Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala 2010

Substrate utilization, ammonium mineralization and phylogenetic relationships of the fungi *Leptodontidium* sp. found in boreal forest soil

Daniel Robert Heinrich Graf-Wimark

Master´s thesis • 30 hec • Advanced E
Masterprogramme Soil and Water Management

Substrate utilization, ammonium mineralization and phylogenetic relationships of the fungi *Leptodontidium* sp. found in boreal forest soil

Daniel Robert Heinrich Graf-Wimark

Supervisor:	Inga T.M. Bödeker, Swedish University of Agriculture, Forest Mycology and Pathology
Assistant Supervisor:	Björn D. Lindahl, Swedish University of Agriculture, Forest Mycology and Pathology
Examiner:	Petra M. A. Fransson, Swedish University of Agriculture, Forest Mycology and Pathology

Credits: 30
Level: Advanced E
Course title: Independent project in Soil Science
Course code: EX0430
Programme/education: Master Programme Soil and Water Management

Place of publication: Uppsala, Sweden
Online publication: http://stud.epsilon.slu.se

Key Words: ***Leptodontidium*** **sp., soil fungi, ammonium mineralization, phylogeny, boreal forest**

Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology

Abstract

Many studies on global elements fluxes neglect biological aspects or simply refer to them unspecifiedly as biological activity. The advent of molecular tools to investigate these activities has although given rise to a further understanding of the matter. In this study an attempt to determine the ecology and function of 11 strains of the fungal genus Leptodontidium *(*L.*) in boreal forest soils is made. In an earlier study* (Lindahl et al. 2010) *strains with affinity to* L. *increased in abundance after the disruption of root carbon transport from host trees to ectomycorrhizal Fungi. At the same time ectomycorrhizal species decreased in abundance which provoked the hypothesis that the senescent ectomycorrhiza is decomposed by* L. *and other opportunistic Fungi. To test this hypothesis* L. *strains were grown on different agar based substrates while substrate utilization was assessed by measuring respiration. Since other studies observed increased soil ammonium levels in disturbed forests (e.g. clear cut areas) another experiment was set up to measure ammonium mineralization of* L. *when decomposing fungal substrate. Finally a phylogeny of the strains based on the rDNA internal spacer and large subunit region was made in order to get more information on taxonomic relationships as well as ecological and functional information.*

Keywords: Leptodontidium, ammonium, phylogeny, forest mycology, soil

Table of contents

1 Introduction

Filamentous Fungi[1] play a key role as decomposers of organic matter in terrestrial ecosystems (Peay et al. 2008). The filamentous lifestyle gives them an advantage over unicellular organisms when competing for resources in the way that they can bridge air filled pores (Boer et al. 2005) in soil and are able to translocate solutes from one part of the mycelium to another (Jennings 1987). These features enable them to exploit detritus of different quality and different nutrient ratios at the same time (Lindahl & Olsson 2004, Boberg et al. 2010) which is indeed a great advantage in an environment as heterogeneous as soil. Another important feature of filamentous Fungi is that they have developed ways to degrade recalcitrant organic matter such as wood and coniferous litter. Hyphal growth provides an advantage also here because the decomposition of organic matter requires penetration of the tissue (Boer et al. 2005). But the perhaps most important trait of filamentous Fungi is the unique enzymatic pathways that BASIDIOMYCOTA and ASCOMYCOTA have developed to degrade complex plant derived material like lignin (Rayner & Boddy 1988). Lignin is a very stable material that withstands both physical and chemical degradation under normal environmental conditions and thus would accumulate in soils restricting nutrient availability if it would not be degraded by Fungi (Taylor & Osborne 1996).

Ecologically decomposing filamentous Fungi can be grouped into three major groups – free living saprotrophs, opportunistic saprotrophs and plant mutualists. The latter group is called mycorrhiza Fungi and contains two major types - arbuscular or endomycorrhiza (AM) Fungi and ectomycorrhiza (EM) Fungi. 92 % of all land plants engage in mycorrhizal mutualism (Wang et al. 2006) whereby the main

[1] The term Fungi refers to all species belonging to the kingdom Fungi. It excludes organisms such as Oomycota and Actinobacteria which previously belonged to the Kingdom. Because of their fungal characteristics they are still often referred to as fungi. For further details see McLaughlin et al. (2009)

characteristic is that photosynthetically fixed C from the plant is exchanged for plant available forms of e.g. P and N from the fungus. Trappe (1987) defined AM as the oldest form of mycorrhiza in angiosperms dating back to 400 million years ago and Wang et al. (2006) widened the definition to all land plants. According to them both EM and nonmycorrhiza Fungi have developed after AM. All extant forms of AM belong to the *Glomeromycota* while EM Fungi can both be found among *Basidiomycota* and *Ascomycota.* Wang et al. (2006) state that EM has developed convergently from nonmycorrhizal Fungi to EM and back thus indicating that evolution of EM is highly dependent on environmental conditions. They further hypothesize that EM comprises an evolutionary short-term adaptation to harsh environmental conditions such as nutrient deficiency or aridity. This is in contrast to AM which established an evolutionary long-term relation with plants when they conquered the land in the Ordovician age.

Soils in coniferous forests are comparatively nutrient poor with a thick humus layer that is dominated by EM species (O'Brien et al. 2005, Lindahl et al. 2007). The fact that they have outsourced carbon derivation to their host plants seems to give them a competitive advantage over free living saprotrophs which have to derive both carbon and mineral nutrients from the soil substrate. Lindahl et al. (2001) showed in a microcosm experiment that the EM species *Suillus variegatus* is outcompeted by the free living *Hypholoma fasciculare* when the latter is provided with a comparatively larger C source while the opposite was the case when *Hypholoma fasciculare* was provided with a smaller C source. The competitive advantage of EM species is however lost when the hyphal connection to the host is physically disrupted or when the host is killed or impeded in transporting C to the fungal mycelium. Studies on the effect of root disruption have shown significant decreases in fungal biomass that could be attributed to negative effects on mycorrhizal species (Siira-Pietikäinen et al. 2001), Brant et al. 2006). Yarwood et al. (2009) cut off the carbon flux to the mycorrhiza through the phloem by girdling the host tree which had long-term detrimental effects on mycorrhizal while the relative abundance of nonmycorrhiza Fungi increased. Lindahl et al. (2010) disrupted carbon transport in a field study by inserting plastic tubes into forest soil thus cutting off mycorrhiza hyphal connections with the plant hosts. Within these cores they observed an increase in hyphal densities and an increase in the share of the overall DNA pool by free living saprotrophic Fungi belonging to the genera *Capronia*, *Penicillium* and *Leptodontidium*. They also observed significant increases of laccase, cellulase and N-acetylglucosaminidase (NAG) activities the

latter of which is involved in chitin degrading (Lindahl & Finlay 2006). With chitin being a major component of fungal cell walls this indicates degradation of fungal substrate. In this context Lindahl et al. (2010) state the idea that the free living saprotrophs take advantage of the severed mycorrhiza using the mycorrhizal mycelium itself as a substrate. The increase of laccases supports this idea because laccases have earlier been observed to be involved in antagonistic interactions of white-rot fungi with other fungi and bacteria (Baldrian 2004).

Under undisturbed conditions free living litter saprotrophs are nitrogen limited in low productivity boreal forest (Boberg et al. 2008) which makes N mineralization unlikely to occur. As stated above the decomposing community in the humus layer of coniferous forests is dominated by EM Fungi. As mutualists they transport nutrients directly to their hosts constituting a short cut in nutrient circulation that may circumvent the need for mineralization (Lindahl et al. 2002; Lindahl et al. 2007). The situation changes however when C translocation from host plants to mycorrhiza is disrupted.

Rosén et al. (1996) who compared net release of nutrients into a catchment area from adjacent clear cut areas with the release from undisturbed forest areas found a particular increase of K^+, NH_4^+, NO_3^-, organic N and total N in the runoff from the clear cut areas but only comparatively small amounts of these minerals from undisturbed forest areas. In addition Sulkava & Huhta (2003) found that NH_4^+ levels increase in combination with frequent freeze-thaw cycles in boreal forest soil. Rosén et al. (1996) attribute the changes to increased runoff, increased insulation and increased biological activity in the soil. Going back to what has been found above this nebulous "biological activity" might be opportunistic saprotrophic fungi decomposing mycorrhizal mycelium. Given the fact that about 8% of the Swedish N emissions into the Baltic Sea come from clear cut areas (Löfgren et al. 2009) it is important to investigate the biological mechanisms involved in nitrogen cycling and retention.

Lindahl et al. (2010) hypothesize that the opportunists living on dead mycelium and other easily available compounds in the aftermath of a perturbation are likely to become carbon limited assuming that they are specialized on mycelium and have limited access to other sources, either due to low degradation capacity or to limited competitive strength and thus mineralize nitrogen. As stated above one of the main actors in their experiment were fungal strains with affinity to members of

the anamorphic genus *Leptodontidium*. Within an earlier study at the same site (Lindahl et al. 2007) these strains were not found in decaying litter but as previously shown increased significantly in abundance subsequent to perturbation. This strengthens the hypothesis that at least some *Leptodontidium* strains follow an opportunistic lifestyle or to say it in terms of ecological theory an R strategy.

Thus one aim of this study is to verify this hypothesis and to determine substrate preferences of different *Leptodontidium* species. Another aim is to verify whether those *L.* species that prefer a fungal substrate mineralize ammonium in the course of breaking down fungal mycelium. The third aim of this study is to investigate the taxonomic relationship of DNA sequences from environmental samples (Lindahl et al. 2010, Menkis et al. 2004, Yarwood et al. 2009) with those from described and cultivated *L.* species and to determine whether the strains with affinity to *Leptodontidium* mentioned above can be classified as *L.*

Apart from *L. orchidicola* and *L. quercuum,* which are not included in this study, all *L.* were described by de Hoog (1979). L. belongs to the order of HELOTIALES which belongs to the class of LEOTIOMYCETES which is under the subdivision of the PEZIZOMYCOTINA which in turn is under the division of ASCOMYCOTA. Fungal classification was originally based on structures associated with sexual reproduction (see below) however many species of the ASCOMYCOTA only reproduce asexually while others know both sexual and asexual stages. These stages do not occur simultaneously but at different points in time and/or under different conditions which is why it is difficult to assign the two stages to one species (Guarro et al. 1999). The sexual stage is called teleomorph and the asexual stage anamorph.The stage of *L.* described by de Hoog is the anamorph. A description for *L. beauverioides* can be seen in the textbook below. Detailed descriptions for other *L.* species and strains are available at the Central Bureau voor Schimmelcultures (CBS) in Utrecht the Netherlands (www.mycobank.org).

"Leptodontium beauverioides de Hoog, sp. nov.

...

Colonies attaining a diameter of 7 mm in 14 days, flat or slightly elevated in the middle, at first smooth, soon becoming farinose to velvety at the centre, finally somewhat funiculose, grey, with an olivaceous black centre and a whitish, straight and sharp margin. Reverse dark grey to black, whitish towards the margin. Exudate and odour absent. Submerged hyphae at first hyaline, smooth, thin-walled, 1-2 µm wide, later becoming dark brown and rather thick-walled, up to 3 µm wide, forming a compact mycelium. Aerial hyphae dark brown, strongly fasciculate. Conidiogenous cells arising in dense clusters from short, inflated, flexuose side branches or apical parts of undifferentiated hyphae, hyaline, smooth, thin-walled, subglobose to elongate, often strongly curved, 3-6 x 1.8-3 µm, in the apical region producing a small cluster of conidia more or less sympodially; scars flat, inconspicuous. The conidiogenous cells may be detached during conidiation. Conidia hyaline, smooth, thin-walled, subglobose to short cylindrical, 2.4-4.3 x 1.6-2.2 µm, with a truncate base. At the centre of old cultures dark brown, thick-walled, ellipsoidal, about 7 x 4 µm chlamydospores may be found."

Source: http://www.mycobank.org/BioloMICSServer.aspx?Link=T&Rec=316568 2010/06/10

Between 1866 and the 1950´s fungal classification was a matter of comparing morphological traits such as spores or fruiting bodies (McLaughlin et al. 2009). Anton de Bary who first introduced evolution to fungal classification included Fungi in the kingdom of plants (Ainsworth 1976). He also introduced the class PHYCOMYCETES – algae like fungi – a class that was later divided into members that belonged to the Fungi and members that were more closely related to algae, namely the OOMYCOTA (Sparrow 1958). Later Sparrow and his contemporary Whittaker (Whittaker 1969) used ultrastructural features of cells and tissues that had become possible to observe by the advent of electron microscopy, to classify Fungi and further on Fungi were separated from plants into a kingdom of their own. But classification on the basis of ultrastructural data is laborious and it takes very well trained mycologists to perform it. Subsequently ultrastructural data is available for only a few species in every phylum (McLaughlin et al. 2009). But since molecular methods such as PCR and Sanger sequencing have become available in the late 1980´s there is another way to classify Fungi – phylogenetics. Phylogenetics is the study of the relatedness of organisms on the basis of their genetic code. Ideally one would sequence whole genomes and compare them but this is time consuming and very expensive. What one resorts to instead is to compare a sample of genes or loci. Since genes mutate at different rates depending on whether they are subject to natural selection or not, not all genes are suitable for phylogenetic comparison (Dawkins 2009). For mycologists genes coding for ribo-

somal RNA (rDNA) have been proven to be the most useful (Bruns & Shefferson 2004). Within the rDNA the internal transcribed spacer region (ITS) can be used to distinguish Fungi from other organism groups in environmental samples and also provides a reasonable taxonomic resolution on genus level whereas genes encoding for the nuclear small subunit and the nuclear big subunit of the ribosome can give phylogenies with lower taxonomic resolution that is to say on order or class level (Bruns & Shefferson 2004). The latter can be useful to study the relationships of different genera with each other and their relative distance to their last common ancestor. The data obtained in this manner can be put into a phylogenetic tree where, in short, the length of the branches symbolizes evolutionary time and distance and the root of the tree is the most recent common ancestor of all species in the tree provided it is monophyletic. Phylogenetic trees can be calculated according to many different algorithms but work in principle on the basis of parsimony where the most parsimonious tree is the tree that needs to claim the minimal amount of evolutionary change and is thus expected to be closest to reality (Dawkins 2009).

2 Material and methods

2.1 Material

11 strains of *Leptodontidium* sp. (see Table 1) were ordered from the Centraalbureau voor Schimmelcultures (CBS) in Utrecht/the Netherlands. The strains were chosen according to the following criteria: 1) in order to create a phylogeny on CBS *Leptodontidium* sp. strains that have no ITS sequence published on genbank (NCBI), 2) to be able to draw conclusions from the phylogeny of the genus *Leptodontidium* at least one strain from every species or subspecies respectively were chosen, 3) since another aim of the study was to investigate whether *Leptodontidium* sp. a) use dead mycelium of other fungi and b) whether they mineralize NH_4^+ during mycelium decomposition, strains that had originally been found on a fungal substrate were preferred over those who were not, 4) in order to exclude adaptive differences due to a different climate or habitat, strains originating from boreal forests or soil under coniferous trees in temperate climate were selected.

Table 1. CBS cultures included in the study

No	CBS acc.	Name	Country of origin	Substrate of origin	C-source study	ITS phylogeny	LSU phylogeny
1	315.85	*L. elatius* var. *ovalisporum*	Germany	Fungus: *Trichaptum abietinum*	YES	YES	NO
2	851.73	*L. irregulare*	Sweden	Forest soil, *Picea abies*	YES	YES	YES
3	394.76	*L. elatius var. ovalisporum*	Netherlands	Fungus: *Hypochinicium punctatum*	NO	YES	NO
4	624.69	*L. elatius* var. *elatius*	USA	Forest soil	NO	YES	YES

5	508.77	*L. camptobactrum*	Sweden	Coniferous wood	YES	YES	YES
6	833.69	*L. elatius* var. *elatius*	Germany	Fungus: *Piptoborus betulinus* (old)	YES	YES	NO
7	405.85	*L. obscurum*	Netherlands	Forest soil, *Picea abies*	YES	YES	NO
8	672.76	*L. beauverioides*	Finland	Wood of *Picea abies*	YES	YES	YES
9	582.81	*L. irregulare*	Germany	Rhizosphere of *Abies alba*	NO	YES	YES
10	683.84	*L. boreale*	Chile	Forest soil	NO	YES	NO
11	240.74	*L. obscurum*	Sweden	Forest soil, *Picea abies*	NO	NO	NO

The first 10 cultures arrived as freeze-dried mycelia in glass ampoules. According to CBS's instructions for revival of freeze-dried cultures the glass ampoules were sterilized with 70 % ethanol and opened on a sterile bench with the help of an ampoule file whereupon the freeze dried material was solved in 2 ml of sterile (deionised) water and left at room temperature for 6 hours. After that the suspension was divided between two petri dishes with solid agar medium in order to spread the contamination risk whereas a third part was kept in the freezer for later DNA-extraction. In accordance with CBS's recommendations the cultures were stored at 6°C for three weeks whereupon they were moved to an incubation chamber that holds 20°C. The agar medium used (called Hagem agar) was created by adding 10 g of malt dextrose, 10 g of glucose, 0.5 g of NH_4NO_3, 0.5 g of K_2PO_4, 0.5 g of $MgSO_4$*7aq and 20 g of Agar to 1 l of deionised water. While the agar medium within this mixture merely serves as a solidifying agent malt dextrose and glucose provides easily accessible carbon for the fungi to grow and the mineral additions provide the necessary nutrients (N, P, K, Mg and S). Deionised water was used to ensure a predictable mineral content of the substrate. The so produced mixture was than sterilized by autoclaving it for 15 min at 125°C.

The 11th culture from CBS arrived rather late, two months after the others as living mycelium on broth in a glass conduit. The mycelium was inoculated on agar plates prepared as mentioned above while some of it was spared for DNA-extraction.

2.2 Methods

2.2.1 Project 1 – Preferred carbon source

In order to investigate which kind of carbon source the different *Leptodontidium* strains prefer an experiment was set up with 7 different solid agar media that were prepared as above except that the malt dextrose was substituted with a) 10 g of glucose b) 10 g of cellulose c) 10 g of chitin d) 10 g of dried and ground needles of *Pinus sylvestris* e) 10 g of freeze dried and ground *Agaricus bisporus* fruit bodies f) 10 g of sawdust of *Picea abies* g) without carbon source, serving as a negative control. Here the glucose was thought to serve as a positive control. Cellulose was included in the study to see whether *Leptodontidium* sp. might derive carbon from plant litter with easily accessible cellulose such as leaves or whether they act as a secondary decomposers of more recalcitrant material such as wood were they rely on other fungi with ligninolytic abilities to gain access to the cellulose. Chitin is the main component of fungal cell walls and was included in the study in order to see if *L.* sp. are mainly after the chitin in the cell walls or if they mainly decompose it in order to access the cell contents when decomposing fungal mycelium. The fruit bodies from *Agaricus bisporus* were included in order to verify the hypothesis whether *L.* sp. act as decomposers of dead fungal mycelium/tissue in forest soils. In contrast the pine needle and the sawdust media were added to investigate the probability of *L.* sp. being litter or wood decomposers respectively.

Pilot study

While the CBS cultures were incubated a pilot study was set up to test the procedure using four different isolates that were available at the institution the first of which is called JÄDRÅS21 and was obtained by Rosling (person. comm.) in a previous study, the second and the third AURIM127 and AURIM688 are cultures that were isolated in the study on dark septate fungi by Menkis et al (2004). These three had been close to *Leptodontidium sp.* in a preliminary unpublished phylogeny by Lindahl et al (person. comm.) which is why they were chosen for this prior investigation. The fourth isolate is a strain of *Marasmius androsaceus* that Boberg et al. (2010) used in their research on fungal C translocation. *Marasmius a.* has well recorded cellulolytic and ligninolytic capabilities (Lindeberg 1944; Cox et al. 2001) and was chosen as a control species as a primary decomposer, mainly decomposing wood and litter in contrast to the other three which were expected to behave as secondary decomposers mainly decomposing fungal fruit bodies, cellulose and chitin.

In order to set up this preliminary experiment, the 4 isolates were inoculated on the seven different media. Thereby using very small inoculates (≈ 2 mm in diameter) in order to minimize nutrient transfer from the original medium. The first approach to measure growth on the agar plates was to measure horizontal expansion. After one week the expansion was measured with a ruler in four directions with an angle of 90° between each point of measurement. From this data the arithmetic mean was calculated. During the following weeks three more measurements were made each week until we had to abandon the method since the fungi did not only grow horizontally but also vertically, with some showing aerial hyphae and others remaining rather compact. Especially those growing on glucose medium did grow very little in horizontal direction, which made the medium obsolete as a positive control within this approach to assess growth.

The second approach to estimate substrate utilization was to measure respiration by measuring CO_2 emissions in a closed chamber. This was undertaken with the EGM-4® (PP-Systems, Amesbury, USA) environmental gas monitor for CO_2. The machine works on the basis of infra-red gas analysis. Since di-atomic gases such as CO_2 absorb photons in the infra-red range the apparatus can measure CO_2 concentrations by allowing air into a sample cell where there is a light bulb on one side and a sensor that is sensitive to photons on the other side (EGM-4® Users Manual Version 4.4 pg 9).

In order to measure the respiration of the cultures one petri dish at a time was put into a small closed chamber that was connected to the machine with both, an in and out tube. Before each measurement the lock of the chamber was removed and the ZERO function of the apparatus was used to ensure stability of the CO_2 signal. Than the petri dish was opened and put into the measurement chamber whose lock was closed. Per measurement, 8 readings were taken every 30^{th} second so that each measurement took 4 minutes.

Main study

When most of the cultures from CBS had grown sufficiently after reviving (after about 3 weeks) they were inoculated on solid agar without nutrients to starve them so that their growth response would be quicker once inoculated on the testing media mentioned above. After two weeks of incubation at 20°C, 6 cultures were selected and inoculated on the 7 different media. A selection was necessary because

inoculating and measuring all 11 cultures would have been too time consuming and because not all cultures had grown sufficiently yet. *Leptodontidium boreale* CBS 683.48 for instance took two more weeks to grow on the initial agar plates. These constraints aside cultures were selected so that each species or subspecies was included as long as this was possible.

The strains selected were CBS 315.85 *Leptodontidium elatius* var. *ovalisporum*, CBS 851.73 *Leptodontidium irregulare*, CBS 508.77 *Leptodontidium camptobactrum*, CBS 833.69 *Leptodontidium elatius* var. *elatius*, CBS 405.85 *Leptodontidum obscurum* and CBS 672.76 *Leptodontidum beauverioides*. When inoculated on the growth media the inoculation piece had a diameter of 0.5 cm on average. The respiration was measured after three weeks of incubation according to the same procedure as the cultures in the preliminary study (see above).

Data analysis

The measurement series data for each replicate was plotted on a graph with respiration as the dependent variable and time as the independent variable. When $\gamma = \alpha x + \beta$, α is the inclination which in this case is ppm CO_2/s. To avoid falsification due to CO_2 from the medium only the last four measurement points when most cultures yielded a linear increase in CO_2 concentration were plotted. A trend line was set and the α-value read and plotted in staple diagrams (see Results). On this data a two factor ANOVA analysis was performed with the Microsoft Excel® (Microsoft, Redmond, USA) data analysis tool. Furthermore a Tukey-test with STATISTICA® (Statsoft, Tulsa, USA) was performed to test the statistical significance of the differences in substrate utilization especially the difference between the substrates and the negative control. This revealed although merely statistical support for some strains on some substrates (see Results). One of the assumptions a Tukey-test is based on is that variation is equally distributed over the range of values. Many respiration values were however very close to cero which does not allow much variation and which resulted in an unequal distribution of variation over the range. The problem was aggravated by the fact that number of replicates of the negative control was rather small. In order to correct for this the substrates that showed values close to cero- cellulose, chitin and wood were summarized with the values of the negative control and treated as a super ordinate negative control in a new Tukey-test (see Results).

2.2.2 Project 2 – Ammonium mineralization

Experiment setup

In order to investigate whether the fungi, that in the first data analysis had shown to prefer fungal substrate over others, would mineralize NH_4^+, another experiment was set up. The two *Leptodontidium* strains chosen were *L. elatius* var. *elatius* CBS 833.69 and *L. beauverioides* CBS 672.76. Sand that was to serve as a matrix was autoclaved two times at 125°C for 15 minutes. *Agaricus bisporus* fruit bodies that should constitute the fungal substrate were freeze dried and ground to powder in liquid nitrogen. The powder was then sterilized by suspending it in 90 % ethanol and subsequently dried under sterile conditions. Sand and mushroom powder were mixed and filled onto 23 petri dishes – 10 for each culture and 3 as negative controls. The *L.* cultures that were to be inoculated on the petri dishes were first grown on agar medium without nutrients. Subsequently, the fully outgrown mycelium was mixed with 40ml of sterile deionised water in a blender. Of this mixture 2 ml were inoculated on each petri dish. The plates were then saturated to field capacity with 7-9 ml of sterile deionised water in order to moisturize the substrate and thus provide optimal growing conditions.

Respiration measurement

After three weeks CO_2 respiration was measured as described in Project 1 except that only 4 measurements were taken. This was done to be able to correlate biological activity to mineralization.

Mineralization measurement

To measure the actual mineralization the solution in the plates was first extracted by putting a small amount of material from the plates onto a filter paper (Munktell/Noax lab, Oslo, Norway) adding 40 ml of sterile deionised water and pumping the solution through the filter into a falcon tube. The extracted solution was then analyzed with a FIAStar™ 5000 system (FOSS, Höganäs, Sweden). The system works on the principle that a sample solution containing Ammonium ions is injected into a carrier stream. This stream is then merged with a Sodium Hydroxide stream. In the subsequent stream gaseous Ammonium is formed that diffuses through a gas permeable membrane into an indicator stream. The indicator stream contains a mixture of acid-base indicators that react with the gaseous Ammonium

which results in colour shifts that can be measured photometrically (Foss, Application Note 5220, 2008). The machine requires several reagents to work that were prepared according to protocol (Foss, Application Note 5220, 2008). The samples had to be diluted 100 times because the initial values were beyond the machines measurement range.

Data analysis

Samples had been weighed before and after drying as well as before and after burning in order to be able to determine dry weight, water content and organic matter per gram dry weight. From these values and the NH_4^+ measurement values the ammonium content per gram organic matter could be calculated. The values in the negative control showed rather high values so that the arithmetic average of these values was subtracted from all other values. All measurement values for *L. beauverioides* were very high except one that was removed because the high difference pointed at a measurement mistake for this replicate. The values were plotted against respiration values that were derived from the measurement series as described above with respiration as the independent value. A one factor ANOVA test was performed on the data with the Microsoft Excel® (Microsoft, Redmond, USA) data analysis tool.

2.2.3 Project 3 – Phylogeny

DNA extraction

First DNA was extracted from a subsample of the freeze dried CBS mycelium according to the same protocol that is described later in this section. This yielded however in only very little or no DNA which is why new DNA extracts were made from the cultures grown on liquid Hagem medium. This was prepared according to the recipe for Hagem agar mentioned above except without the agar. After two weeks of incubation at 20°C they had grown sufficiently for extraction. The mycelium was skimmed off the medium and freeze dried for 24 hours. The dried mycelium powder was then first lysed by adding small glass pearls (diameter ≈ 1 mm) and 3 % CTAB-Mix (see recipe below) to the assays and putting them into a FastPrep® instrument which “shakes” them rapidly in a vertical, angular motion. The settings chosen were: speed: 5.5 m/s; duration: 30 s; cycles: 2.

The 3 % CTAB -MIX contains hexadecyltrimethylammonium bromide (CTAB, FeF chemicals, Køge, Denmark) NaCl, EDTA (ethylenediaminetetraacetic acid) and TRIS-HCL (tris base tromethamine). CTAB is a detergent that dissolves fatty acids and proteins, NaCL makes DNA soluble in CTAB, EDTA inactivates enzymes and TRIS-HCL stabilizes DNA during the extraction process (Doyle & Doyle 1990). In order to optimize the conditions for the CTAB-mix, the assays were incubated at 65°C for 90 min after lysing. Then they were centrifuged at 13000 rpm for 10 minutes. After centrifuging, 700 µl supernatant was transferred to a new tube and 500 µl chloroform was added to denature lipids and enzymes (Doyle & Doyle 1990). After another centrifuge round for 7 min at 10000 rpm the denatured lipids formed an interphase that divided the fluids in the tube into two phases. The upper phase contains the DNA and 500 µl of it was transferred to a new Eppendorf tube where it was precipitated by adding 1.5 volumes of ice-cold isopropanol, mixing and putting the tubes into the freezer for 1½ hours. This was followed by yet another centrifuging for 13 min at 13000 rpm and the supernatant was poured out. The remaining DNA pellet in the tube was washed by adding 200 µl of 70 % cold Ethanol centrifuging at 6500 rpm for 10 min and pouring off of the supernatant. After drying at 65°C for 5 minutes the pellet was resuspended in 50 µl milliQ-water. DNA from CBS 240.74 that arrived late was extracted in the same way except that the mycelium was not freeze dried before lysing and that after precipitating the assay was stored in the freezer over night.

PCR preparation

An analysis of the resulting amounts of DNA with a spectrophotometer showed rather high amounts of DNA with up to 683 ng/µl which is why a dilution series was made with an assay of 10.5 and 3 ng/µl. This was done to ensure a good quality PCR (see below) since too high amounts of template DNA can cause missannealing and put the reaction to early into saturation (Muehlhardt 2009).

PCR

The first phylogeny was to be done on the ITS rRNA region whose DNA was amplified with a PCR using the primers ITS1F (forward) and ITS4 (reverse). The common polymerase chain reaction involves three steps: 1.) denaturation – the separation of the double-stranded DNA into two strands; 2.) annealing – the hybridization of oligonucleotide primers with the target DNA; 3.) amplification –

replication of the primer-target hybrid by the polymerase enzyme taq from the thermophilic bacterium *Thermus aquaticus* (Chien et al. 1976). These steps are regulated by a temperature regime, a special temperature range for each step stated above: 92-96°C – denaturation; 42-65°C – annealing; 72°C – amplification. Within this cycle the target DNA is ideally duplicated and can be multiplied exponentially when several cycles are performed (Muehlhardt 2009). The annealing temperature depends on the melting temperature of the primers used. It can be calculated according to their GC content, length and the expected percentage of mismatch (Muehlhardt 2009). The taq polymerase has its optimum activity temperature between 70°C and 85°C which is why the amplification step is held at a temperature within this range, commonly 72°C (Chien et al. 1976). The temperature regime used for the PCR with ITS1F and ITS4 was: denaturing at 95°C for 30 s, annealing at 57°C for 30 s and amplification at 72°C for 30 s, the number of cycles was set to 35.

Besides primers and polymerase several other reagents need to be added for amplification in a PCR. A given addition is desoxyribonukleosidtriphosphates (dNTPs) which are the building blocks for the polymerase during amplification. Another reagent to add is the PCR-buffer which gives the reaction a pH above 8, where the Taq-polymerase has its optimum. At last MgCl is added because Mg_2^+ stabilizes the enzyme, influences primer annealing, separation of strands during denaturation, product specifity and impedes the formation of primer dimers (Muehlhardt 2009). The reagents for this PCR were proportionally mixed in 1 µl "Mastermix": 0.45 µl sterile deionised water, 0.2 µl 10xBuffer, 0.2 µl dNTP, 0.04 µl ITS1F primer, 0.04 µl ITS4 primer, 0.06 µl MgCl, 0.01 DreamTaq™ (Fermentas Life Sciences, St. Leon-Rot, Germany) DNA polymerase.

To test whether the PCR had amplified the target DNA successfully an electrophoresis gel was cast. The principle of an electrophoresis gel is that DNA is loaded into wells in an agarose gel that is set under electric current, which causes the negatively polarized DNA to move through the gel towards the anode. The gel hereby constitutes a permeable mesh where longer nucleotide fragments move slower than shorter fragments. Thus the fragments are separated and can be compared with a standard of known fragments (a ladder) that is run parallely within the same gel usually at one or both sides. The length of the amplified fragment depends on the type of organism and the primers used (Muehlhardt 2009). The characteristic length of the ITS1F-ITS4 amplicons of ASCOMYCETES is between

500-600 bp (Lindahl, B. person. comm.) and the bands on the gel were around this band length which is why I considered the PCR to be successful.

PCR purification

The next step on the way to obtain DNA sequences from the fungal material was to purify the PCR products. Since a PCR product contains a number of other reagents beside the amplicons (see above) it needs to be purified to assure good quality sequencing results. Purification was performed with the GeneJET™ (Fermentas Life Sciences, St. Leon-Rot, Germany) PCR Purification Kit. The principle of the Kit is to 1.) bind the DNA in a reaction mixture to a membrane in a spin column with a binding buffer 2.) remove the other reagents by centrifuging and washing the DNA on the membrane with a washing buffer and 3.) elute the DNA with an elution buffer (Fermentas GeneJET™ PCR Purification Kit #K0701,#K0702 2008).This was performed according to the protocol of the Kit.

Preparation for sequencing – Internal transcribed spacer rDNA

In order to sequence the ITS region samples were sent to the Department of genetics and pathology at Rudbecks laboratory at Uppsala University in Uppsala. The laboratory works with an automatic sequencing machine that works on the principle that primers are fluorescently labeled during a so called cycle sequencing procedure similar to PCR prior to sequencing which is done with a machine that uses a laser to read the fluorescence of the nucleotides and thus can read the sequences (Muehlhardt 2009).

The labelling was done with fluorescent didesoxyribonucleotides namely BigDye® v. 3.1 (Applied Biosystems, Carlsbad, USA). In contrast to the PCR reaction mentioned above only one primer was used per reaction and template. This is done to achieve two complementary reads for each template that can later be aligned and verified against each other (see Sequence Analysis). The PCR reaction here contained 1.5 µl sterile deionised water, 4 µl BigDye® v. 3.1 diluted with 5x Sequencing Buffer and sterile deionised water, 1 µl 1.67 mM primer and 3.5 µl template (BigDye® Terminator v3.1 and v1.1 Cycle Sequencing Kits, 2002).The 10 mM primers ITS1F and ITS4 were diluted with sterile deionised water to 1.67 mM. The PCR temperature regime used was 1.) initial denaturation 96°C for 1 min 2.) denaturation within each cycle 96°C for 10 s 3.) annealing 50°C for 5 s 4.)

amplification 60°C for 4 min. 35 cycles were performed. Afterward the products were purified in two steps by first adding 40 µl 75 % isopropanol, incubating for 30 min and centrifuging at 3000xg for 45 min. Then the supernatant was removed by turning the plate upside down over the sink and letting it rest on a folded tissue. After that 150 µl 75 % isopropanol was added to each sample and the plate was centrifuged at 2000xg for 10 min and the supernatant was discarded. At last the samples were covered with tin foil and sent to Rudbecks laboratory for sequencing.

Preparation for sequencing – Large Subunit rDNA

The large subunit was sequenced using the Beckman sequencer (Beckman Coulter, Brea, USA) at the department using the primers ITS3 forward, LR0R forward, LR5 reverse and LR7 reverse. The Beckman sequencer works on the same principle as described above. It also requires a cycle sequencing step prior to reading in the machine. In this case cycle sequencing reaction was done with the GenomeLab™ DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (DTCS) (Beckman Coulter, Brea, USA). A mix was prepared with 2 µl DTCS, 2.5 µl primer and 0.34 µl sterile deionised water for each reaction whereupon 2.5 µl purified PCR product was added. The thermal cycling program was 96°C for 20 s, 50°C for 20 s and 60°C for 4 min. 30 cycles were performed. The products were this time purified using Quick Spin Columns for radiolabeled DNA purification; Sephadex G-50, fine (Roche Applied Science, Indianapolis, USA). For this 1 g Sephadex G-50 was added to 16 ml sterile deionised water and the solution was autoclaved and put into the fridge for precipitation. Of a 96 well plate, each well was filled with 400 µl and then centrifuged at 1500 g for 2 min. After centrifuging it down at 1500g for 2 min the template volume of 5 µl was diluted with 15 µl deionised sterile water and then filled up with 15 µl Sample Loading Solution (Beckman Coulter, Brea, USA) to an end volume of 30 µl and pipetted onto the middle of each column. The plate was then centrifuged another time at 1500g for 2 min on the plate that was to be put into the Beckman sequencer so that the cleaned templates would be centrifuged directly into it. At last the templates in each well were covered with a drop of mineral oil to avoid drying out and deterioration of DTCS and at last put into the machine for sequencing.

BLAST analysis and tree creation – Internal Transcribed Spacer region (ITS)

In order to check whether Lindahl et al.'s (2010) sequences with affinity to Leptodontidium actually matched with those sequenced here and to create a meaningful phylogenetic tree Lindahl et al.'s (2010) sequence CO4 was taken as a basis for a BLASTn analysis (Altschul et al. 1997) at the GenBank database of the National Center for Biotechnology Information (NCBI), Rockville/USA. All sequences that matched the sequence to at least 89 % were downloaded. Also all internal spacer sequences named *Leptodontidium* were downloaded in order to see if those sequences actually could be assigned to the genus. At last ITS sequences from a phylogenetic tree of the order Helotiales that had been inferred earlier by Lindahl, B.D. were added in order to be able to put the *L*. sequences into a greater taxonomic context. The downloaded sequences were checked whether they had been put into the database in 5'-3' direction and were reversed if this was not the case. Then the sequences were aligned with the computer program MegAlign® (DNASTAR, Madison, USA). Those sequences that were to short or of bad quality were removed.

The sequences that had been sequenced by Rudbecks laboratory were aligned into contigs which are overlapping DNA fragments from one genetic source. In this case there were two fragments for each Fungus one of the forward and one of the reverse primer. The contigs were aligned with the computer program SeqMan® (DNASTAR, Madison, USA). Normally not all parts of every sequence are of good quality and the alignment helps to find a good quality consensus. Unfortunately a good consensus could not be found for all sequences. The sequence of *Leptodontidium obscurum* was of too bad quality and could not be taken into the general alignment.

As a next step all sequences were aligned in a general alignment yet again using MegAlign®. After too long sequences had been cropped the final alignment resulted in 480 aligned basepairs covering the ITS region whereof 140 covered the most conserved 5.8S region.

At last the resulting phylogenetic tree from MegAlign® was analyzed using the computer program PAUP 4.0 (Sinauer Associates, Sunderland, USA). At first a neighbour joining tree was calculated to get a general overview whereupon a bootstrap analysis using the DISTANCE algorithm was performed with *Peltigera neopolydactyla* defined as an out group, MAXTREES set to autoincrease and TBR

branch swapping. Since the DISTANCE algorithm infers phylogenetic relationships based on pair wise comparism of sequences, single sequences that do not match with a very close related partner within the tree can contort the tree and give low bootstrap values. A bootstrap value can be compared with the R^2 value in a regression analysis it depicts how much of reality in percentage can be explained with the model namely the phylogenetic tree. Since there were some sequences that might not find a match in the tree another BLAST analysis was performed with the sequence of L. beauverioides as a basis. From the result 6 sequences of cultured Fungi with a CBS reference number that matched at least to 97% were downloaded and added to the sequences used to infer the bootstrap values for the phylogenetic tree based on the ITS region. The sequences added were AF281395 *Neofabrea perennans*, AF281399 *Pezicula cinnamomea*, AF176756 *Cryptosporiopsis rhizophila*, *AF141168 Scleropezicula alnicola* and EF596821 *Phialea strobilina*.

BLAST analysis – Large Subunit region (LSU)

To put the LSU sequences into context 50 sequences from Fungi listed by Wang et al. (2006) in a phylogenetic tree of the order Helotiales were downloaded at least one from each clade. The Beckman sequences were aligned in contigs as described above except that this time the contigs contained up to 4 fragments due to sequencing with 4 primers. The sequences where however of rather bad quality so that only 5 consensuses could be accepted the criteria being that a sequence had to be confirmed by at least two fragments and that the resulting sequence was longer than 600bp. Further analysis was performed in the same manner as with the sequences of the ITS region.

3 Results and Discussion

3.1 Project 1 carbon source

As described in 2.2.2 the preferred carbon source of six different *L.* species was determined by measuring CO_2 respiration. Table 2 depicts a two factor ANOVA test that shows that the null hypothesis can be rejected (p-value samples < 0.05) which means that that there was a significant difference in respiration rate between different substrates, and that different isolates differed in their substrate preferences.

Table 2. Two factor ANOVA

Source of Variation	KvS	fg	MKv	F	p-value	F-crit
Substrate	5,211977	6	0,868663	29,20662	1,23E-18	2,208554
Isolate	0,141268	5	0,028254	0,949955	0,453331	2,323126
Interaction	3,060011	30	0,102	3,429507	4,76E-06	1,594962
Within	2,498327	84	0,029742			
Total	10,91158	125				

Figure 1 depicts CO_2 respiration of six *L.* strains and seven different substrates. A Tukey-test revealed that compared to the negative control there is significantly higher respiration on glucose for *L. elatius* var. *ovalisporum*, *L. irregulare* and *L. camptobactrum*. There is furthermore significantly higher respiration on the mushroom substrate compared to the negative control by *L.elatius* var. *elatius.* In addition the figure gives at least indications for that also other *L.* strains seem to take the mushroom substrate as a good carbon source. *L. beauverioides* and L. *elatius* var. *elatius* even seem to prefer it over glucose. *L. elatius* var. *ovalisporum* shows comparatively high respiration on litter, wood and cellulose all of which are plant derived substrates which might indicate that it follows a lifestyle as a free living

decomposer. The same seems to apply to *L. obscurum* which seems to be able to take advantage of both litter and wood but not cellulose. No fungus could efficiently use chitin as a substrate.

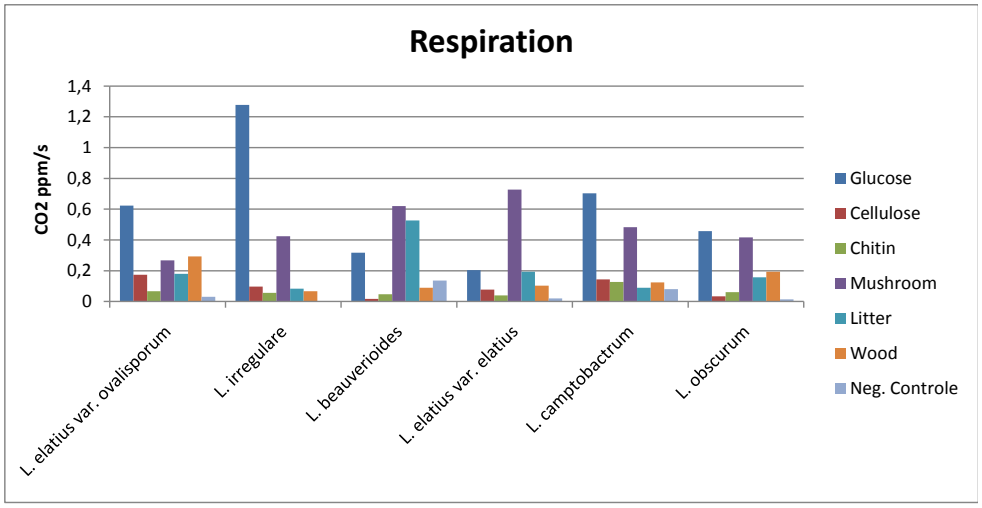

Figure 1. CO_2 respiration

Based on the assumption that both cellulose, chitin and wood supported so little growth that they can qualify as negative control a new Tukey-test was performed that resulted in significant compares for at least some data. This data is indicated with an orange star in Figure 2.

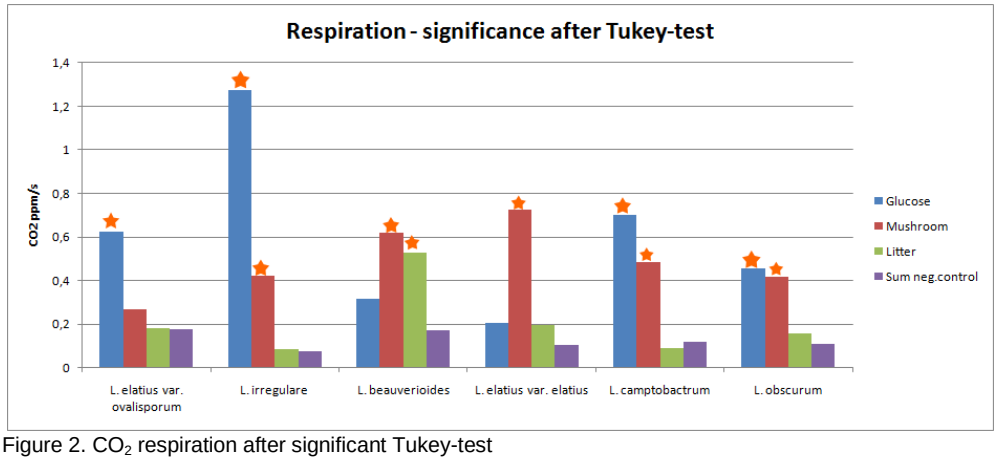

Figure 2. CO_2 respiration after significant Tukey-test

Except for *L. elatius* var. *ovalisporum* preference of mushroom substrate as compared with the super ordinate negative controls was significant. Glucose was also

significantly preferred by all Fungi except *L. beauverioides* and *L. elatius* var. *elatius*. *L. beauverioides* showed in addition a significant preference of litter.

For further comparison the respiration data of the documented litter decomposer *Marasmius adrosaceus* (Boberg et al. 2010) and the fungal strain Jädraås21that was isolated from the roots of Pine seedling and is probably mycorrhizal (Rosling, A, person. comm.) was added to the *L.* strains in Figure 4. The data is however derived from one replicate only that did not show satisfying p-values when a two factor ANOVA was performed. The comparison nevertheless gives at least some information about substrate utilization that are to be expected of a litter decomposer (*Marasmius adrosaceus*) and the substrate utilization that is to be expected of an ectomycorrhizal fungus (JÄDRAÅS21).

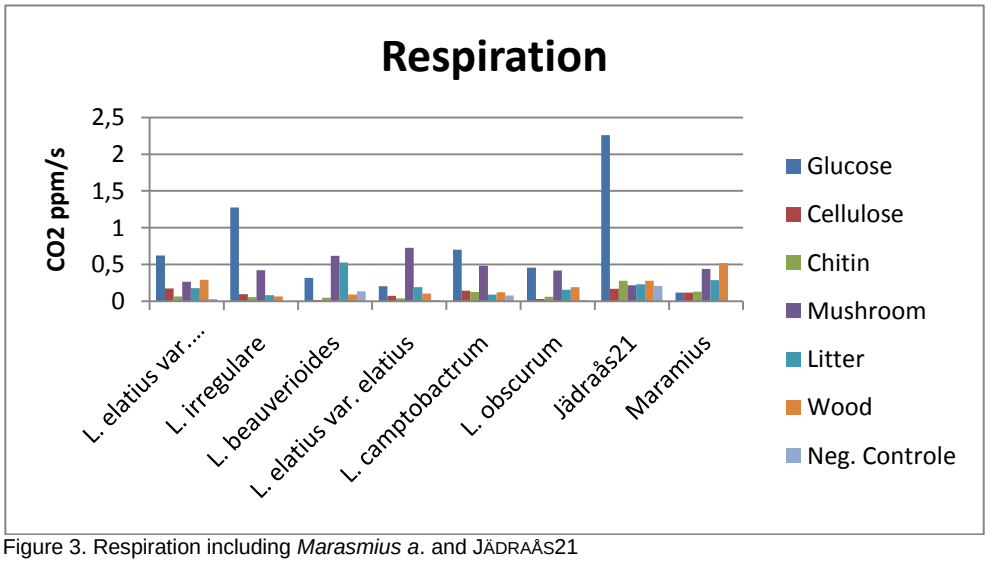

Figure 3. Respiration including *Marasmius a.* and JÄDRAÅS21

To summarize all *L.* species except *L. elatius* var. *ovalisporum* seem to have a significant preference for fungal substrate compared to all other substrates besides glucose. Thus our hypothesis that the *L.* strains preferably use fungal substrate is confirmed by the data for 5 out of 6 strains. What is apparent though is that compared to the respiration data in Project 2 the respiration data here was very low. This could be explained by the fact that sand is much more similar to the natural habitat of the Fungi than agar medium. Since higher overall respiration data could mediate the statistical problems with values close to nil a similar experiment with sand as the basic medium instead of agar might give better results. In addition respiration was only measured once because that procedure was very time consuming. Since it is hard to assess the exact point in time where the respiration peak occurs on a substrate the peak could have been missed for some substrates. In this

regard one can assume that the peak on less utilizable substrates is reached earlier that on those that exhibited to be more usable so that in a future study the measurements should be performed approximately after 10 days and then again after 3 weeks.

3.2 Project 2 Ammonium mineralization

Since *L. beauverioides* and *L. elatius* var. *elatius* clearly preferred mushroom substrate over all others they were the given candidates to investigate whether *L.* species mineralize Ammonium from fungal substrate. Figure 5 depicts Ammonium content in the sample solution per gram organic matter versus CO_2 respiration for *L. beauverioides*.

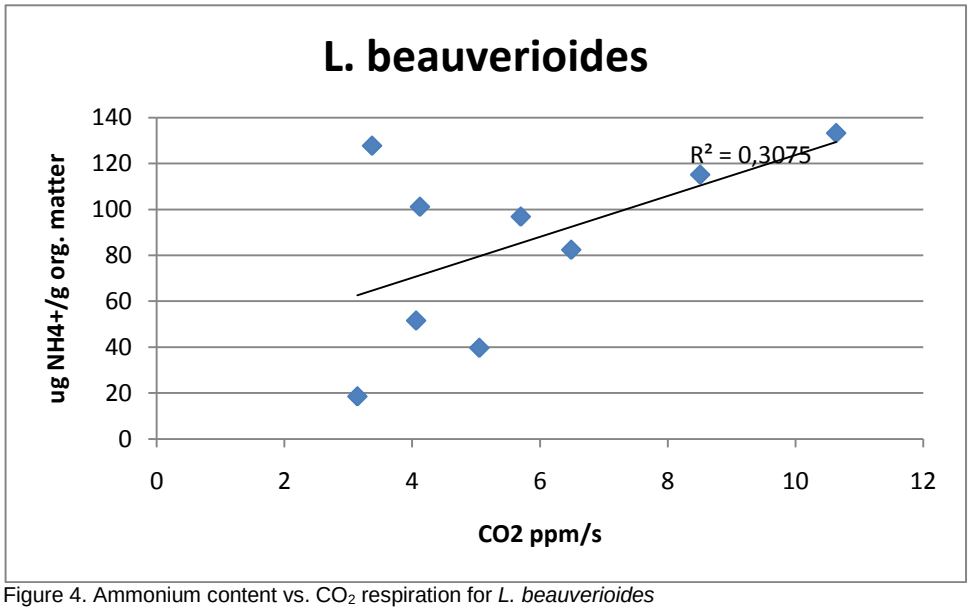

Figure 4. Ammonium content vs. CO_2 respiration for *L. beauverioides*

As of the R^2 value about 30% of the Ammonium content can be explained with respiration. A regression analysis resulted although in a p-value of 0.12 so that the null hypothesis could not be discarded.

Figure 6 shows the results for *L. elatius* var. *elatius*. The R^2 value is very close to nil so that respiration can not at all be accepted as an explanation for the Ammonium content in the sample solution. The p-value for this dataset was even higher with 0.9.

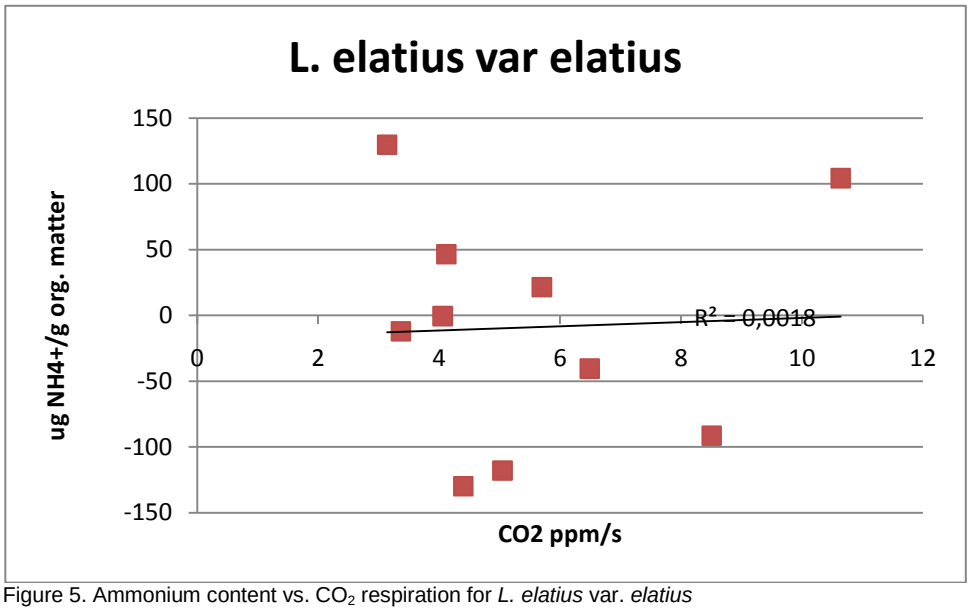

Figure 5. Ammonium content vs. CO_2 respiration for *L. elatius* var. *elatius*

Although the results are not statistically significant there is at least an indication that *L. beauverioides* is capable of mineralization and because no replica showed negative values compared to the negative control it can be hypothesized that they were carbon limited and thus could not make use of all available nitrogen. In the case of *L. elatius* var. *elatius* the situation is more complicated some replica seem to mineralize and some replica seem to immobilize nitrogen thus indicating that some were limited in carbon and some in nitrogen. Nevertheless the results are very hard to interpret. In the future better results and statistical support could probably be achieved with more replicates to account for the obviously high variation.

3.3 Project 3 Phylogeny

As of McLaughlin et al. (2009) a meaningful phylogeny needs to be created from several loci. Here phylogenies were created based on the rRNA encoding regions ITS and LSU.

3.3.1 Internal transcribed spacer phylogeny

A tree computed with a neighbour joining algorithm and based on the ITS region is depicted in Figures 7 and 8. The Leptodontidium species that were sequenced within this study are indicated with a number according to Table 1, abbreviated name and CBS accession number. Other sequences are indicated with GenBank

accession number, their name if available, the substrate they were found on, the country or geographic area of origin and the publication (see also Table 3).

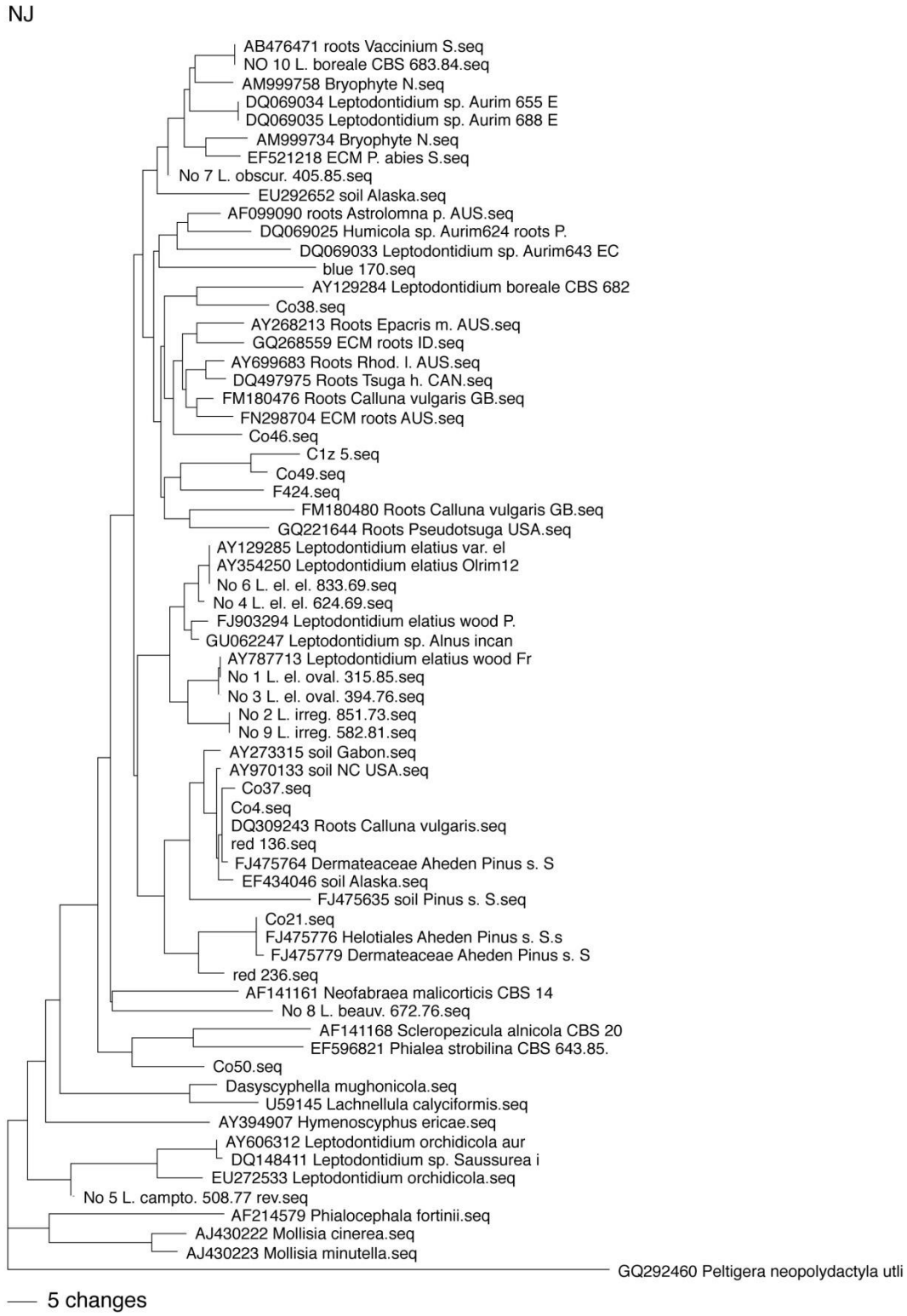

Figure 6 Neighbour joining tree based on ITS

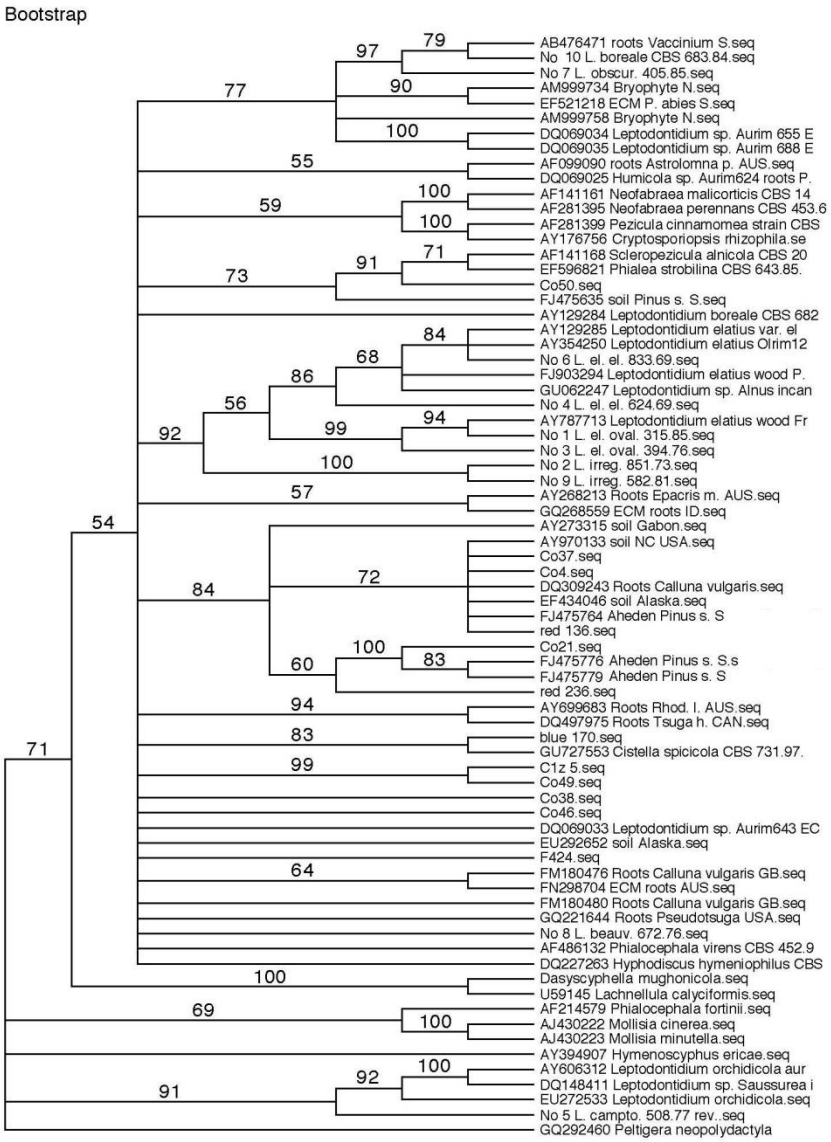

Figure 7 Bootstrap for neighbour joining tree based on ITS

As a reminder the trees are based on sequences with 89% similarity to CO4 (Lindahl et al. 2010) sequences named *Leptodontidium* and species from a previous alignment with CO4 and Helotialean species by Lindahl, B.D.

Perhaps the most striking result is that the L. sequences derived here do not cluster in one group but are actually divided into 4 groups. *L. boreale* (No.10) and *L. obscurum* (No. 7) form a well supported cluster (BS[2]= 77-97) with environmental samples found in boreal forests in Sweden and Norway at the top of tree. In the centre of the tree the *L. elatius* var. *elatius* (No. 4 & 6), the *L. elatius* var. *ovalisporum* (No. 1 & 3) and the *L. irregulare* (No. 2 & 9) strains form a big cluster with good support (BS=92) with other both uncultured and cultured *L.* sequences. The third group is comprised of *L. beauverioides* (No. 8) which could not be grouped with any other sequence and the fourth group is *L. camptobactrum* (No. 5) in a cluster with *L. orchidicola* and an environmental *L.* sequence from China. This division into several clusters reveals that the genus *Leptodontidium* is polyphyletic meaning that not all species in the genus share a common ancestor. In consequence I suggest that only the central *L.* elatius/*irregulare* cluster should keep the name *Leptodontidium* since it encompasses the most strains while the other should be renamed.

In this respect *L. boreale* and *L.obscurum* are so close matches that they can be regarded as one species to be renamed. The environmental sequence AB476471 can also be assigned to this species. The fact that there is another L. boreale sequence (AY129284) in the middle of the tree far from No. 10 speaks for the fact that this strain is rather a *L.obscurum* strain than a *L.boreale* strain.

The splitting of *L. elatius var. elatius* and *L. elatius var. ovalisporum* is not too well supported with a bootstrap value of 56% for this furcating. The furcating does however exist which gives room for discussion whether the species is righteously split into two varieties.

Furthermore AY129285 is correctly named *L. elatius* var. *elatius* and GU062247, AY354250 and FJ903294 can be more specifically named so (BS=86%). AY787713 can be assigned to *L. elatius* var. *ovalisporum* (BS=99%).

[2] Bootstrap value

The split between the L. elatius varieties and *L. irregulare* is very well supported with a BS of 92%.

Within the fourth group DQ148411 can be identified as *L. orchidicola* (BS=92%) whereas No. 5 can be assigned to the group as an independent species (BS=91%).

Interestingly many sequences close to the *L.* species sequenced in this study were derived from samples associated with *Ericacean* shrubs both in boreal forests of the northern hemisphere as well as in Australia. This could indicate an association with the plants themselves in an ericoid mycorrhiza a feature which has been observed for some *Capronia* strains (Allen et al. 2003). This might seem unlikely given the fact that *L.* has never been reported as a mycorrhiza mutualist but if we bear in mind that ECM evolution goes back and forth between symbiotic and free living lifestyles (Wang & Qiu 2006) it is not impossible. Another possible explanation closer to our hypothesis with *L.* as an opportunistic mycelium decomposer might be that L. is not associated to the plants but to their mycorrhizal symbionts waiting for them to be weakened by perturbation. After all present DNA does not necessarily imply that the fungus was active at the time of collection. It was also found on the roots rather than inside. The third possible reason for the findings could simply be that *Ericaceas* and *L.* prefer the same habitat namely humus rich soils with low pH and that there is no ecological relation between them whatsoever.

Both *L. elatius* varieties that were isolated from wood associated fungi cluster with fungal strains found on decaying wood thus supporting our assumption as *L.* as a fungal decomposer except this time not mycorrhiza but wood decomposers. However *L. elatius* var. *ovalisporum* seemed to be able to make at least some use of the wood substrate in Project 1 (Figure 1) so that the possibility that it might be a wood decomposer cannot be definitely excluded. The fungal strain CO4 and other strains isolated by Lindahl, B.D. (named Co# or red#) did not cluster with *L.* so that they could not be identified as *L.* species. However not every branch in the tree has high enough bootstrap values to serve as an acceptable explanation for evolutionary relationships (see Figure 8). This is probably due to low sequence quality combined with relatively short sequence length whereof a substantial part is covered by the 5.8S region which is highly conserved and thus not suitable for the high resolution phylogeny that was aimed to achieve with this tree. The impor-

tant regions are the adjacent ITS1 and ITS2 that are intron loops that vary more frequently and are therefore the actual molecular clock.

Another reason might be the incorporation of *Molisia cinerea*, *Neofabraea malicorticis* and other species which with their comparatively long evolutionary distance to *L*. contorted the tree. Very close sequences combined with very distant ones might give incorrect results during neighbour joining and bootstrapping. This was however mediated in the second tree (Figure 8) by incorporating closely related sequences to these species (see Methods).

Table 3. ITS sequence information

Accession No.	Name	Place of Origin	Substrate	Publication
AB476471	Uncultured fungus, clone: E103	Västerbotten, Sweden	finest root fragment of *Vaccinium*	unpublished
AF099090	Epacrid root endophyte AP-3	West Victoria, Australia	roots of *Astrolomna pinifolius*	Lawrie et al. 1999
AF141161	Neofabrea malicorticis CBS141.22	n/a	*Malus sylvestris*, fruit	unpublished
AF141168	Scleropezicula alnicola strain CBS 200.46	Canada	n/a	Verkley 1999
AF214579	Phialocephala fortinii strain UAMH9525	n/a	n/a	Currah et al. 2000
AJ430222	Mollisia cinerea	Norway	n/a	Schumacher et al. 2002
AJ430223	Mollisia minutella	Norway	n/a	Schumacher et al. 2002
AM999734	Uncultured fungus, clone F3211B	Telemark, Norway	Bryophyte	Kauserud et al. 2008
AM999758	Uncultured fungus, clone B111G	Telemark, Norway	Bryophyte	Kauserud et al. 2008
AY129284	Leptodontidium boreale CBS 682.76	Sweden	Pine wood pole in ground contact	Summerbell et al. 2005
AY129285	Leptodontidium elatius var. elatius CBS 329.53	France/New Foundland, Canada	*Betula*, decaying wood/ *Abies balsamea* decaying wood	Summerbell et al. 2005
AY268213	Epacris microphylla root associated fungus 29	n/a	Roots of *Epacrid microphylla*	unpublished
AY273315	Uncultured ascomycete clone ot1c3	Gabon	soil from gallery forest dominated by *Aucoumea klaineana*	Roose-Amsaleg et al. 2004
AY354250	Leptodontidium elatius isolate olrim127	Lithuania	4 yr old stump of *Betula pendula* xylem	Lygis et al. 2005

Accession No.	Name	Place of Origin	Substrate	Publication
AY394907	Hymenoscyphus ericae	n/a	n/a	unpublished
AY606312	Leptodontidium orchidicola isolate aurim623	Lithuania	Root tip *Picea abies*	Menkis et al. 2004
AY699683	Fungal sp. R8	Queensland, Australia	Roots of *Rhododendron lochiae*	Bougoure & Cairney 2005
AY787713	Leptodontidium elatius	Lithuania	xylem in root collar of dead *Fraxinus excelsior*	Lygis et al. 2005
AY970133	Uncultured ascomycete isolate dfmo1059_122	North Carolina, USA	mixed hardwood A-horizon soil	O'Brien et al. 2005b
DQ069025	Humicola sp. Aurim624	Lithuania	mycorrhizal root tips of *Picea abies*	Menkis et al. 2005
DQ069033	Leptodontidium sp. Aurim643	Lithuania	mycorrhizal root tips of *Picea abies*	(Menkis et al. 2005)
DQ069034	Leptodontidium sp. Aurim655	Lithuania	mycorrhizal root tip of *Picea abies*	(Menkis et al. 2005)
DQ069035	Leptodontidium sp. Aurim688	Lithuania	mycorrhizal root tip of *Picea abies*	(Menkis et al. 2005)
DQ148411	Leptodontidium sp.	China	*Saussurea involucrata*	unpublished
DQ309243	Uncultured fungus isolate RFLP-62	n/a	roots *calluna vulgaris*	unpublished
DQ497975	Uncultured ectomycorrhiza (Helotiales) clone SWUBC331	Vancouver Island, Canada	Rhizosphere of *Tsuga heterophylla*	Wright et al. 2009
EF434046	Uncultured fungus clone P19_OTU131	Alaska, USA	humic horizon soil	Taylor et al. 2007
EF521218	Uncultured fungus clone OTU16	Sweden	*Picea abies* forest	Hedh et al. 2008
EF596821	Phialea strobilina strain CBS 643.85	Norway	*Picea abies* cone	unpublished
EU272533	Leptodontidium orchidicola	Andes, Colombia	*Espeletia* sp.	unpublished
EU292652	Uncultured fungus clone IH_Tag126_3702	Alaska, USA	soil	Taylor et al. 2008
FJ475635	Uncultured fungus clone AhedenB28	Sweden	*Pinus sylvestris* forest soil	Yarwood et al. 2009
FJ475764	Uncultured Dermateaceae clone AhedenJ4	Sweden	*Pinus sylvestris* forest soil	Yarwood et al. 2009
FJ475776	Uncultured Helotiales clone AhedenJ25	Sweden	*Pinus sylvestris* forest soil	Yarwood et al. 2009
FJ475779	Uncultured Dermateaceae clone AhedenL11	Sweden	*Pinus sylvestris* forest soil	Yarwood et al. 2009
FJ903294	Leptodontidium elatius isolate A39	Latvia	decayed wood of *Picea abies*	unpublished

Accession No.	Name	Place of Origin	Substrate	Publication
FM180476	Helotiales sp. 392567	n/a	Roots of *Calluna vulgaris*	Alexander et al. 2009
FM180480	Helotiales sp. IMI 392750	Exeter, UK	ericoid mycorrhizal root of *Calluna vulgaris*	Alexander et al. 2009
FN298704	Uncultured fungus isolate H026x_L3123	Tasmania, Australia	Ectomycorrhizal root-tips	Tedersoo et al. 2009
GQ221644	Uncultured Pezizales clone OTU33	California; USA	Roots from soil in *Pseudotsuga*, *Quercus*, and *Lithocarpus* forest	Wolfe et al. 2009
GQ268559	Uncultured Helotiales clone LH12	Borneo, Malaysia	Ectomycorrhizal root-tips	Peay et al. 2010
GQ292460	Peltigera neopolydactyla (out group)	n/a	n/a	unpublished
GU062247	Leptodontium sp. I169	Latvia	*Alnus incana*	unpublished
U59145	Lachnelulla calyciformis	n/a	n/a	unpubished

3.3.2 Large subunit phylogeny

Figure 9 depicts the neighbour joining tree for the LSU region containing sequences of Wang et al. (2006) who made an rDNA phylogeny on the order Helotiales based on small subunit, internal transcribed spacer and large subunit DNA. For this tree only the LSU sequences were taken and aligned with those sequenced in this study. As mentioned before only five *L.* species gave sequences of justifiable quality. However they cluster with *Hyaloscypha daedalae* and *Mitrula paludosa* or with *Hydrocina chaetocladia* respectively. *Mitrula p.* and *Hydrocina c.* are classed by Wang et al. (2006) as Mitrula clade while *Hyaloscypha d.* is classed as VIBRISSEA-LORAMYCES clade, two clades adjacent to each other in their phylogeny. Anyhow the bootstrapping in Figure 10 cannot support this neighbourhood even though the *L.* sequences seem to be fairly well supported. This might be because of the relatively short partial sequence of LSU that came as a result of the final alignment. Since the large subunit is very highly conserved differences in single base pairs can make a difference so that long sequences are required to bal-

ance this. The consensus here was however only about 700 basepairs which was probably too short.

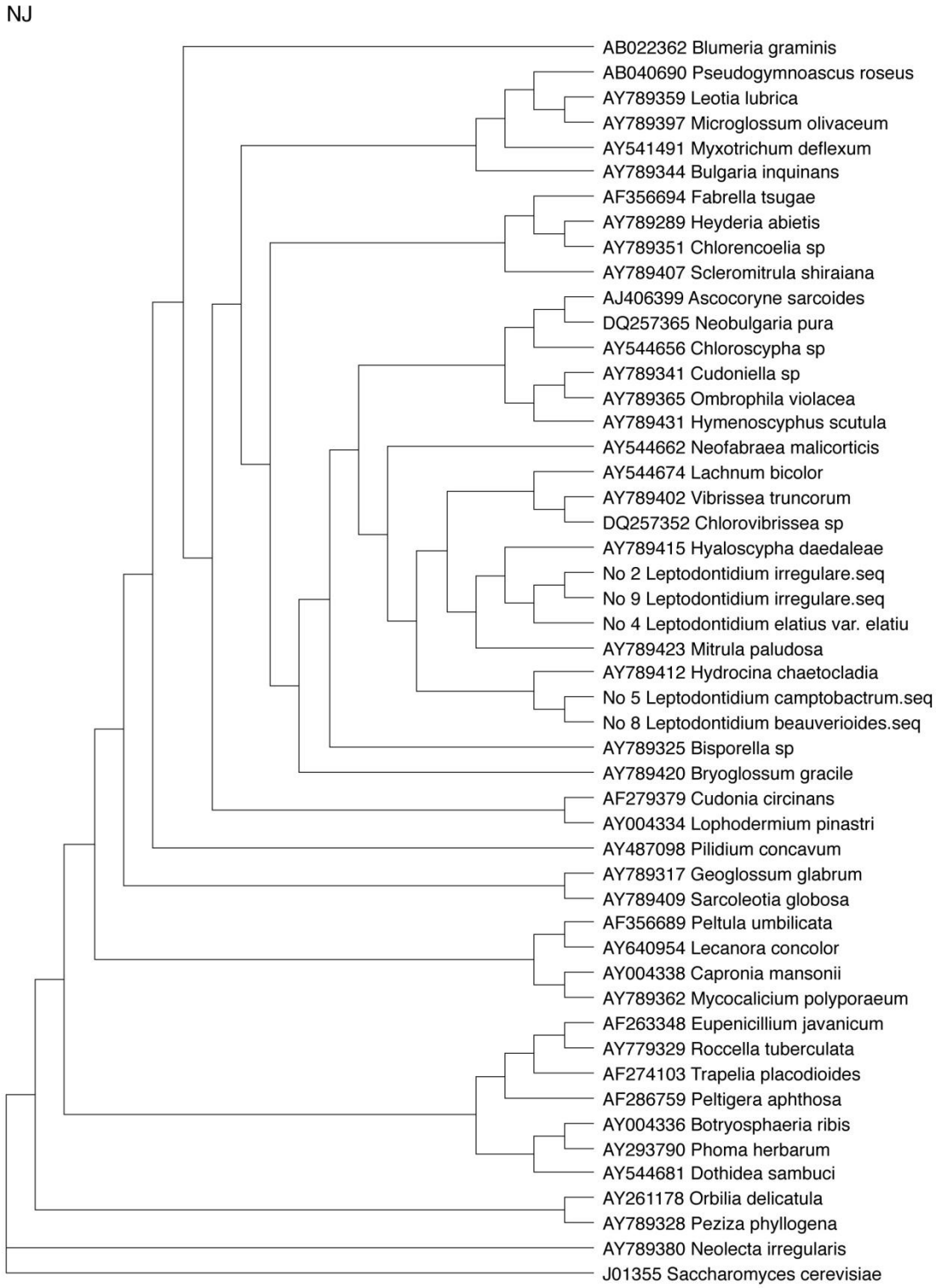

Figure 8 Neighbour joining tree based on LSU

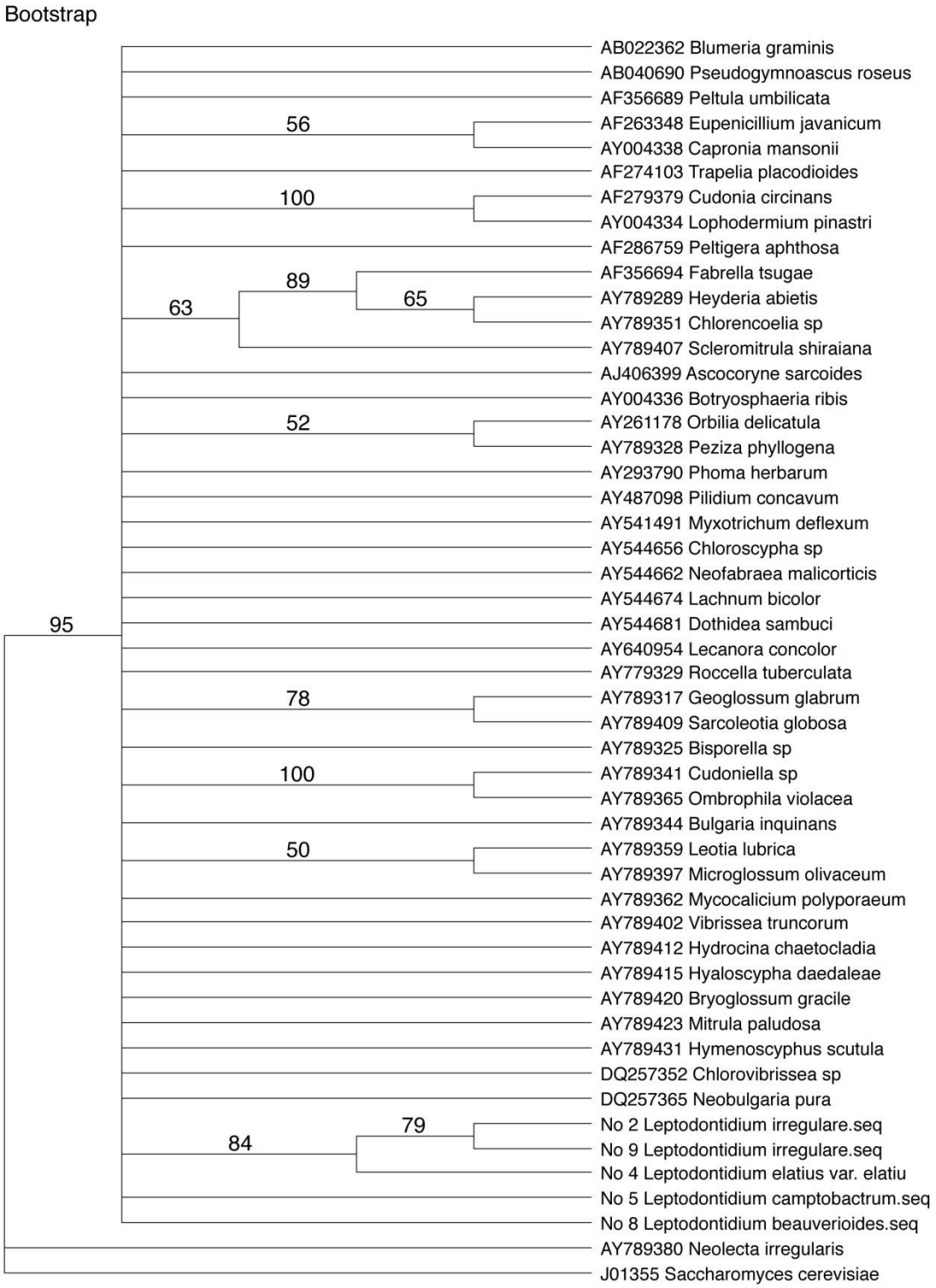

Figure 9 Bootstrap for neighbour joining tree based on LSU

4 Conclusions

4.1 General conclusions

In order to be able to draw proper conclusions one need to remember the situation in forest soil. Fungi live in a patchy environment surrounded by substrates with different C:N ratios. To prosper they need the right amount of both. If a conclusion should be drawn from Project 2 it is that fungal substrate can have a rather high C:N ratio. Fungi that only have this kind of substrate at their deposition soon become carbon limited. Subsequently follows that Fungi that want to make best use of this substrate need an additional carbon source. Lindahl et al. (2010) observed heightened cellulase activities in combination with the disruption of root carbon transport into forest humus. *L. beauverioides* showed significant preferences for mushroom and litter substrate. Putting these two observations together one could hypothesize that *L.beauveriodes* decomposes mushroom in order to get nitrogen and simultaneously litter to get carbon. It has after all been shown previously that filamentous Fungi possess the ability to translocate solutes from one part of the mycelium to another. It could thus been said that *L.beauveriodes* is a free living saprotroph taking advantage of senescing mycelium of other fungi subsequent to disturbance. If the Fungus lives in forest soil that is. Both its closeness to Neofabraea malicorticis which is known to be a tree parasite (Braun et al. 2001) in the ITS tree (Figure 7) and the wood substrate it has initially been found on (Figure 1) could however indicate that it is an endophytic parasite. The definition of opportunists seems more to apply to *L. elatius var. elatius, L. irregulare, L. camptobactrum* and *L. obscurum*. The fact that not all replicates of *L. elatius var. elatius* mineralize ammonium in Project 2 might indicate that they can make better use of the fungal substrate and do not need an additional carbon source. The respiration

data of the other three Fungi clearly shows that they prefer mushroom substrate and that they can not make much use of the other substrates. So if candidates for a further investigation of the matter should be chosen I would choose these four. *L. elatius* var. *ovalisporum* makes for another story though. The corrected respiration data could not show a significant difference between mushroom substrate and the summarized negative controls. Moreover the initial respiration data points towards a better use of the wood substrate than mushroom. This is emphasized by its position in the ITS tree next to a fungal strain that was found on wood in France. Unfortunately Project 1 and 2 could not be conducted with all 11 L. strains due to the scope of this study so that no conclusions as to the ecology of L. boreale can be drawn. Even though its position in the ITS tree next to a strain isolated from *Vaccinium* roots and the fact that it was initially found in soil says at least so much that it lives in soil.

To summarize four *L.* species can be confirmed as opportunists. Mineralization can not be statistically confirmed but is indicated by the data. The phylogeny revealed the genus *Leptodontidium* as polyphyletic thus raising the need to rename some species. Here I suggest that the *L. elatius* strains and *L. irregular* should keep the name since they constitute the majority of strains and form a monophyletic group. *L. camptobactrum* and *L. orchidicola* should be grouped in a new genus and *L. obscurum* and the *L. boreale* strain CBS 683.84 should be treated as one species in yet another genus. Some uncultured strains are so close to *L.* species that they can be classified as them.

Unfortunately the environmental sequences CO4, CO37 and RED136 (Lindahl et al. 2010) could not be confirmed to be part of the genus *L.* They did not group with any other cultured sequence either so that their identity remains unknown. The phylogenetic tree based on ITS has however a very high taxonomic resolution so that similar ecological strategies between the CO4 cluster and *L.* strains should not be unlikely.

As a whole the capability of L. strains to break down fungal substrate has been confirmed as well as their capability to mineralize ammonium during the course so that the decomposition of ectomycorrhizal mycelium by opportunistic fungal strains could very well be an explanation for heightened nitrogen emissions from clear cut areas in boreal forests.

4.2 Future prospects

It is now undoubted that Fungi play a major role in forest ecosystems. It is thus of great importance to further investigate fungal interactions with each other, soil microorganisms and protozoa to further understand their ecological strategies and roles in element cycling. Especially the impact of human perturbations such as forest fertilization, clear cuts and increased atmospheric CO_2 should be in focus for future research activities. To further clarify fungal interactions after clear cut soil cores could be taken before and after clear cut, the fungal community profiled with e.g. TRFLP (Terminal Restriction Length Polymorphism) and then screened for RNA encoding for chitinases and laccases. The latter in order to see whether any antagonistic interactions take place (laccase production) or whether fungal material is decomposed (chitinase production). RNA should be used instead of DNA to make sure that the interactions take place at that very moment.

A future phylogeny should be performed on SSU, ITS and LSU as described in Wang et al. (2006) to gain better support. Here new rapid sequencing technologies such as 454 sequencing will proof to be invaluable leading to a successive change in fungal classification from a morphological based taxonomy to a fungal tree of life based on genomic information.

5 References

5.1 Literature

Ainsworth, G., 1976. *Introduction to the History of Mycology* I., Cambridge UK: Cambridge University Press.

Alexander, I. et al., 2009. Small genetic differences between ericoid mycorrhizal fungi affect nitrogen uptake by Vaccinium. *The New phytologist*, 181(3), 708-18.

Allen, T.R. et al., 2003. Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. *New Phytologist*, 160(1), 255-272.

Altschul, S. et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389 - 3402.

Baldrian, P., 2004. Increase of laccase activity during interspecific interactions of white-rot fungi. *FEMS microbiology ecology*, 50(3), 245-53.

Boberg, J. et al., 2008. Glucose and ammonium additions affect needle decomposition and carbon allocation by the litter degrading fungus Mycena epipterygia. *Soil Biology and Biochemistry*, 40, 995-999.

Boberg, J.B. et al., 2010. Fungal C translocation restricts N-mineralization in heterogeneous environments. *Functional Ecology*, 24(2), 454-459.

Boer, W.D. et al., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS microbiology reviews*, 29(4), 795-811.

Bougoure, D.S. & Cairney, J.W., 2005. Fungi associated with hair roots of Rhododendron lochiae (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. *Environmental Microbiology*, 7, 1743-1754.

Brant, J.B., Myrold, D.D. & Sulzman, E.W., 2006. Root controls on soil microbial community structure in forest soils. *Oecologia*, 148(4), 650-9.

Braun, P. et al., 2001. Phylogenetic relationships among Neofabraea species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the beta-tubulin gene. *Mycological research*, 105, 658 - 669.

Bruns, T.D. & Shefferson, R.P., 2004. Evolutionary studies of ectomycorrhizal fungi : recent advances and future directions 1. , 1132(1), 1122-1132.

Chien, A., Edgar, D.B. & Trela, J.M., 1976. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol*, (174), 1550–1557.

Cox, P., Anderson, J. & Wilkinson, S., 2001. Effects of fungal inocula on the decomposition of lignin and structural polysaccharides in Pinus sylvestris litter. *Biology and fertility of soils*, 33(3), 246 - 251.

Currah, R., Addy, H. & Hambleton, S., 2000. Distribution and molecular characterization of the root endophyte Phialocephala fortinii along

an environmental gradient in the boreal forest of Alberta. *Mycological research*, 104, 1213 - 1221.

Dawkins, R., 2009. The greatest show on earth: The evidence for evolution. In London, UK: Free Press Transworld, pp. 285-337.

Doyle, J.J. & Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. *Focus*, (12), 13-15.

Guarro, J., Gené, J. & Stchigel, A.M., 1999. Developments in fungal taxonomy. *Clinical microbiology reviews*, 12(3), 454-500.

Hedh, J., Wallander, H. & Erland, S., 2008. Ectomycorrhizal mycelial species composition in apatite amended and non-amended mesh bags buried in a phosphorus-poor spruce forest. *Mycological research*, 112(Pt 6), 681-8.

Hoog, G.d., 1979. Nomenclatural Notes on Some Black Yeast-like Hyphomycetes. *Taxon*, 28(4), 347-348.

Jennings, D., 1987. Translocation of solutes in fungi. *Biol. Rev.*, 62, 215–243.

Kauserud, H., Mathiesen, C. & Ohlson, M., 2008. High diversity of fungi associated with living parts of boreal forest bryophytes. *Botany*, 86(11), 1326-1333.

Lawrie, A., McLean, C. & Cunnington, J., 1999. Molecular diversity within and between ericoid endophytes from the Ericaceae and Epacridaceae. *The New phytologist*, 144(2), 351 - 358.

Lindahl, B., Finlay, R. & Stenlid, J., 2001. Effects of resource availability on mycelial interactions and P-32 transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. *FEMS microbiology ecology*, 38(1), 43 - 52.

Lindahl, B.D. & Finlay, R.D., 2006. Activities of chitinolytic enzymes during primary and secondary colonization of wood by basidiomycetous fungi. *The New phytologist*, 169(2), 389-97.

Lindahl, B.D. & Olsson, S., 2004. Fungal translocation - creating and responding to environmental heterogeneity. *Mycologia*, 18(May), 79-88.

Lindahl, B.D. et al., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *The New phytologist*, 173(3), 611-20.

Lindahl, B.D., de Boer, W. & Finlay, R.D., 2010. Disruption of root carbon transport into forest humus stimulates fungal opportunists at the expense of mycorrhizal fungi. *The ISME journal*, 1-10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20220789.

Lindahl, B.O., Taylor, A.F. & Finlay, R.D., 2002. Defining nutritional constraints on carbon cycling in boreal forests – towards a less ‘ phytocentric ’ perspective. *Plant and Soil*, 123-135.

Lindeberg, G., 1944. On the physiology of lignin-decomposing soil Hymenomycetes. Studies on Swedish species of Marasmius. *Symbolae botanicae upsalienses*, 8(2), 183.

Lygis, V. et al., 2005. Wood-inhabiting fungi in stems of Fraxinus excelsior in declining ash stands of northern Lithuania, with particular reference to Armillaria cepistipes. *Scandinavian Journal of Forest Research*, 20(4), 337-346.

Löfgren, S. et al., 2009. Short-term effects of clear-cutting on the water chemistry of two boreal streams in northern Sweden: a paired catchment study. *Ambio*, 38(7), 347-56.

McLaughlin, D.J. et al., 2009. The search for the fungal tree of life. *Trends in microbiology*, 17(11), 488-97.

Mclaughlin, D.J. et al., 2009. The search for the fungal tree of life. *Trends in Microbiology*, (September).

Menkis, A. et al., 2004. Ecology and molecular characterization of dark septate fungi from roots, living stems, coarse and fine woody debris. *Mycological Research*, 108(8), 965-973.

Menkis, A. et al., 2005. Fungal communities in mycorrhizal roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by morphotyping, direct sequencing and mycelial isolation. *Mycorrhiza*, 16(1), 33-41.

Muehlhardt, C., 2009. *Der Experimentator Molekularbiologie/Genomics* 6 ed., Heidelberg: Spektrum Akademischer Verlag.

O'Brien, H.E. et al., 2005. Fungal Community Analysis by Large-Scale Sequencing of Environmental Samples †. *Society*, 71(9), 5544-5550.

O'Brien, H.E. et al., 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied and environmental microbiology*, 71(9), 5544-50.

Peay, K.G. et al., 2010. Potential link between plant and fungal distributions in a dipterocarp rainforest: community and phylogenetic structure of tropical ectomycorrhizal fungi across a plant and soil ecotone. *The New phytologist*, 185(2), 529-42.

Peay, K.G., Kennedy, P.G. & Bruns, T.D., 2008. Fungal Community Ecology: A Hybrid Beast with a Molecular Master. *BioScience*, 58(9), 799.

Rayner, A. & Boddy, L., 1988. *Wood Decomposition: its Biology and Ecology.*, Chichester: John Wiley.

Roose-Amsaleg, C., Brygoo, Y. & Harry, M., 2004. Ascomycete diversity in soil-feeding termite nests and soils from a tropical rainforest. *Environmental Microbiology*, 6, 462-469.

Rosen, K., Aronson, J. & Eriksson, H., 1996. Effects of clear-cutting on streamwater quality in forest catchments in central Sweden. *Forest Ecology and Management*, 83(3), 237-244.

Schumacher, T., Vralstad, T. & Myhre, E., 2002. Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. *The New phytologist*, 155(1), 131 - 148.

Siira-Pietikäinen, A. et al., 2001. Responses of decomposer community to root-isolation and addition of slash. *Soil Biology and Biochemistry*, 33(14), 1993-2004.

Sparrow, F.K., 1958. Interrelationships and Phylogeny of the Aquatic Phycomycetes. *Mycologia*, 50(6), 797 - 813.

Sulkava, P. & Huhta, V., 2003. Effects of hard frost and freeze-thaw cycles on decomposer communities and N mineralisation in boreal forest soil. *Applied Soil Ecology*, 22(3), 225-239.

Summerbell, R. et al., 2005. The hyphomycete Teberdinia hygrophila gen. nov., sp nov and related anamorphs of Pseudeurotium species. *Mycologia*, 97(3), 695 - 709.

Taylor, D.L. et al., 2008. Increasing ecological inference from high throughput sequencing of fungi in the environment through a tagging approach. *Molecular Ecology Resources*, 8(4), 742-752.

Taylor, D.L. et al., 2007. TOPO TA is A-OK : a test of phylogenetic bias in fungal environmental clone library construction. *Environmental Microbiology*, 9, 1329-1334.

Taylor, T. & Osborne, J., 1996. The importance of fungi in shaping the paleoecosystem. *Rev. Palaeobot. Palynol.*, 90, 249–262.

Tedersoo, L. et al., 2009. Ascomycetes associated with ectomycorrhizas: molecular diversity and ecology with particular reference to the. *Environmental Microbiology*, 11, 3166-3178.

Trappe, J., 1987. Phylogenetic and ecologic aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In G. Safir *Ecophysiology of VA mycorrhizal plants*. Boca Rato: CRC, p. 5–25.

Verkley, G., 1999. A monograph of the genus Pezicula and its anamorphs. *Studies in mycology*, (44).

Wang, B. & Qiu, Y., 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, 16(5), 299-363.

Wang, Z. et al., 2006. Evolution of helotialean fungi (Leotiomycetes, Pezizomycotina): a nuclear rDNA phylogeny. *Molecular phylogenetics and evolution*, 41(2), 295-312.

Whittaker, R., 1969. New concepts of kingdoms of organisms. *Science*, 163(3863), 150-160.

Wolfe, B.E. et al., 2009. Distribution and abundance of the introduced ectomycorrhizal fungus Amanita phalloides in North America. *The New phytologist*, (2009), 803-816.

Wright, S.H., Berch, S.M. & Berbee, M.L., 2009. The effect of fertilization on the below-ground diversity and community composition of ectomycorrhizal fungi associated with western hemlock (Tsuga heterophylla). *Mycorrhiza*, 19, 267-276.

Yarwood, S., Myrold, D. & Högberg, M., 2009. Termination of belowground C allocation by trees alters soil fungal and bacterial communities in a boreal forest. *FEMS microbiology ecology*, 70(1), 151-62.

5.2 Personal Communication

Lindahl, Björn D., Department of Forest Mycology and Pathology, Swedish University of agriculture, Uppsala, e-mail: Bjorn.Lindahl@mykopat.slu.se

Rosling, Anna, Department of Forest Mycology and Pathology, Swedish University of agriculture, Uppsala, e-mail: Anna.Rosling@slu.se

6 Acknowledgement

I would like to thank Inga Bödecker for her invaluable help, support, advice and patient explanations; Björn Lindahl for inspiration, helping me to see over the rim of my teacup and guiding me through the cliffs and shallows of statistics, Katarina Ihrmark, Maria Jonsson and Rena Gadjieva for patiently answering all my questions in the laboratory and finally my husband Thomas Wimark for all his support and for tolerating my grumpiness when things had gone wrong.