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Heredity of zinc tolerance in *Suillus luteus* and impact of metal pollution on ectomycorrhizal fungal communities

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VOORWOORD

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SAMENVATTING

Verhoogde concentraties aan zware metalen in bodems, zoals zink en cadmium, zijn toxisch voor de meeste organismen. Vele organismen kunnen dan ook niet overleven in bodems verontreinigd met zware metalen. In enkele zeldzame gevallen zorgt deze selectiedruk voor evolutionaire aanpassingen aan metaalhomeostase mechanismen. Dit fenomeen is goed bestudeerd voor planten, maar slechts weinig is geweten over adaptieve metaaltolerantie bij schimmelsoorten die in associatie met deze planten leven. In hoofdstuk twee wordt een genetische studie gepresenteerd met als doel een beter inzicht te krijgen in de genetische structuur van zinktolerantie bij *Suillus luteus*. In deze studie werden verschillende zinktolerantie fenotypes waargenomen in dikaryote ouderlijke stammen, in monokaryote nakomelingen van deze stammen en in kruisingen tussen monokaryote nakomelingen. Op basis van deze fenotypes werd geconcludeerd dat zinktolerantie in *S. luteus* wordt overgedragen door onvolledige dominantie. Enkele mogelijke modellen die de genetische architectuur van zinktolerantie in *S. luteus* kunnen verklaren, werden in deze studie getest en werden besproken in hoofdstuk twee. Verder heeft een door metalen-geïnduceerde selectiedruk tot gevolg dat populaties van gevoelige organismen plaats zullen ruimen voor meer resistente soorten of soorten die adaptieve metaaltolerantie hebben ontwikkeld. Hierdoor zullen op de meest verontreinigde bodems zich slechts een beperkt aantal soorten kunnen vestigen, waardoor specifieke gemeenschappen ontstaan. Zulke metaal-specifieke gemeenschappen zijn ook sinds lange tijd bekend in het plantenrijk. Met deze plantengemeenschappen zijn vermoedelijk ook specifieke schimmelgemeenschappen geassocieerd. Hiernaar is echter aanzienlijk minder onderzoek verricht in het verleden omdat het bestuderen van (hoofdzakelijk) ondergrondse schimmelgemeenschappen veel moeilijker is dan het bestuderen van plantengemeenschappen. Echter, recente ontwikkelingen in moleculaire biologie hebben gedetailleerde

beschrijvingen van schimmelgemeenschappen mogelijk gemaakt. In het derde hoofdstuk wordt een technische studie gepresenteerd waarin het optimaliseren van de vereiste moleculaire technieken voor het bestuderen van ondergrondse schimmelgemeenschappen als doel werd gesteld. De amplificatie-eigenschappen van de primers die het best presteerden in deze verkennende studie werden getest en vergeleken met andere primers die courant worden gebruikt voor het bestuderen van schimmelgemeenschappen. In hoofdstuk vier worden de in hoofdstuk drie geoptimaliseerde technieken gebruikt voor het bestuderen van schimmelgemeenschappen in de bodem van een pioniersdennenbos in een met zink en cadmium vervuild studiegebied. Naast het karakteriseren van de schimmelgemeenschappen aanwezig in dit vervuilde gebied, werd ook de invloed van metaalverontreiniging op de diversiteit en structuur van de schimmelgemeenschapen bestudeerd. Hoewel duidelijke correlaties tussen de concentraties aan zware metalen en de samenstelling van schimmelgemeenschappen werden waargenomen, werden geen consistente effecten vastgesteld op de schimmeldiversiteit. De aanwezigheid van de meest dominante soorten in dit studiegebied werd besproken. In het vijfde hoofdstuk wordt een studie gepresenteerd waarin de schimmelgemeenschappen die voorkomen in vervuild gebied werden vergeleken met schimmelgemeenschappen in een controlegebied, eveneens in pioniersdennenbos. De soortensamenstellingen van de schimmelgemeenschappen in beide studiegebieden kwamen grotendeels overeen, maar de relatieve abundanties duidden op een verschuiving in de dominantie van aanwezige soorten. Opnieuw werden geen effecten van gemeten omgevingsfactoren op de schimmeldiversiteit vastgesteld. Veranderingen in de relatieve abundanties van aanwezige schimmels over verschillende jaren, duidden bovendien op dynamische ecosystemen in de bestudeerde pioniersbossen. Een opvallende dynamiek is de verdringing van ascomyceet fungi door basidiomyceet fungi in de ectomycorrhizatips van *Pinus* in de aanplanting op verontreinigde bodem. Mogelijk heeft het dynamische karakter van deze ecosystemen een belangrijke rol gespeeld

in het ontstaan van metaaltolerante schimmels zoals *S. luteus*. Ten slotte worden in het zesde en laatste hoofdstuk de meest belangrijke bevindingen van de vier studies die tijdens dit project werden uitgevoerd samengevat en bediscussieerd. Ook worden perspectieven geboden waarop toekomstige studies zich zouden kunnen toespitsen.

SUMMARY

High concentrations of heavy metals in soils, such as zinc and cadmium, are toxic to most organisms. Many organisms are unable to survive in metal-polluted soils. In rare occasions, this selection pressure may cause evolutionary adaptations in the metal homeostasis mechanisms of organisms exposed to increased concentrations of heavy metals. This phenomenon is well studied for plants, but only limited information is available on adaptive metal tolerance in symbiotic fungi that live together with these plants. In chapter two, a genetics study is presented aiming to provide insight in the genetic architecture of zinc tolerance in *Suillus luteus*. In this study, contrasting zinc tolerance phenotypes were observed in dikaryotic, parental strains, in monokaryotic offspring of these strains and in crosses between monokaryotic strains. Based on these phenotypes, it was concluded that zinc tolerance in *S. luteus* is inherited through incomplete dominance. Some potential models for the genetic architecture of zinc tolerance in *S. luteus* were tested and are discussed in chapter two. Furthermore, metal-induced selection pressure may cause populations of sensitive organisms to collapse, leaving only adapted species. Hence, on most polluted sites, only a limited number of organisms may thrive, giving rise to unique communities. Such (pseudo-)metallophyte plant associations have been studied extensively in the past. However, also specific fungal communities are likely to be associated with these plant communities. Nevertheless, much less research has been conducted on these fungal communities in the past, since studying (mainly) belowground fungal communities is more difficult than studying plant communities. Recent developments in molecular biology, however, enabled detailed identification of fungal communities. In the third chapter, a technical study is presented aiming to optimize the required molecular tools for studying belowground fungal communities. The amplification-efficiencies of the primers that were found to be the most efficient in amplifying a broad range of fungal taxa were compared to other primers that are frequently used to

study fungal communities. In chapter four, the optimized techniques described in chapter three were used to study fungal communities in soils of pioneer pine forests thriving on a zinc and cadmium polluted site. Beside characterizing the fungal communities in this study site, also the influence of metal pollution on fungal diversity and fungal community structures were analysed. Whereas clear correlations were found between fungal community compositions and metal concentrations, no consistent effects on fungal diversity were detected. The presence of the most dominant species in this site were discussed. In the fifth chapter, a study is presented in which the fungal communities in a metal polluted site were compared to fungal communities in a control site, both associated with a pioneer pine forest. The species composition of the fungal communities in both sites was very similar. However, the relative abundances of these species indicated a dominance shift. Striking community dynamics in which basidiomycetes replaced ascomycetes in the ectomycorrhizal root tips of pine trees were observed. Again, no effects of measured environmental factors on fungal diversity were observed. Moreover, changes in the relative abundances of present species indicated that the studied pioneer ecosystems are very dynamic. The dynamics of these pioneer ecosystems potentially played an important role in the evolution of metal-tolerant fungi such as *S. luteus*. Finally, in the sixth, and last chapter, the most important conclusions of the current project were summarized and discussed and perspectives for future studies are given.

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Chapter 1: **Introduction**

History of metal pollution in the Campine region

Since the onset of the Industrial Revolution in the mid-18th century, metals such as zinc (Zn) and lead (Pb) have been extracted on an industrial scale through pyrometallurgical processes. The first zinc smelters were built in the United Kingdom around 1740 (Morgan, 1985). When the Industrial Revolution spread to mainland Europe, the pyrometallurgical industry initially started growing around Liège in Belgium and in Silesia (Silesia corresponded to an area covering a large part of current Poland, Germany and the Czech Republic). Flue gasses exited the plants unfiltered and had a devastating impact on the health of many people in densely populated areas such as Liège. Therefore, new zinc smelters were built in low-populated areas such as the Campine region in Belgium and the Netherlands in the early and mid-nineteenth century. The pyrometallurgical industry continued to operate in these areas until the mid-20th century. In Belgium, zinc smelters were built in Overpelt (1880), Balen (1885) and Lommel (1904) and near the Belgian border in the Netherlands in Budel (1892) (Colpaert *et al*., 2004). The pyrometallurgical process developed in Belgium was initially adopted throughout Europe and was later also adopted in the United States. It was the main pyrometallurgical method that was used during the first 100 years of large-scale Zn and Pb production. In this so-called Belgian-type horizontal retort process, ores containing ZnS, PbS, ZnO and PbO were heated together with charcoal to 1400 °C in ceramic retorts. In the ceramic retorts, charcoal reduced Zn and Pb containing minerals to Zn + CO and Pb + CO to prevent their oxidation. At 1400 °C, most Zn and Pb are converted to gasses and S is converted to SO_2. Gaseous Zn and Pb were subsequently collected in water-cooled condensers and CO and SO_2 gasses exited the plants as flue gas (Morgan, 1985). Unfortunately, this early pyrometallurgical process was very inefficient. It proved to be very difficult to tightly regulate the temperatures during the entire process. Consequently, large quantities of Zn and Pb gasses exited

the plants as flue gas as well. Beside Zn and Pb, the ores used for metal extraction, typically also contained high concentrations of cadmium (Cd). Since Cd has a melting point of 321 °C and a boiling point of 767 °C, also most Cd was converted to a gas in the ceramic retorts. During the 18th and 19th century, Cd was not collected because its physical properties did not allow Cd to be used in construction or to manufacture tools and equipment. Only later, in the 20th century, Cd would be collected in metal smelters and used in Nickel-Cadmium batteries and to stabilize plastics. As a consequence, large quantities of S, Zn, Pb and Cd were emitted from zinc smelters in the Campine region during the period between 1880 and 1973. Through precipitation, metal ions in the industrial vapours settled in the near vicinity of zinc smelters, polluting vast areas in the vicinity of the zinc smelters. Moreover, despite its high metal concentrations, water that had been used to cool condensers was discharged in nearby rivers and creeks. Used retorts, condensers and ores (metal slugs) still contained large quantities of Zn, Pb and Cd as well. In the 20th century, this industrial waste has been widely used for road and railway construction, on school and farm yards, for stabilization of industrial terrains or private properties, spreading the pollution even further throughout Belgium and the Netherlands (Verlaek and Weynants, 2006). It is estimated that 290 km of roads have been constructed with metal-polluted waste from zinc smelters and that a total area of 700 km^2 has been polluted with high concentrations of metals in the Campine region of Belgium alone (Hogervorst *et al*., 2007). Since 1973, electrochemical processes replaced pyrometallurgical processes. Since the switch to this new technology, Cd emissions from the zinc smelter in Overpelt, for example, decreased from 125,000 kg per year to 130 kg per year (Verlaek and Weynants, 2006). However, since metal ions are not degradable, they remain present in high concentrations in the environment to date. The toxicity of these metals is most visible on the vegetation at polluted sites. So-called metal deserts in the Campine region, some of which span several square kilometres, are still devoid of most vegetation, decades after emissions from zinc smelters have been cut.

Why are metal ions toxic?

To understand why metals are toxic at specific concentrations, first a distinction between two groups of metals needs to be made. Some metal ions are required for growth and repair of cells. They are, for example, co-factors in many enzymes or they can be structural components of cell walls, cell organelles and cell- or endomembranes. These metals are collectively called "essential nutrients" and their concentrations in cells are tightly regulated. However, when the concentrations of these metals in cells become too high, they become toxic. Examples of essential metal ions are zinc (Zn), iron (Fe), copper (Cu), magnesium (Mg), manganese (Mn), etc. (Gadd, 1993). Other metal ions are not required for normal growth and repair by most organisms and they are denoted as "non-essential nutrients". Examples include lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg), caesium (Cs), etc. (Gadd, 1993). They can become toxic at even lower concentrations when compared to essential elements.

Toxic concentrations of metal ions exert high levels of stress to cells mainly because they replace other bound metals from their binding sites. Hence, proteins my become inhibited or denatured and cell structures may become disrupted, potentially causing membrane leakage (Hall, 2002). Indirectly, metal ions may also cause oxidative stress to cells by engaging in chain reactions, with for example cell membranes, causing lipid peroxidation. This potentially results in cell leakage and eventually cell death (Dietz *et al*., 1999). Finally, metal ions that are present in excess may replace essential metal ions, resulting in deficiency effects (Van Assche and Clijsters, 1990).

It is clear that increased concentrations of metals in cells can cause high levels of stress in various ways. This increased cellular stress, may in turn lead to a decrease in the fitness of organisms living in metal polluted environments. If the metal-induced stress indeed causes a reduction in the fecundity of an organism, this organism will have a selective disadvantage compared to other organisms of the same species and its genotype will be selected against. This metal-induced selection pressure does not only act on individual species, it will also select for those species in a community

that have the capacity to adapt to toxic environments. Even though the impact of metal pollution is also widely studied for invertebrates such as nematodes (Salamun *et al*., 2012) and Collembola (Janssens *et al*., 2009) and for prokaryotes (Porter and Rice, 2013), the current project will focus on the effects of metal pollution on fungi in terrestrial environments and, where possible, comparisons to what is known about the impact of metal pollution on plant species and plant communities will be made.

Effects of metal-induced selection pressure at the species level

Both fungi and plants will typically avoid the accumulation of toxic concentrations in the cytoplasm, rather than develop proteins or cell-structures that resist high concentrations of metals or reactive oxygen species (Hall, 2002). A large number of genes are known to be involved in preventing the build-up of toxic concentrations of metal ions or to reduce or repair damage caused by metal ions in both plants and fungi. A detailed description of known genes involved in these mechanisms is beyond the scope of the current introduction and can be found in Schat *et al*. (1996), Schat and Vooijs (1997), Hall (2002) and Verbruggen *et al*. (2009) for plants, in Bellion *et al*. (2006) for filamentous fungi and in Li *et al*. (1997) and Clemens and Simm (2003) for yeast. Instead, an overview will be given of the potential mechanisms that may be selected for under metal-induced selection pressure.

A first defence mechanism that can be employed by plants and fungi is the exudation of organic acids, such as oxalic acid, to sequester and precipitate metal ions extracellularly. However, it needs to be noted that many exudates in fact increase the availability of metals and are therefore not likely to occur as defence mechanisms against high concentrations of metals (Li *et al*., 2005). Next, the cell wall and cell membrane can also prevent the accumulation of excess metal ions in the cytoplasm. Negative charges on proteins and polysaccharides in the cell wall can bind large amounts of metal ions and cell wall components, such as pectins, may become more methylated in the cell wall, reducing its metal binding

capacity, in response to metal exposure (Colzi *et al*., 2011). In *Paxillus involutus*, a reduction in the production of hydrophobins suggested that cysteine, that would be used in the production of hydrophobins under normal growth conditions, was used for the production of cysteine-rich molecules in the cytoplasm. These cysteine-rich molecules could subsequently sequester excess metal ions in the cytoplasm of *Paxillus involutus* cells exposed to high metal concentrations (Jacob *et al*., 2004). Moreover, transport proteins in the cell membrane of both plants and fungi have been shown to be actively involved in the efflux of metal ions, preventing the build-up of high intracellular metal concentrations (Hall, 2002; Bellion *et al*., 2006). However, as soon as too many metal ions enter the cell, they have to be sequestered to prevent them from causing damage. Both in plants and fungi, metallothioneins, glutathione and phytochelatins can perform this role in the cytoplasm. Metal-ligand complexes can subsequently be stored intracellularly in vacuoles (Hall, 2002; Bellion *et al*., 2006). When cells are exposed to such high concentrations of metals that even these mechanisms are insufficient to protect the cell, damage will be inflicted to cell structures and DNA and the redox balance of the cell will be disrupted. In these cases, repair mechanisms such as the production of heat shock proteins may be upregulated to protect and repair damaged proteins. The redox balance, in turn, can be restored through superoxide dismutases, ascorbate, glutathione, peroxidases and catalases (Cuypers *et al*., 2011). The up- or down regulation of genes involved in the general homeostasis, such as the ones describes above, are considered to be **metal resistance** mechanisms and they are present in all plants and fungi.

Random mutations and genetic recombination in species with sexual reproduction cause new genotypes to be formed in natural populations continuously. Hence, individuals that can tolerate increased concentrations of metals can be present at low frequencies in natural populations. This has, for example, been shown for grasses where 0.1% to 0.5% of grass populations growing on non-polluted soils were found to be metal- tolerant

(Bradshaw and McNeilley, 1981; Al-Hiyaly *et al*., 1993). However, when metal-induced selection pressure is present, genotypes of a given species that exhibit metal tolerance may have an increased fecundity relative to other genotypes of that same species. Consequently, the genotype with an increased fecundity may become more frequent in a population. The negative impact of metals on spore production or mycelial growth in fungi and flower or seed production in plants have been shown in the past, indicating that increased metal concentrations may indeed reduce the fecundity of plants (Hancock *et al*., 2012) or fungi (Andrade *et al*., 2004; Sridhar *et al*., 2005). As soon as individuals of a given species with a particular genetic trait become frequent in a population, a new **ecotype** is said to have been formed. Only in those cases where substantial differences exist in the metal tolerance between different ecotypes of a given species, that species is said to have developed **adaptive metal tolerance**. Only few examples of true adaptive metal tolerance in mycorrhizal fungi have been reported (Colpaert, 2008). The few known examples of adaptive metal tolerance in mycorrhizal fungi include: aluminium tolerance, which was found in *Pisolithus tinctorius* (Egerton-Warburton and Griffin, 1995); nickel tolerance in *Pisolithus albus* (Jourand *et al*., 2010) and *Cenococcum geophilum* (Goncalves *et al*., 2009) and zinc tolerance in *Suillus bovinus*, *Rhizopogon luteolus* and *Suillus luteus* (Colpaert *et al*., 2000; 2004). In higher plants, the most well-known examples of true adaptive metal tolerance can be found in grass species such as *Agrostis capillaris* (Al-Hiyaly *et al*., 1993) but also a few dicotyledonous plants have been found to develop adaptive metal tolerance. Examples of the latter group are *Silene vulgaris*, *Noccaea caerulescens* (formerly known as *Thlaspi caerulescens*) and *Arabidopsis halleri* (Alford *et al*., 2010). The genetic basis for adaptive metal tolerance has been thoroughly studied in plants and was reviewed by Schat *et al*. (1996), Schat and Vooijs (1997), Hall (2002) and Verbruggen *et al*. (2009), but the genetic architecture for adaptive metal tolerance in fungi is poorly understood. Therefore, in the current project, a genetic study was conducted to investigate the genetic architecture of zinc

tolerance in *Suillus luteus* (L.: Fr.) Roussel (Chapter 2). *Suillus luteus* is a basidiomycete that forms ectomycorrhizal associations with trees of the genus *Pinus*. Fruiting bodies of this fungus can be frequently found on sandy soils both in metal-polluted and non-polluted young pioneer pine forests in the Campine region of Belgium. Zn- and Cd- tolerant ecotypes of this species have been described from different populations in the northern part of Limburg, Belgium in the past (Colpaert *et al*., 2004; Krznaric *et al*., 2009) and Cu- tolerant ecotypes have been described from Cu mine spoils in Norway (Adriaensen *et al*., 2005). In most cases, adaptive metal tolerance in plants and fungi has been found to be metal-specific. Hence, different metals may select for different genes, even within the same species. Also for *Suillus luteus*, Zn and Cd tolerance appear not to be linked to one another. The current project will be focused on the genetic architecture of Zn tolerance in *Suillus luteus*. In this study, two major questions were investigated. (i) How many genes are involved and to what extent does every gene affect the zinc tolerance trait? Theoretically, a continuous number of genes may be involved in an adaptive metal tolerance mechanism and the contribution of each gene may be scaled on a continuous scale as well. However, in practice one would attempt to distinguish between a discrete set of possible genetic determinants to gain an idea of the genetic organization of adaptive metal tolerance in a given species. Three discrete models may be distinguished and compared to observed heredity patterns (following Macnair, 1993). (a) The tolerance trait may be determined by one major gene with the possibility of minor modifier genes adjusting the expression of that major gene. (b) A multi-genic model may be found in which a small number of genes strongly determine the tolerance trait. (c) A polygenic model in which a more or less large number of genes determine the tolerance trait and in which each of these genes has a small relative contribution to that tolerance trait. (ii) The second question that can be investigated is how different alleles of a gene and the different genes of a trait interact with each other. For example: are alleles dominant, recessive, co-dominant or is there incomplete dominance

and do different genes act additively or epistatically? The study featured in chapter 2 attempts to give an answer to both questions for the zinc tolerance trait in *S. luteus*.

Effects of metal-induced selection pressure at the community level

The effects of metal pollution are most visible on plant communities. Plant communities thriving on metal-enriched soils largely consist of well-adapted plant species and/or ecotypes unique to metalliferous soils. Across different metals and over a wide geographical range, it is striking that the same plant species are capable of developing pioneer metallophytes (Antonovics *et al*., 1971). For example *Silene vulgaris*, *Arabidopsis halleri* and *Noccaea caerulescens* can be frequently found in metal-polluted sites where other plant species fail to build stable populations. An example of a unique plant community that is specifically associated with metal polluted sites can be found in La Calamine in Belgium. In La Calamine, a number of zinc mines were constructed since rock formations rich in Zn and Pb surface in this region. The plant community that thrives on these metalliferous soils was first described by Schwickerath (1944). This so-called *Violetum calaminariae* association harbours plant species with ecotypes endemic to a single or only a few sites in La Calamine. Examples are *Viola lutea* subsp. *calaminaria*, *Noccaea cearulescens* var. *calaminaria*, *Armeria maritima* var. *calaminaria*, *Festuca aquisgranensis*, *Minuartia verna* subsp. *hercynica* and *Silene vulgaris* subsp. *humilis*. Some other plant species that can be found in this association, but that are not restricted to metal-polluted sites in La Calamine, include *Cochleaira pyrenaica*, *Cardaminopsis halleri* and *Festuca ovina* subsp. *ophioliticola*. In Germany, in Siegerland and eastern Westphalia, specific zinc grassland flora can be found as well. This plant association is characterized by the occurrence of other subspecies of *Armeria maritima* such as *Armeria maritima* subsp*. bottendorfensis* at Bottendorf in Saxony-Anhalt and *Armeria maritima* subsp*. hornburgensis* at Eisleben in Sachen-Anhalt. *Armeria maritima* subsp*. halleri* also occurs in Mechernich in the Eifel. This plant association is called Armerietum halleri

and can not only be recognized by the occurrence of this typical *A. maritima* subspecies, but also by the lack of *Viola lutea* subsp*. calaminaria* and *Noccaea caerulescens* subsp*. calaminaria*. These examples illustrate that only a limited number of plant species can specifically adapt to metal-rich environments, giving rise to specific plant associations. Both examples of well-characterized plant communities also illustrate our vast and long-standing knowledge of (pseudo-) metallophytes. In sharp contrast, very little is known about which fungal species occur in metal-polluted soils since the lifestyle of most fungi is much more cryptic than that of most vascular plants. Nevertheless, mycorrhizal fungi have been shown to be able to protect their plant partners from excessive metals and they may even promote the growth of host plants under stressful conditions (Adriaensen *et al*., 2003). Especially during plant establishment and early growth of saplings in metal-polluted environments, mycorrhizal fungi are vital partners (Wilkinson and Dickinson, 1995). Hildebrandt (1999), for example described that *Viola lutea* subsp. *calaminaria* from the *Violetum calaminariae* association is unable to grow on metal-polluted soils without the presence of a specific arbuscular mycorrhizal fungal strain (*Glomus* Br1). To better understand ecosystem development and functioning in stressful environments, it would therefore be interesting to also gain an idea of the fungal species that occur in metal-polluted sites and which species are unable to colonize these sites. To further understand the development of mycorrhizal and plant communities in metal-polluted environments, also the impact of high metal concentrations on fungal communities needs to be assessed.

Metal pollution or the experimental introduction of high concentrations of metal ions to soil-born fungal communities have been shown to adversely affect fungal communities in numerous ways. A strong reduction in, for example, microbial activity (Azarbad *et al*., 2013), microbial biomass (Azarbad *et al*., 2013; Rieder and Frey, 2013), number of cultivable fungi (Bååth *et al*., 2005), soil fungal diversity (Hui *et al*., 2012), soil fungal richness (Hui *et al*., 2012; Xu *et al*., 2012), fungal growth and root

colonization (Hagerberg *et al*., 2011; Xu *et al*., 2012) and enzyme activity (Mackie *et al*., 2013) have been observed in previous studies. Also changes in the fungal community composition have been observed (Chodak *et al*., 2013; Corneo *et al*., 2013; Hui *et al*., 2011; Jumpoonen *et al*., 2010; Macdonald *et al*., 2011). An equally large number of studies, however, indicated that metal pollution may also have a more neutral impact on fungal communities. For example, Anderson *et al*. (2008) and Kandeler *et al*. (2000) both observed that fungal community compositions remain unaltered under metal stress. Furthermore, neutral effects of metal pollution have been observed by Huang *et al*. (2012), where fungal richness, diversity, rank abundance and root colonization remained unaffected by metal pollution. In some cases, even an increase in soil fungal presence (PLFA: Frostegaard *et al*., 1993; 1995), diversity (Wu *et al*., 2010) and enzyme activity (Rajapaksha *et al*., 2004) have been found due to increased metal concentrations. Whereas, many of these studies mainly focused on the impact of metals on fungal communities, studies also taking the variation into account of present vegetation, found that plant species present in polluted sites and plant diversity may be more important factors shaping fungal communities in metal-polluted sites than the present metal concentrations (Shao *et al*., 2012; Thion *et al*., 2012). Also soil pH (Chodak *et al*., 2013), soil moisture, soil structure (Corneo *et al*., 2013) and organic matter content in metal-polluted soils (Gomes *et al*., 2010) may alter the way fungal communities respond to high concentrations of metal ions. From these studies, it is clear that the interaction of fungi with their environment is very complex and dependant on a number of biotic and abiotic factors. Very few of these studies, however, also identified the fungal species that were present in polluted soils or on mycorrhizal roots of plants. Since the number of cases of true adaptive metal tolerance in fungi is as limited as is the case for plants, probably only few fungal species are capable to adapt to growth in metalliferous soils. Hence, it would be interesting to obtain a picture of which fungal species increase or decrease in abundance with increasing metal pollution. With the development of a whole new range of

molecular techniques (collectively called next-generation sequencing), determining the composition of microbial communities at a species-level has recently become possible. One of these techniques, 454 pyrosequencing (Margulies *et al*., 2005), was employed in the current project and optimized for studying below-ground fungal communities. 454 pyrosequencing requires the amplification of DNA barcode regions, followed by sequencing of the obtained amplicons ("metabarcoding": Taberlet *et al*., 2012). The fungal internal transcribed spacer region (ITS) of the rDNA operon has been recognized as the formal fungal barcode (Schoch *et al*., 2012). Recent 454 pyrosequencing studies usually relied on primer pairs that were designed in the 90s to amplify target rDNA regions. However, these primers were designed based on a limited number of known fungal ITS sequences and were shown to efficiently amplify fungal ITS sequences only of DNA extracted from pure cultures. In order to amplify target rDNA regions in environmental samples, primer pairs have to overcome a number of constraints that could reduce PCR efficiency, such as the presence of humic acids and fenols that may be co-extracted with DNA. Moreover, they have to efficiently and specifically amplify target DNA regions in the presence of high concentrations of non-target DNA. Hence, in the current project, different primers were initially tested using 454 pyrosequencing, real-time PCR (qPCR) and *in silico* analyses. The most promising primer pair was shown to amplify the ITS2 region of the fungal rDNA much more efficiently and robustly than other tested primer pairs (Ihrmark *et al*., 2012; Toju *et al*., 2012) and was therefore selected to be used in our fungal community studies (Chapter 3).

Using the results of 454 pyrosequencing optimization, the impact of soil metal pollution on fungal communities thriving in a site polluted with Zn and Cd and in a control site was investigated. Both sites had very similar soil structures (dry sandy soils) and the dominating plant species was Scots pine (*Pinus sylvestris*). Beside the impact of metal pollution, also local spatial and temporal variations and the impact of physical and chemical soil parameters on fungal communities were investigated to understand the

importance of metal pollution in shaping the studied fungal communities. In a first study, a selected polluted site in Lommel-Maatheide was investigated, allowing for a detailed comparison between fungal communities thriving in soil patches that mainly differed in their Zn- and Cd-content, but that were very similar for other environmental factors (Chapter 4). In chapter 5, this study site was compared to a control site, located in Hechtel-Eksel. This allowed us to compare the fungal communities of two pioneer pine forests with contrasting soil chemistry and to get a picture of naturally occurring fungal communities associated with pioneer pine forests in the Campine region.

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Chapter 2: **Heredity of zinc tolerance in *Suillus luteus*: an ectomycorrhizal basidiomycete adapted to heavy metal tolerance**

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SUMMARY

In rare cases, selection pressure imposed by high metal concentrations in soils triggers evolutionary adaptation of metal homeostasis mechanisms. This phenomenon has been studied thoroughly in some plant species but hardly any knowledge is available on adaptive metal tolerance in mycorrhizal fungi. To gain further insight in adaptive metal tolerance in mycorrhizal fungi, the genetic architecture of zinc tolerance in *Suillus luteus* was investigated using dose-response experiments. The ratios of zinc-tolerant versus zinc-sensitive monokaryotic isolates were studied for thirty families of spore cultures. Crosses between monokaryotic strains were established *in vitro* and tested as well. Varying proportions of zinc-tolerant monokaryotic sibling strains were found for eighteen out of thirty families. Six families consisted of only zinc-sensitive siblings and six families consisted of only tolerant siblings. A strong correlation was found between the average EC_{50}-values of contributing monokaryotic strains and the EC_{50}-values of resulting crosses. Zinc tolerance in *S. luteus* was found to be inherited through incomplete dominance and is under control of a limited number of genes. Possible models for the genetic architecture of zinc tolerance in *S. luteus* are discussed.

Keywords: adaptive metal tolerance, heredity, metals, mycorrhiza, zinc

CHAPTER 2

INTRODUCTION

Soils polluted with high concentrations of metals induce high levels of stress not only to plants, but also to the plant-associated soil-borne micro-organisms. A large number of studies have investigated how plants cope with toxic levels of metal ions (reviewed by Hall, 2002; Verbruggen *et al*., 2009 and Nagajyoti *et al*., 2010). Some well-studied examples of plant species that can tolerate and in some cases even hyper-accumulate metal ions include *Noccaea cearulescens* (formerly *Thlaspi caerulescens*), *Silene vulgaris* and *Arabidopsis halleri* (Alford *et al*., 2010). Relatively few studies have investigated the contribution of plant-associated micro-organisms, including mycorrhizal fungi, in the plant defence against elevated metal concentrations (reviewed by Meharg, 2003 and Colpaert *et al*., 2011). Soil micro-organisms playing an active role in alleviation of metal-induced stress in plants, need themselves sufficient protection against metal toxicity. A number of mechanisms can help to protect mycorrhizal fungi from toxicity at intermediate concentrations of metal ions. Such mechanisms are considered to be resistance mechanisms and they include up- or down regulation of genes involved in metal homeostasis and stress responsive genes. Binding of metal ions to the fungal cell wall or extracellular sequestration of metal ions with organic chelators may also help to reduce metal exposure (Bellion *et al*., 2006). However, eventually, high concentrations of metal ions in the cytoplasm result in severe toxicity, leading to a decreased fitness. Reduced growth and reduced spore production due to elevated levels of metal ions have been reported for a number of fungal species (Andrade *et al*., 2004; Sridhar *et al*., 2005). Elevated concentrations of metal ions are also known to reduce fruit and seed production in plants (Hancock *et al*., 2012). Hence, it is clear that metal toxicity imposes a strong selection pressure. In few cases, this selection pressure gives rise to metal-tolerant ecotypes. Only those cases where marked differences exist in the metal tolerance of different ecotypes of a species are considered to be cases of true adaptive metal tolerance

(Meharg, 2003). Adaptive metal tolerance and associated molecular mechanisms are, again, better documented for plant species (Macnair, 1993; Verbruggen *et al*., 2009; Willems *et al*., 2010) than for their associated symbionts. In most cases, a few "strategic" plant genes are found to play important roles in adaptive metal tolerance and tolerance mechanisms in plants are typically found to be metal specific (Schat *et al*., 1996; Schat & Vooijs, 1997). Only few examples of true adaptive metal tolerance in mycorrhizal fungi have been reported (Colpaert, 2008) and underlying molecular mechanisms are poorly understood. The few known examples of adaptive metal tolerance in mycorrhizal fungi include: aluminium tolerance, which was found in *Pisolithus tinctorius* (Egerton-Warburton & Griffin, 1995); nickel tolerance in *Pisolithus albus* (Jourand *et al*., 2010) and *Cenococcum geophilum* (Goncalves *et al*., 2009) and zinc tolerance in *Suillus bovinus*, *Rhizopogon luteolus* and *Suillus luteus* (Colpaert *et al*., 2000; 2004). To gain more insight in the genetic basis of adaptive metal tolerance in mycorrhizal fungi, the current study aimed to characterize the genetic architecture of zinc tolerance in *S. luteus*. We hypothesized that zinc tolerance in *S*. *luteus* is a heritable trait and that this trait is controlled by only one or a few genes. We assume that the key mechanism of metal tolerance in *S. luteus* is relatively simple as the evolution of zinc tolerance in the studied population should have started no more than 150 years ago.

MATERIALS AND METHODS

Culturing of *Suillus luteus* strains

The standard growth medium used for growing *S*. *luteus* cultures was a slightly modified Fries medium (Fries, 1978). The medium used in the current study contained 5.43 mM $(NH_4)_2$•tartrate, 0.41 mM $MgSO_4$•$7H_2O$, 0.22 mM KH_2PO_4, 0.18 mM $CaCl_2$•$2H_2O$, 0.34 mM NaCl, 1.34 mM KCL, 0.24 mM H_3BO_3, 20 µM $ZnSO_4$•$7H_2O$, 5.01 µM $CuSO_4$•$5H_2O$, 50.29 µM $MnSO_4$•H_2O, 0.16 µM $(NH_4)_6Mo_7O_{24}$•$7H_2O$, 73.99 µM $FeCl_3$•$6H_2O$, 33.3 mM

D-glucose, 55.51 µM myo-inositol, 0.3 µM thiamine•HCl, 0.1 µM biotin, 0.59 µM pyridoxine, 0.27 µM riboflavin, 0.82 µM nicotinamide, 0.73 µM p-aminobenzoic acid, 0.46 µM Ca-pantothenate. The pH was corrected to 4.8. Agar was added to 1% (w/v). Cultures were grown at 23°C.

Isolation of *Suillus luteus* cultures and description of study sites

Suillus luteus (L.: Fr.) Roussel is a basidiomycete that forms ectomycorrhizal associations with conifers from the genus *Pinus*. Both partners typically occur during early colonization stages on nutrient-poor sandy soils. *S*. *luteus* cultures used in this study were collected during the autumns of 2009, 2010 and 2011 from three populations in the northern part of Limburg, Belgium. Two populations were sampled growing on metal-contaminated sites in Lommel-Maatheide (LM; 51°14'12.12" N, 5°15'45.90" E) and Lommel-Sahara (LS; 51°14'43.85" N, 5°16'42.99" E). Both sites are contaminated mainly with zinc, cadmium and lead due to pyrometallurgical activities in the area that started at the end of the nineteenth century. The third population was sampled in Paal (P; 51° 3'33.62" N, 5°10'30.96" E). This last site has no elevated concentrations of metal ions and isolates collected here served as a control population. The LS and P populations were previously sampled in 1992 (Colpaert *et al*., 2004), though present isolates are probably from different genets (young trees). The Lommel-Maatheide population must be different from our previous studies because the original pine plantation at LM was clear cut. New pine trees were planted on an adjacent plot in 2008. A more detailed description of the study sites can be found in Colpaert *et al*., 2004. Zinc concentrations in pine needles collected from the LM site ranged between 170 and 550 µg g^{-1} Zn. Pine needles collected from LS contained between 100 and 200 µg g^{-1} Zn and in the control site in P, pine needles contained between 20 and 50 µg g^{-1} Zn.

Ten basidiocarps were collected from each population. In the field, stems and any debris were removed from the caps. Caps were subsequently sealed in plastic bags to protect them from desiccation during

transportation. In the lab, cleaned caps were placed over Petri dishes containing solid Fries medium for five minutes to collect spores. This was repeated ten times for each cap. The resulting culture plates were incubated for twenty to thirty days at 23°C. Between day twenty and thirty, spores started germinating and germinating spores were isolated under a binocular loupe. Isolated spores were put on fresh solid Fries media resulting in monokaryotic, haploid strains. Monokaryotic strains from the same cap (parent) are denoted as sibling strains in the remainder of the text. Twenty sibling strains were isolated from each basidiocarp. After collecting spores, sterile mycelium was isolated from parent caps and cultured on solid Fries medium to obtain dikaryotic cultures. These cultures are denoted as parental strains. A dikaryotic parental strain together with its twenty monokaryotic sibling strains are denoted as a family. Crosses between monokaryotic strains were obtained by placing two monokaryotic strains, originating from different families, on culture media at a distance of 0.5 cm from each other. Crosses were made between zinc-tolerant monokaryons of different families, between zinc-sensitive monokaryons of different families and between zinc-tolerant and zinc-sensitive monokaryons of different families. In this way, three crossing groups were created: sensitive x sensitive, sensitive x tolerant and tolerant x tolerant. For this experiment, four zinc-tolerant and four zinc-sensitive monokaryotic strains were selected from each of three families (one family for each of the three studied *S. luteus* populations). All crosses between monokaryotic strains of different families were established for each of these 24 selected monokaryons. No crosses between siblings were established (Fig. S2.1). After ten days of incubation, the contact zone was transferred to fresh Fries medium. After another ten days of incubation, dikaryotized mycelium was transferred to fresh Fries medium. To ensure complete dikaryotization, mycelia were subcultured another three times before cultures were tested for their ploidy and zinc tolerance. All pair-wise crosses resulted in 192 potentially crossed strains (there are eight crossing combinations for each of the 24 monokaryons that were selected for this experiment). From these,

120 fully dikaryotized crosses were selected and tested, including 37 crosses between two tolerant monokaryons, 35 crosses between two sensitive monokaryons and 48 crosses between a zinc-sensitive and a zinc-tolerant monokaryon.

Testing the ploidy of cultures

To ensure that all dikaryotic strains (parental strains and crosses) were diploid and to ensure that all monokaryotic strains (sibling strains) were haploid, the ploidy of all strains was checked using microsatellite markers. To this end, DNA was extracted from cultures using the DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Venlo, Netherlands). Isolated DNA was amplified with microsatellite markers Sulu08 and Sulu10 using PCR (Table S2.1; Muller et al., 2006). PCR conditions were as follows: initial denaturation at 95°C for two minutes, followed by 35 cycles of 95°C (30s), 55°C (30s) and 72°C (60s) and a final extension phase at 72°C for 10 minutes. Reactions were carried out in 25 µl reaction volumes using the FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). Each reaction contained 2.75 µl FastStart 10x reaction buffer, 1.8 mM MgCl, 0.2 mM dNTP mix, 0.4 µM of each primer, 1.25 U FastStart HiFi polymerase and 50 ng template DNA (as measured by a Nanodrop spectrophotometer). Amplified DNA was separated using gel electrophoresis on 4% agarose gels. Gels were run at 70 V for 6 hours.

Dose-response experiments

Monokaryotic strains were grown for fourteen days on cellophane covered Fries medium enriched with zinc in the following concentrations: 0 mM, 1.5 mM, 3.1 mM, 6.2 mM, 12.3 mM and 18.5 mM. Zinc was added to the standard growth medium as $ZnSO_4 \bullet 7H_2O$. Dikaryotic strains (parental strains and crosses) were exposed to the same zinc concentrations for ten days. All strains were tested in triplicate. At harvest, mycelia were collected from the treatment plates, lyophilized and their dry weights were measured

with an accuracy of 0.1 mg. EC_{50}-values (the zinc concentrations which inhibit growth by 50%; Colpaert *et al*., 2004) were calculated from the dry weights using non-linear regression in R 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). No extrapolations were allowed, setting the maximum for EC_{50}-values at 18.5 mM. After weighing, the dried mycelia that had been exposed to 1.5 mM additional zinc were wet digested at 120°C three times with HNO_3 65% and finally with HCl 37%. The mycelia of the 1.5 mM Zn treatment were selected for element analyses because of the low toxicity of this treatment, allowing for good growth of all isolates. Concentrations of zinc (Zn), iron (Fe), copper (Cu), manganese (Mn), magnesium (Mg), calcium (Ca), phosphorus (P) and sulphur (S) were measured in the mycelium digests using inductively-coupled plasma optical emission spectroscopy (ICP-OES).

Statistical analyses

Statistical analyses were performed in R 3.0.3. Normal distributions of the residuals of models were checked with the Shapiro-Wilk test, while the homoscedasticity of variances was analysed using either Bartlett's or the Fligner-Killeen test. Depending on the distribution of the estimated parameters, either ANOVA or the Kruskal-Wallis Rank Sum Test was used to check for significant differences in variances of parameters. Two-by-two comparisons were performed using either Tukey Honest Significant Differences (HSD) tests or Pairwise Wilcoxon Rank Sum Tests. Box-Cox power transformations were performed using the package MASS 7.3-29 in R. Cluster analyses were performed using the package mclust 4.2 (Fraley and Raftery, 2002) in R.

RESULTS

Testing the ploidy of cultures

Of the microsatellite locus targeted by Sulu8, 5 alleles are known from the *S. luteus* populations studied here, whereas 16 alleles are known for the microsatellite locus targeted by Sulu10 (Muller *et al*., 2006). A dikaryon can be recognized by the presence of two DNA bands after gel-electrophoresis if both nuclei carried a different allele for the tested locus. Both microsatellite alleles differ in sequence length and therefore appear as two separate bands on a gel. In case both nuclei carry the same allele for a given microsatellite locus, only a single band is visible. Monokaryons display only a single band for each microsatellite after gel-electrophoresis since they carry only a single allele of each microsatellite locus. Crossed strains contain both microsatellite alleles of the haploid strains that were used to establish the respective crossed strain. Using the Sulu08 and Sulu10 microsatellite markers (Muller *et al*., 2006), monokaryotic and dikaryotic strains could be successfully distinguished from each other using gel electrophoresis. These results also confirmed the successful *in vitro* dikaryotization in selected crossed strains (Fig. S2.2).

Description of zinc tolerance phenotypes

Zn tolerance phenotypes were determined based on EC_{50}-values and mycelial Zn concentrations. Both parameters were plotted relative to each other for the parental strains (Fig. 2.1A) and monokaryotic strains (Fig. 2.1B). The EC_{50}-values and mycelial Zn concentrations had a relationship best described by a power function (Box-Cox power transformation: $\lambda = 0$). Indeed, when both the EC_{50}-values and mycelial Zn concentrations were log transformed, a significant linear correlation was observed, both for the monokaryotic (Pearson's product moment correlation coefficient (R^2): $R^2 = -0.71$, $p < 0.01$) and for the parental strains ($R^2 = -0.88$, $p < 0.01$).

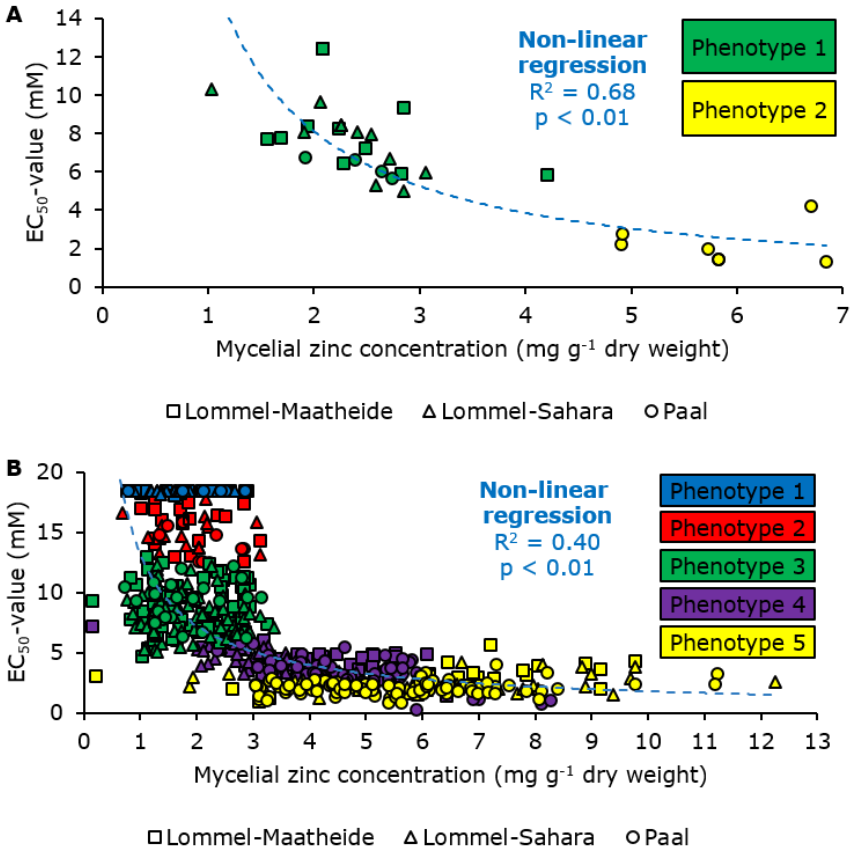

Figure 2.1 Correlation between EC_{50}-values and mycelial zinc concentrations measured by inductively-coupled plasma - optical emission spectroscopy (ICP-OES) on acid digested *Suillus luteus* mycelia. A. Parental strains (n = 30). B. Monokaryotic strains (n = 600). Results from non-linear regressions are given in insets. Clusters identified by Cluster Analysis are indicated in different colours. Strains originating from Paal are indicated with circles. Strains originating from Lommel-Sahara are indicated with triangles and strains originating from Lommel-Maatheide are indicated with squares.

Using model-based (Bayesian Information Criterion) Cluster Analysis, discrete clusters were determined (Fig. 2.1). The optimal number of clusters to describe collected data was 5 for the monokaryotic strains and 2 for the parental strains. Assuming these clusters can be interpreted as

Zn tolerance phenotypes, the following phenotypes were distinguished for the monokaryotic strains: strains clustering into phenotype 1 had EC_{50}-values ranging from 18 mM to 18.6 mM and mycelial Zn concentrations of 0.1 to 3 mg g^{-1} dry weight; strains belonging to phenotype 2 had EC_{50}-values ranging from 12.5 to 18 mM and mycelial Zn concentrations of 0.1 to 3 mg g^{-1} dry weight; strains belonging to phenotype 3 had EC_{50}-values ranging from 5 to 12.5 mM and mycelial Zn concentrations ranging from 0.1 to 3 mg g^{-1} dry weight; phenotype 4 strains had EC_{50}-values ranging between 3 to 5 mM and mycelial Zn concentrations between 3 to 6 mg g^{-1} dry weight; phenotype 5 strains had EC_{50}-values ranging between 0 to 3 mM and mycelial Zn concentrations ranging between 3 to 12 mg g^{-1} dry weight. The tolerance phenotypes observed for the parental strains were the following: phenotype 1 with EC_{50}-values ranging between 5 and 13 mM and mycelial Zn concentrations ranging between 1.6 and 4.5 mg g^{-1} dry weight and phenotype 2 with EC_{50}-values ranging between 0 and 5 mM and mycelial Zn concentrations ranging between 4.5 to 7 mg g^{-1} dry weight. Whereas EC_{50}-values differed significantly between all five phenotypes of monokaryons (Tukey HSD: $p < 0.01$ for all pairwise comparisons; Fig. 2.2C), mycelial Zn concentrations were the same for phenotypes 1, 2 and 3 (Tukey HSD: $p > 0.05$ for all pairwise comparisons; Fig. 2.2D). The two phenotypes observed for parental strains also differed significantly from each other in their EC_{50}-values (t-test: $p < 0.01$; Fig. 2.2A) and mycelial Zn content (t-test: $p < 0.01$; Fig. 2.2B). Based on our previous population studies, tolerance phenotypes 1, 2 and 3 can be described as Zn-tolerant phenotypes (having EC_{50}-values > 5 mM and mycelial Zn concentrations < 3 mg g^{-1} dry weight), whereas phenotypes 4 and 5 are Zn-sensitive phenotypes (having EC_{50}-values < 5 mM and mycelial Zn concentrations between 3 and 12 mg g^{-1} dry weight) (Colpaert *et al*., 2004). For a small portion of the data (< 5%) the description of phenotypes as either Zn-tolerant or Zn-sensitive was not unambiguous. Beside differences in EC_{50}-values and mycelial Zn concentrations, also mycelial Fe, Mg, Mo and S content differed between phenotypes (one-way ANOVA: $p < 0.01$; Fig.

S2.3). Furthermore, a significant positive correlation was found between mycelial Zn and Fe concentrations (R^2 = 0.80, $p < 0.01$ for parental strains and R^2 = 0.51, $p < 0.01$ for monokaryotic strains; Fig. S2.4).

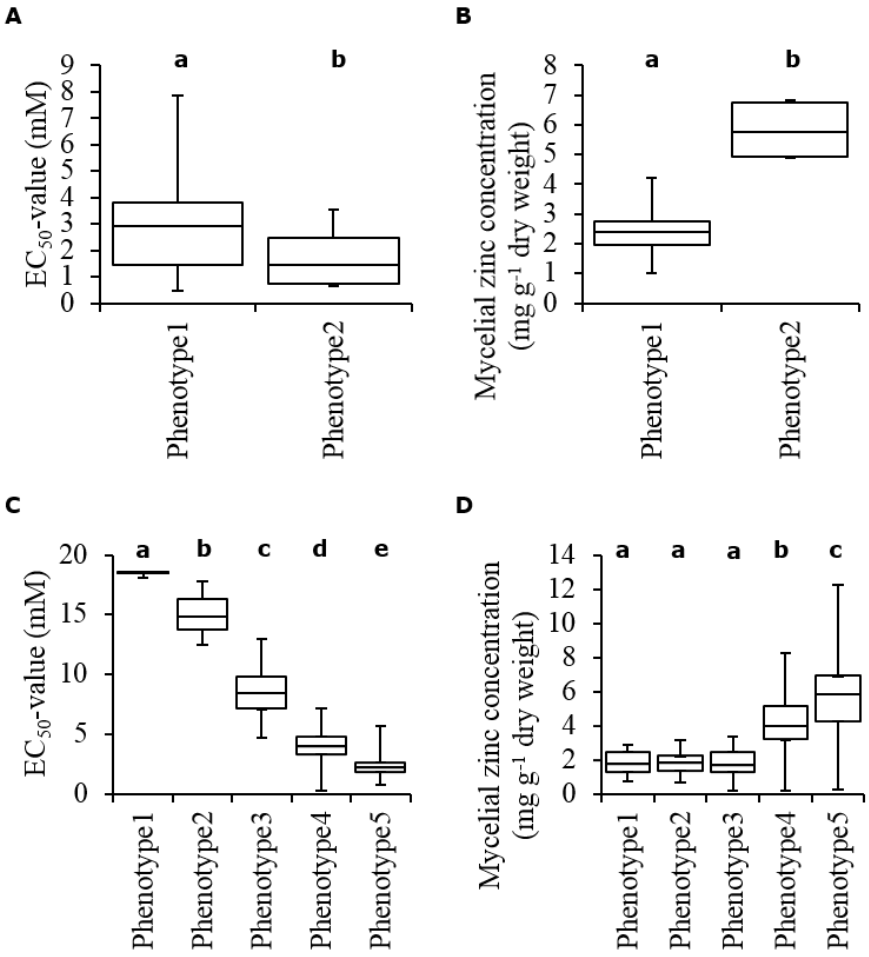

Figure 2.2 Differences in mycelial zinc concentration and EC_{50}-values between observed zinc tolerance phenotypes. A. EC_{50}-values of parental strains (n = 30). B. Mycelial zinc concentrations of parental strains. C. EC_{50}-values of monokaryotic strains (n = 600). D. Mycelial zinc concentrations of monokaryotic strains. Significant differences at the 95% confidence level are indicated with different letters.

Calculation of the genetic component of phenotypic variation

Large variations in EC_{50}-values were observed, especially within Zn-tolerant phenotypes. To estimate the relative contribution of the Zn tolerance trait to observed variations in EC_{50}-values, two-way ANOVA analyses were performed for each family. In this way, the variation in EC_{50}-values was divided into four components: a genetic component attributable to Zn tolerance ("Genotype"), a genetic component that contains part of the genetic component of Zn tolerance but that also contains other genetic factors that influence the growth of isolates ("Genotype:Treatment interaction"), variation introduced by Zn treatments ("Treatment") and random variation introduced by environmental variables ("Environment") (Table 2.1). On average, a large proportion (91% ± 1% SE) of the phenotypic variation in families with only Zn-sensitive siblings could be explained by the Zn treatment. For families with mostly tolerant siblings and families with varying proportions of tolerant siblings, Zn treatment was responsible for a smaller proportion of the total phenotypic variation (49% ± 7% SE and 53% ± 3% respectively). 26% ± 6% SE and 25% ± 2% SE of the phenotypic variation was accounted for by differences in genotypes (differences in Zn tolerance) in families with mainly tolerant siblings and families with both tolerant and sensitive sibling strains respectively, whereas only 3% ± 1% SE of the phenotypic variation was accounted for by genotypic differences in families with only Zn-sensitive siblings. 23% ± 4% SE, 21% ± 1% SE and 5% ± 1% of the total variation was accounted for by genotype:treatment interactions in families with mainly Zn-tolerant siblings, families with varying proportions of Zn-tolerant siblings and families with only sensitive siblings respectively. Only a small proportion of the phenotypic variation could be explained by environmental factors (3% ± 0.4% SE, 1% ± 0.2% SE and 1% ± 0.1% SE for families with mainly tolerant siblings, families with only sensitive siblings and families with varying proportions of tolerant siblings respectively). Results from two-way ANOVAs can be found in Table S2.2 for the parental strains and in Table S2.3 for the monokaryotic strains.

Table 2.1 Proportion of tolerant offspring for each tested family of *Suillus luteus* cultures and the relative contribution to differences in EC_{50}-values of the factors Genotype (G), Treatment (T) and Environment (E) (see Results section for more details). Strains originating from the sampled populations in Paal, Lommel-Maatheide and Lommel-Sahara are indicated with "P", "LM" and "LS" respectively.

Family	Parental phenotype	Proportion zinc tolerant siblings (%)	G (%)	T (%)	G X T interaction (%)	E (%)
LM07	Tolerant	45	23	56	19	1
LM08	Tolerant	45	35	36	27	2
LM10	Tolerant	65	10	73	16	1
LM17	Tolerant	90	41	38	19	2
LM19	Tolerant	100	19	56	22	3
LM22	Tolerant	85	12	71	14	3
LM24	Tolerant	100	50	17	32	2
LM26	Tolerant	55	13	72	14	1
LM32	Tolerant	45	26	50	22	1
LM33	Tolerant	50	18	55	24	2
LS01	Tolerant	35	22	57	20	1
LS02	Tolerant	35	15	65	18	1
LS03	Tolerant	55	29	49	21	1
LS04	Tolerant	50	29	52	19	1
LS05	Tolerant	45	33	39	27	1
LS06	Tolerant	45	22	59	18	1
LS08	Tolerant	55	27	47	25	1
LS11	Tolerant	100	19	66	13	2
LS20	Tolerant	40	21	59	19	1
LS26	Tolerant	85	15	44	36	5
P01	Tolerant	50	45	34	20	1
P05	Tolerant	50	40	36	23	1
P07	Tolerant	45	22	56	20	2
P28	Tolerant	40	17	62	19	1
P02	Sensitive	0	7	85	8	1
P08	Sensitive	0	2	93	4	1
P13	Sensitive	0	2	94	4	1
P14	Sensitive	0	2	93	5	0
P23	Sensitive	0	2	91	5	1
P30	Sensitive	0	3	89	6	2

Phenotypic variation in mycelial Zn concentrations was evaluated with one-way ANOVAs. Variations in mycelial Zn concentrations between parental genotypes accounted for 98% of the total variation in mycelial Zn concentrations for the Lommel-Maatheide population and for 98% and 99% of variation in mycelial Zn concentrations for the Lommel-Sahara and Paal population respectively. For the monokaryotic strains, differences in genotypes accounted for 92.7% to 99.6% of the total variation in mycelial Zn concentrations. Results from one-way ANOVAs can be found in Table S2.4 for the parental strains and in Table S2.5 for the monokaryotic strains.

Model testing to determine the potential genetic architecture of zinc tolerance in *Suillus luteus*

The distribution of the above-described phenotypes of monokaryotic strains were studied for each family (n = 30) of *S. luteus* strains individually, taking into account that phenotypes 1, 2 and 3 are considered to be Zn-tolerant phenotypes and phenotypes 4 and 5 are considered to be Zn-sensitive phenotypes. The number of Zn-tolerant phenotypes were subsequently counted for each family and potential segregation patterns were tested against observed proportions of Zn-tolerant phenotypes (Table 2.1). Theoretically, a continuous number of genes could be involved in the Zn tolerance mechanism in *Suillus luteus*. However, from a practical point of view, Mendelian heredity patterns can be studied, considering a discrete number of genes, in order to obtain an overview of the genetic architecture of metal tolerance (Macnair, 1993). Therefore, in the following analyses, two possible Mendelian heredity patterns were tested against observed segregation patterns. Both models were selected based on the observed proportions of Zn-tolerant phenotypes in each family. *Model 1*: According to a first potential model, the observed Zn tolerance phenotypes are determined by a single gene with two tolerance alleles: a Zn-sensitive allele and a Zn-tolerant allele, resulting in two phenotypes. According to this model, three segregation patterns could be observed, resulting in either 0%, 50% or 100% tolerant siblings per family. *Model 2*: According to a

second potential Mendelian heredity model, two genes could be involved, each with two different alleles. This last model would give rise to five phenotypes, each phenotype with an increasing level of Zn tolerance. Possible segregation patterns in families according to this model include: 0%, 25%, 50%, 75% and 100% tolerant siblings per family. For 25 out of 30 tested families, chi-squared tests revealed that model 1 was a highly suitable model to describe the genetic architecture of Zn tolerance in *S. luteus*. For the remaining 5 families (the ones with 65% to 85% tolerant offspring), model 2 was more suitable although model 1 still had a significant fit to the observed data (Chi-squared tests: all $p > 0.05$). For six families collected in the control-site in Paal, the parental strains had Zn-sensitive phenotypes and 100% of the siblings were sensitive to Zn. For another six families (two collected in Lommel-Sahara and four in Lommel-Maatheide), the parental strains were found to be tolerant to Zn and nearly 100% of their offspring were Zn-tolerant as well. For the remaining 18 families, varying proportions of tolerant siblings were found (range: 35% to 65%; Table 2.1).

Crossing-experiments

Crosses were established between Zn-tolerant monokaryons (phenotypes 1, 2 and 3) and Zn-sensitive monokaryons (phenotypes 4 and 5). The averages of the EC_{50}-values of the monokaryotic strains that were used to establish a specific crossed strain were calculated as the "expected EC_{50}-values". The actual EC_{50}-values of the crossed strains were calculated from the dose response curves as the "observed EC_{50}-values". Expected and observed mycelial Zn concentrations were calculated in the same way. A strong positive correlation was found between observed and expected EC_{50}-values ($R^2 = 0.91$; $p < 0.01$; Fig. 2.3A) and observed and expected mycelial Zn concentrations ($R^2 = 0.73$; $p < 0.01$; Fig. 2.3B). This indicates that the tolerance phenotypes of two contributing monokaryotic strains are averaged out in the Zn tolerance phenotype of the resulting crossed strain.

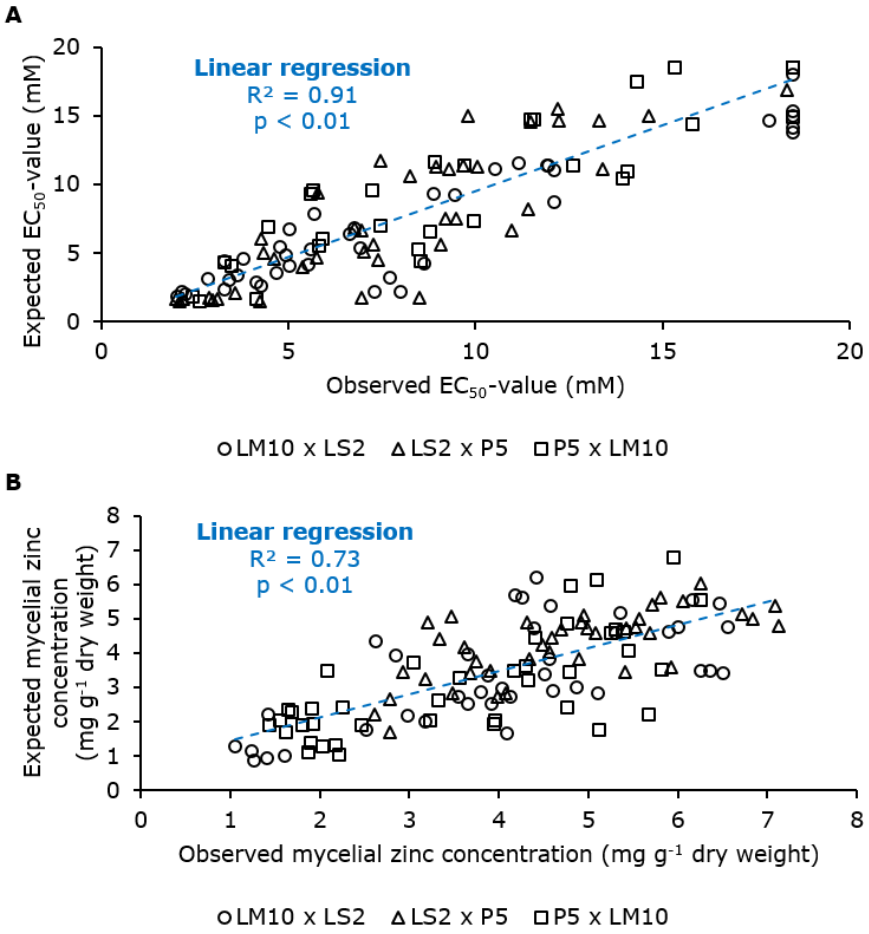

Figure 2.3 Correlations between observed (experimentally measured) and expected (average of tolerance measure of two monokaryotic strains that contributed to the crossed strain) EC_{50}-values and mycelial zinc concentrations of crosses between monokaryotic *Suillus luteus* strains. A. Correlation between observed and expected EC_{50}-values of crossed strains (n = 120). B. Correlation between observed and expected mycelial zinc concentrations of crossed strains (n = 120). The population a family originated from is indicated as follows: "P" for Paal, "LM" for Lommel-Maatheide and "LS" for Lommel-Sahara. Results from linear regressions are given in insets. Crosses between monokaryons from families LM10 and LS2 are indicated with circles. Crosses between monokaryons from families P5 and LM10 are indicated with squares. Crosses between monokaryons from families LS2 and P5 are indicated with triangles.

Hence, there is an equal contribution of each nucleus in the Zn tolerance and Zn uptake in *S. luteus* (incomplete dominance of potential "tolerance alleles"). From Fig. 2.4 it is also clear that, on average, crosses between sensitive monokaryons resulted in sensitive crosses with low EC_{50}-values and high mycelial Zn concentrations. Crosses between two Zn-tolerant strains resulted in highly tolerant crossed strains with high EC_{50}-vlaues and low mycelial Zn concentrations. Crosses between sensitive and tolerant strains had intermediate EC_{50}-values and Zn concentrations (Fig. 2.4).

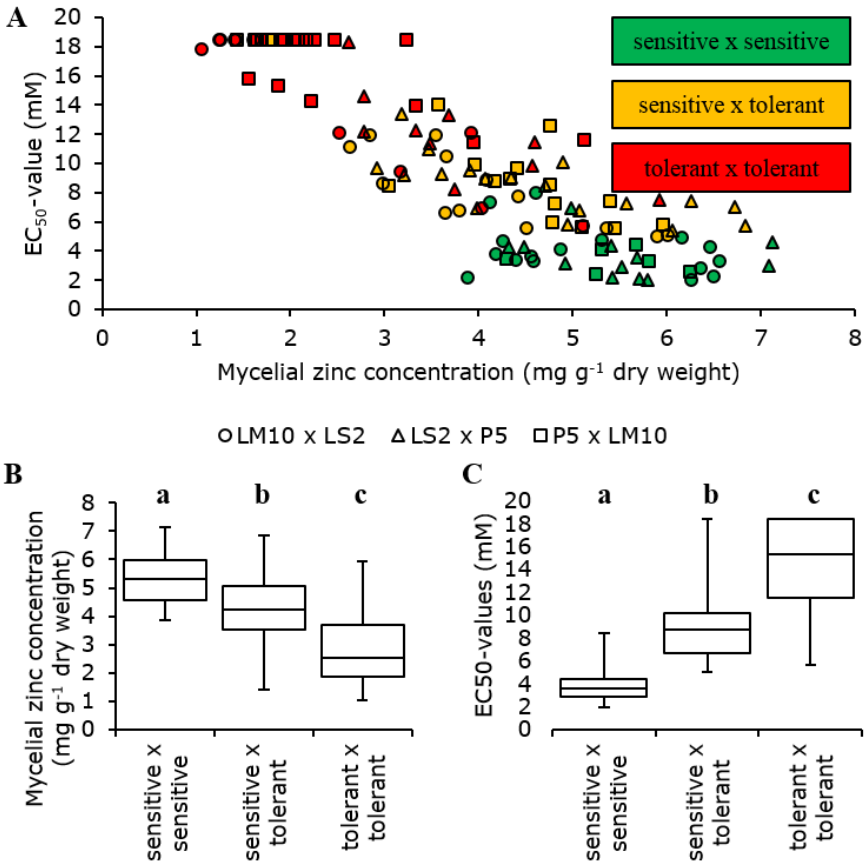

Figure 2.4 Results from dose-response experiments conducted on crosses of monokaryotic *Suillus luteus* strains. A. Relationship between EC_{50}-values and mycelial zinc concentrations for crossed strains (n = 120). Sensitive x sensitive crosses are indicated in green. Sensitive x tolerant crosses are indicated in orange. Tolerant x tolerant crosses are indicated in red. B. Differences in mycelial zinc concentrations between crossing types (sensitive x sensitive n = 35, sensitive x tolerant n = 48, tolerant x tolerant n = 37). C. Differences in EC_{50}-values between crossing types (sensitive x sensitive n = 35, sensitive x tolerant n = 48, tolerant x tolerant n = 37). Significant differences at the 95% significance level are indicated with different letters.

DISCUSSION

Previous research indicated the existence of Zn- and Cd-tolerant ecotypes of the ectomycorrhizal basidiomycete *Suillus luteus* in the northern part of Limburg, Belgium (Colpaert *et al*., 2000; 2004; Krznaric *et al*., 2009) and of Cu-tolerant ecotypes growing on mine spoils in Norway (Adriaensen *et al*., 2005). Metal tolerance mechanisms were shown to be metal specific for *S. luteus* (Adriaensen *et al*., 2005). Element profiles, furthermore, suggested that Zn tolerance in *Suillus* species is based on an exclusion mechanism (Colpaert *et al*., 2005; Ruytinx *et al*., 2013). To gain insight in the genetic architecture of Zn tolerance in *S. luteus*, the heredity of Zn tolerance in *S. luteus* was investigated in the current study.

Prior to studying the heredity of Zn tolerance in *S. luteus*, the ploidy of tested strains was checked. Using the Sulu08 and Sulu10 microsatellite markers described by Muller *et al*. (2006) it was possible to distinguish monokaryotic (haploid) strains from dikaryotic (diploid) strains (Fig. S2.2). These results confirmed the successful isolation of monokaryotic strains grown from single spores. They also confirmed that the *in vitro* crossing of monokaryotic strains was successful, resulting in dikaryotic strains. *S. luteus* is known to have a bipolar (unifactorial) mating system (Fries & Neumann, 1990) as opposed to most other Basidiomycota, who have a tetrapolar (bifactorial) mating system. The inbreeding potential (potential crossing ratio between siblings) therefore is 50%, but the outbreeding potential (potential crossing ratio between two monokaryons originating from a different family) is nearly 100% (Carlile et al., 2006).

To study the genetic architecture of Zn tolerance in *S. luteus*, we collected sporulating basidiocarps from Zn-polluted and non-polluted areas. Although we expected to find homogeneously Zn-tolerant or Zn-sensitive *S. luteus* populations in all areas, as was observed in our previous study (Colpaert *et al*., 2000), some reciprocal introgression was detected in this study. Six out of ten parental strains that originated from the control population in Paal, were found to be sensitive to elevated Zn

concentrations, whereas four strains showed some Zn tolerance. The fact that four tolerant isolates were collected in the control population in Paal is not too surprising however. *S. luteus* is a typical pioneer species that relies mainly on wind and animals for the dispersal of its spores. Spores can therefore be easily dispersed over several kilometres. Muller *et al*. (2004, 2007) investigated the genetic variability of the same *S. luteus* populations investigated here. They found surprisingly large genetic variability in the Paal and Lommel populations as well as a significant gene-flow between the *S. luteus* populations. Therefore, it is possible that Zn tolerance genes got established in the gene pool of the control population in Paal at the time of sampling, as long as these genes do not reduce the fitness of individuals. Parental strains collected in Lommel-Maatheide and Lommel-Sahara were all found to be tolerant to some degree to increased concentrations of Zn (Fig. 2.1A).

The heredity models that were most suitable to explain our data imply either the presence of two genes, each potentially having two alleles or the presence of a single gene with multiple alleles. However, a heredity model implying the presence of a single gene with two alleles could not be rejected based on our data. Whereas significant differences between EC_{50}-values of all monokaryotic phenotypes were observed, no differences were found in the mycelial Zn content of tolerant phenotypes (Fig. 2.2C). These results suggest the presence of a single gene locus that is responsible for the major differences in mycelial Zn concentrations and EC_{50}-values between Zn-tolerant and Zn-sensitive strains, whereas a second gene locus could be responsible for additional differences in EC_{50}-values. A genetic architecture based on the presence of a single or two Zn tolerance loci would correspond to the genetic patterns of metal tolerance described for many plant species (Macnair, 1993). However, the significant contribution of genotype:treatment interactions (Table 2.1) to differences in EC_{50}-values in families with mainly tolerant siblings and families with varying proportions of tolerant siblings indicate that other genetic factors, which are not necessarily linked to the observed Zn tolerance trait, further

influenced the response of *S. luteus* strains to increasing Zn concentrations. For example, intracellular sequestration of Zn with metallothioneins, glutathione or phytochelatins, storage in vacuoles, etc. may influence the growth of both sensitive and tolerant *S. luteus* strains, but these mechanisms are not necessarily part of the adaptive zinc tolerance trait in *S. luteus*. Increased concentrations of S in phenotype 3 of the monokaryons suggest that, at least for this phenotype, sulphur-rich compounds could be present in higher concentrations than in other phenotypes (Fig. S2.3H). Since Zn is an essential nutrient, intracellular Zn concentrations are tightly regulated. Hence, minor modifier genes are expected to play an important role in maintaining Zn homeostasis as well. Furthermore, it is also possible that multiple copies of a "tolerance gene" exist in the genome of *S. luteus*. In metal-tolerant plants, variability in metal tolerance and accumulation among different populations has been partly explained by copy-number expansion of genes involved in metal tolerance and homeostasis. Most of these multi-copy genes code for metal transporters (Craciun *et al*., 2012). To elucidate which genes are involved and to what extent they contribute to adaptive Zn tolerance in *S. luteus*, targeted molecular and proteomics studies need to be conducted in the future. Element profiles of the parental and monokaryotic strains revealed a correlation between mycelial Zn and Fe contents (Fig. S2.4). Since Zn and Fe ions resemble each other chemically and use similar transporters, it is possible that Zn transport mechanisms also translocate some Fe through low-affinity transport and vice versa (Gadd, 1993). EC_{50}-values and mycelial Zn concentrations were strictly inversely correlated, both in parental (Fig. 2.1A) and monokaryotic strains (Fig. 2.1B). The presence of low Zn concentrations in tolerant isolates and high Zn concentrations in sensitive isolates supports previous findings that metal tolerance in *Suillus* species can be attributed to an exclusion mechanism (Colpaert *et al*., 2004; Ruytinx *et al*., 2013).

To study the contribution of individual nuclei in the Zn tolerance of *S. luteus*, crosses were established between monokaryons of different families. When the average EC_{50}-values and mycelial Zn concentrations of

monokaryons that were crossed, were plotted against the measured EC_{50}-values and mycelial Zn concentrations of the resulting dikaryons, a strong positive correlation was found for the EC_{50}-values (Fig. 2.4A) as well as for the mycelial Zn concentrations (Fig. 2.4B). These results indicate that the tolerance phenotype of a dikaryon is the result of the phenotypes of both contributing monokaryotic strains and hence, that Zn tolerance in *S. luteus* is inherited through incomplete dominance.

Conclusions

The goal of the current study was to gain insight in the genetic architecture of the Zn tolerance trait in *S. luteus*. Based on the segregation patterns observed in this study, it is expected that either one or two genes are responsible for the major difference in Zn tolerance between different isolates of *S. luteus*. Other genes or variable copy numbers of a tolerance gene could account for variations in the tolerance level of isolates. Furthermore, dose-response experiments conducted on crosses between monokaryotic strains indicated that Zn tolerance is inherited through incomplete dominance in *S. luteus*. Finally, support was found for the exclusion mechanism that was shown to be responsible for Zn tolerance in *Suillus* in previous studies.

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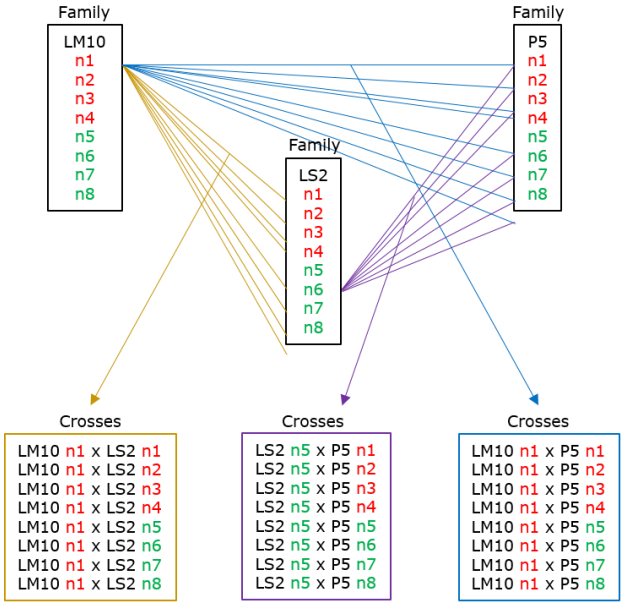
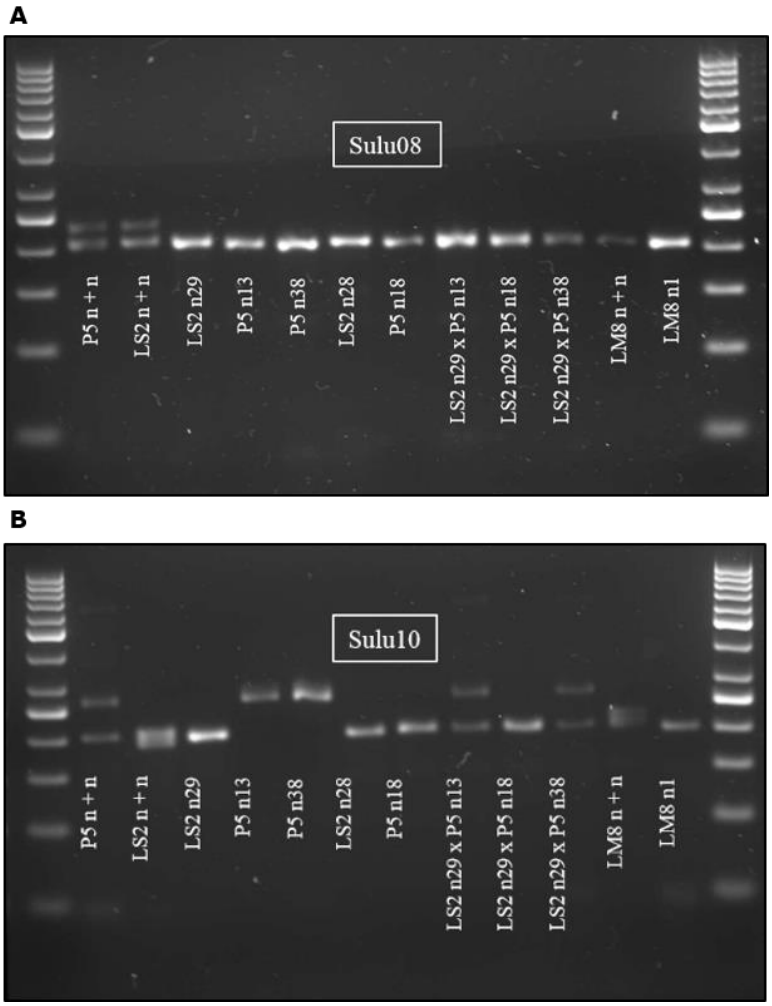
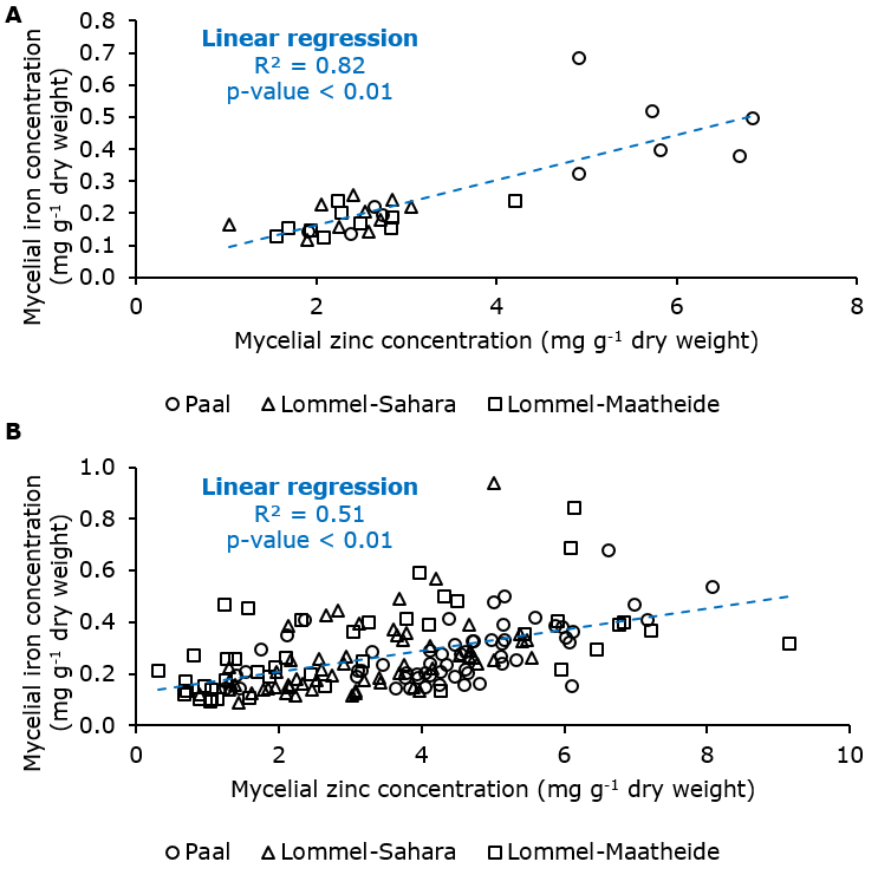
SUPPORTING INFORMATION



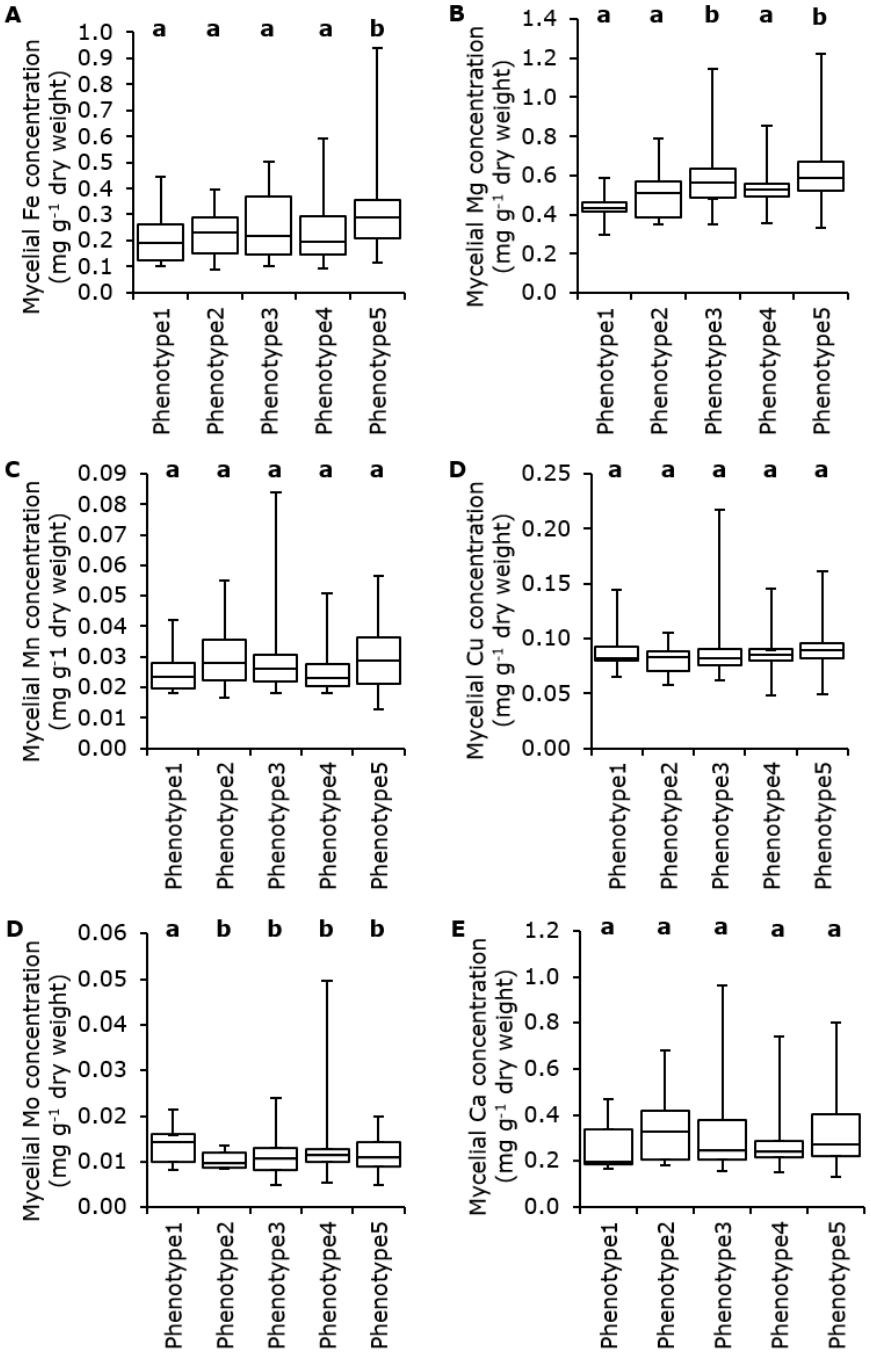
Supporting Information Figure S2.1 Schematic representation of the crossing scheme used to establish diploid crosses between haploid *Suillus luteus* strains. All pairwise crosses (n = 192) were established between haploid strains of different families. No crosses between siblings were established. All pairwise combinations are illustrated for three out of twenty-four haploid strains. Haploid strains are indicated with "n". Crosses are indicated with "x".

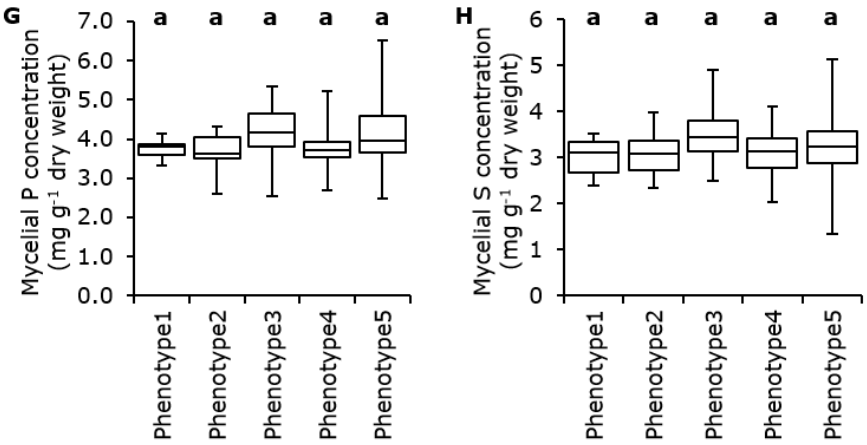


Supporting Information Figure S2.2 Gel-electrophoresis results of PCR amplification with Sulu08 and Sulu10 microsatellite markers. These microsatellite markers were used in the current study to determine the ploidy of parental, monokaryotic and crossed *Suillus luteus* strains in the current study (Muller *et al.*, 2006). A. Microsatellite marker Sulu08. B. Microsatellite marker Sulu10. "n+n" indicates a dikaryotic, parental strain. "n" indicates a monokaryotic strain. "x" indicates a crossed strain. Two bands in a single lane indicate the presence of two alleles of the specified marker in a strain, while the presence of a single band is indicative for a single allele of a given microsatellite marker. Left and right of the sample lanes are 50 base pair (bp) ladders.



Supporting Information Figure S2.3 Correlations between mycelial zinc and iron concentrations of *Suillus luteus* strains. A. Parental strains (n = 30). B. Monokaryotic strains (n = 161). Results of linear regressions are given in insets.


A
Mycelial Fe concentration (mg g-1 dry weight)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a a a a b
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5
B
Mycelial Mg concentration (mg g-1 dry weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a a b a b
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5
C
Mycelial Mn concentration (mg g-1 dry weight)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a a a a a
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5
D
Mycelial Cu concentration (mg g-1 dry weight)
0.25
0.20
0.15
0.10
0.05
0.00
a a a a a
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5
D
Mycelial Mo concentration (mg g-1 dry weight)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a b b b b
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5
E
Mycelial Ca concentration (mg g-1 dry weight)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a a a a a
Phenotype1
Phenotype2
Phenotype3
Phenotype4
Phenotype5



Supporting Information Figure S2.4 Differences in element concentrations between sensitive and tolerant monokaryotic *Suillus luteus* strains (n = 161). A. Iron. B. Magnesium. C. Manganese. D. Copper. E. Molybdenum. F. Calcium. G. Phosphorus. H. Sulphur. Differences in mycelial element concentrations at the 95% confidence level are indicated with different letters.

Supporting Information Table S2.1 Selected microsatellite markers from *Suillus luteus* (Muller *et al*., 2006). Both microsatellite markers were used in this study to check the ploidy of strains used in dose-response experiments.

Locus	Array	p.o. [1]	Primer sequence (5'-3')	a.s.r. [4] (bp) [5]	T_a [6]	EMBL a.n. [7]
Sulu08	$(GAC)_8$	F [2]	GATAGCTTTCATGCCAATCG	196–238	56	AM055723
		R [3]	GACTAGGCGTGTTGGAGACG			
Sulu10	$(GAT)_{23}$	F [2]	CGAGCTCCAGCAGTTACACG	189–264	56	AM055725
		R [3]	AAAACGCTTCTTCTGGTTGG			

[1] p.o.: primer orientation

[2] F: forward

[3] R: reverse

[4] a.s.r.: allele size range

[5] bp: base pairs

[6] T_a: annealing temperature (°C)

[7] EMBL a.n.: EMBL accession number.

Supporting Information Table S2.2 Results of two-way ANOVAs used to calculate the relative contribution of genotype-, treatment-, or environmentally-induced variation in observed EC_{50}-values between dikaryotic *Suillus luteus* strains (n = 30) collected from two metal-polluted sites (Lommel-Maatheide: LM and Lommel-Sahara: LS) and a control site (Paal: P).

Family	Part of phenotypic variation	Variance	F-value	p-value	Relative contribution to phenotypic variation (%)
LM	Genotype	37.65	53.09	< 0.01	7
	Treatment	428.01	965.75	< 0.01	79
	Genotype:Treatment	66.99	18.89	< 0.01	12
	Environment	11.17			2
LS	Genotype	95.70	177.85	< 0.01	9
	Treatment	757.97	5634.69	< 0.01	75
	Genotype:Treatment	155.36	57.75	< 0.01	15
	Environment	6.78			1
P	Genotype	71.54	256.69	< 0.01	10
	Treatment	520.20	3733.00	< 0.01	76
	Genotype:Treatment	93.75	67.28	< 0.01	14
	Environment	3.68			1

Supporting Information Table S2.3 Results of two-way ANOVAs used to calculate the relative contribution of genotype-, treatment-, or environmentally-induced variation in observed EC_{50}-values between monokaryotic *Suillus luteus* strains (n = 600) collected from two metal-polluted sites (Lommel-Maatheide: LM and Lommel-Sahara: LS) and a control site (Paal: P).

Family	Part of phenotypic variation	Variance	F-value	p-value	Relative contribution to phenotypic variation (%)
LM07	Genotype	373.60	200.25	< 0.01	23
	Treatment	909.13	2213.73	< 0.01	56
	Genotype:Treatment	311.95	34.14	< 0.01	19
	Environment	23.18			1
LM08	Genotype	633.01	213.10	< 0.01	35
	Treatment	662.92	1071.21	< 0.01	36
	Genotype:Treatment	495.64	33.37	< 0.01	27
	Environment	37.13			2
LM10	Genotype	180.18	111.98	< 0.01	10
	Treatment	1320.06	3117.70	< 0.01	73
	Genotype:Treatment	285.16	35.45	< 0.01	16
	Environment	20.32			1
LM17	Genotype	470.80	310.66	< 0.01	41
	Treatment	442.11	1050.20	< 0.01	38
	Genotype:Treatment	224.12	29.58	< 0.01	19
	Environment	19.20			2
LM19	Genotype	101.76	79.57	< 0.01	19
	Treatment	299.51	936.77	< 0.01	56
	Genotype:Treatment	120.81	18.89	< 0.01	22
	Environment	16.11			3
LM22	Genotype	92.45	43.23	< 0.01	12
	Treatment	547.40	972.62	< 0.01	71
	Genotype:Treatment	109.37	10.23	< 0.01	14
	Environment	27.01			3

LM24	Genotype	568.83	419.09	< 0.01	50
	Treatment	192.34	453.47	< 0.01	17
	Genotype:Treatment	358.93	52.89	< 0.01	32
	Environment	17.31			2
LM26	Genotype	210.40	165.26	< 0.01	13
	Treatment	1211.43	3615.83	< 0.01	72
	Genotype:Treatment	242.87	38.15	< 0.01	14
	Environment	16.08			1
LM32	Genotype	339.38	311.65	< 0.01	26
	Treatment	654.95	2202.88	< 0.01	50
	Genotype:Treatment	286.06	50.62	< 0.01	22
	Environment	17.99			1
LM33	Genotype	230.07	113.05	< 0.01	18
	Treatment	694.48	1160.20	< 0.01	55
	Genotype:Treatment	304.05	29.88	< 0.01	24
	Environment	25.86			2
LS1	Genotype	291.54	295.29	< 0.01	22
	Treatment	766.80	2395.37	< 0.01	57
	Genotype:Treatment	263.87	50.01	< 0.01	20
	Environment	16.30			1
LS2	Genotype	218.43	209.67	< 0.01	15
	Treatment	935.91	3413.97	< 0.01	65
	Genotype:Treatment	261.81	50.26	< 0.01	18
	Environment	13.16			1
LS3	Genotype	376.14	353.75	< 0.01	29
	Treatment	634.24	2234.16	< 0.01	49
	Genotype:Treatment	273.38	51.09	< 0.01	21
	Environment	15.30			1
LS4	Genotype	415.18	506.57	< 0.01	29
	Treatment	744.02	3449.62	< 0.01	52
	Genotype:Treatment	269.79	65.84	< 0.01	19
	Environment	10.35			1

LS5	Genotype	228.43	491.35	< 0.01	33
	Treatment	274.92	1537.45	< 0.01	39
	Genotype:Treatment	190.67	82.03	< 0.01	27
	Environment	6.01			1
LS6	Genotype	203.81	361.91	< 0.01	22
	Treatment	532.40	3592.55	< 0.01	59
	Genotype:Treatment	166.44	59.11	< 0.01	18
	Environment	7.11			1
LS8	Genotype	384.26	473.77	< 0.01	27
	Treatment	665.38	3117.39	< 0.01	47
	Genotype:Treatment	355.87	87.75	< 0.01	25
	Environment	10.25			1
LS11	Genotype	124.88	94.39	< 0.01	19
	Treatment	443.08	1272.57	< 0.01	66
	Genotype:Treatment	86.41	13.06	< 0.01	13
	Environment	16.71			2
LS20	Genotype	345.48	359.40	< 0.01	21
	Treatment	964.77	4014.66	< 0.01	59
	Genotype:Treatment	303.64	63.18	< 0.01	19
	Environment	12.11			1
LS26	Genotype	54.98	36.47	< 0.01	15
	Treatment	162.95	410.82	< 0.01	44
	Genotype:Treatment	134.97	17.91	< 0.01	36
	Environment	19.04			5
P1	Genotype	600.99	581.84	< 0.01	45
	Treatment	450.84	1745.88	< 0.01	34
	Genotype:Treatment	260.60	50.46	< 0.01	20
	Environment	13.01			1
P5	Genotype	506.66	388.88	< 0.01	40
	Treatment	452.61	1250.61	< 0.01	36
	Genotype:Treatment	295.35	45.34	< 0.01	23
	Environment	16.50			1

P7	Genotype	294.00	153.33	< 0.01	22
	Treatment	742.23	1470.88	< 0.01	56
	Genotype:Treatment	263.60	27.49	< 0.01	20
	Environment	24.22			2
P28	Genotype	267.11	162.78	< 0.01	17
	Treatment	961.96	2227.63	< 0.01	62
	Genotype:Treatment	298.44	36.37	< 0.01	19
	Environment	20.73			1
P2	Genotype	69.65	70.58	< 0.01	7
	Treatment	895.83	3268.08	< 0.01	85
	Genotype:Treatment	80.31	16.28	< 0.01	8
	Environment	12.50			1
P8	Genotype	19.34	19.32	< 0.01	2
	Treatment	1166.84	4427.80	< 0.01	93
	Genotype:Treatment	51.40	10.27	< 0.01	4
	Environment	12.65			1
P13	Genotype	40.54	30.24	< 0.01	2
	Treatment	1875.78	5317.27	< 0.01	94
	Genotype:Treatment	70.94	10.58	< 0.01	4
	Environment	16.93			1
P14	Genotype	36.35	49.73	< 0.01	2
	Treatment	1710.66	10296.75	< 0.01	93
	Genotype:Treatment	89.37	24.45	< 0.01	5
	Environment	9.17			0
P23	Genotype	36.78	27.03	< 0.01	2
	Treatment	1439.09	3806.87	< 0.01	91
	Genotype:Treatment	84.10	12.36	< 0.01	5
	Environment	17.24			1
P30	Genotype	42.46	25.89	< 0.01	3
	Treatment	1125.96	2471.52	< 0.01	89
	Genotype:Treatment	71.71	8.75	< 0.01	6
	Environment	20.77			2

Supporting Information Table S2.4 Results of one-way ANOVAs used to calculate the relative contribution of genotypic differences in mycelial Zn concentrations between dikaryotic *Suillus luteus* strains (n = 30) collected from two metal-polluted sites (Lommel-Maatheide: LM and Lommel-Sahara: LS) and a control site (Paal: P).

Family	Part of phenotypic variation	Variance	F-value	p-value	Relative contribution to phenotypic variation (%)
LM	Genotype	1.44	107.52	< 0.01	98
	Environment	0.03			2
LS	Genotype	1.18	88.47	< 0.01	98
	Environment	0.03			2
P	Genotype	5.84	230.31	< 0.01	99
	Environment	0.06			1

Supporting Information Figure S2.5 Results of one-way ANOVAs used to calculate the relative contribution of genotypic differences in mycelial Zn concentrations between monokaryotic *Suillus luteus* strains (n = 600) collected from two metal-polluted sites (Lommel-Maatheide: LM and Lommel-Sahara: LS) and a control site (Paal: P).

Family	Part of phenotypic variation	Variance	F-value	p-value	Relative contribution to phenotypic variation (%)
LM10	Genotype Environment	174.53 0.76	483.08	< 0.01	100 0
LM17	Genotype Environment	11.50 0.23	104.05	< 0.01	98 2
LM19	Genotype Environment	22.58 0.46	103.03	< 0.01	98 2
LM22	Genotype Environment	37.98 0.52	154.55	< 0.01	99 1
LM24	Genotype Environment	27.18 0.38	150.08	< 0.01	99 1
LM26	Genotype Environment	374.63 2.12	372.39	< 0.01	99 1
LM32	Genotype Environment	256.61 2.03	266.23	< 0.01	99 1
LM33	Genotype Environment	275.29 1.93	300.73	< 0.01	99 1
LM7	Genotype Environment	269.55 1.98	286.94	< 0.01	99 1
LM8	Genotype Environment	354.07 2.13	350.10	< 0.01	99 1
LS1	Genotype Environment	65.46 1.29	107.29	< 0.01	98 2
LS11	Genotype Environment	5.11 0.17	64.09	< 0.01	97 3
LS2	Genotype Environment	675.30 3.51	404.68	< 0.01	99 1
LS20	Genotype Environment	58.73 0.89	139.74	< 0.01	99 1

LS26	Genotype	9.08	36.69	< 0.01	95
	Environment	0.52			5
LS3	Genotype	17.82	76.58	< 0.01	97
	Environment	0.49			3
LS4	Genotype	196.55	253.78	< 0.01	99
	Environment	1.63			1
LS5	Genotype	75.54	154.94	< 0.01	99
	Environment	1.03			1
LS6	Genotype	359.84	358.06	< 0.01	99
	Environment	2.12			1
LS8	Genotype	497.20	457.96	< 0.01	100
	Environment	2.29			0
P1	Genotype	174.11	253.46	< 0.01	99
	Environment	1.45			1
P13	Genotype	39.92	44.50	< 0.01	95
	Environment	1.89			5
P14	Genotype	107.89	60.97	< 0.01	97
	Environment	3.73			3
P2	Genotype	37.90	26.52	< 0.01	93
	Environment	3.01			7
P23	Genotype	213.21	156.22	< 0.01	99
	Environment	2.87			1
P28	Genotype	287.94	320.58	< 0.01	99
	Environment	1.89			1

P30	Genotype	121.80	100.39	< 0.01	98
	Environment	2.55			2
P5	Genotype	427.30	360.60	< 0.01	99
	Environment	2.49			1
P7	Genotype	321.80	363.01	< 0.01	99
	Environment	1.87			1
P8	Genotype	45.82	42.13	< 0.01	95
	Environment	2.29			5

Chapter 3: **Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies**

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SUMMARY

Current metabarcoding studies aiming to characterize microbial communities generally rely on the amplification and sequencing of relatively short DNA regions. For fungi, the internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) operon has been accepted as the formal fungal barcode. Despite an increasing number of fungal metabarcoding studies, the amplification efficiency of primers is generally not tested prior to their application in metabarcoding studies. Some of the challenges that metabarcoding primers should overcome efficiently are the amplification of target DNA strands in samples rich in non-target DNA and environmental pollutants, such as humic acids, that may have been co-extracted with DNA. In the current study, three selected primer pairs were tested for their suitability as fungal metabarcoding primers. The selected primer pairs include two primer pairs that have been frequently used in fungal metabarcoding studies (ITS1F/ITS2 and ITS3/ITS4) and a primer pair (ITS86F/ITS4) that has been shown to efficiently amplify the ITS2 region of a broad range of fungal taxa in environmental soil samples. The selected primer pairs were evaluated in a 454 amplicon pyrosequencing experiment, real-time PCR (qPCR) experiments and *in silico* analyses. Results indicate that experimental evaluation of primers provides valuable information that could aid in the selection of suitable primers for fungal metabarcoding studies. Furthermore, we show that the ITS86F/ITS4 primer pair outperforms other primer pairs tested in terms of *in silico* primer efficiency, PCR efficiency, coverage, number of reads and number of species-level

operational taxonomic units (OTUs) obtained. These traits push the ITS86F/ITS4 primer pair forward as highly suitable for studying fungal diversity and community structures using DNA metabarcoding.

Keywords: community analysis, soil fungi, internal transcribed spacer (ITS), next generation sequencing, 454 amplicon pyrosequencing, operational taxonomic unit (OTU), real-time PCR (qPCR)

INTRODUCTION

Until the late 1980s, microbial ecologists and taxonomists have relied on culturing and morphological and physiological characteristics to describe microbial communities and members thereof. In the last two decades, DNA sequencing has revolutionized the way microbial communities are being characterized (Stahl *et al*., 1984; Hugenholtz and Pace, 1996). In addition, since the introduction of pyrosequencing by Margulies *et al*. (2005), characterization of microbial communities has undergone a second revolution as this technology (used by e.g. Sogin *et al*. (2006) and Buée *et al*. (2009)) enables detailed microbial community characterization at greater sequencing depth than was deemed possible via cloning and Sanger sequencing. A number of next-generation sequencing technologies now enable researchers to identify a large number of organisms from environmental samples using relatively short DNA sequences. This molecular identification method has been termed metabarcoding (Taberlet *et al*., 2012). Nevertheless, whatever sequencing technology is used, DNA metabarcoding generally depends on the amplification of barcode regions using taxon-specific primers (Hebert *et al*., 2003). Such primers need to be universal enough to cover a large group of taxa (e.g. the fungal kingdom), but at the same time have to result in amplicons that are variable enough to efficiently distinguish between closely related species or to identify operational taxonomic units (OTUs) (Hebert *et al*., 2003; Justé *et al*., 2008). For fungi and oomycetes, the internal transcribed spacer region (ITS; spanning the ITS1, 5.8S and ITS2 regions) in the ribosomal RNA (rRNA) operon has been recognized as the formal DNA barcoding region (Seifert, 2009; Begerow *et al*., 2010; Schoch *et al*., 2012).

The full ITS region in fungi has an average length of 500 and 600 base pairs (bp) for ascomycetes and basidiomycetes, respectively, and an average length of 600 bp across all fungal lineages (Porter and Golding, 2011). As current 454 amplicon pyrosequencing (using Roche's Genome Sequencer FLX (GS-FLX) instrument and Titanium chemistry) generates read lengths

averaging 450 bp, it is impossible to span the entire ITS region in a single run. Even with recent advances in sequencing technologies that enable sequencing across the entire ITS region, it will probably remain desirable for fungal metabarcoding studies to exclude the 5.8S region of the rRNA operon. The inclusion of conserved regions in DNA sequences are known to increase the risk of chimera formation during PCR (Haas *et al*., 2011). Therefore, generally, either the ITS1 or the ITS2 region is used in ecological studies aiming at the characterization of fungal communities.

Primers that will be used in metabarcoding studies should be able to efficiently amplify their target DNA regions in the presence of high concentrations of non-target DNA and contaminants, such as humic acids, that may have been co-extracted with DNA (Kosch and Summers, 2013). Therefore, *in silico* testing of primers is expected to result in an incomplete picture of how primers will behave during amplification of DNA extracted from environmental samples. Comparing the amplification efficiency and robustness of primers used in metabarcoding studies is important because differences in primer efficiency may result in strong biases in favour of more easily amplifiable sequences during PCR reactions, potentially influencing our view on fungal communities (Polz and Cavanaugh, 1998; Jumpponen, 2007; Engelberktson *et al*., 2010). Moreover, a primer set that covers a large proportion of the species that compose a community of interest and that produces a reliable outcome is desired as ecological metabarcoding studies typically rely on a single primer pair to map microbial diversity.

The most commonly used primers in fungal ecology for sequence-based fungal identification at the species level were published by White et al. (1990): ITS1, ITS2, ITS3 and ITS4, and by Gardes and Bruns (1993): ITS1F and ITS4B. Whereas the primers developed by White et al. (1990) had a broad spectrum, ITS1F and ITS4B were developed to be specific for fungi and basidiomycetes respectively (Gardes and Bruns, 1993). ITS1F is most frequently combined with ITS2 to amplify the ITS1 region of the fungal rRNA operon and ITS3 is usually combined with ITS4 to amplify the ITS2 region. These primer pairs have been used in many branches of

mycological research in the past twenty years and are popular tools in recent fungal community studies as well (Buée *et al*., 2009; Jumpponen and Jones, 2009; Amend *et al*., 2010; Ghannoum *et al*., 2010; Jumpponen *et al*., 2010; Tedersoo *et al*., 2010; also reviewed in Hibbett *et al*. (2011)). The aim of the current study was to evaluate the amplification efficiency of these established primer pairs and to compare them to a selected primer pair (ITS86F/ITS4) that has been shown to specifically and efficiently amplify ITS sequences from a broad range of fungal taxa in human blood samples as well as in environmental soil samples (Vancov and Keen, 2009).

MATERIALS AND METHODS

Study site and soil sampling

A pioneer pine forest on a stabilised sand dune in the northern part of Limburg, Belgium (Hechtel-Eksel: 51° 7′ 33″ N, 5° 22′ 22″ E) was selected to obtain samples for this study. The study site is not freely accessible. To gain access to this study site, please contact the responsible authorities (Table S3.1). The soil in this study site is a dry sandy soil without a litter layer, poor in organic matter and slightly acidic. The average organic carbon content for this site is 0.7% and the average pH is 4.7. The pioneer vegetation at the study site is dominated by young Scots pine trees (*Pinus sylvestris* L.), mosses and lichens, with only few grasses and heather shrubs (*Calluna vulgaris* (L.) Hull). Tree ages at the time of sampling ranged from one to five years. The region has an average annual rainfall of 800 mm per square meter and the average annual temperature is 10°C (Royal Meteorological Institute, Ukkel, Belgium).

Soil samples for fungal community characterization were collected in November 2009. Samples were collected at a depth of 0 to 20 cm using a soil corer with a diameter of 1 cm. Four replicate soil samples were collected within a distance of ten centimetres from each other for seven sampling locations. Each sampling location was chosen close to a three to five year old pine tree randomly selected in the field. Selected pine trees were at

least 20 m apart from each other. The 4 replicate soil samples were pooled for each sampling location, resulting in a total of seven pooled samples. Samples were sealed in plastic bags and tightly closed to prevent desiccation during transportation. Upon arrival in the lab, soil samples were sieved using a 2 mm sieve to homogenize the sample and remove roots, large pieces of organic matter and stones. Samples were subsequently stored at -80°C until DNA was extracted. No protected species were sampled during the study.

DNA extraction, PCR amplification and pyrosequencing

Approximately 250 mg of soil was used for each DNA extraction. DNA was extracted in quadruplicate from each pooled sample using the UltraClean Soil DNA Isolation Kit according to the manufacturer's protocol (MoBio, Carlsbad, CA, USA). This resulted in four replicates for each of seven pooled soil samples. Subsequently, amplicon libraries were created using barcode-tagged primers for the primer pairs ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4 (Table 3.1).

Table 3.1 Primers used in the current study.

Primer [(1)]	Primer sequence (5'-3')	rRNA operon binding site	Reference
ITS1F (F)	CTTGGTCATTTAGAGGAAGTAA	SSU	Gardes and Bruns, 1993
ITS2 (R)	GCTGCGTTCTTCATCGATGC	5.8S	White *et al.*, 1990
ITS3 (F)	GCATCGATGAAGAACGCAGC	5.8S	White *et al.*, 1990
ITS4 (R)	TCCTCCGCTTATTGATATGC	LSU	White *et al.*, 1990
ITS86F (F)	GTGAATCATCGAATCTTTGAA	5.8S	Turenne *et al.*, 1999
ITS86R (R)	TTCAAAGATTCGATGATTCAG	5.8S	Vancov and Keen, 2009

ITS86R contains a wrong base at the 3' end. The G should be replaced by a C (see Discussion).

[(1)] Primers are indicated as forward (F) or reverse (R).

Both forward and reverse primers were synthesized with a tail containing the Roche 454 pyrosequencing adaptors and a sample-specific 10 bp barcode (multiplex identifiers: MIDs) (Carlsen *et al*., 2012) enabling sorting out the obtained sequences after sequencing (Roche Applied Science, Mannheim, Germany). Fusion primers were designed according to the scheme provided in Table S3.2.

DNA samples were amplified using a Techne TC-5000 thermocycler (Bibby Scientific Limited, Staffordshire, UK) under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C during 1 minute; a final extension phase was performed at 72°C during 10 minutes. Reactions were carried out in 25 µl reaction volumes using the FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). Each reaction contained 2.75 µl FastStart 10x reaction buffer, 1.8 mM MgCl, 0.2 mM dNTP mix, 0.4 µM of each primer, 1.25 U FastStart HiFi polymerase and 5 ng template DNA (as measured by a Nanodrop spectrophotometer).

Amplified DNA was cleared from PCR primers and primer dimers using the Agencourt AMpure XP System according to the manufacturer's protocol (Beckman Coulter, Brea, CA, USA). Finally, purified dsDNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany) and subsequently pooled in equimolar concentrations. The resulting amplicon pool, containing all 84 samples, was sequenced on one fourth of a Pico Titer Plate on a Roche Genome Sequencer FLX System using Titanium chemistry (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

Bioinformatics processing

The standard flowgram format (SFF) file that resulted from the interpreted flowgrams was deposited in the NCBI Sequence Read Archive under accession number SRP026207 (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra). From the original SFF file, three separate quality and fasta files were created with a custom biopython script according to the three primer pairs used (Table S3.2). Further analyses were carried out in Mothur 1.31.2 on the individual fastq and fasta files (Schloss *et al*., 2009). Quality trimming in Mothur removed reads shorter than 200 bases, reads longer than 600 bases, reads with homopolymers longer than 8 bases and reads containing ambiguous bases. Reads were trimmed when the average Phred quality score dropped below 35 over a window of 50 bases. Next, sequences were compared to each other and duplicate sequences were replaced by a single sequence, while archiving the abundance data of the unique sequences. Subsequently, unique reads were checked for chimeric sequences using the Uchime tool in Mothur followed by their removal from the datasets. Unique reads were aligned with the pairwise alignment tool in Mothur. Finally, species-level OTUs were defined based on a 97% sequence similarity level, which is within the range of intraspecific ITS sequence similarity (Blaalid *et al*., 2013). In order to further remove potential sequencing errors from the analysis, global singletons (i.e. OTUs represented by only a single sequence over an entire dataset) were removed (Tedersoo *et al*., 2010).

Because the primer pairs resulted in different amounts of reads per sample, the number of reads per sample were rarefied to 200 reads per sample. Samples for which less than 200 reads were obtained were removed from the dataset. For ITS1F/ITS2 14 of 28 samples were removed. For ITS3/ITS4 4 samples were removed and for ITS86F/ITS4 no samples were removed. Inter-sample rarefaction curves were constructed based on 10,000 iterations. Subsequently, intra-sample diversity, richness and Good's coverage estimates were calculated in Mothur 1.31.2 based on 10,000 iterations. BLAST searches for a representative sequence of each OTU (as

determined by Mothur) were conducted using the PlutoF v2.0 massBLASTer online tool (Abarenkov *et al*., 2010). Reads were blasted against the UNITE (Kõljalg *et al*., 2005) and INSD (Nakamura *et al*., 2013) databases. Resulting HTML files were combined with the abundance data obtained in Mothur using a custom Python script. This script also acquired the names of species or genera that resemble Latin binomials with the highest BLAST score, avoiding unidentified OTUs in the databases to be seen as best BLAST hits. Unidentified OTUs were indicated as "not applicable (NA)".

Quantitative real-time PCR

To evaluate the performance of the primer pairs amplifying target DNA from a heterogeneous pool of DNA in environmental samples, all primer pairs were tested in a qPCR set-up. A 2-fold dilution series (1:1 to 1:64) was made from twelve DNA samples (ranging from 5 ng μl^{-1} to 78 pg μl^{-1}, including one no-template control (NTC) for each sample). Amplification was performed in optical 96-well plates using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green chemistry. PCR conditions were as follows: initial denaturation at 95°C for two minutes, followed by 40 cycles of 95°C (30s), 55°C (30s) and 72°C (60s) and a final extension phase at 72°C for 10 minutes followed by the generation of a dissociation curve to verify amplification specificity. These qPCR conditions were chosen to mimic the PCR conditions used during the PCR step prior to emPCR and amplicon pyrosequencing. Reactions contained 2.5 µL template DNA, 5 µL 2x Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 µl forward and reverse primers (3.3 µM each) and 1.9 µL nuclease-free H_2O in a total volume of 10 µL. PCR efficiencies (E) were calculated as $E = (10^{-1/slope} - 1) \times 100$.

To assess a potential PCR-bias at the phylum level, DNA was extracted from 15 pure cultures including 5 basidiomycetes (*Lentinula edodes* (MUCL 44827), *Agrocybe praecox* (MUCL 46727), *Coniophora marmorata* (MUCL 39471), *Suillus luteus* (UH-Slu-LM8-n1) and *Antrodia vaillantii* (MUCL 54533)), 5 ascomycetes (*Cladosporium cladosporioides* (MUCL 53652),

Cryptosporiopsis radicicola (MUCL 53485), *Monilinia laxa* (MUCL 30841), *Arthroderma otae* (MUCL 39756) and *Galactomyces geotrichum* (MUCL 52377)), 2 glomeromycetes (*Rhizophagus clareus* (MUCL 46238) and *Rhizophagus* sp. (MUCL 41833)) and 3 zygomycetes (*Mortierella verticillata* (MUCL 9658), *Absidia corymbifera* (MUCL 38907) and *Mucor hiemalis* (MUCL 15439)). DNA was extracted from cultures using the DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Venlo, Netherlands). DNA concentrations extracted from pure cultures used for qPCR ranged from 5 ng μl^{-1} to 20 ng μl^{-1}. PCR bias at the phylum level was tested according to the qPCR protocol described above.

***In silico* evaluation of primer pairs**

To evaluate the primer-to-target mismatches *in silico*, primers were tested with PrimerProspector 1.0.1 (Walters *et al*., 2011) against sequences downloaded from NCBI. Three sets of sequences were downloaded from NCBI containing only full-length fungal 5.8S, 18S and 28S sequences. Duplicate sequences were removed using Mothur 1.31.2. ITS1F was tested against 3,748 18S rDNA sequences. ITS2, ITS3 and ITS86F were tested against 4,421 5.8S rDNA sequences. ITS4 was tested against 4,270 28S rDNA sequences. For comparison, also all primers described by Ihrmark *et al*. (2012) and Toju *et al*. (2012) were tested. All tests were performed as described by Walters *et al*. (2011) using standard settings. Primer scores were calculated based on the following formula: weighted score = non-3' mismatches x 0.40 + 3' mismatches x 1.00 + non-3' gaps x 1.00 + 3' gaps x 3.00. An additional penalty score of 3.00 was assigned if the final 3' base of a primer had a mismatch with its target sequence (Walters *et al*., 2011).

Statistical analysis

Statistical analyses were conducted in R 2.13.0 (The R Foundation for Statistical Computing, Vienna, Austria). Normal distributions of the residuals of models were checked with the Shapiro-Wilk test, while homoscedasticity of variances was analysed using either Bartlett's or the Fligner-Killeen test. Depending on the distribution of the estimated parameters, either ANOVA or the Kruskal-Wallis Rank Sum Test was used to check for significant differences in variances of parameters. Two-by-two comparisons were conducted using either Tukey Honest Significant Differences tests or Pairwise Wilcoxon Rank Sum Tests. Poisson corrections were implemented for abundance data. Distributions of ratios were compared with Pearson's Chi-squared tests. Non-metric multi-dimensional scaling (NMDS) was performed using the Vegan 2.0-8 package in R.

RESULTS

Parametrical analysis of 454 amplicon pyrosequencing data

For the three tested primer pairs, GS-FLX sequencing of the amplicon libraries generated a total of 151,650 reads. For a read to be successfully assigned to a sample, we required that both the forward and the reverse MIDs and primers were identified in a read with no more than one erroneous base in the MIDs and no more than two erroneous bases in the primer sequences. Based on the primer and MID sequences, 65,133 reads were assigned to their respective sample and 86,517 reads remained unassigned. The average length of reads assigned to either ITS1F/ITS2, ITS3/ITS4 or ITS86F/ITS4 prior to quality checking and trimming was 314, 331 and 369 bp respectively (excluding primers). The average read length of the unassigned reads was 116 bp (including primers, data not shown).
Rarefaction curves were constructed showing the rarefied number of OTUs defined at a 97% sequence similarity threshold relative to the number of samples (Fig. 3.1). These results indicate that, on average, a higher OTU richness and a better coverage of the fungal community can be expected

for the ITS86F/ITS4 and ITS3/ITS4 primer pairs. The lowest OTU richness and coverage was predicted for the ITS1F/ITS2 primer pair. As most rarefaction curves tended towards saturation, the sequencing depth was assumed to be sufficient to retrieve the most abundant fungal OTUs in analysed soil samples that are detectable by the respective primers and 454 amplicon pyrosequencing.

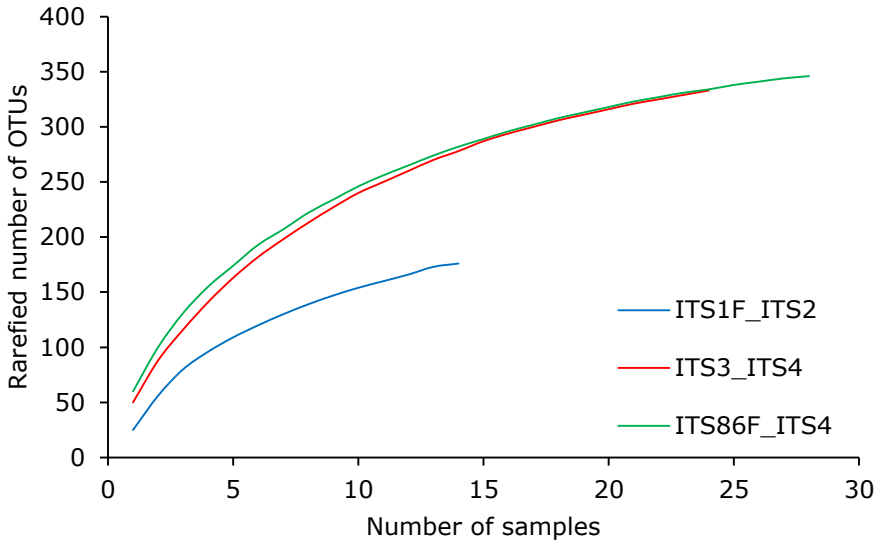

Figure 3.1 Rarefaction curves for each of the three primer pairs used in this study: ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4. In these graphs, the number of samples is plotted against the rarefied number of operational taxonomic units (OTUs) that were created based on a 97% sequence similarity cut-off value.

To compare primer pair performance in the 454 amplicon pyrosequencing experiment, averages of the number of reads were calculated across replicates (four replicates per sample) and samples (seven samples) for each primer pair. The average number of reads per sample obtained by ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4 after quality trimming differed significantly ($p < 0.01$) and primer pairs yielded on average (± standard error) 356 (± 26), 523 (± 43) and 797 (± 34) high quality reads per sample, respectively (Fig. 3.2A). The average number of OTUs found for

each primer pair at a 97% sequence similarity threshold (observed OTU richness) also differed significantly ($p < 0.01$). The highest OTU richness was observed for ITS86F/ITS4 with an average of 62 OTUs per sample (min = 42; max = 106). ITS1F/ITS2 yielded on average 32 OTUs per sample (min = 15; max = 60), whereas ITS3/ITS4 resulted in an average of 50 OTUs per sample (min = 27; max = 76) (Fig. 3.2B). Diversity was estimated with the inverse Simpson index. The inverse Simpson index differed significantly between ITS86F/ITS4 and ITS1F/ITS2, whereas with ITS1F/ITS2 a lower diversity was found than with ITS86F/ITS4 ($p = 0.04$). However, no significant differences were found between ITS3/ITS4 and ITS1F/ITS2 or between ITS3/ITS4 and ITS86F/ITS4 ($p = 0.31$ and $p = 0.53$, respectively) (Fig. 3.2C). The average Good's coverage per sample obtained for ITS1F/ITS2 was 96.8% (min = 93.8%, max = 98.9%), whereas the average Good's coverage obtained for ITS3/ITS4 and ITS86F/ITS4 was 96.5% (min = 93.2%, max = 99.0%) and 97.5% (min = 95.3%, max = 99.6%) respectively (Fig. 3.2D). Significant differences in Good's coverage were found between ITS3/ITS4 and ITS86F/ITS4 ($p < 0.01$). However, no significant differences were found between ITS1F/ITS2 and ITS3/ITS4 ($p = 0.81$) or between ITS1F/ITS2 and ITS86F/ITS4 ($p = 0.31$).

Community similarity compared between primer pairs

To compare the fungal community characterized with ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4 at the species and phylum level, a representative sequence of each OTU (as selected by Mothur) was blasted against the UNITE and INSD databases using the massBLASTer tool in PlutoF v2.0 (Abarenkov *et al.*, 2010). Relative frequency distributions of the obtained species-level OTUs and phyla were analysed with chi-squared tests for the different primer pairs, based on the average abundances across replicates (four) and samples (seven).

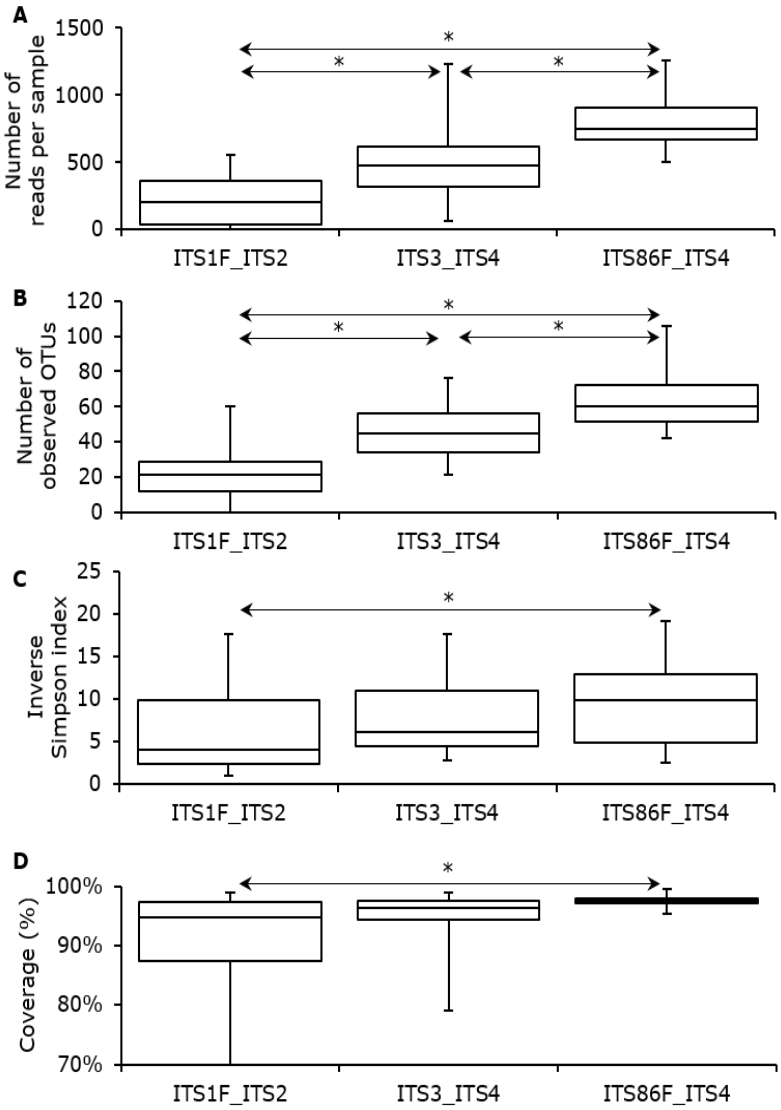

Figure 3.2 Parametrical comparison between the three primer pairs used in this study (ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4). A. Average number of sequences obtained after quality trimming. B. Average number of operational taxonomic units (OTUs), based on a 97% sequence similarity cut-off value. C. Average inverse Simpson index. D. Average Good's coverage. Averages were calculated across replicates (four) and samples (seven) for each primer pair. Differences at the 95% significance level are indicated with an asterisk "*".

Representative reads of OTUs that could not be coupled to an accession of either the UNITE or INSD databases were considered as unidentified OTUs (indicated as not applicable "NA" in Appendix A). A total of 51 unidentified OTUs were found of which 50 were found with ITS86F/ITS4 and 1 with ITS3/ITS4. BLAST scores and corresponding E-values for all OTUs can be found in Appendix A. At the species level, differences were observed between the fungal communities identified by the three primer pairs studied ($p < 0.01$). To give an idea of the fungal communities identified by each primer pair, pie charts displaying the top ten most abundant OTUs were constructed covering 68%, 62% and 64% of all sequences obtained with ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4, respectively (Fig. 3.3). Using the ITS1F/ITS2 primer pair (targeting the ITS1 region) a total of 183 OTUs across all samples were observed, with the most abundant OTUs corresponding to *Sistotrema* sp. Fr. (27%), *Rhizopogon luteolus* Fr. (9%), *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf (8%), *Cladophialophora minutissima* M.L. Davey & Currah (7%), and *Capronia* sp. Sacc. (5%) (Fig. 3.3A). The primer pairs ITS3/ITS4 and ITS86F/ITS4 (targeting the ITS2 region) identified 333 and 346 OTUs across all samples, respectively. In line with ITS1F/ITS2, the fungal communities identified with ITS3/ITS4 and ITS86F/ITS4 were also dominated by *Sistotrema* sp. (21-19%), but the subdominant OTUs were not exactly the same (Fig. 3.3B,C). Interesting to note is that the soil samples are dominated by ectomycorrhizal and ericoid mycorrhizal fungi and mycobionts from lichens. Based on field observations, we assumed that the fungal community in the pioneer forest that was sampled in this study would be relatively species poor compared to old forest soils (Buée *et al*., 2009) and that biotrophic fungi would dominate over saprotrophic ones. These assumptions were confirmed by all three primer pairs (Fig. 3.3). At the phylum level, differences were found between all primer pairs tested ($p < 0.01$ for all comparisons) (Fig. 3.4). Nevertheless, the majority of OTUs identified by all tested primer pairs belonged to the phyla Ascomycota (56% to 71%), followed by Basidiomycota (14% to 17%). A minority of OTUs identified, belonged to the Zygomycota (3% to 4%), Chytridiomycota (3% to 4%) and Glomeromycota (0% to 3%) (Fig. 3.4).

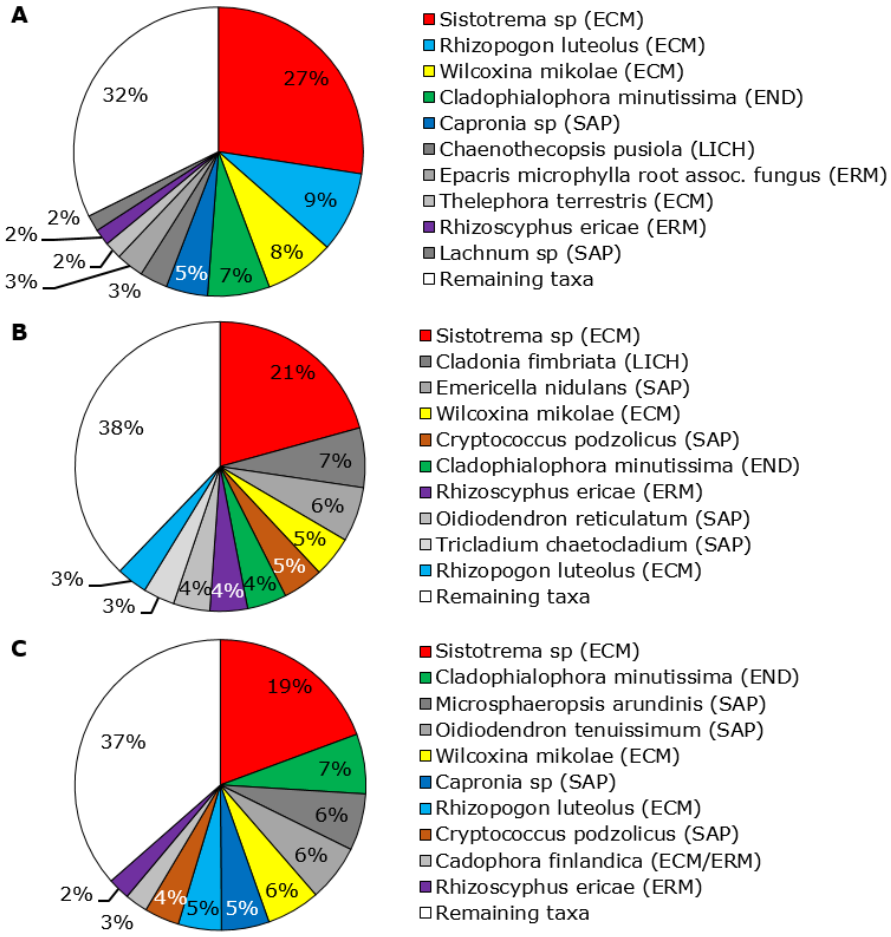

Figure 3.3 Relative abundance for the top ten most abundant species-level operational taxonomic units (OTUs), based on a 97% sequence similarity cut-off value, obtained for each of the three primer pairs studied (ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4). Reads that did not result in a BLAST hit against the UNITE or INSD databases were indicated as "not applicable (NA)". Ecological functions of OTUs are indicated between brackets behind the OTU identities (ECM: ectomycorrhizal, ERM: ericoid mycorrhizal, SAP: saprotrophic, LICH: lichenized, END: endophytic). OTUs not belonging to the top ten most abundant OTUs were pooled in the category "Remaining taxa". OTUs that appear exclusively in a single chart are indicated in grayscale. OTUs that can be found in multiple pie charts are indicated in colour. OTU abundance scores were averaged across replicates (four) and samples (seven). A. ITS1F/ITS2. B. ITS3/ITS4. C. ITS86F/ITS4.

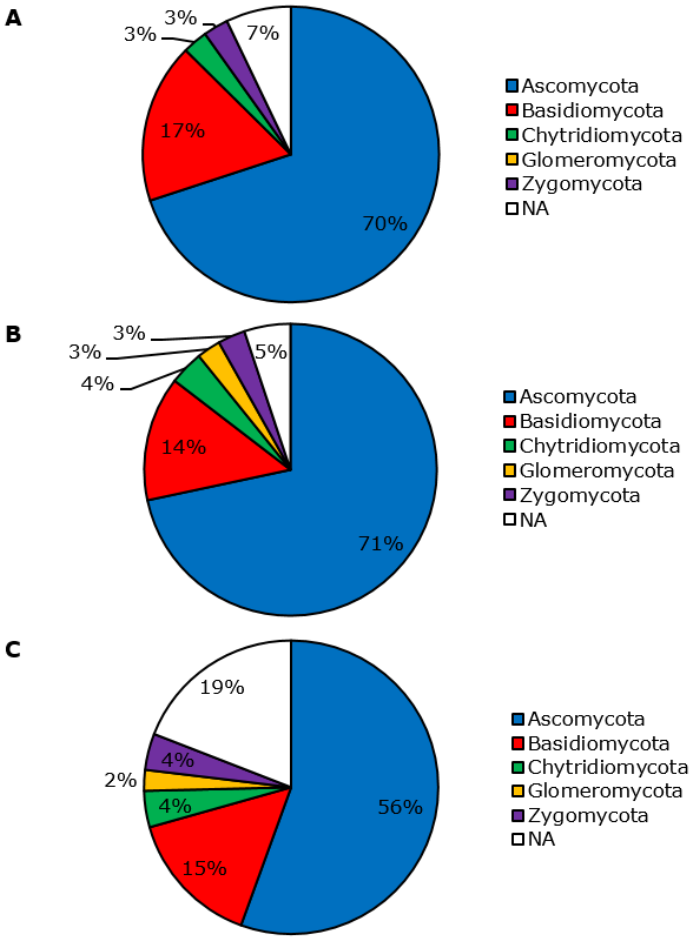

Figure 3.4 Relative number of OTUs belonging to different fungal phyla. OTUs that could not be assigned to a phylum were grouped together under "not applicable (NA)". Averages were calculated across replicates (four) and samples (seven). A. ITS1F/ITS2. B. ITS3/ITS4. C. ITS86F/ITS4.

Repeatability of metabarcoding results

The repeatability of the molecular identification of fungal OTUs from environmental samples was compared between the three tested primer pairs to assess their experimental robustness. Replicates of samples were compared for each primer pair using NMDS with Bray-Curtis dissimilarities. In this analysis, samples with a similar OTU-composition will have smaller

Bray-Curtis distances than samples with more dissimilar OTU compositions. In general, for all three primer pairs, replicates from the same sample grouped closely together (especially for ITS3/ITS4) (Fig. S3.1). Hence, the results of molecular identification of fungal OTUs are fairly consistent between replicated samples using the current experimental set-up. In order to test the possibility that some OTUs are missed in metabarcoding analyses based on the amplification and sequencing of target DNA from a single DNA extraction, results from the four replicated DNA extractions of the same sample were compared (Fig. S3.2). This assessment was performed for the four most abundant OTUs, representing *Sistotrema* sp., *Rhizopogon luteolus*, *Cladophialophora minutissima* and *Wilcoxina mikolae*. From Fig. S3.2, it is clear that in some replicated extractions of the same sample abundant OTUs can be missed. These results indicate that PCR amplification and sequencing can best be performed on multiple DNA extractions from the same environmental sample that are pooled prior to PCR in order to obtain an accurate picture of a fungal community.

Efficiency of primer pairs studied

To test the amplification efficiency of the three primer pairs in a heterogeneous pool of DNA (environmental sample) a qPCR experiment was conducted. More specifically, a 2-fold dilution series, ranging from 1:1 to 1:64 dilutions of twelve randomly selected DNA samples were amplified with randomly selected ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4 primers with MIDs and 454 adaptors attached. For ITS1F/ITS2, exponential amplification was obtained between 24 and 32 PCR cycles for ten out of twelve samples (data not shown). For two samples no exponential amplification phase was obtained within 40 cycles with this primer pair. ITS3/ITS4 showed exponential amplification after 22 to 36 cycles for all twelve samples, whereas ITS86F/ITS4 already showed an exponential amplification phase after 20 to 31 cycles for all samples (data not shown). Average PCR efficiencies (± standard error) were calculated to be 76% (±

4%) for ITS3/ITS4, 82% (± 5%) for ITS1F/ITS2 and 97% (± 6%) for ITS86F/ITS4 (Table 3.2).

Table 3.2 Average PCR amplification efficiencies obtained for twelve environmental DNA samples using quantitative real-time PCR

Primer pair	ITS1F_ITS2	ITS3_ITS4	ITS86F_ITS4
Average (%)	82	76	97
Standard error (%)	4	5	6
Minimum (%)	64	67	78
Maximum (%)	97	103	120

Phylum-level PCR bias

qPCR amplification efficiency did not significantly differ between primer pairs tested (ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4), nor between phyla (Ascomycota, Basidiomycota, Glomeromycota and Zygomycota) (Fig. 3.5). Two-way ANOVA resulted in $p = 0.14$ for phylum and $p = 0.59$ for primer pair. Primer pair - rDNA target combinations with poor PrimerProspector scores tended to have slightly lower PCR efficiencies, but these differences were not significant. Species used for this experiment and PCR efficiencies can be found in Table S3.4 and Table S3.5, respectively.

***In silico* evaluation of primers**

In a final analysis, the primer-to-target mismatches of the three primer pairs used in this study were evaluated with PrimerProspector (Walters *et al*., 2011). PrimerProspector was used to calculate a score for each primer based on mismatches between primers and target DNA sequences. The closer the score of a primer is to 0, the fewer mismatches were detected between primers and target sequences. The average scores (± standard error) for primers used in our study were: ITS1F = 4.55 (± 0.05), ITS2 = 0.70 (± 0.03), ITS3 = 0.58 (± 0.03), ITS4 = 3.96 (± 0.04) and ITS86F = 0.52 (± 0.02) (Table 3.3). Moreover, it was found that 44% of the tested sequences had a mismatch with the last base at the 3' end of primer ITS1F. This particular mismatch between the last base at the 3' end of a primer

sequence and a target sequence occurred with only 9%, 4%, 16% and 3% of the tested sequences for ITS2, ITS3, ITS4 and ITS86F respectively (Table 3.3). For comparison, also the primers suggested by Ihrmark *et al*. (2012) and Toju *et al*. (2012) were tested with PrimerProspector. Also in this analysis, ITS86F was found to have the best primer score of all tested primers (Table S3.5).

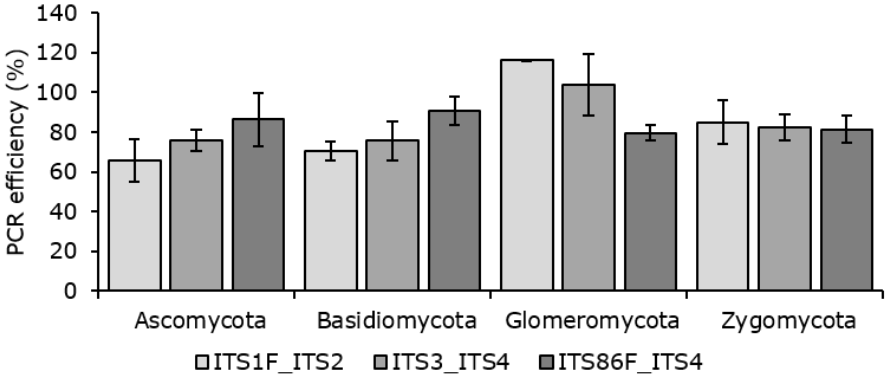

Figure 3.5 Phylum-level PCR bias assessed using qPCR. Average PCR efficiencies were calculated for each phylum using 5 basidiomycetes, 5 ascomycetes, 2 glomeromycetes and 3 zygomycetes. Error bars represent standard errors. No significant differences between primer pairs and phyla were found at the 95% confidence level.

Table 3.3 Results of *in silico* testing of primers using PrimerProspector 1.0.1 (Walters *et al*., 2011).

Primer [(1)]	Number of sequences tested	3' end base mismatch (%)	Average score ± SE
ITS1F (F)	3748	44%	4.6 ± 0.05
ITS2 (R)	4421	9%	0.7 ± 0.03
ITS3 (F)	4421	4%	0.6 ± 0.03
ITS4 (R)	4270	16%	4.0 ± 0.04
ITS86F (F)	4421	3%	0.0 ± 0.00

Average PrimerProspector scores are shown ± standard errors (SE).

[(1)] Primers are indicated as forward (F) or reverse (R).

DISCUSSION

Amplification and sequencing of short, standard DNA regions (metabarcoding) is becoming an increasingly popular tool for the characterization of fungal communities. Nevertheless, in most fungal metabarcoding studies, primers are generally used without being tested for their efficiency to amplify heterogeneous DNA pools, which may affect our view on studied fungal communities. Whereas the most commonly used primers in fungal metabarcoding studies were designed in the 90s for species identification of a limited number of focal species, environmental metabarcoding studies generally aim to characterize diverse communities in environmental samples. Hence, primers used for fungal metabarcoding should be able to amplify a broad range of target DNA sequences in a sample that is also rich in non-target DNA and that may contain environmental contaminants (Kennedy and Oswald, 2011). Even though recent efforts have resulted in new primers that could amplify a large proportion of target fungal DNA sequences (Ihrmark *et al*., 2012; Toju *et al*., 2012), an experimental evaluation of PCR efficiency and primer performance should be performed on real environmental samples.

Initially, also ITS1F/ITS86R was included in our study design, but this primer pair was discarded from the study as no amplification was obtained in exploratory PCR and gel-electrophoresis tests. A plausible explanation for this failure can be found in the fact that the reverse primer (ITS86R) used and reported by Turenne *et al*. (1999) and Vancov and Keen (2009) contains an incorrect base at the 3' end of the primer sequence. In order to be the perfect reverse complement of ITS86F, the sequence of ITS86R should be 5'-TTCAAAGATTCGATGATTCAC-3', and not 5'-TTCAAAGATTCGATGATTCAG-3' as reported. GS-FLX sequencing of the amplicon pool resulted in 151,650 raw reads prior to quality trimming. Of these reads, 65,133 were assigned to their respective sample and 86,517 reads remained unassigned. The unassigned reads were investigated manually revealing that the majority were primer sequences probably

resulting from primer dimers in our sequenced amplicon pool. Most likely, these primer dimers were not sufficiently removed during post-PCR clean-up steps.

Rarefaction curves were constructed for each primer pair (Fig. 3.1). These rarefaction curves indicate that the highest rarefied OTU richness and best coverage of the fungal community can be expected for the ITS86F/ITS4 and ITS3/ITS4 primer pairs. The average observed number of reads and the average observed number of OTUs (derived from these reads at a 97% sequence identity cut-off) indeed were highest for the ITS86F/ITS4 primer pair (797 reads and 62 OTUs on average per sample) and the ITS3/ITS4 primer pair (523 reads, 50 OTUs) and were much lower for the ITS1F/ITS2 primer pair (356 reads and 32 OTUs) (Fig. 3.2). The average observed diversity per sample, estimated by the inverse Simpson index, did not differ between ITS3/ITS4 and ITS86F/ITS4, but was significantly lower for ITS1F/ITS2 (Fig. 3.2). Overall, the low number of OTUs per sample found in the current study, are in sharp contrast with the more than 1000 OTUs per gram of forest soil found by Buée *et al*. (2009) based on amplification with the ITS1F/ITS2 primer pair. This difference in richness may be explained by the fact that pioneer forests probably contain relatively fewer fungal species compared to old forest soils (Buée *et al*., 2009). Additionally, overestimation or underestimation of species richness can also originate from data handling and analysis (Bazzicalupo *et al*., 2013). Based on the *in silico* performance and high Good's coverage calculated for ITS86F, it can be expected that the 62 OTUs found on average per sample by the ITS86F/ITS4 primer pair is close to the real species richness for the pioneer ecosystem growing on stabilised sand dunes which were studied here. The 50 OTUs per sample found by ITS3/ITS4 and the 32 OTUs found by ITS1F/ITS2, are probably underestimations due to a more narrow primer spectrum and/or lower PCR efficiencies. The fact that a high Good's coverage was found for the ITS1F/ITS2 primer pair despite a low observed OTU richness indicates that this primer pair is unable to multiply the ITS1 region of a large number of fungi. This is also supported by the *in silico*

analysis. In this analysis, ITS1F was shown to have the poorest primer score of 4.6 and its sequence was shown to have a mismatch at the final base at the 3' end of the primer (having a detrimental effect on amplification efficiency (Lefever *et al*., 2013)) with no less than 44% of the tested fungal sequences (Table 3.3). The large number of mismatches between the ITS1F primer and its target sequences was previously also addressed by Bellemain *et al*. (2010) and Ihrmark *et al*. (2012). In comparison, the ITS4 primer was given a score of 4.0 and was found to have a primer-to-target mismatch at the 3' end of the primer with only 16% of the tested sequences. For the ITS2, ITS3 and ITS86F primers a score of 0.7, 0.6 and 0.0 was obtained respectively (Table 3.3). These primers were shown to have a mismatch at the 3' end of the primer with only 9%, 4% and 4% of the tested sequences, respectively (Table 3.3), illustrating their broad amplification potential. Furthermore, our *in silico* analyses indicated that the primers suggested by Ihrmark *et al*. (2012) and Toju *et al*. (2012) had more mismatches to their respective target sequences than ITS86F.

To test how these parametrical differences would translate to amplification efficiency during PCR amplification preceding emulsion PCR (emPCR) and pyrosequencing, a first qPCR experiment was conducted. To this end, DNA was extracted from 12 soil samples and amplified with the same primer pairs used in the pyrosequencing experiment. The calculated PCR efficiencies were 82% for ITS1F/ITS2, 76% for ITS3/ITS4 and 97% for ITS86F/ITS4 (Table 3.2). From these PCR efficiencies, it is clear that ITS86F/ITS4 amplified its target ITS regions with greater efficiency than the other two primer pairs. Contrary to our expectations from the *in silico* analysis, ITS3/ITS4 obtained a lower efficiency than the ITS1F/ITS2 primer pair. This could be explained by the fact that also other factors determine the amplification efficiency of PCR reactions beside binding and dissociation of primers to their target DNA sequences. Such factors include the temperature-dependent properties of target DNA sequences and primer sequences in the PCR mixture, the temperature-dependent behaviour of

the used polymerase enzyme mixtures, the use of ROX as an endogenous reference dye, etc. (Kennedy and Oswald, 2011). Alternatively, the range of target sequences that ITS1F and ITS2 may bind to during PCR amplification is smaller, but the sequences that do get bound by these primers are amplified efficiently.

To see whether differences in amplification efficiency between primer pairs would also be reflected in the identities of the OTUs identified in the 454 amplicon pyrosequencing experiment, a representative read for each OTU was blasted against the UNITE and INSD databases and the BLAST hits with the highest score and a species or genus name were used to reconstruct the fungal community for each primer pair (Fig. 3.3). According to all three primer pairs, the soil fungal community was dominated by an OTU corresponding to *Sistotrema* sp. Additionally, all primer sets produced a number of OTUs that were commonly identified by all primer pairs (Fig. 3.3). The community identified by the three tested primer pairs still differed significantly, however. These differences confirm the finding that targeting either the ITS1 or the ITS2 region may result in different pictures of the fungal communities at the OTU level, as was previously assessed by both *in silico* (Nilsson *et al*., 2009) and sequencing studies (Bazzicalupo *et al*., 2013; Monard *et al*., 2013). In addition, it was found that primers targeting the same ITS region do not necessarily result in the same OTU composition (Fig. 3.3), highlighting the importance of primer choice in a given study. However, it needs to be noted that in comparative studies, it has been shown that an ecological signal can be much stronger than the differences in community composition originating from primer choice (Monard *et al*., 2013).

At the phylum level, significant differences between ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4 were found as well (Fig. 3.4). Although in varying proportions, all three primer pairs identified more OTUs belonging to ascomycetes (70%, 71% and 56% respectively) than basidiomycetes (17%, 14% and 15%), but also Chytridiomycota (3%, 4%, 4%), Glomeromycota (0%, 3% and 2%) and Zygomycota (3%, 3% and 4%)

were detected (Fig. 3.4). This might suggest that more ascomycetes were present in the soil at the time of investigation. However, amplification of DNA from ascomycetes may be favoured relative to amplification of DNA from basidiomycetes as the ITS sequences for ascomycetes are generally shorter than basidiomycete ITS sequences (this is especially true for the ITS2 region (Porter and Golding, 2011)) and amplification of shorter DNA fragments is favoured during PCR. Whereas in previous *in silico* analyses indeed a phylum-level bias was expected for some of the primers used (Bellemain *et al*., 2010), no such bias was found in the current study based on experimental data derived from qPCR of DNA extracted from 15 fungal species belonging to the major fungal phyla (Fig. 3.5).

Whatever the aim of a metabarcoding study, results obtained from metabarcoding need to be reliable. To assess the repeatability of the fungal metabarcoding experiment, we analysed four replicate DNA extractions of seven soil samples separately. The analysis of all replicates of samples revealed that replicated analysis of the same sample with a specific primer pair generally results in similar fungal community compositions (Fig. S3.1). This is especially true for the ITS3/ITS4 and ITS86F/ITS4 primer pairs as their replicated samples clustered nicely together. However, this is less true for the ITS1F/ITS2 primer pair, where replicates of samples tend to have greater projected Bray-Curtis distances (Fig. S3.1). Moreover, we have shown that it is possible to miss certain OTUs, even abundant ones, when one sequences amplicon pools that are constructed from a single DNA extraction (Fig. S3.2). It is therefore advisable to extract DNA from environmental samples in multiple replicates, pool the eluates and perform PCR and sequencing on the DNA from the mixed eluate. This observation is in line with other studies performed previously, demonstrating that at least three replicated extractions are required to obtain a DNA pool that is representative for the microbial community present in a given soil sample (Feinstein *et al*., 2009; Lindahl *et al*., 2013).

Apart from the technical issues that were addressed in this study, our data also provided a glimpse at the fungal community present in the studied

site. Based on field observations of above-ground basidiocarps, we assumed that pioneer pine forests in the Campine region in Belgium are dominated by biotrophic species (mostly lichens, ectomycorrhizal and ericoid mycorrhizal fungi) over saprotrophic species. All three primer pairs confirmed this assumption, but they found different fungal OTUs to be dominant. According to the results obtained with ITS1F/ITS2, the fungal community in the studied site was dominated by OTUs corresponding to *Sistotrema* sp. (27%), followed by *Rhizopogon luteolus* (9%), *Wilcoxina mikolae* (8%) and *Cladophialophora minutissima* (7%) (Fig. 3.3) (Davey and Currah, 2007). These OTUs were also found to be very important members of the studied community according to ITS3/ITS4 and ITS86F/ITS4 as they appeared in the top ten of the most abundant OTUs found by both primer pairs, although in varying proportions (Fig. 3.3). *Sistotrema* sp., likely an important member of our studied ecosystem, was recently shown to be polyphyletic, containing both ectomycorrhizal and saprotrophic taxa (Münzenberger *et al*., 2012). The reads that were found in the current study correspond to *Sistotrema* strains that were sampled from ectomycorrhizal root tips of *Pinus contorta* Dougl. growing on coastal sand dunes (Ashkannejhad and Horton, 2006). This genus provides a fine example of the power of molecular tools, such as DNA metabarcoding, to draw attention to ecologically important, cryptic fungal species. Based on field observations alone (basidiocarps observations and root tip morphotying), we never expected this genus to be so abundant in this pioneer ecosystem.

Concluding remarks

In many fungal metabarcoding studies universal primers from previous phylogenetic or ecological studies are used without first performing an evaluation of their spectrum and performance for high-throughput sequencing, potentially resulting in a biased description of fungal communities. Whereas *in silico* PCR analyses on sequences retrieved from sequence databases may suggest promising primers (Ihrmark *et al*., 2012;

Toju *et al*., 2012), we showed that an experimental set-up to evaluate their usefulness in practice provides complementary information on the actual performance of the primers for high-throughput sequencing of environmental samples. Indeed, here we demonstrated that the choice of primers has a significant impact on how fungal communities are translated into OTU communities and subsequent data analysis. As such, before setting up large scale sequencing experiments, we recommend to first test a number of promising primer pairs, e.g. selected with *in silico* analyses, under real PCR conditions for a subset of the samples under investigation. In case an in-depth characterization of a fungal community is desired, the use of more than one primer pair is advisable. We also showed that quantitative real-time PCR, evaluating the efficiency of selected primer pairs, may help in selecting the most efficient primer pairs. After all, using primer pairs that are not very efficient in amplifying DNA from an environmental sample will undoubtedly result in a low number of reads, and consequently in biased community descriptions.

In this study, the primer pair ITS86F/ITS4, which amplifies the ITS2 region of the fungal rRNA operon, was shown to be the most suitable primer pair for the characterization of fungal communities with metabarcoding. This primer pair not only resulted in superior amplification efficiency leading to a significantly higher number of reads, but also yielded a high number of OTUs belonging to different phyla. In addition, this primer pair resulted in a robust amplification reaction for the broadest range of samples and across replicated extractions.

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SUPPORTING INFORMATION

Supporting Information Table S3.1 Contact addresses for access to the study site.

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Supporting Information Table S3.2 Primer and study design used in the 454 amplicon pyrosequencing experiment. Samples (n = 7), replicates of samples (n = 4) and used primers are indicated. Primers used for pyrosequencing are displayed as their respective components (454 adapter, multiplex identifier (MID) and fungal ITS primers).

			Forward primer			Reverse primer		
Sa. (1)	Re. (2)	Am. (3)	454 adapter (4)	MID (5)	Fungal ITS primer (6)	454 adapter (4)	MID (5)	Fungal ITS primer (6)
		1	A	12	ITS1F	B	8	ITS2
	1	2	A	12	ITS3	B	8	ITS4
		3	A	12	ITS86F	B	8	ITS4
		4	A	9	ITS1F	B	12	ITS2
	2	5	A	9	ITS3	B	12	ITS4
1		6	A	9	ITS86F	B	12	ITS4
		7	A	8	ITS1F	B	7	ITS2
	3	8	A	8	ITS3	B	7	ITS4
		9	A	8	ITS86F	B	7	ITS4
		10	A	8	ITS1F	B	8	ITS2
	4	11	A	8	ITS3	B	8	ITS4
		12	A	8	ITS86F	B	8	ITS4
		13	A	12	ITS1F	B	9	ITS2
	1	14	A	12	ITS3	B	9	ITS4
		15	A	12	ITS86F	B	9	ITS4
		16	A	9	ITS1F	B	7	ITS2
	2	17	A	9	ITS3	B	7	ITS4
2		18	A	9	ITS86F	B	7	ITS4
		19	A	8	ITS1F	B	9	ITS2
	3	20	A	8	ITS3	B	9	ITS4
		21	A	8	ITS86F	B	9	ITS4
		22	A	9	ITS1F	B	8	ITS2
	4	23	A	9	ITS3	B	8	ITS4
		24	A	9	ITS86F	B	8	ITS4
		25	A	9	ITS1F	B	9	ITS2
	1	26	A	9	ITS3	B	9	ITS4
		27	A	9	ITS86F	B	9	ITS4
		28	A	10	ITS1F	B	12	ITS2
	2	29	A	10	ITS3	B	12	ITS4
3		30	A	10	ITS86F	B	12	ITS4
		31	A	10	ITS1F	B	7	ITS2
	3	32	A	10	ITS3	B	7	ITS4
		33	A	10	ITS86F	B	7	ITS4
		34	A	8	ITS1F	B	10	ITS2
	4	35	A	8	ITS3	B	10	ITS4
		36	A	8	ITS86F	B	10	ITS4

		37	A	10	ITS1F	B	8	ITS2
	1	38	A	10	ITS3	B	8	ITS4
		39	A	10	ITS86F	B	8	ITS4
		40	A	9	ITS1F	B	10	ITS2
	2	41	A	9	ITS3	B	10	ITS4
4		42	A	9	ITS86F	B	10	ITS4
		43	A	10	ITS1F	B	9	ITS2
	3	44	A	10	ITS3	B	9	ITS4
		45	A	10	ITS86F	B	9	ITS4
		46	A	10	ITS1F	B	10	ITS2
	4	47	A	10	ITS3	B	10	ITS4
		48	A	10	ITS86F	B	10	ITS4
		49	A	12	ITS1F	B	10	ITS2
	1	50	A	12	ITS3	B	10	ITS4
		51	A	12	ITS86F	B	10	ITS4
		52	A	11	ITS1F	B	7	ITS2
	2	53	A	11	ITS3	B	7	ITS4
5		54	A	11	ITS86F	B	7	ITS4
		55	A	8	ITS1F	B	11	ITS2
	3	56	A	8	ITS3	B	11	ITS4
		57	A	8	ITS86F	B	11	ITS4
		58	A	11	ITS1F	B	8	ITS2
	4	59	A	11	ITS3	B	8	ITS4
		60	A	11	ITS86F	B	8	ITS4
		61	A	9	ITS1F	B	11	ITS2
	1	62	A	9	ITS3	B	11	ITS4
		63	A	9	ITS86F	B	11	ITS4
		64	A	11	ITS1F	B	9	ITS2
	2	65	A	11	ITS3	B	9	ITS4
6		66	A	11	ITS86F	B	9	ITS4
		67	A	10	ITS1F	B	11	ITS2
	3	68	A	10	ITS3	B	11	ITS4
		69	A	10	ITS86F	B	11	ITS4
		70	A	11	ITS1F	B	10	ITS2
	4	71	A	11	ITS3	B	10	ITS4
		72	A	11	ITS86F	B	10	ITS4
		73	A	11	ITS1F	B	11	ITS2
	1	74	A	11	ITS3	B	11	ITS4
		75	A	11	ITS86F	B	11	ITS4
		76	A	12	ITS1F	B	12	ITS2
	2	77	A	12	ITS3	B	12	ITS4
7		78	A	12	ITS86F	B	12	ITS4
		79	A	12	ITS1F	B	7	ITS2
	3	80	A	12	ITS3	B	7	ITS4
		81	A	12	ITS86F	B	7	ITS4
		82	A	8	ITS1F	B	12	ITS2
	4	83	A	8	ITS3	B	12	ITS4
		84	A	8	ITS86F	B	12	ITS4

Sequence name	Primer sequence (5'-3')	Reference
A	CGTATCGCCTCCCTCGCGCCATCAG	
B	CTATGCGCCTTGCCAGCCCGCTCAG	
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns, 1993
ITS2	GCTGCGTTCTTCATCGATGC	White *et al*., 1990
ITS3	GCATCGATGAAGAACGCAGC	White *et al*., 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al*., 1990
ITS86F	GTGAATCATCGAATCTTTGAA	Vancov and Keen, 2009
MID7	CGTGTCTCTA	
MID8	CTCGCGTGTC	
MID9	TAGTATCAGC	
MID10	TCTCTATGCG	
MID11	TGATACGTCT	
MID12	TACTGAGCTA	

(1) Sa.: sample

(2) Am.: amplicon

(3) Re.: replicate

(4) A: 454 pyrosequencing adapter A, B: 454 pyrosequencing adapter B

(5) MID: Multiplex identifier

(6) ITS: Internal transcribed spacer

Supporting Information Table S3.3 Taxonomic composition of fungal species (n = 15) used in the current study to assess PCR bias at the phylum level.

Genus/species	Strain	Family	Order	Phylum
Cladosporium cladosporioides	MUCL 53652	Cladosporiaceae	Capnodiales	Ascomycota
Cryptosporiopsis radicicola	MUCL 53485	Dermateaceae	Helotiales	Ascomycota
Monilinia laxa	MUCL 30841	Sclerotiniaceae	Helotiales	Ascomycota
Arthroderma otae	MUCL 39756	Arthrodermataceae	Onygenales	Ascomycota
Galactomyces geotrichum	MUCL 52377	Dipodascaceae	Saccharomycetales	Ascomycota
Lentinula edodes	MUCL 44827	Omphalotaceae	Agaricales	Basidiomycota
Agrocybe praecox	MUCL 46727	Strophariaceae	Agaricales	Basidiomycota
Coniophora marmorata	MUCL 39471	Coniophoraceae	Boletales	Basidiomycota
Suillus luteus	UH-Slu-Lm8-n1	Suillaceae	Boletales	Basidiomycota
Antrodia vaillantii	MUCL 54533	Fomitopsidaceae	Polyporales	Basidiomycota
Rhizophagus clareus	MUCL 46238	Glomeraceae	Glomerales	Glomeromycota
Rhizophagus sp.	MUCL 41833	Glomeraceae	Glomerales	Glomeromycota
Mortierella verticillata	MUCL 9658	Mortierellaceae	Mortierellales	Zygomycota
Absidia corymbifera	MUCL 38907	Cunninghamellaceae	Mucorales	Zygomycota
Mucor hiemalis	MUCL 15439	Mucoraceae	Mucorales	Zygomycota

Supporting Information Table S3.4 PCR efficiencies determined with qPCR of all species and primer pair combinations used to assess phylum-level PCR bias in the current study.

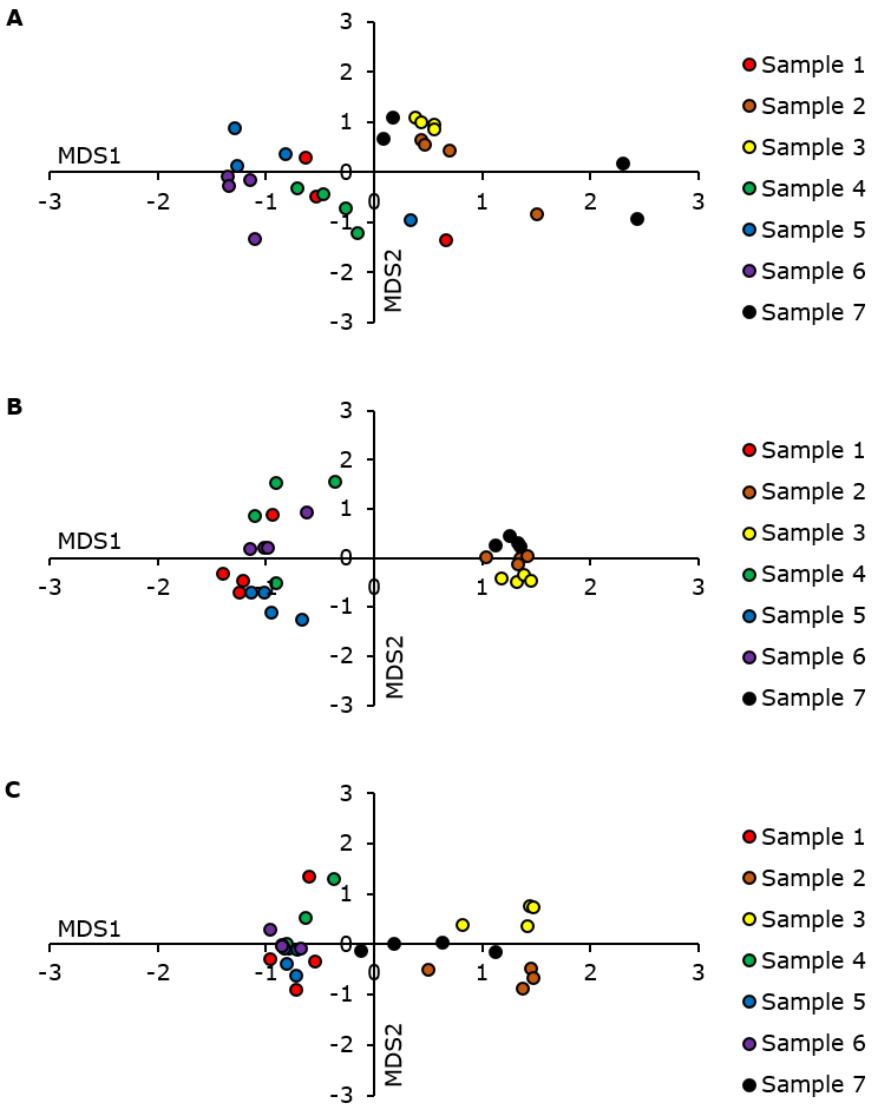
Genus/species	Strain	PCR efficiency (%)		
		ITS1F/ITS2	ITS3/ITS4	ITS86F/ITS4
Cladosporium cladosporioides	MUCL 53652	55	85	92
Cryptosporiopsis radicicola	MUCL 53485	58	56	131
Monilinia laxa	MUCL 30841	74	72	78
Arthroderma otae	MUCL 39756	36	80	91
Galactomyces geotrichum	MUCL 52377	106	87	39
Lentinula edodes	MUCL 44827	70	39	77
Agrocybe praecox	MUCL 46727	73	98	112
Coniophora marmorata	MUCL 39471	73	64	69
Suillus luteus	UH-Slu-Lm8-n1	85	88	94
Antrodia vaillantii	MUCL 54533	51	88	101
Rhizophagus clareus	MUCL 46238	117	126	85
Rhizophagus sp.	MUCL 41833	116	82	74
Mortierella verticillata	MUCL 9658	106	74	86
Absidia corymbifera	MUCL 38907	60	74	65
Mucor hiemalis	MUCL 15439	88	98	93
Average		78	81	86
Standard error		6	5	5

Supporting Information Table S3.5 PrimerProspector (Walters *et al*., 2011) results for all primers investigated in this study and investigated by Ihrmark *et al*. (2012) and Toju *et al*. (2012).

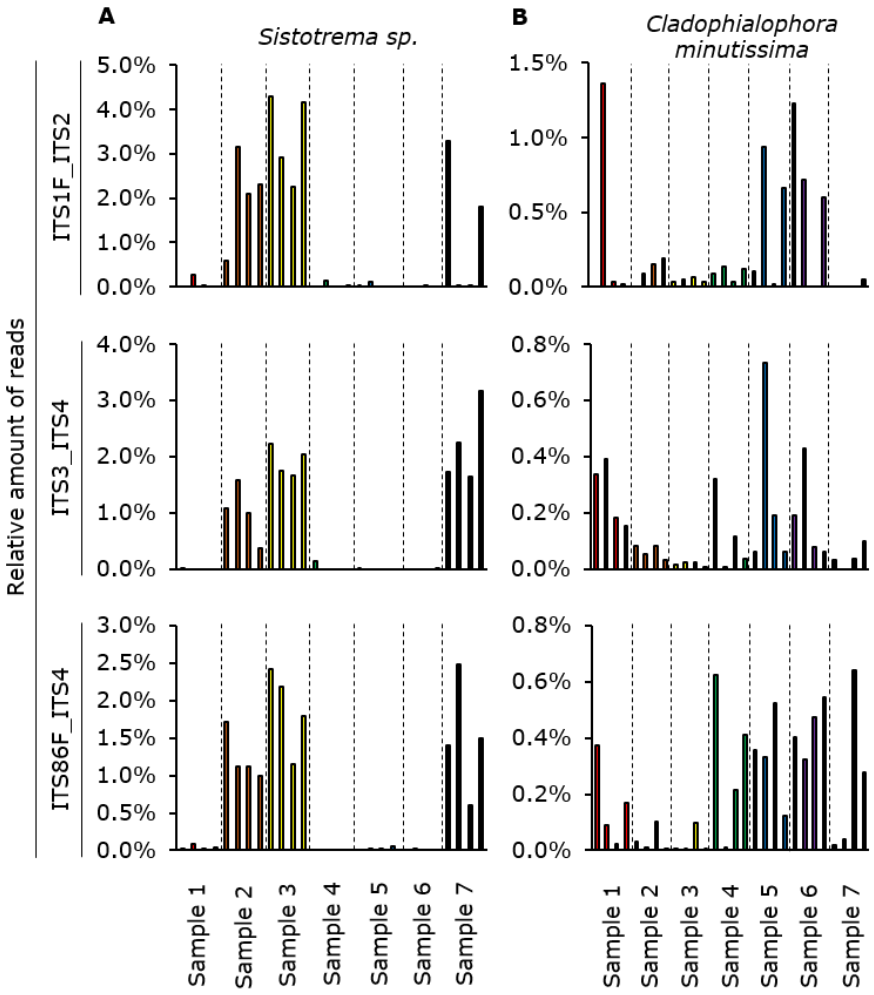
Primer binding site and orientation	Primer name	Primer sequence (5' - 3')	3' mismatch (%)	Average Primer Prospector Score	Standard Error on Score	Reference
5.8S (forward)	ITS86F	GTGAATCATCGAATCTTTGAA	3	0.00	0.00	Turenne *et al*., 1999
	ITS3	GCATCGATGAAGAACGCAGC	4	0.58	0.03	White *et al*., 1990
	gITS9	GAACGCAGCRAAIIGYGA	4	0.41	0.02	Ihrmark *et al*., 2012
	fITS7	GTGARTCATCGAATCTTTG	4	0.49	0.02	Ihrmark *et al*., 2012
	gITS7	GTGARTCATCGARTCTTTG	4	0.49	0.02	Ihrmark *et al*., 2012
	ITS3_KYO1	AHCGATGAAGAACRYAG	5	0.42	0.02	Toju *et al*., 2012
	ITS3_KYO2	GATGAAGAACGYAGYRAA	2	0.40	0.02	Toju *et al*., 2012
	58A1F	GCATCGATGAAGAACGC	5	0.51	0.02	Martin and Rygiewicz, 2005
	58A2F	ATCGATGAAGAACGCAG	6	0.52	0.02	Martin and Rygiewicz, 2005
5.8S (reverse)	ITS2	GCTGCGTTCTTCATCGATGC	9	0.70	0.03	White *et al*., 1990
	ITS10mun	GCTGCGTTCTTCATCGAT	3	0.48	0.02	Egger, 1995
	ITS2_KYO1	CTRYGTTCTTCATCGDT	2	0.37	0.02	Toju *et al*., 2012
	ITS2_KYO2	TTYRCTRCGTTCTTCATC	5	0.49	0.02	Toju *et al*., 2012
	58A2R	CTGCGTTCTTCATCGAT	3	0.47	0.02	Martin and Rygiewicz, 2005

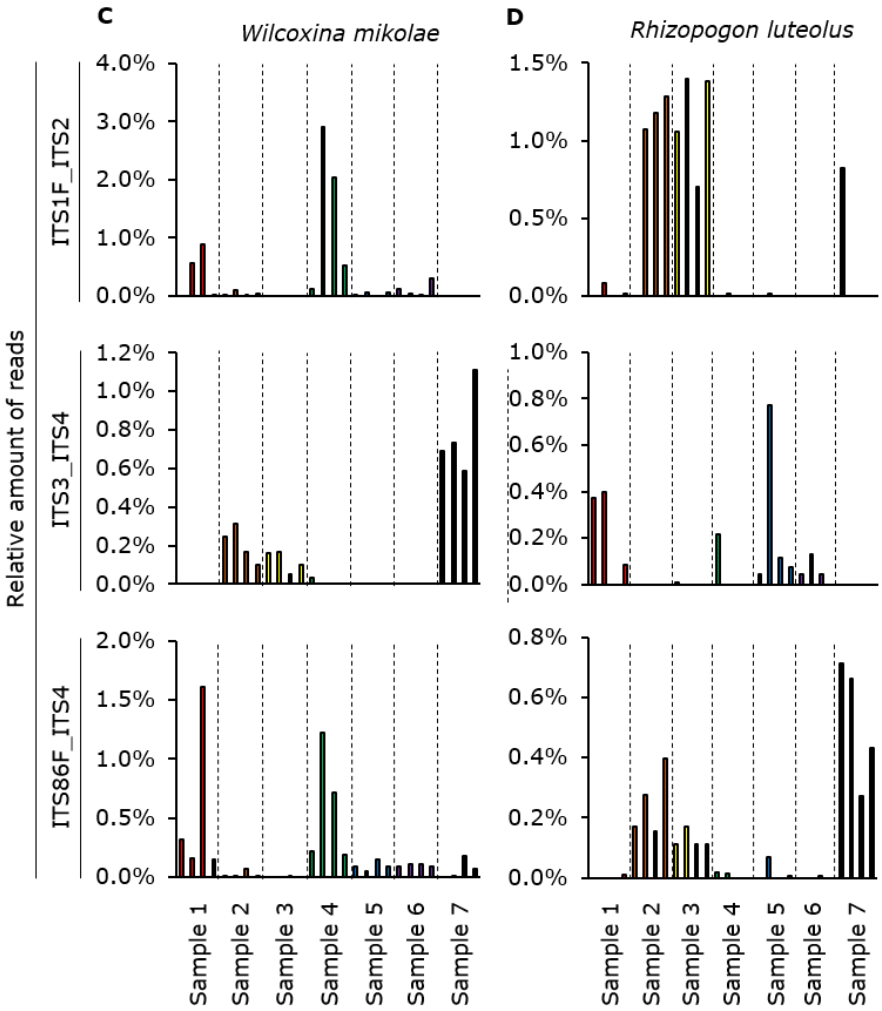
Primer binding site and orientation	Primer name	Primer sequence (5' - 3')	3' mismatch (%)	Average Primer Prospector Score	Standard Error on Score	Reference
28S (reverse)	ITS4B	CAGGAGACTTGTACACGGTCCAG	41	5.75	0.03	Gardes and Bruns, 1993
	ITS4_KYO1	TCCTCCGCTTWTTGWTWTGC	23	3.71	0.04	Toju et al., 2012
	ITS4_KYO2	RBTTTCTTTTCCTCCGCT	44	4.39	0.03	Toju *et al*., 2012
	ITS4_KYO3	CTBTTVCCKCTTCACTCG	42	3.47	0.04	Toju *et al*., 2012
	ITS4	TCCTCCGCTTATTGATATGC	16	3.96	0.04	White *et al*., 1990
	ITS8mun	CTTCACTCGCCGTTACTA	61	4.07	0.03	Egger, 1995
	NL6Amun	CAAGTGCTTCCCTTTCAACA	10	3.49	0.03	Egger, 1995
	NL6Bmun	CAAGCGTTTCCCTTTCAACA	9	3.32	0.03	Egger, 1995
	NLB3	GGATTCTCACCCTCTATGA	13	3.04	0.03	Martin and Rygiewicz, 2005
	NLB4	GGATTCTCACCCTCTATGAC	53	4.17	0.04	Martin and Rygiewicz, 2005
	NLC2	GAGCTGCATTCCCAAACAACTC	25	3.90	0.04	Martin and Rygiewicz, 2005

Primer binding site and orientation	Primer name	Primer sequence (5' - 3')	3' mismatch (%)	Average Primer Prospector Score	Standard Error on Score	Reference
18S (forward)	ITS1	TCCGTAGGTGAACCTGCGG	12	2.29	0.04	White *et al*., 1990
	ITS1F	CTTGGTCATTTAGAGGAAGTAA	44	4.55	0.05	Gardes and Bruns, 1993
	ITS1F_KYO1	CTHGGTCATTTAGAGGAASTAA	36	4.11	0.05	Toju *et al*., 2012
	ITS1F_KYO2	TAGAGGAAGTAAAAGTCGTAA	15	3.43	0.04	Toju *et al*., 2012
	ITS5	GGAAGTAAAAGTCGTAACAAGG	8	2.96	0.04	White *et al*., 1990
	ITS9mun	TGTACACACCGCCCGTCG	27	3.34	0.04	Egger, 1995
	NSI1	GATTGAATGGCTTAGTGAGG	36	4.11	0.04	Martin and Rygiewicz, 2005
	NSA3	AAACTCTGTCGTGCTGGGGATA	40	4.66	0.04	Martin and Rygiewicz, 2005



Supporting Information Figure S3.1 Non-metric multi-dimensional scaling (NMDS) comparing community dissimilarities (based on Bray-Curtis distances) between each replicate (n = 4) of a sample (n = 7). Replicates are indicated by the same icon colour within a graph. Replicates with more similar communities are plotted more closely together than more dissimilar replicates. A. ITS1F/ITS2. B. ITS3/ITS4. C. ITS86F/ITS4.


A
Sistotrema sp.
B
Cladophialophora minutissima
Relative amount of reads
ITS1F_ITS2
ITS3_ITS4
ITS86F_ITS4
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
1.5%
0.5%
0.8%
0.6%
0.4%
0.2%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7



Supporting Information Figure S3.2 Bar charts displaying the relative amount of reads that were assigned to a specific species-level operational taxonomic unit (OTU) by each primer pair used in this study (ITS1F/ITS2, ITS3/ITS4 and ITS86F/ITS4). Only the four most abundant species-level OTUs were displayed. The x-axes display all replicates and samples separately, where replicates appear as bars in the same colour and samples as different colours. The y-axes show the amount of reads found by a specific primer pair for one replicate of a sample relative (%) to the total amount of reads found for that OTU across all primer pairs, replicates and samples. A. *Sistotrema* sp. B. *Cladophialophora minutissima* C. *Wilcoxina mikolae* and D. *Rhizopogon luteolus*.

Chapter 4: **Impact of metal pollution on fungal diversity and community structures**

Op De Beeck M, Lievens B, Busschaert P, Rineau F, Smits M, Vangronsveld J, Colpaert JV

SUMMARY

The impact of metal pollution on plant communities has been studied extensively in the past, but little is known about the effects of metal pollution on fungal communities that occur in metal-polluted soils. Metal-tolerant ecotypes of the ectomycorrhizal fungus *Suillus luteus* are frequently found in pioneer pine forests in the Campine region in Belgium on metal-polluted soils. We hypothesized that metal pollution would play an important role in shaping belowground fungal communities that occur in these soils and that *Suillus luteus* would be a dominant player. To test these hypotheses, the fungal communities in a young pine plantation in soil polluted with zinc and cadmium were studied using 454 amplicon pyrosequencing. Results show that zinc, cadmium and soil organic matter content were strongly correlated with the fungal community composition, but no effects on fungal diversity were observed. As hypothesized, *S. luteus* was found to be a dominant member of the studied fungal communities. However, other dominant fungal species, such as *Sistotrema* sp., *Wilcoxina mikolae* and *Cadophora finlandica* were found as well. Their presence in metal-polluted sites is discussed.

Keywords: mycorrhiza, metabarcoding, metal pollution, zinc, cadmium, fungal diversity

CHAPTER 4

INTRODUCTION

Due to pyrometallurgical industry, vast areas throughout the world have been polluted with high concentrations of heavy metals such as zinc (Zn), cadmium (Cd), lead (Pb) and copper (Cu). Increased concentrations of metals in soils are known to adversely affect biodiversity. At low metal exposure, many organisms can still survive through metabolic adjustments. However, when cellular metal homeostasis becomes disrupted, populations of more sensitive organisms are expected to collapse, leaving only most adapted species (Bradshaw and McNeilly, 1981; Al-Hiyaly *et al*., 1990; Mergeay *et al*., 2003). Ernst (1990) for example, observed a decrease in floristic diversity along transects towards metal smelters. Consequently, specialized plant communities occur on metal-polluted soils, mainly consisting of metal-resistant (plants with sufficient phenotypic plasticity to survive the harsh conditions) and metal-tolerant plant species (plants with genotypes adapted to high metal concentrations through micro-evolution). Whereas some plant species are endemic to metal-polluted sites, others can build stable populations on both polluted and non-polluted sites. Especially grasses are well-known to develop metal-tolerant ecotypes (Schat *et al*., 2000), but also a few dicotyledonous plant species have been found to flourish on metal-polluted soils (Alford *et al*., 2010). In sharp contrast to the vast body of literature that is available on (pseudo-)metallophytes, little is known about the fungal species that occur in metal-polluted soils. Studying fungal diversity is crucial to understand belowground ecosystem functioning. Moreover, the presence or absence of key mycorrhizal fungi may have a strong impact on the establishment and fitness of plants on metal-polluted sites (Hildebrandt *et al*., 1999). Recent studies focusing on phytoremediation of metal-polluted soils have illustrated that mycorrhiza can protect their host plants from metal-toxicity and even enhance the efficiency of phytoremediation by enhancing plant growth, the mobilization of metal ions and the translocation of metals to plants (Leung *et al*., 2013). A better understanding of belowground fungal

community structures and the factors shaping fungal communities therefore provide information that will not only help us to understand fungal ecology in general, they will also help us to develop strategies to reduce or remediate the human impact on the living environment.

In the past, the impact of metal pollution on microbial communities has been studied (e.g. Chodak *et al.,* 2013; Corneo *et al.*, 2013). In general, shifts in microbial community compositions are found, but most studies do not reveal which species dominate belowground communities in metal-polluted soils and which species are unable to colonize highly polluted soil patches. To better understand ecosystem functioning and community dynamics in stressful environments, it is desirable to identify members of communities of interest at a species level. For example, basidiocarps of Zn- and Cd-tolerant strains of the ectomycorrhizal basidiomycete *Suillus luteus* (L.) Roussel, can be frequently found in pioneer conditions on Zn- and Cd-polluted sites in the Campine region in Belgium (Colpaert *et al*., 2000; 2004; Krznaric *et al*., 2009). We therefore hypothesized that *S. luteus* would dominate the belowground fungal communities of metal-polluted sites in the Campine region. Furthermore, we also hypothesized that metal pollution would be a strong driving factor determining fungal communities thriving in these soils. To estimate the relative importance of metal pollution in shaping fungal communities, we also evaluated the effects of a number of environmental variables that are known to potentially affect fungal community composition and fungal diversity at the species level (Hartmann *et al*., 2012; Azarbad *et al*., 2013; Uroz *et al*., 2013).

MATERIALS AND METHODS

Study site and sampling

A study site located in a metal pollution gradient in the Campine region in the northern part of Belgium (Lommel-Maatheide: 51° 14’ 10” N; 5° 15’ 50” E) was selected for this study (Colpaert *et al*., 2004). The study site is part of a large area that has been contaminated by a zinc smelter that was

active from 1904 until 1974. This particular zinc smelter has been shut down for almost forty years, but still hardly any vegetation had re-colonized the polluted area by the start of the current study. One year old nursery seedlings of Scots pine (*Pinus sylvestris* L.) were planted in 2008 in an area where topsoil was removed and where no trees have been present for the past few decades. The disturbance of the topsoil introduced great heterogeneity in the newly exposed soil and resulted in large differences in metal concentrations over short distances. Trees were planted in a grid with a distance of approximately two meters between each tree. Mosses, lichens, and a few grass species form the accompanying primary pioneer vegetation at this site. The soil at the study site is a dry sandy soil without a litter layer, poor in organic matter and slightly acidic. The average soil organic carbon content was 0.8% ± 0.2 % (SE) and the average pH was 4.8 ± 0.1 (SE). Zn concentrations in pine needles, one year after pine trees were planted, ranged from 176 µg g^{-1} dry pine needle weight to 545 µg g^{-1} with an average of 348 µg g^{-1}. Cd concentrations ranged from 0.1 µg g^{-1} to 7 µg g^{-1} dry pine needle weight with an average of 2 µg g^{-1}. In the Campine region, pines growing on non-polluted soils contain roughly 50 µg g^{-1} Zn and < 0.1 µg g^{-1} Cd in their needles. An overview of all measured environmental variables can be found in Fig. 4.1 and Fig. S4.1. The region has an average annual rainfall of 800 mm per square meter and the average annual temperature is 10°C (Royal Meteorological Institute, Ukkel, Belgium). For the current study, twenty-two pine trees were randomly selected and marked in 2009 within an area of 40 m by 400 m. Soils were sampled in November 2009 and again in November 2011. More specifically, for fungal community analysis soil samples were collected with a soil corer with a diameter of 1 cm at a depth of 0 to 20 cm. For each tree, five samples were collected according to the cardinal directions at different distances from the stem. These included samples collected immediately next to the stem and at a distance of 25 cm, 50 cm, 75 cm and 100 cm from the stem bases (Fig. S4.2).

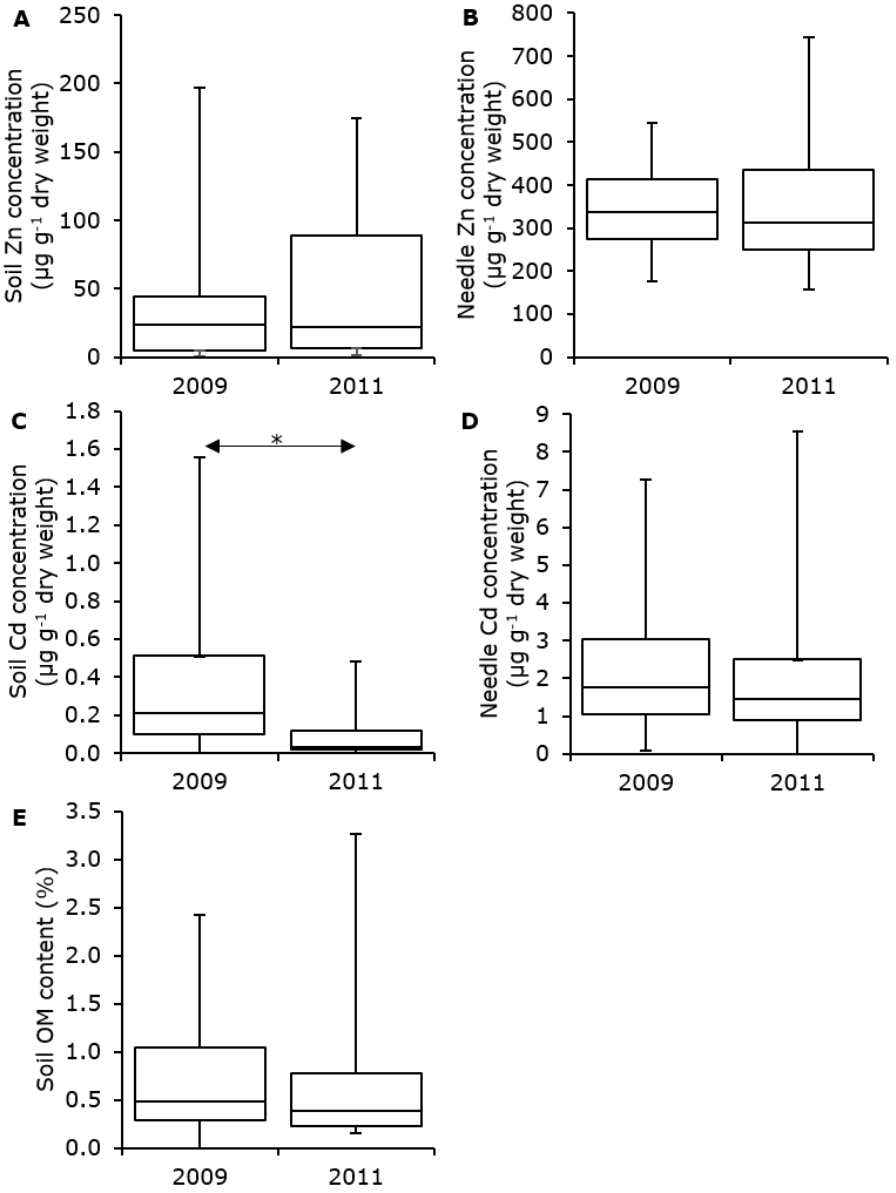


Figure 4.1 Comparison between sampling years (2009 and 2011) of environmental variables that correlated with observed fungal community compositions. Significant differences at the 95% confidence level are indicated with an asterisk (*). A. Soil Zn concentrations. B. Needle Zn concentrations. C. Soil Cd concentrations. D. Needle Cd concentrations. E. Soil organic matter (OM) content.

Samples were pooled for each of these distances and mixed before they were sealed in plastic bags and brought to the laboratory, resulting in a total of five pooled samples for each tree with each sample representing a certain distance from the stem. Additionally, samples for physical and chemical soil characterization were collected next to each selected tree with a soil corer with a diameter of 10 cm at a depth of 0 to 20 cm. To estimate the metal concentrations that pine trees were exposed to in the field, pine needles were collected from the most recently emerged shoots. Following arrival in the lab, soil samples for fungal community analysis were homogenized, sieved with a 2 mm sieve to remove small rocks, roots, twigs and grasses, and stored at -80°C. Samples for soil characterization were dried at ambient temperature for two weeks before physical and chemical analyses were conducted. Collected pine needles were dried for two weeks at 60 °C before being analysed for their metal content.

Soil physical and chemical characterization

pH was measured in both a water extract (10 g soil extracted with 25 ml distilled water) and a KCl extract (10 g soil extracted with 25 ml 1M KCl) of soil samples. Conductivity was measured on the water extracts. Soil organic matter content (OM) was analysed with the Walkley and Black method (Walkley and Black, 1934). Cation exchange capacity (CEC) was measured according to Rhoades' method (Rhoades, 1982). Exchangeable cations were extracted using 0.1 M $Ca(NO_3)_2$ (25 ml for 5 g soil). Dried pine needles were digested with nitric acid (65%) and hydrochloric acid (37%) at 120 °C. Concentrations of zinc (Zn), cadmium (Cd), iron (Fe), magnesium (Mg), potassium (K), copper (Cu) and manganese (Mn) were measured with inductively-coupled plasma - optical emission spectroscopy (ICP-OES) in samples obtained from calcium nitrate extraction and pine needle digestion. Calcium (Ca) concentrations were measured in pine needle digests.

Characterization of the fungal communities

To characterize the fungal communities in soil samples, DNA was extracted using the UltraClean soil DNA isolation kit (MoBio, Carlsbad, CA, USA) from approximately 250 mg of soil according to the manufacturer's instructions. DNA was extracted in quadruplicate from each soil sample (5 per tree) and replicated extracts were pooled per sample prior to PCR amplification using the ITS86F forward primer (Vancov and Keen, 2009) and ITS4 reverse primer (Gardes and Bruns, 1993). This primer pair was shown to efficiently amplify the fungal internal transcribed spacer 2 (ITS2) region and characterize fungal communities using 454 amplicon pyrosequencing (Waud *et al*., 2014; Op De Beeck *et al*., 2014). "Fusion" primers, required for the 454 process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-A sequencing containing the Roche 454 pyrosequencing adapters and a 10-bp multiplex identifier (MID) barcode in between each adapter and primer sequence (Table S4.1). DNA was amplified using a Techne TC-5000 thermocycler (Bibby Scientific Limited, Staffordshire, UK) under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C during 1 minute. A final extension phase was performed at 72°C during 10 minutes. Reactions were carried out in 25 µl reaction volumes using the FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). Each reaction contained 2.75 µl FastStart 10x reaction buffer, 1.8 mM MgCl, 0.2 mM dNTP mix, 0.4 µM of each primer, 1.25 U FastStart HiFi polymerase and 5 ng template DNA (as measured by a Nanodrop spectrophotometer). Amplified DNA was cleared from PCR primers and primer dimers using the Agencourt AMPure XP system according to the manufacturer's protocol (Beckman Coulter, Brea, CA, USA). Next, purified DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany) and pooled into four equimolar amplicon libraries (10^7 molecules per µl) of 55 samples. Each of the four resulting amplicon pools (two pools for the

samples from 2009 and two pools for the samples from 2011) were sequenced on one fourth of a pico titer plate on a Roche Genome Sequencer FLX system using Titanium chemistry (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

The four Standard Flowgram Format (SFF) files that resulted from the interpreted flowgrams were submitted to the NCBI Sequence Read Archive under accession number SRP028404 (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra). The information in the separate SFF files was combined in a single quality and a fasta file using a custom Python script. Further analyses were performed in Mothur 1.31.2 (Schloss *et al*., 2009). Quality trimming in Mothur was used to remove reads shorter than 200 bases, reads longer than 600 bases, reads containing homopolymers longer than 8 bases and reads containing ambiguous bases. Reads were trimmed when the average Phred quality score dropped below 35 over a window of 50 bases (Schloss *et al*., 2009). Next, sequences were compared to each other and duplicate sequences were replaced by a single sequence, while archiving the abundance data of the unique sequences. Unique reads were checked for chimeric sequences with the Uchime software implemented in Mothur and chimeric sequences were removed from the dataset. Unique reads were subsequently aligned with the pairwise alignment tool in Mothur using default settings. Finally, species-level operational taxonomic units (OTUs) were defined based on a 97% sequence similarity cut-off, which is generally within the range of intraspecific ITS sequence similarity (Blaalid *et al*., 2013). In order to further remove potential sequencing errors from the dataset, singletons were removed (Tedersoo *et al*., 2010; Waud *et al*., 2014). Subsequently, rarefaction curves at the level of each tree were constructed and Good's coverage scores were calculated with Mothur for each tree. OTU richness, Pielou evenness scores and inverse Simpson indices were calculated for each tree in R 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). In parallel, diversity parameters were also calculated based on a dataset in which the number of reads per sample was rarefied to 500 reads per sample

(10 samples did not meet this requirement and were omitted for this analysis). However, no differences in fungal diversity patterns were observed between the rarefied and non-rarefied datasets. We therefore opted to keep all sequencing information in our dataset (not rarefying the number of reads per sample). BLAST searches for a representative sequence of each OTU (as determined by Mothur) were performed using PlutoF v2.0 (Abarenkov *et al*., 2010). Reads were blasted against the UNITE (Kõljalg *et al*., 2005) and INSD (Nakamura *et al*., 2013) databases. Resulting HTML files were combined with the abundance data obtained in Mothur using a custom Python script. This script also acquired the names of species and/or genera that resemble Latin binomials with the highest BLAST score, avoiding unidentified OTUs in the databases to be seen as best BLAST hits. OTUs of for which no BLAST hits were found, were indicated as "not applicable (NA)".

Statistical analysis

Statistical analyses were carried out in R 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). Normal distributions of the residuals of models were checked with the Shapiro-Wilk test, while the homoscedasticity of variances were analysed using either Bartlett's or the Fligner-Killeen test. Depending on the distribution of the estimated parameters, either ANOVA or the Kruskal-Wallis rank sum test was used to check for significant differences in variances of parameters. Two-by-two comparisons were conducted using either Tukey's Honest Significant Differences tests or Pairwise Wilcoxon Rank Sum tests. Correlations between diversity parameters and measured soil parameters were calculated based on Pearson's product moment correlation coefficient (R^2). Non-Metric Multi-dimensional Scaling (NMDS) was conducted using the metaMDS() function of the vegan package (version 2.0-10; Oksanen *et al*., 2013) in R. Canonical Correspondence Analysis (CCA) model building was conducted based on the cca() function of the vegan package and CCA analysis was based on the decorana() and envfit() functions of the vegan

package. To analyse distance decay of similarity across different spatial and temporal scales, linear models were fitted to the data and the mantel() function of the vegan package was used to assess the significance of model fits. Mantel tests were conducted using the mantel() function of the vegan package in R. PERMANOVA analyses were conducted in R using the adonis() function of the vegan package. Distance matrices for community data were based on Bray-Curtis distances using read abundances. The distance matrix for the geographical position of trees and distances between samples and years were based on Euclidian distances. Indicator species analysis was conducted using the multipat() function of the indicspecies package (version 1.7.1; De Caceres and Legendre, 2009) in R. Spatial and temporal autocorrelations were tested based on Moran's I, using the Moran.I() function of the ape package (version 3.1-1; Paradis *et al*., 2004) in R.

RESULTS

Belowground fungal communities in a single young Scots pine plantation (Lommel-Maatheide: 51° 14' 10" N; 5° 15' 50" E) were identified for two sampling years (2009 and 2011) using 454 amplicon pyrosequencing (Margulies *et al*., 2005). This pioneer forest is growing on a site where polluted bare topsoil was removed in 2004. This disturbance introduced heterogeneity in the newly exposed sandy soil and resulted in large differences in metal concentrations over short distances. To estimate the overall metal exposure of individual pine trees, soil samples and last-year pine needles from 22 pine trees were collected for element analysis. Measured environmental variables which were found to be important in determining the fungal community compositions in the current study are shown in Fig. 4.1. Variables that did not contribute to the structure of the fungal communities can be found in Fig. S4.1.

Four amplicon libraries were sequenced, each on one fourth of a picotiter plate. These sequencing runs resulted in 368,085 raw reads before quality trimming and assigning the reads to their respective sample. After quality

trimming and assigning reads to the different samples (220 samples, 22 trees, 5 distances, 2 sampling years), 346,364 high-quality reads remained in the dataset. Rarefaction curves assessing the OTU richness per tree generally approached saturation and indicated that 25 to 80 OTUs can be expected in the samples collected in 2009 and between 15 and 70 OTUs for the samples collected in 2011 (Fig. 4.2). Averages of calculated Good's coverage scores were 95% ± 1% (SE) for the 2009 sampling session and 96% ± 0.1% (SE) for the 2011 sampling session, indicating that the sequencing depth was sufficient to accurately describe the fungal communities at the tree level.

Local spatial and temporal variations in fungal diversity

Local variations in fungal diversity were studied at two spatial scales: within a one meter radius of pine trees (using individual samples collected at different distances from a pine stem as sampling units) and across the entire study site (using pine trees as sampling units) and a temporal scale (comparison of two sampling years: 2009 and 2011). No significant differences between OTU richness indices were found for samples collected within a one meter radius from pine stems (three-way ANOVA using fungal diversity measures as dependent variables and sampling locations and sampling period as fixed, dependent variables: $p = 0.38$), nor between inverse Simpson indices ($p = 0.32$) or Pielou evenness indices ($p = 0.70$). Significant differences, however, were found at the level of the study site (using trees as sampling units; three-way ANOVA: $p < 0.01$ for OTU richness indices, inverse Simpson indices and Pielou evenness indices) and between sampling years (three-way ANOVA: $p < 0.01$ for OTU richness indices, inverse Simpson indices and Pielou evenness indices). None of the interaction terms were significant ($p > 0.05$). Spatial and temporal autocorrelation (based on Moran's I) of diversity data were tested on the same scales as described above.

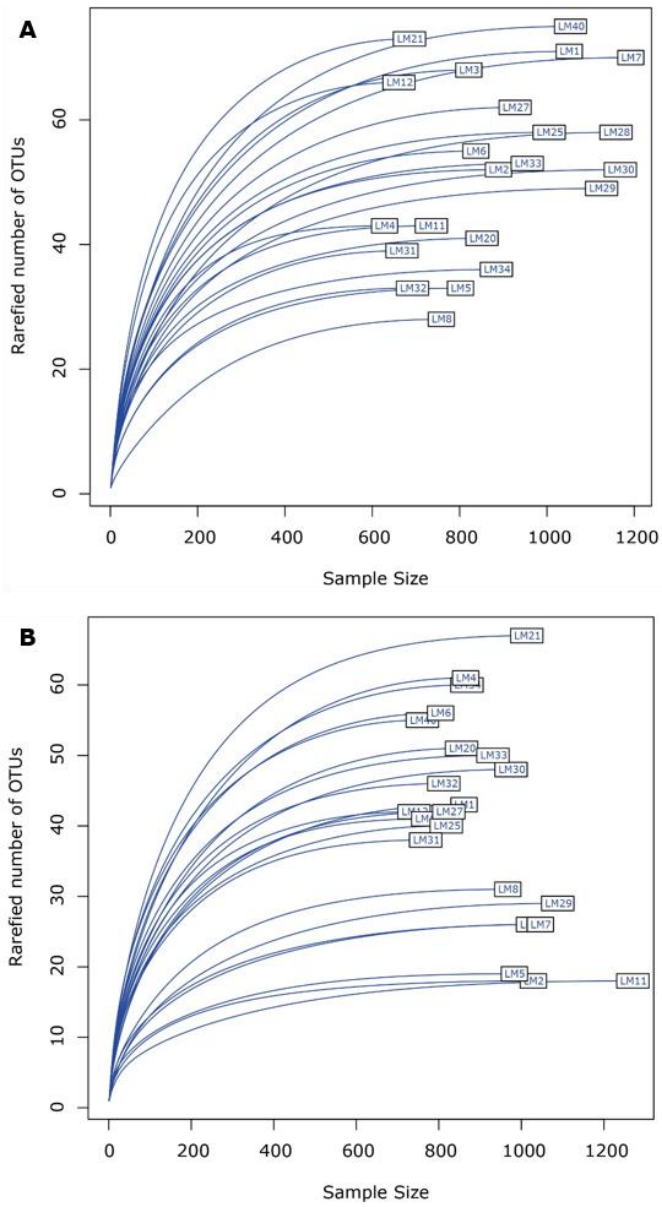


Figure 4.2 Rarefaction curves at the level of individual trees. Rarefaction curves are based on the average number of OTUs of five samples that were collected at different distances from the same pine tree (n = 22). OTUs were generated in Mothur based on a 97% sequence similarity cut-off. A. Rarefaction curves for the 2009 sampling session. B. Rarefaction curves for the 2011 sampling session.

Spatial autocorrelation was not observed for samples collected within a one meter radius from the same pine tree (OTU richness: $p = 0.91$, Pielou evenness: $p = 0.59$ and inverse Simpson indices: $p = 0.83$), nor at the level of the entire study site (OTU richness: $p = 0.95$, Pielou evenness: $p = 0.15$ and inverse Simpson indices: $p = 0.46$). However, temporal autocorrelation was observed between sampling years ($p < 0.01$ for all diversity measures).

Local spatial and temporal variations in fungal community structure

Observed fungal community compositions were investigated on the same spatial and temporal scales as the ones used for fungal diversity analyses. Three-way PERMANOVAs (using community distance matrices based on Bray-Curtis distances as dependent variables and sampling location and sampling period as fixed, independent variables) revealed a significant difference in fungal community composition between samples collected within a one meter radius of pine trees, between individual trees across the study site and between sampling years (all $p < 0.01$).

To analyse community similarities (based on 1 - Bray-Curtis distances (BC)) across spatial and temporal scales, distance decay of similarity analyses were conducted (Fig. S4.3). No significant decay of similarity was observed within a one meter radius from pine trees, between different pine trees across the study site, nor between sampling years (slopes: -0.0003, -0.00008 and -0.01 for distances between individual samples, distances between trees and distances between years respectively). The significance of these decay patterns were verified with Mantel tests (Mantel statistic: $r = -0.06$, $p = 1.00$; $r = -0.07$, $p = 0.84$ and $r = -0.08$, $p = 1.00$ respectively). These results indicate that the fungal communities in samples collected further away from each other (either in space or in time) can be as similar to each other as the fungal communities of samples collected close to each other. Differences in the fungal community composition of samples that were collected at different distances from each other were visualized using non-metric multi-dimensional scaling (NMDS). As can be observed in Fig.

4.3, samples did not cluster according to the distance to the stem they were collected at, but some samples did tend to cluster according to trees. A more detailed comparison of the OTU composition of the fungal community observed in 2009 and the one observed in 2011, revealed that of the 771 OTUs that were observed across both datasets, 240 OTUs (31%) that were present in 2009, no longer were detected in 2011, whereas 182 OTUs (24%) were newly discovered. 349 OTUs (45%) were shared between both sampling years. OTUs that were lost or gained from the datasets, however, all had very low abundances, whereas all OTUs with a relative read abundance > 1% were shared between the datasets. To test whether some fungal species were significantly associated with a specific sampling year or sampling distance to a stem, indicator species analyses were conducted. Using a community matrix excluding fungal OTUs with an average relative read abundance of < 1%, no indicator species were identified. When OTUs with low abundances (< 1%) were included in the dataset, 93 species were found to specifically associate with a particular sampling year or sampling distance from pine stems. Results of indicator species analyses including OTUs with a low average relative read abundance can be found in Table S4.2.

Relationships between observed fungal communities and their environment

Spatial autocorrelation was studied for the main environmental factors (soil and needle Zn and Cd concentrations) based on Moran's I. No spatial autocorrelation was observed for soil Zn ($p = 0.46$), soil Cd ($p = 0.37$), needle Zn ($p = 0.63$) or needle Cd concentrations ($p = 0.12$). Significant correlations between a number of measured environmental variables and fungal diversity measures were observed, but none of the correlations were consistent between the 2009 and 2011 datasets (Table S4.3). To investigate how different environmental factors relate to the observed fungal community compositions, a canonical correspondence analysis (CCA) was performed on the 2009 and 2011 datasets.

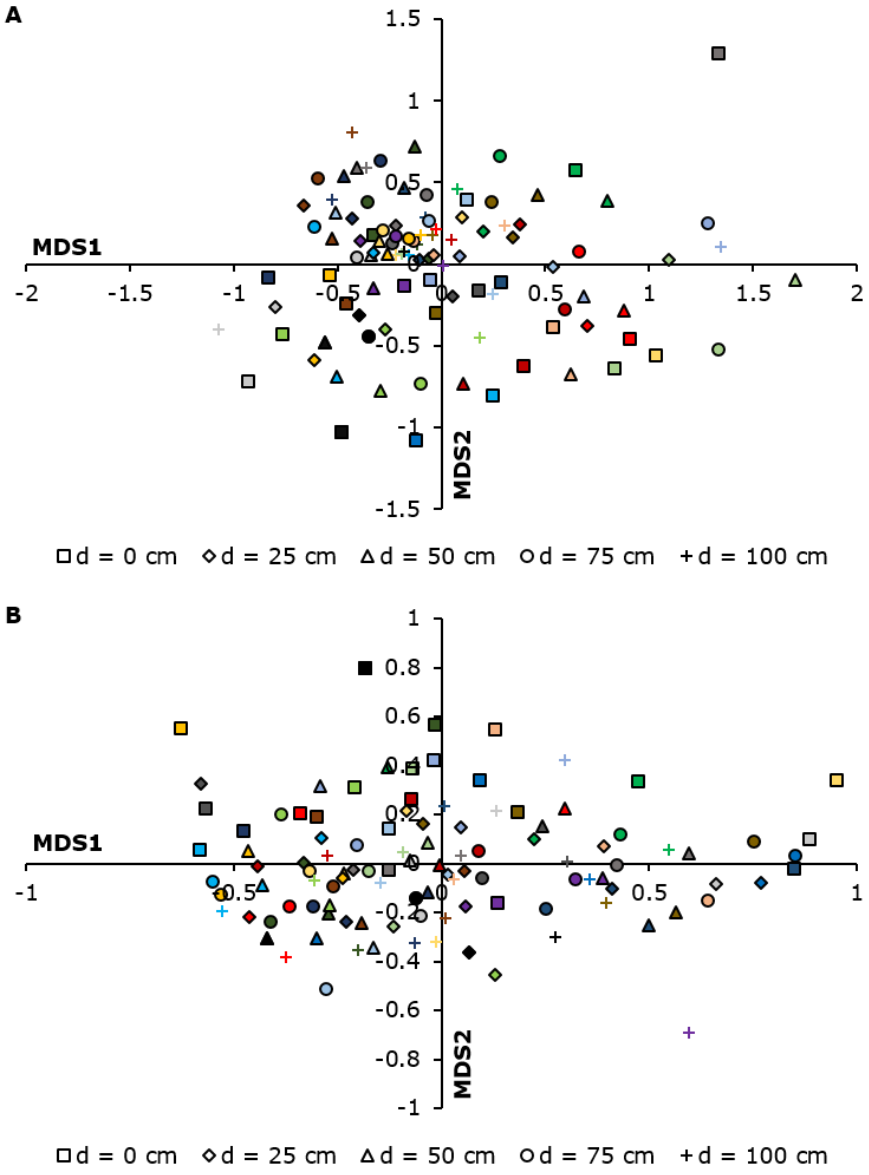
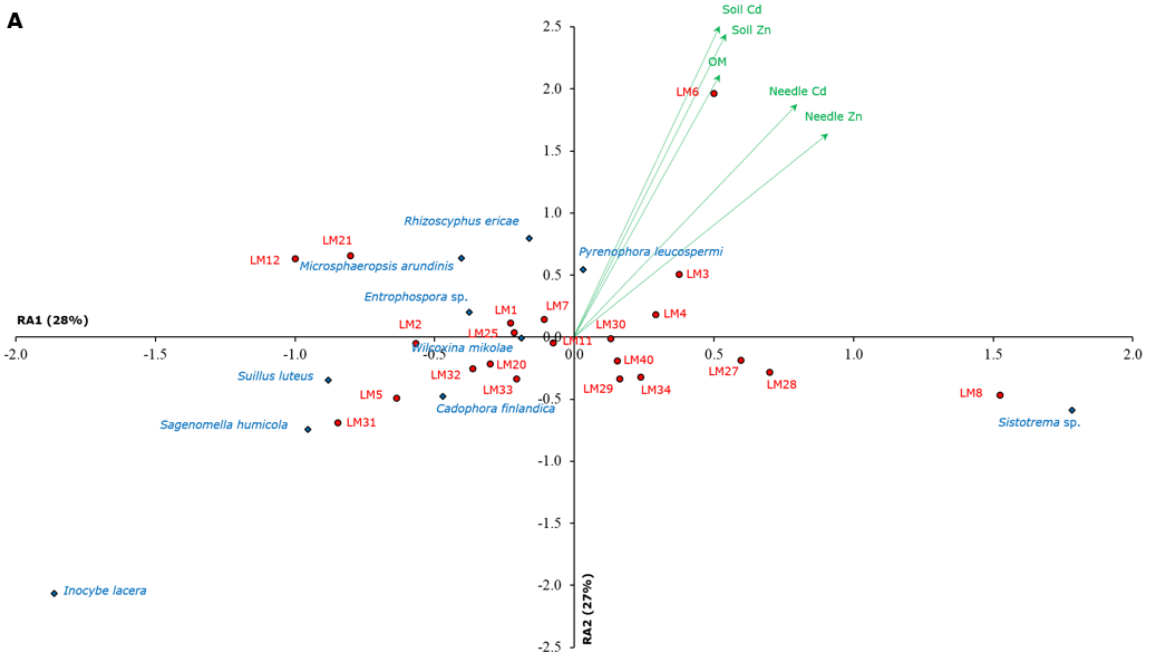


Figure 4.3 Non-metric multi-dimensional scaling (NMDS) plots displaying dissimilarities (based on Bray-Curtis distances) in the fungal community composition of samples that were collected at different distances from the same tree (d = distance). A. Results of the 2009 analysis. B. Results of the 2011 analysis. OTUs were generated in Mothur based on a 97% sequence similarity cut-off.

Both for the 2009 and 2011 sampling sessions, soil organic matter (OM), needle Zn and Cd concentrations and soil Zn and Cd concentrations were found to be significantly correlated with the observed fungal community composition (Permutation tests resulted in $p < 0.01$ for all five parameters; only the top ten most abundant OTUs were displayed to avoid crowding of the graphs; Fig. 4.4). Since all five parameters were strongly correlated with each other (all $p < 0.01$ both for the 2009 and 2011 datasets), it is impossible to separate the individual effects of Zn, Cd and OM. In the 2009 CCA analysis, RA1 explained 28% of the total variance and RA2 explained 27% of the total variance. For the 2011 dataset, RA1 explained 42% of the total variance and RA2 explained 23% of the variance (Fig. 4.4). Nine of the ten most abundant OTUs neither had a strongly increased nor a decreased abundance in more severely polluted samples. Only OTUs corresponding to *Inocybe lacera* (Fr.) P. Kumm. were more abundant in samples with lower metal concentrations and a lower OM content (Fig. 4.4). It is noteworthy that 22% of all OTUs (including low-abundant OTUs) in the 2009 dataset had an increased relative abundance (average read count for a specific OTU per sample relative to the average total read count of each sample) in samples with higher Zn, Cd and OM concentrations, whereas 18% had a decreased relative abundance in these samples. 60% of all OTUs were equally abundant in all samples in the 2009 dataset. In the 2011 dataset, only 1% of all OTUs were less abundant in more polluted samples, whereas 67% were more abundant in more severely polluted samples. The remaining 32% of OTUs were equally abundant in all samples in the 2011 dataset.

The relative abundances of the top ten most abundant OTUs were displayed in pie charts (Fig. 4.5). The remaining, less abundant OTUs, were grouped together as "Remaining taxa" in this figure. Average relative abundances of all OTUs, BLAST Scores, corresponding E-values and species or genera to which the OTUs corresponded can be found in Appendix B.


A
Soil Cd
Soil Zn
OM
Needle Cd
Needle Zn
LM6
Rhizoscyphus ericae
LM21
LM12
Microsphaeropsis arundinis
Pyrenophora leucospermi
LM3
Entrophospora sp.
LM1
LM7
LM4
LM2
LM25
LM30
LM11
Wilcoxina mikolae
LM40
LM20
LM27
Suillus luteus
LM32
LM29
LM34
LM28
LM5
LM33
LM8
Cadophora finlandica
Sagenomella humicola
LM31
Sistotrema sp.
Inocybe lacera
RA1 (28%)
RA2 (27%)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-2.0
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-1.0
-0.5
0.0
0.5
1.0
1.5
2.0

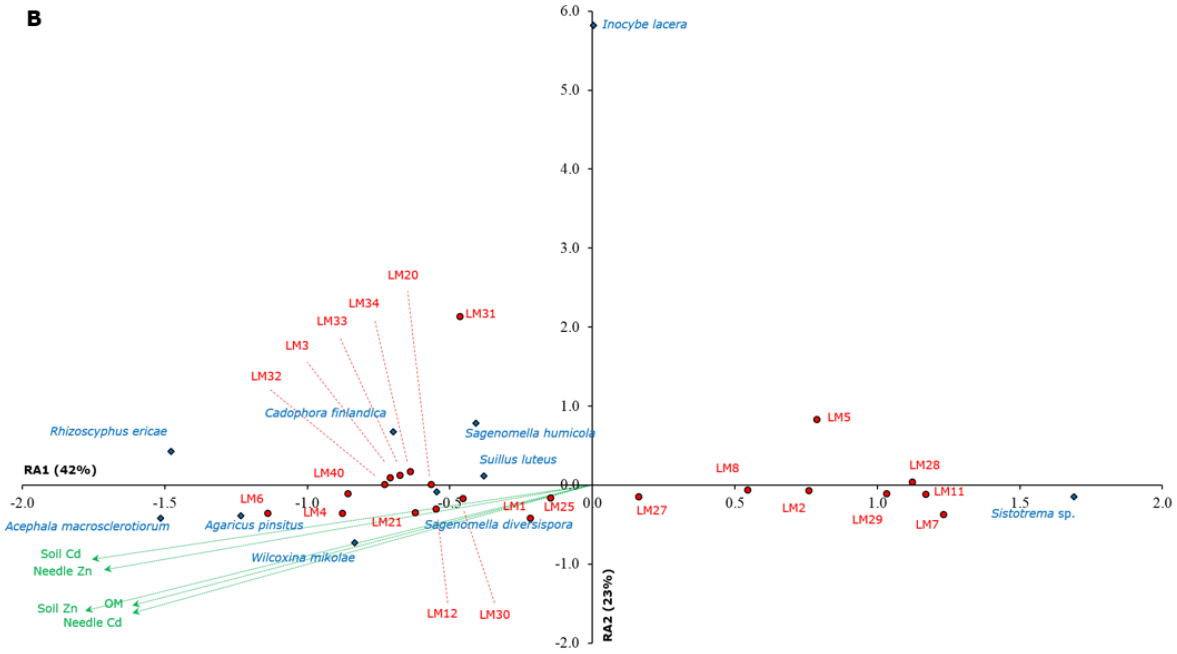

Figure 4.4 Canonical correspondence analysis (CCA) displaying the relationship between fungal community composition and environmental parameters near trees (n = 22). A. CCA of the 2009 sampling session. B. CCA of the 2011 sampling session. OTUs were generated in Mothur based on a 97% sequence similarity cut-off. OTU abundances of five samples that were collected at different distances from the same pine tree were averaged-out. Only those environmental parameters that significantly correlated with fungal community compositions at the 95% confidence level are shown. Of the fungal OTUs, only the top ten most abundant OTUs are shown.

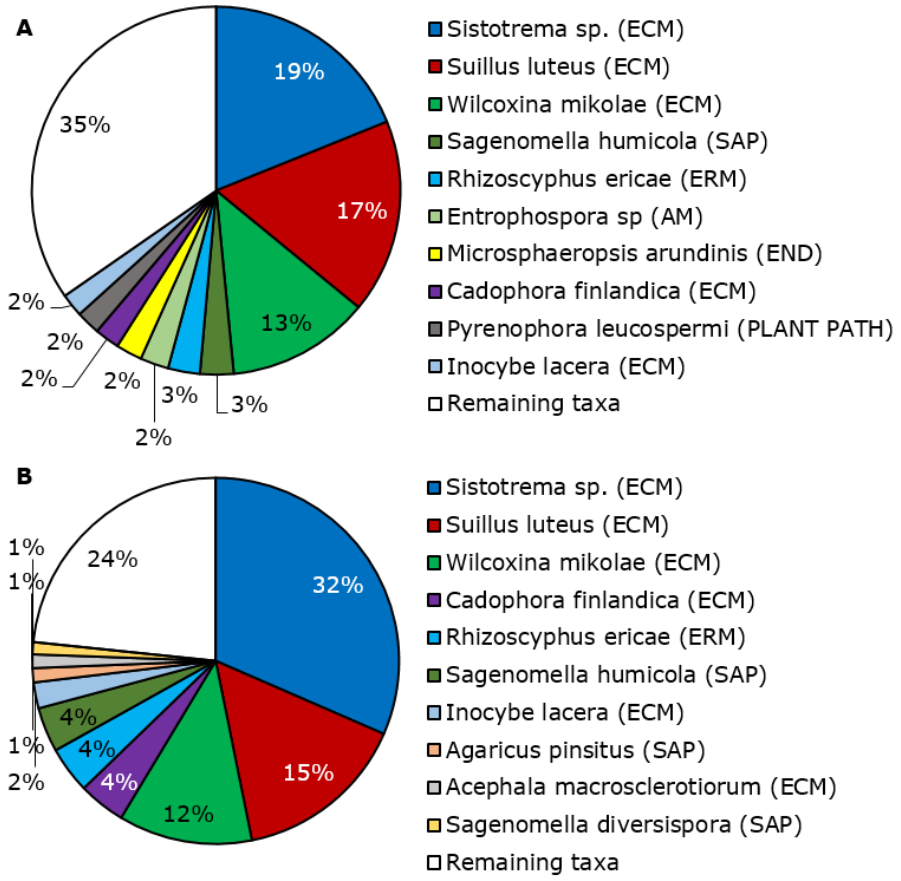

Figure 4.5 Pie charts displaying the top ten most abundant fungal OTUs and their average relative abundance per sample for A. the 2009 dataset and B. the 2011 dataset. OTUs were generated in Mothur based on a 97% sequence similarity cut-off. Abundances of OTUs not appearing in the list of top ten most abundant OTUs were combined under "Remaining taxa". Fungal lifestyles are indicated between brackets behind the genus or species name that corresponds to the respective OTU. ECM: ectomycorrhizal. SAP: saprotrophic. ERM: ericoid mycorrhizal. AM: arbuscular mycorrhizal. END: endophytic. PLANT PATH: plant pathogen.

From Fig. 4.5, it is clear that the same top three most abundant OTUs represented a high proportion (49% and 59%) of all reads identified in the 2009 and 2011 datasets, respectively. These OTUs corresponded to *Sistotrema* sp. Fr., *Suillus luteus* and *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf and on average accounted for 19% (range: 0% to 83%), 17% (range: 0% to 45%) and 13% (range: 0% to 28%) of all reads identified in 2009 and for 32% (range: 3% to 77%), 15% (range: 4% to 32%) and 12% (range: 2% to 36%) of all reads identified in 2011 (Fig. 4.5), respectively. Other OTUs that were frequently encountered in both the 2009 and 2011 datasets corresponded to *Sagenomella humicola* (Onions & G.L. Barron) W. Gams (3% in 2009 and 4% in 2011), *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang & Korf (3% in 2009 and 4% in 2011), *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew (2% in 2009 and 4% in 2011) and *Inocybe lacera* (2% both in 2009 and in 2011) (Fig. 4.5).

DISCUSSION

Local spatial and temporal variations in observed fungal communities

In order to determine the effects of metal pollution on fungal communities, we investigated local spatial and temporal variations in the observed belowground fungal communities. Factors potentially affecting the spatial structure of fungal communities in the immediate vicinity of a tree are decreasing root density (Peay *et al*., 2011) and root age (Last *et al*., 1987). Both factors decrease with increasing distance from the stem base of a tree and may recruit different ectomycorrhizal fungi. However, we found no evidence for such a pattern in the current circumstances Furthermore, fungal populations in the immediate vicinity of the studied communities provide fungal inoculum to the new pine plantation and could therefore cause spatial structuring of the fungal communities at the scale of the study site (Peay *et al*., 2010). Our analyses, however, revealed that samples

collected close to each other (either in space or time) can be very similar to samples collected further apart from each other (Fig. 4.3 and Fig. S4.3). We did not find any significant structuring of the observed fungal communities in time or space and overall observed fungal communities in different years or sampling locations within the study area were very similar to each other. Although introduction of fungal inoculum from surrounding fungal populations is likely (Muller *et al*., 2004), it is considered a minor factor in influencing the structure of the studied fungal communities. Comparison of the OTU composition of the 2009 and 2011 datasets revealed that only a small proportion (47%) of the observed OTUs were shared between the 2009 and 2011 sampling sessions. Nevertheless, all OTUs that were lost from the 2009 dataset or gained in the 2011 dataset had very low abundances suggesting that these OTUs were most likely fungal spores or other propagules that are picked up due to the high sensitivity of 454 amplicon pyrosequencing. Hence, the large proportion of OTUs that were either lost or gained between the two datasets are not necessarily selected against because of high metal concentrations. Probably they reflect random detection of propagules in the environmental samples. A possible approach to reduce the detection of metabolically inactive propagules would be an analysis of environmental precursor ITS rRNA instead of DNA (van der Linde and Haller, 2013). Analyses excluding OTUs with a low abundance, however, still revealed differences in the fungal community compositions observed in 2009 and 2011. Since all abundant OTUs were shared between the communities observed in 2009 and 2011, the differences between the community compositions in both years are mainly due to differences in the relative abundances of OTUs shared between both datasets. Also, the fungal diversity of 2009 differed from the fungal diversity in 2011. These results suggest that the studied pioneer fungal communities are still highly dynamic. The observed decrease in OTU richness from 2009 to 2011 together with the fact that the abundances of more OTUs were positively correlated with soil metal pollution and soil OM content in 2011 (67%) than in 2009 (22%) suggests that a shift towards a

fungal community with more metal-resistant and metal-tolerant ecotypes is taking place. On the other hand, also seasonal fluctuations in climate might be responsible for some differences between the fungal community identified in 2009 and the one observed in 2011 (Jumpponen *et al*., 2010; Davey *et al*., 2012). To confirm the hypothesis that the present metal pollution is indeed selecting for metal-tolerant ecotypes, strains of abundant species will need to be isolated from the field and tested for their Zn- and Cd- tolerance.

Linking observed fungal community composition and diversity to environmental parameters

Both for the 2009 and 2011 sampling sessions, strong correlations were found between Zn and Cd concentrations and soil OM content and fungal community compositions (Fig. 4.4). However, since these environmental parameters were also strongly correlated with each other, it is difficult to separate the individual effects of these parameters. More specifically, the chemical interaction between soil OM content and Zn and Cd concentrations is a well-known process that could be triggered by the effects of metal-toxicity on microbial communities. Because of a reduced microbial decomposition rate, organic matter could accumulate in highly polluted patches of soil (Chodak *et al*., 2013), and on the other hand, any increase in soil OM content could further cause an increased sequestration of metal ions. In our study, the actual range in metal concentrations in soil and pine needles is more pronounced than the range in soil OM content (Fig. 4.1). Anyhow, increased concentrations of metal ions and soil OM content, individually, have been shown to affect the composition of fungal communities in previous studies. Macdonald *et al*. (2007; 2008), for example, have shown that the composition of fungal communities, characterized with terminal restriction fragment length polymorphisms (TRFLP), were altered due to increased concentrations of Zn and Cu. Furthermore, Macdonald *et al*. (2007) found that the abundance of some fungal terminal restriction fragments (TRFs) increased significantly with

increasing Zn concentrations in soils, whereas other fungal TRFs were lost completely in metal-polluted sites. Hui *et al*. (2011) investigated the effects of increased Pb concentrations on ectomycorrhizal communities in *Pinus sylvestris* stands in Southern Finland. Similar to the findings in our study (Fig. 4.4), Hui *et al*. (2011) found that increased metal concentrations in boreal forest soils significantly altered the composition of ectomycorrhizal communities, but the fungal diversity remained unchanged. However, changes in fungal diversity caused by increased metal concentrations and varying soil OM content have been reported in previous studies. High concentrations of metals, for example, have been found to decrease microbial species diversity in metal-polluted fields (e.g. Chodak *et al*., 2013) and soil organic matter content has been shown in the past to strongly affect fungal diversity in various ways (Hartmann *et al*., 2012; Azarbad *et al*., 2013). Although each environmental factor probably did affect fungal diversity on its own, overall, fungal diversity remained unchanged throughout our study site despite considerable variations in metal concentrations and soil OM content. The fact that some authors do find effects of metal pollution on fungal diversity whereas other studies do not, may be due to differences in the metal species involved and the actual toxicity of metal ions. It is, for example, well known that microbial communities may alter the form of metal species (Gadd, 1993) and environmental parameters such as soil pH and cation exchange capacity may strongly affect the availability (and hence the toxicity) of metal ions as well. The Zn and Cd concentrations in our study site were clearly toxic to pine trees as a number of trees with more than 400 μg g^{-1} needle Zn in the study site showed substantial leaf chlorosis.

Dominant fungal species in the investigated communities

From Fig. 4.5, it is clear that three fungal OTUs strongly dominated the fungal community in Lommel-Maatheide. These OTUs corresponded to *Sistotrema* sp., *Suillus luteus* and *Wilcoxina mikolae* and their dominance appears to have increased from 2009 to 2011. Interestingly, *S. luteus* and

W. mikolae are both species that have been associated with metal-polluted soils in previous studies. *S. luteus* is an ectomycorrhizal basidiomycete that typically occurs in pioneer pine stands. Additionally, previous research has shown the presence of Zn- and Cd-tolerant ecotypes of this species in the studied area (Colpaert *et al*., 2004; Krznaric *et al*., 2009). *W. mikolae* is an ectomycorrhizal ascomycete. It is a cosmopolitan species that has been isolated from a wide range of environments, including metal-rich mine spoils (Prabhu *et al*., 1996). The most abundant OTU identified in the current study corresponds to a *Sistotrema* species (strain B216) that has been described as an ectomycorrhizal basidiomycete isolated from sand dunes in the USA (Ashkannejhad and Horton, 2006). Species in this genus have not been described to occur in metal-polluted soils so far. Since it was the most abundant fungal OTU present in our study site, both in 2009 and in 2011, it would be interesting to investigate Zn and Cd tolerance of this *Sistotrema* population. Beside *S. luteus*, two other Suilloid fungi - *S. bovinus* and *Rhizopogon luteolus* - have been found to have developed Zn- and/or Cd-tolerant ecotypes in the Campine region in Belgium (Colpaert *et al*., 2004). *S. bovinus* was detected in only one sample in the 2009 dataset with an abundance of 5% and in five samples in the 2011 dataset with abundances up to 5%. Since *S. bovinus* occurs in later stages of forest succession than *S. luteus*, this species is expected to become more dominant as the pine stand ages. *R. luteolus*, also a typical pioneer species, was detected in 16 samples in 2009 with an abundance ranging between 1% and 5% and in 19 samples in 2011with an abundance ranging between 1% and 9%. On a regional scale, *R. luteolus* is a declining species most likely because of its sensitivity to high nitrogen deposition and soil acidification (Arnolds, 1991). Other interesting OTUs that appeared in the list of top ten most abundant OTUs corresponded to the genus *Entrophospora* R.N. Ames & R.W. Schneid and the species *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew. OTUs corresponding to *Entrophospora* were the sixth most abundant OTU in the 2009 dataset and the eleventh most abundant OTU in the 2011 dataset

(data not shown). This genus has been found in Cu mine spoils in previous studies (da Silva *et al*., 2005). *C. finlandica* is an ascomycete species belonging to the *Rhizoscyphus ericae* aggregate (Vrålstad *et al*., 2000). Fungal species in this species aggregate are believed to be able to form ectomycorrhizal and ericoid mycorrhizal associations with different plant species (Vrålstad, 2004). These fungal species can be frequently found in metal-polluted soils as well (Gorfer *et al*., 2009) where they may improve plant nutrition and enhance plant survival under harsh conditions (Mrnka *et al*., 2009). The abundance of only one of the most abundant OTUs was clearly negatively correlated with increasing Zn, Cd and OM concentrations. This OTU corresponds to the ectomycorrhizal basidiomycete *Inocybe lacera*, a typical basidiomycete of primary successions (Jumpponen *et al*., 2002). Even though some reports indicated the presence of *Inocybe* species on metal-polluted mine spoils (Huang *et al*., 2012), OTUs corresponding to *I. lacera* in our study were more abundant in samples with lower Zn, Cd and OM concentrations. Overall, it is noteworthy that the fungal communities in the studied site were dominated by biotrophic species and in particular by ectomycorrhizal fungi, testifying to the importance of these fungal symbionts in disturbed pioneer pine forests.

Conclusions

No spatial structuring of the studied fungal communities was found within a one meter radius surrounding sampled trees nor at the scale of the entire study site. However, Zn- and Cd concentrations, together with soil OM content, were shown to correlate well with fungal community compositions. Hence, we conclude that metal pollution and soil OM content are the most important factors shaping the studied pioneer fungal communities. None of the measured environmental parameters were found to consistently correlate with fungal diversity indices. This is not to say that these factors do not influence fungal diversity individually, but overall no effect of the microbial environment on fungal diversity was observed. The abundance of most fungal OTUs identified in the current study either positively correlated

with increasing metal pollution and soil OM content or had a similar abundance in all samples. The present results and our previous observations on metal-tolerant Soilloid ecotypes suggest that the harsh environmental conditions in the studied site are selecting for metal-resistant and metal-tolerant genotypes (ecotypes), maintaining a relatively high fungal diversity which presumes sufficient genetic and phenotypic variation in pioneer fungi. Nevertheless, it was shown that a restricted number of, probably well adapted, ectomycorrhizal fungal species dominated the studied communities. *S. luteus* was found to be one of the most abundant species, as was hypothesized. Other species that were found to be dominant in the studied fungal communities, such as *Sistotrema* sp., *Wilcoxina mikolae* and *Cadophora finlandica* provide interesting new opportunities to further investigate the presence of metal-resistance and metal-tolerance in fungi growing in metal-polluted sites.

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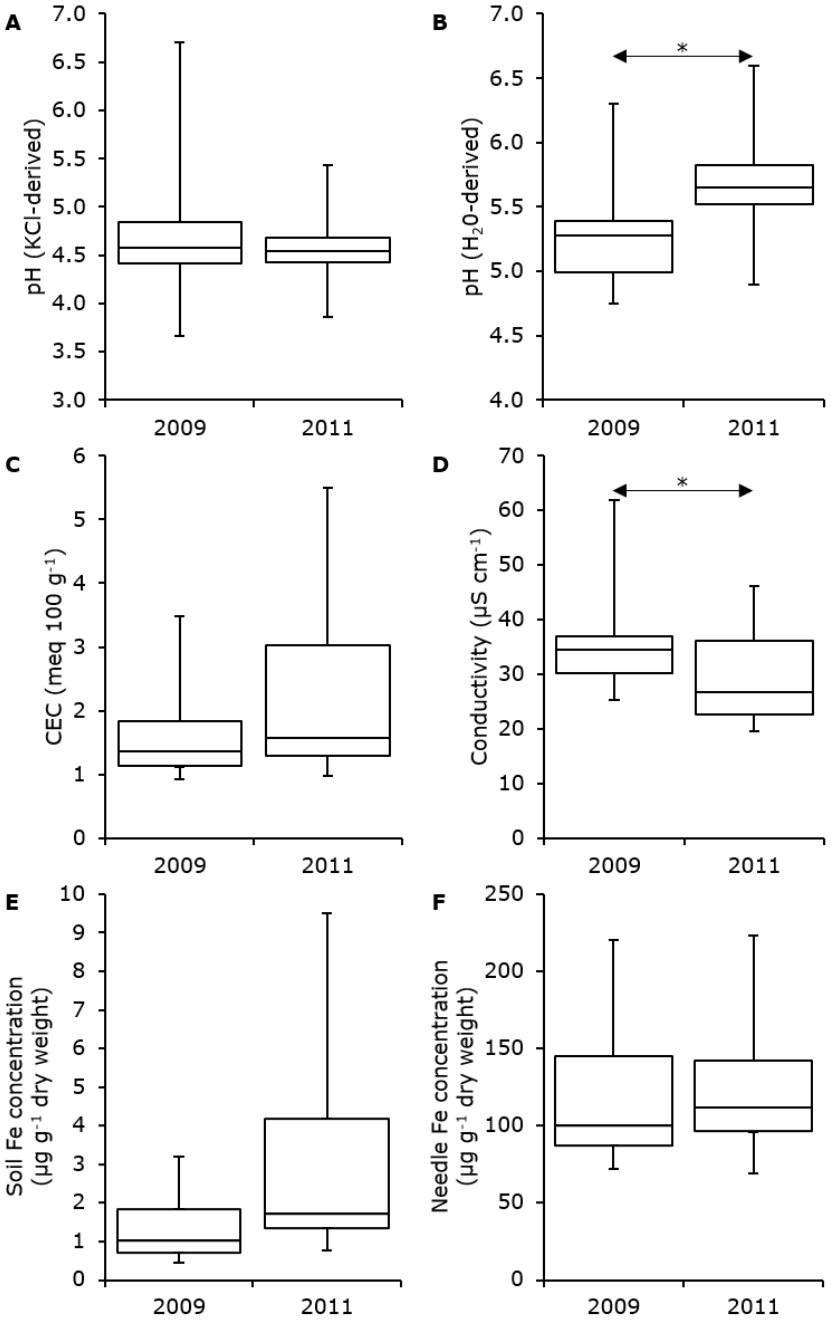
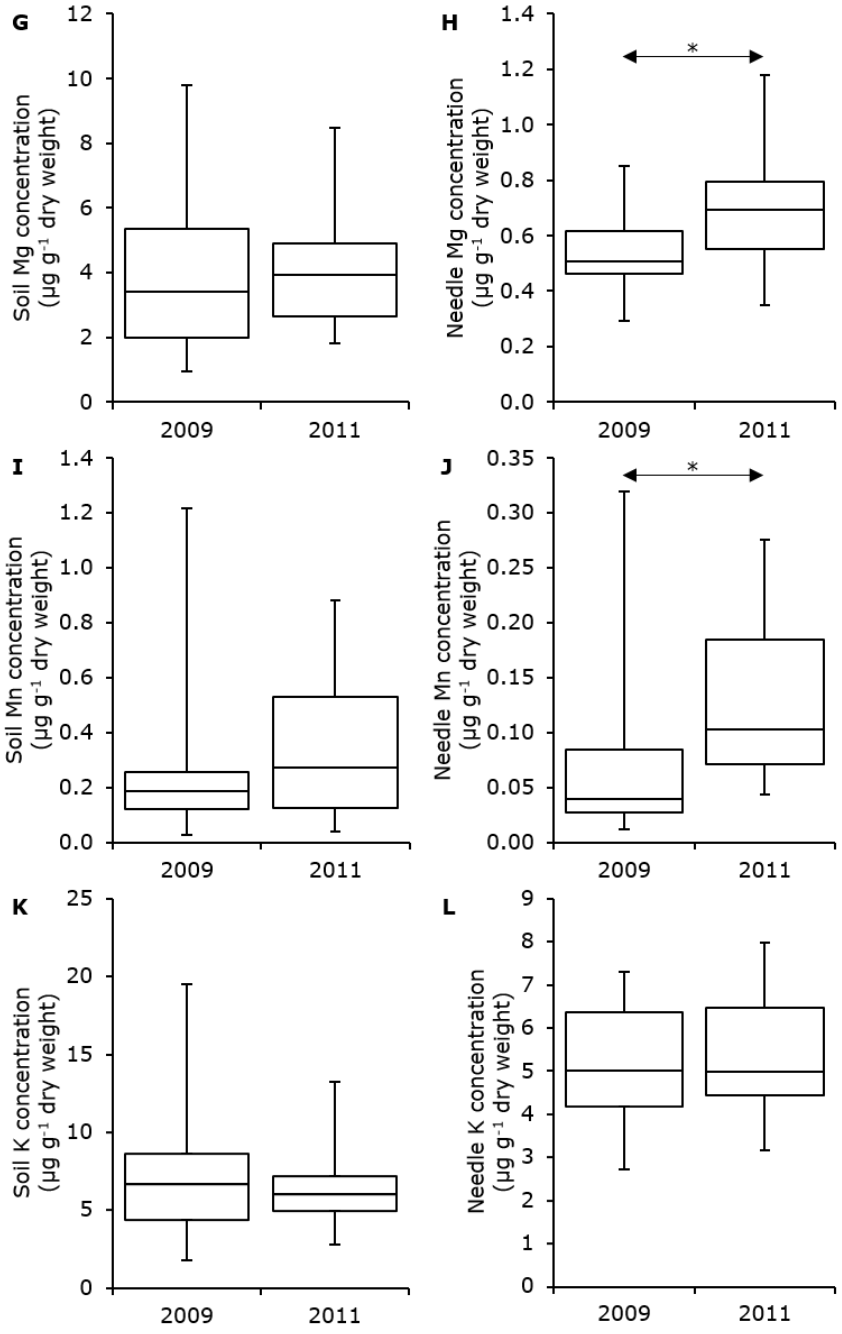
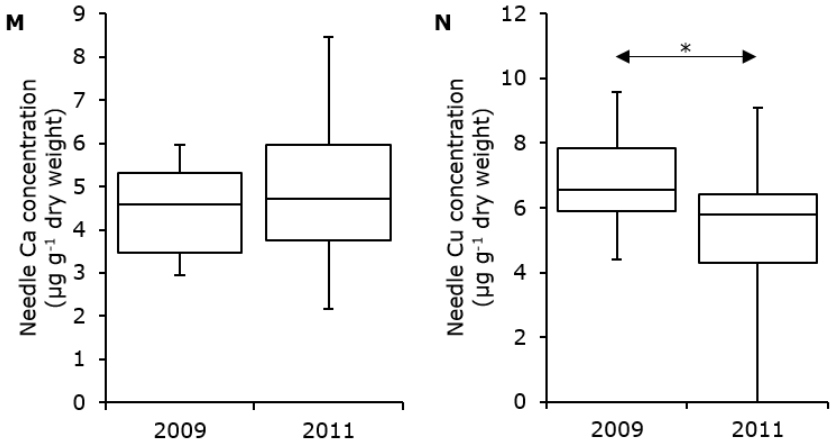
CHAPTER 4

Waud MJ, Busschaert P, Ruyters S, Jacquemyn H, Lievens B (2014). Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. *Molecular Ecology Resources*. doi: 10.1111/1755-0998.12229.

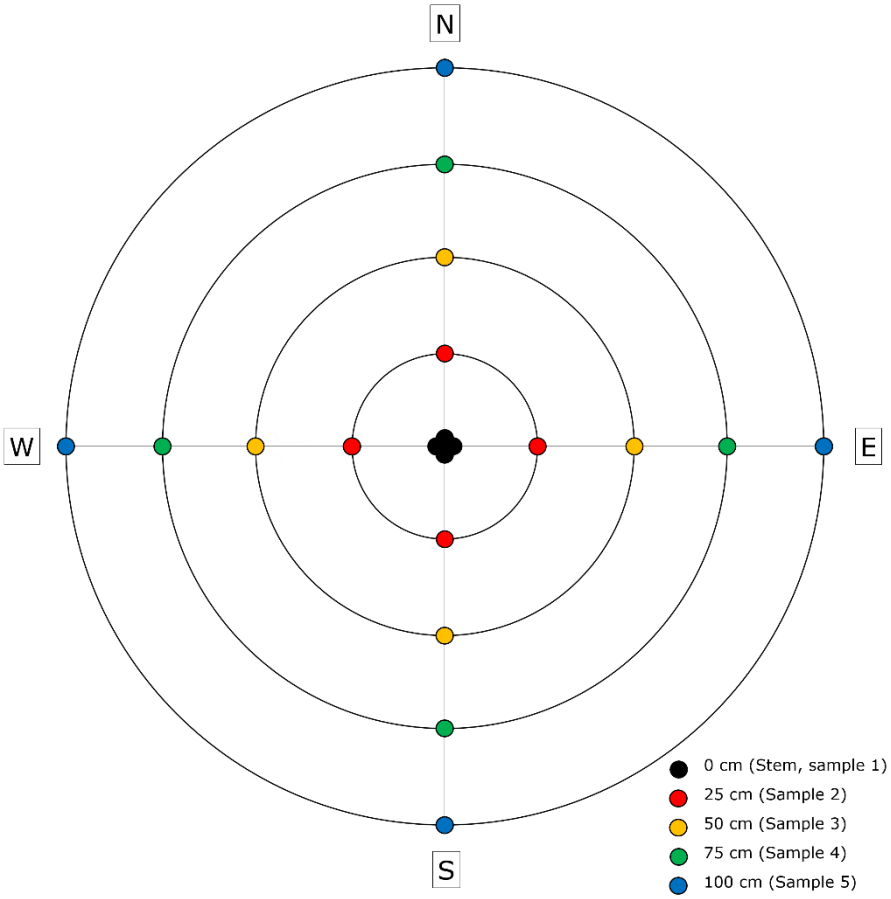
SUPPORTING INFORMATION



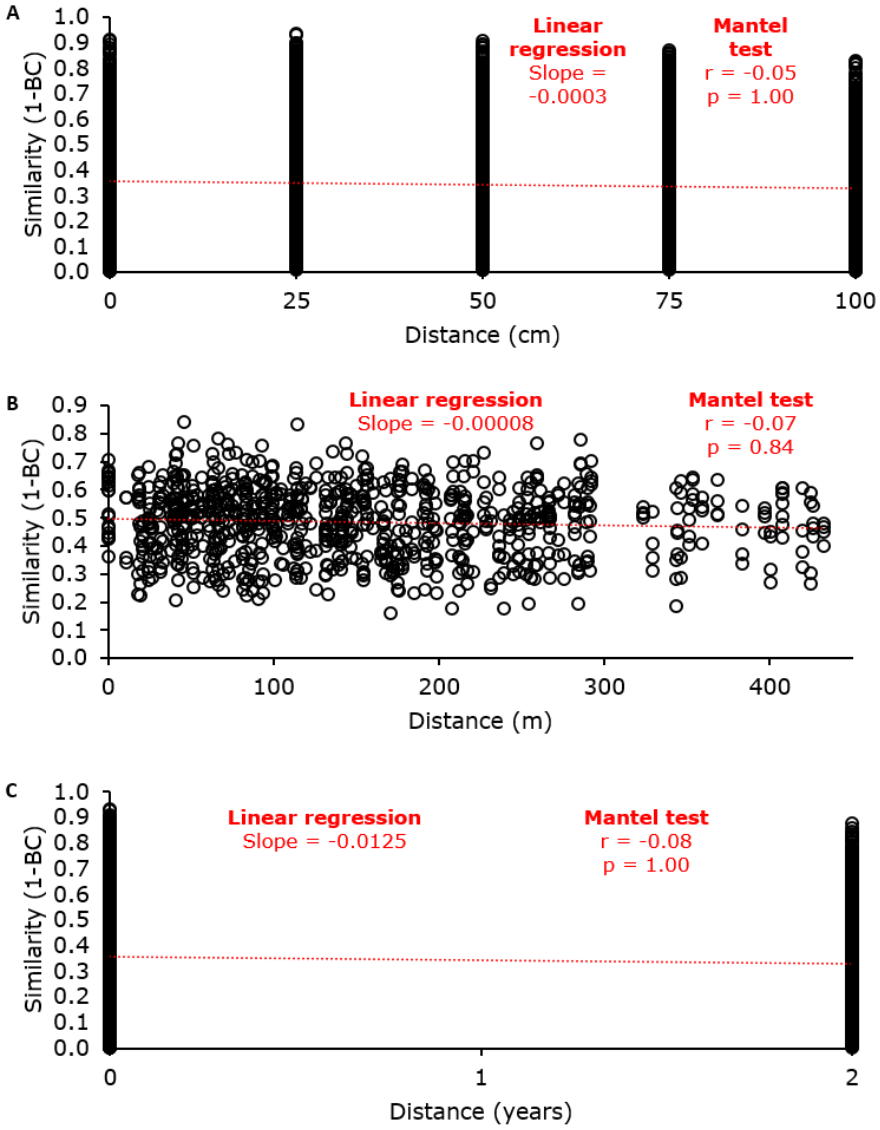

G
Soil Mg concentration (µg g-1 dry weight)
12
10
8
6
4
2
0
2009
2011
H
Needle Mg concentration (µg g-1 dry weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
2009
2011
I
Soil Mn concentration (µg g-1 dry weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2009
2011
J
Needle Mn concentration (µg g-1 dry weight)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
2009
2011
K
Soil K concentration (µg g-1 dry weight)
25
20
15
10
5
0
2009
2011
L
Needle K concentration (µg g-1 dry weight)
9
8
7
6
5
4
3
2
1
0
2009
2011



Supporting Information Figure S4.1 Comparison of measured environmental variables between sampling years (2009 and 2011). Statistically significant differences at the 95% confidence level are indicated with an asterisk (*). Box-plots display the first (25%) and third (75%) quartile, the median, maximum and minimum observed values. Soil Cu concentrations were below the detection limit of 0.1 µg g^{-1}. A. pH (KCl-derived). B. pH (H_20-derived). C. CEC. D. Conductivity. E. Soil Fe concentrations. F. Needle Fe concentrations. G. Soil Mg concentrations. H. Needle Mg concentrations. I. Soil Mn concentrations. J. Needle Mn concentrations. K. Soil K concentrations. L. Needle K concentrations. M. Needle Ca concentrations. N. Needle Cu concentrations.



Supporting Information Figure S4.2 Sampling design used in the current studies to collect soil samples for fungal community analysis. This sampling design was used for each selected tree (n = 22).



Supporting Information Figure S4.3 Results of distance decay of similarity analyses displaying the decay of community similarity (1- Bray-Curtis distances (BC)) A. at the scale of individual samples collected at different distances from a pine stem (d = 0 cm, 25 cm, 50 cm, 75 cm and 100 cm) B. at the scale of the study site (using individual trees as sampling unit) and C. between sampling years (2009 and 2011). Results of linear regressions and Mantel tests are given in insets.

Supporting Information Table S4.1 Results of indicator species analysis displaying fungal species significantly associated with samples collected from different distances from a pine stem (d = 0 cm, 25 cm, 50 cm, 75 cm or 100 cm or combinations thereof) or samples collected in different years (2009 or 2011). The association of all fungal species with specific samples shown in these tables were significant at the 95% confidence level.

Sample	Species	p-value	Rel. abund. (%) [1]	SE on Rel. abund. [2]
0 cm	Heyderia abietis	0.001	0.134	4.244E-06
	Chalara microchona	0.001	0.462	1.268E-05
	Phialocephala virens	0.001	0.259	7.998E-06
	Antarctic yeast CBS 8941	0.001	0.210	1.026E-05
	Rhodotorula philyla	0.001	0.106	5.963E-06
	Xenopolyscytalum pinea	0.001	0.062	3.056E-06
	Pyrenochaetopsis microspora	0.001	0.035	2.051E-06
	Sistotrema alboluteum	0.009	0.050	8.599E-06
	Fusicladium cordae	0.002	0.008	8.035E-07
	Zalerion arboricola	0.009	0.004	6.526E-07
	Dactylaria lanosa	0.031	0.008	1.772E-06
	Exobasidium inconspicuum	0.039	0.003	7.789E-07
	Lecanora polytropa	0.041	0.006	1.451E-06
	Scoliciosporum umbrinum	0.039	0.003	7.789E-07
	Myrmecridium phragmitis	0.048	0.008	1.157E-06
25 cm	Sporobolomyces gracilis	0.029	0.021	1.274E-06
	Clitopilus sp FZ1433	0.035	0.007	1.317E-06
50 cm	Spizellomyces pseudodichotomus	0.002	0.053	2.728E-06
	Spizellomyces plurigibbosus	0.006	0.018	1.507E-06
	Hypholoma fasciculare	0.011	0.015	1.102E-06
	Laetisaria lichenicola	0.036	0.054	6.942E-06
	Rhizophydium sp JEL 385	0.015	0.006	1.524E-06
	Cladonia borealis	0.021	0.011	1.343E-06

0 cm + 25 cm	Acephala macrosclerotiorum	0.001	0.886	1.335E-05
	Lophodermium pinastri	0.011	0.087	2.917E-06
	Teratosphaeria microspora	0.007	0.056	1.664E-06
	Mortierella sp TR158	0.015	0.132	5.258E-06
	Helicodendron websteri	0.009	0.036	2.386E-06
	Cryptosporiopsis ericae	0.01	0.022	1.168E-06
	Bullera miyagiana	0.031	0.015	1.302E-06
0 cm + 100 cm	Microsphaeropsis proteae	0.049	0.023	2.529E-06
25 cm + 75 cm	Xanthoria parietina	0.011	0.016	1.172E-06
0 cm + 25 cm + 50 cm	Meliniomyces bicolor	0.007	0.063	2.381E-06
0 cm + 25 cm + 100 cm	Epacris microphylla root associated fungus 5	0.05	0.023	1.236E-06
50 cm + 75 cm + 100 cm	Collophora rubra	0.001	0.134	3.290E-06
	Caloplaca cerina	0.003	0.066	2.549E-06
	Phaeophyscia exornatula	0.016	0.066	2.549E-06
	Cryptococcus sp MD76 1BY	0.041	0.019	1.044E-06
25 cm + 50 cm + 75 cm + 100 cm	Camarographium koreanum	0.001	0.793	6.305E-06
	Stictis radiata	0.001	0.425	3.607E-06
	Ophiocordyceps sinensis	0.002	0.554	6.160E-06
	Preussia sp SL08070	0.042	0.262	3.584E-06
	Aureobasidium pullulans	0.011	0.228	4.735E-06
	Podospora ellisiana	0.002	0.115	3.201E-06
	Macroconia leptosphaeriae	0.039	0.199	6.203E-06

	Rachicladosporium pini	0.001	0.213	2.630E-06
	Phialophora sp DF36	0.001	0.276	2.710E-06
	Aureobasidium pullulans	0.001	0.228	4.735E-06
	Teratosphaeria capensis	0.001	0.112	1.459E-06
	Mortierella elongata	0.001	0.175	2.168E-06
	Cryptococcus aff amylolyticus AS 22398	0.001	0.117	1.541E-06
	Rhynchostoma proteae	0.001	0.132	1.706E-06
	Macroconia leptosphaeriae	0.001	0.199	6.203E-06
	Pringsheimia euphorbiae	0.001	0.088	1.471E-06
	Phaeosphaeria avenaria	0.001	0.121	2.483E-06
	Phaeosphaeria nodorum	0.001	0.071	1.228E-06
	Porosphaerella cordanophora	0.004	0.144	2.804E-06
	Rhizophlyctis rosea	0.002	0.101	2.091E-06
	Fusarium solani	0.006	0.072	1.317E-06
	Cladosporium cladosporioides	0.005	0.072	1.174E-06
	Ascomycota sp AR 2010	0.001	0.062	1.279E-06
	Lyophyllum sp Cultivar Jpn	0.001	0.042	1.410E-06
	Cryptococcus skinneri	0.003	0.052	1.454E-06
	Cylindrocladiella camelliae	0.001	0.034	1.049E-06
	Microscypha ellisii	0.003	0.043	1.188E-06
	Arnium macrotheca	0.001	0.061	2.896E-06
2009	Strumella coryneoidea	0.001	0.039	2.198E-06
	Articulospora proliferata	0.015	0.051	1.676E-06
	Mortierella cystojenkinii	0.019	0.035	1.494E-06
	Cryptococcus terricola	0.021	0.040	1.495E-06
	Phialemonium curvatum	0.005	0.047	2.241E-06
	Ganoderma applanatum	0.001	0.016	8.468E-07
	Passalora zambiae	0.001	0.019	1.128E-06
	Powellomyces hirtus	0.01	0.023	1.722E-06
	Spizellomyces plurigibbosus	0.003	0.018	1.507E-06
	Cryptococcus sp MD76 1BY	0.039	0.019	1.044E-06
	Xanthoria parietina	0.021	0.016	1.172E-06
	Inocybe ochroalba	0.037	0.023	2.612E-06
	Talaromyces thermophilus	0.006	0.010	1.034E-06
	Hypholoma fasciculare	0.047	0.015	1.102E-06
	Bacidina chloroticula	0.047	0.014	1.108E-06
	Archaeospora sp isa33	0.018	0.012	1.932E-06
	Candida morakotiae	0.025	0.007	9.670E-07
	Dactylaria higginsii	0.023	0.006	8.865E-07
	Myxotrichum carminoparum	0.035	0.007	9.210E-07
	Lophodermium pini excelsae	0.045	0.017	2.015E-06
	Mycena olida	0.047	0.018	4.691E-06
	Pochonia suchlasporia	0.047	0.009	1.784E-06

	Lecythophora mutabilis	0.001	0.119	2.355E-06
	Helicodendron websteri	0.005	0.036	2.386E-06
2011	Leotiomycetes sp NK264	0.003	0.029	1.618E-06
	Cenococcum geophilum	0.013	0.015	1.410E-06
	Mycena corynephora	0.037	0.011	1.245E-06

(1) Rel. abund.: Relative read abundance

(2) SE on Rel. abund.: Standard deviation on relative read abundance

Supporting Information Table S4.2 Pearson's correlation coefficients (R^2) between measured soil parameters and fungal diversity indices. Fungal diversity indices were calculated in R based on OTU abundances. OTUs were generated in Mothur based on a 97% sequence similarity cut-off. Significant correlations at the 95% confidence level are indicated in bold.

Environmental parameter	2009 dataset			2011 dataset		
	Rich. (5)	Pielou evenn. (6)	Inv. Simp. (7)	Rich. (5)	Pielou evenn. (6)	Inv. Simp. (7)
OM (1)	0.41	0.30	-0.41	0.41	0.27	-0.24
CEC (2)	-0.04	-0.17	0.16	0.04	0.02	0.03
Conduct. (3)	**0.47**	**0.47**	-0.37	0.15	0.19	-0.20
pH KCl	0.29	0.37	-0.16	-0.07	0.08	-0.06
pH H_2O	0.36	0.33	-0.16	-0.03	0.07	0.02
Soil Zn (4)	0.24	0.22	-0.19	0.31	0.29	-0.27
Soil Cd (4)	0.24	0.27	-0.23	0.31	0.39	-0.33
Soil Fe (4)	-0.12	-0.09	0.07	-0.31	**-0.55**	**0.61**
Soil Mg (4)	0.33	**0.46**	-0.31	0.07	0.03	-0.05
Soil Mn (4)	0.09	0.15	-0.18	0.01	-0.03	-0.08
Soil K (4)	0.16	0.25	-0.29	-0.04	-0.17	0.09
Soil Cu (4)	0.28	0.14	-0.17	0.05	0.12	-0.17
Needle Zn (4)	0.20	0.25	-0.32	0.37	0.29	-0.28
Needle Cd (4)	0.18	0.07	-0.16	0.24	0.15	-0.18
Needle Fe (4)	0.01	0.07	-0.06	-0.03	-0.08	0.10
Needle Mg (4)	0.20	0.25	-0.29	0.38	0.38	-0.32
Needle Mn (4)	-0.03	-0.12	0.05	-0.07	-0.08	-0.01
Needle K (4)	0.34	0.39	**-0.47**	-0.07	-0.04	-0.11
Needle Cu (4)	0.19	0.30	-0.25	-0.18	-0.24	0.18
Needle Ca (4)	-0.14	0.08	0.11	0.38	0.36	-0.31

(1) OM: Soil organic matter (%)

(2) CEC: Cation exchange capacity (meq 100 g^{-1})

(3) Conduct.: Conductivity (µS cm^{-1})

(4) Measured in µg g^{-1} dry weight

(5) Rich.: OTU richness

(6) Pielou evenn.: Pielou evenness index

(7) Inv. Simp.: Inverse Simpson index

Supporting Information Table S4.3 Primer design used in the current study to construct "fusion primers" for 454 amplicon pyrosequencing.

Amplicon pool	Sample [1]	Forward primer			Reverse primer		
		454 adapter [2]	MID [3]	Fungal ITS primer [4]	454 adapter [2]	MID [3]	Fungal ITS primer [4]
Pool 1	LM_5.1_2009	A	MID6	ITS86F	B	MID6	ITS4
Pool 1	LM_5.2_2009	A	MID6	ITS86F	B	MID7	ITS4
Pool 1	LM_5.4_2009	A	MID7	ITS86F	B	MID6	ITS4
Pool 1	LM_34.1_2009	A	MID7	ITS86F	B	MID7	ITS4
Pool 1	LM_6.1_2009	A	MID6	ITS86F	B	MID8	ITS4
Pool 1	LM_27.5_2009	A	MID8	ITS86F	B	MID6	ITS4
Pool 1	LM_32.2_2009	A	MID7	ITS86F	B	MID8	ITS4
Pool 1	LM_12.1_2009	A	MID8	ITS86F	B	MID7	ITS4
Pool 1	LM_32.1_2009	A	MID8	ITS86F	B	MID8	ITS4
Pool 1	LM_21.5_2009	A	MID6	ITS86F	B	MID9	ITS4
Pool 1	LM_32.3_2009	A	MID9	ITS86F	B	MID6	ITS4
Pool 1	LM_12.4_2009	A	MID7	ITS86F	B	MID9	ITS4
Pool 1	LM_12.5_2009	A	MID9	ITS86F	B	MID7	ITS4
Pool 1	LM_12.2_2009	A	MID8	ITS86F	B	MID9	ITS4
Pool 1	LM_12.3_2009	A	MID9	ITS86F	B	MID8	ITS4
Pool 1	LM_21.1_2009	A	MID9	ITS86F	B	MID9	ITS4
Pool 1	LM_21.3_2009	A	MID6	ITS86F	B	MID10	ITS4
Pool 1	LM_31.3_2009	A	MID10	ITS86F	B	MID6	ITS4
Pool 1	LM_31.5_2009	A	MID7	ITS86F	B	MID10	ITS4
Pool 1	LM_21.2_2009	A	MID10	ITS86F	B	MID7	ITS4
Pool 1	LM_4.1_2009	A	MID8	ITS86F	B	MID10	ITS4
Pool 1	LM_4.2_2009	A	MID10	ITS86F	B	MID8	ITS4
Pool 1	LM_11.1_2009	A	MID9	ITS86F	B	MID10	ITS4
Pool 1	LM_11.2_209	A	MID10	ITS86F	B	MID9	ITS4
Pool 1	LM_31.1_2009	A	MID10	ITS86F	B	MID10	ITS4
Pool 1	LM_31.2_2009	A	MID6	ITS86F	B	MID11	ITS4
Pool 1	LM_27.3_2009	A	MID11	ITS86F	B	MID6	ITS4

Pool 1	LM_27.4_2009	A	MID7	ITS86F	B	MID11	ITS4
Pool 1	LM_5.3_2009	A	MID11	ITS86F	B	MID7	ITS4
Pool 1	LM_5.5_2009	A	MID8	ITS86F	B	MID11	ITS4
Pool 1	LM_30.3_2009	A	MID11	ITS86F	B	MID8	ITS4
Pool 1	LM_31.4_2009	A	MID9	ITS86F	B	MID11	ITS4
Pool 1	LM_11.3_2009	A	MID11	ITS86F	B	MID9	ITS4
Pool 1	LM_11.5_2009	A	MID10	ITS86F	B	MID11	ITS4
Pool 1	LM_4.3_2009	A	MID11	ITS86F	B	MID10	ITS4
Pool 1	LM_32.4_2009	A	MID11	ITS86F	B	MID11	ITS4
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Pool 1	LM_4.4_2009	A	MID12	ITS86F	B	MID6	ITS4
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Pool 1	LM_27.1_2009	A	MID12	ITS86F	B	MID8	ITS4
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Pool 4	LM_6.3_2011	A	MID13	ITS86F	B	MID8	ITS4

(1) LM: Lommel-Maatheide

(2) A, B: Pyrosequencing-specific A- and B- adapter sequences

(3) MID: Multiplex-identifier

(4) ITS: Internal transcribed spacer

Chapter 5: **Comparison of ectomycorrhizal communities of pioneer Scots pine forests (*Pinus sylvestris* L.) on a metal-polluted and non-polluted site**

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SUMMARY

The impact of soil metal pollution on plant communities has been studied extensively in the past. However, very little is known about the fungal species that are associated with these plant communities on metal-polluted soils. To characterize the fungal communities that are associated with plants thriving on metal-polluted soils, the current study aimed to identify the ectomycorrhizal fungi associated with *Pinus sylvestris* (L.) using 454 pyrosequencing on a metal-polluted and a non-polluted site in the Campine region in Belgium. The ectomycorrhizal communities at both study sites were shown to consist mainly of the same fungal species, but a consistent shift in the relative abundances of these species was observed, whereas no differences in fungal diversity were found. In metal polluted soil, roots tips of young pines were initially largely colonised by stress-tolerant Ascomycota that were mostly replaced by metal-tolerant Basidiomycota within 2 years.

Keywords: metal pollution, ectomycorrhiza, pioneer community, metabarcoding, 454 pyrosequencing

CHAPTER 5

INTRODUCTION

Worldwide, vast areas have become contaminated with high concentrations of metals due to pyrometallurgical industry and mining activities. High concentrations of metal ions in soils have been found to have detrimental effects on fungal, plant and bacterial populations. Ernst (1990) for example, observed a decrease in floristic diversity along a metal pollution gradient towards metal smelters. Hence, plant communities thriving on metalliferous soils often consist of highly adapted plant species, some of which may even be endemic to a restricted number of metalliferous sites. For example, in La Calamine in Belgium, a plant community was described in 1931 growing in areas where metal-rich ores surface (Schwickerath, 1944). This so-called *Violetum calaminariae* association harbours plant species that are typical for metal-enriched environments such as *Silene vulgaris* and *Armeria maritima*. Other representatives, for example *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) are typically metal-accumulating plants that have potential to clean up metal-contaminated soils (Reeves and Baker, 2000). Nevertheless, relatively few tree species can survive on metalliferous soils with high metal loads. Metallophyte vegetation on naturally metalliferous soils in western and northern Europe are slowly invaded by ectomycorrhizal *Betula* sp., *Salix* sp., *Pinus* sp. and by arbuscular mycorrhizal *Acer* sp. (Colpaert and Vandenkoornhuyse, 2001). In many sites, tree growth is seriously affected by metal stress and metals in leaves or needles can reach toxic concentrations.

In contrast to our vast and long-standing knowledge on plant communities that thrive on metal-polluted soils, much less is known about the soil-born microorganisms that are associated with these pseudo-(metallophytes). An important reason for this lack of knowledge is the obvious practical difficulty in assessing belowground microbial communities and populations. However, developments in molecular biology now provide us with some tools that allow for the detection and accurate identification of belowground fungi in roots and soil. Ectomycorrhizal (ECM) fungi can be found

everywhere potential host trees are colonising metal-contaminated soils. Knowledge on the fungal communities from these metal-polluted soils is not only important for our general understanding of the functioning of natural ecosystems in stressful environments, this knowledge may also help us in developing strategies to remediate polluted areas (Turnau *et al*., 2008; Solíz-Domínguez *et al*., 2011). Furthermore, a thorough understanding of the impact of metal pollution on fungal diversity and fungal community dynamics is necessary to understand the faith of ECM fungi and plants after their introduction to metal-polluted environments. Therefore, the current study aimed to characterize and compare the ectomycorrhizal community associated with pioneer pine forests (*Pinus sylvestris* L.) growing on a metal-polluted and a non-polluted site in the Campine region in Belgium using 454 pyrosequencing (Margulies *et al*., 2005) and to assess the effects of metal pollution on fungal biodiversity and community composition.

MATERIALS AND METHODS

Study sites and sampling

Fungal communities were sampled in two pioneer Scots pine forests (*Pinus sylvestris* L.) growing on sandy soils in the Campine region in Belgium. The first fungal community was sampled in a metal-polluted site in Lommel-Maatheide (LM: 51° 14' 10" N; 5° 15' 50" E). $Ca(NO_3)_2$ extractable soil Zn and Cd concentrations in this site range from 1 to 197 µg g^{-1} dry weight (d. wt) Zn and <0.1 to 1.56 µg g^{-1} d. wt Cd. The second site is situated in Hechtel-Eksel (HE: 51° 7' 33'' N, 5° 22' 22'' E). This site is hardly polluted by pyrometallurgical activities and is used as a reference site in this study. $Ca(NO_3)_2$ extractable soil Zn concentrations in this site range from 3 to 13 µg g^{-1} d. wt and Cd concentrations were below the detection limit of 0.1 µg g^{-1} d. wt. Mosses, lichens, and a few grass species form the accompanying primary pioneer vegetation at both sites. In HE, also a few *Calluna vulgaris* (L.) Hull. shrubs occur on the study site. The soil at both study sites is a

dry sandy soil without a litter layer, poor in organic matter and slightly acidic. The average soil organic matter (OM) content in HE was 0.7% ± 0.1 % (standard error: SE) and the average pH was 4.5 ± 0.02 (SE). In LM, the average OM content was 0.8% ± 0.1 (SE) and the average pH was 4.6 ± 0.07 (SE). More detailed information on measured environmental variables in LM and HE can be found in Fig. S5.1. The region has an average annual rainfall of 800 mm per square meter and the average annual temperature is 10°C (Royal Meteorological Institute, Ukkel, Belgium).

The pioneer forest in LM is growing on a site where most polluted topsoil was removed. This disturbance introduced heterogeneity in the newly exposed soil and resulted in large differences in metal concentrations over short distances. To estimate the overall metal exposure of individual pine trees in LM, soil samples and last-year pine needles from 22 one-year old trees were collected for Zn and Cd analysis. Finally, a subset of 10 trees containing between 200 and 400 µg Zn g^{-1} d. wt in needles were selected for the fungal community analysis. Needles of these trees were not chlorotic, though the critical leaf tissue concentrations affecting growth in most plants ranges from 200 to 300 µg Zn g^{-1} d. wt (Påhlsson, 1989).

These 10 trees were compared to 10 one-year old trees from HE, containing from 20 to 90 µg Zn g^{-1} d. wt in needles (Fig. S5.1). Selected pine trees were at least 20 m apart from each other.

For the characterization of fungal communities, soil and root tip samples were collected at both sites in November 2009 and in November 2011. Soil samples were collected with a soil corer with a diameter of 1 cm at a depth of 0 cm to 20 cm. For each tree, five samples were collected according to the cardinal directions at different distances from the stem. These included samples collected immediately next to stems and at a distance of 25 cm, 50 cm, 75 cm and 100 cm from stems (Fig. S4.2). Samples were pooled for each of these distances and mixed, resulting in a total of five pooled samples for each tree with each sample representing a certain distance from the stem. Additionally, roots from selected pine trees were collected in both sampling years. Two long roots were unearthed per tree from the

stem base up to the growth tip of the roots. In the lab, roots were washed with tap water to remove most adhering soil. For each tree, all visible short root tips were collected from the entire length of the long roots, pooled and homogenized. Samples for physical and chemical soil characterization were collected next to each tree with a soil corer with a diameter of 10 cm at a depth of 0 to 20 cm. Soil samples for fungal community analysis were homogenized, sieved with a 2 mm sieve to remove small stones, roots, and other debris, and stored at -80°C. Samples for soil characterization were dried at ambient temperature for two weeks before physical and chemical analyses were conducted. Collected pine needles were dried for two weeks at 60 °C before being analysed for their metal content.

Soil physical and chemical characterization

pH was measured in both a water extract (10 g soil extracted with 25 ml distilled water) and a KCl extract (10 g soil extracted with 25 ml 1M KCl) of soil samples. Conductivity was measured on the water extracts. Soil organic matter content (OM) was analysed with the Walkley and Black method (Walkley and Black, 1934). Cation exchange capacity (CEC) was measured according to Rhoades' method (Rhoades, 1982). Exchangeable cations were extracted using 0.1 M $Ca(NO_3)_2$ (25 ml for 5 g soil). Dried pine needles were digested with nitric acid (65%) and hydrochloric acid (37%) at 120 °C. Concentrations of zinc (Zn), cadmium (Cd), iron (Fe), magnesium (Mg), potassium (K), copper (Cu) and manganese (Mn) were measured with inductively-coupled plasma - optical emission spectroscopy (ICP-OES) in samples obtained from calcium nitrate extraction and pine needle digestion. Calcium (Ca) concentrations were measured in pine needle digests.

Characterization of the fungal communities

To characterize the fungal communities in soil and root tip samples, DNA was extracted using the UltraClean soil DNA isolation kit (MoBio, Carlsbad, CA, USA) from approximately 250 mg of soil or root tips according to the manufacturer's instructions. DNA was extracted in quadruplicate from each

sample and replicated extracts were pooled per sample prior to PCR amplification using the ITS86F forward primer (Vancov and Keen, 2009) and ITS4 reverse primer (Gardes and Bruns, 1993). This primer pair was shown to efficiently amplify the fungal internal transcribed spacer 2 (ITS2) region and characterize fungal communities using 454 pyrosequencing (Op De Beeck *et al*., 2014). "Fusion" primers, required for the 454 process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-A sequencing containing the Roche 454 pyrosequencing adapters and a 10-bp multiplex identifier (MID) barcode in between each adapter and primer sequence (Table S5.1). DNA was amplified using a Techne TC-5000 thermocycler (Bibby Scientific Limited, Staffordshire, UK) under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C during 1 minute. A final extension phase was performed at 72°C during 10 minutes. Reactions were carried out in 25 µl reaction volumes using the FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). Each reaction contained 2.75 µl FastStart 10x reaction buffer, 1.8 mM MgCl, 0.2 mM dNTP mix, 0.4 µM of each primer, 1.25 U FastStart HiFi polymerase and 5 ng template DNA (as measured by a Nanodrop spectrophotometer). Amplified DNA was cleared from PCR primers and primer dimers using the Agencourt AMPure XP system according to the manufacturer's protocol (Beckman Coulter, Brea, CA, USA). Next, purified DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany) and pooled into five equimolar amplicon libraries (10^7 molecules per µl) of 40 to 50 samples. Each of the five resulting amplicon pools were sequenced on one fourth of a pico titer plate on a Roche Genome Sequencer FLX system using Titanium chemistry (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

The five Standard Flowgram Format (SFF) files that resulted from the interpreted flowgrams were submitted to the NCBI Sequence Read Archive

under accession number SRP037968 (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra). The information in the separate SFF files was combined in a single quality and a fasta file using a custom Python script. Further analyses were performed in Mothur 1.31.2 (Schloss *et al*., 2009). Quality trimming in Mothur was used to remove reads shorter than 200 bases, reads longer than 600 bases, reads containing homopolymers longer than 8 bases and reads containing ambiguous bases. Reads were trimmed when the average Phred quality score dropped below 35 over a window of 50 bases (Schloss *et al*., 2009). Next, sequences were compared to each other and duplicate sequences were replaced by a single sequence, while archiving the abundance data of the unique sequences. Unique reads were checked for chimeric sequences with the Uchime software implemented in Mothur and chimeric sequences were removed from the dataset. Unique reads were subsequently aligned with the pairwise alignment tool in Mothur, using default settings. Finally, species-level operational taxonomic units (OTUs) were defined based on a 97% sequence similarity cut-off, which is generally within the range of intraspecific ITS sequence similarity (Blaalid *et al*., 2013). In order to further remove potential sequencing errors from the dataset, singletons were removed (Tedersoo *et al*., 2010). Subsequently, rarefaction curves at the level of individual trees were constructed and Good's coverage was calculated with Mothur for each tree. OTU richness, Pielou evenness scores and inverse Simpson indices were calculated for each tree in R 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). Since rarefication of the number of reads per sample did not result in any major changes in the fungal diversity patterns or community structures, we kept all observed reads in samples. BLAST searches for a representative sequence of each OTU (as determined by Mothur) were performed using PlutoF v2.0 (Abarenkov *et al*., 2010). Reads were blasted against the UNITE (Kõljalg *et al*., 2005) and INSD (Nakamura *et al*., 2013) databases. Resulting HTML files were combined with the abundance data obtained in Mothur using a custom Python script. This script also acquired the names of species and/or genera

that resemble Latin binomials with the highest BLAST score, avoiding unidentified OTUs in the databases to be seen as best BLAST hits. Unidentified OTUs were indicated as "not applicable (NA)".

Statistical analysis

Statistical analyses were carried out in R 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). Normal distributions of the residuals of models were checked with the Shapiro-Wilk test, while the homoscedasticity of variances were analysed using either Bartlett's or the Fligner-Killeen test. Depending on the distribution of the estimated parameters, either ANOVA or the Kruskal-Wallis rank sum test was used to check for significant differences in variances of parameters. Two-by-two comparisons were conducted using either Tukey's Honest Significant Differences tests or Pairwise Wilcoxon Rank Sum tests. Correlations between diversity parameters and measured environmental parameters were calculated based on Pearson's product moment correlation coefficient (R^2). Non-Metric Multi-dimensional Scaling (NMDS) was conducted using the metaMDS() function of the vegan package (version 2.0-10; Oksanen *et al.*, 2013) in R. Canonical Correspondence Analysis (CCA) model building was based on the cca() function of the vegan package and CCA analysis was based on the decorana() and envfit() functions of the vegan package. Mantel tests were conducted using the mantel() function of the vegan package in R. PERMANOVA analyses were conducted in R using the adonis() function of the vegan package. Distance matrices for community data were based on Bray-Curtis distances using read abundances.

RESULTS

The 454 amplicon pyrosequencing runs resulted in a total of 460,354 raw reads across soil and root tip samples. After quality trimming and assigning reads to samples, 424,296 high-quality reads remained in the dataset.
Calculation of Good's coverage scores indicated that in 2009, 95% ± 0.9% (SE) and in 2011 96% ± 0.2% (SE) of all fungal OTUs present in soil samples in LM were detected. For the fungal communities sampled in HE, it was calculated that in 2009, 96% ± 0.5% (SE) of the present OTUs were sampled and 96% ± 0.8% (SE) in 2011. Good's coverage scores for root tips collected from LM trees in 2009 and 2011 were 95% ± 0.6% (SE) and 96% ± 0.9 % (SE) respectively. Good's coverage scores for root tips collected from HE trees in 2009 and 2011 respectively were 96% ± 0.2% (SE) and 97% ± 0.1% (SE). These results indicate that the used sampling depth was sufficient to identify most fungal species present in both soil and root tip samples.

Comparison of environmental parameters

Environmental parameters were compared between the metal-polluted site LM and the control site HE using a two-way ANOVA. Environmental parameters were used as dependent variables and study site and sampling year were used as fixed, independent variables. Significant differences in almost all environmental variables were found between study sites. For a few environmental variables (Needle Mg, Needle Mn, Needle K and Needle Cu) differences between sampling years were observed as well. More detailed information about differences in environmental variables between LM and HE are presented in Fig. S5.1.

Comparison of fungal diversity parameters

Fungal diversity parameters (OTU richness, Pielou evenness and inverse Simpson index) were compared among study sites (LM and HE) and sampling years (2009 and 2011) using two-way ANOVA. Fungal diversity

parameters were used as dependent variables and spatio-temporal variables as fixed, independent variables. No significant differences between study sites or sampling years were observed ($p = 0.32$ for sampling year and $p = 0.46$ for study site; Fig. 1).

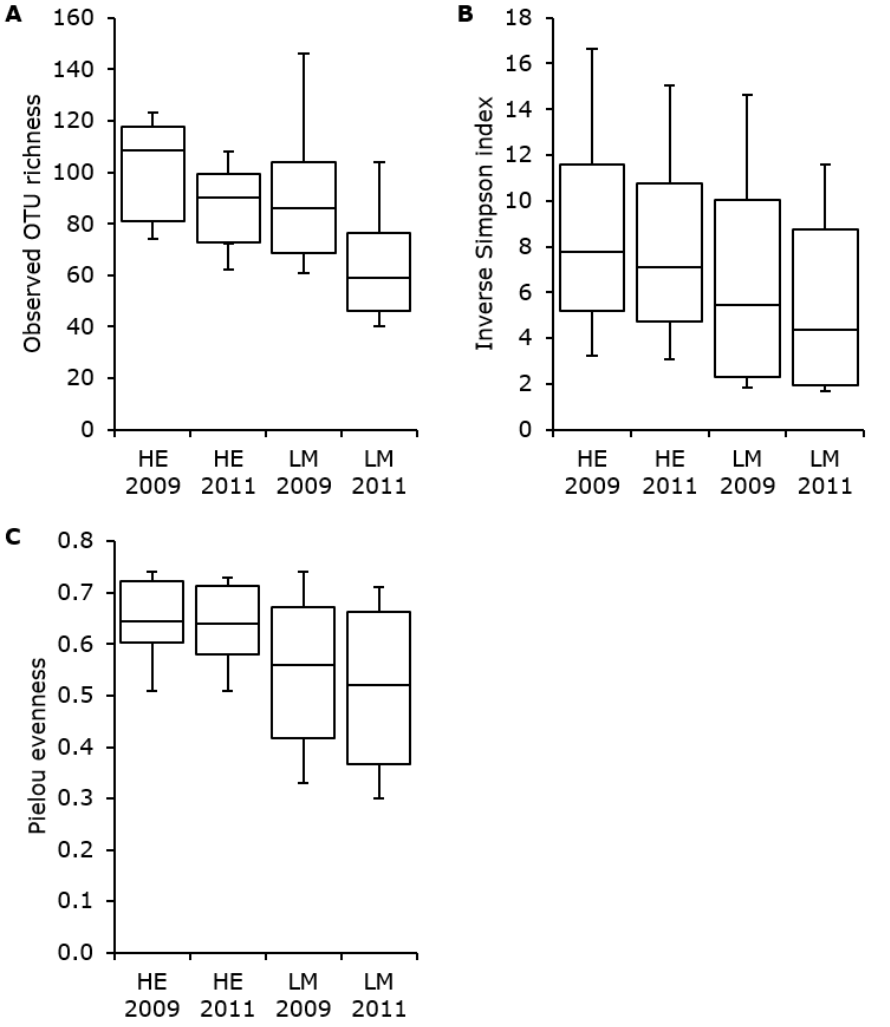


Figure 5.1 Comparison of fungal diversity measures between a metal-polluted site in Lommel-Maatheide (LM) and a control site in Hechtel-Eksel (HE). A. OTU richness. B. Inverse Simpson index. C. Pielou evenness. No significant differences at the 95% confidence level were observed.

Comparison of fungal community compositions

Fungal community compositions were compared between sampling years and study sites using two-way PERMANOVA. A community distance matrix (based on Bray-Curtis distances) was used as dependent variable and spatial and temporal scales were used as independent, fixed effects. Significant differences in the fungal community compositions were observed both between study sites and sampling years ($p < 0.01$).

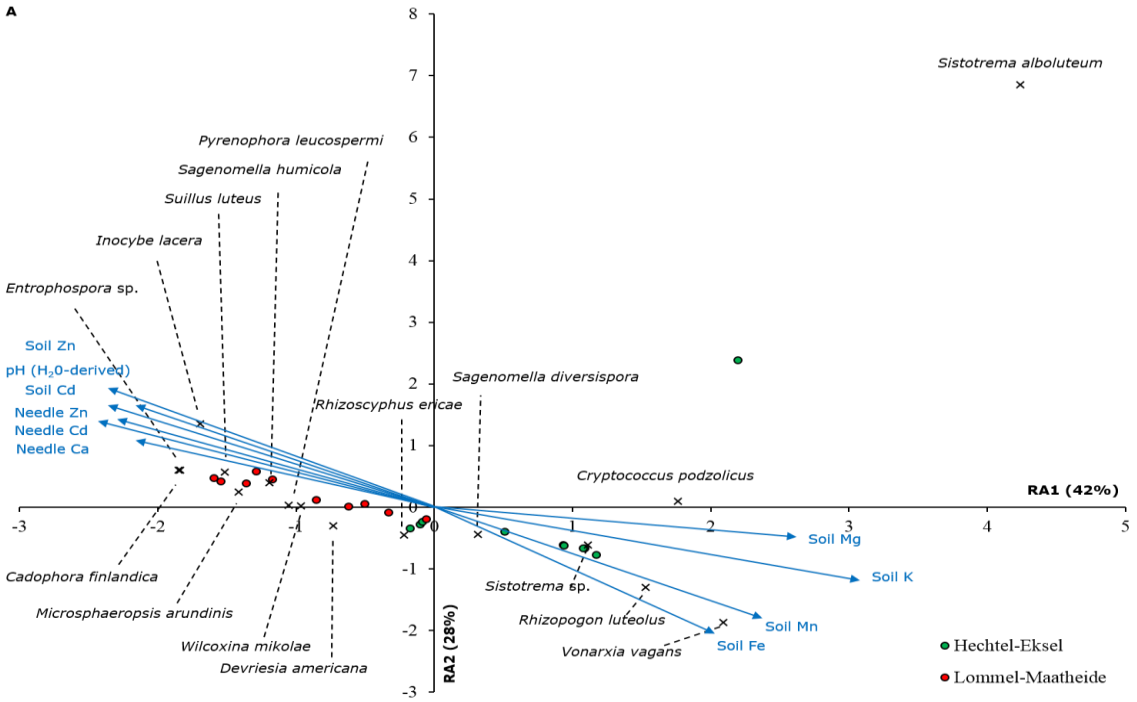
Subsequently, a more detailed comparison was made between the fungal communities based on the presence or absence of individual OTUs. Presence-absence of OTUs in specific combinations of sampling years and study sites are presented as a Venn diagram in Fig. S5.2. From this Venn diagram it is clear that of a total of 719 OTUs identified in this study, a large proportion (48.5%) was uniquely identified in single year-site combinations. Another relatively large proportion of OTUs (18.6%) were shared by all sampling year and study site combinations. Interestingly, all OTUs with an average relative abundance > 1% were shared by all sampling years and study sites.

Effects of environmental parameters on fungal diversity

Correlations between measured environmental parameters and fungal diversity measures were calculated. A number of environmental factors were negatively correlated with OTU richness in the 2011 dataset ($p < 0.05$): CEC, pH (H_2O-derived), needle Zn, needle Cd and needle Cu concentrations. Furthermore, a positive correlation ($p < 0.05$) between OTU richness and soil Fe, Mg and K was observed for the 2011 dataset. Whereas CEC and needle Cu concentrations were also negatively correlated with Pielou evenness indices ($p < 0.05$), soil Mg and K concentrations showed a positive correlation with Pielou evenness indices in the 2011 dataset ($p < 0.05$). In the 2009 dataset, however, none of these correlations were significant. A complete list of Pearson's correlation coefficients between environmental parameters and fungal diversity indices can be found in Table S5.2.

Effects of environmental parameters on fungal community composition

Canonical correspondence analyses (CCA) comparing fungal community dissimilarities (based on Bray-Curtis distances) show that both in 2009 and 2011 the LM and HE communities differ from each other (Fig. 2). Many of the measured environmental factors were found to significantly correlate with the fungal community compositions in both sampling years (Permutation tests; all $p < 0.01$; Fig. 2). Two main gradients were identified, consisting of different inter-correlating factors. A first gradient mainly represented the degree of metal pollution and consisted of needle Zn, Cd and Ca concentrations and soil Zn and Cd concentrations. The second gradient consisted of soil Fe, Mn, Mg and K concentrations. For the 2009 analysis, RA1 represented 42% of the total variation and RA2 represented 28%. For the 2011 analysis, RA1 and RA2 represented 34% and 27% of the total variation, respectively (Fig. 2). On the CCA plots, only the top ten most abundant OTUs for each site were shown to prevent crowding of the graphs. A number of OTUs were consistently more abundant in LM than in HE. These OTUs corresponded to *Suillus luteus*, *Sagenomella humicola*, *Cadophora finlandica*, *Wilcoxina mikolae* and *Inocybe lacera*. OTUs that were consistently more abundant in HE samples corresponded to *Rhizopogon luteolus*, *Cryptococcus podzolicus*, *Rhizoscyphus ericae* and *Vonarxia vagans*. The OTU that was the most abundant OTU in most samples corresponded to *Sistotrema* sp. The relative abundances of the top ten most abundant OTUs per site and sampling year are displayed in Fig. 3. A complete list of all OTUs identified in the current study, their average relative abundances across the entire study, BLAST scores and corresponding E-values can be found in Appendix C.


A
Sistotrema alboluteum
Pyrenophora leucospermi
Sagenomella humicola
Suillus luteus
Inocybe lacera
Entrophospora sp.
Soil Zn
pH (H20-derived)
Soil Cd
Needle Zn
Needle Cd
Needle Ca
Sagenomella diversispora
Rhizoscyphus ericae
Cryptococcus podzolicus
RA1 (42%)
Soil Mg
Soil K
Soil Mn
Soil Fe
Sistotrema sp.
Rhizopogon luteolus
Vonarxia vagans
Cadophora finlandica
Microsphaeropsis arundinis
Wilcoxina mikolae
Devriesia americana
RA2 (28%)
Hechtel-Eksel
Lommel-Maatheide

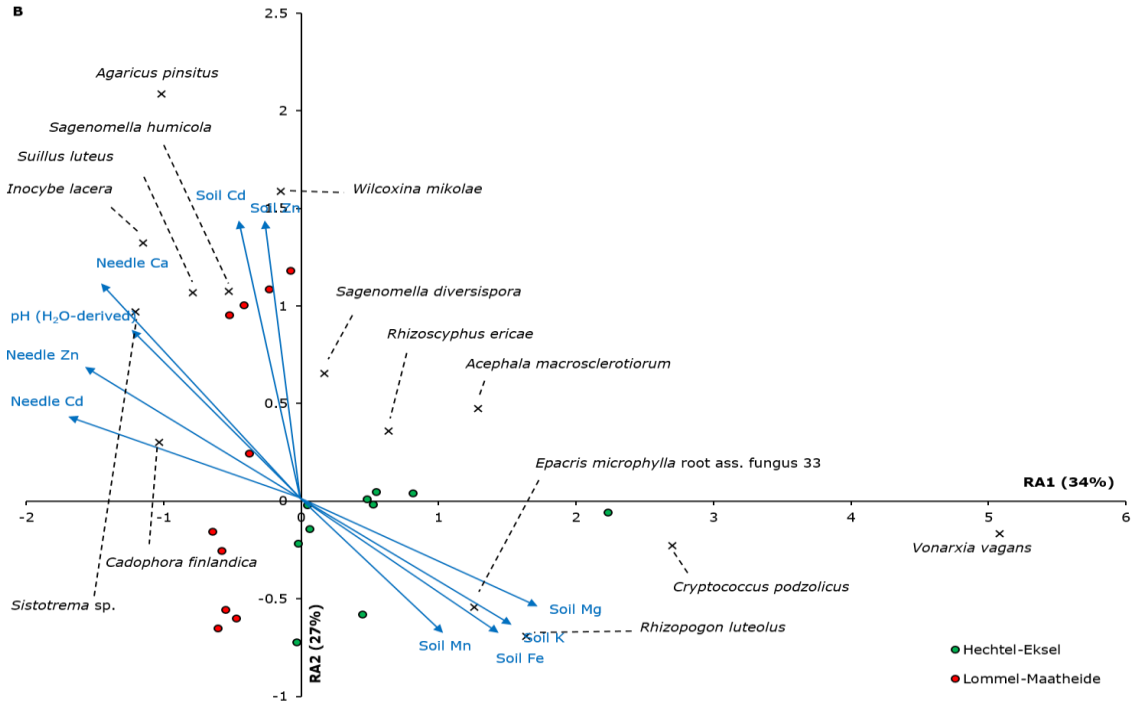
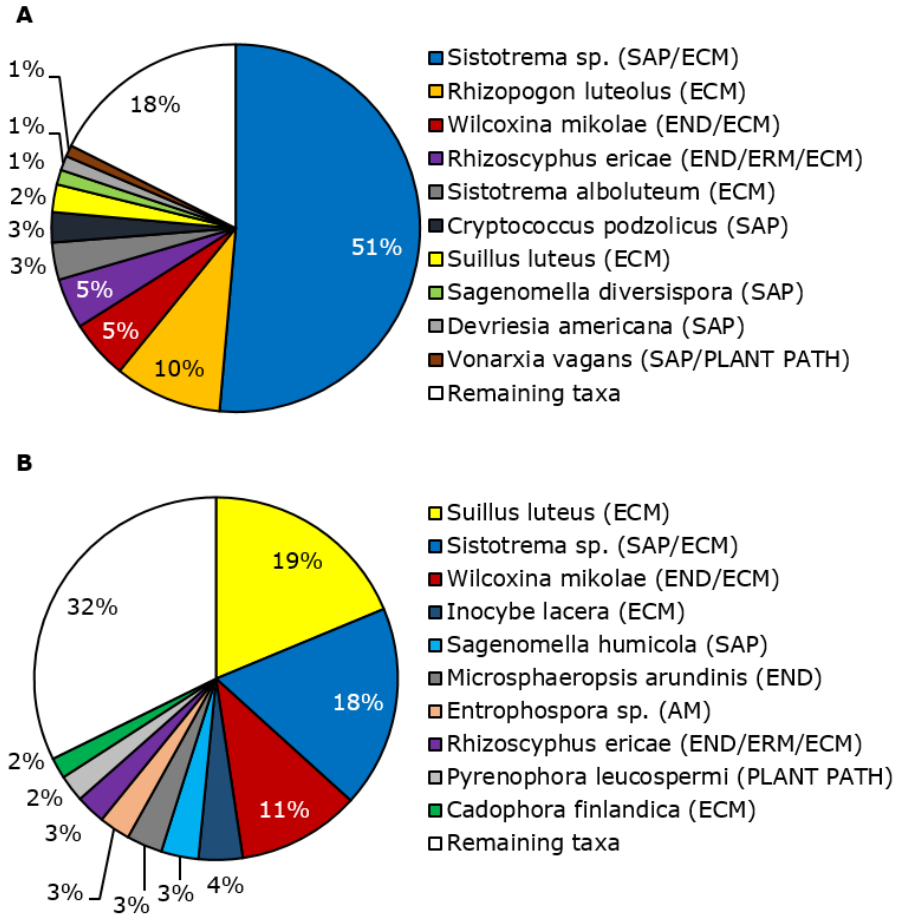


Figure 5.2 Results of Canonical Correspondence Analysis (CCA) correlating fungal community structure (based on Bray-Curtis dissimilarities) with measured environmental parameters for a metal-polluted site in Lommel-Maatheide (LM) and a control site in Hechtel-Eksel (HE) for two sampling years (2009 and 2011). A. CCA plot comparing LM and HE samples collected in 2009. B. CCA plot comparing LM and HE samples collected in 2011.


A
1%
18%
1%
1%
2%
3%
3%
51%
5%
5%
10%
Sistotrema sp. (SAP/ECM)
Rhizopogon luteolus (ECM)
Wilcoxina mikolae (END/ECM)
Rhizoscyphus ericae (END/ERM/ECM)
Sistotrema alboluteum (ECM)
Cryptococcus podzolicus (SAP)
Suillus luteus (ECM)
Sagenomella diversispora (SAP)
Devriesia americana (SAP)
Vonarxia vagans (SAP/PLANT PATH)
Remaining taxa
B
19%
32%
18%
2%
2%
3%
11%
3%
3%
3%
4%
Suillus luteus (ECM)
Sistotrema sp. (SAP/ECM)
Wilcoxina mikolae (END/ECM)
Inocybe lacera (ECM)
Sagenomella humicola (SAP)
Microsphaeropsis arundinis (END)
Entrophospora sp. (AM)
Rhizoscyphus ericae (END/ERM/ECM)
Pyrenophora leucospermi (PLANT PATH)
Cadophora finlandica (ECM)
Remaining taxa

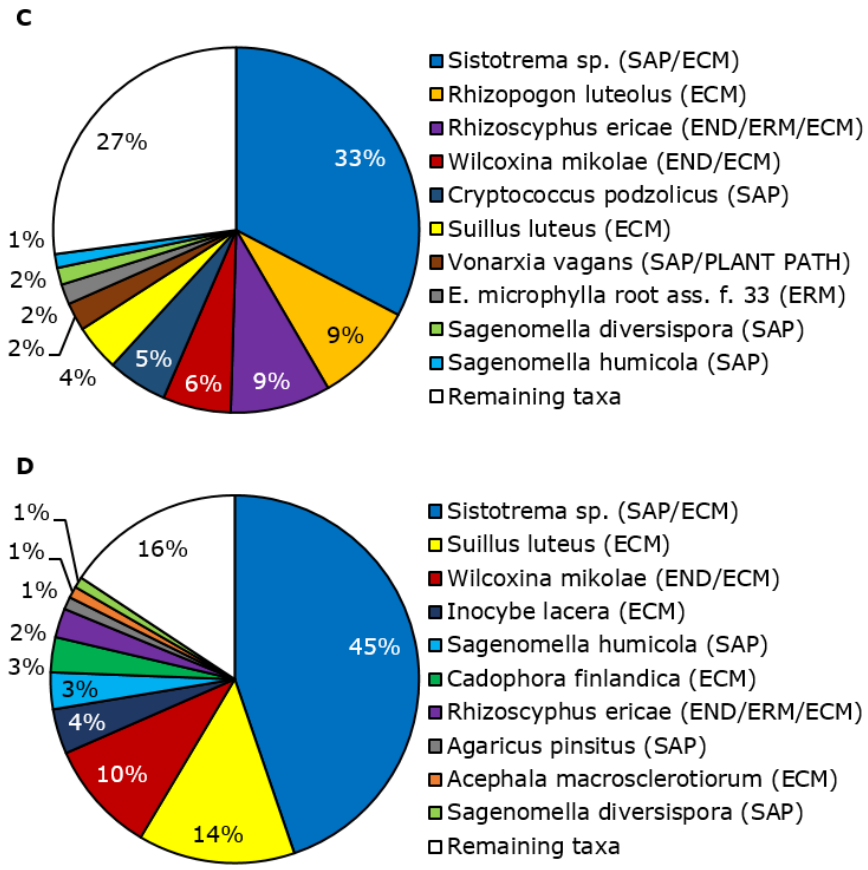
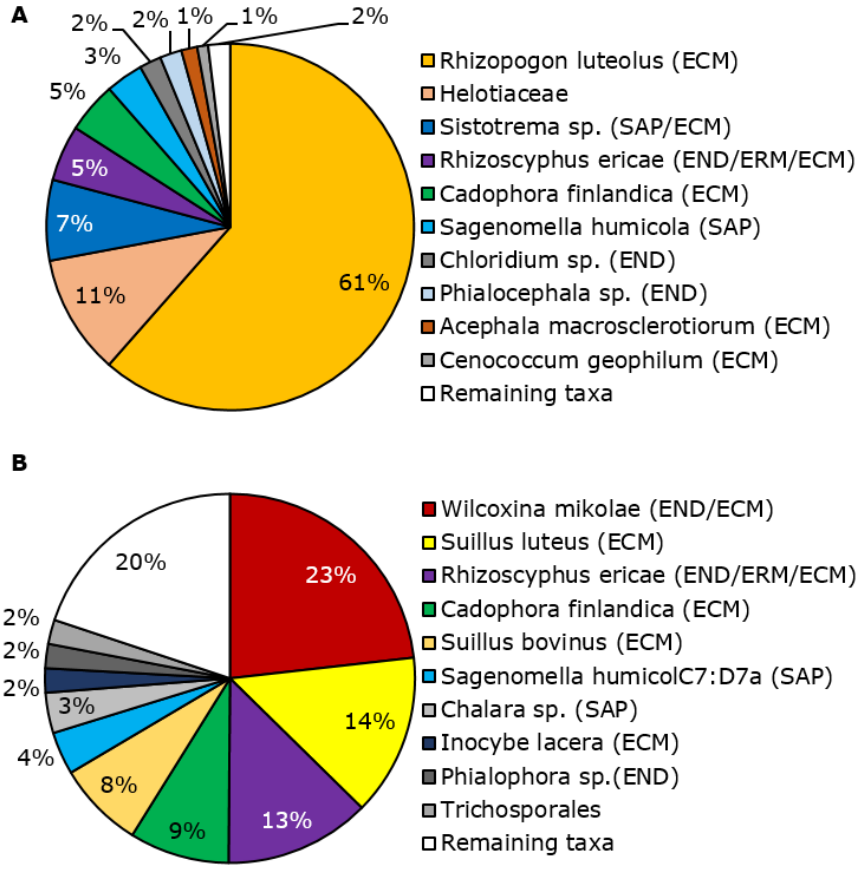


Figure 5.3 Pie charts displaying the top ten most abundant fungal species identified in soil samples using 454 amplicon pyrosequencing. A. Hechtel-Eksel 2009. B. Lommel-Maatheide 2009. C. Hechtel-Eksel 2011. D. Lommel-Maatheide 2011. Species not occurring in the list of top ten most abundant species are grouped together as "Remaining taxa". Lifestyles of fungal species are given between brackets: ECM (ectomycorrhizal), ERM (ericoid mycorrhizal), AM (arbuscular mycorrhizal), SAP (saprotrophic), PATH (pathogenic), END (endophytic).

Fungal species identified from root tip samples

A number of the OTUs that were identified in soil samples were also found in root tip samples (Fig. 4). From Fig. 4 it is clear that even though OTUs corresponding to *Sistotrema* sp. were very dominant in soil samples in both sampling years and sites, some other OTUs were found to dominate the fungal communities identified from root tip samples. Root tips collected in HE were largely dominated by *Rhizopogon luteolus* (accounting for 61% of all reads identified in 2009 and 49% in 2011). Other dominant OTUs identified from root tips in HE corresponded to *Rhizoscyphus ericae* (5% in 2009 and 9% in 2011), *Acephala macrosclerotiorum* (1% and 9% respectively). *Sistotrema* sp. accounted for 7% and 4% of all reads identified from root tips collected in HE in 2009 and 2011 respectively. In LM, *Suillus luteus* dominated root tip samples (accounting for 14% and 55% of all reads identified in 2009 and 2011 respectively). Also *Rhizoscyphus ericae* was frequently identified (13% in 2009 and 10% in 2011), together with *Wilcoxina mikolae* (23% in 2009 and 5% in 2011), *Cadophora finlandica* (9% in 2009 and 6% in 2011), *Suillus bovinus* (8% in 2009 and 3% in 2011) and *Sagenomella humicola* (4% in 2009 and 1% in 2011). At the phylum level, root tips were dominated by Ascomycota in LM in 2009, but Basidiomycota dominated the root tips of pines in 2011 in this study site. In HE, Basidiomycota dominated the root tips in both sampling years (Fig. S5.3).


A
2%
2%
1%
1%
2%
3%
5%
5%
7%
11%
61%
Rhizopogon luteolus (ECM)
Helotiaceae
Sistotrema sp. (SAP/ECM)
Rhizoscyphus ericae (END/ERM/ECM)
Cadophora finlandica (ECM)
Sagenomella humicola (SAP)
Chloridium sp. (END)
Phialocephala sp. (END)
Acephala macrosclerotiorum (ECM)
Cenococcum geophilum (ECM)
Remaining taxa
B
20%
23%
2%
2%
2%
3%
4%
8%
9%
13%
14%
Wilcoxina mikolae (END/ECM)
Suillus luteus (ECM)
Rhizoscyphus ericae (END/ERM/ECM)
Cadophora finlandica (ECM)
Suillus bovinus (ECM)
Sagenomella humicolC7:D7a (SAP)
Chalara sp. (SAP)
Inocybe lacera (ECM)
Phialophora sp.(END)
Trichosporales
Remaining taxa

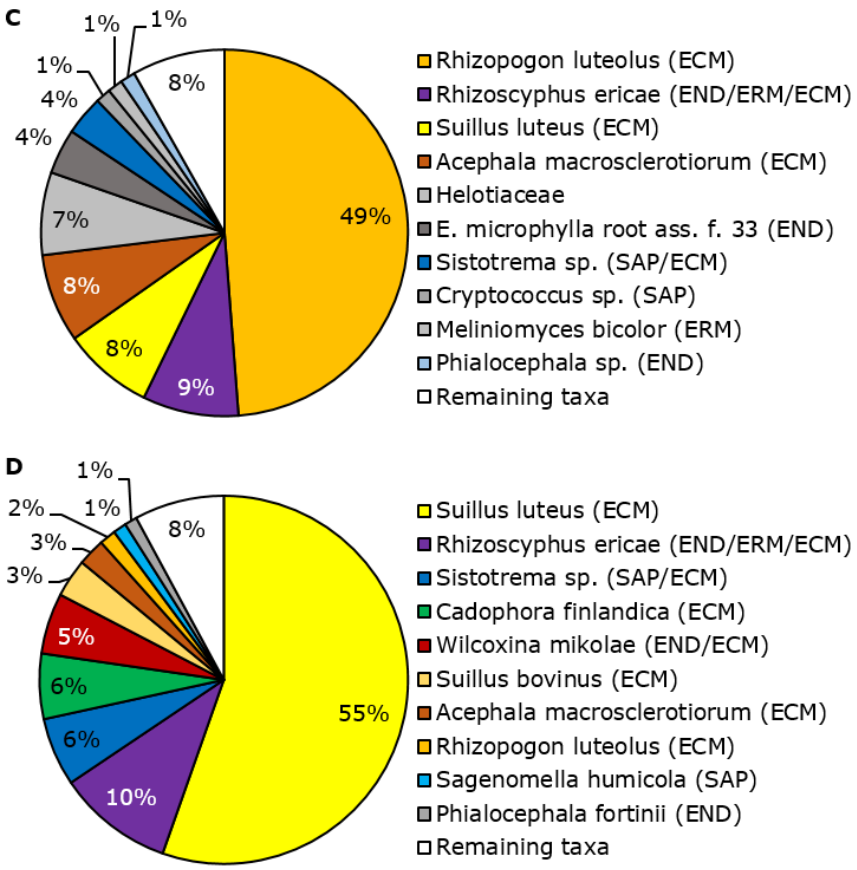


Figure 5.4 Pie charts displaying the top ten most abundant fungal species identified in root tip samples using 454 amplicon pyrosequencing. A. Hechtel-Eksel 2009. B. Lommel-Maatheide 2009. C. Hechtel-Eksel 2011. D. Lommel-Maatheide 2011. Species not occurring in the list of top ten most abundant species are grouped together as "Remaining taxa". Lifestyles of fungal species are given between brackets: ECM (ectomycorrhizal), ERM (ericoid mycorrhizal), AM (arbuscular mycorrhizal), SAP (saprotrophic), PATH (pathogenic), END (endophytic).

DISCUSSION

The effects of metal pollution on plant communities has been studied extensively in the past, but little is known about the fungal symbionts that occur with these plant communities. A number of studies have been conducted to investigate the effect of increased concentrations of metals on microbial communities. Whereas many studies reported shifts in fungal communities caused by increasing concentrations of metal ions, they usually do not reveal which fungal species are associated with plants thriving on metal-polluted sites and which species are unable to colonize polluted soils (e.g. Chodak *et al*., 2013; Corneo *et al*., 2013).

In the current study, we identified the fungal communities associated with Scots pine trees on a metal-polluted and a control site. The fungal communities at both study sites consisted mainly of the same fungal species as all OTUs with a relative read abundance > 1% were shared between both study sites (Fig. S5.2). However, the relative abundances of OTUs differed markedly between the study sites, indicating a shift in the relative abundance of fungal species that are otherwise characteristic for pioneer pine ecosystems in the Campine region in Belgium. Differences between both fungal communities were found to be strongly related to the presence of metal pollution on the one hand and soil mineral content on the other hand (Fig. 2). These results are in line with previous studies where shifts in ectomycorrhizal communities have been linked to changes in nutrient concentrations and the presence of metal-pollution (Toljander *et al*., 2006 and Hui *et al*., 2011, respectively). On the other hand, metal pollution appears to have little or no effect on fungal diversity (Fig. 1 and Hui *et al*., 2011). Differences in the observed fungal communities between sampling years suggest that colonization of soils by fungi in the investigated pioneer pine forests must be very dynamic. Possibly, seasonal fluctuations in fungal colonisation of soils and root systems resulted in different amounts of mycelium in soil samples and on collected pine roots from which DNA was extracted. The variation in frequency of dominant species in soil

and roots suggests that root and soil colonization by mycelia is a rapid process with a high turnover between years in the studied pioneer forests. These results also confirm findings by Pickles *et al*. (2010) where both spatial and temporal differences in ECM fungal communities associated with *Pinus sylvestris* were found at similar spatial and temporal scales as the ones investigated in the current study. A high turn-over rate of fungal mycelium in metal-polluted soils would favour the evolution of adaptive metal tolerance over short time spans, explaining why adaptive metal tolerance is most often observed in r-strategists amongst bacteria, fungi and plants and hardly ever in k-strategists such as woody tree species. The dynamics of the observed fungal communities associated with pine roots were also obvious at the phylum level (Fig. S5.3). Whereas the communities were dominated by Basidiomycota for both sampling years in HE, Ascomycota dominated the primary pioneer fungal communities in LM in 2009. In 2011, however, Basidiomycota became the most abundant phylum on root tips in LM. Since the first sampling session for the current study took place only one year after the pine trees were planted in LM, stress-tolerant pioneer ectomycorrhizal Basidiomycota were probably not sufficiently available to colonize pine roots and to establish their extensive mycelia. In the absence of Basidiomycota, opportunistic and endophytic Ascomycota colonized the available short roots. It remains unclear whether these associations benefit the host plants. By 2011, however, mycelial networks of Suilloids outcompeted the ubiquitous Ascomycota that were dominant in 2009.

The observed OTU richness for all study sites and sampling years was much lower than the observed richness in older forest soils, such as the ones studied by Hartmann *et al*., 2014 and Voříšková *et al*., 2014. In our studies, 518 OTUs were identified at a 97% sequence similarity cut-off in HE across sampling years. In LM, 521 OTUs were identified. In comparison, 1,007 OTUs (at the genus level) were identified in *Fagus sylvatica* (L.) and *Picea abies* (L.) Karst forest soils in Switzerland by Hartmann *et al*. (2014) and 2,534 OTUs (excluding singletons) were identified by Voříšková *et al*.

(2014) in a *Quercus petraea* (Matt.) Liebl. forest stand in the Czech Republic. The relatively low number of OTUs identified in our study is probably typical for young pioneer forests (Kipfer *et al*., 2011).

Fungal species identified in the polluted and non-polluted sites

In both sites, root tip and soil samples were found to be dominated by only a few ECM fungal species, whereas the majority of OTUs had very low relative abundances. This abundance pattern is typical for ECM fungi (Horton and Bruns, 2001). However, the species abundance patterns of the root tip samples differed greatly from the abundance patterns observed in soil samples (Fig. 3,4). Nevertheless, our study confirms that Suilloid fungi are the primary root colonisers of pine trees thriving in pioneer conditions (Ashkannejhad and Horton, 2006). The discrepancy in occurrence of the ECM fungi identified in root tip samples and soil samples in this study probably reflects differences in exploration types and life history traits of the different ECM species (Genney *et al*., 2006). Root tips collected from HE were strongly dominated by *Rhizopogon luteolus*, whereas *Suillus luteus* and *Wilcoxina mikolae* dominated root tips collected in LM. In LM, *S. luteus* becomes more dominant on root tips of the 3-year old trees, mainly at the expense of *W. mikolae* mycorrhizas. Dominance of *Suillus luteus* was expected in the metal-polluted site since this species has been shown in the past to have developed Zn- and Cd-tolerant populations in the LM region (Colpaert *et al*., 2004; Krznaric *et al*., 2009). Interestingly, the dominant position of *Suillus luteus* in LM soil and root samples appears to be taken up by *Rhizopogon luteolus* in HE. *Rhizopogon luteolus* occupies a very similar ecological (belowground) niche compared to *S. luteus*, but it may be less adapted to high metal concentrations at the LM site. In a previous study, we recorded a higher *in vitro* adaptation potential for Zn tolerance in *S. luteus* than in *R. luteolus* (Colpaert *et al*., 2004). Some species, mostly Ascomycota, that appeared in the list of top ten most abundant fungal species in LM have been observed in metal-polluted soils and other stressful environments in previous studies, suggesting that these

species exhibit substantial metabolic resilience to the harsh conditions or that they have developed adaptive metal tolerance mechanisms as did *S. luteus* (Colpaert *et al*., 2004). For example, *Wilcoxina mikolae*, *Cadophora finlandica* and *Inocybe lacera* have all been observed in metal-enriched soils in previous investigations (Prabhu *et al*., 1996, Gorfer *et al*., 2009 and Huang *et al*., 2012 respectively). These fungi are therefore interesting candidates to test for metal-resistance and/or -tolerance mechanisms.

Conclusions

Fungal communities thriving in pioneer pine forests in the Campine region of Belgium were shown to be dominated by a few pioneer ECM fungal species such as *Sistotrema* sp., *Wilcoxina mikolae*, *Suillus luteus* and *Rhizopogon luteolus*. Both in metal-polluted and non-polluted forest soils, the same fungal species were detected, but their relative abundances differed markedly. Statistical analysis indicated the existence of two gradients that correlate well with the observed fungal community compositions. The first gradient corresponded to metal pollution and the second gradient was found to be composed of the soil minerals Fe, Mg, Mn and K. Since for both the metal-polluted and non-polluted sites very similar diversity measures were found and all abundant species were shared between study sites, differences in ECM fungal community structures were attributed to abundance shifts of species that are otherwise probably typical for the studied fungal pioneer communities. Differences between sampling years revealed a highly dynamic fungal community, suggesting that mycelial exploration of pioneer soils is a process with a high turnover rate.

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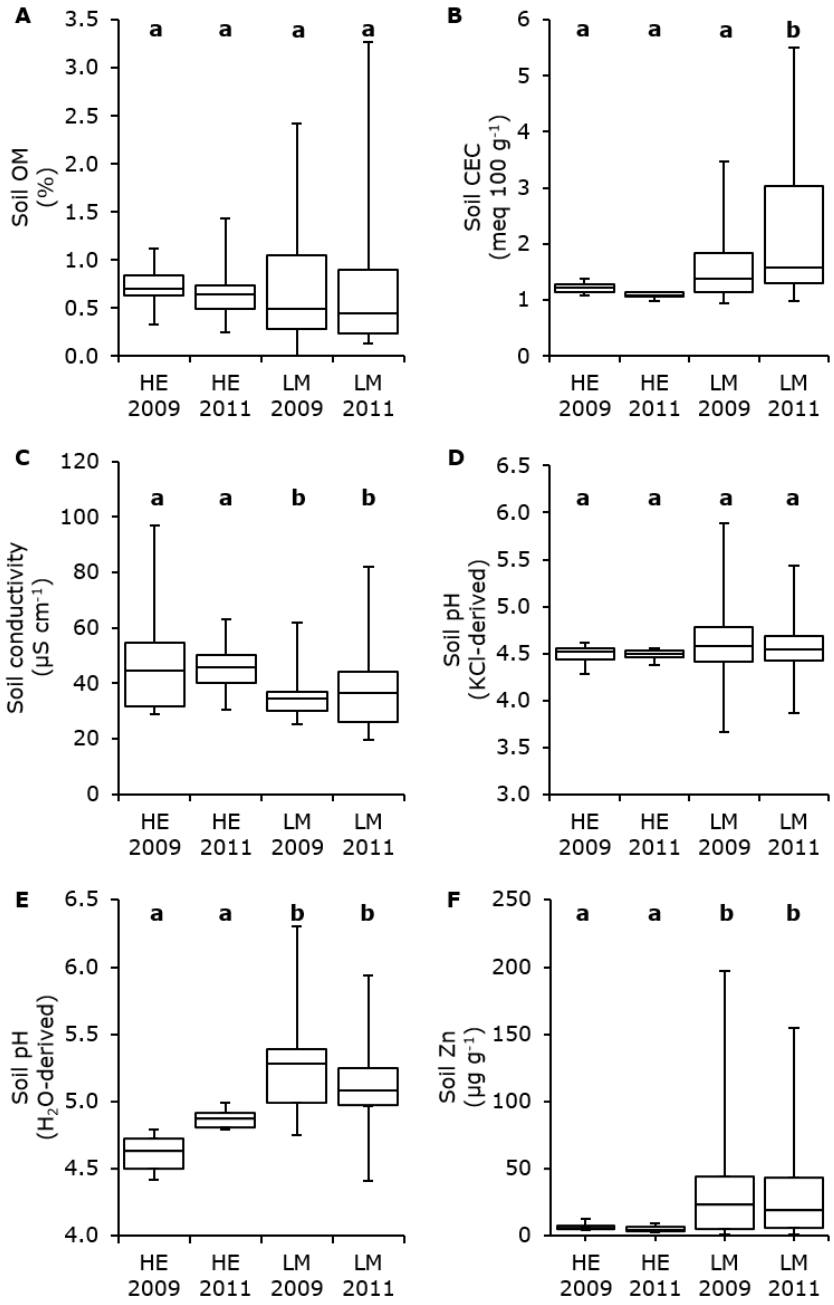
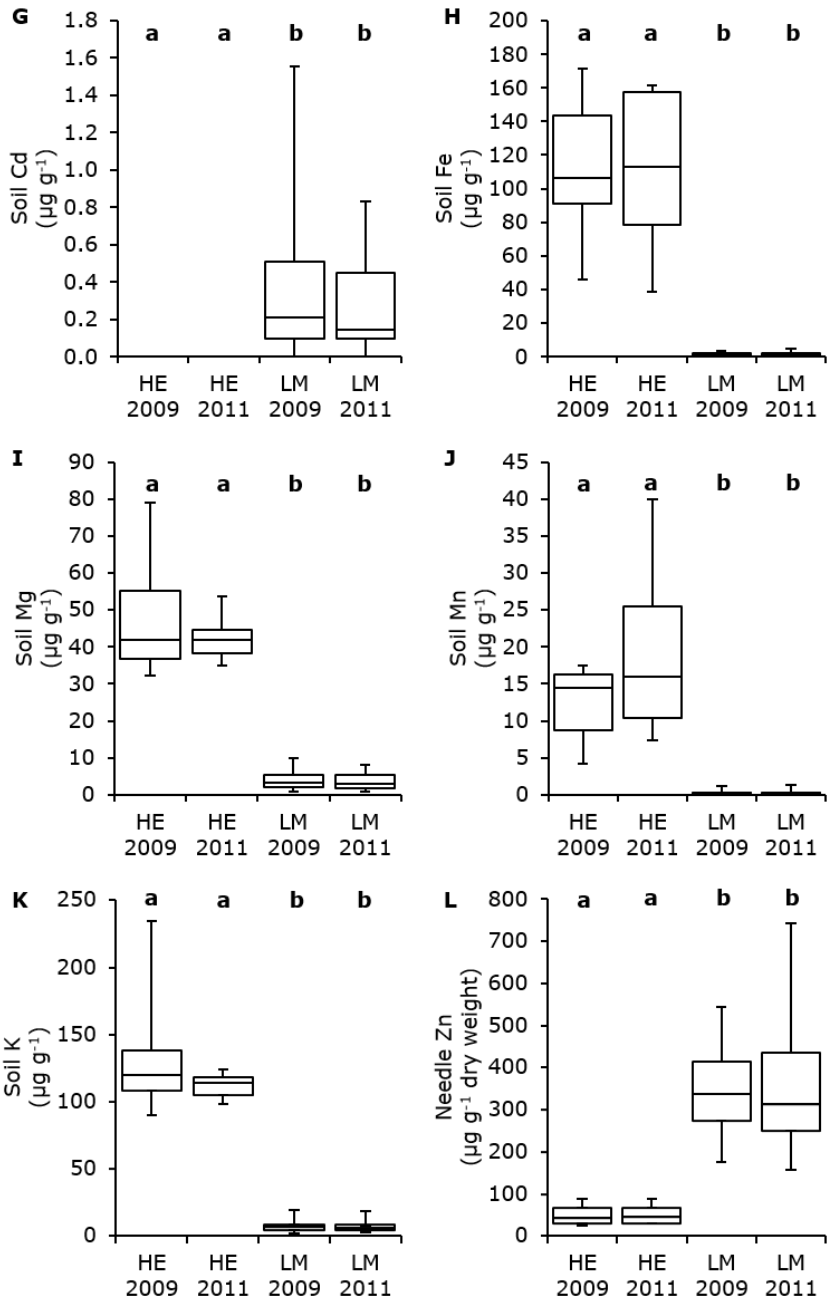
SUPPORTING INFORMATION

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Soil Cd (µg g-1)
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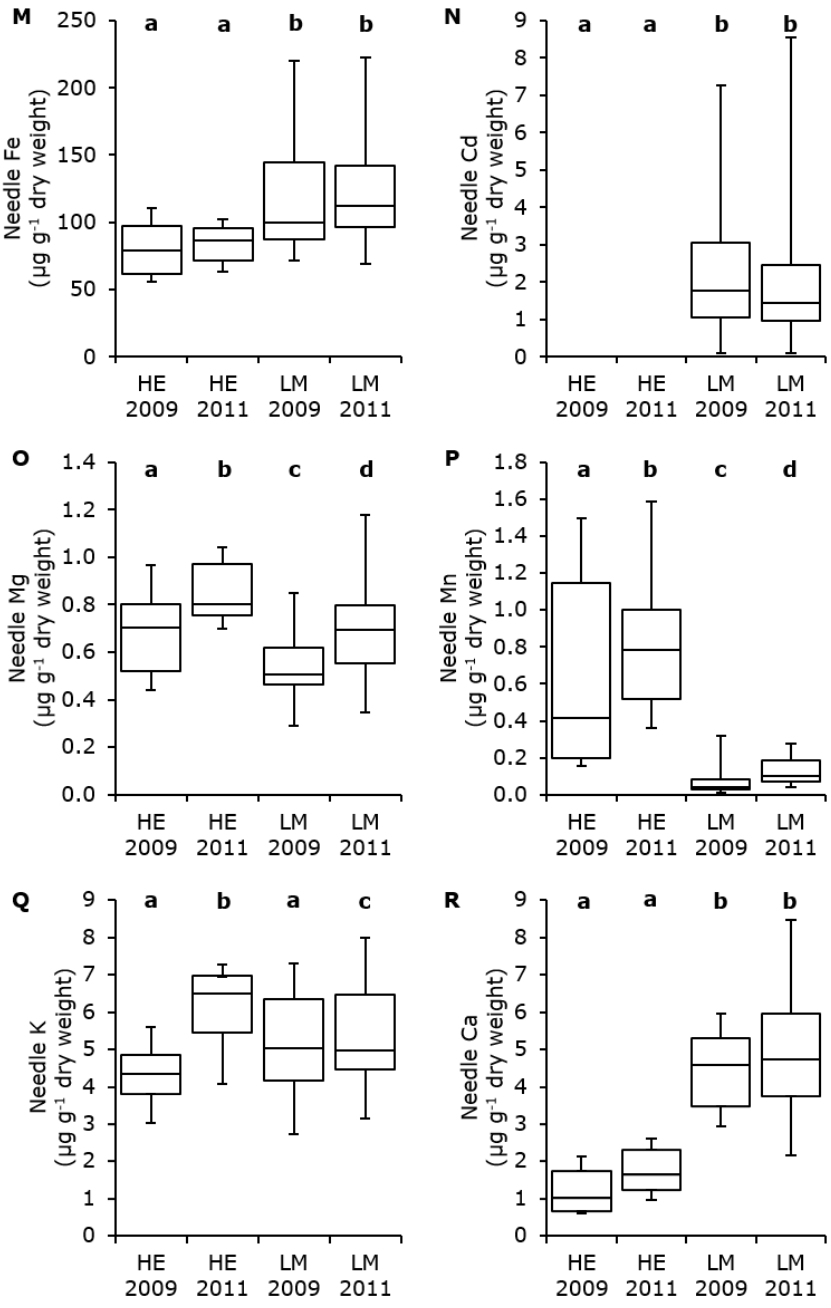
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
H
Soil Fe (µg g-1)
200
180
160
140
120
100
80
60
40
20
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
I
Soil Mg (µg g-1)
90
80
70
60
50
40
30
20
10
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
J
Soil Mn (µg g-1)
45
40
35
30
25
20
15
10
5
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
K
Soil K (µg g-1)
250
200
150
100
50
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
L
Needle Zn (µg g-1 dry weight)
800
700
600
500
400
300
200
100
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011


M
Needle Fe (µg g⁻¹ dry weight)
250
200
150
100
50
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
N
Needle Cd (µg g⁻¹ dry weight)
9
8
7
6
5
4
3
2
1
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011
O
Needle Mg (µg g⁻¹ dry weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a b c d
HE 2009
HE 2011
LM 2009
LM 2011
P
Needle Mn (µg g⁻¹ dry weight)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a b c d
HE 2009
HE 2011
LM 2009
LM 2011
Q
Needle K (µg g⁻¹ dry weight)
9
8
7
6
5
4
3
2
1
0
a b a c
HE 2009
HE 2011
LM 2009
LM 2011
R
Needle Ca (µg g⁻¹ dry weight)
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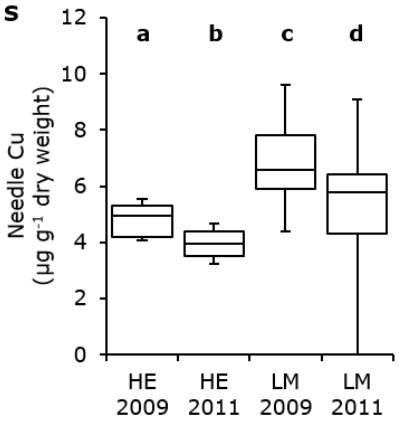
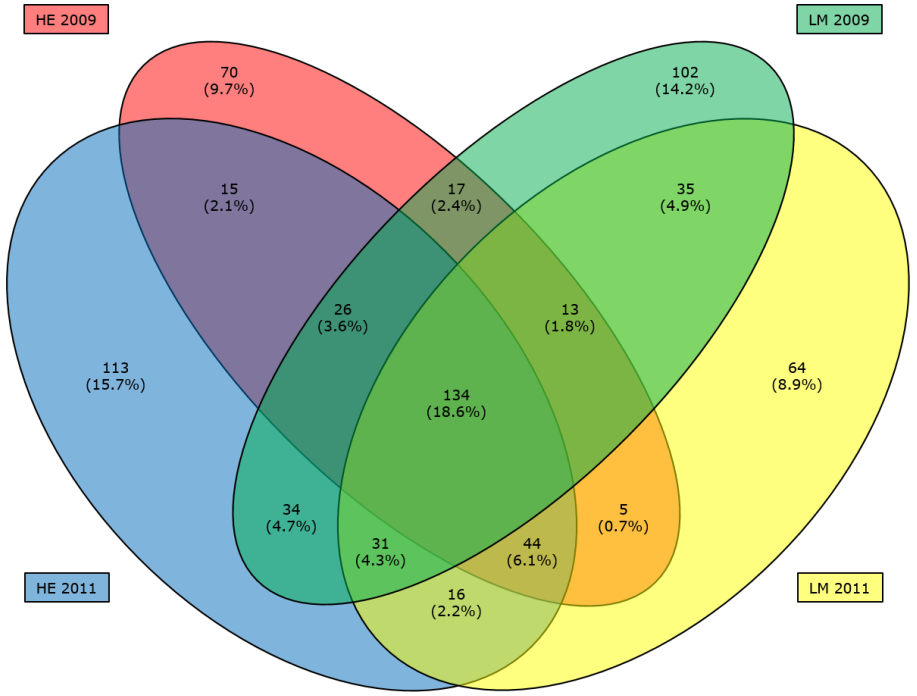
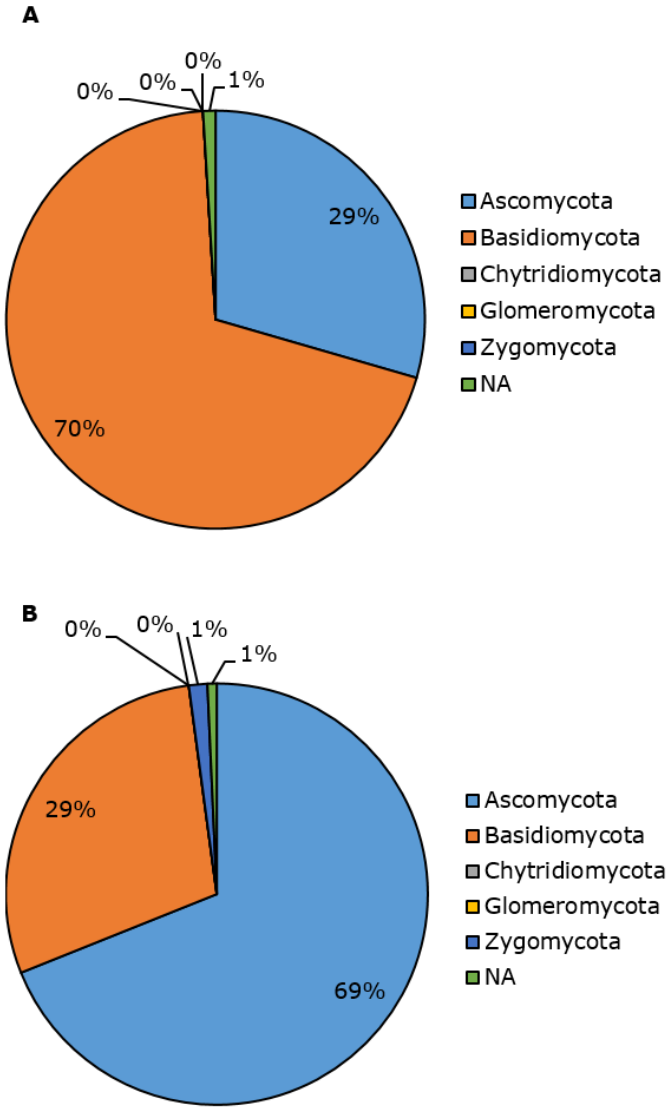
8
7
6
5
4
3
2
1
0
a a b b
HE 2009
HE 2011
LM 2009
LM 2011

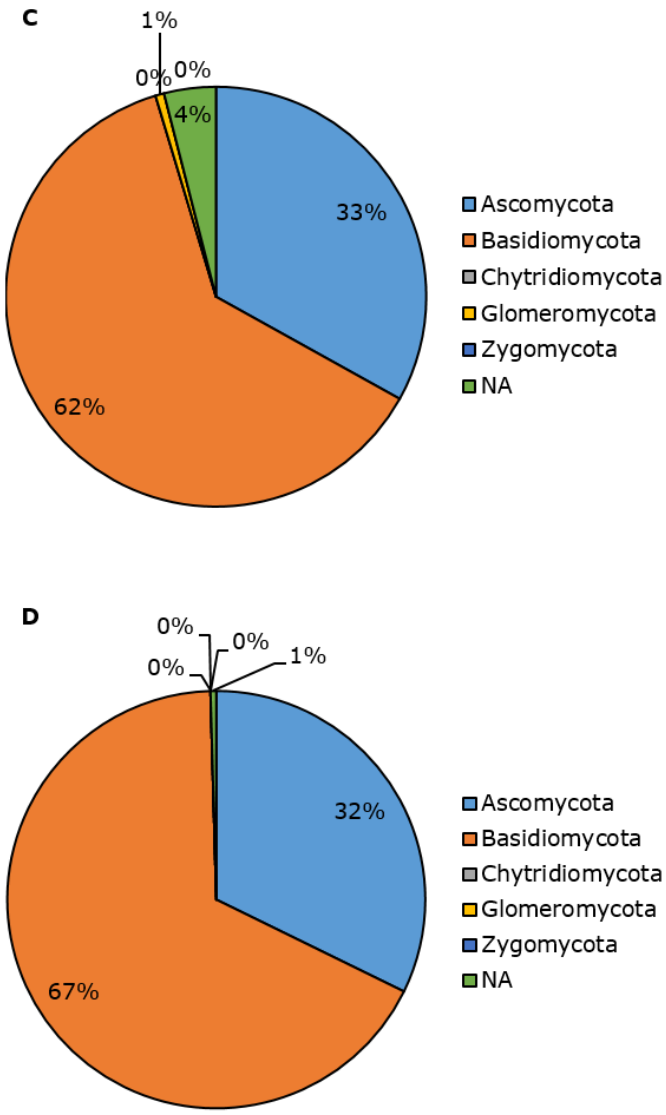


Supporting Information Figure S5.1 Environmental parameters measured in the current study in Lommel-Maatheide (LM: metal-polluted site) and Hechtel-Eksel (HE: control site). All parameters were measured in 2009 and again in 2011. Significant differences at the 95% confidence level are indicated with letters. A. Soil organic matter (OM). B. Cation exchange capacity (CEC). C. Soil conductivity. D. pH (KCl-derived). E. pH (H_2O-derived). F. Soil zinc concentration. G. Soil cadmium concentration. H. Soil iron concentration. I. Soil magnesium concentration. J. Soil manganese concentration. K. Soil potassium concentration. L. Needle zinc concentration. M. Needle iron concentration. N. Needle cadmium concentration. O. Needle magnesium concentration. P. Needle manganese concentration. Q. Needle potassium concentration. R. Needle calcium concentration. S. Needle copper concentration. Cadmium concentrations in pine needles and soil samples and copper concentrations in soil samples were below the detection limit of 0.1 µg g^{-1} for samples collected in HE.



Supporting Information Figure S5.2 Venn diagram showing the overlap in operational taxonomic unit (OTU) composition between study sites (a metal-polluted site in Lommel-Maatheide: LM and a control site in Hechtel-Eksel: HE) and sampling years (2009 and 2011). Numbers represent the number of OTUs identified in a given study site or sampling year. A total of 719 OTUs across study sites and sampling years were identified.


A
0%
0%
0%
1%
29%
70%
Ascomycota
Basidiomycota
Chytridiomycota
Glomeromycota
Zygomycota
NA
B
0%
0%
1%
1%
29%
69%
Ascomycota
Basidiomycota
Chytridiomycota
Glomeromycota
Zygomycota
NA



Supporting Information Figure S5.3 Fungal community composition of root tip samples at the phylum level for the metal polluted site in Lommel-Maatheide (LM) and the control site in Hechtel-Eksel (HE). Samples were collected in 2009 and again in 2011. A. HE 2009 fungal community. B. LM 2009 fungal community. C. HE 2011 fungal community. D. LM 2011 fungal community.

Supporting Information Table S5.1 Primer design used in the current study to construct fusion primers for 454 amplicon pyrosequencing.

Pool	Sample [1]	Forward primer			Reverse primer		
		454 Adapter [2]	MID [3]	Fungal ITS primer [4]	454 Adapter [2]	MID [3]	Fungal ITS primer [4]
1	LM_11.1_2009	A	6	ITS86F	B	6	ITS4
1	LM_11.2_209	A	6	ITS86F	B	7	ITS4
1	LM_11.3_2009	A	7	ITS86F	B	6	ITS4
1	LM_11.4_2009	A	7	ITS86F	B	7	ITS4
1	LM_11.5_2009	A	6	ITS86F	B	8	ITS4
1	LM_12.1_2009	A	8	ITS86F	B	6	ITS4
1	LM_12.2_2009	A	7	ITS86F	B	8	ITS4
1	LM_12.3_2009	A	8	ITS86F	B	7	ITS4
1	LM_12.4_2009	A	8	ITS86F	B	8	ITS4
1	LM_12.5_2009	A	6	ITS86F	B	9	ITS4
1	LM_2.1_2009	A	9	ITS86F	B	6	ITS4
1	LM_21.1_2009	A	7	ITS86F	B	9	ITS4
1	LM_21.2_2009	A	9	ITS86F	B	7	ITS4
1	LM_21.3_2009	A	8	ITS86F	B	9	ITS4
1	LM_21.4_2009	A	9	ITS86F	B	8	ITS4
1	LM_21.5_2009	A	9	ITS86F	B	9	ITS4
1	LM_27.1_2009	A	6	ITS86F	B	10	ITS4
1	LM_27.2_2009	A	10	ITS86F	B	6	ITS4
1	LM_27.3_2009	A	7	ITS86F	B	10	ITS4
1	LM_27.4_2009	A	10	ITS86F	B	7	ITS4
1	LM_27.5_2009	A	8	ITS86F	B	10	ITS4
1	LM_29.3_2009	A	10	ITS86F	B	8	ITS4
1	LM_30.1_2009	A	9	ITS86F	B	10	ITS4
1	LM_30.2_2009	A	10	ITS86F	B	9	ITS4
1	LM_30.3_2009	A	10	ITS86F	B	10	ITS4
1	LM_30.4_2009	A	6	ITS86F	B	11	ITS4
1	LM_30.5_2009	A	11	ITS86F	B	6	ITS4
1	LM_31.1_2009	A	7	ITS86F	B	11	ITS4
1	LM_31.2_2009	A	11	ITS86F	B	7	ITS4
1	LM_31.3_2009	A	8	ITS86F	B	11	ITS4
1	LM_31.4_2009	A	11	ITS86F	B	8	ITS4
1	LM_31.5_2009	A	9	ITS86F	B	11	ITS4
1	LM_5.1_2009	A	11	ITS86F	B	9	ITS4
1	LM_5.2_2009	A	10	ITS86F	B	11	ITS4
1	LM_5.3_2009	A	11	ITS86F	B	10	ITS4
1	LM_5.4_2009	A	11	ITS86F	B	11	ITS4
1	LM_5.5_2009	A	6	ITS86F	B	12	ITS4
1	LM_2.2_2009	A	12	ITS86F	B	6	ITS4
1	LM_2.3_2009	A	7	ITS86F	B	12	ITS4

1	LM_2.4_2009	A	12	ITS86F	B	7	ITS4
1	LM_2.5_2009	A	8	ITS86F	B	12	ITS4
1	LM_28.1_2009	A	12	ITS86F	B	8	ITS4
1	LM_28.2_2009	A	9	ITS86F	B	12	ITS4
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2	LM_11.3_2011	A	6	ITS86F	B	7	ITS4
2	LM_11.4_2011	A	7	ITS86F	B	6	ITS4
2	LM_11.5_2011	A	7	ITS86F	B	7	ITS4
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2	LM_21.4_2011	A	11	ITS86F	B	10	ITS4
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4	HE_10.1_2011	A	8	ITS86F	B	6	ITS4
4	HE_10.2_2011	A	7	ITS86F	B	8	ITS4
4	HE_10.3_2011	A	8	ITS86F	B	7	ITS4
4	HE_10.4_2011	A	8	ITS86F	B	8	ITS4
4	HE_10.5_2011	A	6	ITS86F	B	9	ITS4
4	HE_12.1_2011	A	9	ITS86F	B	6	ITS4
4	HE_12.2_2011	A	7	ITS86F	B	9	ITS4
4	HE_12.3_2011	A	9	ITS86F	B	7	ITS4
4	HE_12.4_2011	A	8	ITS86F	B	9	ITS4
4	HE_12.5_2011	A	9	ITS86F	B	8	ITS4
4	HE_17.1_2011	A	9	ITS86F	B	9	ITS4
4	HE_17.2_2011	A	6	ITS86F	B	10	ITS4
4	HE_17.3_2011	A	10	ITS86F	B	6	ITS4
4	HE_17.4_2011	A	7	ITS86F	B	10	ITS4
4	HE_17.5_2011	A	10	ITS86F	B	7	ITS4
4	HE_18.1_2011	A	8	ITS86F	B	10	ITS4
4	HE_18.2_2011	A	10	ITS86F	B	8	ITS4
4	HE_18.3_2011	A	9	ITS86F	B	10	ITS4
4	HE_18.4_2011	A	10	ITS86F	B	9	ITS4
4	HE_18.5_2011	A	10	ITS86F	B	10	ITS4
4	HE_23.1_2011	A	6	ITS86F	B	11	ITS4
4	HE_23.2_2011	A	11	ITS86F	B	6	ITS4
4	HE_23.3_2011	A	7	ITS86F	B	11	ITS4
4	HE_23.4_2011	A	11	ITS86F	B	7	ITS4
4	HE_23.5_2011	A	8	ITS86F	B	11	ITS4

4	HE_24.1_2011	A	11	ITS86F	B	8	ITS4
4	HE_24.2_2011	A	9	ITS86F	B	11	ITS4
4	HE_24.3_2011	A	11	ITS86F	B	9	ITS4
4	HE_24.4_2011	A	10	ITS86F	B	11	ITS4
4	HE_24.5_2011	A	11	ITS86F	B	10	ITS4
4	HE_27.1_2011	A	11	ITS86F	B	11	ITS4
4	HE_27.2_2011	A	6	ITS86F	B	12	ITS4
4	HE_27.3_2011	A	12	ITS86F	B	6	ITS4
4	HE_27.4_2011	A	7	ITS86F	B	12	ITS4
4	HE_27.5_2011	A	12	ITS86F	B	7	ITS4
4	HE_29.1_2011	A	8	ITS86F	B	12	ITS4
4	HE_29.2_2011	A	12	ITS86F	B	8	ITS4
4	HE_29.3_2011	A	9	ITS86F	B	12	ITS4
4	HE_29.4_2011	A	12	ITS86F	B	9	ITS4
4	HE_29.5_2011	A	10	ITS86F	B	12	ITS4
4	HE_30.1_2011	A	12	ITS86F	B	10	ITS4
4	HE_30.2_2011	A	11	ITS86F	B	12	ITS4
4	HE_30.3_2011	A	12	ITS86F	B	11	ITS4
4	HE_30.4_2011	A	12	ITS86F	B	12	ITS4
4	HE_30.5_2011	A	6	ITS86F	B	13	ITS4
5	HE_03_roots_2009	A	6	ITS86F	B	6	ITS4
5	HE_03_roots_2011	A	6	ITS86F	B	7	ITS4
5	HE_10_roots_2009	A	7	ITS86F	B	6	ITS4
5	HE_10_roots_2011	A	7	ITS86F	B	7	ITS4
5	HE_12_roots_2009	A	6	ITS86F	B	8	ITS4
5	HE_12_roots_2011	A	8	ITS86F	B	6	ITS4
5	HE_17_roots_2009	A	7	ITS86F	B	8	ITS4
5	HE_17_roots_2011	A	8	ITS86F	B	7	ITS4
5	HE_18_roots_2009	A	8	ITS86F	B	8	ITS4
5	HE_18_roots_2011	A	6	ITS86F	B	9	ITS4
5	HE_23_roots_2009	A	9	ITS86F	B	6	ITS4
5	HE_23_roots_2011	A	7	ITS86F	B	9	ITS4
5	HE_24_roots_2009	A	9	ITS86F	B	7	ITS4
5	HE_24_roots_2011	A	8	ITS86F	B	9	ITS4
5	HE_27_roots_2009	A	9	ITS86F	B	8	ITS4
5	HE_27_roots_2011	A	9	ITS86F	B	9	ITS4
5	HE_29_roots_2009	A	6	ITS86F	B	10	ITS4
5	HE_29_roots_2011	A	10	ITS86F	B	6	ITS4
5	HE_30_roots_2009	A	7	ITS86F	B	10	ITS4
5	HE_30_roots_2011	A	10	ITS86F	B	7	ITS4
5	LM_02_roots_2009	A	8	ITS86F	B	10	ITS4
5	LM_02_Roots_2011	A	10	ITS86F	B	8	ITS4
5	LM_05_roots_2009	A	9	ITS86F	B	10	ITS4
5	LM_05_roots_2011	A	10	ITS86F	B	9	ITS4
5	LM_11_roots_2009	A	10	ITS86F	B	10	ITS4
5	LM_11_roots_2011	A	6	ITS86F	B	11	ITS4
5	LM_12_roots_2009	A	11	ITS86F	B	6	ITS4

5	LM_12_roots_2011	A	7	ITS86F	B	11	ITS4
5	LM_21_roots_2009	A	11	ITS86F	B	7	ITS4
5	LM_21_roots_2011	A	8	ITS86F	B	11	ITS4
5	LM_27_roots_2009	A	11	ITS86F	B	8	ITS4
5	LM_27_roots_2011	A	9	ITS86F	B	11	ITS4
5	LM_28_roots_2009	A	11	ITS86F	B	9	ITS4
5	LM_28_roots_2011	A	10	ITS86F	B	11	ITS4
5	LM_29_roots_2009	A	11	ITS86F	B	10	ITS4
5	LM_29_roots_2011	A	11	ITS86F	B	11	ITS4
5	LM_30_roots_2009	A	6	ITS86F	B	12	ITS4
5	LM_30_roots_2011	A	12	ITS86F	B	6	ITS4
5	LM_31_roots_2009	A	7	ITS86F	B	12	ITS4
5	LM_31_roots_2011	A	12	ITS86F	B	7	ITS4

Primer component name	Primer sequence (5'-3')
A	CGTATCGCCTCCCTCGCGCCATCAG
B	CTATGCGCCTTGCCAGCCCGCTCAG
ITS86F	GTGAATCATCGAATCTTTGAA
ITS4	TCCTCCGCTTATTGATATGC
MID6	ATATCGCGAG
MID7	CGTGTCTCTA
MID8	CTCGCGTGTC
MID9	TAGTATCAGC
MID10	TCTCTATGCG
MID11	TGATACGTCT
MID12	TACTGAGCTA
MID13	CATAGTAGTG

(1) LM: Lommel-Maatheide. HE: Hechtel-Eksel

(2) A: 454 pyrosequencing adapter A, B: 454 pyrosequencing adapter B

(3) MID: Multiplex identifier

(4) ITS: Internal transcribed spacer

Supporting Information Table S5.2 Pearson's correlation coefficients (R^2) correlating measured environmental variables and fungal diversity indices for the control site in Hechtel-Eksel and the metal-polluted site in Lommel-Maatheide. Fungal diversity indices were calculated in R based on OTU abundances. OTUs were generated in Mothur based on a 97% sequence similarity cut-off. Significant correlations at the 95% confidence level are indicated in bold. Cadmium concentrations in pine needles and soil samples collected in HE and copper concentrations in soil samples collected from both sites were below the detection limit of 0.1 µg g^{-1}.

Environmental parameter	2009 dataset			2011 dataset		
	Rich. (5)	Pielou evenn. (6)	Inv. Simp. (7)	Rich. (5)	Pielou evenn. (6)	Inv. Simp. (7)
OM (1)	0.01	-0.16	-0.08	0.19	-0.03	-0.01
CEC (2)	-0.02	-0.03	-0.04	**-0.57**	**-0.58**	-0.39
Conduct. (3)	0.22	0.22	0.23	0.38	0.18	0.21
pH KCl	0.34	0.28	0.27	0.11	0.13	0.00
pH H2O	-0.10	-0.27	-0.11	**-0.45**	-0.40	-0.35
Soil Zn (4)	0.42	0.43	0.44	0.17	0.26	0.23
Soil Cd (4)	0.34	0.26	0.33	-0.05	0.08	0.04
Soil Fe (4)	0.18	0.34	0.20	**0.54**	0.37	0.26
Soil Mg (4)	0.39	0.37	0.23	**0.61**	**0.53**	0.43
Soil Mn (4)	0.16	0.25	0.05	0.24	0.25	0.06
Soil K (4)	0.31	0.33	0.18	**0.58**	**0.47**	0.35
Needle Zn (4)	-0.31	-0.41	-0.31	**-0.49**	-0.33	-0.27
Needle Cd (4)	-0.24	-0.36	-0.26	**-0.46**	-0.38	-0.30
Needle Fe (4)	-0.04	-0.32	-0.25	-0.29	-0.38	-0.25
Needle Mg (4)	-0.01	0.21	-0.01	0.37	0.31	0.16
Needle Mn (4)	-0.10	0.08	-0.17	0.35	0.28	0.05
Needle K (4)	-0.24	0.07	-0.03	0.07	0.33	0.28
Needle Ca (4)	-0.15	-0.27	-0.17	-0.36	-0.23	-0.19
Needle Cu (4)	-0.23	-0.36	-0.30	**-0.48**	**-0.45**	-0.34

(1) OM: Soil organic matter (%)
(2) CEC: Cation exchange capacity (meq 100 g^{-1})
(3) Conduct.: Conductivity (µS cm^{-1})
(4) Measured in µg g^{-1} dry weight
(5) Rich.: OTU richness
(6) Pielou evenn.: Pielou evenness index
(7) Inv. Simp.: Inverse Simpson index

Chapter 6: **Conclusions and future perspectives**

GENETIC ARCHITECTURE OF ZINC TOLERANCE IN *SUILLUS LUTEUS*

Previous studies indicated the presence of zinc-tolerant ecotypes in the ectomycorrhizal basidiomycete *Suillus luteus,* in different populations in the northern part of Limburg, Belgium (Colpaert et al., 2004). The ecotypes from metal-polluted soils were found to have higher EC_{50}-values than ecotypes from non-polluted soils, when exposed to increasing Zn concentrations. Whereas dikaryotic isolates sampled in Lommel-Maatheide and Lommel-Sahara had EC_{50}-values exceeding 6 mM, isolates collected from Paal had EC_{50}-values below 6 mM (Colpaert et al., 2004). To study the genetic architecture of this Zn-tolerance trait, the same sites were sampled in 2008, 2009 and 2010 for the current study. Since most isolates collected for the current study were sampled near young pine trees, the genets collected for are most likely different from the ones studied by Colpaert et al. (2004). Evaluation through microsatellite markers indicated the successful isolation of monokaryotic cultures from single spores, dikaryotic, parental strains from basidiocarps and the successful *in vitro* dikaryotisation of crosses between monokaryons. Based on dose-response experiments, five hypothetical Zn-tolerance phenotypes were identified. Whereas all five phenotypes had different EC_{50}-values, their mycelial Zn content suggested the existence of only two distinct phenotypes (one phenotype having mycelial Zn concentrations below 3 mg g^{-1} d. wt. and one phenotype having mycelial Zn concentrations well above 3 mg g^{-1} d. wt.). These results suggest that one gene could be responsible for the regulation of internal Zn concentrations in *S. luteus* isolates. If this is the case, this gene would be pleiotropic, influencing both the mycelial Zn concentration and growth (EC_{50}-values) of studied isolates. Differences in EC_{50}-values could then be explained by genetic factors that are not necessarily linked to the hypothesized Zn-tolerance gene locus. In a number of plant species, for example, metal tolerance is regulated by a

single major gene locus and the expression of this major gene locus is further fine-tuned by minor modifier genes (Macnair, 1993). Alternatively, a single Zn-tolerance gene could be present in multiple copies in the genome of *S. luteus*. Differences in mycelial Zn concentrations could then be explained by the presence or absence of Zn-tolerance alleles in all copies of the gene for a given isolate and variations in EC_{50}-values could be caused by differences in the expression levels of copies or differences in the Zn transportation efficiency of proteins produced by these genes. This too is a very plausible situation as in metal-tolerant plants, variability in metal tolerance and accumulation among different plant populations has been partly explained by copy-number expansion of genes involved in metal-tolerance and -homeostasis as well (Craciun *et al*., 2012). Dividing the total variation of EC_{50}-values into different components such as genotype-induced variation, Zn-treatment-induced variation and variation induced by the environment, a large proportion of the total phenotypic variation was found to be explained by differences between genotypes (on average 25% ± 2% SE), but also a large proportion of this phenotypic variation was explained by genetic factors that are not necessarily linked to differences in Zn-tolerance genotypes (21% ± 1%). Since Zn is an essential nutrient, cytoplasmic Zn concentrations are tightly regulated by a wide spectrum of cellular processes. For example, intracellular sequestration of excess Zn ions with metallothioneins, glutathione, phytochelatins or variations in vacuolar storage efficiency could be responsible for a better growth of *S. luteus* isolates exposed to high Zn concentrations, but such mechanisms would not influence Zn exclusion from cells. Hence, a number of Zn-homeostasis mechanisms potentially affect EC_{50}-values, partially obscuring true Zn-tolerance phenotypes, without altering mycelial Zn concentrations. Another confounding factor that may influence the interpretation of phenotypic differences in Zn-tolerance between *S. luteus* strains is the way EC_{50}-values are typically calculated. In the current study, EC_{50}-values were calculated based on the dry weights of mycelia. Growth, however, is a polygenic trait and typically results in a continuum of possible mycelium

sizes. A discrete number of Zn-tolerance phenotypes could therefore be obscured by the continuous nature of mycelial growth. Similarly, early studies of metal-tolerance in plants led to the conclusion that metal tolerance in plants must be a polygenic trait. Polygenic inheritance of metal tolerance has been suggested, for example, for Zn tolerance in *Silene vulgaris* (Bröker, 1963), *Anthoxanthum odoratum* (Gartside and McNeilly, 1974a) and *Agrostis capillaris* (Gartside and McNeilly, 1974b), Pb tolerance in *Festuca ovina* (Wilkins, 1960) and Al tolerance in *Zea mays* (Magnavaca et al., 1987). On the other hand, studies designed for testing Mendelian segregation of metal tolerance concluded that adaptive metal tolerance is generally governed by a single or a few major genes. Examples can be found in Cu tolerance in *Mimulus guttatus* (Macnair and Watkins 1983), *Silene vulgaris* (Schat and ten Bookum, 1992) and *Agrostis capillaris* (Macnair, 1990) and As tolerance in *Holcus lanatus* (Macnair et al., 1992) and *Agrostis capillaris* (Watkins and Macnair, 1991). In a number of these cases, evidence was found for the presence of one or two minor modifier genes as well (see Table 1 in Macnair, 1993). In parallel, scaling of Zn-tolerance phenotypes of *S. luteus* to mycelial weights in our experiments, undoubtedly introduced at least some degree of continuity, making it more difficult to identify a discrete genetic architecture for the Zn-tolerance trait in *S. luteus*. To obtain more insight in the genetic architecture of the Zn-tolerance trait in *S. luteus*, we opted to test the fit of two Mendelian heredity patterns that were selected based on observed Zn-tolerance phenotypes. According to a first possible model, a single gene locus could be responsible for the Zn-tolerance trait in *S. luteus*. If two alleles exist for this gene locus (a Zn-tolerance and a Zn-sensitivity allele), this model predicts the existence of two distinct Zn-tolerance phenotypes. According to a second model, two genes could be responsible for Zn-tolerance, each locus having two alleles. In this case, five different phenotypes would exist, ranging from highly Zn-sensitive, over intermediary Zn-tolerant to highly Zn-tolerant phenotypes. At first sight, our experimental data appear to support the second model. Chi-squared tests confirmed that the second model indeed

had a better fit to the experimental data than the first model, but model 1 still had a significant fit to all observed data and could therefore not be discarded. If Zn-tolerance in *S. luteus* would indeed be governed by two genes, one gene could, for example, be responsible for Zn-exclusion and another one could be responsible for additional variations in growth rates. Also a model where a single gene is responsible for Zn-tolerance, however, could fit model 2, if this gene has multiple copies that result in contrasting tolerance phenotypes. Alternatively, a genetic architecture comprised of two Zn-tolerance loci could result in segregation patterns that would fit model 1 instead of model 2 if both loci are tightly coupled on the same chromosome. Because it is currently still impossible to acquire progeny from *in vitro* created crosses of *S. luteus*, it is impossible to further evaluate the suitability of either model. Hence, future targeted molecular and proteomics studies will need to be conducted to further unravel which genes are exactly responsible for each of the observed phenotypes. Furthermore, our data indicated that the phenotype of a crossed strain is determined by both monokaryons that contributed to this crossed strain. Hence, Zn-tolerance in *S. luteus* is inherited through incomplete dominance of the gene(s) that is (are) responsible for the Zn-tolerance trait. The strictly inverse relationship between EC_{50}-values and mycelial Zn concentrations observed for all isolates, suggest that Zn-exclusion is responsible for the Zn-tolerance trait in *Suillus luteus*. Zn-exclusion has recently been shown to infer Zn-tolerance in *Suillus bovinus* (Ruytinx et al., 2013), suggesting parallel evolution of Zn-tolerance in these closely related species. However, it needs to be noted that currently no experimental data have been collected that would indicate that the Zn-tolerance trait in *S. luteus* is based on the same gene loci in the different populations that were investigated, nor that Zn-tolerance in *S. luteus* and *S. bovinus* are based on the same gene loci. Recently, the genome of *S. luteus* has been sequenced. Future re-sequencing of a number of strains with contrasting Zn-tolerance phenotypes could be conducted and comparative genomics could subsequently reveal more details about the genetic architecture of Zn-

tolerance in different *S. luteus* and *S. bovinus* populations. Results obtained from targeted gene expression analysis and functional proteomics of genes and proteins involved in the Zn-homeostasis in *S. luteus* could be linked to the results obtained in the current study to estimate the importance of individual genes and proteins for metal-homeostasis in *S. luteus*. It would also be interesting to compare the genetic architecture of Zn tolerance and Cd tolerance in both *Suillus* species. Since Cd is a non-essential element, little or no genetic factors, other than the genes involved in Cd tolerance, are expected to affect mycelial Cd concentrations and mycelial growth. Hence, studies investigating Cd tolerance are probably not confounded by cellular metal-homeostasis and a more clear picture of the genes involved in Cd tolerance could be obtained. Comparative genomics could finally reveal which genes are most susceptible to metal-induced selection pressure across different fungal species, providing key information about the micro-evolutionary processes that shape natural communities in disturbed environments.

EFFECT OF METAL POLLUTION ON FUNGAL BIODIVERSITY AND COMMUNITY STRUCTURES

Local spatial and temporal variations in ectomycorrhizal communities

Besides differences in metal concentrations, a number of other environmental factors are known to affect fungal diversity and community compositions. These confounding factors may alter our view on the effects of metal pollution on fungal communities. To obtain a clearer picture of the impact of metal pollution, the effects of a number of environmental variables on fungal diversity and community compositions were investigated before focusing on the effects of metal pollution. Local variations in root density and root age of host tree species have been found to alter mycorrhizal community structures (Peay et al., 2010 and Last et al., 1987). Whereas young, newly emerging, root tips may be colonized by

typical pioneer species, older parts of root systems may be colonized by fungi that typically appear in later stages of succession. On the other hand, senescent parts of root networks may be primarily occupied by saprotrophic species. Results obtained in our studies for both a metal-polluted site (Lommel-Maatheide) and a control site (Hechtel-Eksel), however, indicated that the studied fungal communities near stems of pine trees are not necessarily more similar to each other than they are to fungal communities that were sampled further away from stems. In line with these results, Branco et al. (2013) did not find any correlations between fungal community composition (detected in in-growth bags) and sampling distance from pine stems either, even though the ages of the pine trees investigated by Branco et al. (2013) were much older (trees were either 16 years old or between 50 and 80 years old) than the pine trees investigated in our studies (ranging from 2 to 5 years old). Individual pine trees in our studies were found to harbor slightly different fungal communities across the study sites, but no spatially-explicit structuring was observed at the scale of the study sites. Also between sampling years (2009 and 2011) some differences in the fungal communities were found, but in general, fungal communities were composed of the same fungal species across sampling sessions. The main differences in fungal communities were attributable to changes in the relative abundances of the fungal species over the two-year period. Since the pioneer communities investigated in our studies have hardly any plant cover and the sandy soils contain almost no organic matter, there is very little buffer capacity for the ecosystems against climatic variations and therefore moisture and temperature fluctuations are probably strong factors influencing the growth of mycelia in soils. The presence of seasonal dynamics in fungal communities have been reported in previous studies as well (Jumpponen et al., 2010; Davey et al., 2012; Vořísková et al., 2014). Davey et al. (2012), for example, observed a clear decrease in fungal richness during summer and winter months, whereas during late spring and late autumn, the OTU richness peaked. Also the total fungal biomass in soils appears to follow this trend

(Voříšková et al., 2014). Even at the phylum-level large changes in the fungal community compositions across sampling years were obvious in our studies, both in soil samples and on root tips of pine trees. Whereas Ascomycota dominated the metal-polluted study site in 2009, Basidiomycota took over this dominant position in 2011. In the control site, Basidiomycota dominated the fungal communities in both sampling years. Since the studied primary pioneer ecosystem in the metal-polluted site is very young (trees were planted in this study site one year before the first sampling session of our studies took place), the dominance of Ascomycota in 2009 is attributable to the dominant presence of spores and other fungal propagules and ascomycete mycelia in the study site and the absence of larger mycelial networks of Basidiomycota that would take much longer to get established. It is not until two years later, in 2011, that Basidiomycota appear to have formed their large mycelial networks and start to outcompete the smaller mycelia and propagules of some opportunistic Ascomycetes. It is clear that the studied pioneer ecosystems are still highly dynamic. Such highly dynamic communities may favor the development of metal-tolerant ecotypes and could, at least partly, explain the rapid evolution of Zn- and Cd tolerant ecotypes of *Suillus luteus* (Colpaert et al., 2004; Krznaric et al., 2009). Finally, the absence of spatial structuring of fungal communities at the level of the study sites suggested that mainly local environmental variables are responsible for structuring the studied communities. Whereas input of fungal inoculum from surrounding population is undoubtedly a factor that influences which fungal species are present in the study sites (Muller et al., 2004), it is probably not the strongest driving factor in determining the structure of the studied fungal communities.

Relationship between ectomycorrhizal communities and their abiotic environment

Overall, the studied fungal communities consisted largely of the same fungal species across study sites and sampling years and OTU richness remained fairly constant over sampling years. Despite clear similarities in the overall composition, differences in the relative abundances of present species were observed, indicating shifts in the abundance patterns of species that are otherwise characteristic for pioneer pine forests thriving on sandy Campine soils. The differences in fungal community structures across study sites were found to be mainly correlated with metal-pollution and soil mineral content. More specifically, a metal-pollution gradient, mainly consisting of Zn and Cd pollution, was found to be an important factor correlating with the fungal community composition in the metal-polluted site, whereas soil Fe, Mg, Mn and K concentrations were found to correlate well with fungal community compositions in the control site. These results confirm earlier findings that soil mineral content may be an important factor in determining fungal community composition (Toljander et al., 2006), as is the presence of metal-pollution (Hui et al., 2011). Similar to the findings in our studies, Hui et al. (2011) did not observe a strong effect of metal pollution on fungal diversity. This discrepancy between effects of metal pollution on fungal community composition and the absence of an effect on fungal diversity suggests that in metal-polluted sites certain fungal species are replaced by fungi with similar ecological niches, but with a greater capacity to adapt to high concentrations of metal ions. The shift from a community dominated by (among others) *Suillus luteus* in the metal-polluted site to a dominance of its sister species, *Rhizopogon luteolus*, in the control site could be an example of the selection by heavy metal pollution for adapted species. However, it needs to be noted that some studies did report detrimental effects of metal pollution on fungal diversity (Chodak et al., 2013). Differences in reports on the effects of metal pollution on fungal diversity may be due to differences in the metal species involved and the actual toxicity of metal species in certain ecosystems. For

example, microbial communities and abiotic variables, such as soil moisture content, pH, cation exchange capacity, etc. may influence the availability, and hence, the toxicity of metal ions (Gadd, 1993). In our study site in Lommel-Maatheide, Zn and Cd concentrations were clearly phytotoxic as a number of the present pine trees were already fully necrotic within one year after their plantation in 2008.

Dominant species of studied ectomycorrhizal communities

Overall, the observed OTU richness in our studies was low compared to the richness of fungal communities that are reported from older forest soils. Whereas in our studies between 500 and 800 fungal OTUs were detected across study sites and sampling years, Hartmann et al. (2014) identified well over 1000 OTUs from a *Fagus sylvatica* and *Picea abies* forest in Switzerland and Vořísková et al. (2014) identified over 2500 OTUs in a *Quercus petrea* forest in the Czech Republic. However, a relatively low number of OTUs is probably typical for young pioneer forests (Kipfer et al., 2011). Furthermore, all study sites were clearly dominated by only a few OTUs with the remaining OTUs having low relative abundances. Such abundance patterns are considered to be typical for ectomycorrhizal fungal communities (Horton and Bruns, 2001). Both from root tip samples and soil samples it was also clear that the studied fungal communities were dominated by Suilloid fungi, confirming their importance as primary root colonizers of pine trees thriving in pioneer conditions (Ashkannejhad and Horton, 2006). Substantial differences were observed between the fungal communities identified in soils samples and those detected in root tip samples. These differences most likely reflect differences in exploration types and life history traits of the fugal species present in the investigated sites (Genney et al., 2006). Interestingly, a number of the fungal species that were found to be dominant members of the communities thriving in metal-polluted soils have been identified from metal-polluted areas or mine spoils in previous studies. Almost invariably, fungal communities identified in soil samples were dominated by a *Sistotrema* sp. strain (B216). This

strain was described as an ectomycorrhizal fungus occurring in pioneer sand dunes in the USA (Ashkannejhad and Horton, 2006), but in general *Sistotrema* species are more often associated with wood and pine needle decay. Species in this genus appear to be frequently associated with heath vegetation and typically occur in very dry habitat (Ashkannejhad and Horton, 2006; Ryberg et al., 2011). *Sistotrema* species show hardly any formation of true mycorrhizal root tips and their status as true ectomycorrhizas is still under debate (Potvin et al., 2012). In our studies, this genus was readily detected in soil samples but it was shown to be far less dominant on collected pine roots, suggesting that this genus may indeed not form substantial ectomycorrhizal mycelium on pine roots. Nevertheless, this species was almost equally abundant in soil samples in the metal-polluted site than in the control site. The abundance of this *Sistotrema* strain makes it an interesting subject to study metal-resistance and/or metal-tolerance mechanisms. *Suillus luteus* was shown to be a dominant member of the fungal community identified in soil samples and on root tips in the metal-polluted site as well. In the control site, however, it was found to be far less dominant. Instead, *Rhizopogon luteolus* appears to have replaced the dominant position of *S. luteus* in the control site. Both ectomycorrhizal species are typical primary pioneer species that can rapidly colonize young pine forests. The dominant position of *S. luteus* was expected, since fruiting bodies of this species can be frequently found on the metal-polluted sites in the Lommel area (Colpaert et al, 2004; Krznaric et al., 2009). A relatively low abundance of *R. luteolus* in the polluted site in Lommel-Maatheide could be explained by the fact that it is less adapted to high metal concentrations. Indeed, in a previous study we recorded a higher *in vitro* adaptation potential for Zn tolerance in *S. luteus* than in *R. luteolus* (Colpaert et al., 2004). On the other hand, extensive gene-flow between different populations of *S. luteus* in Lommel was detected previously (Muller et al., 2004), indicating frequent exchange of spores between heavy metal-adapted and unadapted populations. In contrast to *S. luteus*, *R. luteolus* forms hypogeous fruiting bodies and dispersal of

metal-tolerance genes may be therefore be more limited than is the case for *S. luteus*. Population genetics studies could cast some light on the dispersal of metal-tolerance genes in the future. The species that were found to be dominant members of the fungal communities thriving in the metal-polluted soils in our community studies are potential candidates for such population genetics studies, given that they do indeed possess adaptive metal tolerance and that they can be cultured *in vitro*. *Wilcoxina mikolae* is an ectomycorrhizal ascomycete that has been identified on pine roots in a number of studies. In accordance with observations from other studies, this species is often found to be a dominant member of ectomycorrhizal fungal communities (Nguyen, 2012). Even though *W. mikolae* has been detected in mine-spoils in the past (Prabhu et al 1996) and in our studies this species was found to be the third most abundant species, both in metal-polluted and non-polluted soil samples, its resistance or tolerance to increased concentrations of metal ions has not yet been assessed. *Cadophora finlandica* is an ascomycete species that can form mycorrhizal structures with ectomycorrhizal and ericoid mycorrhizal plants and is part of a species complex termed the *Rhizoscyphus ericae* aggregate (REA; Vrålstad et al., 2000). This species is frequently isolated from metal-polluted soils (Gorfer et al., 2009) and potential Zn- and Cd detoxification mechanisms have been studied, attributing metal-resistance to several extracellular proteins with unknown functions and plasma-membrane and endomembrane localized metal-transport proteins (Gorfer et al., 2009). *C. finlandica* was found to be more dominant in root tips than in soil samples and occurred frequently in the metal-polluted site in Lommel-Maatheide. However, it was not specifically associated with this site as it also appeared in the list of top ten most abundant OTUs identified from root tips in the control site in Hechtel-Eksel. Similar to *C. finlandica*, *Rhizoscyphus ericae* is a member of the REA aggregate (Vrålstad et al., 2000) and it is frequently identified in conifer roots. *R. ericae* is known to be able to form both ectomycorrhizal and ericoid mycorrhizal symbioses with different plant species (Grelet et al., 2010). Our studies are the first to report a frequent

occurrence of this species in metal-polluted soils. In our studies more reads belonging to this species were found on pine roots than in soil samples, but reads identified as *R. ericae* were almost equally abundant in the metal-polluted site than in the control site. Finally, also *Inocybe lacera* appeared in the list of top ten most abundant OTUs for both sampling years, but it was clearly more abundant in the metal-polluted site than in the control site. Both for soil and root tip samples, similar abundance patterns were observed. *Inocybe* species are ectomycorrhizal basidiomycetes that typically occur during early stages of primary and secondary succession (Jumpponen et al., 2002). Even though sister species of *I. lacera* (*I. curvipes*) have been identified in Pb-Zn mine sites in China (Huang et al., 2012), *I. lacera* itself has not yet been reported from metal-polluted sites. Since the above-mentioned fungal species are probably resistant to some degree to high concentrations of metal ions and since they typically occur during early stages of plant development, they could be very useful to improve phytoremediation and/or phytostabilisation of metal-polluted areas. Recent studies focusing on phytoremediation of metal-polluted soils have illustrated that mycorrhiza can protect their host plants from metal-toxicity and even enhance the efficiency of phytoremediation by enhancing plant growth, the mobilization of metal ions and the translocation of metals to plants (Leung et al., 2013).

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APPENDIX A

BLAST hit References, E-values and corresponding BLAST scores for all OTUs identified at a 97% sequence similarity cut-off in the study presented in chapter 3. OTUs were blasted against the UNITE and INSD databases using the massBLASTer tool available from PlutoF v2.0 (http://unite.ut.ee/workbench.php). OTUs that did not have a BLAST hit in the UNITE or INSD databases were indicated as "not applicable (NA)".

Reference name	Score	E-value
Abrothallus suecicus	318	5.E-86
Absconditella lignicola	168	1.E-40
Absidia caerulea	470	1.E-131
Acanthostigma perpusillum	311	8.E-84
Acephala macrosclerotiorum	436	1.E-121
Acremonium sp BCC 14080	340	1.E-92
Acremonium strictum	411	1.E-113
Agaricus pinsitus	542	4.E-153
Alatospora acuminata	180	3.E-44
Aliquandostipite khaoyaiensis	87	2.E-16
Alternaria alternata	412	2.E-114
Alternaria consortialis	481	5.E-135
Alternaria rosae	484	5.E-136
Alternaria tenuissima	486	2.E-136
Alternaria triticina	462	2.E-129
Amanita muscaria	586	2.E-166
Anhellia nectandrae	355	7.E-97
Antarctic yeast CBS 8941	473	1.E-132
Anthracothecium prasinum	88	2.E-16
Arachnopeziza aurata	396	3.E-109
Arnium gigantosporum	288	5.E-77
Arnium macrotheca	467	9.E-131
Arthrinium sacchari	477	1.E-133
Arthrobotrys oligospora	86	1.E-15
Articulospora proliferata	431	8.E-120
Ascobrunneispora aquatica	229	4.E-59
Ascomycota sp 6 RB 2011	383	1.E-105
Ascomycota sp AM12	374	2.E-102
Ascomycota sp AR 2010	420	2.E-116
Ascomycota sp ARIZ OCAsh3 11	393	2.E-108
Ascomycota sp CH Co12	457	1.E-127
Ascomycota sp GMU LL 02 B3	443	1.E-123
Ascomycota sp I306	391	6.E-108
Ascomycota sp r433	150	2.E-35
Aspergillus viridinutans	497	8.E-140
Aspicilia cinerea	252	7.E-66
Aspicilia dendroplaca	265	6.E-70
Aspicilia verruculosa	320	2.E-86
Asteridiella obesa	75	2.E-12

Asterophora sp aurim714	332	3.E-90
Athelia epiphylla	420	2.E-116
Athelopsis lembospora	484	6.E-136
Aulographina pinorum	361	8.E-99
Aureobasidium pullulans	480	1.E-134
Bacidina chloroticula	392	3.E-108
Bacidina flavoleprosa	142	5.E-33
Barriopsis fusca	237	1.E-61
Basidiodendron caesiocinereum	163	4.E-39
Beauveria pseudobassiana	468	5.E-131
Bellemerea alpina	196	2.E-49
Belonopsis eriophori	359	3.E-98
Betamyces americaemeridionalis	193	5.E-48
Bipolaris sorokiniana	391	6.E-108
Bjerkandera fumosa	510	9.E-144
Boletus edulis	732	0.E+00
Botryobambusa fusicoccum	375	3.E-103
Botryosporium longibrachiatum	97	3.E-19
Botryotinia fuckeliana	462	2.E-129
Bovista dermoxantha	547	7.E-155
Brunneodinemasporium brasiliense	337	1.E-91
Bryoglossum gracile	348	4.E-95
Bullera globispora	245	5.E-64
Bullera sakaeratica	226	3.E-58
Cadophora finlandica	454	6.E-127
Calcarisporiella thermophila	161	2.E-38
Calcarisporium arbuscula	336	4.E-91
Calyptrozyma arxii	351	5.E-96
Camarographium koreanum	320	2.E-86
Candida homilentoma	86	8.E-16
Candida novakii	240	2.E-62
Candida ontarioensis	103	6.E-21
Candida santamariae	527	7.E-149
Capronia pulcherrima	337	1.E-91
Capronia sp 94003b	420	1.E-116
Capronia sp 94006a	288	8.E-77
Capronia sp 96003a	366	2.E-100
Catenulifera brachyconia	426	2.E-118
Catenulifera brevicollaris	329	2.E-89
Catenulostroma hermanusense	444	8.E-124
Catenulostroma microsporum	465	3.E-130
Catenulostroma protearum	391	7.E-108
Cenococcum geophilum	431	8.E-120
Ceratocystis paradoxa	480	1.E-134
Cercophora sulphurella	168	1.E-40
Cercospora sophorae	551	5.E-156
Chaenothecopsis pusiola	65	1.E-09
Chaetomidium arxii	453	1.E-126
Chaetomium aureum	486	2.E-136
Chaetomium cupreum	431	6.E-120
Chaetomium jodhpurense	370	1.E-101
Chaetomium nigricolor	473	1.E-132
Chaetosphaeria bombycina	253	2.E-66
Chaetosphaeria dilabens	331	9.E-90

Chaetothyriales sp 16708	484	5.E-136
Chalara microspora	462	2.E-129
Chalara pseudoaffinis	410	1.E-113
Chlamydotubeufia khunkornensis	180	2.E-44
Chlorociboria argentinensis	215	4.E-55
Chrysosporium merdarium	415	5.E-115
Chrysosporium pseudomerdarium	401	5.E-111
Cistella acuum	294	8.E-79
Cistella grevillei	391	7.E-108
Cistella spicicola	344	8.E-94
Cladonia borealis	446	2.E-124
Cladonia coniocraea	63	6.E-09
Cladonia diversa	407	1.E-112
Cladonia fimbriata	458	8.E-128
Cladonia foliacea	416	2.E-115
Cladonia furcata	350	1.E-95
Cladonia gracilis	443	1.E-123
Cladonia gracilis subsp elongata	448	5.E-125
Cladonia grayi	491	7.E-138
Cladonia merochlorophaea	340	1.E-92
Cladophialophora chaetospira	391	6.E-108
Cladophialophora humicolae	665	0.E+00
Cladophialophora minutissima	442	3.E-123
Cladophialophora modesta	207	1.E-52
Cladophialophora scillae	285	6.E-76
Cladosporium oxysporum	367	8.E-101
Claroideoglomus claroideum	57	5.E-07
Claviradulomyces dabeicola	237	2.E-61
Clitocybe vermicularis	223	3.E-57
Clitopilus hobsonii	434	1.E-120
Clonostachys rosea	497	8.E-140
Coccomyces mucronatus	131	8.E-30
Coemansia asiatica	58	1.E-07
Coleophoma eucalyptorum	364	9.E-100
Collophora hispanica	386	2.E-106
Collophora paarla	388	6.E-107
Coniochaeta gigantospora	442	4.E-123
Coniochaeta prunicola	169	5.E-41
Coniothyrium fuckelii	465	3.E-130
Conlarium duplumascospora	390	1.E-107
Conocybe echinata	545	3.E-154
Coprinellus disseminatus	559	2.E-158
Coprinellus verrucispermus	523	1.E-147
Coprinopsis atramentaria	546	1.E-154
Coprinopsis cinerea	545	4.E-154
Cordana pauciseptata	291	1.E-77
Cordyceps memorabilis	380	1.E-104
Cortinarius odorifer	168	2.E-40
Cortinarius parvannulatus	516	2.E-145
Corynascus kuwaitiensis	516	2.E-145
Cosmospora vilior	245	4.E-64
Cryptococcus	324	6.E-88
Cryptococcus aerius	559	2.E-158
Cryptococcus aff amylolyticus AS 22398	484	6.E-136

Cryptococcus aff laurentii D 0721a1	191	1.E-47
Cryptococcus cylindricus	560	1.E-158
Cryptococcus dimennae	204	2.E-51
Cryptococcus flavus	198	7.E-50
Cryptococcus laurentii	492	3.E-138
Cryptococcus paraflavus	258	9.E-68
Cryptococcus podzolicus	489	2.E-137
Cryptococcus randhawii	578	4.E-164
Cryptococcus sp BI20	196	2.E-49
Cryptococcus sp VPCI 1367 B1	111	8.E-24
Cryptococcus terricola	604	5.E-172
Cryptococcus victoriae	416	2.E-115
Cryptococcus wieringae	599	2.E-170
Cryptosporiopsis actinidiae	380	2.E-104
Cudoniella acicularis	399	3.E-110
Cudoniella clavus	378	4.E-104
Curreya pityophila	369	3.E-101
Cyphellophora hylomeconis	385	6.E-106
Dactylaria lanosa	340	1.E-92
Dactylella oviparasitica	163	4.E-39
Dactylellina drechsleri	504	1.E-141
Dactylellina ellipsospora	478	4.E-134
Dactylellina phymatopaga	174	1.E-42
Daldinia fissa	396	2.E-109
Davidiella tassiana	436	1.E-121
Debaryomyces hansenii	529	2.E-149
Debaryomyces polymorphus var polymorphus	543	1.E-153
Degelia gayana	313	2.E-84
Devriesia pseudoamericana	369	3.E-101
Dinemasporium morbidum	480	1.E-134
Dinemasporium pseudostrigosum	439	4.E-122
Dinemasporium strigosum	400	8.E-111
Dioszegia athyri	425	3.E-118
Dioszegia rishiriensis	415	4.E-115
Dokmaia monthadangii	459	2.E-128
Dothideomycetes sp DC2167	448	6.E-125
Dothideomycetes sp genotype 188	313	2.E-84
Drechslera erythrospila	475	4.E-133
Drechslera nobleae	329	3.E-89
Drechslera poae	381	6.E-105
Elsinoe ampelina	320	2.E-86
Emericella nidulans	381	6.E-105
Emericella purpurea	125	7.E-28
Emmonsia parva	519	2.E-146
Entoloma conferendum	439	4.E-122
Epacris microphylla root associated fungus 12	453	2.E-126
Epacris microphylla root associated fungus 17	472	3.E-132
Epacris microphylla root associated fungus 21	431	8.E-120
Epacris microphylla root associated fungus 26	425	3.E-118
Epacris microphylla root associated fungus 33	488	4.E-137
Epacris pulchella root associated fungus EP20	490	1.E-137
Epacris pulchella root associated fungus EP26	167	2.E-40
Epacris pulchella root associated fungus EP54	448	5.E-125
Epacris pulchella root associated fungus EP55	401	5.E-111

Epicoccum nigrum	347	1.E-94
Eudarluca caricis	212	5.E-54
Exobasidium kishianum	455	4.E-127
Exobasidium rostrupii	443	1.E-123
Exophiala bergeri	418	5.E-116
Exophiala equina	215	6.E-55
Exophiala eucalyptorum	491	7.E-138
Exophiala placitae	389	2.E-107
Exophiala sideris	462	3.E-129
Filobasidium uniguttulatum	622	2.E-177
Friedmanniomyces endolithicus	367	1.E-100
fungal endophyte	340	1.E-92
fungal sp 2747 YZ 2011	416	2.E-115
fungal sp ARIZ AZ0780	433	2.E-120
fungal sp NLEndoHerit 014 2008N5 26 2N	327	8.E-89
Funneliformis geosporum	112	7.E-24
Fusarium biseptatum	355	7.E-97
Fusarium culmorum	355	5.E-97
Fusarium equiseti	322	5.E-87
Fusarium merismoides	457	1.E-127
Fusarium oxysporum	448	6.E-125
Fusarium torulosum	497	8.E-140
Fusicladium cordae	502	3.E-141
Fusicladium phillyreae	213	2.E-54
Gaertneriomyces semiglobifer	81	4.E-14
Gammamyces ourimbahensis	86	6.E-16
Ganoderma adspersum	538	4.E-152
Ganoderma applanatum	542	4.E-153
Geoglossum atropurpureum	174	1.E-42
Geomyces pannorum	462	2.E-129
Geomyces vinaceus	442	3.E-123
Geopora clausa	82	6.E-15
Geosmithia langdonii	183	2.E-45
Geosmithia putterillii	137	2.E-31
Glomerella graminicola	453	1.E-126
Glomus custos	126	4.E-28
Glomus intraradices	106	6.E-22
Glomus sp 3 SUN 2011	95	9.E-19
Guignardia citricarpa	95	8.E-19
Helicodendron multiseptatum	440	1.E-122
Helotiaceae sp IV GK 2010	320	1.E-86
Helotiales sp 1 CG 2012	312	4.E-84
Helotiales sp 1 MV 2011	432	3.E-120
Helotiales sp 16 MV 2011	434	9.E-121
Helotiales sp 2 BB 2010	378	4.E-104
Helotiales sp 27 MV 2011	444	8.E-124
Helotiales sp 5 CG 2012	318	4.E-86
Helotiales sp I12F 02299	305	4.E-82
Helotiales sp MU 2009 3	345	4.E-94
Helotiales sp ODKB3	440	1.E-122
Helotiales sp PIMO 265	247	1.E-64
Helotiales sp REF045	405	3.E-112
Helotiales sp REF055	315	4.E-85
Helotiales sp SC3 4	467	9.E-131

Helotiales sp SC9 3	388	5.E-107
Helotiales sp SL11101	233	2.E-60
Helotiales sp WMM 2012b	443	1.E-123
Helotiales sp WMM 2012g	336	3.E-91
Helvella maculata	291	8.E-78
Herpotrichiellaceae sp RB 2011	337	1.E-91
Heterobasidion annosum	415	4.E-115
Hirsutella minnesotensis	497	8.E-140
Homortomyces combreti	215	6.E-55
Humicolopsis cephalosporioides	404	1.E-111
Hyaloscypha hepaticola	385	4.E-106
Hyaloscypha sp 2 13c	366	3.E-100
Hydnotrya tulasnei	551	5.E-156
Hyphodontia breviseta	385	6.E-106
Hypholoma fasciculare	567	9.E-161
Hypocrea lixii	510	1.E-143
Hypocrea voglmayrii	259	3.E-68
Hypogymnia inactiva	148	6.E-35
Hypomyces cervinigenus	326	3.E-88
Infundichalara microchona	405	4.E-112
Inocybe lacera var lacera	559	2.E-158
Inocybe ochroalba	320	2.E-86
Johansonia chapadiensis	150	3.E-35
Knufia chersonesos	449	2.E-125
Kockovaella schimae	383	2.E-105
Kurtzmanomyces nectairei	215	8.E-55
Lachnum brevipilosum	301	7.E-81
Lachnum sp 1 MV 2011	453	2.E-126
Lachnum sp 252	318	5.E-86
Lachnum virgineum	150	2.E-35
Lasiosphaeria ovina	248	6.E-65
Lecanicillium psalliotae	492	2.E-138
Lecythophora sp BESC803p	446	2.E-124
Lecythophora sp YP363	271	1.E-71
Lenzites betulinus	556	2.E-157
Leotiomycetes sp ASR H18 12A	307	1.E-82
Leotiomycetes sp F21	321	7.E-87
Leotiomycetes sp genotype 134	364	6.E-100
Leotiomycetes sp NK264	462	2.E-129
Leotiomycetes sp NK266	420	2.E-116
Lepraria aff obtusatica BRY C56005	94	3.E-18
Lepraria elobata	228	9.E-59
Leptosphaeria doliolum	296	3.E-79
Leptosphaeria korrae	351	5.E-96
Leptosphaerulina chartarum	465	3.E-130
Leuconeurospora sp T11Cd2	377	1.E-103
Limnoperdon incarnatum	257	1.E-67
Linderina macrospora	71	2.E-11
Lobaria retigera	241	1.E-62
Lobariella pallida	267	1.E-70
Lophiostoma chamaecyparidis	374	1.E-102
Lophiostoma cynaroidis	294	8.E-79
Lophodermium baculiferum	79	8.E-14
Lophodermium conigenum	248	5.E-65

Lophodermium pinastri	464	8.E-130
Lophodermium seditiosum	451	5.E-126
Lycoperdon aff pyriforme Scl 6 1 1L	553	2.E-156
Lyophyllum sp Cultivar Jpn	57	6.E-07
Macroconia leptosphaeriae	250	1.E-65
Magnaporthe poae	147	3.E-34
Malassezia globosa	579	3.E-164
Malassezia restricta	678	0.E+00
Malassezia sympodialis	651	0.E+00
Massariosphaeria typhicola	536	2.E-151
Melanelixia piliferella	168	1.E-40
Melanocarpus albomyces	386	2.E-106
Melanotaenium euphorbiae	103	6.E-21
Meliniomyces bicolor	459	2.E-128
Meliniomyces sp GK 2010	183	2.E-45
Meliniomyces sp SM7 2	392	3.E-108
Meliniomyces variabilis	383	2.E-105
Meliniomyces vraolstadiae	351	5.E-96
Metarhizium flavoviride	340	1.E-92
Metschnikowia hawaiiensis	87	2.E-16
Metschnikowia pulcherrima	89	8.E-17
Micarea denigrata	296	2.E-79
Micarea hedlundii	225	8.E-58
Microdochium bolleyi	391	6.E-108
Microscypha ellisii	375	3.E-103
Microsphaeropsis arundinis	459	2.E-128
Mollisia incrustata	237	1.E-61
Monoblepharis hypogyna	183	2.E-45
Mortierella alpina	573	1.E-162
Mortierella angusta	617	6.E-176
Mortierella bainieri	381	1.E-104
Mortierella cystojenkinii	603	2.E-171
Mortierella elongata	636	0.E+00
Mortierella jenkinii	141	2.E-32
Mortierella lignicola	612	3.E-174
Mortierella macrocystis	595	3.E-169
Mortierella parvispora	616	2.E-175
Mortierella polycephala	77	5.E-13
Mortierella turficola	436	2.E-121
Mortierellaceae sp PDKB9	612	3.E-174
Mucor moelleri	122	1.E-26
Mycena arcangeliana	569	3.E-161
Mycena corynephora	154	2.E-36
Mycena epipterygia	562	3.E-159
Mycena galopus	569	3.E-161
Mycena maurella	94	4.E-18
Mycena meliigena	423	3.E-117
Mycena metata	416	2.E-115
Mycena simia	547	7.E-155
Mycoblastus sanguinarioides	202	4.E-51
Myrmecridium banksiae	402	3.E-111
Myrmecridium phragmitis	293	3.E-78
Myrmecridium schulzeri	445	4.E-124
Myrothecium gramineum	206	5.E-52

NA	NA	NA
Naemacyclus niveus	464	8.E-130
Neocallimastix sp H GFM 2	97	4.E-19
Noosia banksiae	388	7.E-107
Ochrolechia juvenalis	153	2.E-36
Ogataea chonburiensis	126	3.E-28
Oidiodendron	0	0.E+00
Oidiodendron chlamydosporicum	439	3.E-122
Oidiodendron griseum	324	6.E-88
Oidiodendron pilicola	435	3.E-121
Oidiodendron reticulatum	454	6.E-127
Oidiodendron tenuissimum	97	2.E-19
Ophiocordyceps entomorrhiza	148	5.E-35
Ophiocordyceps irangiensis	63	4.E-09
Ophiocordyceps nutans	98	1.E-19
Ophiocordyceps prolifica	502	3.E-141
Ophiostoma bicolor	100	5.E-20
Paraconiothyrium brasiliense	145	7.E-34
Paraconiothyrium sporulosum	455	4.E-127
Paraphaeosphaeria michotii	383	1.E-105
Parastagonospora nodorum	361	7.E-99
Passalora zambiae	252	5.E-66
Penicillium aculeatum	457	1.E-127
Penicillium adametzii	494	7.E-139
Penicillium bialowiezense	423	2.E-117
Penicillium brevicompactum	228	9.E-59
Penicillium canescens	480	1.E-134
Penicillium cecidicola	477	8.E-134
Penicillium citreonigrum	393	2.E-108
Penicillium concentricum	150	2.E-35
Penicillium corylophilum	483	2.E-135
Penicillium herquei	351	5.E-96
Penicillium janthinellum	412	4.E-114
Penicillium lanosum	492	2.E-138
Penicillium montanense	490	1.E-137
Penicillium namyslowskii	494	7.E-139
Penicillium ochrochloron	497	8.E-140
Penicillium raistrickii	403	1.E-111
Penicillium simile	422	4.E-117
Penicillium tularense	423	2.E-117
Penidiella ellipsoidea	147	1.E-34
Peniophora lycii	444	9.E-124
Pesotum fragrans	82	5.E-15
Pezicula carpinea	258	8.E-68
Peziza ostracoderma	448	7.E-125
Pezizella discreta	294	7.E-79
Pezizomycetes sp genotype 454	443	1.E-123
Phaeoacremonium griseorubrum	224	1.E-57
Phaeococcomyces catenatus	542	4.E-153
Phaeococcomyces chersonesos	201	1.E-50
Phaeococcomyces eucalypti	307	1.E-82
Phaeococcomyces nigricans	381	5.E-105
Phaeosphaeria avenaria	453	2.E-126
Phaeosphaeria herpotrichoides	449	2.E-125

Phaeosphaeria nodorum	451	5.E-126
Phaeosphaeria phragmitis	414	6.E-115
Phaeosphaeriopsis sp C652	453	2.E-126
Phaeosphaeriopsis sp TMS 2011	323	2.E-87
Phaeotheca fissurella	377	8.E-104
Phellinopsis conchata	183	2.E-45
Phellinus pomaceus	92	1.E-17
Phialemonium curvatum	275	5.E-73
Phialocephala fortinii	436	1.E-121
Phialocephala fusca	396	2.E-109
Phialocephala virens	337	1.E-91
Phialophora lignicola	336	3.E-91
Phialophora phaeophora	532	2.E-150
Phialophora sp DF36	446	1.E-124
Phlebia livida subsp tuberculata	161	1.E-38
Phlyctochytrium africanum	195	1.E-48
Phlyctochytrium palustre	180	3.E-44
Pholiota populnea	573	1.E-162
Phoma betae	292	3.E-78
Phoma caloplacae	396	3.E-109
Phoma herbarum	294	8.E-79
Phoma macrostoma	320	1.E-86
Phyllachora phyllostachydis	345	5.E-94
Pichia kudriavzevii	57	3.E-07
Piriformospora sp X 30	90	3.E-17
Placynthiella icmalea	442	4.E-123
Platismatia stenophylla	320	2.E-86
Plectania rhytidia	104	2.E-21
Pleopsidium chlorophanum	187	3.E-46
Pleospora herbarum	446	2.E-124
Pleosporales sp 28e	410	1.E-113
Pleosporales sp 5 TMS 2011	190	2.E-47
Pochonia bulbillosa	527	7.E-149
Pochonia suchlasporia	329	2.E-89
Podoscypha venustula	97	5.E-19
Podospora ellisiana	413	1.E-114
Podospora formosana	448	5.E-125
Podospora miniglutinans	420	1.E-116
Polyphlyctis unispina	161	1.E-38
Porosphaerella cordanophora	321	7.E-87
Powellomyces hirtus	94	5.E-18
Preussia australis	470	1.E-131
Preussia dubia	415	5.E-115
Preussia minima	343	1.E-93
Pringsheimia euphorbiae	294	8.E-79
Pseudocercosporella fraxini	367	1.E-100
Pyrenochaetopsis microspora	435	3.E-121
Pyrenophora tetrarrhenae	272	3.E-72
Pyrenula macrospora	115	7.E-25
Pyxine limbulata	99	7.E-20
Rachicladosporium pini	443	1.E-123
Ramaria abietina	111	8.E-24
Ramichloridium strelitziae	252	5.E-66
Rhexocercosporidium panacis	461	7.E-129

Rhizophlyctis harderi	86	7.E-16
Rhizophlyctis rosea	605	4.E-172
Rhizophydium globosum	82	9.E-15
Rhizophydium laterale	82	8.E-15
Rhizophydium sp JEL 385	79	9.E-14
Rhizoplaca chrysoleuca	110	2.E-23
Rhizopogon luteolus	654	0.E+00
Rhizopycnis vagum	87	2.E-16
Rhizoscyphus ericae	507	1.E-142
Rhodosporidium babjevae	340	1.E-92
Rhodotorula bloemfonteinensis	329	3.E-89
Rhodotorula cassiicola	257	1.E-67
Rhodotorula eucalyptica	386	2.E-106
Rhodotorula glutinis	483	2.E-135
Rhodotorula mucilaginosa	578	4.E-164
Rhynchostoma proteae	86	5.E-16
Rufoplaca tristiuscula	185	6.E-46
Saccharata intermedia	125	1.E-27
Sagenomella diversispora	448	6.E-125
Sagenomella humicola	489	2.E-137
Sagenomella striatispora	462	2.E-129
Sarcoscypha hosoyae	180	2.E-44
Sarea difformis	163	4.E-39
Sarea resinae	442	4.E-123
Scedosporium apiospermum	388	5.E-107
Schizothecium glutinans	271	1.E-71
Sclerotium delphinii	64	3.E-09
Scolecobasidium terreum	183	2.E-45
Scorias leucadendri	165	6.E-40
Scutellospora calospora	316	2.E-85
Scytalidium vaccinii	316	1.E-85
Sebacina grisea	92	1.E-17
Septoria digitalis	305	4.E-82
Septoria escalloniae	222	4.E-57
Septoria lamii	350	2.E-95
Sesquicillium microsporum	418	4.E-116
Siphula ceratites	183	2.E-45
Sistotrema diademiferum	290	3.E-77
Sistotrema sp B216	526	2.E-148
Sistotrema sp.	339	3.E-92
Skyttea nitschkei	283	2.E-75
Sordaria fimicola	446	2.E-124
Sordariales sp Pi GPB	261	9.E-69
Sordariomycetes sp 11344	418	5.E-116
Sordariomycetes sp DC2118	467	9.E-131
Sordariomycetes sp genotype 106	228	8.E-59
Sorocybe resinae	234	1.E-60
Spadicoides bina	212	4.E-54
Sphaerobolus iowensis	569	2.E-161
Sphaeropsis pyriputrescens	326	3.E-88
Sphaeropsis sapinea	464	6.E-130
Spizellomyces acuminatus	152	1.E-35
Spizellomyces dolichospermus	294	8.E-79
Spizellomyces lactosolyticus	121	2.E-26

Spizellomyces palustris	209	7.E-53
Spizellomyces plurigibbosus	163	3.E-39
Spizellomyces pseudodichotomus	264	1.E-69
Spizellomyces sp JEL 148	81	3.E-14
Sporendocladia foliicola	269	3.E-71
Sporobolomyces gracilis	553	2.E-156
Sporobolomyces inositophilus	445	5.E-124
Sporobolomyces lactophilus	290	3.E-77
Sporobolomyces tsugae	499	3.E-140
Sporormiella sp FBl04	472	3.E-132
Squamarina gypsacea	180	2.E-44
Staphylotrichum boninense	232	2.E-60
Stictis radiata	291	9.E-78
Stilbum vulgare	123	2.E-27
Strelitziana mali	247	2.E-64
Stropharia cyanea	553	2.E-156
Suillus bovinus	611	5.E-174
Suillus luteus	611	5.E-174
Sydowia polyspora	488	6.E-137
Sympodiella acicola	464	8.E-130
Syzygospora bachmannii	291	1.E-77
Syzygospora effibulata	108	1.E-22
Talaromyces verruculosus	480	1.E-134
Talaromyces wortmannii	480	1.E-134
Teratosphaeria capensis	477	1.E-133
Teratosphaeria jonkershoekensis	394	8.E-109
Teratosphaeria persoonii	136	3.E-31
Thelephora	581	5.E-165
Thelephora terrestris	577	1.E-163
Thelotrema lepadinum	71	1.E-11
Thielavia fragilis	348	5.E-95
Tolypocladium cylindrosporum	451	5.E-126
Tolypocladium inflatum	434	1.E-120
Trechispora hymenocystis	390	1.E-107
Trechispora stevensonii	418	7.E-116
Trechispora subsphaerospora	423	2.E-117
Tremella brasiliensis	206	4.E-52
Tremella diploschistina	225	8.E-58
Tremella giraffa	240	2.E-62
Tremella taiwanensis	126	3.E-28
Trichocladium asperum	375	3.E-103
Trichoderma atroviride	38	1.E-01
Trichoderma koningiopsis	481	6.E-135
Trichoderma petersenii	412	4.E-114
Trichomerium deniqulatum	434	1.E-120
Trichomerium gleosporum	331	1.E-89
Trichopezizella otanii	176	3.E-43
Trichosporon debeurmannianum	147	3.E-34
Trichosporon porosum	494	7.E-139
Trichothecium roseum	133	6.E-30
Tricladium chaetocladium	191	9.E-48
Truncatella angustata	462	2.E-129
Tuber cistophilum	90	3.E-17
Umbelopsis autotrophica	548	5.E-155

Umbelopsis isabellina	543	1.E-153
Umbelopsis ramanniana	548	5.E-155
Umbelopsis sp I GK 2010	226	2.E-58
Umbilicaria calvescens	242	5.E-63
uncultured Archaeospora	520	1.E-146
uncultured Archaeosporales	580	1.E-164
uncultured Archaeosporales	531	7.E-150
uncultured Chytridiaceae	53	8.E-06
uncultured Cladosporium	446	2.E-124
uncultured Cladosporium	470	1.E-131
uncultured Dermateaceae	438	4.E-122
uncultured ectomycorrhizal fungus	386	2.E-106
uncultured fungus	576	1.E-163
uncultured fungus	664	0.E+00
uncultured fungus	400	8.E-111
uncultured Helotiaceae	418	5.E-116
uncultured Helotiaceae	453	2.E-126
uncultured Hypocreales	88	1.E-16
uncultured Leotiomycetes	462	2.E-129
uncultured Leptodontidium	469	3.E-131
uncultured Mortierella	183	3.E-45
uncultured Phaeococcomyces	229	3.E-59
uncultured Phialophora	367	8.E-101
uncultured Sebacina	489	2.E-137
uncultured Sebacina mycobiont of Trifolium pratense	418	5.E-116
uncultured Sebacinaceae	497	7.E-140
uncultured soil fungus	600	1.E-170
uncultured soil fungus	223	3.E-57
uncultured soil fungus	610	1.E-173
uncultured Trechisporales	554	6.E-157
uncultured Trechisporales	547	7.E-155
Unguiculariopsis lettaui	200	2.E-50
Urocystis agropyri	619	2.E-176
Vermispora fusarina	263	4.E-69
Verrucaria subcrustosa	231	1.E-59
Verrucariales sp RB 2011	241	1.E-62
Verticillium leptobactrum	518	5.E-146
Wallemia sebi	486	2.E-136
Wilcoxina mikolae	475	4.E-133
Wojnowicia sp NW 2013	331	8.E-90
Xanthoria parietina	172	5.E-42
Xenobotrytis acaducospora	250	2.E-65
Xenochalara juniperi	437	1.E-121
Xenopolyscytalum pinea	416	2.E-115
Xylaria globosa	97	3.E-19
Xylaria intracolorata	313	2.E-84
Xylodon sambuci	381	7.E-105
Zeloasperisporium hyphopodioides	213	2.E-54
Zopfiella tabulata	171	2.E-41
Zychaea mexicana	62	1.E-08

APPENDIX B

BLAST hit References, average relative read abundances, E-values and corresponding BLAST scores for all OTUs identified at a 97% sequence similarity cut-off in the study presented in chapter 4. OTUs were blasted against the UNITE and INSD databases using the massBLASTer tool available from PlutoF v2.0 (http://unite.ut.ee/workbench.php). OTUs that did not have a BLAST hit in the UNITE or INSD databases were indicated as "not applicable (NA)".

Reference name	Average relative abundance %	Standard error on average relative abundance	Score	E-value
Abrothallus suecicus	0.001	6.6E-07	318	4.E-86
Absidia caerulea	0.001	9.9E-07	492	2.E-138
Acanthostigma chiangmaiensis	0.001	6.6E-07	189	3.E-47
Acanthostigma perpusillum	0.007	8.0E-07	311	6.E-84
Acaulospora lacunosa	0.001	6.6E-07	420	1.E-116
Acephala applanata	0.001	6.6E-07	344	6.E-94
Acephala macrosclerotiorum	0.890	1.3E-05	436	9.E-122
Acremonium cavaraeanum	0.040	3.5E-06	298	5.E-80
Acremonium cellulolyticus	0.004	2.6E-06	457	1.E-127
Acremonium psammosporum	0.018	1.4E-06	104	1.E-21
Acremonium strictum	0.002	8.4E-07	492	2.E-138
Acrodontium crateriforme	0.001	9.9E-07	425	2.E-118
Acrostalagmus luteoalbus	0.001	6.6E-07	507	8.E-143
Agaricus pinsitus	1.000	2.4E-05	529	2.E-149
Agrocybe pediades	0.002	6.6E-07	547	5.E-155
Agrocybe vervacti	0.001	9.9E-07	298	6.E-80
Alatospora acuminata	0.001	6.6E-07	427	6.E-119
Alternaria alternata	0.002	6.6E-07	455	3.E-127

Alternaria lini	0.014	1.1E-06	462	2.E-129
Alternaria linicola	0.001	9.9E-07	90	3.E-17
Alternaria longipes	0.012	1.4E-06	464	5.E-130
Alternaria triticina	0.005	1.3E-06	457	8.E-128
Amanita muscaria	0.013	1.4E-06	586	1.E-166
Amauroascus mutatus	0.001	6.6E-07	455	3.E-127
Ambispora leptoticha	0.001	6.6E-07	512	2.E-144
Amphinema sp 7 UK 2011	0.140	3.5E-05	507	8.E-143
Antarctic yeast CBS 8941	0.210	1.0E-05	442	3.E-123
Antennariella placitae	0.012	2.2E-06	375	2.E-103
Anthostomella eucalyptorum	0.036	1.7E-05	344	6.E-94
Anthostomella pinea	0.008	1.1E-06	424	8.E-118
Aphanoascus fulvescens	0.056	4.8E-06	455	3.E-127
Aphyllophorales sp EXP0530F	0.001	6.6E-07	327	6.E-89
Apiosordaria otanii	0.014	1.6E-06	353	1.E-96
Apiospora montagnei	0.007	1.2E-06	453	1.E-126
Apodospora peruviana	0.110	3.6E-06	213	2.E-54
Aquaticola hongkongensis	0.057	2.7E-06	230	2.E-59
Archaeospora sp	0.001	9.9E-07	484	6.E-136
Archaeospora sp isa33	0.012	1.9E-06	189	4.E-47
Archaeospora trappei	0.002	6.6E-07	496	2.E-139
Armillaria novae zelandiae	0.002	8.4E-07	158	1.E-37
Arnium macrotheca	0.061	2.9E-06	438	3.E-122
Arthrinium arundinis	0.028	2.5E-06	453	1.E-126
Arthrinium phaeospermum	0.002	1.6E-06	455	3.E-127
Articulospora proliferata	0.051	1.7E-06	390	7.E-108
Ascochyta hordei	0.013	1.2E-06	451	3.E-126
Ascochyta manawaorae	0.002	1.6E-06	355	3.E-97
Ascomycota sp AR 2010	0.062	1.3E-06	446	2.E-124
Ascomycota sp BBC	0.001	6.6E-07	303	1.E-81
Ascomycota sp CH Tc23	0.002	6.6E-07	241	8.E-63
Ascomycota sp Da PoC C4	0.001	9.9E-07	387	1.E-106
Ascomycota sp E8013g	0.002	1.3E-06	449	1.E-125
Ascomycota sp X69	0.001	6.6E-07	387	1.E-106

Aspicilia aquatica	0.015	9.0E-07	178	7.E-44
Aspicilia cinerea	0.002	6.6E-07	195	7.E-49
Aspicilia dendroplaca	0.005	9.1E-07	265	5.E-70
Athelia pyriformis	0.001	9.9E-07	294	8.E-79
Aulographina pinorum	0.004	7.5E-07	425	2.E-118
Aureobasidium pullulans	0.230	4.7E-06	457	7.E-128
Auricularia americana	0.007	4.6E-06	187	2.E-46
Auxarthron alboluteum	0.012	7.9E-06	283	1.E-75
Bacidina chloroticula	0.014	1.1E-06	392	2.E-108
Bahusakala australiensis	0.002	6.6E-07	435	4.E-121
Barriopsis fusca	0.004	1.5E-06	237	1.E-61
Beauveria bassiana	0.021	1.6E-06	468	4.E-131
Beauveria caledonica	0.470	4.4E-05	483	1.E-135
Bellemerea alpina	0.120	2.2E-06	202	4.E-51
Belonopsis eriophori	0.240	2.6E-06	368	4.E-101
Bensingtonia ciliata	0.002	8.4E-07	88	2.E-16
Bensingtonia musae	0.001	6.6E-07	178	9.E-44
Bensingtonia yamatoana	0.005	8.3E-07	531	5.E-150
Bionectria ochroleuca	0.010	1.3E-06	477	6.E-134
Blastobotrys adeninivorans	0.004	1.0E-06	86	5.E-16
Boletus edulis	0.002	8.4E-07	732	0.E+00
Botryosphaeria obtusa	0.007	9.4E-07	204	1.E-51
Botryotinia fuckeliana	0.006	2.4E-06	440	7.E-123
Botryozyma mucatilis	0.003	1.0E-06	94	4.E-18
Bovista dermoxantha	0.064	7.0E-06	553	1.E-156
Bryoglossum gracile	0.001	6.6E-07	401	3.E-111
Bullera globispora	0.001	6.6E-07	411	6.E-114
Bullera miyagiana	0.015	1.3E-06	335	4.E-91
Bulleromyces albus	0.002	1.3E-06	496	2.E-139
Byssoascus striatosporus	0.048	1.6E-06	191	8.E-48
Cadophora fastigiata	0.001	6.6E-07	412	2.E-114
Cadophora finlandica	3.000	2.2E-05	436	1.E-121
Calcarisporiella thermophila	0.036	1.9E-06	215	7.E-55
Caloplaca cerina	0.066	2.5E-06	126	3.E-28

Caloplaca chlorina	0.001	6.6E-07	117	2.E-25
Caloplaca stillicidiorum	0.001	9.9E-07	111	7.E-24
Calyptrozyma arxii	0.130	2.0E-06	351	3.E-96
Camarographium carpini	0.001	6.6E-07	95	8.E-19
Camarographium koreanum	0.790	6.3E-06	281	5.E-75
Camarosporula persooniae	0.001	9.9E-07	344	6.E-94
Candida morakotiae	0.007	9.7E-07	69	5.E-11
Candida psychrophila	0.001	9.9E-07	529	2.E-149
Capnobotryella sp	0.001	6.6E-07	372	4.E-102
Capronia sp 94003b	0.370	2.6E-06	425	2.E-118
Capronia sp 96003a	0.012	1.0E-06	418	4.E-116
Castanedomyces australiensis	0.001	6.6E-07	427	6.E-119
Catenulifera brachyconia	0.001	9.9E-07	374	7.E-103
Catenulifera brevicollaris	0.001	6.6E-07	331	5.E-90
Catenulostroma hermanusense	0.650	3.3E-06	449	1.E-125
Catenulostroma protearum	0.006	9.3E-07	444	6.E-124
Cenococcum geophilum	0.015	1.4E-06	399	1.E-110
Ceraceomyces fouquieriae	0.003	2.3E-06	102	6.E-21
Ceratobasidiaceae sp M468	0.001	6.6E-07	505	3.E-142
Ceratobasidium sp AG A	0.033	6.3E-06	174	1.E-42
Cercophora appalachianensis	0.002	6.6E-07	215	5.E-55
Cercospora sophorae	0.006	2.4E-06	551	4.E-156
Cetraria aculeata	0.003	2.3E-06	135	4.E-31
Cetraria sepincola	0.001	6.6E-07	239	3.E-62
Ceuthospora pinastri	0.007	9.9E-07	303	1.E-81
Chaetodermella luna	0.002	6.6E-07	156	4.E-37
Chaetomium aureum	0.040	1.9E-06	459	2.E-128
Chaetomium cupreum	0.053	3.6E-06	459	2.E-128
Chaetomium globosum	0.015	3.0E-06	453	1.E-126
Chaetomium jodhpurense	0.047	4.0E-06	370	1.E-101
Chaetomium rectangulare	0.001	9.9E-07	444	6.E-124
Chaetomium truncatulum	0.002	1.6E-06	196	2.E-49
Chaetomium undulatulum	0.001	6.6E-07	147	2.E-34
Chaetosphaeria bombycina	0.001	6.6E-07	152	4.E-36

Chalara microchona	0.460	1.3E-05	427	6.E-119
Chalara microspora	0.001	6.6E-07	431	4.E-120
Chaunopycnis alba	0.001	9.9E-07	335	4.E-91
Chaunopycnis pustulata	0.001	6.6E-07	401	4.E-111
Chlamydotubeufia huaikangplaensis	0.001	6.6E-07	161	7.E-39
Chlamydotubeufia khunkornensis	0.002	6.6E-07	191	8.E-48
Chloridium sp	0.001	6.6E-07	385	5.E-106
Chlorociboria halonata	0.005	2.1E-06	279	2.E-74
Chrysosporium keratinophilum	0.001	6.6E-07	457	7.E-128
Chrysosporium merdarium	0.003	6.5E-07	370	1.E-101
Chrysosporium pseudomerdarium	0.015	1.4E-06	407	7.E-113
Chytridiomycota sp Mori B3	0.011	4.2E-06	97	3.E-19
Cistella grevillei	0.005	6.5E-07	350	1.E-95
Cladia aggregata	0.001	6.6E-07	147	2.E-34
Cladonia borealis	0.011	1.3E-06	446	2.E-124
Cladonia coniocraea	0.001	6.6E-07	451	4.E-126
Cladonia fimbriata	0.056	1.8E-06	444	7.E-124
Cladonia foliacea	0.001	6.6E-07	416	1.E-115
Cladonia furcata	0.021	4.5E-06	475	2.E-133
Cladonia grayi	0.002	1.3E-06	466	2.E-130
Cladonia pyxidata	0.001	6.6E-07	448	5.E-125
Cladophialophora chaetospira	0.006	8.0E-07	424	8.E-118
Cladophialophora minutissima	0.910	4.1E-06	455	3.E-127
Cladophialophora modesta	0.003	1.0E-06	207	8.E-53
Cladophialophora scillae	0.002	8.4E-07	315	5.E-85
Cladosporium cladosporioides	0.072	1.2E-06	446	2.E-124
Claroideoglomus claroideum	0.003	2.0E-06	57	6.E-07
Claroideoglomus drummondii	0.002	8.4E-07	117	2.E-25
Clavaria argillacea	0.003	2.0E-06	414	8.E-115
Clavaria falcata	0.099	1.0E-05	411	8.E-114
Clitopilus hobsonii	0.360	9.1E-06	525	2.E-148
Clitopilus prunulus	0.025	4.3E-06	401	4.E-111
Clitopilus scyphoides	0.001	6.6E-07	436	2.E-121
Clitopilus sp FZ1433	0.007	1.3E-06	69	4.E-11

Clonostachys candelabrum	0.001	6.6E-07	387	1.E-106
Coccomyces dentatus	0.002	6.6E-07	340	8.E-93
Codinaeopsis gonytrichoides	0.011	1.1E-06	254	1.E-66
Coemansia sp ID05 F0205	0.001	6.6E-07	57	4.E-07
Coleophoma eucalyptorum	0.003	9.8E-07	300	1.E-80
Collophora africana	0.130	3.3E-06	340	7.E-93
Collophora capensis	0.043	3.1E-06	315	5.E-85
Collophora paarla	0.001	9.9E-07	261	6.E-69
Collophora pallida	0.012	1.6E-06	289	3.E-77
Collophora rubra	0.130	3.3E-06	327	6.E-89
Collybia cirrhata	0.001	6.6E-07	553	1.E-156
Coniochaeta africana	0.150	6.3E-06	418	4.E-116
Coniochaeta gigantospora	0.001	9.9E-07	398	7.E-110
Coniochaeta prunicola	0.260	2.1E-06	302	4.E-81
Coniochaeta sp 2 ICMP 18911	0.002	1.6E-06	259	2.E-68
Coniophora olivacea	0.002	8.4E-07	372	3.E-102
Coniosporium sp	0.002	1.3E-06	261	9.E-69
Coniothyrium fuckelii	0.470	2.3E-06	449	1.E-125
Coniothyrium nitidae	0.001	9.9E-07	265	5.E-70
Conoplea aff elegantula Kurogi sn	0.001	6.6E-07	165	6.E-40
Coprinellus disseminatus	0.001	9.9E-07	553	1.E-156
Coprinellus micaceus	0.003	6.5E-07	545	2.E-154
Coprinellus radians	0.001	6.6E-07	545	2.E-154
Coprinopsis cinerea	0.006	2.6E-06	538	3.E-152
Coprinopsis radiata	0.001	6.6E-07	540	9.E-153
Coprinus foetidellus	0.001	6.6E-07	523	9.E-148
Cordana ellipsoidea	0.003	1.0E-06	224	8.E-58
Cordyceps cylindrica	0.007	2.5E-06	383	1.E-105
Cortinarius ochraceopallescens	0.001	6.6E-07	514	5.E-145
Cortinarius uraceus	0.001	6.6E-07	82	8.E-15
Corynascus kuwaitiensis	0.002	8.4E-07	416	1.E-115
Corynespora olivacea	0.003	6.5E-07	287	1.E-76
Crepidotus mollis	0.001	6.6E-07	451	4.E-126
Cryptococcus aerius	0.074	1.3E-06	551	4.E-156

Cryptococcus aff amylolyticus AS 22398	0.120	1.5E-06	462	2.E-129
Cryptococcus aff gilvescens IMUFRJ 51979	0.001	6.6E-07	204	1.E-51
Cryptococcus aff laurentii D 0721a1	0.001	6.6E-07	191	9.E-48
Cryptococcus arrabidensis	0.001	9.9E-07	161	9.E-39
Cryptococcus dimennae	0.006	1.1E-06	418	3.E-116
Cryptococcus laurentii	0.079	1.4E-06	499	1.E-140
Cryptococcus nemorosus	0.004	7.5E-07	148	6.E-35
Cryptococcus paraflavus	0.032	3.0E-06	198	6.E-50
Cryptococcus podzolicus	0.590	3.4E-06	468	4.E-131
Cryptococcus randhawii	0.076	2.1E-06	569	1.E-161
Cryptococcus saitoi	0.004	1.5E-06	580	6.E-165
Cryptococcus skinneri	0.052	1.5E-06	353	1.E-96
Cryptococcus sp MD76 1BY	0.019	1.0E-06	250	2.E-65
Cryptococcus terreus	0.001	9.9E-07	592	3.E-168
Cryptococcus terricola	0.040	1.5E-06	604	3.E-172
Cryptococcus wieringae	0.002	8.4E-07	604	3.E-172
Cryptodiscus rhopaloides	0.004	8.9E-07	244	7.E-64
Cryptosporiopsis actinidiae	0.001	6.6E-07	335	3.E-91
Cryptosporiopsis ericae	0.022	1.2E-06	333	1.E-90
Cryptosporiopsis radicicola	0.001	6.6E-07	237	1.E-61
Cryptosporiopsis rhizophila	0.012	2.1E-06	241	8.E-63
Cudoniella acicularis	0.097	2.8E-06	351	3.E-96
Cudoniella clavus	0.001	6.6E-07	353	1.E-96
Curreya grandicipis	0.005	1.3E-06	292	2.E-78
Curvularia trifolii	0.001	9.9E-07	470	1.E-131
Cylindrocladiella camelliae	0.034	1.0E-06	68	2.E-10
Cyphellophora hylomeconis	0.004	1.0E-06	209	2.E-53
Cystocoleus ebeneus	0.006	1.7E-06	176	2.E-43
Dactylaria higginsii	0.006	8.9E-07	174	9.E-43
Dactylaria lanosa	0.008	1.8E-06	340	8.E-93
Dactylella oviparasitica	0.001	6.6E-07	97	2.E-19
Dactylellina cionopaga	0.006	1.4E-06	479	2.E-134
Dactylellina ellipsospora	0.003	7.8E-07	440	8.E-123
Dactylellina lobata	0.005	7.3E-07	503	1.E-141

Dactylellina phymatopaga	0.018	1.5E-06	95	8.E-19
Daedaleopsis confragosa	0.001	9.9E-07	534	4.E-151
Davidiella tassiana	0.170	1.9E-06	446	2.E-124
Debaryomyces hansenii	0.008	9.0E-07	529	2.E-149
Degelia gayana	0.062	1.3E-06	259	2.E-68
Devriesia americana	1.100	7.4E-06	335	3.E-91
Devriesia lagerstroemiae	0.001	6.6E-07	185	4.E-46
Devriesia pseudoamericana	0.002	1.6E-06	298	7.E-80
Diaporthe batatas	0.008	1.3E-06	161	7.E-39
Diaporthe eres	0.002	1.3E-06	448	4.E-125
Dictyonema sp R04	0.001	9.9E-07	62	7.E-09
Dictyosporium toruloides	0.001	6.6E-07	276	2.E-73
Dioszegia crocea	0.001	9.9E-07	73	5.E-12
Dissoconium aciculare	0.002	1.3E-06	442	2.E-123
Dissoconium dekkeri	0.004	9.3E-07	460	6.E-129
Dokmaia monthadangii	0.026	1.7E-06	427	6.E-119
Dothideomycetes sp DC2167	0.110	2.5E-06	448	4.E-125
Drechslera erythrospila	0.620	6.5E-06	460	6.E-129
Drechslera fugax	0.005	1.9E-06	372	3.E-102
Elsinoe ampelina	0.001	6.6E-07	311	6.E-84
Elsinoe banksiae	0.003	6.5E-07	302	4.E-81
Embellisia lolii	0.008	1.1E-06	215	5.E-55
Entoloma ameides	0.001	9.9E-07	44	3.E-03
Entoloma platyphylloides	0.001	6.6E-07	407	9.E-113
Entorrhiza aff fineranae PDD70949	0.002	1.6E-06	64	3.E-09
Entrophospora sp	0.003	2.3E-06	344	9.E-94
Entrophospora sp JJ61	0.010	1.2E-06	451	3.E-126
Entrophospora sp shylm131	1.600	6.7E-06	451	3.E-126
Epacris microphylla root associated fungus 1	0.006	3.9E-06	442	2.E-123
Epacris microphylla root associated fungus 12	0.150	1.4E-06	435	3.E-121
Epacris microphylla root associated fungus 20	0.017	2.3E-06	228	7.E-59
Epacris microphylla root associated fungus 21	0.270	2.9E-06	398	4.E-110
Epacris microphylla root associated fungus 26	0.590	3.8E-06	431	4.E-120
Epacris microphylla root associated fungus 3	0.002	1.3E-06	183	1.E-45

Epacris microphylla root associated fungus 31	0.001	9.9E-07	383	1.E-105
Epacris microphylla root associated fungus 33	0.410	4.0E-05	431	4.E-120
Epacris microphylla root associated fungus 5	0.023	1.2E-06	494	6.E-139
Epacris microphylla root associated fungus 6	0.003	6.5E-07	122	3.E-27
Epacris pulchella root associated fungus EP19	0.015	4.1E-06	433	1.E-120
Epacris pulchella root associated fungus EP2	0.002	6.6E-07	431	4.E-120
Epacris pulchella root associated fungus EP20	0.350	4.1E-06	484	4.E-136
Epacris pulchella root associated fungus EP23	0.008	1.5E-06	399	1.E-110
Epacris pulchella root associated fungus EP26	0.051	2.5E-06	211	6.E-54
Epacris pulchella root associated fungus EP42	0.001	6.6E-07	331	6.E-90
Epicoccum nigrum	0.530	3.8E-06	459	2.E-128
Erythrobasidium hasegawianum	0.001	9.9E-07	193	3.E-48
Eucasphaeria capensis	0.004	1.5E-06	510	6.E-144
Eupenicillium hirayamae	0.001	6.6E-07	243	2.E-63
Eutypella scoparia	0.009	6.2E-06	462	2.E-129
Exidia recisa	0.001	6.6E-07	414	5.E-115
Exobasidium inconspicuum	0.003	7.8E-07	409	2.E-113
Exophiala eucalyptorum	0.001	6.6E-07	464	5.E-130
Exophiala salmonis	0.002	6.6E-07	516	1.E-145
Exophiala sp EXP0542F	0.002	6.6E-07	399	1.E-110
Faurelina indica	0.001	9.9E-07	84	1.E-15
Fellomyces lichenicola	0.003	1.0E-06	453	1.E-126
Fibulobasidium inconspicuum	0.004	1.0E-06	254	1.E-66
Fibulobasidium murrhardtense	0.003	7.8E-07	252	4.E-66
Filobasidium capsuligenum	0.002	1.6E-06	261	8.E-69
Flagelloscypha minutissima	0.001	6.6E-07	577	8.E-164
Fomes fomentarius	0.001	9.9E-07	538	3.E-152
Fulgensia schistidii	0.002	1.3E-06	169	4.E-41
Fusarium cerealis	0.005	8.3E-07	449	1.E-125
Fusarium incarnatum	0.001	6.6E-07	217	1.E-55
Fusarium larvarum	0.002	1.6E-06	473	8.E-133
Fusarium lateritium	0.004	2.6E-06	473	8.E-133
Fusarium merismoides var crassum	0.006	3.9E-06	291	8.E-78
Fusarium oxysporum	0.070	2.1E-06	448	4.E-125

Fusarium solani	0.072	1.3E-06	477	6.E-134
Fusicladium cordae	0.008	8.0E-07	484	4.E-136
Fusicladium pini	0.001	9.9E-07	481	5.E-135
Fusidium griseum	0.002	8.4E-07	486	1.E-136
Gaertneriomyces semiglobifer	0.003	6.5E-07	81	3.E-14
Gaertneriomyces tenuis	0.002	1.3E-06	97	3.E-19
Gaeumannomyces cylindrosporus	0.008	8.2E-07	490	8.E-138
Galerina marginata	0.001	6.6E-07	534	4.E-151
Ganoderma applanatum	0.016	8.5E-07	529	2.E-149
Ganoderma sp E7091	0.001	6.6E-07	73	3.E-12
Geastrum triplex	0.001	6.6E-07	497	5.E-140
Geomyces pannorum	0.006	8.0E-07	438	3.E-122
Geopora clausa	0.002	8.4E-07	82	6.E-15
Geopora cooperi	0.001	9.9E-07	82	5.E-15
Gibberella avenacea	0.008	2.0E-06	473	8.E-133
Gibberella pulicaris	0.003	2.0E-06	92	9.E-18
Gibberella zeae	0.014	2.3E-06	449	1.E-125
Gigasperma americanum	0.001	6.6E-07	64	3.E-09
Glomerobolus gelineus	0.017	2.7E-06	292	2.E-78
Gonapodya prolifera	0.005	8.6E-07	100	2.E-20
Gongronella butleri	0.001	6.6E-07	505	3.E-142
Guignardia mangiferae	0.002	1.6E-06	73	4.E-12
Gyalecta hypoleuca	0.001	9.9E-07	115	5.E-25
Gymnopilus penetrans	0.002	8.4E-07	564	5.E-160
Haptocillium balanoides	0.001	6.6E-07	425	2.E-118
Hebeloma cavipes	0.024	4.4E-06	556	9.E-158
Helicodendron multiseptatum	0.002	1.3E-06	440	7.E-123
Helicodendron websteri	0.036	2.4E-06	435	3.E-121
Helicoma chlamydosporum	0.001	6.6E-07	147	2.E-34
Helicoma isiola	0.003	7.8E-07	250	1.E-65
Helicoon aff maioricense ICMP14920	0.002	6.6E-07	340	7.E-93
Helminthosporium velutinum	0.001	6.6E-07	172	3.E-42
Helotiaceae sp II GK 2010	0.037	2.5E-06	379	2.E-104
Helotiaceae sp III GK 2010	0.005	1.9E-06	370	1.E-101

Helotiaceae sp IV GK 2010	0.004	1.6E-06	372	3.E-102
Helotiaceae sp VI GK 2010	0.001	6.6E-07	165	5.E-40
Helotiales sp	0.016	1.1E-05	444	8.E-124
Helotiales sp 16 MV 2011	0.006	2.2E-06	353	1.E-96
Helotiales sp 27 MV 2011	0.130	6.7E-06	444	6.E-124
Helotiales sp 31 MV 2011	0.001	9.9E-07	121	1.E-26
Helotiales sp ARDLJ 2009d	0.001	9.9E-07	375	2.E-103
Helotiales sp CWG F1 E3	0.001	6.6E-07	322	3.E-87
Helotiales sp SC4 4	0.031	2.1E-06	302	4.E-81
Helvella maculata	0.002	1.3E-06	196	2.E-49
Hemibeltrania mitrata	0.001	6.6E-07	113	2.E-24
Herpotrichia juniperi	0.001	6.6E-07	102	6.E-21
Heyderia abietis	0.130	4.2E-06	130	2.E-29
Hirsutella minnesotensis	0.002	6.6E-07	484	4.E-136
Hirsutella rhossiliensis	0.009	9.0E-07	472	3.E-132
Hirsutella thompsonii	0.002	1.3E-06	102	4.E-21
Holtermanniella festucosa	0.001	9.9E-07	544	7.E-154
Hyaloscypha sp	0.001	6.6E-07	420	1.E-116
Hymenoscyphus scutula	0.001	6.6E-07	143	2.E-33
Hyphoderma puberum	0.001	6.6E-07	555	3.E-157
Hyphodiscus hymeniophilus	0.001	9.9E-07	366	1.E-100
Hyphodontia radula	0.001	9.9E-07	507	8.E-143
Hypholoma fasciculare	0.015	1.1E-06	558	2.E-158
Hypocenomyce scalaris	0.001	6.6E-07	132	5.E-30
Hypocrea ceramica	0.004	6.5E-07	492	2.E-138
Hypocrea koningii	0.002	6.6E-07	492	2.E-138
Hypocrea lixii	0.017	1.1E-06	492	2.E-138
Hypocrea rufa	0.002	6.6E-07	496	2.E-139
Hypocrea virens	0.019	1.9E-06	481	5.E-135
Hypoderma cordylines	0.004	6.5E-07	335	4.E-91
Hypoderma rubi	0.001	6.6E-07	86	6.E-16
Hypoxylon howeanum	0.001	6.6E-07	440	8.E-123
Ilyonectria radicicola	0.008	3.5E-06	472	3.E-132
Inocybe lacera	2.100	2.8E-05	556	9.E-158

Inocybe lacera var helobia	0.006	8.9E-07	540	9.E-153
Inocybe lacera var lacera	0.033	1.5E-05	545	2.E-154
Inocybe ochroalba	0.023	2.6E-06	241	8.E-63
Itersonilia perplexans	0.003	2.0E-06	592	3.E-168
Kappamyces sp ARG063	0.005	6.5E-07	152	6.E-36
Knufia chersonesos	0.003	7.8E-07	322	3.E-87
Kochiomyces dichotomus	0.002	1.6E-06	507	1.E-142
Laccaria proxima	0.170	5.4E-05	553	1.E-156
Lachnum sp	0.001	6.6E-07	425	3.E-118
Lachnum sp 1 MV 2011	0.036	1.6E-06	420	9.E-117
Lactarius rufus	0.001	6.6E-07	652	0.E+00
Laetiporus cincinnatus	0.001	9.9E-07	529	2.E-149
Laetisaria lichenicola	0.054	6.9E-06	405	4.E-112
Lasiodiplodia gonubiensis	0.001	9.9E-07	182	5.E-45
Lasiodiplodia theobromae	0.003	1.2E-06	195	6.E-49
Lasiosphaeria ovina	0.001	6.6E-07	174	8.E-43
Lecanicillium attenuatum	0.001	9.9E-07	462	2.E-129
Lecanicillium fusisporum	0.002	8.4E-07	464	5.E-130
Lecanicillium psalliotae	0.043	1.3E-06	472	3.E-132
Lecanora polytropa	0.006	1.5E-06	294	6.E-79
Lecidea cancriformis	0.003	1.0E-06	392	2.E-108
Lecidella carpathica	0.001	6.6E-07	331	5.E-90
Lecythophora hoffmannii	0.020	1.2E-06	435	3.E-121
Lecythophora mutabilis	0.120	2.4E-06	355	3.E-97
Leohumicola minima	0.001	6.6E-07	252	4.E-66
Leohumicola sp 1 MV 2011	0.001	6.6E-07	364	4.E-100
Leotiomycetes sp NK264	0.029	1.6E-06	436	9.E-122
Leptosphaeria contecta	0.002	8.4E-07	313	2.E-84
Leptosphaeria doliolum	0.002	1.6E-06	226	2.E-58
Leptosphaeria korrae	0.092	2.2E-06	351	4.E-96
Leptosphaerulina australis	0.004	1.1E-06	457	7.E-128
Leptosphaerulina chartarum	0.013	9.0E-07	455	3.E-127
Leucopaxillus tricolor	0.002	8.4E-07	551	4.E-156
Leucosporidiella muscorum	0.001	6.6E-07	193	3.E-48

Lewia infectoria	0.006	8.0E-07	464	5.E-130
Limnoperdon incarnatum	0.001	6.6E-07	268	5.E-71
Limonomyces culmigenus	0.001	6.6E-07	396	2.E-109
Linderina macrospora	0.009	1.3E-06	71	2.E-11
Linderina pennispora	0.002	1.6E-06	75	1.E-12
Lindgomyces apiculatus	0.058	2.0E-06	250	1.E-65
Lindgomyces lemonweirensis	0.001	6.6E-07	226	2.E-58
Liroa emodensis	0.002	1.6E-06	355	4.E-97
Lobaria linita	0.017	1.1E-06	244	6.E-64
Lobaria retigera	0.001	6.6E-07	230	2.E-59
Lophiostoma chamaecyparidis	0.051	1.5E-06	350	1.E-95
Lophiostoma cynaroidis	0.004	1.5E-06	424	8.E-118
Lophium mytilinum	0.001	6.6E-07	237	1.E-61
Lophodermium pinastri	0.087	2.9E-06	438	3.E-122
Lophodermium pini excelsae	0.017	2.0E-06	449	1.E-125
Lycoperdon lambinonii	0.001	6.6E-07	544	7.E-154
Lycoperdon niveum	0.002	1.6E-06	555	3.E-157
Lyophyllum semitale	0.006	2.4E-06	69	6.E-11
Lyophyllum sp Cultivar Jpn	0.042	1.4E-06	57	4.E-07
Macroconia leptosphaeriae	0.200	6.2E-06	261	6.E-69
Macrophomina phaseolina	0.001	9.9E-07	185	4.E-46
Malassezia globosa	0.008	1.9E-06	701	0.E+00
Malassezia restricta	0.097	4.0E-06	678	0.E+00
Malassezia sympodialis	0.001	6.6E-07	250	2.E-65
Melampsora pinitorqua	0.001	6.6E-07	580	6.E-165
Meliniomyces bicolor	0.063	2.4E-06	431	4.E-120
Meliniomyces sp	0.001	6.6E-07	326	3.E-88
Meliniomyces sp SM7 2	0.001	9.9E-07	375	2.E-103
Meliniomyces variabilis	0.003	6.5E-07	375	2.E-103
Meliniomyces vraolstadiae	0.006	1.1E-06	368	3.E-101
Metarhizium anisopliae	0.016	3.5E-06	496	2.E-139
Micarea denigrata	0.043	1.7E-06	383	1.E-105
Microdiplodia hawaiiensis	0.019	1.2E-06	357	8.E-98
Microdochium bolleyi	0.039	1.8E-06	481	5.E-135

Microglossum olivaceum	0.003	2.0E-06	141	8.E-33
Microscypha ellisii	0.043	1.2E-06	377	6.E-104
Microsphaeropsis arundinis	1.600	7.3E-06	429	2.E-119
Microsphaeropsis proteae	0.023	2.5E-06	451	3.E-126
Mollisia cinerea	0.005	1.9E-06	435	4.E-121
Mollisia fusca	0.001	6.6E-07	496	2.E-139
Mollisia melaleuca	0.002	8.4E-07	496	2.E-139
Monacrosporium doedycoides	0.001	9.9E-07	81	2.E-14
Monilinia jezoensis	0.005	3.3E-06	75	8.E-13
Monoblepharella mexicana	0.001	6.6E-07	104	2.E-21
Monoblepharis macrandra	0.008	9.2E-07	110	3.E-23
Mortierella alpina	0.045	3.4E-06	634	0.E+00
Mortierella angusta	0.005	3.3E-06	614	6.E-175
Mortierella cystojenkinii	0.035	1.5E-06	586	1.E-166
Mortierella dichotoma	0.002	6.6E-07	92	1.E-17
Mortierella elasson	0.002	1.3E-06	270	2.E-71
Mortierella elongata	0.180	2.2E-06	636	0.E+00
Mortierella fimbricystis	0.011	1.3E-06	573	1.E-162
Mortierella gamsii	0.005	7.3E-07	575	3.E-163
Mortierella gemmifera	0.014	2.5E-06	623	1.E-177
Mortierella hyalina	0.008	2.3E-06	77	4.E-13
Mortierella lignicola	0.009	1.1E-06	556	1.E-157
Mortierella macrocystis	0.026	3.3E-06	601	4.E-171
Mortierella minutissima	0.005	1.9E-06	281	7.E-75
Mortierella paraensis	0.001	6.6E-07	272	4.E-72
Mortierella parvispora	0.002	8.4E-07	566	2.E-160
Mortierella sp CZ 2011	0.001	6.6E-07	274	1.E-72
Mortierella sp TR065	0.002	1.3E-06	599	2.E-170
Mortierella sp TR158	0.130	5.3E-06	490	9.E-138
Mortierella verticillata	0.001	6.6E-07	586	1.E-166
Mycena arcangeliana	0.009	6.2E-06	560	7.E-159
Mycena corynephora	0.011	1.2E-06	154	1.E-36
Mycena galopus	0.001	6.6E-07	560	1.E-158
Mycena olida	0.018	4.7E-06	124	1.E-27

Mycena silvae nigrae	0.001	6.6E-07	139	4.E-32
Mycena tenax	0.001	6.6E-07	459	3.E-128
Mycoblastus sanguinarius	0.001	9.9E-07	77	2.E-13
Mycosphaerella delegatensis	0.001	6.6E-07	217	1.E-55
Myrmecridium phragmitis	0.008	1.2E-06	438	3.E-122
Myrmecridium schulzeri	0.001	6.6E-07	132	5.E-30
Myxotrichum carminoparum	0.007	9.2E-07	95	6.E-19
Myxotrichum chartarum	0.001	6.6E-07	189	3.E-47
Myxotrichum deflexum	0.001	9.9E-07	121	1.E-26
NA	4.900	NA	NA	NA
Naemacyclus minor	0.004	6.5E-07	448	4.E-125
Nemania abortiva	0.001	6.6E-07	117	2.E-25
Neofusicoccum arbuti	0.002	1.6E-06	202	4.E-51
Noosia banksiae	0.003	6.5E-07	370	1.E-101
Occultifur aff externus IMUFRJ 52019	0.001	6.6E-07	195	9.E-49
Ochrocladosporium frigidarii	0.001	6.6E-07	394	6.E-109
Octospora wrightii	0.030	3.4E-06	99	8.E-20
Oidiodendron chlamydosporicum	0.001	6.6E-07	359	2.E-98
Oidiodendron flavum	0.001	6.6E-07	416	1.E-115
Oidiodendron truncatum	0.004	1.5E-06	81	2.E-14
Omphalina foliacea	0.002	6.6E-07	148	7.E-35
Ophiocordyceps paracuboidea	0.001	9.9E-07	409	3.E-113
Ophiocordyceps sinensis	0.550	6.2E-06	383	1.E-105
Ophiostoma sp X ES 1996	0.004	3.0E-06	518	4.E-146
Orbilia luteorubella	0.002	1.3E-06	79	8.E-14
Paecilomyces carneus	0.001	6.6E-07	488	4.E-137
Paecilomyces marquandii	0.015	2.4E-06	510	6.E-144
Paraconiothyrium brasiliense	0.001	6.6E-07	436	1.E-121
Paraconiothyrium cyclothyrioides	0.003	1.2E-06	414	5.E-115
Paraconiothyrium sp HS 03	0.012	5.4E-06	449	1.E-125
Paraphaeosphaeria sp	0.001	6.6E-07	455	4.E-127
Paraphoma chrysanthemicola	0.005	1.3E-06	213	2.E-54
Passalora zambiae	0.019	1.1E-06	252	4.E-66
Penicillium aculeatum	0.002	8.4E-07	457	7.E-128

Penicillium adametzii	0.003	7.8E-07	472	3.E-132
Penicillium biourgeianum	0.002	8.4E-07	468	4.E-131
Penicillium canescens	0.011	1.3E-06	473	8.E-133
Penicillium cecidicola	0.001	6.6E-07	407	8.E-113
Penicillium glabrum	0.001	9.9E-07	451	4.E-126
Penicillium islandicum	0.001	6.6E-07	460	6.E-129
Penicillium montanense	0.001	6.6E-07	411	6.E-114
Penicillium namyslowskii	0.003	1.2E-06	460	6.E-129
Penicillium ochrochloron	0.001	6.6E-07	472	3.E-132
Penicillium pinophilum	0.002	1.6E-06	448	4.E-125
Penicillium proteolyticum	0.009	2.6E-06	460	6.E-129
Penicillium pulvillorum	0.001	6.6E-07	466	1.E-130
Penicillium purpurogenum	0.013	9.2E-06	435	4.E-121
Penicillium purpurogenum var rubrisclerotium	0.003	1.2E-06	459	2.E-128
Penicillium raistrickii	0.001	6.6E-07	466	1.E-130
Penicillium ramulosum	0.003	2.3E-06	477	6.E-134
Penicillium sclerotigenum	0.001	6.6E-07	390	8.E-108
Penicillium verruculosum	0.150	5.3E-06	457	7.E-128
Penidiella aggregata	0.003	6.5E-07	316	1.E-85
Penidiella ellipsoidea	0.006	1.5E-06	401	3.E-111
Penidiella strumelloidea	0.001	9.9E-07	381	4.E-105
Peniophora cinerea	0.002	8.4E-07	477	7.E-134
Pestalotiopsis besseyi	0.001	6.6E-07	431	4.E-120
Pestalotiopsis caudata	0.003	1.0E-06	473	8.E-133
Pestalotiopsis citrina	0.003	7.8E-07	444	6.E-124
Pezicula carpinea	0.002	1.3E-06	233	1.E-60
Peziza ostracoderma	0.007	9.2E-07	448	5.E-125
Pezizomycotina sp 11302	0.001	9.9E-07	99	6.E-20
Phacidiopycnis pyri	0.001	6.6E-07	226	2.E-58
Phacidiopycnis washingtonensis	0.019	1.1E-06	302	4.E-81
Phaeoacremonium aleophilum	0.001	6.6E-07	92	9.E-18
Phaeoacremonium griseorubrum	0.010	1.2E-06	224	8.E-58
Phaeoacremonium mortoniae	0.012	1.5E-06	104	1.E-21
Phaeoacremonium parasiticum	0.001	9.9E-07	183	1.E-45

Phaeobotryosphaeria visci	0.001	6.6E-07	355	3.E-97
Phaeococcomyces catenatus	0.003	1.2E-06	525	2.E-148
Phaeococcomyces nigricans	0.036	1.0E-06	344	6.E-94
Phaeocollybia sipei	0.001	6.6E-07	71	1.E-11
Phaeomoniella effusa	0.007	2.5E-06	305	3.E-82
Phaeophyscia exornatula	0.064	3.4E-06	172	3.E-42
Phaeophyscia squarrosa	0.003	1.0E-06	169	4.E-41
Phaeosclera dematioides	0.001	6.6E-07	180	2.E-44
Phaeosclera sp TRN524	0.001	6.6E-07	209	2.E-53
Phaeosphaeria avenaria	0.120	2.5E-06	425	2.E-118
Phaeosphaeria eustoma	0.006	1.6E-06	337	1.E-91
Phaeosphaeria herpotrichoides	0.006	1.7E-06	442	2.E-123
Phaeosphaeria nodorum	0.071	1.2E-06	427	6.E-119
Phaeosphaeria phragmitis	0.005	1.6E-06	427	6.E-119
Phaeosphaeria sp	0.001	9.9E-07	438	4.E-122
Phaeosphaeriopsis sp	0.001	6.6E-07	366	2.E-100
Phaeothecoidea melaleuca	0.002	8.4E-07	346	2.E-94
Phaeothecoidea proteae	0.002	1.3E-06	425	2.E-118
Phanerochaete sordida	0.001	6.6E-07	84	2.E-15
Phialemonium aff dimorphosporum II 0563b	0.001	6.6E-07	206	3.E-52
Phialemonium curvatum	0.047	2.2E-06	202	4.E-51
Phialocephala fluminis	0.064	2.0E-06	350	1.E-95
Phialocephala fortinii	0.072	1.9E-06	438	3.E-122
Phialocephala helvetica	0.002	1.6E-06	409	2.E-113
Phialocephala virens	0.260	8.0E-06	346	2.E-94
Phialophora sessilis	0.001	6.6E-07	499	1.E-140
Phialophora sp DF36	0.280	2.7E-06	422	3.E-117
Phialophora sp MLB Phi	0.003	2.3E-06	313	2.E-84
Phlebia radiata	0.001	9.9E-07	527	7.E-149
Phoma eupyrena	0.001	6.6E-07	449	1.E-125
Phoma herbarum	0.510	3.8E-06	383	1.E-105
Phoma macrostoma	0.001	6.6E-07	250	1.E-65
Phoma moricola	0.001	6.6E-07	339	3.E-92
Phomopsis liquidambari	0.001	9.9E-07	106	3.E-22

Physcia adscendens	0.001	6.6E-07	457	7.E-128
Physisporinus vitreus	0.001	6.6E-07	327	7.E-89
Placynthiella icmalea	0.008	1.1E-06	390	8.E-108
Placynthiella uliginosa	0.025	1.9E-06	327	6.E-89
Platygloea disciformis	0.064	6.3E-06	287	1.E-76
Pleopsidium chlorophanum	0.001	6.6E-07	230	2.E-59
Pleospora herbarum	0.001	9.9E-07	464	5.E-130
Pleosporales sp 5 TMS 2011	0.009	1.4E-06	381	5.E-105
Pleosporales sp HF 10	0.004	1.7E-06	307	8.E-83
Pleosporales sp LH70	0.003	6.5E-07	377	6.E-104
Pleurotus eryngii	0.004	7.5E-07	99	5.E-20
Pleurotus salmoneostramineus	0.004	1.5E-06	156	4.E-37
Pochonia bulbillosa	0.010	9.1E-07	512	2.E-144
Pochonia suchlasporia	0.009	1.8E-06	503	1.E-141
Podospora curvicolla	0.005	3.3E-06	372	3.E-102
Podospora decidua	0.003	7.8E-07	381	5.E-105
Podospora ellisiana	0.120	3.2E-06	357	8.E-98
Podospora minicauda	0.001	6.6E-07	209	2.E-53
Podospora miniglutinans	0.008	1.2E-06	425	2.E-118
Podospora petrogale	0.010	1.0E-06	246	2.E-64
Podospora pyriformis	0.001	6.6E-07	158	9.E-38
Polyplosphaeria fusca	0.001	6.6E-07	195	6.E-49
Porosphaerella cordanophora	0.140	2.8E-06	239	3.E-62
Powellomyces hirtus	0.023	1.7E-06	82	7.E-15
Powellomyces sp DAOM 226231	0.001	9.9E-07	81	3.E-14
Preussia dubia	0.001	6.6E-07	381	5.E-105
Preussia isomera	0.005	1.1E-06	230	2.E-59
Preussia polymorpha	0.001	9.9E-07	355	3.E-97
Preussia pseudominima	0.002	6.6E-07	424	7.E-118
Preussia sp 2 ICMP 18938	0.001	9.9E-07	446	2.E-124
Preussia sp SL08070	0.260	3.6E-06	448	4.E-125
Pringsheimia euphorbiae	0.088	1.5E-06	300	1.E-80
Pseudaegerita corticalis	0.001	9.9E-07	311	6.E-84
Pseudaegerita viridis	0.001	9.9E-07	165	5.E-40

Pseudaleuria quinaultiana	0.002	6.6E-07	86	5.E-16
Pseudeurotium bakeri	0.002	6.6E-07	442	2.E-123
Pseudeurotium desertorum	0.002	8.4E-07	374	8.E-103
Pseudocyphellaria clathrata	0.006	1.0E-06	134	1.E-30
Psilocybe montana	0.012	1.2E-06	547	5.E-155
Purpureocillium lilacinum	0.005	1.3E-06	468	4.E-131
Pyrenochaeta inflorescentiae	0.002	1.6E-06	416	1.E-115
Pyrenochaeta sp	0.001	9.9E-07	387	1.E-106
Pyrenochaeta sp ZLY 2010b	0.001	6.6E-07	353	1.E-96
Pyrenochaetopsis microspora	0.035	2.1E-06	392	2.E-108
Pyrenophora leucospermi	1.400	6.6E-06	460	6.E-129
Quambalaria cyanescens	0.011	2.0E-06	71	1.E-11
Rachicladosporium pini	0.210	2.6E-06	425	2.E-118
Raffaelea montetyi	0.015	5.2E-06	569	1.E-161
Ramularia sp KACC 42532	0.001	6.6E-07	215	6.E-55
Rhexocercosporidium panacis	0.180	2.3E-06	431	4.E-120
Rhizoctonia sp 266	0.001	6.6E-07	566	2.E-160
Rhizoctonia sp KW214	0.002	6.6E-07	540	9.E-153
Rhizophlyctis rosea	0.100	2.1E-06	612	2.E-174
Rhizophydium chaetiferum	0.036	1.2E-05	86	5.E-16
Rhizophydium globosum	0.026	1.7E-06	82	6.E-15
Rhizophydium littoreum	0.003	2.0E-06	66	8.E-10
Rhizophydium sp JEL 385	0.006	1.5E-06	79	7.E-14
Rhizopogon arctostaphyli	0.001	9.9E-07	99	7.E-20
Rhizopogon luteolus	0.490	8.3E-06	654	0.E+00
Rhizopogon subcaerulescens	0.001	6.6E-07	99	6.E-20
Rhizoscyphus ericae	3.300	1.5E-05	479	2.E-134
Rhodotorula auriculariae	0.001	6.6E-07	309	3.E-83
Rhodotorula bacarum	0.003	2.3E-06	595	2.E-169
Rhodotorula cassiicola	0.008	7.0E-07	257	1.E-67
Rhodotorula diffluens	0.007	7.0E-07	283	2.E-75
Rhodotorula eucalyptica	0.004	8.9E-07	337	1.E-91
Rhodotorula fujisanensis	0.002	1.3E-06	562	2.E-159
Rhodotorula graminis	0.001	6.6E-07	538	3.E-152

Rhodotorula mucilaginosa	0.001	6.6E-07	571	3.E-162
Rhodotorula philyla	0.110	6.0E-06	368	5.E-101
Rhynchostoma proteae	0.130	1.7E-06	92	1.E-17
Rickenella fibula	0.003	2.0E-06	507	9.E-143
Rickenella mellea	0.016	1.7E-06	510	7.E-144
Saccharomycopsis microspora	0.001	6.6E-07	62	9.E-09
Sagenomella diversispora	0.810	8.1E-06	448	4.E-125
Sagenomella humicola	3.300	1.3E-05	460	6.E-129
Sagenomella striatispora	0.042	1.4E-06	460	6.E-129
Sarcodon scabrosus	0.001	6.6E-07	81	3.E-14
Sarcoleotia globosa	0.270	5.0E-06	316	1.E-85
Sarea difformis	0.001	6.6E-07	156	3.E-37
Sarocladium strictum	0.001	6.6E-07	296	2.E-79
Scedosporium apiospermum	0.004	8.9E-07	532	1.E-150
Scedosporium aurantiacum	0.002	6.6E-07	542	2.E-153
Scedosporium prolificans	0.004	6.5E-07	350	1.E-95
Schizophyllum commune	0.010	6.9E-06	614	6.E-175
Scleroderma citrinum	0.052	7.9E-06	621	3.E-177
Sclerostagonospora opuntiae	0.002	6.6E-07	453	1.E-126
Sclerotium delphinii	0.005	8.6E-07	64	2.E-09
Scolecobasidium excentricum	0.001	6.6E-07	436	1.E-121
Scoliciosporum umbrinum	0.003	7.8E-07	381	5.E-105
Scutellinia superba	0.001	6.6E-07	143	3.E-33
Scutellospora calospora	0.003	2.3E-06	309	2.E-83
Scytalidium lignicola	0.002	8.4E-07	195	6.E-49
Sebacina grisea	0.003	6.5E-07	92	1.E-17
Sebacina vermifera	0.018	2.6E-06	329	2.E-89
Sistotrema alboluteum	0.050	8.6E-06	322	4.E-87
Sistotrema brinkmannii	0.001	9.9E-07	246	2.E-64
Sistotrema coronilla	0.017	3.2E-06	222	4.E-57
Sistotrema muscicola	0.002	1.6E-06	137	1.E-31
Sistotrema sp B216	23.000	1.0E-04	551	6.E-156
Sordaria alcina	0.076	1.7E-06	446	2.E-124
Sordariales sp G9i88H	0.002	6.6E-07	394	6.E-109

Sordariales sp MU 2009 1	0.017	1.6E-06	224	8.E-58
Sordariomycetes sp	0.001	9.9E-07	228	9.E-59
Sordariomycetes sp DC2118	0.081	1.4E-06	440	7.E-123
Sordariomycetes sp E8924B	0.001	6.6E-07	104	1.E-21
Sphaceloma protearum	0.001	6.6E-07	298	5.E-80
Sphaerobolus iowensis	0.001	9.9E-07	366	2.E-100
Sphaeropsis pyriputrescens	0.003	6.5E-07	246	2.E-64
Sphaeropsis sapinea	0.001	6.6E-07	464	5.E-130
Sphaerostilbella aureonitens	0.001	6.6E-07	385	4.E-106
Spiromastix tentaculatu	0.002	1.6E-06	316	2.E-85
Spizellomyces dolichospermus	0.003	6.5E-07	100	2.E-20
Spizellomyces lactosolyticus	0.120	1.1E-05	200	2.E-50
Spizellomyces palustris	0.003	1.2E-06	568	4.E-161
Spizellomyces plurigibbosus	0.018	1.5E-06	102	5.E-21
Spizellomyces pseudodichotomus	0.053	2.7E-06	154	1.E-36
Sporobolomyces falcatus	0.002	6.6E-07	342	3.E-93
Sporobolomyces gracilis	0.021	1.3E-06	547	5.E-155
Sporobolomyces inositophilus	0.003	2.0E-06	390	1.E-107
Sporobolomyces lactophilus	0.001	9.9E-07	254	1.E-66
Sporobolomyces lactosus	0.001	9.9E-07	429	2.E-119
Sporobolomyces roseus	0.005	1.2E-06	551	4.E-156
Sporobolomyces subbrunneus	0.011	1.1E-06	73	3.E-12
Sporobolomyces symmetricus	0.001	6.6E-07	538	3.E-152
Squamarina gypsacea	0.014	8.1E-07	399	1.E-110
Staphylotrichum coccosporum	0.001	6.6E-07	154	1.E-36
Steccherinum fimbriatum	0.008	5.6E-06	545	2.E-154
Stictis radiata	0.430	3.6E-06	217	1.E-55
Strumella coryneoidea	0.039	2.2E-06	104	1.E-21
Suillus bovinus	0.530	3.1E-05	612	2.E-174
Suillus luteus	15.000	3.7E-05	608	3.E-173
Sydowia polyspora	0.060	2.1E-06	464	5.E-130
Syncephalastrum racemosum	0.120	3.1E-06	457	7.E-128
Talaromyces helicus var major	0.001	9.9E-07	411	6.E-114
Talaromyces proteolyticus	0.001	9.9E-07	488	4.E-137

Talaromyces purpureus	0.002	6.6E-07	344	6.E-94
Talaromyces thermophilus	0.010	1.0E-06	497	5.E-140
Taphrina carnea	0.001	9.9E-07	542	2.E-153
Teratosphaeria capensis	0.110	1.5E-06	453	1.E-126
Teratosphaeria jonkershoekensis	0.021	3.2E-06	363	2.E-99
Teratosphaeria karinae	0.001	6.6E-07	134	2.E-30
Teratosphaeria knoxdaviesii	0.002	6.6E-07	355	3.E-97
Teratosphaeria mexicana	0.001	9.9E-07	231	5.E-60
Teratosphaeria microspora	0.056	1.7E-06	438	3.E-122
Teratosphaeria persoonii	0.001	6.6E-07	165	5.E-40
Tetracladium apiense	0.001	6.6E-07	81	2.E-14
Texosporium sancti jacobi	0.001	9.9E-07	88	1.E-16
Thanatephorus cucumeris	0.001	9.9E-07	95	1.E-18
Thanatephorus fusisporus	0.006	3.9E-06	577	7.E-164
Thanatephorus theobromae	0.001	6.6E-07	113	3.E-24
Thedgonia ligustrina	0.001	9.9E-07	228	6.E-59
Thelebolaceae sp BEA 2010	0.005	1.3E-06	444	6.E-124
Thelebolus sp UFMGCB 3742	0.003	2.3E-06	195	6.E-49
Thelephora terrestris	0.920	1.4E-05	577	7.E-164
Thermomyces lanuginosus	0.001	6.6E-07	473	8.E-133
Thielavia microspora	0.002	8.4E-07	250	2.E-65
Thielavia terrestris	0.003	1.2E-06	81	2.E-14
Thozetella havanensis	0.008	1.4E-06	250	1.E-65
Tolypocladium cylindrosporum	0.001	9.9E-07	433	1.E-120
Torrendiella brevisetosa	0.001	6.6E-07	84	2.E-15
Trapelia involuta	0.001	9.9E-07	363	2.E-99
Trechispora hymenocystis	0.020	3.2E-06	390	1.E-107
Trechispora subsphaerospora	0.002	8.4E-07	387	1.E-106
Trechisporales	0.004	3.0E-06	560	1.E-158
Tremella encephala	0.001	6.6E-07	86	6.E-16
Tremella foliacea	0.008	9.0E-07	346	2.E-94
Tremella giraffa	0.001	6.6E-07	110	2.E-23
Trichocladium asperum	0.005	1.3E-06	453	1.E-126
Trichoderma atroviride	0.008	1.6E-06	492	2.E-138

Trichoderma inhamatum	0.003	6.5E-07	488	3.E-137
Tricholoma imbricatum	0.013	2.3E-06	562	2.E-159
Trichosporon jirovecii	0.001	9.9E-07	453	1.E-126
Trichosporon moniliiforme	0.003	1.2E-06	475	2.E-133
Trichosporon porosum	0.001	9.9E-07	473	8.E-133
Trimmatostroma cordae	0.001	9.9E-07	425	2.E-118
Trimorphomyces papilionaceus	0.011	2.2E-06	128	6.E-29
Truncatella angustata	0.006	3.9E-06	451	3.E-126
Tubaria furfuracea	0.001	9.9E-07	538	3.E-152
Tubeufia cerea	0.006	1.0E-06	294	6.E-79
Udeniomyces pyricola	0.001	6.6E-07	595	2.E-169
Umbelopsis autotrophica	0.400	4.5E-06	536	1.E-151
Umbelopsis isabellina	0.018	1.3E-06	523	1.E-147
Umbelopsis ramanniana	0.032	1.4E-06	534	4.E-151
Umbelopsis vinacea	0.001	6.6E-07	523	1.E-147
Umbilicaria esculenta	0.062	1.4E-06	235	5.E-61
Umbilicaria nylanderiana	0.001	6.6E-07	135	4.E-31
Urocystis agropyri	0.001	6.6E-07	623	1.E-177
Ustilago davisii	0.001	6.6E-07	686	0.E+00
Varicosporium elodeae	0.008	7.6E-07	442	2.E-123
Vascellum pratense	0.002	1.3E-06	556	9.E-158
Vermispora fusarina	0.089	3.2E-06	195	8.E-49
Vestigium trifidum	0.001	9.9E-07	353	1.E-96
Volvariella pusilla	0.002	1.6E-06	442	2.E-123
Vonarxia vagans	0.045	1.9E-06	94	2.E-18
Wilcoxina mikolae	12.000	4.0E-05	470	1.E-131
Woollsia root associated fungus X	0.001	9.9E-07	490	8.E-138
Xanthoria parietina	0.016	1.2E-06	161	7.E-39
Xenobotrytis acaducospora	0.003	6.5E-07	250	1.E-65
Xenochalara juniperi	0.001	6.6E-07	401	3.E-111
Xenopolyscytalum pinea	0.062	3.1E-06	440	7.E-123
Xerocomus parasiticus	0.001	6.6E-07	669	0.E+00
Ypsilina graminea	0.003	2.0E-06	433	1.E-120
Zalerion arboricola	0.004	6.5E-07	444	6.E-124

Zalerion varium	0.003	7.8E-07	438	3.E-122
Zopfiella karachiensis	0.006	1.1E-06	350	1.E-95
Zopfiella tabulata	0.015	1.1E-06	176	2.E-43
Zygophiala cryptogama	0.001	9.9E-07	224	8.E-58
Zygorhynchus moelleri	0.001	9.9E-07	497	5.E-140

APPENDIX C

BLAST hit References, average relative read abundances, E-values and corresponding BLAST scores for all OTUs identified at a 97% sequence similarity cut-off in the study presented in chapter 5. OTUs were blasted against the UNITE and INSD databases using the massBLASTer tool available from PlutoF v2.0 (http://unite.ut.ee/workbench.php). OTUs that did not have a BLAST hit in the UNITE or INSD databases were indicated as "not applicable (NA)".

Reference name	Average relative abundance (%)	Standard error on average relative abundance	Score	E-value
Abrothallus suecicus	0.006	1.1E-06	318	4.E-86
Absidia caerulea	0.001	7.5E-07	512	2.E-144
Acanthostigma perpusillum	0.002	7.5E-07	305	3.E-82
Acephala applanata	0.002	7.5E-07	353	1.E-96
Acephala macrosclerotiorum	0.404	8.7E-06	436	9.E-122
Acremonium cavaraeanum	0.011	1.0E-06	298	5.E-80
Acremonium cereale	0.003	2.3E-06	490	8.E-138
Acremonium psammosporum	0.005	8.6E-07	102	4.E-21
Acremonium sp YX	0.013	5.0E-06	315	5.E-85
Agaricus pinsitus	0.447	2.1E-05	529	2.E-149
Agrocybe pediades	0.001	7.5E-07	547	5.E-155
Allophylaria campanuliformis	0.019	1.4E-05	261	1.E-70
Alternaria alternata	0.001	7.5E-07	455	3.E-127
Alternaria lini	0.012	1.4E-06	462	2.E-129
Alternaria longipes	0.011	1.6E-06	464	5.E-130
Alternaria triticina	0.004	8.9E-07	457	8.E-128
Amanita muscaria	0.049	7.9E-06	586	1.E-166
Amauroascus mutatus	0.001	7.5E-07	455	3.E-127
Ambispora leptoticha	0.001	7.5E-07	512	2.E-144

Antarctic yeast CBS 8941	0.223	9.2E-06	442	3.E-123
Antennariella placitae	0.005	1.2E-06	375	2.E-103
Anthostomella eucalyptorum	0.039	1.9E-05	344	6.E-94
Anthostomella pinea	0.006	1.5E-06	424	8.E-118
Aphanoascus fulvescens	0.065	5.9E-06	455	3.E-127
Aphanoascus verrucosus	0.001	7.5E-07	364	5.E-100
Aphyllophorales sp EXP0530F	0.001	7.5E-07	327	6.E-89
Apiosordaria otanii	0.010	2.1E-06	353	1.E-96
Apiospora montagnei	0.001	7.5E-07	433	1.E-120
Apodospora peruviana	0.046	2.5E-06	213	2.E-54
Aquaticola hongkongensis	0.030	4.5E-06	230	2.E-59
Archaeospora sp isa33	0.001	7.5E-07	180	2.E-44
Archaeospora trappei	0.002	7.5E-07	496	2.E-139
Armillaria novae zelandiae	0.005	1.2E-06	163	2.E-39
Arnium macrotheca	0.017	1.4E-06	438	3.E-122
Arthrinium arundinis	0.017	2.8E-06	453	1.E-126
Arthrinium phaeospermum	0.002	1.5E-06	455	3.E-127
Articulospora proliferata	0.033	1.8E-06	390	7.E-108
Ascochyta hordei	0.003	7.5E-07	448	4.E-125
Ascochyta manawaorae	0.008	1.9E-06	355	3.E-97
Ascomycota sp AR 2010	0.040	1.7E-06	431	4.E-120
Ascomycota sp BBC	0.001	7.5E-07	303	1.E-81
Ascomycota sp CH NI20	0.001	7.5E-07	121	1.E-26
Ascomycota sp CH Tc23	0.004	3.0E-06	241	8.E-63
Ascomycota sp X33	0.002	1.1E-06	300	1.E-80
Aspergillus flavipes	0.006	4.1E-06	246	2.E-64
Aspergillus niger	0.001	7.5E-07	86	5.E-16
Aspicilia aquatica	0.008	8.9E-07	176	3.E-43
Aspicilia cinerea	0.017	4.3E-06	200	1.E-50
Aspicilia dendroplaca	0.002	1.1E-06	265	5.E-70
Aspicilia verruculosa	0.001	7.5E-07	204	1.E-51
Astrocystis sublimbata	0.001	7.5E-07	337	1.E-91
Aulographina pinorum	0.004	8.9E-07	425	2.E-118
Aureobasidium pullulans	0.154	4.7E-06	457	7.E-128

Bacidina arnoldiana	0.002	1.1E-06	294	6.E-79
Bacidina chloroticula	0.008	1.8E-06	392	2.E-108
Bahusakala australiensis	0.001	7.5E-07	435	4.E-121
Barriopsis fusca	0.001	7.5E-07	237	1.E-61
Beauveria bassiana	0.017	2.0E-06	457	8.E-128
Beauveria caledonica	0.033	1.9E-06	483	1.E-135
Bellemerea alpina	0.086	2.5E-06	202	4.E-51
Belonopsis ericae	0.005	3.4E-06	150	3.E-37
Belonopsis eriophori	0.242	2.9E-06	368	4.E-101
Bensingtonia ciliata	0.003	9.6E-07	88	2.E-16
Bionectria ochroleuca	0.001	7.5E-07	477	6.E-134
Blastobotrys terrestris	0.001	7.5E-07	219	4.E-56
Boletus edulis	0.103	8.9E-06	732	0.E+00
Botryosphaeria obtusa	0.011	1.1E-06	204	1.E-51
Botryozyma mucatilis	0.003	1.2E-06	94	4.E-18
Bovista dermoxantha	0.067	9.0E-06	553	1.E-156
Bullera miyagiana	0.013	1.6E-06	335	4.E-91
Bullera unica	0.002	1.1E-06	126	3.E-28
Byssoascus striatosporus	0.037	1.9E-06	185	4.E-46
Cadophora finlandica	1.386	1.8E-05	435	3.E-121
Calcarisporiella thermophila	0.028	1.7E-06	215	7.E-55
Caloplaca cerina	0.050	2.8E-06	121	1.E-26
Caloplaca chlorina	0.001	7.5E-07	117	2.E-25
Calyptrozyma arxii	0.103	3.5E-06	351	3.E-96
Camarographium koreanum	0.745	7.8E-06	283	1.E-75
Candida morakotiae	0.008	1.1E-06	69	5.E-11
Candida psychrophila	0.001	7.5E-07	529	2.E-149
Candida santamariae var membranifaciens	0.030	2.5E-06	516	1.E-145
Candida valdiviana	0.024	2.8E-06	152	4.E-36
Capnobotryella sp MA 4701	0.002	1.1E-06	379	2.E-104
Capronia sp 94003b	0.485	9.5E-06	420	1.E-116
Capronia sp 96003a	0.017	1.5E-06	416	1.E-115
Capronia sp 97003b	0.001	7.5E-07	444	6.E-124
Catenulifera brevicollaris	0.002	1.5E-06	346	2.E-94

Catenulostroma hermanusense	0.545	3.7E-06	449	1.E-125
Catenulostroma protearum	0.001	7.5E-07	444	6.E-124
Cenococcum geophilum	0.106	3.2E-06	399	1.E-110
Ceraceomyces fouquieriae	0.004	2.6E-06	102	6.E-21
Ceratobasidium sp AG A	0.027	8.7E-06	174	1.E-42
Cercophora appalachianensis	0.004	1.4E-06	215	5.E-55
Cercospora piaropi	0.003	1.9E-06	294	6.E-79
Cetraria aculeata	0.004	2.6E-06	135	4.E-31
Cetraria sepincola	0.001	7.5E-07	217	1.E-55
Cetrelia braunsiana	0.001	7.5E-07	86	5.E-16
Ceuthospora pinastri	0.005	1.3E-06	303	1.E-81
Chaetodermella luna	0.004	8.9E-07	156	4.E-37
Chaetomium aureum	0.122	2.4E-06	459	2.E-128
Chaetomium cupreum	0.004	1.0E-06	431	5.E-120
Chaetomium globosum	0.004	1.7E-06	448	4.E-125
Chaetomium jodhpurense	0.051	4.4E-06	370	1.E-101
Chaetomium truncatulum	0.003	1.9E-06	196	2.E-49
Chalara microchona	0.165	3.9E-06	433	1.E-120
Chaunopycnis pustulata	0.001	7.5E-07	407	8.E-113
Chlamydotubeufia khunkornensis	0.001	7.5E-07	191	8.E-48
Chrysosporium merdarium	0.001	7.5E-07	381	4.E-105
Chrysosporium pseudomerdarium	0.017	1.6E-06	407	7.E-113
Chytridiomycota sp Mori B3	0.012	4.8E-06	97	3.E-19
Cistella acuum	0.015	1.7E-06	300	1.E-80
Cistella grevillei	0.005	9.5E-07	350	1.E-95
Cladia aggregata	0.001	7.5E-07	147	2.E-34
Cladobotryum semicirculare	0.001	7.5E-07	156	6.E-39
Cladonia borealis	0.018	2.0E-06	446	2.E-124
Cladonia chlorophaea	0.001	7.5E-07	390	8.E-108
Cladonia coniocraea	0.004	8.9E-07	464	5.E-130
Cladonia diversa	0.007	2.0E-06	446	2.E-124
Cladonia fimbriata	0.080	2.7E-06	444	7.E-124
Cladonia foliacea	0.007	1.0E-06	416	1.E-115
Cladonia furcata	0.024	4.7E-06	475	2.E-133

Cladonia gracilis subsp elongata	0.001	7.5E-07	368	4.E-101
Cladonia gracilis subsp turbinata	0.021	2.0E-06	429	2.E-119
Cladonia pulvinata	0.001	7.5E-07	424	8.E-118
Cladophialophora chaetospira	0.007	9.9E-07	448	5.E-125
Cladophialophora minutissima	0.632	3.5E-06	455	3.E-127
Cladophialophora scillae	0.003	9.6E-07	315	5.E-85
Cladosporium cladosporioides	0.055	1.5E-06	446	2.E-124
Clitopilus hobsonii	0.252	6.9E-06	525	2.E-148
Clitopilus prunulus	0.009	2.2E-06	401	4.E-111
Clitopilus scyphoides	0.001	7.5E-07	351	8.E-98
Clitopilus sp FZ1433	0.004	1.7E-06	69	4.E-11
Clonostachys candelabrum	0.018	1.3E-05	387	1.E-106
Coccomyces dentatus	0.002	7.5E-07	340	8.E-93
Codinaeopsis gonytrichoides	0.001	7.5E-07	248	5.E-65
Coemansia sp ID05 F0205	0.001	7.5E-07	57	4.E-07
Coleophoma empetri	0.001	7.5E-07	307	8.E-83
Coleophoma eucalyptorum	0.009	1.0E-06	311	6.E-84
Collophora africana	0.127	4.8E-06	363	2.E-99
Collophora capensis	0.040	3.8E-06	315	5.E-85
Collophora paarla	0.006	1.1E-06	370	1.E-101
Collophora pallida	0.016	1.6E-06	298	5.E-80
Collophora rubra	0.331	7.0E-06	327	6.E-89
Collybia cirrhata	0.001	7.5E-07	553	1.E-156
Coniochaeta africana	0.035	1.9E-06	418	4.E-116
Coniochaeta prunicola	0.370	3.1E-06	204	1.E-51
Coniochaeta sp 2 ICMP 18911	0.003	1.9E-06	259	2.E-68
Coniophora olivacea	0.001	7.5E-07	372	3.E-102
Coniothyrium fuckelii	0.441	4.4E-06	449	1.E-125
Conoplea aff elegantula Kurogi sn	0.002	7.5E-07	165	6.E-40
Coprinellus disseminatus	0.002	1.1E-06	553	1.E-156
Coprinellus micaceus	0.002	7.5E-07	545	2.E-154
Coprinopsis cinerea	0.007	3.0E-06	538	3.E-152
Coprinus foetidellus	0.001	7.5E-07	523	9.E-148
Cordana ellipsoidea	0.005	1.7E-06	233	1.E-60

Cordyceps cylindrica	0.004	2.6E-06	316	1.E-85
Cortinarius uraceus	0.001	7.5E-07	82	8.E-15
Corynascus kuwaitiensis	0.003	1.1E-06	416	1.E-115
Corynespora olivacea	0.001	7.5E-07	287	1.E-76
Crepidotus mollis	0.001	7.5E-07	451	4.E-126
Crocicreas complicatum	0.027	2.0E-05	130	4.E-31
Crocicreas furvum	0.008	5.6E-06	172	6.E-44
Cryptococcus aerius	0.060	1.7E-06	551	4.E-156
Cryptococcus aff amylolyticus AS 22398	0.090	1.8E-06	462	2.E-129
Cryptococcus arrabidensis	0.002	1.1E-06	161	9.E-39
Cryptococcus dimennae	0.004	8.9E-07	418	3.E-116
Cryptococcus elinovii	0.002	1.1E-06	544	7.E-154
Cryptococcus flavus	0.006	1.2E-06	202	4.E-51
Cryptococcus laurentii	0.038	1.2E-06	499	1.E-140
Cryptococcus nemorosus	0.017	2.6E-06	148	6.E-35
Cryptococcus paraflavus	0.002	1.5E-06	193	3.E-48
Cryptococcus podzolicus	2.056	1.4E-05	468	4.E-131
Cryptococcus randhawii	0.044	1.2E-06	568	4.E-161
Cryptococcus saitoi	0.004	1.7E-06	580	6.E-165
Cryptococcus skinneri	0.053	1.8E-06	353	1.E-96
Cryptococcus sp MD76 1BY	0.019	1.3E-06	250	2.E-65
Cryptococcus terreus	0.002	1.5E-06	592	3.E-168
Cryptococcus terricola	0.034	1.2E-06	604	3.E-172
Cryptodiscus rhopaloides	0.004	8.9E-07	244	7.E-64
Cryptosporiopsis actinidiae	0.001	7.5E-07	261	6.E-69
Cryptosporiopsis ericae	0.012	1.3E-06	329	2.E-89
Cryptosporiopsis rhizophila	0.009	1.5E-06	246	2.E-64
Cudoniella acicularis	0.036	1.8E-06	348	4.E-95
Curvularia trifolii	0.002	1.1E-06	470	1.E-131
Cyathus striatus	0.001	7.5E-07	81	5.E-16
Cylindrocladiella camelliae	0.025	1.3E-06	68	2.E-10
Cylindrocladiella lageniformis	0.001	7.5E-07	106	3.E-22
Cyphellophora hylomeconis	0.006	1.2E-06	209	2.E-53
Cystocoleus ebeneus	0.008	1.7E-06	176	2.E-43

Dactylaria higginsii	0.006	1.1E-06	174	9.E-43
Dactylaria lanosa	0.006	2.3E-06	340	8.E-93
Dactylellina cionopaga	0.002	1.5E-06	479	2.E-134
Dactylellina ellipsospora	0.002	1.1E-06	435	4.E-121
Dactylellina lobata	0.002	7.5E-07	503	1.E-141
Dactylellina phymatopaga	0.019	1.6E-06	95	8.E-19
Daedaleopsis confragosa	0.006	2.5E-06	545	2.E-154
Daldinia concentrica	0.002	1.5E-06	479	2.E-134
Davidiella tassiana	0.143	2.3E-06	446	2.E-124
Debaryomyces hansenii	0.007	1.1E-06	529	2.E-149
Degelia gayana	0.036	1.5E-06	219	4.E-56
Devriesia americana	0.952	9.0E-06	335	3.E-91
Devriesia lagerstroemiae	0.001	7.5E-07	185	4.E-46
Diaporthe eres	0.004	1.5E-06	448	4.E-125
Dictyonema sp R04	0.002	1.1E-06	62	7.E-09
Dictyosporium toruloides	0.003	9.6E-07	278	6.E-74
Didymostilbe echinofibrosa	0.002	1.5E-06	250	1.E-65
Dissoconium dekkeri	0.002	1.5E-06	460	6.E-129
Dokmaia monthadangii	0.016	2.0E-06	427	6.E-119
Dothideomycetes sp DC2167	0.075	2.9E-06	448	4.E-125
Drechslera erythrospila	0.388	6.7E-06	460	6.E-129
Duddingtonia flagrans	0.001	7.5E-07	77	3.E-13
Elaphomyces muricatus	0.001	7.5E-07	115	1.E-26
Elsinoe ampelina	0.012	8.2E-07	303	1.E-81
Elsinoe banksiae	0.003	7.5E-07	302	4.E-81
Elsinoe mimosae	0.001	7.5E-07	53	6.E-06
Embellisia lolii	0.009	1.4E-06	215	5.E-55
Endogone lactiflua	0.001	7.5E-07	388	4.E-107
Endogone pisiformis	0.003	9.6E-07	84	2.E-15
Endomelanconiopsis endophytica	0.002	1.5E-06	174	8.E-43
Entoloma ameides	0.002	1.1E-06	44	3.E-03
Entoloma cetratum	0.002	1.5E-06	388	3.E-107
Entoloma platyphylloides	0.001	7.5E-07	407	9.E-113
Entoloma saundersii	0.001	7.5E-07	470	1.E-131

Entorrhiza aff fineranae PDD70949	0.002	1.5E-06	64	3.E-09
Entrophospora sp JJ61	0.004	1.1E-06	451	3.E-126
Entrophospora sp shylm131	1.076	7.3E-06	446	2.E-124
Entyloma linariae	0.001	7.5E-07	512	2.E-144
Entyloma polysporum	0.001	7.5E-07	592	3.E-168
Epacris microphylla root associated fungus 12	0.136	1.7E-06	435	3.E-121
Epacris microphylla root associated fungus 20	0.014	2.9E-06	233	1.E-60
Epacris microphylla root associated fungus 21	0.214	3.1E-06	398	4.E-110
Epacris microphylla root associated fungus 26	0.335	4.1E-06	431	4.E-120
Epacris microphylla root associated fungus 33	0.575	6.4E-06	444	6.E-124
Epacris microphylla root associated fungus 5	0.048	2.6E-06	494	6.E-139
Epacris microphylla root associated fungus 6	0.002	7.5E-07	122	3.E-27
Epacris pulchella root associated fungus EP12	0.003	2.3E-06	95	7.E-19
Epacris pulchella root associated fungus EP19	0.014	6.7E-06	433	1.E-120
Epacris pulchella root associated fungus EP20	0.297	4.1E-06	490	8.E-138
Epacris pulchella root associated fungus EP23	0.005	1.9E-06	399	1.E-110
Epacris pulchella root associated fungus EP26	0.014	9.9E-07	211	6.E-54
Epacris pulchella root associated fungus EP42	0.001	7.5E-07	331	6.E-90
Epacris pulchella root associated fungus EP52	0.001	7.5E-07	204	1.E-51
Epacris pulchella root associated fungus EP55	0.002	7.5E-07	449	1.E-125
Epicoccum nigrum	0.364	3.5E-06	459	2.E-128
Eucasphaeria capensis	0.004	1.7E-06	510	6.E-144
Eutypa lata	0.002	1.1E-06	403	1.E-111
Exobasidium inconspicuum	0.003	9.6E-07	403	1.E-111
Exobasidium japonicum	0.002	1.5E-06	368	4.E-101
Exophiala eucalyptorum	0.002	7.5E-07	459	2.E-128
Exophiala salmonis	0.001	7.5E-07	481	5.E-135
Exophiala sp EXP0542F	0.005	9.9E-07	399	1.E-110
Fellomyces lichenicola	0.015	2.3E-06	453	1.E-126
Fibulobasidium inconspicuum	0.003	1.2E-06	254	1.E-66
Fibulobasidium murrhardtense	0.001	7.5E-07	252	4.E-66
Filobasidium capsuligenum	0.003	1.9E-06	261	8.E-69
Fomes fomentarius	0.002	1.1E-06	538	3.E-152
Friedmanniomyces endolithicus	0.001	7.5E-07	315	5.E-85

Fulgensia schistidii	0.002	1.5E-06	169	4.E-41
Fusarium cerealis	0.002	1.1E-06	449	1.E-125
Fusarium flocciferum	0.002	1.1E-06	468	4.E-131
Fusarium lichenicola	0.002	1.5E-06	425	2.E-118
Fusarium merismoides var crassum	0.006	4.5E-06	291	8.E-78
Fusarium oxysporum	0.043	2.6E-06	448	4.E-125
Fusarium solani	0.031	1.3E-06	477	6.E-134
Fusicladium cordae	0.007	1.1E-06	484	4.E-136
Fusidium griseum	0.003	9.6E-07	486	1.E-136
Gaertneriomyces semiglobifer	0.001	7.5E-07	81	3.E-14
Gaertneriomyces tenuis	0.002	1.5E-06	97	3.E-19
Gaeumannomyces cylindrosporus	0.009	1.0E-06	490	8.E-138
Ganoderma applanatum	0.011	1.1E-06	529	2.E-149
Ganoderma sp E7091	0.026	4.8E-06	73	3.E-12
Geastrum triplex	0.001	7.5E-07	497	5.E-140
Geoglossum arenarium	0.030	2.1E-05	119	8.E-28
Geomyces pannorum	0.010	1.7E-06	438	3.E-122
Geopora clausa	0.001	7.5E-07	82	6.E-15
Geopora cooperi	0.002	1.1E-06	82	5.E-15
Gibberella avenacea	0.003	1.9E-06	473	8.E-133
Gibberella pulicaris	0.007	1.4E-06	99	6.E-20
Gibberella zeae	0.009	1.5E-06	449	1.E-125
Gigasperma americanum	0.001	7.5E-07	64	3.E-09
Glomerobolus gelineus	0.021	3.3E-06	292	2.E-78
Glomus versiforme	0.001	7.5E-07	440	9.E-123
Gonapodya prolifera	0.003	1.2E-06	100	2.E-20
Gymnopilus penetrans	0.002	7.5E-07	564	5.E-160
Gymnopus fusipes	0.003	2.3E-06	556	2.E-159
Gymnopus ocior	0.011	7.5E-06	95	1.E-20
Hapalopilus nidulans	0.006	4.5E-06	77	7.E-15
Hebeloma cavipes	0.026	5.0E-06	556	9.E-158
Helicodendron luteoalbum	0.001	7.5E-07	359	2.E-98
Helicodendron multiseptatum	0.002	1.5E-06	440	7.E-123
Helicodendron websteri	0.017	2.5E-06	435	3.E-121

Helicoma isiola	0.003	9.6E-07	250	1.E-65
Helicoon aff maioricense ICMP14920	0.001	7.5E-07	340	7.E-93
Helotiaceae sp II GK 2010	0.360	6.6E-06	379	2.E-104
Helotiaceae sp III GK 2010	0.005	9.9E-07	438	3.E-122
Helotiaceae sp IV GK 2010	0.002	1.5E-06	322	3.E-87
Helotiales sp 16 MV 2011	0.004	1.7E-06	353	1.E-96
Helotiales sp 27 MV 2011	0.046	3.1E-06	444	6.E-124
Helotiales sp 31 MV 2011	0.002	1.1E-06	111	7.E-24
Helotiales sp CWG F1 E3	0.001	7.5E-07	322	3.E-87
Helotiales sp EMF22	0.002	1.1E-06	433	1.E-120
Helotiales sp SC4 4	0.016	1.9E-06	298	5.E-80
Helotiales sp SC7 1	0.001	7.5E-07	246	2.E-64
Helvella maculata	0.003	1.2E-06	196	2.E-49
Hemicarpenteles ornatus	0.005	1.9E-06	237	1.E-61
Hemicarpenteles thaxteri	0.043	1.4E-05	252	4.E-66
Hemimycena gracilis	0.002	1.1E-06	124	1.E-27
Heyderia abietis	0.055	4.3E-06	313	2.E-84
Hirsutella minnesotensis	0.002	7.5E-07	479	2.E-134
Hirsutella rhossiliensis	0.007	8.2E-07	472	3.E-132
Hyaloscypha aureliella	0.002	1.1E-06	359	2.E-98
Hydnotrya tulasnei	0.064	6.1E-06	538	3.E-152
Hygrocybe coccinea	0.001	7.5E-07	92	2.E-19
Hygrophoropsis aurantiaca	0.002	1.1E-06	569	1.E-161
Hymenoscyphus monotropae	0.003	1.9E-06	154	1.E-36
Hyphodiscus hymeniophilus	0.005	2.0E-06	366	1.E-100
Hyphodontia radula	0.003	9.6E-07	507	8.E-143
Hypholoma fasciculare	0.016	2.7E-06	558	5.E-160
Hypocenomyce scalaris	0.003	9.6E-07	137	1.E-31
Hypocrea ceramica	0.014	3.5E-06	492	2.E-138
Hypocrea koningii	0.004	1.4E-06	492	2.E-138
Hypocrea lixii	0.035	1.7E-06	492	2.E-138
Hypocrea pilulifera	0.001	7.5E-07	470	1.E-131
Hypocrea rufa	0.022	1.4E-06	496	2.E-139
Hypoderma cordylines	0.001	7.5E-07	335	4.E-91

Hypomyces aconidialis	0.005	3.4E-06	121	2.E-28
Hypomyces lateritius	0.003	1.9E-06	113	4.E-26
Hypoxylon howeanum	0.001	7.5E-07	440	8.E-123
Inocybe lacera	2.236	3.3E-05	556	9.E-158
Inocybe lacera var helobia	0.004	1.1E-06	540	9.E-153
Inocybe ochroalba	0.020	3.8E-06	241	8.E-63
Itersonilia perplexans	0.004	1.4E-06	597	6.E-170
Kappamyces sp ARG063	0.001	7.5E-07	143	3.E-33
Knufia chersonesos	0.003	9.6E-07	322	3.E-87
Kockovaella machilophila	0.001	7.5E-07	285	4.E-76
Laccaria proxima	0.001	7.5E-07	547	5.E-155
Lachnum sp 1 MV 2011	0.026	1.5E-06	420	9.E-117
Lachnum virgineum	0.002	1.1E-06	436	9.E-122
Lactarius rufus	0.001	7.5E-07	652	0.E+00
Laetisaria lichenicola	0.060	7.9E-06	405	4.E-112
Lasiodiplodia gonubiensis	0.002	1.1E-06	182	5.E-45
Lasiodiplodia theobromae	0.004	1.5E-06	195	6.E-49
Lasiosphaeria ovina	0.003	7.5E-07	183	1.E-45
Lecanicillium fusisporum	0.009	6.4E-06	470	1.E-131
Lecanicillium psalliotae	0.031	1.2E-06	472	3.E-132
Lecanora polytropa	0.002	1.5E-06	285	4.E-76
Lecidea cancriformis	0.006	1.1E-06	396	2.E-109
Lecythophora hoffmannii	0.008	8.0E-07	429	2.E-119
Lecythophora mutabilis	0.087	2.1E-06	361	6.E-99
Lentinula edodes	0.003	1.9E-06	75	9.E-13
Leohumicola incrustata	0.001	7.5E-07	119	4.E-26
Leotiomycetes sp NK264	0.021	1.2E-06	436	9.E-122
Leptosphaeria contecta	0.001	7.5E-07	263	2.E-69
Leptosphaeria korrae	0.082	2.8E-06	351	4.E-96
Leptosphaeria sp SF99	0.001	7.5E-07	217	1.E-55
Leptosphaerulina australis	0.003	1.9E-06	444	6.E-124
Leptosphaerulina chartarum	0.007	8.2E-07	455	3.E-127
Lewia infectoria	0.007	9.9E-07	464	5.E-130
Limonomyces culmigenus	0.001	7.5E-07	396	2.E-109

Linderina macrospora	0.005	9.5E-07	71	2.E-11
Linderina pennispora	0.005	1.7E-06	75	1.E-12
Lindgomyces apiculatus	0.051	2.3E-06	250	1.E-65
Lindgomyces lemonweirensis	0.001	7.5E-07	226	2.E-58
Lobaria linita	0.028	1.5E-06	250	1.E-65
Lophiostoma chamaecyparidis	0.086	2.0E-06	350	1.E-95
Lophium mytilinum	0.001	7.5E-07	237	1.E-61
Lophodermium nitens	0.001	7.5E-07	143	2.E-33
Lophodermium pinastri	0.112	3.0E-06	438	3.E-122
Lophodermium pini excelsae	0.011	1.6E-06	449	1.E-125
Lophodermium seditiosum	0.002	1.5E-06	451	3.E-126
Lycoperdon lambinonii	0.001	7.5E-07	549	1.E-155
Lyophyllum sp Cultivar Jpn	0.038	1.6E-06	64	3.E-09
Macroconia leptosphaeriae	0.106	8.0E-06	259	2.E-68
Malassezia globosa	0.007	1.9E-06	669	0.E+00
Malassezia restricta	0.111	3.4E-06	678	0.E+00
Melanops tulasnei	0.002	1.1E-06	167	1.E-40
Meliniomyces bicolor	0.048	3.3E-06	431	4.E-120
Meliniomyces sp SM7 2	0.005	2.0E-06	392	2.E-108
Meliniomyces variabilis	0.001	7.5E-07	355	3.E-97
Meliniomyces vraolstadiae	0.009	1.6E-06	363	2.E-99
Metarhizium anisopliae	0.021	7.8E-06	496	2.E-139
Meyerozyma guilliermondii	0.001	7.5E-07	79	1.E-13
Micarea denigrata	0.027	2.0E-06	383	1.E-105
Microdiplodia hawaiiensis	0.013	1.2E-06	357	8.E-98
Microdochium bolleyi	0.022	2.5E-06	481	5.E-135
Microglossum olivaceum	0.003	2.3E-06	141	8.E-33
Microscypha ellisii	0.023	1.4E-06	375	2.E-103
Microsphaeropsis arundinis	1.275	9.2E-06	424	7.E-118
Microsphaeropsis proteae	0.017	2.3E-06	451	3.E-126
Mollisia cinerea	0.015	2.5E-06	435	4.E-121
Mollisia fusca	0.001	7.5E-07	496	2.E-139
Mollisia melaleuca	0.002	1.1E-06	427	6.E-119
Mollisia minutella	0.007	2.0E-06	436	9.E-122

Monacrosporium doedycoides	0.002	1.1E-06	81	2.E-14
Monoblepharella mexicana	0.002	7.5E-07	104	2.E-21
Monoblepharis macrandra	0.003	1.2E-06	110	3.E-23
Mortierella alpina	0.037	3.9E-06	634	0.E+00
Mortierella angusta	0.023	1.3E-06	619	1.E-176
Mortierella armillariicola	0.001	7.5E-07	102	6.E-21
Mortierella cystojenkinii	0.011	1.1E-06	586	1.E-166
Mortierella dichotoma	0.001	7.5E-07	92	1.E-17
Mortierella elasson	0.023	2.2E-06	278	1.E-73
Mortierella elongata	0.106	2.8E-06	636	0.E+00
Mortierella fimbricystis	0.012	1.4E-06	590	1.E-167
Mortierella gamsii	0.007	1.0E-06	580	6.E-165
Mortierella gemmifera	0.012	2.1E-06	623	1.E-177
Mortierella hyalina	0.005	9.5E-07	608	3.E-173
Mortierella lignicola	0.048	2.5E-06	520	1.E-146
Mortierella macrocystis	0.012	2.0E-06	601	4.E-171
Mortierella minutissima	0.004	3.0E-06	281	7.E-75
Mortierella parvispora	0.003	9.6E-07	608	3.E-173
Mortierella sp CZ 2011	0.003	1.2E-06	274	1.E-72
Mortierella sp TR065	0.031	1.2E-06	632	2.E-180
Mortierella sp TR158	0.059	3.6E-06	488	3.E-137
Mortierella verticillata	0.015	1.3E-06	612	2.E-174
Mucor fragilis	0.001	7.5E-07	503	1.E-141
Mycena arcangeliana	0.022	6.6E-06	560	7.E-159
Mycena corynephora	0.019	3.4E-06	154	1.E-36
Mycena galopus	0.054	1.1E-05	560	7.E-159
Mycena olida	0.003	9.6E-07	124	1.E-27
Mycena silvae nigrae	0.001	7.5E-07	139	4.E-32
Mycena tenax	0.001	7.5E-07	459	3.E-128
Mycoblastus sanguinarius	0.004	1.3E-06	77	2.E-13
Mycocalicium victoriae	0.001	7.5E-07	303	1.E-81
Mycosphaerella delegatensis	0.002	1.1E-06	217	1.E-55
Mycosphaerella punctiformis	0.005	1.9E-06	435	3.E-121
Myrmecridium phragmitis	0.006	1.5E-06	209	2.E-53

Myxotrichum chartarum	0.001	7.5E-07	189	3.E-47
NA	4.071	NA	NA	NA
Naemacyclus minor	0.009	1.9E-06	448	4.E-125
Naohidea sebacea	0.001	7.5E-07	217	2.E-55
Neofusicoccum arbuti	0.003	1.9E-06	202	4.E-51
Niptera dilutella	0.007	4.9E-06	374	1.E-104
Noosia banksiae	0.006	1.8E-06	370	1.E-101
Occultifur aff externus IMUFRJ 52019	0.001	7.5E-07	195	9.E-49
Ochrocladosporium frigidarii	0.001	7.5E-07	394	6.E-109
Ochroconis gamsii	0.002	1.1E-06	108	1.E-22
Octospora wrightii	0.031	4.5E-06	99	8.E-20
Ogataea chonburiensis	0.010	4.6E-06	126	3.E-28
Oidiodendron chlamydosporicum	0.002	7.5E-07	359	2.E-98
Oliveonia pauxilla	0.007	3.2E-06	185	5.E-46
Omphalina foliacea	0.001	7.5E-07	148	7.E-35
Ophiocordyceps sinensis	0.506	7.6E-06	377	6.E-104
Ophiostoma sp X ES 1996	0.005	3.4E-06	518	4.E-146
Paecilomyces carneus	0.001	7.5E-07	488	3.E-137
Paecilomyces marquandii	0.003	1.9E-06	510	6.E-144
Panaeolus papilionaceus	0.005	3.8E-06	79	2.E-15
Pannaria pallida	0.001	7.5E-07	86	4.E-16
Paraconiothyrium cyclothyrioides	0.004	1.3E-06	414	5.E-115
Paraconiothyrium sp HS 03	0.013	6.1E-06	449	1.E-125
Paraphoma chrysanthemicola	0.004	1.7E-06	213	2.E-54
Passalora zambiae	0.013	1.1E-06	252	4.E-66
Penicillium aculeatum	0.035	3.0E-06	457	7.E-128
Penicillium adametzii	0.009	9.4E-07	473	8.E-133
Penicillium biourgeianum	0.002	1.1E-06	468	4.E-131
Penicillium canescens	0.012	1.7E-06	468	4.E-131
Penicillium cecidicola	0.009	2.0E-06	477	6.E-134
Penicillium glabrum	0.002	1.1E-06	387	1.E-106
Penicillium islandicum	0.013	1.9E-06	472	3.E-132
Penicillium lividum	0.001	7.5E-07	377	6.E-104
Penicillium melinii	0.001	7.5E-07	363	2.E-99

Penicillium montanense	0.046	1.9E-06	460	6.E-129
Penicillium namyslowskii	0.004	8.9E-07	473	8.E-133
Penicillium ochrochloron	0.001	7.5E-07	472	3.E-132
Penicillium primulinum	0.001	7.5E-07	396	2.E-109
Penicillium proteolyticum	0.016	3.0E-06	475	2.E-133
Penicillium pulvillorum	0.001	7.5E-07	466	1.E-130
Penicillium purpurogenum	0.015	1.1E-05	435	4.E-121
Penicillium purpurogenum var rubrisclerotium	0.005	2.0E-06	459	2.E-128
Penicillium ramulosum	0.004	2.6E-06	477	6.E-134
Penicillium verruculosum	0.102	4.2E-06	457	7.E-128
Penidiella aggregata	0.002	7.5E-07	316	1.E-85
Penidiella ellipsoidea	0.008	1.4E-06	401	3.E-111
Peniophora cinerea	0.003	9.6E-07	477	7.E-134
Peniophorella praetermissa	0.003	1.9E-06	545	2.E-154
Pestalotiopsis besseyi	0.001	7.5E-07	431	4.E-120
Pestalotiopsis caudata	0.003	1.2E-06	473	8.E-133
Pestalotiopsis citrina	0.012	1.3E-06	449	1.E-125
Pezicula carpinea	0.002	1.5E-06	233	1.E-60
Pezicula cinnamomea	0.002	1.5E-06	407	7.E-113
Peziza ostracoderma	0.003	9.6E-07	442	2.E-123
Pezizomycotina sp 11302	0.002	1.1E-06	99	6.E-20
Phacidiopycnis pyri	0.003	1.2E-06	244	6.E-64
Phacidiopycnis washingtonensis	0.084	9.2E-06	302	4.E-81
Phaeoacremonium aleophilum	0.001	7.5E-07	92	9.E-18
Phaeoacremonium griseorubrum	0.003	9.6E-07	224	8.E-58
Phaeoacremonium mortoniae	0.012	1.8E-06	104	1.E-21
Phaeobotryon mamane	0.003	1.9E-06	172	3.E-42
Phaeobotryosphaeria visci	0.001	7.5E-07	355	3.E-97
Phaeococcomyces catenatus	0.004	1.4E-06	531	5.E-150
Phaeococcomyces nigricans	0.032	1.3E-06	342	2.E-93
Phaeocollybia sipei	0.001	7.5E-07	71	1.E-11
Phaeophyscia exornatula	0.052	4.7E-06	172	3.E-42
Phaeophyscia squarrosa	0.001	7.5E-07	169	4.E-41
Phaeosclera dematioides	0.001	7.5E-07	180	2.E-44

Phaeosclera sp TRN524	0.001	7.5E-07	209	2.E-53
Phaeosphaeria avenaria	0.092	3.2E-06	425	2.E-118
Phaeosphaeria herpotrichoides	0.006	2.3E-06	442	2.E-123
Phaeosphaeria nodorum	0.041	1.3E-06	427	6.E-119
Phaeosphaeria phragmitis	0.008	1.9E-06	431	4.E-120
Phaeothecoidea melaleuca	0.011	1.2E-06	351	4.E-96
Phaeothecoidea proteae	0.002	1.5E-06	425	2.E-118
Phallus impudicus	0.002	1.5E-06	363	2.E-99
Phanerochaete sordida	0.001	7.5E-07	84	2.E-15
Phialemonium aff dimorphosporum II 0563b	0.001	7.5E-07	206	3.E-52
Phialemonium curvatum	0.008	1.1E-06	213	2.E-54
Phialocephala fluminis	0.028	1.3E-06	350	1.E-95
Phialocephala fortinii	0.051	2.3E-06	438	3.E-122
Phialocephala virens	0.118	6.0E-06	344	6.E-94
Phialophora sessilis	0.001	7.5E-07	499	1.E-140
Phialophora sp DF36	0.156	2.5E-06	422	3.E-117
Phialophora sp MLB Phi	0.005	1.9E-06	313	2.E-84
Phlebia radiata	0.002	1.1E-06	527	7.E-149
Phlebiella vaga	0.002	1.1E-06	99	1.E-21
Phoma eupyrena	0.001	7.5E-07	449	1.E-125
Phoma herbarum	0.412	3.3E-06	383	1.E-105
Phoma macrostoma	0.002	1.1E-06	320	1.E-86
Phoma moricola	0.001	7.5E-07	339	3.E-92
Piptoporus betulinus	0.001	7.5E-07	531	5.E-150
Placynthiella uliginosa	0.010	1.2E-06	327	6.E-89
Platygloea disciformis	0.021	2.6E-06	263	2.E-69
Pleopsidium chlorophanum	0.001	7.5E-07	230	2.E-59
Pleospora herbarum	0.002	1.1E-06	464	5.E-130
Pleosporales sp 5 TMS 2011	0.006	1.5E-06	303	1.E-81
Pleosporales sp HF 10	0.001	7.5E-07	265	5.E-70
Pleosporales sp Rx 1 1	0.002	1.1E-06	324	8.E-88
Pleurotus eryngii	0.001	7.5E-07	540	9.E-153
Pochonia bulbillosa	0.023	2.0E-06	514	5.E-145
Pochonia suchlasporia	0.007	1.7E-06	492	2.E-138

Poculum henningsianum	0.001	7.5E-07	403	1.E-111
Podospora decidua	0.004	8.9E-07	381	5.E-105
Podospora ellisiana	0.084	2.4E-06	357	8.E-98
Podospora minicauda	0.001	7.5E-07	209	2.E-53
Podospora miniglutinans	0.014	1.3E-06	425	2.E-118
Podospora petrogale	0.009	1.3E-06	246	2.E-64
Podospora pyriformis	0.001	7.5E-07	158	9.E-38
Polyphlyctis unispina	0.001	7.5E-07	200	2.E-50
Porosphaerella cordanophora	0.051	2.0E-06	250	1.E-65
Powellomyces hirtus	0.012	1.6E-06	94	3.E-18
Powellomyces sp DAOM 226231	0.001	7.5E-07	81	3.E-14
Preussia dubia	0.001	7.5E-07	381	5.E-105
Preussia isomera	0.004	1.0E-06	230	2.E-59
Preussia pseudominima	0.004	1.1E-06	424	7.E-118
Preussia sp 2 ICMP 18938	0.002	1.1E-06	446	2.E-124
Preussia sp SL08070	0.163	3.5E-06	448	4.E-125
Preussia tetramera	0.002	1.1E-06	307	8.E-83
Pringsheimia euphorbiae	0.077	1.7E-06	300	1.E-80
Pseudaegerita corticalis	0.001	7.5E-07	333	1.E-90
Pseudaegerita viridis	0.007	1.5E-06	161	6.E-39
Pseudaleuria quinaultiana	0.002	7.5E-07	86	5.E-16
Pseudeurotium desertorum	0.001	7.5E-07	355	3.E-97
Pseudeurotium ovale var milkoi	0.003	9.6E-07	276	2.E-73
Pseudoclathrosphaerina spiralis	0.003	9.6E-07	350	1.E-95
Pseudocyphellaria clathrata	0.002	1.1E-06	132	5.E-30
Pseudogymnoascus roseus	0.003	7.5E-07	442	2.E-123
Pseudotomentella tristis	0.002	1.5E-06	82	1.E-16
Psilocybe montana	0.102	6.1E-06	542	2.E-153
Pulveroboletus curtisii	0.007	4.9E-06	104	3.E-23
Pyrenochaeta inflorescentiae	0.003	1.9E-06	416	1.E-115
Pyrenochaetopsis microspora	0.020	1.7E-06	392	2.E-108
Pyrenophora leucospermi	1.052	7.3E-06	460	6.E-129
Pyrenophora seminiperda	0.003	1.9E-06	366	1.E-100
Pyxine limbulata	0.001	7.5E-07	99	5.E-20

Quambalaria cyanescens	0.006	2.4E-06	71	1.E-11
Rachicladosporium pini	0.194	2.9E-06	425	2.E-118
Raffaelea montetyi	0.001	7.5E-07	512	2.E-144
Ramalina farinacea	0.001	7.5E-07	252	4.E-66
Ramaria abietina	0.001	7.5E-07	121	2.E-26
Ramaria gracilis	0.001	7.5E-07	92	1.E-17
Ramularia sp KACC 42532	0.001	7.5E-07	215	6.E-55
Rhexocercosporidium panacis	0.151	3.0E-06	431	4.E-120
Rhizoctonia sp 266	0.001	7.5E-07	566	2.E-160
Rhizophlyctis rosea	0.092	3.5E-06	617	4.E-176
Rhizophydium chaetiferum	0.033	1.6E-05	86	5.E-16
Rhizophydium globosum	0.032	1.8E-06	88	2.E-16
Rhizopogon arctostaphyli	0.002	1.1E-06	99	7.E-20
Rhizopogon luteolus	4.652	3.1E-05	654	0.E+00
Rhizopogon subcaerulescens	0.001	7.5E-07	99	6.E-20
Rhizoscyphus ericae	4.226	3.1E-05	479	2.E-134
Rhodotorula aff marina IMUFRJ 52025	0.002	1.5E-06	94	3.E-18
Rhodotorula auriculariae	0.001	7.5E-07	309	3.E-83
Rhodotorula cassiicola	0.008	8.0E-07	257	1.E-67
Rhodotorula diffluens	0.006	8.3E-07	283	2.E-75
Rhodotorula mucilaginosa	0.002	1.5E-06	571	3.E-162
Rhodotorula philyla	0.025	2.6E-06	374	1.E-102
Rhodotorula yarrowii	0.002	1.1E-06	460	7.E-129
Rhynchostoma proteae	0.120	1.8E-06	86	5.E-16
Rickenella mellea	0.002	1.5E-06	510	7.E-144
Russula consobrina	0.002	1.5E-06	62	1.E-10
Saccharata sp 1 ICMP 18939	0.002	7.5E-07	207	8.E-53
Saccharicola bicolor	0.001	7.5E-07	436	1.E-121
Saccharomyces cerevisiae	0.010	7.2E-06	599	2.E-170
Saccharomycopsis microspora	0.001	7.5E-07	62	9.E-09
Sagenomella diversispora	1.037	6.6E-06	448	4.E-125
Sagenomella humicola	2.078	1.2E-05	460	6.E-129
Sagenomella striatispora	0.034	1.8E-06	460	6.E-129
Sarcoleotia globosa	0.341	5.3E-06	322	3.E-87

Sarea difformis	0.002	7.5E-07	156	3.E-37
Scedosporium apiospermum	0.003	9.6E-07	529	2.E-149
Scedosporium aurantiacum	0.002	7.5E-07	542	2.E-153
Scedosporium prolificans	0.003	7.5E-07	350	1.E-95
Schizoxylon albescens	0.001	7.5E-07	100	1.E-20
Scleroderma citrinum	0.057	9.1E-06	621	3.E-177
Sclerostagonospora opuntiae	0.002	7.5E-07	453	1.E-126
Sclerotium delphinii	0.007	2.6E-06	64	2.E-09
Scolecobasidium excentricum	0.001	7.5E-07	436	1.E-121
Scoliciosporum chlorococcum	0.001	7.5E-07	390	7.E-108
Scoliciosporum umbrinum	0.001	7.5E-07	377	6.E-104
Sebacina grisea	0.003	7.5E-07	100	2.E-20
Sebacina vermifera	0.025	3.3E-06	355	3.E-97
Septobasidium kameii	0.001	7.5E-07	401	4.E-111
Septoria digitalis	0.001	7.5E-07	230	2.E-59
Sesquicillium microsporum	0.002	1.5E-06	448	4.E-125
Sistotrema alboluteum	0.862	2.6E-04	387	2.E-108
Sistotrema brinkmannii	0.002	1.1E-06	246	2.E-64
Sistotrema coronilla	0.002	7.5E-07	217	2.E-55
Sistotrema muscicola	0.004	1.5E-06	137	1.E-31
Sistotrema sp B216	35.491	1.2E-04	551	6.E-156
Sordaria alcina	0.040	2.4E-06	446	2.E-124
Sordariales sp G9i88H	0.003	7.5E-07	394	6.E-109
Sordariales sp MU 2009 1	0.007	1.4E-06	224	8.E-58
Sordariomycetes sp DC2118	0.045	1.5E-06	440	7.E-123
Sordariomycetes sp E8924B	0.001	7.5E-07	104	1.E-21
Sowerbyella imperialis	0.002	1.1E-06	81	4.E-16
Sowerbyella radiculata var. kewensis	0.001	7.5E-07	86	8.E-18
Sphaceloma protearum	0.002	7.5E-07	303	1.E-81
Sphaerobolus iowensis	0.047	3.5E-06	569	1.E-161
Sphaeropsis pyriputrescens	0.003	7.5E-07	246	2.E-64
Sphaeropsis sapinea	0.003	7.5E-07	464	5.E-130
Sphaerostilbella aureonitens	0.001	7.5E-07	385	4.E-106
Spiromastix tentaculatum	0.005	1.3E-06	326	2.E-88

Spizellomyces dolichospermus	0.001	7.5E-07	100	2.E-20
Spizellomyces lactosolyticus	0.061	5.6E-06	200	2.E-50
Spizellomyces palustris	0.004	1.4E-06	568	4.E-161
Spizellomyces plurigibbosus	0.014	1.8E-06	102	5.E-21
Spizellomyces pseudodichotomus	0.043	2.9E-06	148	7.E-35
Sporobolomyces dracophylli	0.001	7.5E-07	244	8.E-64
Sporobolomyces falcatus	0.002	7.5E-07	342	3.E-93
Sporobolomyces gracilis	0.008	1.6E-06	547	5.E-155
Sporobolomyces inositophilus	0.006	1.3E-06	390	1.E-107
Sporobolomyces lactophilus	0.002	1.1E-06	254	1.E-66
Sporobolomyces lophatheri	0.001	7.5E-07	307	1.E-82
Sporobolomyces marcillae	0.001	7.5E-07	523	9.E-148
Sporobolomyces roseus	0.004	8.9E-07	551	4.E-156
Sporobolomyces subbrunneus	0.011	1.3E-06	73	3.E-12
Sporobolomyces symmetricus	0.001	7.5E-07	538	3.E-152
Squamarina gypsacea	0.038	1.5E-06	377	6.E-104
Staphylotrichum coccosporum	0.005	1.9E-06	159	2.E-38
Stictis radiata	0.292	4.6E-06	294	6.E-79
Strumella coryneoidea	0.013	1.6E-06	104	1.E-21
Suillus bovinus	0.146	1.9E-05	612	2.E-174
Suillus luteus	9.093	3.8E-05	608	2.E-173
Sydowia polyspora	0.041	2.1E-06	464	5.E-130
Syncephalastrum racemosum	0.084	3.9E-06	457	7.E-128
Syzygospora sorana	0.001	7.5E-07	274	1.E-72
Talaromyces helicus var major	0.002	1.1E-06	411	6.E-114
Talaromyces purpureus	0.001	7.5E-07	344	6.E-94
Talaromyces thermophilus	0.008	1.3E-06	497	5.E-140
Taphrina purpurascens	0.001	7.5E-07	551	4.E-156
Teratosphaeria capensis	0.098	1.7E-06	453	1.E-126
Teratosphaeria encephalarti	0.001	7.5E-07	298	5.E-80
Teratosphaeria jonkershoekensis	0.027	3.0E-06	377	6.E-104
Teratosphaeria karinae	0.001	7.5E-07	134	2.E-30
Teratosphaeria knoxdaviesii	0.001	7.5E-07	355	3.E-97
Teratosphaeria mexicana	0.001	7.5E-07	150	2.E-35

Teratosphaeria microspora	0.033	1.6E-06	438	3.E-122
Tetracladium apiense	0.005	8.6E-07	81	2.E-14
Texosporium sancti jacobi	0.003	9.6E-07	88	1.E-16
Thanatephorus cucumeris	0.002	1.1E-06	95	1.E-18
Thanatephorus theobromae	0.001	7.5E-07	113	3.E-24
Thedgonia ligustrina	0.001	7.5E-07	228	6.E-59
Thelebolaceae sp BEA 2010	0.001	7.5E-07	444	6.E-124
Thelebolus sp UFMGCB 3742	0.001	7.5E-07	200	1.E-50
Thelephora terrestris	0.236	6.4E-06	577	1.E-165
Thermomyces lanuginosus	0.001	7.5E-07	473	8.E-133
Thielavia microspora	0.003	9.6E-07	250	2.E-65
Thielavia terrestris	0.021	4.0E-06	81	2.E-14
Thozetella havanensis	0.004	8.9E-07	250	1.E-65
Tolypocladium cylindrosporum	0.002	1.1E-06	433	1.E-120
Torrendiella brevisetosa	0.001	7.5E-07	84	2.E-15
Trechispora confinis	0.001	7.5E-07	529	2.E-149
Trechispora hymenocystis	0.003	1.2E-06	390	1.E-107
Trechispora subsphaerospora	0.017	2.3E-06	387	1.E-106
Tremella foliacea	0.006	1.2E-06	346	2.E-94
Tremella polyporina	0.004	3.0E-06	97	2.E-19
Trichocladium asperum	0.005	1.4E-06	453	1.E-126
Trichoderma atroviride	0.027	1.6E-06	492	2.E-138
Trichoderma inhamatum	0.002	7.5E-07	488	3.E-137
Tricholoma albobrunneum	0.001	7.5E-07	562	4.E-161
Tricholoma imbricatum	0.007	2.7E-06	562	2.E-159
Tricholoma scalpturatum	0.002	1.1E-06	544	7.E-154
Tricholoma vaccinum	0.001	7.5E-07	538	3.E-152
Trichophaea gregaria	0.095	6.8E-05	289	6.E-79
Trichosporon jirovecii	0.002	1.1E-06	453	1.E-126
Trichosporon moniliiforme	0.002	1.5E-06	475	2.E-133
Trichosporon porosum	0.005	1.9E-06	473	8.E-133
Trimmatostroma betulinum	0.003	2.3E-06	398	4.E-110
Trimmatostroma cordae	0.002	1.1E-06	425	2.E-118
Trimorphomyces papilionaceus	0.011	2.9E-06	128	6.E-29

Tubeufia cerea	0.006	1.1E-06	294	6.E-79
Umbelopsis autotrophica	0.160	2.6E-06	536	1.E-151
Umbelopsis isabellina	0.111	2.9E-06	518	4.E-146
Umbelopsis ramanniana	0.301	4.8E-06	577	7.E-164
Umbilicaria esculenta	0.048	1.7E-06	196	2.E-49
Varicosporium elodeae	0.005	9.5E-07	442	2.E-123
Vascellum pratense	0.004	1.3E-06	556	9.E-158
Vermispora fusarina	0.015	1.1E-06	195	8.E-49
Verticillium leptobactrum	0.001	7.5E-07	497	5.E-140
Volvariella pusilla	0.003	1.9E-06	442	2.E-123
Vonarxia vagans	0.840	2.3E-05	94	2.E-18
Wilcoxina mikolae	7.655	4.3E-05	470	1.E-131
Woollsia root associated fungus X	0.010	4.3E-06	490	8.E-138
Xanthoparmelia mexicana	0.001	7.5E-07	135	4.E-31
Xanthoria parietina	0.012	1.3E-06	161	7.E-39
Xenobotrytis acaducospora	0.008	1.8E-06	250	1.E-65
Xenochalara juniperi	0.007	1.2E-06	416	1.E-115
Xenopolyscytalum pinea	0.016	2.2E-06	440	7.E-123
Xylaria globosa	0.002	1.1E-06	97	2.E-19
Xylaria polymorpha	0.002	1.5E-06	158	2.E-39
Zalerion arboricola	0.011	2.9E-06	444	6.E-124
Zalerion varium	0.006	1.5E-06	438	3.E-122
Zopfiella karachiensis	0.006	1.5E-06	339	3.E-92
Zopfiella tabulata	0.003	7.5E-07	171	1.E-41