenness varied across horizons in DNA and RNA from 0.7 to 0.8, which is in a good agreement with Evenness I had obtained. Fungal sequences from forest topsoil belonged mainly to Dikarya (49.2% *Basidiomycota* and 47.2% *Ascomycota*). *Mucoromycotina* were represented by 1.68% of the sequences, *Glomeromycota* by 1.08%, *Chytridiomycota* by 0.3%, *Zoopagomycotina* by 0.09%, *Blastocladiomycota* by 0.05%, *Entomophthoromycotina* by 0.13%, *Oomycetes* 0.02%, *Cryptomycota* 0.01%, plants and animals sequences by 0.17% of ITS sequences.

In this study significantly higher abundances in summer were recorded for genera: *Amorphotheca, Cladophialophora, Sympodiella, Gymnopilus, Piloderma, Rhynchostoma* and *Lachnellula*. On the other hand, *Amanita, Cenococcum, Mortierella, Xenochalara, Xerocomus, Cadophora Verrucaria* and *Cryptococcus* were more abundant in winter. The dominance of *Cenococcum geophilum* in winter has also been reported (Baier *et al.* 2006).

Lindhal *et al.* (2007) found the fungi belonging to the *Dothideomycetes* in the ‘early’ fungal community, defined as the taxa occurring with a higher frequency in litter samples compared with older organic matter and mineral soil. In this study, there were only few members recorded from *Dothideomycetes* and two of them preferred L horizon: *Botryosphaeria* (0.18%) and V*enturia* (0.35%). In this study, the member of *Dothideomycetes* – *Cenococcum*

occured more frequently in the H horizon which would be rather typical for a ‘late’ fungus. This is in good compliance with the results of Courty *et al.* (2008), which isolated DNA from root tips of oak trees and found *Cenococcum geophilum* to occur in the A2 (organic) horizon. According to Baldrian *et al.* (2012) *Botryosphaeriales, Lecanorales* and *Eurotiales* were significantly more abundant in the L horizon. In this study, the order *Lecanorales* was represented by the lichenised fungus *Cladonia* occurring in the L horizon. Representatives of the *Eurotiales* order *Elaphomyces* and *Penicillium* were more frequent in the H horizon. Representatives of the order *Chaetothyriales* were found in the L horizon by Baldrian *et al.* (2012) and here the sequences from *Amorphotheca* were recovered from litter and these of *Rhinocladiella* from the organic horizon. The whole order showed higher abundance in the L horizon and in the summer.

Lindhal *et al.* (2007) found in the ‘late’ fungal community taxa from within the *Helotiales*, but these were different taxa from the helotialean fungi detected in the surface litter. Baldrian *et al.* (2012) had found the members of the *Helotiales* in the H horizon which was confirmed in this study. Other *Helotiales* species were, however, more frequent in the litter and the members of the *Helotiales* representing the ‘early’ fungal community were also isolated from needles on fallen trees or litterbags: *Phacidiopycnis sp*., *Chalara longipes, Cistella acuum, Ceuthospora pinastri.* In agreement with Lindhal *et al.* (2007) I have found the genera *Marasmius* and *Mycena* mostly in the L horizon. According to Lindhal *et al.* (2007) the *Mortierellomycotina* and *Mucoromycotina* fungi (*Mortierella* and *Umbelopsis spp.)* also belong to the ‘late’ community. In this study, *Mortierella* (1.6%) and *Umbelopsis spp*. (0.033%) occured mostly in H horizon. In contrary to Lindhal`s *et al.* (2007) findings, *Rhizoscyphus ericae,* the most thoroughly studied ericoid mycorrhizal fungus, was more abundant in this study (0.25%) than other ericoid mycorrhizal fungus *Capronia taxa* (0.008%). *Rhizoscyphus ericae* did not show any significant dependence on any horizon maybe because *Vaccinium myrtillus*, which is most abundant ericoid plant in Šumava spruce forest, has shallow roots rooting in thin layer of organic horizon as well as in the litter horizon. Among fungi found in the ‘late’ fungal community, ectomycorrhizal basidiomycetes *Amanita* (1.2%), *Elaphomyces* (0.73%), *Hygrophorus* (2.3%), *Piloderma(8.3%) Russula* (3.4%), *Tricholoma* (4%) *Xerocormus* (1%) and *Piloderma* (8.3%) dominated with high frequencies in H horizon. Most of the ECM fungi including the genera *Cortinarius, Laccaria, Amanita, Tricholoma, Hygrophorus,* and *Piloderma* were confirmed from A1 = H horizon and *Inocybe sp.* from A2 horizon = mineral horizon in oak forest, when mycorrhizal root tips were examined (Courty *et al.* 2008). *Piloderma* was confirmed to be highly abundant species

in coniferous forests by several studies (Baldrian *et al.* 2012, Lindhal *et al.* 2007, Taylor 2010) and together with ectomycorrhizal *Tylospora* were also the most abundant in the boreal *Picea abies* forests in Finland and Sweden (Korkama *et al.* 2006, Rosling *et al.*2003, Wallander *et al.*2010). Here Tylospora represented 3.7% of all sequences and did not show any significant trends in it occurrence. *Piloderma* was found to be indicator of the season. In this study, it showed higher abundance in summer (September), but according to Taylor *et al.* (2010) it should rather indicate spring (May) and winter (February). *Cortinarius* was showed to be abundant (Bueé *et al.* 2009) in H horizon (Lindhal *et al.* 2007, Taylor *et al.* 2010) and was significantly abundant in spring (Taylor *et al.* 2010). In this study *Cortinarius* (0.17%) had slightly higher abundance in the H horizon and was found in both seasons. The most abundant ectomycorrhizal basidiomycete - *Nolanea* (10% of all sequences) was significantly more abundant in the L horizon than H. This contradiction can be probably explained by shallow rooting of spruce trees. Saprotrophe *Mycena* was abundant in L horizon in O`Brien *et al.* (2005) study. In this thesis, *Mycena* (0.18%) was found mostly in L horizon and slightly more in the winter. Another abundant saprotrophic species found by classical ITS cloning channeled by O`Brien *et al.* (2005) was *Cryptococcus* found in litter. I have found the fungus (0.91%) in all horizons and seasons but it was significantly more abundant in HW. *Russula* showed strong association with H horizon (this study, Baldrian *et al.* 2012, Lindhal *et al.* 2007, O`Brien *et al.* 2005, Taylor *et al.* 2010). O`Brien *et al.* (2005) had recorded sequences of ECM fungi *Tomentella, Tylospora, Hygrocybe* from mineral horizon. I have found *Tomentella* (0.16%) exclusively in HW on one site; *Tylospora* (3.7%) in H horizon but also in LS with high abundance and saprotroph *Hygrocybe* (0.49%) in H horizon. Although *Tylospora sp.* is considered to be an ectomycorrhizal fungus it was highly abundant in L horizon sequenced by Baldrian *et al.* (2012).

In terms of cellulolytic community, there were 51-97 clusters recorded when *cbhI* sequences were resampled at 267 per sample. L horizon was richer in OTUs and show more seasonal variance than H horizon. It seems that H horizon is more balanced in terms of species and seasonal impact on them. Evenness indicates that species were distributed evenly through the population of cellulolytic fungi and there were no extremely dominant species.

From the 56 *cbhI* most abundant clusters included in the BioNj nucleotide tree, 53 clusters were assigned to phyla, of which 31 belonged to the *Ascomycota* and 22 to the *Basidiomycota*. Štrusová *et al.* (2012) observed that 94% of *cbhI* sequences in microcosms containing organic horizon from a similar environment belonged to the *Ascomycota*, while I

did not find any differences in the occurrence of *Ascomycota* clusters among soil horizons, but *Basidiomycota* were slightly more abundant in the L horizon.

Among fungi isolated from spruce needles and whose *cbhI* sequence was cloned successfully, *Hypocrea* is a typical fast-growing non-basidiomycetous fungi that are able to efficiently degrade cellulose (Baldrian *et al.* 2011, Baldrian and Valášková 2008, Deacon *et al.* 2006, Štrusová *et al.* 2012). In the study of Štrusová *et al.* (2012) *Geomyces* belonged to the most abundant genera among fungi utilizing cellulose, but I was not able to clone any *cbhI* sequence from *Geomyces panorum* neither did I find any *cbhI* sequences similar to *Geomyce*s in the environmental sequences. It is possible that *Geomyces* in Štrusová *et al.* (2012) study was just paraziting on other fungi, which were truly able to decompose cellulose and was feeding on simple sugars released into the microcosms. Alternatively, the fungus can use another enzyme for cellulose decomposition or contain an intron in the sequence of primers used for *cbhI* amplification. Basidiomycetous cord-formers represented mainly by the genera *Marasmius* and *Mycena* were found to decompose cellulose in Štrusová *et al.* (2012) and Baldrian *et al.* (2011). This study confirmed the presence of the *cbhI* gene in *Mycena galopus* but not in *Marasmius androsaceus.* Although the basidiomycetous yeast *Cryptococcus* was abundant in H horizon in the total fungal community (0.9%) its abundance in soil can reach as much as 1/3 of all sequences (Bueé *et al.* 2009).

According to Štrusová *et al.* (2012) *Russula emetica* was an abundant species in litter microcosms but showed only low incorporation of cellulose-derived C. In a previous study, members of the *Russulales* have been found to be inactive during winter, considered to be the period of prevailing organic matter decomposition (Baldrian *et al.*, 2012). I have identified 9 *cbhI* OTUs clustering with *Russula* species. This finding can suggest that ectomycorhizzal fungi may be more capable to decompose litter than was originally thought and have highly abundant cellulolytic genes in the environmental DNA. Štrusová *et al.* (2012) showed that members of the *Dothideales, Leotiomycetes, Tremellales* and *Chaetothyriales* orders were also incorporating cellulose-derived carbon.

Some of the fungi isolated from *Picae abies* needles where cellobiohydrolase activity was recorded and the *cbhI* gene was sequenced were also present in the total fungal community from the topsoil. One of these fungi was *Mycena galopus,* which was present mostly in the LW but also in the LS, less frequently also from the HS. *Mycena* is a saprotroph found in upper layers of soil - litter horizon (Frankland *et al.* 1995). Members of the genus *Chalara* was isolated from needles on fallen tree, litterbags and form soil. It was found mostly in from the LS and LW, but also in the HS. *Chalara* is a saprothrophic fungi and it was also isolated

from the litter in the same ecosystem. *Thysanophora penicillioides* was isolated from litterbags and was also present in the metagenomic DNA. Finding *Thysanophora penicillioides* in the organic horizon was unpredicted because this saprothrophic fungus was previously found mostly in the litter (Iwamoto *et al.* 2005). Fungi *Sirococcus conigenus* (isolated from the needles of a fallen tree in a similar ecosystem), *Hormonema dematioides* (litterbags), *Ceuthospora pinastri* (litterbags), *Cylidrocarpon magnusianum* (needles on fallen tree and litterbags), *Phacidiopycnis sp*.(needles on fallen tree), *Trichoderma pleuroticola* (litterbags) were not recorded from the soil by 454-pyrosequencing. *Cistella acuum* was found on needles on fallen tree and litterbags and also in the soil in the litter by pyrosequencing. *Marasmius* was found in the litter horizon on *Picea abies* needles and also detected in the litter by pyrosequencing. *Fomitopsis pinicola* and *Peniophora sp.* were isolated from needles on fallen tree but were not found in the topsoil DNA, which is understandable due to the fact that they are ligninolytic wood-decomposing fungi. Some fungal strains from which *cbhI* gene was cloned were found in metagenome of total fungal community: *Russula, Cadophora, Phialophora, Leptodontium, Oidiodendron* and *Mucor.* In this study, *Russula ochroleuca* was found in H horizon but also in L horizon, which is surprising because it is an ectomycorrhizal fungus occurring mostly in an organic horizon (Courty *et al.* 2008). A parasitic fungus *Cadophora malorum* was found in H horizon, which is in discrepancy with its ecology but *Cadophora finlandica* found in both horizons, can form ERM with ericoid hosts and ECM with ectomycorrhizal hosts (Vrålstad *et al.* 2002). Fungi isolated from litterbags or litter horizon were recorded by 454-pyrosequencing also from the litter horizon.

Multiple isoforms of the *cbhI* gene were identified in fungal isolates. *Ascomycota* and *Basidiomycota* possess more than one gen isoform, suggesting that they may play bigger role in cellulose decomposition than originally thought. We can only argue whether different isoforms have different functions. The exocellulase activity in the studied fungi was confirmed by enzymatic tests. We may guess that some of these isoforms, if they are functional, would act differently in cellulose decomposition. Until now, there is only one record on differences among isolated *CBHI* enzyme isoforms. Medve *et al.* (1998) showed that the isoenzymes of *Trichoderma reesei* had different isoelectric points (pI) but their catalytic and substrate-binding properties were similar. I have shown that *CbhI* isoenzymes are probably orthologs, but also horizontal transfer might theoretically take place resulting in different placing of *cbhI* isoforms of the same fungus in a phylogenetic tree and its similarity to the genes of unrelated taxa. We do not know whether *CbhI* enzymes have the same or

different function just based on their partial sequence, but *cbhI* genes in constructed phylogenetic trees isolated from *basidiomycota* were clustering with *basidiomycota* and from *ascomycota* with *ascomycota*, thus seems to be conserved amongst phyla.

The assessment of diversity by molecular methods presents several experimental and informatics hitches that introduce uncertainties into diversity estimation (Huse *et al.* 2007, Kunin *et al.* 2010, Quince *et al.* 2009) and which are further complicated by the huge amount of unknown fungal taxa likely to be found in soils (Buée *et al.* 2009, Schmit and Mueller 2007). From an experimental point of view, PCR and sequencing errors, as well as alignment and clustering methods based on approximate algorithms, may create artificial OTUs which may inflate the diversity estimation (Huse *et al.* 2007 and 2010, Kunin *et al.* 2010, Quince *et al.* 2009). In this study, 62% of PlutoF ITS hits were assigned sufficiently, meaning that OTU had at least 80% similarity with hit and was aligned at least on 70% of its length. The question remains about the OTUs showing lower similarity to known taxa that may represent so far unknown species or genera. For these fungi, further efforts should be oriented towards isolation and characterization of their traits.

7. Conclusion

Fungi from different decomposition stages from spruce needles were isolated into pure cultures. This collection of fungi represents different succession stages of fungal community on *Picea abies* needles. I supposed that if different species were present on the litter material in different time of succession they would possess different enzymatic equipment for litter degradation. Although species from different succession stages differ in ecology, they did not differ significantly in production of enzymes, either their quality or quantity. To verify ability of isolated fungi to decompose cellulose – the main component of spruce needles, *cbhI* genes were amplified and cloned. The *cbhI* gene as cellulolytic proxy were also cloned from species of fungal collection and from sporocarps previously showed to be able to degradate cellulose (Štrusová *et al.* 2012). The analysis of the *cbhI* genes of fungal isolates obtained in this study confirmed the previous findings of Edwards *et al.* (2008) that most fungal species contain more than one gene (typically 2-3) and that these are very often highly dissimilar. The most of isolated fungi from spruce litter were *Ascomycota* and proved to be particularly

important for cellulose hydrolysis and that the fungi involved in cellulose decomposition contain the *cbhI* gene.

To follow the succession in later stages of decomposition, analysis of fungal community in litter and organic horizons of the soil were examined by 454-pyrosequencing. Previous studies of Baldrian *et al.* (2012), Bueé *et al.* (2009), Izzo *et al.* (2004), Lindhal *et al.* (2007), O`Brien *et al.* (2005), Taylor *et al.* (2010) indicate horizontal distribution of fungal communities in the soil. Total fungal community was examined with ITS1 and ITS4 primer. From 11866 sequences were constructed 1267 clusters and identified 275 species. Among the 61 most abundant genera recovered, 73% showed clear preference for one soil horizon and 37% exhibited differences in abundance among the summer and winter. From 26 saprotrophic fungi 11 were more frequent in the L horizon and 7 in the H horizon, while from the 22 mycorrhizal fungi 12 were more frequent in the H horizon and only 3 in the L horizon. Generally, mycorrhizal fungi were found mostly in the H horizon and saprotrophic, parasitic or lichenised fungi in the L horizon. Studies of Buée *et al.* (2005), Courty *et al.* (2008), Izzo *et al.* (2005), Taylor *et al.* (2010) and Walker *et al.* (2008) proved seasonal differences between fungal communities. I supposed that mycorrhizal fungi should have higher biomass in summer season when they have enough root exudates from the photosynthesizing trees. Saprotrophic fungi should then outcompete the mycorhizzal fungi in the upper layers of soil in winter due to the better enzymatic equipment for litter decomposition and the reduction of nutrient availability in the mycorhizzal fungi. Contrary to the expectation, six mycorrhizal fungi were found to be more abundant in winter and only one in summer, while six saprotrophic fungi were dominant in summer and 7 in winter. The higher preference for winter can be due to the stable temperature and water content during the winter when snow layer is present (the temperature of soil is >0°C under the snow cover). The *cbhI* gene was demonstrated to be present in ectomycorrhizal fungi, so it is possible that in times of low nutrient support from trees ECM fungi can partially switch to saprotrophic way of life.

Fungal community was also explored in terms of functional *cbhI* gene as proxy representing cellulolytic community. Cellulolytic community should respond flexibly to changes in cellulose availability during the year and in different soil horizons representing very different environments when comes to nutrient availability. Although cloned *cbhI* genes from isolated fungi helped a lot, identification of cellulolytic OTUs was demanding because of the limited amount of sequences present in the public databases. Like total fungal community, there were OTUs significantly dependent on some horizon or season and resilient part of community. From 56 clusters involved into analyses, 62% of *cbhI* OTUs depend on some

soil horizon and 21% on some season. Of 31 *Ascomycota* 6 OTUs were dependent on LS, one on LW, 2 on HW, 10 on H horizon without seasonal influence and 6 only on L horizon. From 22 identified *Basidiomycota* 2 were significantly dependent on HW, 2 on H and 7 on L horizon only. Unidentified cluster 4 was positively influenced by HW. *CbhI* community seems to be more stable trough the year and not so much affected by soil horizons as the total fungal community.

The analyses of environmental samples were strongly influenced by differences of fungal community composition among sampling sites. This is an important finding for the future research showing that ecosystems must be described by analyzing many independent samples. The influence of environmental factors on fungal communities – in particular the seasonality – is expectable for the active part of the community, which can be analyzed as a pool of RNA or the metatranscriptome. Since *cbhI* is only one of multiple genes involved in ligno-cellulose decomposition, more genes have to be included into future analyses to describe the complexity of cellulolytic communities. One of recently reviewed enzyme - polysaccharide monooxygenase, seems to have great potential in decomposition of ligno-cellulose also of other organic compounds (Žifčákova and Baldrian 2012).These analyses are planned in my future research.

8. Buffers and solutions

Buffers and solutions used for enzyme assays:

100 mM succinate-lactate buffer, pH 4.5:

100 ml deionised H_2O

1.64 g DL-lactic acid, sodium salt 60%

0.146 g succinic acid

100 mM citrate – 200 mM phosphate buffer, pH 5:

100 ml deionised H_2O

3.56 g $Na_2HPO_4.2H_2O$

2.1 g citric acid, monohydrate

100 mM phosphate buffer, pH=6.5 (for DMAB dissolution):

100 ml deionised H_2O

0.95 g KH2PO4
0.54 g Na2HPO4.2H2O

50 mM Acetate buffer,pH 5:
1000 ml distilled H_2O
900 µl acetic acid
2.78 g sodium acetate

0.08% (w/v) ABTS
25 mM DMAB
1 mM MBTH
2 mM MnSO4
5 mM H_2O_2
2 mM EDTA

ZYM A
25 g Tris-hydroxymethyl-aminomethane
11 ml 37 % HCl
10 g sodium lauryl sulfate
100 ml H2O

ZYM B
0.12 g Fast Blue BB
50 ml methanol
50 ml dimethyl sulfoxide

Azo-CM cellulose:
40 ml H_2O heated to 85-90ºC
5 ml deionised H_2O
5 ml 2 M acetate buffer pH 5
1 g Azo-CM-cellulose
0.02 g sodium azide
Azo-CM-cellulose and hot water was mixed 15 min before adding the rest of chemicals.

Azo-Xylan :

Azo-Xylan (1% w/v final concentration) was dissolved in 11.428 ml acetic acid and mixed with 800 ml of deionised water.pH was adjusted to 4.5 with HCl. Than 200 ml of water was added.

Precipitant:

40 ml deionised H_2O

10 g sodium acetate x 3 H_2O

1 g zinc acetate

pH 5 was achieved by adding HCl and the volume was complete to 50 ml. Then 200 ml of 95% ethanol was added to the final solution.

Enzymes stated below, were measured by following procedures:

Laccase (EC 1.10.3.2)

In a 96-well microplate were mixed:

150 µl citrate-phosphate buffer, pH 5

50 µl sample

50 µl ABTS solution

We observed the spectrophotometric change of absorbance at 420 nm for 3 minutes in 30 s intervals and than for 12 minutes in 2 minutes intervals (Šnajdr *et al*. 2008).

Mn-peroxidase (EC 1.11.1.13)

In a 96-well microplate were mixed:

200 µl AR or AP or AB

50 µl sample

AR, AP and AB consist of:

AB: succinate-lactate buffer, DMAB, MBTH, EDTA, water (15:2:1:1:1 vol/vol)

AP: succinate-lactate buffer, DMAB, MBTH, EDTA and H_2O_2 (15:2:1:1:1 vol/vol)

AR: succinate-lactate buffer, DMAB, MBTH, $MnSO_4$ and H_2O_2 (15:2:1:1:1 vol/vol)

We examined the spectrophotometric change of absorbance at 590 nm (Ngo and Lenhoff 1980) for 3 minutes in 30 s intervals and than for 12 minutes in 2 minutes intervals (Šnajdr *et al*. 2008). The activity of all oxidases is defined by the measurement of sample with the AB solution. The activity of peroxidases, without MnP, is defined by the measurement of sample with the AP solution minus measured activity of the AB solution. The activity of MnP is

defined by the measurement of sample with the AR solution minus measured activity of the AP solution. EDTA in the AP and the AB solutions replaced MnSO4 to chelate any Mn ions present in the measured solution.

Endo-1,4-β-glucanase (EC 3.2.1.4)

The test substrate supplier protocol was followed (Megazyme, Ireland).150 µl of a sample (as a blank was used distilled water) was mixed with 150 µl Azo-CM cellulose, the chromogenic substrate. The mixture was vortexed and incubated for 120 minutes at 40ºC. Then 750 µl of ethanol-based precipitant was added. An eppendorf tube with mixture was vortexed 10 s and centrifugated 10 min at 10000 x g. The absorbance of supernatant was measured at 595 nm in a spectrofotometr (Šnajdr *et al*. 2008).

Endo-1,4- β -xylanase (EC 3.2.1.8)

The protocol is identical with the endoglucanase protocol, but like a chromogenic substrate was used Azo-xylan.

Measuring of enzymes using MUF and AMC as substrates:

In a 96-well microplate were mixed:

160 µl substrate

40 µl sample

Chromogenic substrates for fluorescently measured enzymes:

1,4-β-N-acetylglucosaminidase (EC 3.2.1.52) 1.2 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide

cellobiohydrolyse (EC 3.2.1.91) 1.2 mM p-nitrophenyl-β-D-cellobioside

1,4-β-glucosidase (EC 3.2.1.21) 1.2 mM pnitrophenyl-β-D-glucoside

1,4-β-xylosidase (EC 3.2.1.37) 1.2 mM p-nitrophenyl-β-D-xyloside

arylsulfatase (EC 3.1.6.1) 4 mM p-nitrophenylsulfate

acid phosphatase (EC 3.1.3.1) 2 g / l p-nitrophenylphosphate

leucine aminopeptidase (EC 3.4.11.1) L-leucine-7-AMC

alanine aminopeptidase (EC 3.4.11.2) L-alanine-7-AMC

8.1 Special chemicals

ABTS Sigma

AC-Axo-CM cellulose M e gazyme

AC-Axo-Xylan M e g azyme

Agarose United States Biochemical

BSA New England Biolabs

10x buffer for DyNAzyme DNA Polymeraze Finnzymes

Citric acid Sigma

CloneJETTM PCR Cloning Kit Fermentas

CTAB Serva

Cyclohexane Chromservis

DL-lactic acid, sodium salt Sigma

DMAB Sigma

6X DNA Loading Dye Fermentas

DyNAzyme II DNA polymerase Finnzymes

EDTA Amersham

Ethidium bromide Fluka

Formamide Serva

Glass beads 0.5 mm and 0.1mm Biospec products Inc

HEPES Serva

Isoamyl alcohol Sigma

Isopropanol Sigma

MBTH Sigma

Methanol Sigma

Mini Elute Purification Quiagen

O'GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use Fermentas

PCR Nucleotide Mix Roche

Phenol for molecular biology Serva

pJET2.1forward Generi Biotech

pJET2.1reverse Generi Biotech

p-nitrophenyl-N-acetyl-β-D-glucosaminide S i g m a

p-nitrophenyl phosphate S i g m a

p-nitrophenyl sulfate S i g m a

p-nitrophenyl-β-D-cellobioside S i g m a

p-nitrophenyl-β-D-glucoside S i g m a

p-nitrophenyl-β-D-glucoside S i g m a

Primer ITS1F Generi Biotech

Primer ITS4 Generi Biotech

Primer ITS4B Generi Biotech

Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) S i g m a

Wizard SV Gel and PCR Clean Up System Promega

9. References

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