Linking biodiversity and ecology of fungi from pine and spruce needles

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HABILITATION THESIS

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CONTENTS

PREFACE (INCL. ACKNOWLEDGEMENTS)

This habilitation thesis is composed of sixteen papers published in international peer-reviewed journals and one monography that resulted from my research carried out during the past decade. Since my very first beginnings as a master degree student at the Department of Botany of the Faculty of Science at Charles University in Prague, I was interested in microfungi associated with pines. As the time passed, my interests widened to include topics like fungal biodiversity and succession, taxonomy, nutrient transformation, and interactions with other organisms. I also got involved in studies of fungi associated with spruces. Together, these studies represent a rather heterogeneous mixture of methodical approaches, yet the main underlying question remains the same: **What is the biodiversity of fungi colonizing pine and spruce needles, and what is the role of these fungi in these habitats?** I nevertheless divided this thesis into four chapters covering different topics:

Diversity patterns, concerning the diversity of needle endophytes and the diversity of fungi colonizing needles in pine and spruce litter.

Overlooked, rare and cryptic species, concerning the diversity previously not recognized using morphological species concepts with an example of how well-delimited morphological species may consist of several phylogenetic lineages and the difficulties in interpreting these results.

Nutrient transformation during decomposition, concerning the role of individual fungal species in litter decomposition and nutrient transformation with emphasis on carbon sources, phosphorus forms and humic compounds.

Interaction with other organisms, where special attention was paid to one highly diverse group of microarthropods and one protistan group directly affecting fungal communities colonizing coniferous litter and vice versa.

I would like to express my gratitude to my university friends, who later became my co-workers (Miroslav Kolařík, Jan Mourek and David Svoboda), to my colleagues from the Department of Mycorrhizal Symbioses of the Institute of Botany, Czech Academy of Sciences (Marcela Kovářová, Libor Mrnka, David Püschel, Jana Rydlová and Martin Vohník), to my supervisors, who were always helpful during my master and postgraduate studies (Karel Prášil and Miroslav Vosátka), and to my co-workers, whose expertise in fields completely unknown to me proved indispensable for my research (Petr Baldrian, Milan Gryndler, Věroslava Hadincová, Richard Hrabal, Zdeněk Janovský, David Minter, František Novák). Most of the work would be impossible without the painstaking and time-consuming work of my students that helped me during all phases of research, including field sampling, fungal isolations, setting up of experiments, and DNA and statistical analyses. A special thank goes to Fred Rooks, who kindly read and improved the final version of this thesis.

The list would not be complete without special thanks to my ever-patient and understanding wife.

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INTRODUCTION AND GENERAL DISCUSSION

Pines (*Pinus* spp., Pinaceae, Pinophyta) represent the largest genus of conifers. With more than 110 currently recognized species, pines are key and frequently dominant trees of temporal, boreal, subalpine and tropical forests, as well as arid woodlands of the Northern Hemisphere. They are an important source of wood, paper, resins, charcoal and food, and have been planted for economic reasons or as components of parks and gardens on all continents except Antarctica (Gernandt et al. 2005). Among them, *Pin. sylvestris* represents the most widely distributed pine species in the world with enormous ecological and economic importance in Europe (Eckenwalder 2009). Spruces (*Picea* spp., Pinaceae, Pinophyta), with more than 30 species, represent the most prominent trees of cold regions and montane habitats in the Northern Hemisphere due to their adaptation to snowy winters. Owing to exceptionally long wood fibres, they have high economic importance in timber production (Eckenwalder 2009). In Europe, *Pic. abies* is one of the dominant tree species with a natural distribution in the boreal zone and in higher-altitude forests in temperate zones; currently, however, spruce is spreading south as a result of natural expansion and artificial planting (Seppä et al. 2009).

DIVERSITY PATTERNS

The current number of recognized fungal species exceeds 100,000, but estimates of real fungal diversity reach 5–6 million (Blackwell 2011, Taylor et al. 2014). Pine- and spruce-dominated forests are not obvious hot-spots of fungal diversity compared to e.g. tropical forests (Hawksworth & Rossman 1997). On the other hand, pine and spruce needles, which persist for several seasons and decompose only slowly due to a high content of recalcitrant chemical substances (which also applies to twigs, branches, bark, wood and roots), offer enormous microhabitat diversity supporting a rich community of fungi (above all microfungi). In connection with the common occurrence of these conifers in Europe and North America, that is, regions with a long tradition of "mycofloristic" studies (Gremmen 1960, Minter 1980), our knowledge about the diversity of fungi associated with pines is paralleled in only a few other plant species (such as *Eucalyptus* species among trees and *Urtica dioica* among herbs). More or less systematic biodiversity surveys (Gremmen 1960, Minter 1980, Minter & Holubová-Jechová 1981) supplemented with the first surveys involving the culturing of litter needles (Kendrick 1958a, Maanen & Gourbière 1997, Tokumasu 1998a, b, Tokumasu et al. 1994) together with taxonomical studies (Minter 1981) yielded several hundred species records in association with pines. Hawksworth (2001) mentioned 893 species known from *Pin. sylvestris*, 186 out of which are known from this species only. A more recent and comprehensive on-line list of fungal species associated with pines (Marmolejo & Minter 2006) offers a more detailed perspective on this species richness. Depending on the host pine species or substrate, the database, for example, contains 1,200 species associated with *Pin. sylvestris* out of which 950 species are associated with needles and needle litter. This figure, which is based on scientific literature, specimens in fungal reference collections, and unpublished field observations, is still underestimated, considering the recent surge in records of fungi new to pines from hitherto understudied pine species (Prihatini et al. 2015a, Sanz-Ros et al. 2015). Unfortunately, no such project has been conducted for spruces, but the species list probably contains at least several hundred species.

Substantial species diversity is present already in living needles that are colonized by horizontally transmitted endophytes. Though the first complex studies of endophytic fungal communities in needles of spruces (Carroll & Carroll 1978) historically preceded those on pines (Helander et al. 1994), the "fascination" by pines has lasted to this day, owing to the higher diversity of pine species and ecosystems where they occur. Budding pine needles are virtually endophyte-free, and the rate of infection and fungal richness grow with increasing needle age (Deckert & Peterson 2000, Hata et al. 1998, Helander et al. 1994, Kowalski & Zych 2002), though the effect is not simply additive; i.e., the frequency of occurrence of particular fungal species may show decreasing tendency with time. Depending on the climatic conditions, endophytic fungal communities at different sites markedly differ in richness, species composition and the identity of the dominant endophyte species (Arnold 2007, Botella et al. 2010). In some cases, differences between sites are clearly attributable to a single environmental factor such as rainfall intensity (Prihatini et al. 2015b). Rainfall was also found to be the most important factor promoting higher species richness when different seasons were compared (Zamora et al. 2008), though season was also found to have a negligible effect (Guo et al. 2008, Helander et al. 1994). When viewing the infection frequency and species richness along the latitudinal gradient and comparing with leaves of broadleaved trees, pines and spruces often harbour more diverse communities than their latitudinal position would suggest, probably due to their long-lived foliage (Arnold & Lutzoni 2007). Last but not least, human activities also affect needle endophytes. Air pollution by heavy metals has had a negative effect on endophytic fungal communities, decreasing fungal infection rates and diversity (Helander 1995, Jurc et al. 1996, Kowalski & Zych 2002), though some species were not affected.

Considering the phylogenetic context of endophytes, they belong almost exclusively to the ascomycetes, though each time members of different classes dominate. Botella et al. (2010) and Prihatini et al. (2015b) identified the Dothideomycetes as the richest and most abundant class, though Arnold (2007), Hata et al. (1998), Helander (1995) and Kowalski & Zych (2002) found endophytes to be dominated by the Leotiomycetes. Unlike the biodiversity-oriented surveys aimed at pines, similar studies of endophytes in spruces are rare (Higgins et al. 2007, Lorenzi et al. 2006, Sokolski et al. 2007, Stefani & Bérubé 2006) , and most of the research has been aimed at specific questions, such as of the link between needle endophytes and aquatic fungi (Sokolski et al. 2006a) and the participation of endophytes in early stages of decomposition (Müller et al. 2001). To fill this knowledge gap, we looked into the biodiversity of endophytes in needles of *Pic. abies* in a montane forest in the Šumava National Park (Czech Republic). During our preliminary tests of the suitability of the sampling and cultivation method, we found that needles still attached to wind-fallen trees harboured a rich fungal community. As summarised in **PAPER I** (Koukol et al. 2012), the fungal community differed from those obtained in previous studies aimed at spruce needle endophytes. The montane climate (at elevations above 1,000 m), a dramatic change in nutrient composition (sudden cessation of assimilation) and microclimatic conditions (exposed parts of the crown) were identified as the main factors responsible for this uniqueness. The identified fungal species (mostly belonging to the Helotiales) showed substantial overlap in host affinities, most prominently between *Pinus* and *Picea*, and also among species from distant plant lineages (Figure 1). We also noted a substantial overestimation of species counts

based on delimited morphospecies. Especially in case of sterile cultures, we were too conservative, and the final ratio of originally recognized morphospecies to species defined based on a combination of phenotypic and molecular data (ITS and 28S rDNA) reached 3.6:1. Such a complex and critical approach is important to avoid overestimating the number of taxonomic units based solely on gross cultural morphology or one DNA region only (Arnold et al. 2007). Nevertheless, the greater number of strains used for the sequencing was beneficial for further demarcation of intra- and interspecific boundaries between newly described fungal species (Koukol 2011b).

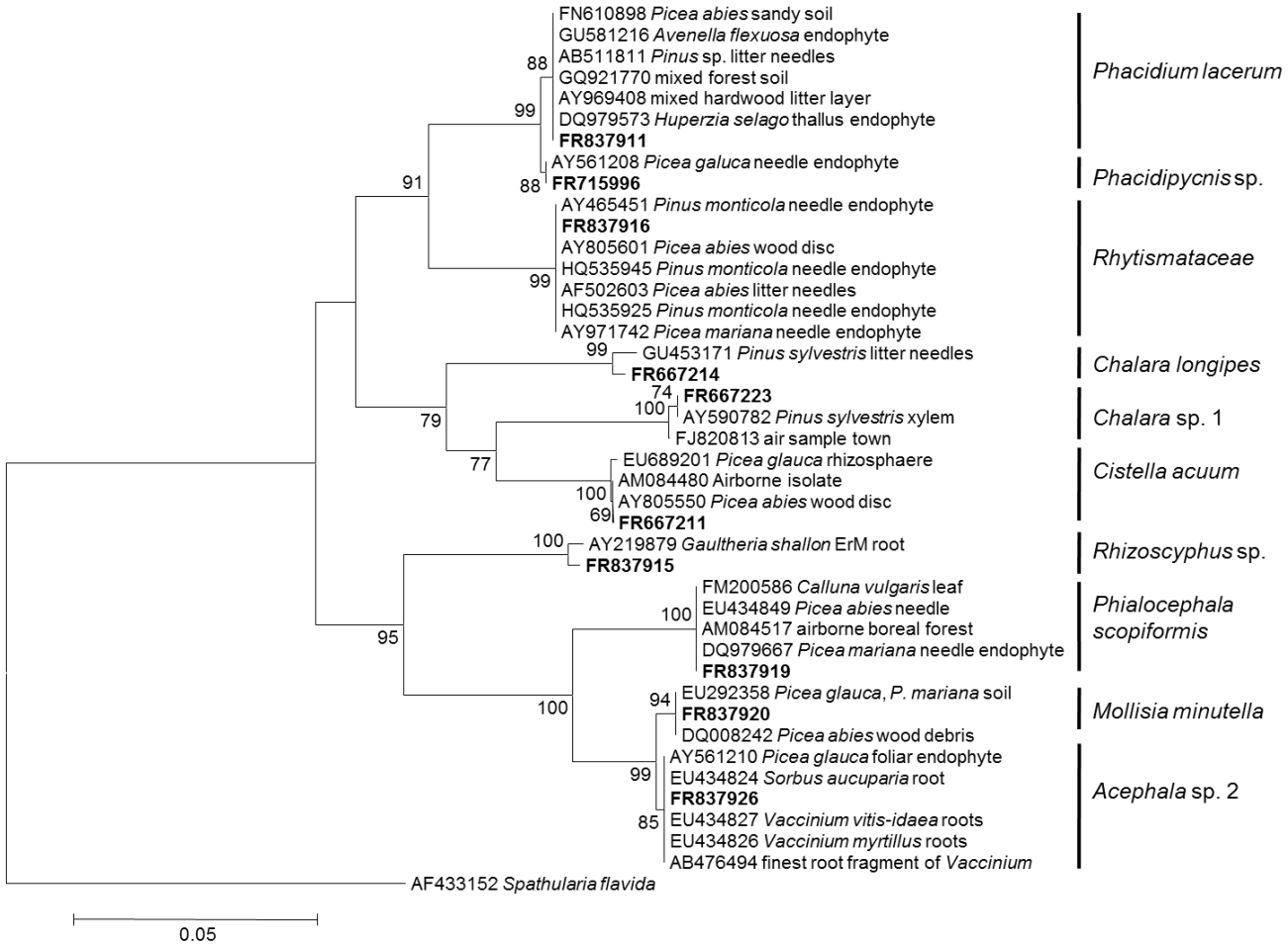

Figure 1: A phylogeny of species of the Helotiales based on analysis of ITS and 28S rDNA showing that conspecific fungi were isolated as endophytes or saprotrophs from pine needles and litter and endophytes of ericoid shrubs, grasses and lycopodiophytes (from **PAPER I**)

From the overview above, it is clear that whenever an endophytic fungal community in pine or spruce needles is surveyed, the species composition is mostly unique with a few dominating fungal species (usually not recorded as dominant in other studies), a certain proportion of fungal species with low incidence (recorded also in other studies) and the majority of species recorded only rarely, mostly as singletons (some of them recorded also in other studies). Local environmental conditions affect the community as a whole (species richness and composition), but there is limited evidence that they allow to predict the incidence of individual fungal species. The occurrence and incidence (or absence) of a particular species are merely stochastic because horizontally transmitted endophytes mirror

the available airborne inoculum. The majority of them are specifically associated with their host trees with a later shift towards pathogens (Botella et al. 2010) or early saprotrophs (Osono & Hirose 2011), but a proportion of the endophyte community are actually fungi whose spores landed on a non-host plant (Porras-Alfaro & Bayman 2011).

When pine and spruce needles enter the litter, they are exposed to a community of fungi accompanied by other (micro)organisms. Depending on whether they are naturally senescent, prematurely fallen due to fungal pathogens, insect attack or following mechanical detachment, they are already pre-colonized by endophytes or foliar pathogens (Minter & Millar 1980). These fungi may limit colonization by other fungal species via competition (Gourbière et al. 2001), participate in decomposition (Müller et al. 2001, Osono & Hirose 2011) or be soon replaced in succession by litter-dwelling fungi (Mitchell & Millar 1978). Besides fungi, needles are attacked also by numerous invertebrate organisms that facilitate their decomposition (Ponge 1991). Due to a combination of numerous biotic and abiotic factors, the majority of fungi specific to coniferous needles sporulate or produce fruiting bodies in litter, which enables their targeted sampling and identification (Minter & Holubová-Jechová 1981) or isolation into pure cultures (Gremmen 1960).

In **PAPER II** (Koukol 2007), I compared the diversity and frequency of occurrence of microfungi colonizing needles in the litter of two pine species, the native *Pin. sylvestris* and the introduced (and invasive) *Pin. strobus* growing in the sandstone area of the Bohemian Switzerland National Park (Czech Republic). Both pine species hosted a comparable number of fungal species (35 and 33 species). Most of them (29 species) were recorded on needles of both pines, though with different frequencies of occurrence. The only species that was obviously co-introduced together with *Pin. strobus* was *Meloderma desmazieri* (Rhytismatales, Leotiomycetes), a weak foliar pathogen of *Pin. strobus* (Deckert & Peterson 2000) causing sporadic local outbreaks of needle cast in plantations (Bednářová et al. 2013). Comparable species richness was recorded also on needles in the litter of one local and four introduced pine species in Japan (Tokumasu 1978). Surprisingly, the invasive *Pin. strobus* hosted similar root-associated ectomycorrhizal fungi as *Pin. sylvestris* growing in the same area (Kohout et al. 2011). These results point to the limited effect of introduced congeneric coniferous species on local fungal communities and suggest that saprotrophs and ectomycorrhizal species are adaptable to closely related hosts.

Temperature of cultivation may also affect the observed diversity of microfungi, as I found out in **PAPER III** (Koukol 2011). Needles of *Pin. sylvestris* in litter sampled at two sites differing in the extent of snow cover remaining in early spring revealed higher fungal diversity when cultivated at a temperature close to the natural conditions. Even if it seems a trivial finding, diversity data from studies using cultivation at room temperature (even when needles were collected during cold periods) (Tokumasu 1998a) might have been underestimated.

To characterize in more detail the fungal community in needles of montane spruce forest outlined in **PAPER I**, we further focused on studying the succession of decomposing needles and compared two different methodical approaches in **PAPER IV** (Haňáčková et al. 2015). Isolation of fungi after surface sterilization of needles and terminal restriction fragment length polymorphism analysis of the total DNA extracted from the needles revealed similar dominant species over the course of fungal succession. Unexpectedly, we recorded a shift in

the dominant fungal group when compared to needles still attached to wind-fallen trees at the same locality. During the initial stage of decomposition, members of the order Dothideales were dominant over members of the Helotiales. We further investigated whether fungal succession on needles in litter differs between sites where bark-beetle attacks had killed adult trees, stopping the yearly input of fresh litter, and control sites. The negative effect of the bark beetle attack was only apparent during the initial stages of decomposition, i.e., in the absence of fungi typical for freshly fallen needles. These two studies were succeeded by a survey of metabolic activities of dominant fungal species (**PAPER I**) and is discussed below.

What links **PAPERS I-IV** with the other above-mentioned studies of fungal communities in pine and spruce needles is the culture-based approach. This approach has been used systematically to study pine- and spruce-associated microfungi since the late 1950s (Hayes 1965, Kendrick 1958b), and its limits are well known: restriction to culturable species only, underestimation of fungal diversity due to different growth rates in primary isolations (slow growers may be overgrown and remain unnoticed) and its overestimation by records of contaminating fungi present only as spores (surviving the sterilization procedure). The culture-based approach seems to specifically restrict basidiomycetes, but on the other hand, when compared to the environmental DNA-based method, it can also lead to severe underestimation of the diversity of particular ascomycete classes (Arnold et al. 2007). Still, the culture-based approach is indispensable, especially in the context of linking the biodiversity of endophytic and litter-dwelling fungi with their taxonomy and function. An adequate substrate sterilization method, suitable cultivation conditions, precise delimitation of isolated strains into morphospecies and their identification based on thorough characterization of phenotypic and molecular data are the preconditions for further treatment of these strains in taxonomic (Koukol 2011b, Sokolski et al. 2006b) and ecological studies (Korkama-Rajala et al. 2008, Müller et al. 2001) or prospecting of isolated strains for bioactive secondary metabolites (Qadri et al. 2014).

As concerns fungi colonizing needles in litter and soil, the phenotype-based approach (direct sampling and isolation or combination of both of them) has the potential to reveal only a small proportion of their diversity, the “apparent” fungal diversity. The “actual” diversity is obviously much higher due to the existence of overlooked rare fungi, unculturable fungi and fungi that produce only vegetative mycelium disabling their recognition on the substrate or identification in culture. Unravelling the “actual” diversity of fungi may be possible with surveys adopting DNA sequence–based characterization of fungal communities from environmental DNA (Buée et al. 2009, Lindahl et al. 2007, O'Brien et al. 2005, Schadt et al. 2003). Besides detailed semiquantitative analyses of fungal communities in large sample sets, these methods have led to discoveries of previously unknown fungal lineages (Rosling et al. 2011) and have the potential to provide ecological information in detail and magnitude exceeding previous methods (Baldrian et al. 2012, Lindahl et al. 2013). Because of my previous focus on the culture-based approach only, the advantages and disadvantages of sequence-based methods and their comparison with the culture-based approach are beyond the scope of this thesis.

OVERLOOKED, RARE AND CRYPTIC SPECIES

Considering that pine and spruce litter in Europe has been systematically studied for almost sixty years (Kendrick 1958a, Söderström 1975) it may seem surprising that species new to science are still described from these habitats. These species represent either hitherto overlooked species (Crous & Groenewald 2010) or very rare species recorded frequently as single colonies (Crous et al. 2012, Koukol & Kolářová 2010). Recent additions to the list of such species include also cryptic species, i.e. species that are phylogenetically distinct, reproductively isolated, but lacking or with subtle morphological differences. The limitations of applying morphological species concepts to fungi became apparent already when mating tests diagnosed more species than recognized by morphology (Shear & Dodge 1927), i.e. long before molecular data became available. Mating tests were successfully used to define biological species especially within basidiomycetes (Petersen & Hughes 1999), but this approach cannot be applied universally to all fungi because of limits in their culturability and due to the absence of meiospores (Taylor et al. 2000). Easier acquisition of molecular data speeded the recognition of cryptic species and significantly altered the overall estimates of fungal diversity.

Cryptic speciation among fungi colonizing coniferous needles is mainly documented in fungal pathogens of pines and spruces in connection with surveys of their distribution ranges, host specificity and pathogenicity (Alamouti et al. 2011, De Wet et al. 2003, Schneider et al. 2009, Smith et al. 2003). Most attention has been paid to cryptic speciation in the genus *Lophodermium* (Rhytismatales, Leotiomycetes), which comprises more than 30 described species colonizing pine needles. They are distributed worldwide and show both host specificity and host universality as well as pathogenic and saprotrophic strategies (Minter 1981). They are also frequently present as endophytes in living needles (Müller et al. 2001). The similar morphology of ascomata and the co-occurrence of different species in the same host and geographic area (Oono et al. 2014) hinder the identification of species, and rare or hitherto undescribed species may be easily overlooked (Sokolski et al. 2004). Proper identification of species may be thus done only based on a combination of ecological data (geography, host pine, effect on the host), phenotypic data (morphology of ascomata on needles, morphology and growth in culture) and molecular data (mostly sequences of ITS rDNA). Numerous sequences from strains isolated mostly as endophytes were obtained in the last decade and frequently attributed to *L. pinastri*. Reignoux et al. (2009) thoroughly characterized strains of *Lophodermium* isolated from three locations in Scotland using the multi-locus phylogenetic species recognition approach and population genetic analysis. They recognized three cryptic taxa within *L. pinastri* that were well supported by a sequence-based phylogeny and phenotypic data in culture. Rather than describing two new species, Reignoux et al. (2014) pointed to potential conspecificity of one of these cryptic taxa with *L. staleyi*, a rare species specific to *Pin. sylvestris* that may be easily misidentified as *L. pinastri.* This species occurring in native pinewoods in Scotland (Minter 1981) is not represented by a reference sequence in the GenBank database. In **PAPER V** (Koukol et al. 2015), we provided evidence of two more cryptic species within the *L. pinastri* aggregate and also within *L. conigenum*. However, our main goal was to thoroughly characterize fungal strains with a morphology typical of members of the genus *Lophodermium* originating from prematurely fallen needles of *Pin. mugo* in the Karkonosze National Park, Poland (Pusz et al. 2013).

Rather surprisingly, though the species is morphologically almost indistinguishable from *L. pinastri*, analysis of ITS rDNA revealed that it forms a distinct lineage closely related to the parasitic species *L. conigenum* and *L. seditiosum* and largely unrelated to *L. pini-mugonis*, which has recently been described from needles of *Pin. mugo* in the Austrian Alps (Hou et al. 2008). This result was partly supported by an analysis of sequences for the actin gene, though it was less informative because only few actin sequences of *Lophodermium* are available in the GenBank database. The newly described species *L. corconticum* seems to be rare and endemic growing on mountain pine in the Krkonoše mountain range, though its potential occurrence in other regions should be verified by fresh sampling.

Another genus with an increasing number of known species despite its limited morphological features is *Fusicladium* (Venturiales, Dothideomycetes). As currently circumscribed based on phylogenetic affinities, this genus mostly includes biotrophic, leaf-spotting pathogens whose host affinity may represent one of the speciation factors, and also saprotrophs (Crous et al. 2007). Among saprotrophs, two species (*F. pini* and *F. ramoconidii*) differing only in the presence/absence of ramoconidia were described based on single isolations from pine needles in litter. I found a striking similarity of these two species with a fungus that I observed relatively frequently colonizing litter needles of *Pin. strobus* and identified as *Septonema ochraceum* (*Incertae sedis*, Dothideomycetes) in **PAPER II**. In **PAPER VI** (Koukol 2009), I found that though this fungus was morphologically indistinguishable from both *F. pini* and *F. ramoconidii* and was found in the same habitat, a phylogeny based on ITS and 28S rDNA separated it clearly from the two other pine colonizers. The species was described as *F. cordae* and might have been considered cryptic. The identity of *S. ochraceum* remained obscure in the absence of type material and with the recognition of two other strains supposedly representing *S. ochraceum* as distinct species in the genera *Fuscicladium* and *Cladophialophora* (*Incertae sedis*, Dothideomycetes).

Cryptic speciation is obviously more frequent than expected and remains unnoticed until multiple strains of a supposedly single species from different hosts or geographical localities are thoroughly characterized by molecular data. This is the case of *Desmazierella acicola* (Pezizales, Pezizomycetes), which is frequently found on pine needles in litter layers of European and Asian forests, and irregularly in pine plantations in North America (Maanen & Gourbière 1997, Pfister et al. 2008, Tubaki & Saitô 1969). In **PAPER VII** (Martinović et al. 2016), we characterised multiple strains of *D. acicola* based on their phenotypic characteristics and molecular data. Multiple phylogenetic lineages identified by analyses of three gene regions were recognized among thirty strains of *D. acicola* obtained from pine and spruce forests in various parts of the world, albeit with an ambivalent interpretation. On the one hand, the phylogenies derived from two coding genes (*TEF-1α* and *RPB2*) yielded incongruent topologies, and the morphology of the anamorph was highly variable among as well as within the lineages. On the other hand, some of the lineages exhibited clear host and geographical specificity, similar growth characteristics in culture, and the most distant ones shared only an 88% similarity in their ITS rDNA, which is far less than the lowest general estimate for species delimitation (Kõljalg et al. 2013). This inconsistency could not have been resolved in absence of the teleomorph, which is formed only during a very short period in early spring, and its morphological characteristics, so that we finally decided to treat the lineages as distinct populations of a single species.

NUTRIENT TRANSFORMATION DURING DECOMPOSITION

Coniferous litter, with its high carbon/nitrogen and carbon/phosphorus ratios and high amounts of nutrients fixed in recalcitrant compounds, selects for fungi able to acquire these sources and mobilize the limiting resources (above all nitrogen and phosphorus). A prominent role in the decomposition of needles and turnover of organic nutrients has been attributed to basidiomycetes. They represent key actors in carbon cycling, especially in boreal forests (Lindahl et al. 2002). Saprotrophic basidiomycetes producing strongly hydrolytic and oxidative enzymes (polyphenoloxidase and peroxidase enzymes) also possess long-lived mycelia that are adapted to colonization of large areas within litter, its decomposition and efficient translocation of nutrients (Boberg et al. 2014, Boddy 1999, Cairney 2005). Moreover, degradation of own senescent mycelium and recycling of the nitrogen contained within it is of substantial advantage in this nitrogen-limiting environment (Lindahl & Finlay 2006).

Coniferous litter is not homogeneous and besides needles contains cones, twigs, pieces of barks and wood. Naturally senescent needles are mixed with prematurely fallen green needles, needles fallen due to fungal pathogens and needles still attached to twigs. These different needle types may be colonized by different communities of microfungi (Minter & Millar 1980), and supposedly might have different attractiveness for saprotrophic basidiomycetes. In **PAPER VIII** (Koukol et al. 2008), we hypothesized that *Gymnopus androsaceus* (Agaricales, Agaricomycetes) would be affected by spruce needles of different quality and would decompose green needles (originating from premature defoliation) more slowly due to their higher integrity and intact cuticle than naturally senescent brown needles. Nevertheless, the fungus caused higher mass loss of green needles, obviously taking advantage of the high content of easily degradable carbon sources. The high content of phenolics (*p*-hydroxyacetophenone and catechin) in fresh needles even seemed to be stimulating, and *G. androsaceus* degraded the phenolics efficiently. We did not observe faster degradation of green needles when combined with brown needles, which was later confirmed by Boberg et al. (2014), who observed reallocation of nitrogen from old litter into freshly fallen litter, but without change in the decomposition rate of freshly fallen litter.

Considering that saprotrophic basidiomycetes redistribute rather than release nutrients (Boberg et al. 2014), their mycelium itself represents a substantial pool (Figure 2). Towards deeper soil horizons, the decreasing quality of needles (limited accessible carbon sources) promotes their colonization by ectomycorrhizal species supplied with carbon from plant assimilates (Lindahl et al. 2007). The competitive advantage of ectomycorrhizal species may also lead to capture of organic nutrients such as phosphorus from the saprotrophic basidiomycetes (Lindahl et al. 1999). However, this transfer of phosphorus may be also reverted (Lindahl et al. 2001).

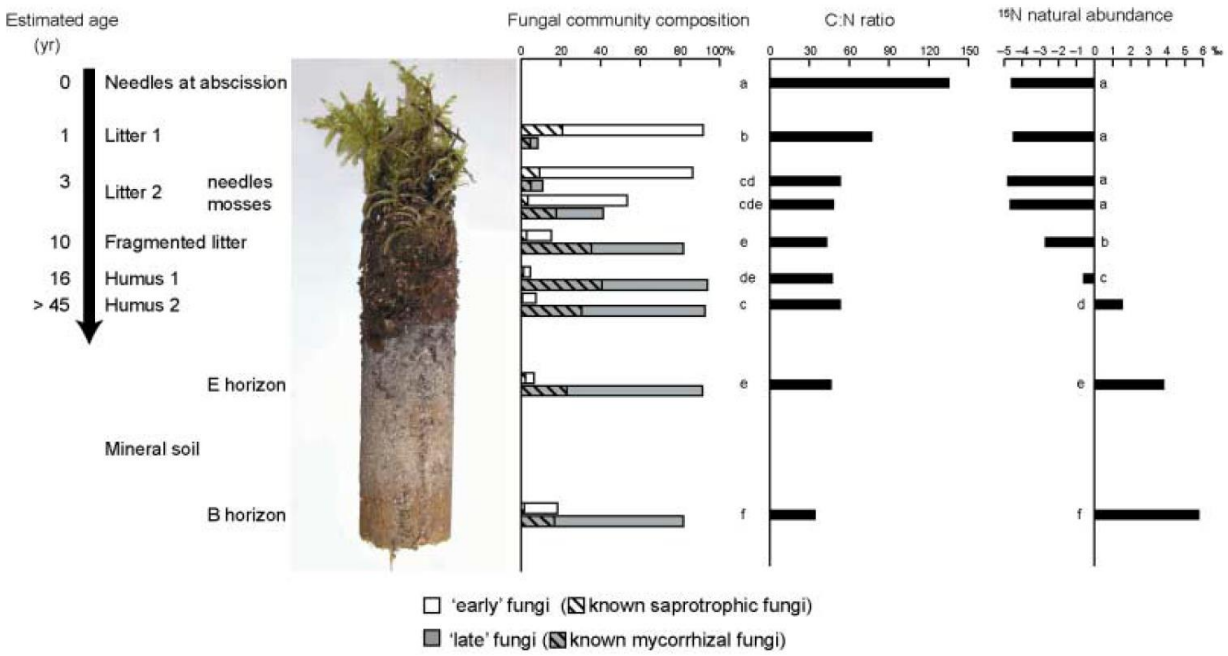

Figure 2: Fungal community composition depending on time and litter characteristics in boreal *Pinus sylvestris* forest (after Lindahl & al. 2007)

Different forms of phosphorus are present in fresh plant litter, deeper soil horizons and fungal mycelia. Microorganisms, including fungi, are involved in their transformation (Turner et al. 2004). Therefore, in **PAPER IX** (Koukol et al. 2006), we analysed phosphorus forms derived from spruce needles colonized and decomposed by six individual autochthonous fungal species and their mixture *in vitro*. All fungi were directly involved in phosphorus transformation and transformed orthophosphate monoesters and diesters derived from spruce litter needles into diphosphates and polyphosphates. We also recorded fungal production of phosphonates, a peculiar form of phosphorus with a C-P bond, most prominent in the metabolic activities of *G. androsaceus* and *Phacidium lacerum* (Helotiales, Leotiomycetes). The presence of phosphonates in soil had been obscure, and their origin had been attributed to various microorganisms (Turner et al. 2004, Turner et al. 2003), but we provided direct evidence of their fungal origin for the first time. This was confirmed also in **PAPER X** (Koukol et al. 2008), where we regularly detected phosphonates in fruiting bodies of six basidiomycetes (saprotrophic and ectomycorrhizal) from a mountain spruce forest.

By contrast, the majority of ascomycetes colonizing coniferous litter is mostly unit-restricted and spread by their spores to new substrate units (e.g. single needles) (Gourbière & Gourbière 2002). Considered solely as decomposers, thy are supposed to play a limited role with the ability to degrade relatively easily accessible carbon source such as cellulose (Osono et al. 2003, Osono & Takeda 2006, Virzo De Santo et al. 2002) and only exceptionally may degrade minor fractions of lignin (Boberg et al. 2011, Osono & Hirose 2011). To avoid competition for nutrients with basidiomycetes, ascomycetes benefit from their presence in needles as endophytes prior to their abscission and participate only in early stages of litter decomposition (Boberg et al. 2011, Müller et al. 2001), though some of them may still be active even after two years (Korkama-Rajala et al. 2008). In **PAPER XI** (Žifčáková et al. 2011), we found that the decomposition abilities of fungi retrieved from the endophytic

community in needles and representatives of later litter colonizers were comparable. There was no significant difference in the overall enzyme production between these two ecological groups; ascomycetes differed from basidiomycetes only by the absence of activity of Mn-peroxidase. This study was complemented by **PAPER XII** (Koukol & Baldrian 2012), where we analysed the decomposition abilities of twelve phylogenetically unrelated ascomycetes representing the autochthonous mycobiota of pine needles in litter. Most of the strains produced enzymes involved in cellulose degradation, but their activities differed. *Desmazierella acicola* produced most of the tested enzymes with activities comparable to enzymes of saprotrophic basidiomycetes. Also remarkable was its production of N-acetylglucosaminidase with a potential to recycle nitrogen from own hyphae or senescent mycelia of other fungi. Also in this respect, *D. acicola* was functionally more similar to basidiomycetes than to other ascomycetes.

Considering these seemingly contradictory results between expected and observed decomposing abilities (ascomycetes vs basidiomycetes), it is rather difficult to find a general pattern distinguishing supposedly different early vs later decomposers and ascomycetes vs basidiomycetes. In view of the huge diversity of fungal species and limited types of substrates in coniferous forests, one has to expect a strong overlap in functional traits. Different constraints limiting the occurrence of particular fungal species may thus be (micro)climatic, geographical and biotic (competition with other fungi), rather than nutritional. Substantial phenotypic differences among particular strains of a single species (Osono & Hirose 2011) should be taken into consideration as well. Thus, the selection of a strain of a given fungal species for an experimental trial is a matter of serendipity and may easily lead to results contradicting previous studies with a different strain of the same species (Boberg et al. 2011). Unfortunately, in the context of increasing use of environmental sequences to define functional traits of fungi, the previous emphasis on reliable identification of the fungus under study to the species level (Christensen 1989) has been relaxed, and functional traits are being attributed to taxa identified to various taxonomic levels (Treseder et al. 2014) or even to pooled unidentified species (McGuire et al. 2010).

In deeper soil horizons, ascomycetes may occupy different niches and benefit from localized disturbances of the mycelia of basidiomycetes connected with nutrient release (Brabcová et al. 2016, Lindahl et al. 2002, Osono et al. 2006). Some of them are recognized as key symbionts providing nutrients to particular plant groups (ericaeous mycorrhizal members of the *Rhizoscyphus ericeae* complex) or are otherwise associated with plant roots (Lindahl et al. 2007, Mitchell & Gibson 2006). Potentially, they also participate in the turnover of humic substances, i.e. a large pool of organic nutrients fixed in highly complex compounds transformed from residues of decomposition of plant, animal and microbial biomass in soils (Stevenson 1994, Tan 2014). The contribution of fungal biomass (especially components of fungal cell walls) to the formation of soil humic substances received much attention in past. Melanins, i.e., polymeric phenolic compounds complexed with proteins in fungal cell walls (Butler & Day 1998), have a similar elemental composition, similar infrared spectra and are composed of similar functional groups as soil-derived humic substances (Haider & Martin 1967, Knicker et al. 1995, Paim et al. 1990). Nevertheless, interest in the function of fungal melanins in the genesis of humic substances decreased over the decades and received only very little attention in recent years (Tsyganova et al. 2011), although it still

remains a point of discussion (Tan 2014). Soil fungi have also been considered in connection with degradation of humic substances (Řezáčová et al. 2006), but the effect of ascomycetes is supposed to be limited only to their modification (Grinhut et al. 2007). In **PAPER XIII** (Koukol et al. 2004), we found that the ascomycete *Chalara longipes* (Helotiales, Leotiomycetes) is able to degrade humic acids from spruce litter in liquid culture and partially utilize them as a source of nitrogen. Interestingly, this strain did not produce polyphenol oxidase and peroxidase (Koukol et al. 2006). Instead, it significantly decreased the pH of the medium by producing organic acids. Our results thus support the view of humic substances as complex mixtures of small biopolymers bound together by weak dispersion forces (Piccolo 2002) rather than large complex molecules linked by covalent bonds (Stevenson 1994).

INTERACTIONS WITH OTHER ORGANISMS

Besides the high diversity of fungi in coniferous litter, this habitat is occupied also by a rich community of edaphic organisms, such as collembolans, oribatid mites and nematodes. Their interactions with fungi may affect fungal distribution, competition and also decomposition processes (Crowther et al. 2012). One of the best-documented examples is the restricted vertical distribution of *G. androsaceus* and *Mycena galopus* (Agaricales, Agaricomycetes) due to preferential grazing of *G. androsaceus* by the collembolan *Onychiurus latus*. *Gymnopus androsaceus* is more competitive in the colonization of litter needles than *M. galopus*, but this ability is reverted when being grazed (Newell 1984). One of the most important and species-rich decomposer groups are oribatid mites (Acari, Oribatida) (Maraun & Scheu 2000). They reach high species and population densities and occupy different feeding niches, ranging from carnivores to primary decomposers and secondary decomposers (Schneider et al. 2004). Multiple choice feeding experiments indicated overlaps in the preferences for the same fungal species of secondary decomposers and fungivorous (omnivorous) oribatid mites, rather than a strict species-to-species dependency resulting from co-evolution (Maraun et al. 2003, Schneider & Maraun 2005). Nevertheless, a distinct separation of preferences exists between oribatid mites and collembolans preferring ascomycetes and basidiomycetes, respectively. In previous experimental trials, insufficiently accurate identification of fungi precluded the identification of ecological traits of individual fungal species that could be important for their preferences. In **PAPER XIV** (Koukol et al. 2009), we tested whether oribatid mites inhabiting pine litter show a higher preference for microfungi specific to this substrate or ubiquitous species. *Cladosporium cladosporioides* (Capnodiales, Dothideomycetes) representing the ubiquitous saprotrophs was grazed significantly most intensively. However, in its absence, different mite species showed relatively stable preferences for particular microfungi specific to pine litter. These diverse preferences may explain the high diversity of microarthropods and their low level of competition for food. The factors affecting the palatability and attractiveness of microfungi specific to pine litter were not elucidated, but obviously are very complex. For example, the previously supposed preference for fungi producing more extracellular enzymes (Maraun et al. 1998a) was not supported in our study, because two highly preferred species, *D. acicola* and *Sympodiella acicola* (Venturiales, Dothideomycetes), strongly differed in their enzymatic activities (**PAPER XII**). On the other hand, we observed that several tested fungi apparently protected important structures from grazing. Tested mites grazed moderately on two fungal

species, *P. lacerum* and *Allantophomopsis lycopodina* (Helotiales, Leotiomyetes), but always selected their mycelium, whilst conidia produced from pycnides and embedded in slime remained untouched. The slime seemed to protect the conidia from grazing similarly to crystals on hyphal surface of some basidiomycetes protects from grazing by collembolans (Böllmann et al. 2010).

Besides obvious negative effects on fungal biomass, oribatid mite grazing can also benefit fungi by aiding their dispersal. Undamaged fungal spores and fragments of hyphae were found in mite faeces (Smrž & Norton 2004), and numerous microfungi were isolated from the surface of mite bodies (Renker et al. 2005). Oribatid mites may thus be responsible for changes in the composition of microfungal communities or even its recovery after severe disturbance (Maraun et al. 1998b). In **PAPER XV** (Koukol et al., unpublished), we subjected *F. cordae* and *S. acicola* to oribatid mite grazing in two different intensities to evaluate the effect of grazing on the dispersal of microfungi. This experimental trial confirmed a slightly beneficial effect of low-intensity mite grazing on fungal fitness. The number of fungal propagules giving rise to new colonies showed an increasing tendency compared to the ungrazed control (Figure 3).

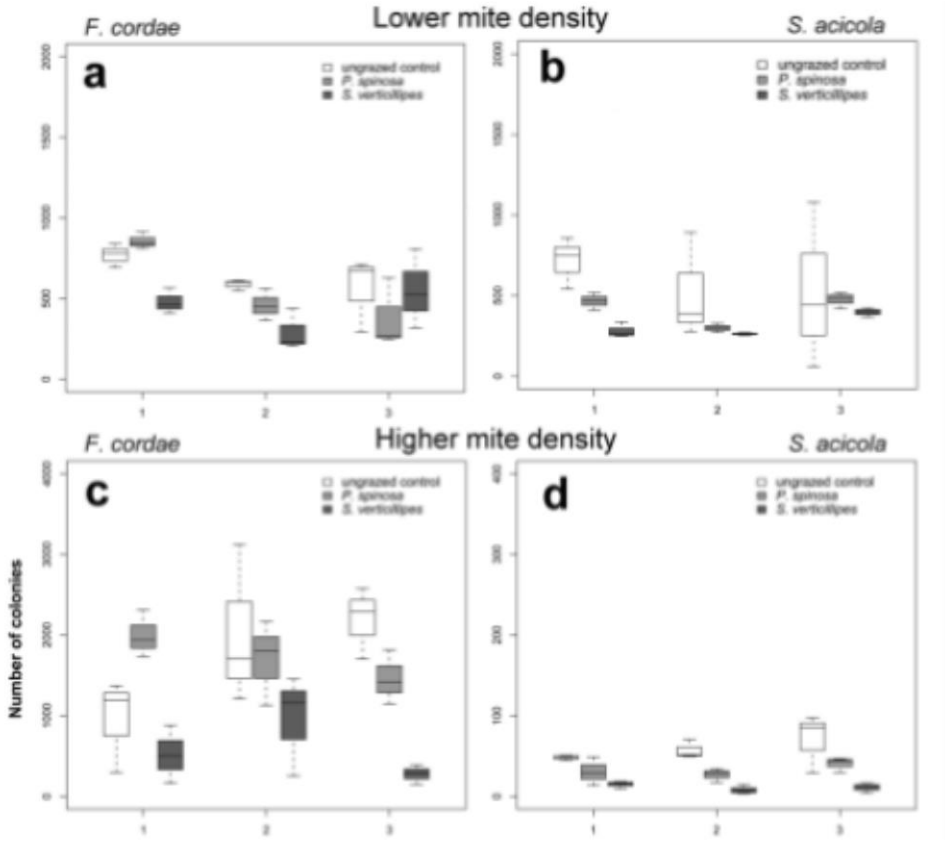

Figure 3: Number of colonies of *Fusicladium cordae* (a, c) and *Sympodiella acicola* (b, d) growing from propagules in suspension from precolonized needles after grazing of the needles by mites at lower (a, b) and higher (c, d) density.

The implications of this result suggest that low-intensity grazing should not be viewed as merely destructive, but also as a factor that positively affects fungal distribution. Selective grazing of senescent mycelia of saprotrophic basidiomycetes by collembolans may have a

similar positive effect. Under low densities of collembolans, grazing may result in increased hyphal coverage (Bretherton et al. 2006), stimulation of enzyme activities involved in decomposition (Crowther et al. 2011b), nutrient uptake from wood (Crowther et al. 2011a), and it may also affect the outcome of fungal interactions (Rotheray et al. 2011).

Compared to our knowledge of microarthropods, our understanding of interactions between microfungi and other soil microorganisms, such as protists, is very limited. Protistan diversity has mostly been studied in aquatic environments, and soil-dwelling protists have hitherto been overlooked. Only recently has the diversity of soil protists been comprehensively surveyed using next generation sequencing (Bates et al. 2013). Nonetheless, some of them, such as testate amoebae, also include fungivores and therefore may be directly affected by the composition and density of fungal communities (Krashevska et al. 2008). I observed a close association of testate amoebae and microfungi outgrowing from pine litter needles cultivated in damp chambers already in 1990s (O. Koukol, unpublished results), but could not offer an interpretation of their coexistence. Soil-dwelling testate amoebae actually received very little attention, and their ecology remained largely unknown (Wilkinson 2008). Vohník et al. (2009) carried out a detailed survey of communities of testate amoebae associated with the roots of ericaceous plants and provided an experimental evidence of fungal utilization of testate amoebae shells. In connection with this study, I returned to using damp chambers in our next study in **PAPER XVI** (Vohník et al. 2011). We showed that the communities of testate amoebae reached their highest density and species richness on pine needles from litter. These values decreased on filter paper colonized by mycelium of *Anavirga laxa* (*Incertae sedis*, Pezizomycotina) growing out of needles and reached minimal values on uncolonized filter paper. Though the association of testate amoebae (above all of *Phryganella acropodia*) with mycelium of *A. laxa* was evident, we concluded that testate amoebae utilized bacteria associated with fungal hyphae rather than melanized hyphae.

In conclusion, the present thesis provides an overview of current trends and findings in the study of diversity and function of fungi in coniferous needles, including the advantages and limitations of different approaches. In research topics such as fungal diversity in litter, our knowledge benefits from the development and growing accessibility of methods of high-throughput sequencing. However, before these methods will allow us to draw a reliable picture of fungal diversity and elucidate the functional traits of individual fungal species, culture-based surveys remain indispensable. The availability of DNA-based identification of fungi is hugely important for gaining deeper insights into the diversity and taxonomy of culturable fungi. The enormous diversity of fungi in coniferous litter and their microscopic nature do not allow experiments to be carried out under natural conditions. Though simplifying the natural conditions, laboratory studies represent the most powerful tool advancing our understanding of the role these fungi play in ecosystems.

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