University of Alberta

Fungal communities associated with roots of the Betulaceae

by

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"Feeling desolate, Ememquut walks through the bleak camps of the lower world. Ememquut meets Mold-Woman. She is sullen and coarse looking; wrapped in dirty and torn rags, she is also covered with a heavy layer of mold. She smells fetid and foul. But to Ememquut's surprise, the soft and tender voice in which she addresses him lets him forget her unpleasant appearance. Ememquut is glad to have found a tender and sympathetic being in the lower world. He suggests marriage..."

– excerpt from a Koriak love story[1]

[1]**Rethmann P. 2001.** *Tundra Passages: history and gender in the Russian Far East.* University Park, PA, USA: Pennsylvania State Press.

Abstract

Communities of fungi associated with roots and nodules of *Alnus incana* subsp. *tenuifolia* and the roots of *Betula papyrifera* collected at four sites in the aspen parkland ecosystem around Edmonton, Alberta, were described using taxonomic and physiological approaches. Patterns in community structure, diversity, and composition and their relationship to collection site and host were analyzed. Comparisons of overall community structure showed the collection site has greater influence than the type of host, despite fungal communities associated with alder nodules having lower species diversity than roots of either host species. Fungal species that appeared to be specific to one of the three habitats investigated were fewer than generalist species. The root endophyte *Leptodontidium orchidicola* isolated at one site produced fertile apothecia in culture. This novel teleomorph is *Mollisia rhizophila sp. nov.*

Dedication

This thesis is dedicated to John and Margerite Skinner. My grandfather, John, died while I was editing this thesis. My grandmother, Margerite, misses him as only a person married over seventy years to one person can. Both taught the importance of the outdoors, of perseverance, and of trying new things.

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Finally, I would like to thank my family. My parents, Patti and Bud, fostered my interest in the sciences and provided support. My sister Sue never ceased in her encouragement and shared her knowledge and advice. Beatrice encouraged me to get exercise every day and made me laugh. I express my deepest gratitude to Polly, who not only relocated to be with me, but was understanding and supportive at every step.

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Chapter 1 - Introduction

Introduction

My thesis consists of two projects: one described communities of fungi associated with certain roots, and the other a new fungal species discovered in some of these communities. Both projects involved the ecology of microfungi, in particular those associated with roots, and their plant hosts. These concepts will be introduced before discussing the two projects in more detail.

A working definition of "root-associated fungi"

In contrast to saprobic fungi, which degrade dead material, mycorrhizal, pathogenic and endophytic fungi can at some point in their life grow within the living tissues of a plant. Mycorrhizal fungi are recognized by forming distinctive structural features in root tissues, such as arbuscules, vesicles, mantles, or Hartig nets. Roots bearing such structures are generally assumed to be receiving nutrients from their fungal partner(s) such as phosphates (Koide & Kabir, 2000) and nitrogen (Lindahl *et al.*, 2005) in exchange for photosynthate. Pathogenic fungi use a range of structural and/or chemical mechanisms to destroy or parasitize tissues of the host. Their effects on the host create symptoms of disease, including necrosis, chlorosis or some otherwise obvious reductions or interruptions in host vigor. The term "fungal endophyte" is somewhat more difficult to define (Wilson, 1995), but the term typically refers to species that live asymptomatically within a living plant (Carroll,

1986; Wilson, 1995): they do not cause any symptoms of disease in their hosts, such as necrosis or decreased vigor, nor do they form structures typical of mycorrhizae. Many researchers have concluded that endophytes are mutualistic symbionts that confer some advantage to their host, in some conditions, because their hosts have not evolved mechanisms to eliminate them. A well known example of such an advantage is the antagonism of plant pathogens or herbivores by some endophytes (Carroll, 1986; Wilson, 1993). Some endophytes may also benefit their host by assisting in the degradation or pruning of senescent organs (Kowalski & Kehr, 1992; Wilson, 1993), or through the formation of "cryptic mycorrhizae" (Jumpponen, 2001).

It is difficult to study communities of endophytes directly because they occur within opaque habitats at microscopic scales. Endophytic community composition is typically determined by isolating and identifying fungi growing from a surface-sterilized plant organ that has been incubated on agar medium (Carroll, 1986), or, more recently, by amplifying and processing a region of all fungal DNA from such a substrate (Seghers *et al.*, 2004). Although the majority of endophytic research is based on these approaches, two issues must be kept in mind. First, fungal species not typically considered endophytes may be detected because it is inevitable that some propagules or hyphae of epiphytic fungi may survive surface sterilization (especially when the substrate has complex surfaces or dead tissues). Second, the exact location, extent, and nature of the association are difficult to assess using cultural or molecular methods alone. These two problems may be

reduced through microscopic observations of cleared and stained plant tissue (Addy *et al.*, 2005). However, this approach can only show, within field-collected substrates, the presence and distribution of endophytes in general, because most endophytic species cannot be differentiated *in situ*. Endophytic species may be observed in this way if they are individually inoculated into axenically grown plants; however this approach is seldom used because it is very labour intensive.

A single sample of field-collected roots could conceivably host fungi involved in all three types of associations with their host, i.e., as mycorrhizal, pathogenic and endophytic associates, and, at the same time, the root surface and contiguous moribund tissues could also be supporting a diverse array of saprobic species. For community level studies that focus on the fungi in roots, it is expedient to refer initially to all species isolated or otherwise detected as "root-associated". The nature of the relationship each species has with the host can be deduced or explored subsequently.

Definition of microfungi

Here, the term microfungi refers to taxa that are recovered from their natural habitat or substrate and grown on agar media. Under these artificial circumstances, they may produce morphologically distinctive (and usually microscopic) structures or features that can be used for identification purposes. Microfungi, like fungi generally, can be identified and named scientifically according to their sexual morphology (teleomorph) or according to one (or more) distinctive asexual morphologies (anamorph(s)) if the teleomorph is absent or unknown, or if the use of

this name is impractical or creates ambiguity. These so-called pleomorphic fungi include basidiomycetes and ascomycetes and are the only taxa in the whole of biological nomenclature permitted to have more than one legitimate scientific name (Gams *et al.*, 2003).

In culture, most microfungi reproduce via asexual propagules or conidia. Morphology of these conidia, the conidiogenous cells, and the structures bearing them, i.e., conidiophores, are generally sufficient for identification, assuming that the species in hand has been described and named. However, in the case of many endophytic species, identification is often complicated by the diminutive size and frequent lack of distinctive characteristics of their reproductive structures (Summerbell, 2005). Identification is further complicated by variations in reproductive structures that are, in part, substrate and strain dependant (Petrini, 1986). Increasingly, evidence based on DNA sequence data (or molecular data) shows that some morphologically identified species, or form-species, may actually contain several cryptic species (Addy *et al.*, 2005). Finally, some endophytic species do not readily sporulate in culture. For these reasons, studies of endophytic communities increasingly rely on molecular methods.

While most microfungal species grown in culture are identified by their anamorphic characters, their wider taxonomic placement is based on teleomorphic characters. Before the advent of DNA characterization, observations of teleomorphic structures forming within cultures with a known anamorph were the only way to link definitively an anamorphically described species to a

teleomorphically described one. Anamorph-teleomorph connections may be applied more generally at the generic level or higher. The teleomorphic genus *Mollisia,* for example, has been connected to numerous anamorphically described genera (or form-genera) (Hennebert & Bellemére, 1979). Such connections may be used to generate and test genus-level taxonomic or ecological hypotheses.

Ecological concepts

For the last two decades, scientists, conservationists, policy makers, and the general public have become increasingly concerned with biodiversity. This concern stems from anthropogenic decreases in biodiversity, and from the links between biodiversity and ecosystem stability, resiliency (Loreau *et al.*, 2002), and functioning (Hooper *et al.*, 2002). The term biodiversity is prone to being used in several ways because of the variety of its users. Like any other concept or metric, it also has its own implications, assumptions, and limitations. One definition of biodiversity is that it is the synthesis of the interrelated elements of species, genetic, and functional diversity, within a defined system or geographic space (Solbrig, 1991; Zak *et al.*, 1994). All too often, species diversity (the number of species moderated by their relative abundances) or species richness (the number of species) are used interchangeably with the term biodiversity (Spellerberg & Fedor, 2003; e.g., Mueller & Schmit, 2007). Some researchers (Zak & Visser, 1996; Hooper *et al.*, 2002) suggest that little understanding of gross ecosystem processes can be gleaned from studies based solely on species diversity or richness in the absence of detailed ecological knowledge of most species detected. This situation clearly

applies to most fungal communities, since only a maximum of 13% of fungal species have been scientifically described (Schmit & Mueller, 2007), most without much ecological information.

A longstanding theme in plant ecology is to look at relationships between species diversity and other factors including productivity or soil nutrients (Loreau *et al.*, 2001; Tilman, 1986). Recently, researchers have started to look more closely at plant functional diversity. Tilman *et al.* (1997) demonstrated clearly the validity of functional ecology when they noted that the functional diversity of artificial plant communities had a greater influence on primary productivity than species diversity. Another study linked functional profiles of fungal communities to different disturbance and climatic regimes (Sobek & Zak, 2003). In the latter study, the authors postulated that the linkage they reported was likely due to a combination of environmental conditions and vegetation assemblages that covaried from site to site. A next step would be to investigate the importance of the species, function and organ types of plants, relative to external site factors, on the composition and function of their associated fungal communities. A better understanding of the links between plant communities and their associated fungal communities could improve fungal diversity estimates and overall understanding of fungal ecology.

Thesis Objectives

The first purpose of my research was to determine the composition of the fungal communities associated with the roots of two plant species. The second was to determine the importance of host species, type of root or nodule, and site factors on

the composition, structure and general function of these communities. This study used a physiological approach, substrate utilization profiles (SUPs), as proxies for fungal community function. The final purpose was to describe any new species encountered. My major hypothesis was that different roots and nodules and/or different collection sites would shift both the taxonomic and physiological composition and structure of the associated fungal communities, and that new fungal species would be detected. To limit the scope of this study, two host species were sampled at four sites in the aspen parkland ecosystem of central Alberta, Canada. These species were *Alnus incana* (L.) Moench subsp. *tenuifolia* (Nutt.) Breitung (mountain alder) and *Betula papyrifera* Marsh (paper birch). Three plant organs were studied: the nodules of *Alnus incana* subsp. *tenuifolia*, organs housing the nitrogen-fixing symbiotic bacterium *Frankia*, and the fine roots of both *Alnus incana* subsp. *tenuifolia* and *Betula papyrifera*.

Synopsis

Chapter 2 describes, for the first time, a small discomycetewhich was isolated from the nodules of *Alnus incana* subsp. *tenuifolia*, and from the roots of both *Alnus incana* subsp. *tenuifolia* and *Betula papyrifera* collected at one site. This new species, *Mollisia rhizophila*, sp. nov., was compared to the type species, *M. cinerea*, as well as *M. cinerea f. minutella.* Morphological evidence showed the anamorph of this new species is the frequently isolated root endophyte *Leptodontidium orchidicola.* Phylogenetic analysis of one gene, the ITS1-5.8s-ITS2 region of ribosomal DNA, supported this conclusion. The endophytic nature

of *L. orchidicola* compared with the reportedly saprobic nature of the genus *Mollisia* supported my hypothesis that *M. rhizophila* is capable of occupying these two putatively distinct niches. Preliminary observations of the asci of *M. rhizophila* indicated subapical dehiscence. Ascus dehiscence of this type has not been noted within the *Helotiales*.

Chapter 3 describes research done to test the hypothesis that the taxonomic and physiological composition and structure of fungal communities associated with roots and nodules would vary by sample site, by host species, and/or by type of host organ. A traditional culture-based study was done in parallel to a study using substrate utilization profiles to detect differences in taxonomic and functional community structures. The results of both approaches were compared not only to test the hypothesis, but also to assess the value of each approach in describing fungal community dynamics.

The final chapter, Chapter 4, summarizes the major findings of Chapters 2 and 3, and suggests some future research directions.

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Chapter 2 - *Mollisia rhizophila*, sp. nov.: the teleomorph of *Leptodontidium orchidicola*

Introduction

Since its initial isolation from roots of boreal orchids and subsequent description (Currah *et al.*, 1987), *Leptodontidium orchidicola* Sigler & Currah has been reported as a Dark Septate Endophyte (DSE) from the roots of many different species (Jumpponen & Trappe, 1998; Addy *et al.*, 2005). Colonies are grey to olive and often sterile although the minute, globose to tear-drop shaped conidia, which are solitary and lateral on undifferentiated hyphae or arise sympodially to form small terminal clusters, occur sporadically and are easily overlooked. A teleomorph is unknown but is expected among the Dermateaceae based on affiliations inferred from DNA base sequence comparisons of the small subunit of the rDNA among inoperculate taxa (Wilson *et al.*, 2004).

During a survey of the communities of microfungi associated with the roots of *Alnus incana* subsp. *tenuifolia* (Nutt.) Breitung., and *Betula papyrifera* Marsh. near Edmonton, Alberta, Canada, a series of DSE isolates that had been identified as *L. orchidicola* produced apothecia that appeared to represent a hitherto undescribed species of *Mollisia*. However, with over 500 species and subspecies (CBS database, July 11, 2006) and no comprehensive monograph, the genus is a difficult one in which to delineate new taxa. Most species are known from field collections and consist of apothecia that have developed ostensibly as saprobes on

the stems of herbaceous plants. A variety of form-genera have been reported in isolates derived from these collections, including *Cadophora* (identified as *Phialophora*; Le Gal & Mangenot, 1956, 1958, 1961; see Harrington & McNew, 2003), *Chalara* (Arendholz & Sharma, 1984), and *Cystodendron* (Aebi, 1972). As far as I am aware, a *Leptodontidium* state has never been reported for any cultured species in this genus.

Given the necessity for precise anatomical characters for the identification of *Mollisia* species, I prepared a detailed examination of apothecial development in these new isolates. Also, using sequence data from the ITS1-5.8s-ITS2 region of ribosomal DNA, a phylogeny was prepared using putatively similar species of *Mollisia* and species of *Leptodontidium*, including *L. orchidicola*, deposited in GenBank. In this chapter, I describe how these data were used to challenge the hypothesis that my fertile isolates represent a hitherto unknown species of *Mollisia*, and provide a formal description of the new taxon.

Materials and methods

Sample collection

Roots and nodules of *A. incana* subsp. *tenuifolia* and roots of *B. papyrifera* were sampled from four sites by excising a roughly 30 X 30 X 15 cm sample of root-containing topsoil adjacent to or directly under (depending on the size of the plant) selected trees. Only some samples of each of these substrates, i.e., those collected at the Lily Lake Natural Area (UTM 11N 655620E 5954235N), NW of Edmonton,

Alberta, yielded pleomorphic isolates of *L. orchidicola* that eventually produced apothecia, while samples from two other sites yielded isolates of *L. orchidicola* that did not produce a teleomorph. All sites were characterized by moist or wet soils and were adjacent to wetlands and water bodies in the local aspen parkland ecosystem; however, at 3-4 cm below the soil surface, the Lily Lake site appeared to have the highest water table.

Sample preparation

Samples of fine roots and nodules were excised from washed root balls and agitated with a magnetic stirrer in two serial baths of distilled water (sdH_2O) in 100 X 80 mm deep culture dishes (with lid), soaked for one minute in ~3% hydrogen peroxide, and rinsed with sdH_2O. These organs were placed in 18 mm diameter test tubes containing 10 ml of a 1% buffer of sodium and potassium phosphates (pH 7.4) (Canadian Laboratory Supplies, Vancouver, BC, Canada) and ground for 30 seconds using a 17 mm homogenizer. The resulting slurries were rinsed through two sieves (500 μm & 250 μm) and particulates between 500 μm and 250 μm in size were swabbed into a vial containing 15 ml of 0.2% water agar (amended with 100 mg l^{-1} of streptomycin sulfate (Sigma-Aldrich, Canada) and 50 mg l^{-1} of oxytetracycline HCl (Sigma-Aldrich)) to prepare a suspension of 70% transparency. A 100 μL aliquot of suspension was plated on modified benomyl-free BAF media (Hutchison, 1991) using oxytetracycline HCl and $MnCl_2$ (6 g l^{-1}) instead of chlortetracycline HCl and $MnSO_4$ (5 g l^{-1}). After incubation at room temperature

(RT, ~23 °C) in the dark, colonies were transferred to CMA (corn meal agar, Acumedia, Baltimore, MD, USA) amended with oxytetracycline HCl (100 mg l^{-1}) and then to CMA, MEA (15.0 g Difco Bacto malt extract (Difco, Sparks, MD, Canada), 17.0 g Difco Bacto agar, 1 L dH_2O), PDA (potato dextrose agar, Difco), slide cultures consisting of blocks of OA (20.0 g powdered oatmeal, 20.0 g agar, 1 L dH_2O) or Pablum cereal agar (CER) (100 g dry pablum (Pablum Canada, North York, ON, Canada), 20.0 g Difco Bacto agar, 1 L dH_2O) on TWA (20.0 g Difco Bacto agar, 1 L tap water), and on sterile aspen (*Populus tremuloides* Michx) wood chips in a moist chamber. Subsequent transfers were grown at RT in ambient light (diffuse natural light combined with light from standard fluorescent lab lighting) or, for select cultures on PDA or aspen chips, in a "black light" regime combining a fluorescent "gro-light" (Gro-Lux Sylvania F20T12, 20 W; Osram Sylvania, Mississauga, ON, Canada) with a UV light (Philips F20T12-BL, 20 W; Philips Lighting, NJ, USA) (Rice & Currah, 2005) on a 12 hour cycle.

Microscopy

For transmission electron microscopy (TEM), specimens were prepared according to Tsuneda & Currah (2004): fixed in glutaraldehyde, postfixed in OsO_4, dehydrated, embedded in Spurr's resin, and post sectioning stained in uranyl acetate and lead citrate. Transmission electron micrographs were taken with a Hitachi H-7000 electron microscope at ~75 kV. For compound light microscopy using bright field and Nomarski Interference Microscopy (NIM), slide cultures, mycelium, and some hand-sectioned apothecia were unstained or stained with acid fuchsin before

mounting in polyvinyl alcohol (PVA) or glycerin jelly. Coverslips from slide cultures often had mycelium growing on both sides; mycelium on the unmounted side was observed using brightfield microscopy. Other hand-sectioned apothecia were stained with Melzer's reagent, and were observed without any mounting medium. Additional apothecia were fixed, dehydrated, embedded in araldite, sectioned (to about 1 μm), and stained with a slightly alkaline solution of toluidine blue (10 g l^{-1}) in borax (10 g l^{-1}) (Meek, 1970; Tsuneda & Currah, 2004) before viewing with bright field microscopy. Compound light microscopy was performed using an Olympus BX50 light microscope fitted with Olympus UPlanFl objectives; images were captured with an Olympus DP12 digital camera. Low-magnification incident light microscopy of macroscopic material was performed using a Wild M3B stereo microscope (Wild Leitz Canada Ltd.) and photographed with a Nikon Coolpix 950 digital camera.

Molecular analyses

Isolates were grown on MEA or PDA overlaid with a Cellophane™ membrane (UCB Films, Bridgwater, Somerset, UK). DNA extraction, amplification and sequencing of the targeted regions, ITS1, 5.8s, ITS2, and flanking portions of the SSU and LSU regions, and subsequent analysis were modified from the methods of Gibas *et al.* (2002). Mycelium scraped free of the membrane was placed in a precooled sterile mortar with sterilized sand and liquid nitrogen, and ground to a powder. One milliliter of extraction buffer [20 g l^{-1} cetyl-trimethyl ammonium bromide (CTAB); 1.5 M NaCl; 100 mM TRIS HCl; 20 mM EDTA] was mixed with

the pulverized mycelium; this slurry was then incubated in a 2 ml screw-capped microcentrifuge tube with 2 µl of β-Mercaptoethanol for 30 to 120 minutes at 65 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and mixed by inverting 40 times. After centrifugation at room temperature for 20 minutes at >10 000 g (10 000 rpm, r_{av} 9.5 cm), the resultant aqueous phase (crude DNA solution) was removed and purified using the QIAquick DNA purification kit (QIAgen Inc., Mississauga, Ont., Canada). The purified DNA was stored at -20 °C.

The targeted regions, ITS1, 5.8s, ITS2, and flanking portions of the SSU and LSU regions, were amplified from the purified DNA by a polymerase chain reaction (PCR) using the primers BMB-CR (Lane *et al.*, 1985) and ITS4 (White *et al.*, 1990) (primers manufactured at the Molecular Biology Service Unit, University of Alberta). Reaction mixtures contained 26 µl dH_2O, 5 µl 10 X buffer [500 mM KCl, 100 mM TRIS HCl pH 8.3], 4 µl 10 mM DNTPs, 3 µl 1 M $MgCl_2$, 5 µl of each primer (5 µM), 1 µl of DNA template, 2 µl DMSO, 1 µl Taq DNA polymerase, and cycled 30 times according to the following parameters: denaturation at 94 °C for one minute, annealing at 55 °C for one minute, and extension at 72 °C for two minutes. Initial denaturation was 94 °C for two minutes, and the final extension was at 72 °C for seven minutes. Crude PCR product was purified using the QIAquick DNA purification kit, then quantified using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA).

A BigDye™ 3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions for all forward and reverse

sequencing reactions; 10 µl reaction mixtures were prepared using 0.5 µl of primer (5 µM), 1.0 µl BigDye™ 3.1, 3.0 µl of sequencing buffer [200 mM TRIS HCl pH 9.0, 5 mM $MgCl_2$], amplified DNA (55 ng was targeted, however masses varied), and dH_2O (enough to bring the total volume to 10 µl). These mixtures were cycled 25 times according to the following parameters: denaturation at 94 °C for 20 seconds, annealing at 50 °C for two minutes, and extension at 60 °C for one minute. Sequencing reactions were primed using the primers BMB-CR (Lane *et al.*, 1985), ITS1, ITS2, ITS4, and, when needed, ITS3 (White *et al.*, 1990). While these reactions were cycling, Sephadex columns were prepared by centrifuging (2250 g, or 4600 rpm, r_{av} 9.5 cm) first 600 µl of 60 g l^{-1} Sephadex (G-50) then 150 µl dH_2O in UV-sterilized spin columns for one minute each at room temperature. Finished sequencing products were mixed with 10 µl dH_2O, and passed through a fresh Sephadex column. Cleaned sequencing products were dried at 40 °C in a centrifugal vacuum dryer, resuspended in 1.5 µl of loading dye/formamide mix, denatured at 70 °C for five minutes, snap cooled on ice, then 0.75 µl was loaded onto a tine of a 64+4 paper comb. Loaded combs were then run on an ABI 377 automated sequencer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Consensus sequences, assembled and edited using Sequencher™ for Windows 4.0.2 (Gene Codes Corp. Ann Arbor, MI, USA), were aligned manually using Se-Al v1.0a1 Fat (Rambaut, 1995). Phylogenetic analysis was done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b8 (Swofford, 2001); robustness of the resultant phylogenetic tree was tested by bootstrapping using 1000

resamplings (Felsenstein, 1985). Taxa included in the phylogenetic analysis were chosen based on affinity as shown by BLAST searches or by morphological similarity. *Phialocephala fortinii* (GenBank AY880935) was chosen as the outgroup taxon.

Taxonomy

Mollisia rhizophila Skinner & Currah, sp. nov. (Figs 1-23)

Etym.: Root lover.

Anamorph: Leptodontidium orchidicola Sigler & Currah

Apothecia pallide-grisea, leviter cupulata, 300-700 μm, margo regularis, in maturitate crenulata. Excipulum nigrescens, cum textura angulari per superficies externas ad texturam prismaticam fastigata, cellulae 6-12 X 9-15 μm. Margo excipularis vallum laxum ex cellulis cylindricis ad clavatis. Asci inoperculati, octospori, pedo subtensi, cylindrici (30-40 X 3.5-5 μm) ad piriformes (25-30 X 8 μm). Ascosporae ellipsoideae, interdum leviter asymmetricae, 6.5-9 X 1.5-2.5 μm. Paraphyses cylindricae ad clavatae, saepe septatae et ramosae. Apothecia exsiccata fusce-grisea.

Anamorphosis: *Leptodontidium orchidicola* Sigler & Currah.

Typus: **Canada**: *Province of Alberta*: Lac Ste. Anne County: Lily Lake Natural Area, *ca* UTM 11N 655620E 5954235N, in nodules of *Alnus incana* subsp. *tenuifolia*, 20 July 2004, *S. Skinner* 3-ALNn-2-2 (UAMH 10779 – ex Holotypus)

Paratypus: **Canada**: *Province of Alberta*: Lac Ste. Anne County: Lily Lake Natural Area, *ca* UTM 11N 655620E 5954235N, in roots of *Alnus incana* subsp. *tenuifolia*, 20 July 2004, *S. Skinner* 3-ALNr-2-4 (UAMH 10780 – ex Paratypus)

Paratypus: **Canada**: *Province of Alberta*: Lac Ste. Anne County: Lily Lake Natural Area, *ca* UTM 11N 655620E 5954235N, in roots of *Betula papyrifera*, 20 July 2004, *S. Skinner* 3-BET-2-1 (UAMH 10781 – ex Paratypus)

Teleomorph

Apothecia slightly concave, 300 to 700 μm across, stipitate, margin regular (Fig. 1), becoming crispate to irregular with increasing age and size (Fig. 2). Hymenium pale gray or blue gray, excipulum and stipe darkly dematiaceous and hirsute (Fig. 3). Occasionally forming in clusters (Fig. 4). Stipe 40-150 μm long and 100-135 μm thick. Setose hyphae 2-3 μm broad, ~200 μm long hyaline at 16 x, but clearly melanized when viewed at 400 x. Excipulum melanized, *textura angularis* (Fig. 5) grading outwards to *textura prismatica*, cells 6-12 X 9-15 μm. Excipular margin of cylindrical to clavate cells with free tips (Figs 6, 7) that give a distinctly asperulate appearance. Asci inoperculate, 8-spored (Fig. 8), subtended by a crozier (Fig. 9), irregularly biseriate, cylindrical (30-40 X 3.5-5 μm) to bowling-pin shaped (25-30 X 8 μm) (Fig. 10), with an apical plug blueing in Melzer's reagent (Fig. 11). Dehisced asci with sub-apical slits (Figs 12, 13). Ascospores ellipsoidal, sometimes slightly asymmetric, 6.5-9 X 1.5-2.5 μm, non-septate (Fig. 14). Paraphyses

cylindrical to clavate, often septate and branched at the base, 25 X 1.8 μm (Fig. 15). Dried apothecia slate gray.

Apothecial primordia black, spherical, 75-200 μm in diameter, sparsely setose (Fig. 16). Hymenium first exposed through the formation of a small apical pore that broadens as the apothecium becomes cupulate. Straight, setose hyphae, melanized and 2-3 μm in diameter, radiate from the stipe and basal exipulum (Fig. 17) but become obscured as the apothecium expands and flattens (Fig. 2). Ascospores are ejected while the apothecia are slightly concave. With increasing age, apothecia become convex, dark brown, and finally hygrophanous as they degenerate (Fig. 18). Apothecia on agar media and wood chips identical.

Anamorphic/microscopic features

Hyphae septate, smooth or occasionally slightly asperulate, olive brown. Aerial hyphae, 1.5-2.5 μm, occasionally forming loops. Fertile hyphae infrequent, most common along the exposed surfaces of the coverslip in slide cultures or the margin of the Petri dish, scarcely differentiated from submerged vegetative hyphae, 1.5-2.5 μm wide, bearing solitary, or oppositely paired, sessile lateral conidia, or terminal conidia in groups of 2-4 forming sympodially from swollen hyphal tips (Figs19, 20). Conidia globose, ellipsoidal, ovate, obovate, or pyriform, 1.5-2.5 X 2-7.5 μm. Detached conidia not observed.

Cultural features

On PDA after 10 days, mycelium gray brown or olive-brown, black or dark olive-brown below, felted, slightly sulcate near inoculation point, margin broad, white, submerged, after 20 days 56-58 mm in diam (Fig. 21) (~0.059 mm $hour^{-1}$). On CMA, mycelium somewhat radially striate, translucent, olive or grayish brown with sparse tufts of white aerial mycelium, ~75 mm after 20 days (Fig. 21) (~0.078 mm $hour^{-1}$).

Results

Morphology

Apothecia in UAMH 10779 forming on CMA, PDA or TWA with OA blocks within one month of inoculation, while taking ~3 months to form alone or in pairs on wood chips or in small clusters on MEA. The remaining isolates (UAMH 10780, UAMH 10781) took >6 months to form a few apothecia on CMA. No apothecia formed on cultures irradiated with UV. Black spherical bodies of melanized pseudoparenchymatous tissue, 30-150 μm in diameter, were numerous and submerged in CMA, PDA, or TWA with OA blocks (Fig. 22). Similar structures, 20 to 40 μm in diam, were often found in, or associated with, degenerating apothecia (Fig. 23).

Phylogenetic analyses

Alignment of the target region yielded a total length of 1237 bases, of which 1044 were constant, 82 variable but parsimony-uninformative, and 111 parsimony-

informative. A heuristic random sequence stepwise addition search treating gaps as missing characters yielded 14 most parsimonious trees, one of which is shown in Fig. 24. Table 1 describes the sequences used in this tree. The tree length was 301 steps with a consistency index of 0.777, a retention index of 0.806 and a homoplasy index of 0.223. This phylogenetic tree shows the three isolates identified as *M. rhizophila* clustering together with a bootstrap value of 87%. Base-pair differences among these isolates are as follows: UAMH 10780 and UAMH 10779 differed at 10 of 609 positions (98.36% identical), UAMH 10780 and UAMH 10781 differed at 1 of 568 positions (99.82% identical), and UAMH 10779 and UAMH 10781 differed at 1 of 593 positions (99.83% identical). This clade was clustered with *Leptodontidium orchidicola* Sigler & Currah (both sequences taken from GenBank and sequences obtained from solely anamorphic isolates of *L. orchidicola* collected at other nearby sites) with a bootstrap value of 82%. Average base-pair similarity among members within each clade only was 99.15%, while the average base-pair similarity between the clades was 98.59%. The average base-pair similarity between this *L. orchidicola/ M. rhizophila* clade and *M. dextrinospora* Korf & Greenleaf (the closest representative of *Mollisia* in Figure 24) was 93.41%.

Discussion

The sequential development of the apothecium, from a small, spherical, pseudoparenchymatous, melanized primordium with a collar of fine radiating hyphae, to the appearance and exposure of the concave hymenium, and finally to senescent stages in which the apothecium becomes convex with an irregular

margin, closely matches that described by Le Gal & Mangenot (1958) for *Mollisia cinerea* (Batsch) P. Karst., the type species of the genus. However, *M. cinerea* has longer asci, and an anamorph characterized by dense clusters of branched phialidic conidiophores (Le Gal & Mangenot, 1956), possibly assignable to *Cystodendron* or *Phialocephala*. *Mollisia cinerea f. minutella* Sacc. (Breitenbach & Kränzlin, 1981) is also similar but has longer asci and lacks setose hairs. Since all observations of apothecial characters and development in *M. rhizophila* were consistent on all agar media and on wood chips, it seems likely that apothecia found in natural conditions would be similar. Type of medium seems only to affect the clustering of the apothecia. The phylogenetic analysis using ITS sequences also indicated that *M. rhizophila* is not conspecific with these two taxa.

The blueing of the ascus apex in Melzer's reagent, a feature associated with numerous inoperculate discomycetes including some *Helotiales*, is typical of *Mollisia* (Korf, 1973). However, the sub-apical slits of dehisced asci of *M. rhizophila* resemble those of some members of the operculate order *Pezizales* (Korf, 1973), more specifically those with *Octospora* or *Thelebolus* type asci (Brummelen, 1981), and do not resemble the everted apical ring typical of many inoperculate discomycetes (Beckett, 1981). The presence of very similar sub-apical slits in the *Rhytismataceae*, a family with morphological characters more typical of inoperculate discomycetes, was deemed sufficient to erect the order *Rhytismatales* within the *Leotiomycetes* (Minter & Cannon, 1984). Though there are no descriptions of the ultrastructure or dehiscence of ascus apical apparati in the

Dermateaceae, other work within the *Helotiales* shows dehiscence results in an eversion of the apical apparatus, with no indication of a slit or operculum (Verkley, 1993, 1994). Researchers (Minter & Cannon, 1984; Nannfeldt, 1976) have hypothesized that simplified modes of dehiscence, including apical slits, coincide with reduced non-amyloid apical apparati. However, the possibility of apical slits in *M. rhizophila* in conjunction with its complex amyloid apical apparatus may illustrate an exception to this hypothesis. More ultrastructural work is needed to determine the nature of the dehiscence mechanism of *M. rhizophila* and related taxa.

This is the first report of a *Leptodontidium* state in a species of *Mollisia*, a genus that otherwise includes species that produce either conidia from phialides with collarettes including the genera *Cadophora* (formerly *Phialophora*; Le Gal & Mangenot, 1956, 1958, 1961; see Harrington & McNew, 2003), *Cystodendron* (Aebi, 1972), and *Chalara* (Arendholz & Sharma, 1984), or large disarticulating Ingoldian conidia including *Coniothecium* (Le Gal & Mangenot, 1956), *Anguillospora* (Webster, 1961), and *Helicodendron* (Fisher & Webster, 1983). The anamorphic form of *M. rhizophila* matches the original and subsequent descriptions of *L. orchidicola* (Currah *et al.*, 1987; Fernando & Currah, 1995) in several aspects; their conidial morphology, conidiogenesis, and rarity of detached conidia are nearly identical. Cultural morphology and colour of *M. rhizophila* also match those of the original description of *L. orchidicola* (Currah *et al.*, 1987) on both PDA and CMA. My isolates of *M. rhizophila* differ from the type of *L. orchidicola* in three ways.

The average growth rate of *M. rhizophila* on PDA at room temperature is slower (0.059 mm hour^{-1} versus 0.078 mm hour^{-1}, respectively). This difference can be attributed to strain variation or subtle differences in cultural conditions and is unlikely to be taxonomically significant. Isolates of *M. rhizophila* form melanized spherical bodies in agar media while these are absent from the description of the type of *L. orchidicola*. These bodies appear to be apothecial initials, and thus may not be expected in sexually infertile isolates of *L. orchidicola*. These putative initials are found throughout the agar medium and only those arising near the surface develop further. The final difference is that the type of *L. orchidicola* is described as having a "dense layer of sclerotic tissue composed of chains of short, swollen, globose to teardrop to dumbbell-shaped cells" (Currah *et al.*, 1987); such a layer was not observed in my fertile isolates. It is possible that this sclerotic layer is homologous to the putative apothecial initials of fertile isolates of *L. orchidicola*.

The putative ecology of the genus *Mollisia* and that of *L. orchidicola* are at odds: the teleomorph is often described as a saprobe (Gremmen, 1954; Korf, 1973; Dix & Webster, 1995), while the anamorph has been shown to grow as an endophyte in the roots of a range of plants (Fernando & Currah, 1995). It is possible that this species (in a holomorphic sense) is a root-pruning fungus with an "endophytic phase" and a "saprobic phase" as hypothesized for the "branch-pruning fungi" studied by Kowalski & Kehr (1992). They hypothesized that the "endophytic phase" of such fungi mediates the senescence of redundant branches; their host thus benefits from improved pruning. Once the branch has been pruned,

these "branch-pruning fungi" are free to begin their "saprobic phase" characterized by substrate decomposition and, often, their teleomorph. Kowalski & Kehr (1992) identified *M. cinerea* (among others) as being such a "branch-pruning" fungus, and noted that *M. cinerea*, like *M. rhizophila*, was not strictly host specific. Dix & Webster (1995) also discussed a similar life-history of *M. acerina* in leaves. The hypothesis that *M. rhizophila* may be a root-pruning fungus is also supported by the production of polyphenolic oxidases, typical of many saprobic fungi, by *L. orchidicola*, an endophytic species that shows no signs of pathogenicity on host plants in resynthesis experiments (Fernando & Currah, 1995). This hypothesis also predicts fruiting soon after senescence of host structures as was observed in an unidentified *Mollisia* by Gremmen (1954) who noted that "...these fungi in nature thrive on dead stems and leaves of last year..." More specifically, the *in situ* fruiting of *M. rhizophila* likely occurs on the surface of recently dead substrates beneath the soil because substrates *in vitro* exposed to UV light did not yield apothecia, while non-irradiated substrates did. Despite evidence supporting this root-pruning hypothesis, we are not yet able to determine the benefits of root-pruning to the host in relation to other possible benefits of root endophytes, including the deterance of herbivory and pathogens, and the improvement nutrient acquisition and environmental tolerance (Mandyam & Jumpponen, 2005).

The phylogenetic analysis showed two subclades within the *L. orchidicola/M. rhizophila* clade. This dichotomy, supported by a moderate bootstrap value (87%), clearly divides isolates that produced the teleomorphic state

from those that did not. However, it is reasonable to view these subclades as intraspecific, because there are no discernable differences between the anamorphs of these two subclades, and because the average base-pair similarity between these subclades (98.59%) was only slightly less than that within each subclade (99.15%) and much higher than that between this entire clade and the closest related *Mollisia* species (*Mollisia dextrinospora*) found in GenBank (93.41%).

Using a combination of morphological characters of the *Mollisia* teleomorph and the *Leptodontidium* anamorph, and using molecular evidence, I have described the new species *M. rhizophila*, as well as a new anamorph connection. Anamorphic characters, often overlooked in many studies of ascomycetes, were important in describing this new species. As Hennebert & Bellemére (1979) argued, taxonomy would be much more informative if mycologists took as much care in studying and describing anamorphic forms they do with teleomorphic forms.

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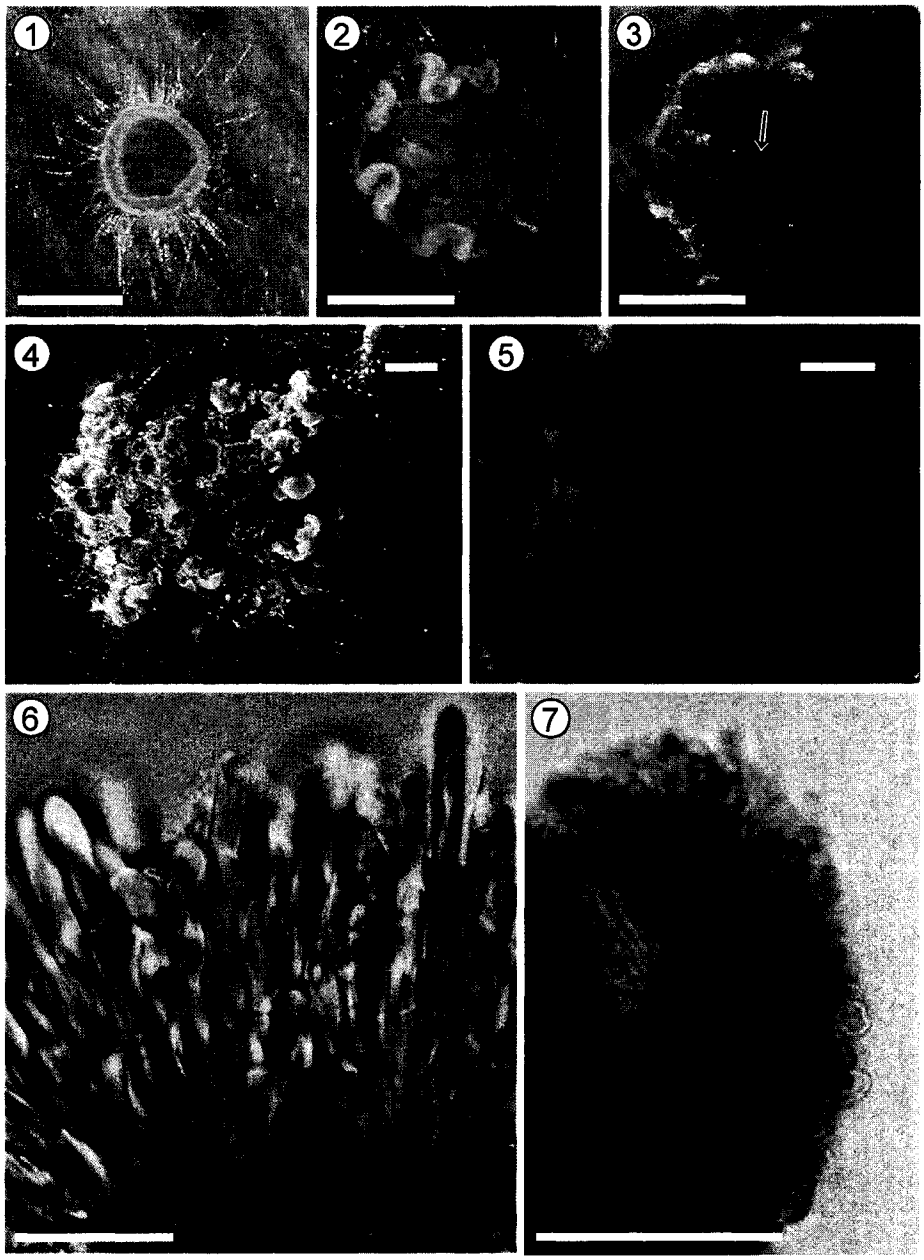
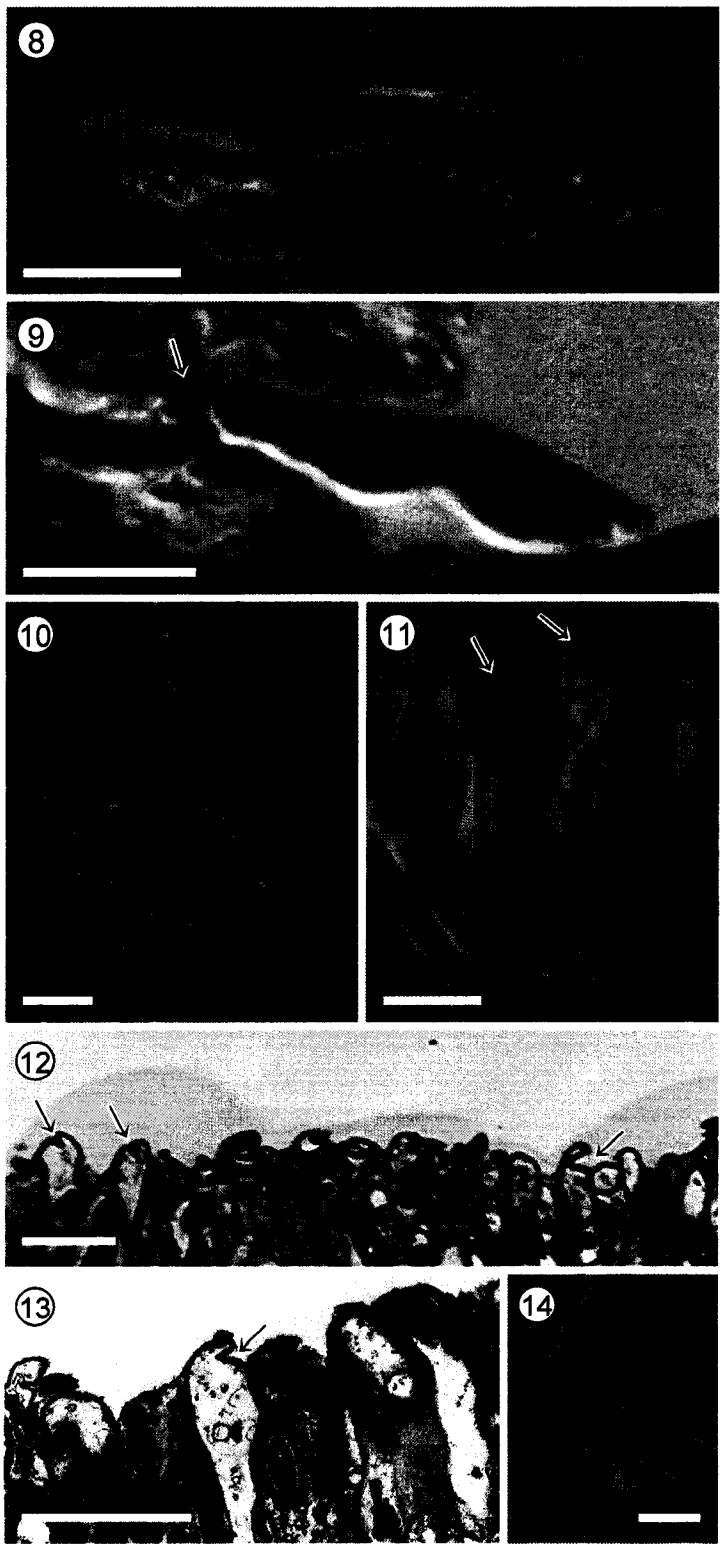
Table 2-1. Description of sequences used in Fig. 24. State codes (State) describe the state for the species: A = anamorph, T = teleomorph.

Species Name	State	NCBI Accession Number	Collection Number	Notes
Phialocephala fortinii	A	AY880935		Outgroup
Lachnum pteridophyllum	T	U58635		
Leptodontidium elatius	A	AF475152		
L. elatius var. elatius	A	AY129285		Type species of *Leptodontidium*
Leptodontidium boreale	A	AY129284		
Mollisia dextrinospora	T	AY259134		
Cadophora gregata	A	U66729		
Cadophora malorum	A	AF083202		
Leptodontidium orchidicola	A	AF486133		Collected in Alberta
Leptodontidium orchidicola	A	AF214576		Authentic, collected in Alberta
Leptodontidium orchidicola	A		1-ALNn-3-6	From alder nodules
Leptodontidium orchidicola	A		5-ALNr-4-4	From alder roots
Leptodontidium orchidicola	A	AF214578		Authentic, collected in Alberta
Mollisia rhizophila	T		3-ALNn-2-2	Ex type, from alder nodules, UAMH 10779
Mollisia rhizophila	T		3-BET-3-1	From birch roots
Mollisia rhizophila	T		3-ALNr-2-4	Ex paratype, from alder roots, UAMH 10780
Mollisia cinerea f. minutella	T	AJ430223		
Mollisia cinerea	T	AJ430222		Type species of *Mollisia*
Mollisia melaleuca	T	AY259136		
Mollisia cinerea	T	AY259135		Type species of *Mollisia*
Mollisia fusca	T	AY259138		

Figures 2-1 to 2-7. Apothecia and excipular details of *Mollisia rhizophila* (ex Type UAMH 10779). **1**. Mature apothecium on CMA. Incident light microscopy. **2**. Older mature apothecium with crispate margin on CMA. Incident light microscopy. **3**. Undersurface of crispate apothecium revealing darkly dematiaceous excipulum, hirsute minute stipe (arrow). On aspen wood, incident light microscopy. **4**. Cluster of apothecia on CMA 5.5 months after inoculation. Incident light microscopy. **5**. Melanized central excipulum featuring *textura angularis*. Unstained squash mount in PVA. **6**. Excipular margin of cylindrical to clavate cells with free tips. Unstained quash mount in PVA. **7**. Excipular margin of cylindrical to clavate cells with free tips. Unstained hand-section in PVA.
Scale bars in 1, 2, 3, 4 = 400 μm; in 5, 6, 7 = 20 μm.



Figures 2-8 to 2-14. Asci and ascospores of *Mollisia rhizophila* (ex Type UAMH 10779). **8.** Ascus containing 8 irregularly arranged ascospores. Unstained squash mount in glycerine gelly, NIM. **9.** Ascus subtended by a crozier (arrow). Squash mount in Melzer's reagent, NIM. **10.** Bowling-pin shaped asci. Squash mount in Melzer's reagent, NIM. **11.** Asci with apical plug blueing in Melzer's reagent (arrows). **12.** Dehisced asci with sub-apical slits (arrows). Embedded in araldite, sectioned to about 1 μm, bright field microscopy. **13.** Dehisced ascus with sub-apical slit (arrow). TEM. **14.** Ascospores. Melzer's reagent, bright field microscopy. Scale bars = 10 μm.



Figures 2-15 to 2-20. Paraphyses, some developmental stages and anamorph of *Mollisia rhizophila* (ex Type UAMH 10779, except 17). **15**. Branched and septate paraphyses. Squash mount stained with acid fuchsin, in PVA. **16**. Apothecial primordium on aspen wood. Incident light microscopy. **17**. Young, cupulate apothecium with radiating setose hyaphae. Incident light microscopy, ex Paratype UAMH 10781. **18**. Over-mature, degenerated, convex apothecium. Side view on CMA, incident light microscopy. **19**. Sessile lateral conidia. Unmounted slide cultured material. **20**. Conidia born laterally and sympodially on slightly swollen hyphal tips. Unmounted slide cultured material.
Scale bars in 15, 19, 20 = 10 μm; 16, 17, 18 = 300 μm.

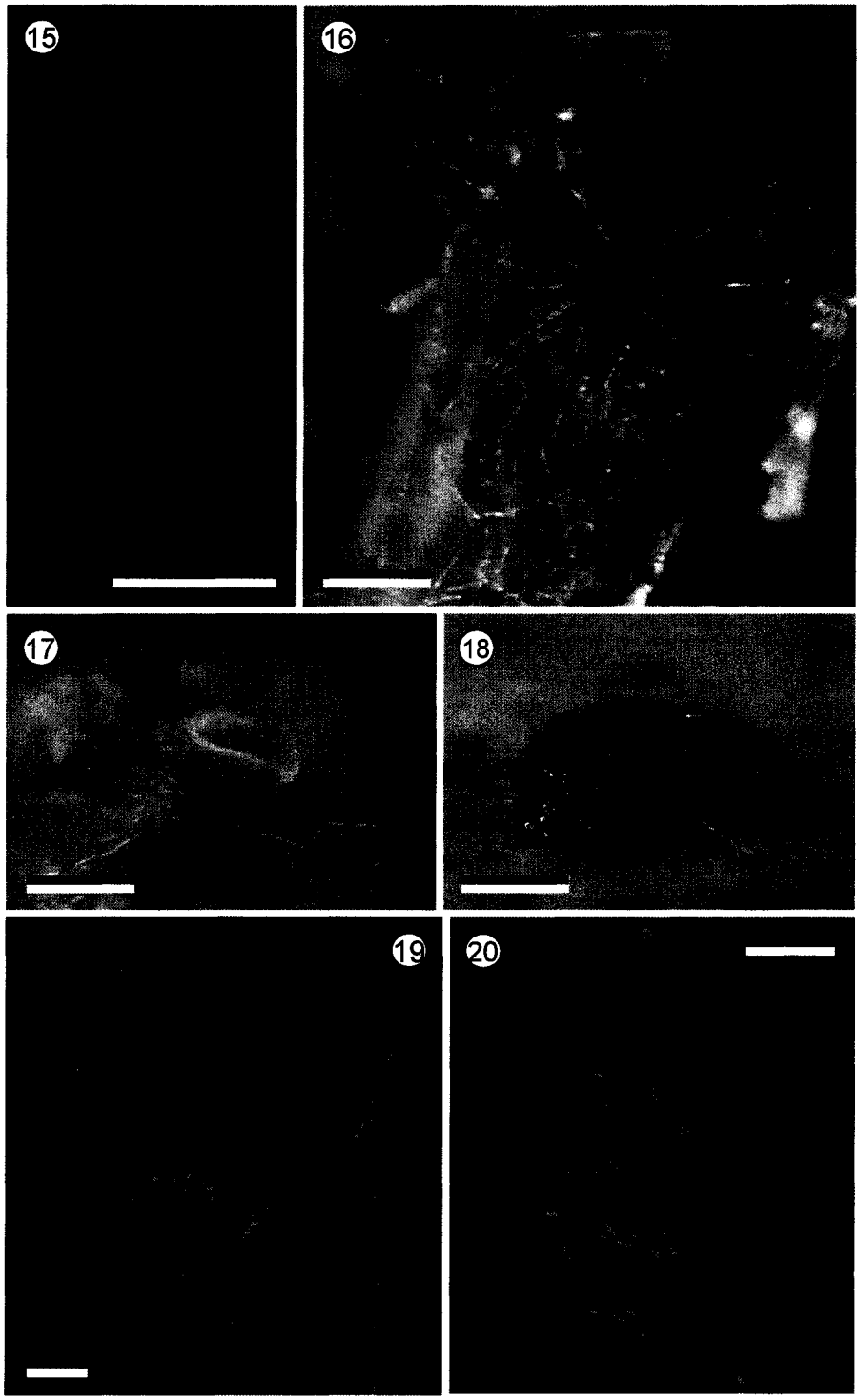
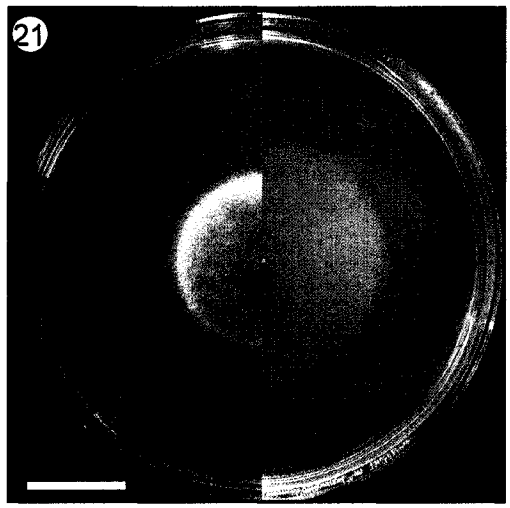
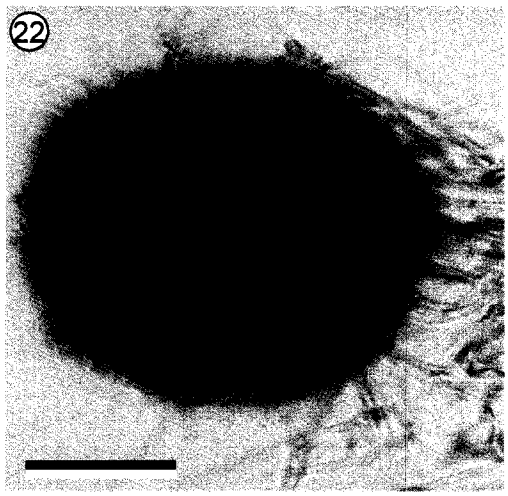


Figure 2-21 to 2-23. Cultural morphology and melanized spherical bodies that are likely apothecial primordia (ex Type UAMH 10779). **21**. Plan view of culture. Left: on PDA. Right: on CMA. **22**. Melanized spherical bodies submerged in an agar medium. Unstained squash mount. **23**. Melanized spherical bodies within degrading apothecium (arrows). Acid fuchsin stained hand-section in PVA.
Scale bars in 21 = 2 cm; 22, 23 = 30 μm.





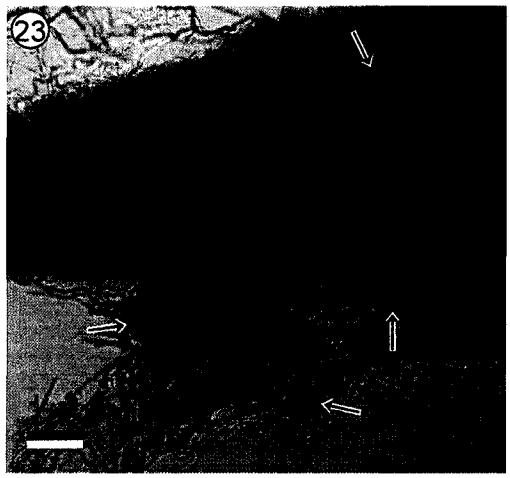
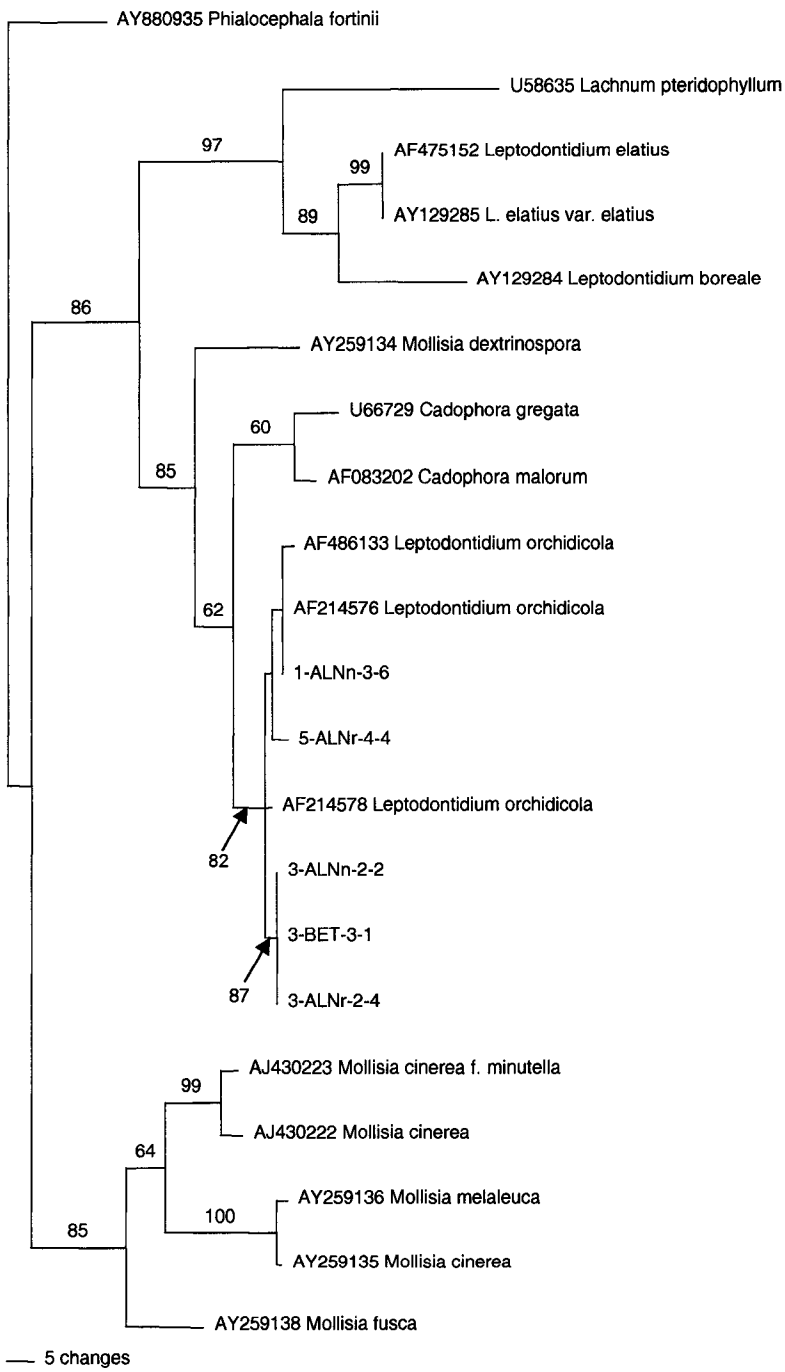


Figure 2-24 Phylogenetic placement of *Mollisia rhizophila* isolates relative to those taxa with morphological or molecular (as shown by BLAST searches) affinities. Phylogenetic tree resulted from maximum parsimony analyses using the heuristic search algorithm in PAUP (random addition) and bootstrapping (1000 bootstrap replicates) of the partial LSU-ITS-5.8s-ITS2-partial SSU rDNA sequences. Bootstrap values >50 are shown. *Phialocephala fortinii* (GenBank AY880935) was chosen as the outgroup taxon. Accession numbers adjacent to taxa refer to sequences retrieved from GenBank.



Chapter 3 - Taxonomic and functional comparisons of communities of microfungi associated with the roots of birch and alder

Introduction

Classifying the constituents of a plant community functionally allows ecologists to preserve ecologically relevant information while greatly simplifying its description (Noble & Gitay, 1996). How individual plant species are functionally classified depends on the objectives of the study (Gitay *et al.*, 1999). Classification can be based on physical characters, resource use, response to perturbations (Gitay *et al.*, 1999), primary productivity, carbon and nitrogen retention and decomposition (Wright *et al.*, 2006). Furthermore, this classification can be subjective, where classes are chosen subjectively then defined using induction, or deductive, where an *a priori* statement about the ecological process of interest allows categories to be chosen deductively, or data-driven, where multivariate analyses show clusters of species (Gitay *et al.*, 1999). One of the more used functional classification of plants is the subjective grass/forb/legume (GFL) scheme (Wright *et al.*, 2006); however, in the last decade, the use of alternatives has been explored (Noble & Gitay, 1996; Wright *et al.*, 2006). Functional approaches can be useful in land-use management (Tilman *et al.*, 1997), in the prediction of community response to disturbance or environmental change (Hooper *et al.*, 2002), and in ecological modeling at broad scales where direct taxonomic comparisons are difficult (Gitay *et al.*, 1999).

For fungal communities, functional diversity includes their enzymatic versatility in degrading macromolecules, e.g., lignin, cellulose, peptides, etc. (Zak & Visser, 1996), their range of secondary metabolites (Zak & Visser, 1996) and structural components such as chitins and ergosterols, and their tolerance to environmental variables e.g., temperature, salinity, pH, etc. Studies of fungal biodiversity usually omit functional attributes because most species are poorly understood in this respect. In addition, assessing the functional diversity of fungal communities is difficult or impossible *in situ* because of the opaque and chemically complex nature of natural substrates. In some instances, fungi have been isolated from a targeted habitat and crude degradative abilities have been assessed on a species-by-species basis (Schulz & Thormann, 2005). A second proxy for estimating the functional diversity of a fungal community can be obtained by observing more comprehensive and detailed substrate utilization profiles (SUPs) of taxonomically undefined assemblages, rather than individual identified or characterized isolates. While this physiological approach is still biased towards culturable species and may mask actual diversity when groups of species give similar test results (Konopka *et al.*, 1998), other research (Sobek & Zak, 2003; Zak & Visser, 1996) has shown that these bulk assessments of fungal function in soil can be related to plant community composition and human impacts. Other studies have detected differences among microbial communities on roots of different plants (Garland, 1996; Grayston & Campbell, 1996). The relationship of such bulk enzymatic assessments to fungal taxonomic diversity has not yet been tested.

Despite several studies of the links between plant communities and their diversity with fungal communities and their diversity (Nantel & Neumann, 1992; Zak *et al.*, 2003; Unterseher & Tal, 2006), there have been few attempts to link plant function to fungal community ecology. The objective of this study was to determine the relative influences of host species function, type of host organ, and site, on the fungal communities associated with the roots of two similar, yet functionally different, focal plant species. Here, I compared, using both taxon-based and functional approaches, fungal communities associated with the fine roots and nodules of the plant species *Alnus incana* subsp. *tenuifolia* and *Betula papyrifera*. These species are both single- or multi-stemmed shrubs or trees in the Betulaceae and co-occur in wet soils. They have one obvious functional difference: *A. incana* subsp. *tenuifolia* fixes nitrogen with root nodules bearing the nitrogen-fixing actinomycete *Frankia*, while *B. papyrifera* is nodule-free. This study also aimed to evaluate both approaches by comparing their results.

Materials and methods

Sample collection

During summer of 2004 (late June to late August), four sites where both focal plant species (*Alnus incana* (L.) Moench ssp. *tenuifolia* (Nutt.) Breitung and *Betula papyrifera* Marsh., hereafter referred to as alder and birch) co-occurred were sampled within 150 km of Edmonton, Alberta (Table 1). Site one was resampled the following summer to repeat faulty Biolog® assays of 2004. All sites were in the

local aspen parkland ecosystem and appeared to have mesic-to-wet soils; three were adjacent to wetlands or water bodies. At each site, a 50 m transect was laid through the middle of a stand containing both focal plant species. Three plots at the 10, 30 and 50 m positions of the transect were established at each site. At each plot, the nearest birch and alder plant to the plot centre was located; a roughly 30 X 30 X 15 cm sample of root-containing topsoil adjacent to, or directly under (depending on the size of the specimen), each plant was excised. In this way, six samples were collected from all four sites; one additional birch and three additional alder samples were collected at site four. All samples were refrigerated within 8 hours. Fifteen alder and thirteen birches were sampled in all four sites combined.

Sample preparation

The "Soil FungiLog" procedure, developed by Sobek & Zak (2003) to describe catabolic profiles of soil fungal communities, was adapted to profile root- and nodule- associated fungi. After washing coarse debris from root balls using tap water, most of the fine roots, <1 mm diameter, and all alder nodules were excised. These excisions yielded 43 organ samples, counting the birch and alder roots and the alder nodules from each sample, over the course of the sampling season. These samples, considered as sample units in subsequent analyses, were agitated twice in sterile distilled water (sdH_2O) in a 100 X 80 mm deep culture dish (with lid) using a magnetic stirrer, soaked in ~3% hydrogen peroxide for one minute, and rinsed with sdH_2O. They were then placed in an 18 mm diameter test tube containing 10 ml of sodium/potassium phosphate buffer (pH 7.4) (Canadian Laboratory Supplies,

Vancouver, BC, Canada) and ground for thirty seconds with a 17 mm homogenizer. Using sdH_2O, the resulting slurries were rinsed through two sieves (500 μm & 250 μm) to recover particles in this size range. These were transferred to screw-capped vial containing 15 ml of 0.2% water agar (amended with 100 mg l^{-1} of streptomycin sulfate (Sigma-Aldrich, Canada) and 50 mg l^{-1} of oxytetracycline HCl (Sigma-Aldrich)) to make a suspension with ~70% transparency which yielded approximately 75 particles per well, a ratio considered optimum for balancing low light distortion with high fungal activity and richness (Sobek & Zak 2003). Each suspension was used to inoculate one 32-well section of a Biolog® EcoPlate® using 100 μl of suspension per well. The optical density at 490, 590 and 750 nm for each well was recorded immediately after inoculation and periodically thereafter for up to 20 days using a Biolog MicroStation™ microtiter plate reader. A 100 μl aliquot of each suspension was also streaked across each of two 100 mm diameter Petri plates containing modified BAF medium (Hutchison, 1991) one of which was amended with 2 mg l^{-1} of Later's Benomyl 50 (Richmond, BC, Canada). Modifications of BAF included using oxytetracycline HCl (2 mg l^{-1}) and $MnCl_2$ (6 g l^{-1}) instead of chlortetracycline HCl (2 mg l^{-1}) and $MnSO_4$ (5 g l^{-1}). These plates were incubated at room temperature (RT ~23 °C) in the dark. Colonies were transferred to corn meal agar (CMA) (Acumedia, Baltimore, MD, USA) amended with 100 mg l^{-1} oxytetracycline HCl, and incubated at RT in ambient light (diffuse natural light combined with light from standard fluorescent lab lighting), and were stored for up to a year at 4 °C in the dark prior to complete identification.

Fungal identification

Fungi were identified primarily on the basis of microscopic characters and secondarily using DNA sequence data when needed. Nomenclature follows the Index Fungorum (www.indexfungorum.org). The methods of Gibas et al. (2002) were used for DNA extraction, amplification, sequencing, and analysis, and are outlined below with modifications described. Isolates were grown on MEA (15.0 g Difco Bacto malt extract (Difco, Sparks, MD, USA), 17.0 g Difco Bacto agar, 1 l dH_2O) or PDA (Difco) overlaid with a Cellophane^{TM} membrane (UCB Films, Bridgwater, Somerset, UK). Mycelium scraped free of the membrane was placed in a precooled sterile mortar with sterilized sand and liquid nitrogen, and ground to a powder. One milliliter of extraction buffer [20 g l^{-1} cetyl-trimethyl ammonium bromide (CTAB); 1.5 M NaCl; 100 mM TRIS HCl; 20 mM EDTA] was mixed with the pulverized mycelium; this slurry was then incubated in a 2 ml screw-capped microcentrifuge tube with 2 µl of β-mercaptoethanol for 30 to 120 minutes at 65 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and mixed by inverting 40 times. After centrifugation for 20 minutes at >10,000 g (10 000 rpm, r_{av} 9.5 cm), the resultant aqueous phase (crude DNA solution) was removed and purified using the QIAquick DNA purification kit (QIAgen Inc., Mississauga, ON, Canada). The purified DNA was stored at -20 °C.

The targeted regions, ITS1, 5.8s, ITS2, and flanking portions of SSU and LSU, were amplified from the purified DNA by a polymerase chain reaction (PCR) using the primers BMB-CR (Lane *et al.*, 1985) and ITS4 (White *et al.*, 1990)

(primers manufactured at the Molecular Biology Service Unit, University of Alberta). Reaction mixtures contained 26 µl dH_2O, 5 µl 10 X buffer [500 mM KCl; 100 mM TRIS HCl, pH 8.3], 4 µl 10 mM DNTPs, 3 µl 1 M $MgCl_2$, 5 µl of each primer (5 µM), 1 µl of DNA template, 2 µl DMSO, 1 µl Taq DNA polymerase, and cycled 30 times according to the following parameters: denaturation at 94 °C for one minute, annealing at 55 °C for one minute, and extension at 72 °C for two minutes. Initial denaturation was 94 °C for two minutes, and the final extension was at 72 °C for seven minutes. Crude PCR product was purified using the QIAquick DNA purification kit, then quantified using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

A BigDye™ 3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions for all forward and reverse sequencing reactions; 10 µl reaction mixtures were prepared using 0.5 µl of primer (5 µM), 1.0 µl BigDye™ 3.1, 3.0 µl of sequencing buffer [200 mM TRIS HCl, pH 9.0; 5 mM $MgCl_2$], approximately 55 ng amplified DNA, and dH_2O (enough to bring the total volume to 10 µl). These mixtures were cycled 25 times according to the following parameters: denaturation at 94 °C for 20 seconds, annealing at 50 °C for two minutes, and extension at 60 °C for one minute. Sequencing reactions were primed using the primers BMB-CR (Lane *et al.*, 1985), ITS1, ITS2, ITS4, and, when needed, ITS3 (White *et al.*, 1990). While these reactions were cycling, Sephadex columns were prepared by centrifuging (at 2250 g, or 4600 rpm, r_{av} 9.5 cm, for 1 minute) first 600 µl of 60 g l^{-1} Sephadex (G-50), then 150 µl dH_2O in UV

sterilized spin columns. Sequencing products were mixed with 10 µl dH_2O, and centrifuged (at 2250 g, or 4600 rpm, r_{av} 9.5 cm, for 1 minute) through a fresh Sephadex column. Cleaned sequencing products were dried at 40 °C in a centrifugal vacuum dryer, resuspended in 1.5 µl of loading dye/formamide mix, denatured at 70 °C for five minutes, snap cooled on ice; 0.75 µl was then loaded onto a tine of a 64+4 paper comb. Loaded combs were then run on an ABI 377 automated sequencer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Consensus sequences, assembled and edited using Sequencher™ for Windows 4.0.2 (Gene Codes Corp. Ann Arbor, MI, USA), were aligned manually using Se-Al v1.0a1 Fat (Rambaut, 1995). Resultant sequences were compared with those in GenBank using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN) algorithm. Generic names were applied to isolates when sequences matched a named sequence in GenBank at <98% base-pair similarity, and species names were applied to isolates when sequences matched at >98% base-pair similarity, and when matches were unanimous. In cases where GenBank identifications were not unanimous, or when isolates appeared to be con-specific but did not have a match in GenBank (i.e., at >98% base-pair similarity), phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b8 (Swofford, 2001); robustness of the resultant phylogenetic trees was tested by bootstrapping (Felsenstein, 1985). Sequence accessions included in these analyses for comparison were chosen based either on their

similarity to the unidentified isolates according to the BLAST search, or on their representation of adjacent taxa.

Community analyses – taxonomic approach

Isolate identification data were collated into a table showing presence/absence of each species for each sample before subsequent analyses (Appendix 1). Using PC-Ord version 4 (McCune & Mefford, 1999), Shannon's diversity index (Shannon, 1948) was calculated for each sample and then expressed as an average for birch roots, alder roots, and alder nodules. Differences in average diversity and richness between fungal communities associated with alder roots and those of birch roots and alder nodules, were tested using two-tailed T-tests (not assuming equal variance) (Table 2). Two-tailed T-tests were used for comparisons of the differences between birch and alder roots, and the differences between alder roots and nodules rather than an ANOVA testing for differences among all three groups because these pair-wise comparisons addressed ecological questions that had been identified *a priori*. However, I did use an ANOVA to detect differences in diversity and richness among sites. Species accumulation curves (Fig. 1) and first-order jackknife estimates (Heltshe & Forrester, 1983) of total number of species (Table 2) for each site, for each of birch and alder roots and alder nodules, and all samples pooled together were generated using a Sørensen distance matrix (Sørensen, 1948) in PC-Ord.

Ordinations were used to explore the complex multidimensional patterns of fungal species composition and substrate utilization in the taxonomic and

physiological data sets, respectively. These relationships and patterns are referred to here as community structure. Before proceeding, rarely isolated species were removed from the data in order to increase the resolution of these ordinations. The threshold of rarity was determined using the method suggested by McCune & Grace (2002): species were removed in order of decreasing rarity until a peak in resolution among groups identified *a priori* was maximized using multi-response permutation procedures (MRPP, Mielke, 1979). Multi-response permutation procedures test for differences among groups, and are nonparametric (McCune & Grace, 2002). Using this procedure, I tested two potentially ecologically meaningful groupings, i.e., roots and nodules (three types), and site (four sites), and found that removing species isolated from only one or two sample units was optimal for the resolution of both. Results of these MRPP tests were tabulated in Table 3, along with results of MRPP testing for specific differences between alder roots and alder nodules, and between alder roots and birch roots.

A non-metric multi-dimensional scaling (NMDS, Shepard, 1962a, b; Kruskal, 1964a, b) ordination of samples in species space using a Sørensen distance matrix (Sørensen, 1948) was then performed on the reduced community data set using PC-Ord. This ordination does not have the underlying assumptions of multivariate normality or of linear patterns of species abundance, and when used with a Sørensen distance matrix, is less sensitive to outliers and more compatible with presence/absence data than other approaches to ordination (McCune & Mefford, 1999; McCune & Grace, 2002). This algorithm starts with a random

"solution", and attempts to refine it so that the "stress" (roughly the percent departure from a "perfect" representation of the data) of this iteration is lower. This iterative process stops when there is little variation (or instability) in stress among the most recent 20 iterations. The final NMDS ordination was restricted to a maximum of two axes (as appeared optimal on a scree plot of preliminary ordinations), selected from the best of 500 hundred runs, and was compared to 150 runs of randomized data for a Monte Carlo test of significance. The outcome of this test is the probability (*P*) that an NMDS of randomized data would yield a stronger ordination. The best ordination was then rotated using a varimax rotation (simultaneous rotation). Differences in community structure were visualized using scatterplots coded according to type of root of nodule (Fig. 2a) and site (Fig. 2b). Scatterplots were overlaid with 68% confidence ellipses for each group, as generated by the groupEllipse function (ellipse library) of the software package R (R Development Core Team, 2005).

Indicator species analysis (Dufrêne & Legendre, 1997), referred hereafter as "indicator analysis", was performed with PC-Ord, for both types of roots, for nodules, and for site, using the reduced data set. The indicator value (IV) reflects the proportional abundance of a particular species within a given group compounded with the fidelity of that species to that group. Indicator values are reported as percentages; 100 indicates an implausibly perfect IV. This value is tested using a Monte Carlo method (1000 repetitions) to find the probability (*P*) that it could be equaled or exceeded by an IV generated from randomly shuffled data.

Community analyses – physiological approach

Based on preliminary analyses (not shown) that indicated a close correspondence between absorption at 590 nm and turbidity, I based subsequent analyses solely on 590 nm absorption data. Other studies, both those using a tetrazolium dye to indicate metabolism and those that did not, concluded that a wavelength at or near 590 nm adequately reflected fungal growth (Sobek & Zak, 2003; Langvad, 1999). To focus subsequent analyses on functional differences among fungal community types, and to avoid possible confounding effects of differences in inoculum density, average well colour development (AWCD, Garland, 1997) for each observation of each sample unit was calculated by averaging the absorbance of all 32 wells. From all the observations, three compilations were assembled; each included only the observations of each plate with AWCD closest to 0.5, 0.6, 0.7 respectively (Buyer *et al.*, 2001). Though Buyer *et al.* (2001) used the arbitrary value of 0.5, preliminary analyses (not shown) suggested that an AWCD of 0.7 would improve the resolution of communities; therefore, all subsequent analyses were performed using this compilation (Appendix 2). Data were adjusted by dividing absorbencies of each well by their AWCD in order to compensate for the residual variation in AWCD among plates.

These adjusted data were subsequently analyzed in the same way as the taxonomic data; a brief overview follows. The average Shannon's diversity index for each sample was calculated, and differences in average diversity between alder roots and that of alder nodules and birch roots were again tested using a two-tailed

T-test (not assuming equal variance) (Table 2). Differences in diversity (H') among sites were tested using an ANOVA followed by a Tukey test (Tukey, 1953). Again, MRPP was used to detect differences among roots and nodules, between just alder roots and alder nodules, between just alder roots and birch roots, and among all sites (Table 3). An NMDS using a Sørensen distance matrix (Sørensen, 1948) was then performed on the complete physiological data using 200 runs of randomized data, and again visualized using scatterplots and 68% confidence ellipses coded by birch roots, alder roots, and alder nodules (Fig. 3a), or by site (Fig. 3b). Indicator analysis was performed with substrates substituted for species, for roots and nodules, and for site. Finally, the physiological and taxonomic data matrices were compared using a Mantel test (Mantel, 1967) of the two Sørensen distance matrices (Sørensen, 1948) and evaluated using Monte Carlo randomization (1000 replicates) in PC-Ord. This procedure tests the null hypothesis that there is no relationship between two matrices (McCune & Grace, 2002).

Results

Results from taxonomic data

From the 43 samples, 55 different fungi were isolated (Tables 4 & 5). The number of fungi per sample varied from zero to seven, with an average of 3.7. Sixteen species were identified solely on the basis of molecular characters, 34 were identified solely on the basis of morphological characters, and five were identified using a combination of the two approaches. Assembled sequences were typically

550 to 770 base-pairs in length. Forty species were detected in only one or two samples, nine from three or more samples but showing no apparent pattern in providence, one was found only in alder roots and nodules, one was found only in birch roots, three were found only in birch and alder roots, one was found only at site four in birch roots. No species was detected more than twice solely in nodules.

These taxonomic data showed that fungal communities from roots of both alder and birch had Shannon's diversity indices (H') and species richness that were not significantly different, while fungal communities associated with alder nodules had significantly lower H' and richness than alder roots (Table 2). Species-accumulation curves (Fig. 1a) and the number of species estimated by a first-order jackknife procedure (Table 2) showed a lower rate of species accumulation and fewer estimated species, respectively, for alder nodules than for either type of root. The taxonomic data did not show significant differences among sites for both H' and richness, despite sites one and four being more diverse and species rich, having more estimated species (Table 2), and having steeper species-accumulation curves (Fig. 1b) than sites two and three.

Ordination of the taxonomic data separated most samples, illustrated 74.9% of the variation (Fig. 2), had a final stress of 23.15 that was reached after 43 iterations ($P = 0.033$), and a final instability of 0.00426. The distribution of samples of birch roots, alder roots, and alder nodules within this ordination showed extensive overlap, however, sites two and three appeared separated from each other and both had more limited distributions within the ordination compared to those of

sites one and four. Multi-response permutation procedures showed differences in community structure among roots and nodules to be non-significant, but significant among sites (Table 3).

Indicator analysis of birch roots, alder roots, and alder nodules showed no significant indicator species at $\alpha = 0.05$, but did show that *Oidiodendron maius* and *Penicillium canescens* were significant indicator species of birch roots at $\alpha = 0.10$ ($P = 0.062$ and IV = 23.1 for both). *Lachnum pygmaeum* and *Cadophora malorum* were significant indicators of site three at $\alpha = 0.05$ ($P = 0.0070$ and IV = 37.4, $P = 0.018$ and IV = 36.1 respectively), while *Cylindrocarpon magnusianum* and *Leptodontidium orchidicola* were significant indicators of sites four and two, respectively, at $\alpha = 0.10$ ($P = 0.095$ and IV = 23.1, $P = 0.056$ and IV = 30.4 respectively). *Phialocephala fortinii* was ubiquitous; it scored the lowest indicative probability for type of root or nodule ($P = 1.000$) and near the lowest for site ($P = 0.923$).

Results from physiological data

Analysis of the physiological data showed no significant differences in diversity (H') between alder roots and birch roots nor between alder roots and alder nodules (Table 2), however, it showed a significant difference among sites (at $\alpha = 0.05$). A subsequent Tukey test showed that site three was significantly more physiologically diverse than the remaining sites.

Ordination of the physiological data displayed 65.3% of the variation (Fig. 3), had a final stress of 25.38 that was reached after 76 iterations ($P = 0.0050$), and

a final instability of 0.00044. Within this ordination, the distributions of samples of birch roots, alder roots, and alder nodules greatly overlapped and were poorly differentiated, while samples from site three were clearly more clustered and somewhat differentiated from the remaining sites. Multi-response permutation procedures showed no significant differences in community structure among roots and nodules, but significant differences among sites (Table 3). Indicator values were less variable than those from the taxonomic data, however indicator analysis of birch roots, alder roots, and alder nodules showed one significant indicator substrate, phenylethylamine, at $\alpha = 0.05$ ($P = 0.0020$ and IV = 48). The same analysis of the sites showed several significant indicator substrates (at $\alpha = 0.05$): site two: D-xylose ($P = 0.047$ and IV = 31), alpha-cyclodextrin ($P = 0.008$ and IV = 41); site three: water (control well) ($P = 0.001$ and IV = 38), D-galacturonic acid ($P = 0.037$ and IV = 31), D-glucosaminic acid ($P = 0.012$ and IV = 36), itaconic acid ($P = 0.004$ and IV = 39), glucose-1-phosphate ($P = 0.001$ and IV = 43), L-alpha-glycerol phosphate ($P = 0.001$ and IV = 39); site four: L-asparagine ($P = 0.038$ and IV = 32), L-serine ($P = 0.037$ and IV = 34). The Mantel test rejected the null hypothesis at $\alpha = 0.10$ ($r = 0.13$, $P = 0.055$), thus showing a significant relationship between taxonomic and physiological data.

Discussion

This study, using eight statistical procedures on two parallel sets of data, yielded several patterns in diversity, composition, and structure among the root- and nodule-associated fungal communities. This section first discusses each pattern as

it relates to their supporting statistics, data, examples in the literature, and implications, then discusses these findings in broader contexts. Some observations are relevant to more than one pattern, and consequently are discussed more than once.

Using both taxonomic and physiological data, analysis of the community structure with NMDS showed no obvious separation of either root type or nodules. However, samples from site three were distinguished from the rest by their tight clustering and off-center position. This pattern was also noted in exploratory ordinations using other distance matrices, ordinations, and arrangements of the data (exploratory results not shown), and is supported by the results of the MRPP of both data sets. The detection of more taxonomic and physiological indicators of site, in particular of site three, than of roots and nodules, substantiates this pattern. In contrast, NMDS of both data sets resulted in high stress values, indicating a lack of strong patterns, or community structure (McCune &Grace, 2002). Despite this weak structure, I feel the similarity of the patterns in the exploratory ordinations, the agreement of the ordinations with the results of the MRPP and indicator analyses, and both ordinations being significantly stronger than expected by chance ($P<0.05$), imply that both taxonomic and physiological approaches are reporting a real, yet subtle, pattern in fungal community structure. The relatively tight clustering of samples from site three in the ordinations may reflect less variation of fungal community composition among samples from this site than from the remaining ones. For site three, its high numbers of indicators of site three, together

with its peripheral position in the ordinations, suggest that its community structure differs the most different among sites. This may indicate that drier locations, like site three, have community structures comparable to those of wetter locations (i.e., distribution of dry locations overlaps, yet is not concentric with, that of wet locations in an ordination). Drier sites may also have less variation in structure among samples (i.e., dry locations are more tightly clustered in an ordination than wet locations).

Site three was better distinguished in analyses of physiological data compared to taxonomic data, as shown by comparisons of the ordinations (Fig. 2b vs. Fig. 3b), diversities (Table 2), and number of indicators. So, despite relatively less intersample variation in physiological composition (Fig. 3b), fungal communities at site three, and possibly by extension, at dry sites in general, are more physiologically diverse. Discrepancies between the results of the physiological and taxonomic approaches may be explained by the suggestion of Petrini *et* al. (1992) that single, widely distributed, morphologically defined endophytic species may have several site-specific, physiologically distinct, cryptic strains. However, any link between soil moisture and physiological diversity should be tested further.

The low numbers of species in communities associated with alder nodules compared to those of connected alder roots was apparent during primary isolation steps: samples of alder nodules yielded fewer fungal colonies. However, this pattern was not reflected in the ordination results, likely because rarely isolated

species had been removed. Comparisons of the apparent physiological diversity between alder roots and nodules also did not show this pattern possibly because overlap of physiological profiles of nodule- and root-associated fungi may mask any taxonomic differences between these fungal communities. However, the smaller number of fungi and reduced taxonomic richness and diversity in nodules maybe explained by the presence of frankiamide. Haansuu *et al.* (2001) demonstrated that this compound, derived from the nodule inhabiting symbiont, *Frankia*, has antifungal properties. Using a disc diffusion method, they found that species of *Phytophthora*, *Heterobasidion*, *Fusarium*, *Botrytis*, and *Rhizoctonia*, all plant pathogens, were inhibited, but the yeast, *Candida albicans*, was unaffected. A second mechanism could be based the unique chemistry and physiology within alder nodules that lead to strongly reducing conditions (Benson *et al.*, 1980; Berry *et al.*, 1993; Beckwith *et al.*, 2002) likely inhospitable to many fungi.

Contrary to expectations, there were no significant differences in community structure or diversity between the fungal communities associated with the two species of roots. Despite both host species being chosen for their overall similarity, it was expected that differences in nitrogen dynamics due to the *Frankia*-associated nodulation in alder, and its lack thereof in birch, would shift their associated fungal communities. It is possible that exogenous *Frankia* associated with birch roots (Smolander, 1990) would result in nitrogen dynamics similar to alder, and would therefore lead to the assembly of similar fungal communities. Alternatively, communities may respond more to functional differences in their

hosts other than to nitrogen dynamics. Wright *et al.* (2006) suggested that because there is no "universal" functional classification scheme, the "ecosystem function" of interest should dictate the categories used. Since the "ecosystem function" in this case is the diversity and structure of root-associated fungal communities, the most appropriate classification scheme is not obvious. A scheme based on growth rates, mycorrhizal associates, lignin or resin content in the roots, or growth form could yield stronger differences among these communities. Classification schemes better able to resolve differences among root communities would yield improved insights into the ecology of these fungi.

Six of the 15 species detected in two or more samples were found solely with either root type or nodules; one of these fungi was isolated at only one site. Specificity for a particluar plant organ has also been detected in several studies (Fisher & Petrini, 1987; Kumar & Hyde, 2004; see also Petrini *et al.*, 1992), as has specificity for a single host (Carroll & Carroll, 1978; Kowalski & Kehr, 1992; Arnold *et al.*, 2001). However, the findings discussed in previous paragraphs suggest that fungal community structure is more influenced by conditions outside of the host. Fisher *et al.* (1991) also suggested that root endophytes in general are more specific to environment than to host. Other concurring studies found that species composition of root endophyte communities is shifted according to site factors in *Picea abies* (Holdenrieder & Sieber, 1992), and was more greatly affected by site factors than by root health in *Quercus* species (Halmschlager & Kowalski, 2004).

Arnold *et al.* (2001) suggested limited dispersal capabilities led to spatial heterogeneity among endophyte communities in leaves of the same woody tropical plant species. A similar conjecture can be made for root- and nodule-associated fungal communities since broad-scale dispersal may be more limiting in a hypogeous environment. Another possible contribution to this heterogeneity is the higher landscape-level spatial variation in the exogenous environment (e.g., soil pH and nutrient and oxygen content) of hypogeous endophytes compared to that experienced by most epigeous endophytes. A related hypothesis, where site conditions select for site-specific endophytic communities, was postulated by Petrini *et al.* (1992).

There were discrepancies between the results of the indicator analysis and the possible root and nodule or site specificities noted in Table 4. The approaches agreed that *Oidiodendron maius* and *Penicillium canescens* are specific to birch roots, however the other putative indicator species (i.e., with significantly higher indicator values) were not strictly specific to only one site, host organ, or host species. Other species that were specific to one host were not significant indicators. These discrepancies may be explained in part by my limited sample size, and by the different goals of each approach: indicator values (IV) take into account the proportional abundance within a group and among groups (McCune and Grace, 2002), while specificity, as defined here, refers to species detected more than twice only in a given site or plant sample.

Typical of community studies, species accumulation curves for all sites and all roots and nodules never reached their asymptote, indicating that further sampling would yield more species. Based on first-order jackknife estimates, ~63% of species present in each type of root and nodule were identified. By fitting second-order polynomial curves to the species-accumulation curves, I estimate that theoretically all detectable species predicted by the first-order jackknife estimates would be encountered if the sampling effort was doubled, and 80% would be detected if there was 46% more sampling (e.g., one sample of each root and nodule type from 22 individual plants). In contrast, Petrini *et al.* (1992) suggested that 30 to 40 samples per organ type from each of 40 individuals would be sufficient to detect 80% of the species. The 55- to 73-fold difference between my estimate and that of Petrini *et al.* (1992), i.e., 22 versus 1200-1600 samples, seems larger than can be attributed to the inherent inaccuracies of species-accumulation curve-based estimates (Chiarussi *et* al., 2003) compounded by a low sample number. A portion of this discrepancy may be attributed to differences in sampling protocols; each of my samples bulked hundreds of roots (or nodules) from one clod of soil, while Petrini *et al.* (1992) presumably sampled individual organs. This bulk approach therefore may be an attractive method when presence/absence data are required for fungal community descriptions; however, it may not be suitable for quantitative studies because of the difficulty of standardizing these bulked samples.

Aspects of both taxonomic and physiological data were comparable because analyses were almost identical. With some exceptions, interpretations of these two

data sets are congruous. This congruity was supported by the results of the Mantel test which showed a significant similarity between the two data sets. This agreement thus suggests that both methods may be suitable for some comparisons of various fungal communities. It further suggests that the composition of a fungal community is closely tied to its function. However, because I used SUPs as proxies for community function, several aspects of fungal functional diversity were ignored. Other components, such as the production of secondary metabolites (Zak & Visser, 1996) and structural components, and environmental tolerances, may not be as tightly connected to community composition as is substrate utilization. This ambiguity is common to all studies of functional diversity because of their need to reduce the breadth of "function" to a few (usually one) quantifiable aspect or "ecosystem function" (Wright *et al.*, 2006).

This study is likely the first to evaluate using SUPs for fungal community description by comparing results with those of traditional taxonomic approaches. However, this is not the first time Biolog®-derived SUPs of microbial communities have been evaluated by comparison to another method. Analysis of SUPs of communities of rhizosphere bacteria are comparable to results of fatty acid methyl ester (FAME) assays (Fang *et al.*, 2001) and phospholipids fatty acid (PLFA) assays (Khalil *et al.*, 2001), while Haack *et al.* (1995) and Verschuere *et al.* (1997) successfully demonstrated that SUPs can distinguish model communities of bacteria. Wolfaardt & van der Merwe (2002) had only moderate success comparing SUPs and sporocarp diversity of soil fungal communities; this is not surprising

given only a subset of soil fungi form sporocarps. Another study compared the rhizosphere of two tree species at three different sites using SUPs of bacterial communities, general quantification of bacteria, actinomycetes, filamentous fungi, and yeasts, and identification of pseudomonads and fungi (Grayston & Campbell, 1996). Unlike my study, it only identified "weedy" fungal species to genus, and did not compare those results to fungal SUPs.

This project also appears to be the first to study fungal communities using Biolog® EcoPlates®. EcoPlates® were not designed for use with fungal communities: they use a tetrazolium dye not optimized for fungal metabolism (Dobranic & Zak, 1999), and therefore may introduce a bias towards fungi able to reduce it. Despite this possible bias, I noted a correspondence between absorption at 590 nm and turbidity, and therefore felt that these data adequately reflected fungal growth. Another critique of the use of Biolog® plates for community profiling is that the substrates included are not representative of the environment being tested (Konopka *et al.*, 1998). This specific criticism was reserved for 95-substrate GN and GP plates that were designed for the identification of individual species of Gram-negative and Gram-positive bacteria, respectively, and not for discrimination of microbial communities. While no SUP-based approach can represent all the substrates possible in any environment, EcoPlates®, designed for microbial community profiling, contain more ecologically relevant substrates (Preston-Mafham *et al.*, 2002). Unlike other Biolog® plates with 95 organic substrates, EcoPlates® have only 31. Despite relatively less detail, this approach

allows more sampling or more replication for a given number of plates. Studies with a low sample size (a common situation in fungal ecology) using 95-substrate Biolog® plates often resort to pooling absorbancy data into chemical guilds (Zak *et al.*, 1994) to improve power of statistical analyses (Sobek & Zak, 2003). This pooling often is unnecessary when using 31-substrate EcoPlates®. The encouraging findings presented here show that EcoPlates® are adequate for some studies of fungal communities; however, they would likely be improved for this purpose if they were available without a tetrazolium dye, or with MTT, a tetrazolium dye suitable for fungal SUPs (Dobranic & Zak, 1999).

Biodiversity can be defined as the synthesis or sum of species, genetic, and functional diversity within a defined system or geographic space (Solbrig, 1991; Zak & Visser, 1996). Unlike many studies that only use species diversity or richness (Hooper *et al.*, 2002), this study used both taxonomic and functional approaches to address the biodiversity of a community. The taxonomic approach found that nodule associated fungal communities are smaller and less diverse than those associated with adjacent roots, while the physiological approach better detected site influences on community structure. However, the overall congruence of the results of these approaches in comparing community structure suggested that both approaches are valid. This correspondence, albeit based on only two focal plant hosts, implied that plant function, isolated from other factors, may not always correlate with structural or compositional changes in these fungal communities. This link between plant and fungal ecological theories may have implications in

large scale models of fungal diversity. Since the current estimated number of fungal species is largely based on the relationships between host plants and their associated fungi (Schmit & Mueller, 2007; see also Hyde *et al.*, 2007), future refinements of this estimation may weigh spatial and environmental variation more heavily than the functional diversity of their hosts.

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Table 1 Description of field sites

Site #	Date collected	General description	Ecological description	UTM zone *	Easting	Northing
1	24/06/2004 (taxonomic) 10/07/2005 (functional)	Private land E of Elk Island National Park	Adjacent to a small wetland	12N	384270	5947040
2	20/07/2004	Lily Lake Natural Area	Wetland margin; site with the highest water table	11N	655620	5954235
3	26/07/2004	Lloyd Creek Natural Area	Gently sloping opening in forest; site farthest from open water	11N	680290	5868965
4	31/08/2004	Redwater Natural Area	Adjacent to a slowly moving stream	12N	367220	5975820

*UTM coordinates were obtained using a GIS; locations are therefore not exact.

Table 2 Comparison of fungal richness and diversity among roots and nodules and among sites. Comparisons with alder root richness and diversity (Shannon's diversity index — H') were performed with two-tailed T-tests; p values of these tests are presented. Insignificant differences among sites as tested by ANOVA: * $P = 0.34$, ** $P = 0.26$. Superscripted letters indicate significant differences detected by a Tukey test ($P < 0.05$).

Community:	# of Samples	Taxonomic data:				Physiological data:
		Average H'	Average richness	Observed number of species	Estimated number of species[§]	Average H'
Alder nodules	15	0.751, $P = 0.0035$	2.33, $P = 0.0012$	20	33.1	3.3, $P = 0.51$
Alder roots	15	1.38	4.47	37	59.4	3.28
Birch roots	13	1.42, $P = 0.79$	4.38, $P = 0.89$	29	44.7	3.3, $P = 0.54$
Site 1	9	1.36*	4.11**	23	37.2	3.25^{bc}
Site 2	9	0.96*	2.89**	14	22	3.22^{c}
Site 3	9	1.02*	3.22**	15	22.1	3.39^{a}
Site 4	16	1.28*	4.19**	32	49.8	3.29^{b}

[§]First-order jackknife estimate

Table 3 Results of multi-response permutation procedures (MRPP) testing for difference among all sites, among all root and nodule types, between alder roots and nodules (organ comparison), and between alder and birch roots (host species comparison), using both taxonomic and functional data. Values shown are *P* (probability of falsely rejecting similar structures among groups) and A (effect size).

	Taxonomic data	Physiological data
Site	0.012 (A = 0.045)	< 0.0001 (A = 0.076)
Root and nodule type (all three compared)	0.22 (A = 0.0098)	0.44 (A = 0.00019)
Organ (root vs. nodule)	0.42 (A = 0.00013)	0.17 (A = 0.0056)
Host species (alder vs. birch)	0.33 (A = 0.0040)	0.39 (A = 0.0010)

Table 4 Possible host specificities and identification method of all species detected. Specificity codes (Specificity) are as follows: 0 = isolated only once or twice, 1 = no obvious specificity, 2 = alder specific, 3 = birch specific, 4 = root specific, 5 = nodule specific, 6 = birch and site four specific. Identification codes (ID Code) describe the characters used for identification: percentages are the highest percent base-pair matching by BLAST to a named sequence in GenBank or to sequences demonstrated to be monophyletic by a phylogenetic tree, a = anamorphic evidence used to identify a teleomorphic species, M = morphological (morphological evidence alone sufficient for identification), m = morphological (morphological evidence alone insufficient for identification, but corroborates molecular evidence), t = phylogenetic tree used to test conspecificity of isolates to each other or to GenBank sequences.

Species	Authority	Specificity	ID Code
Acremonium crotocinigenum	(Schol-Schwarz) W. Gams	0	100%, m
Acremonium kiliense	Grütz	0	M
Acremonium strictum	W. Gams	0	M
Beauveria bassiana	(Bals.-Criv.) Vuill.	0	M
Cadophora luteo-olivacea	(J.F.H. Beyma) T.C. Harr. & McNew	0	99%, m, t
Cadophora affin. malorum	(Kidd & Beaumont) W. Gams	1	98%, M, t
affin. Chalara	(Corda) Rabenh.	0	94%
Chalara longipes	(Preuss) Cooke	0	M
Cladorrhinum *c.f.* foecundissimum	Sacc. & Marchal	0	M
Cladosporium cladosporioides	(Fresen.) G.A. de Vries	0	M
Cladosporium sphaerospermum	Penz.	0	M
Clonostachys *c.f.* compactiuscula	(Sacc.) D. Hawksw. & W. Gams	0	M
Cryptosporiopsis radicicola	Kowalski & C. Bartnik	1	100%, m, t
Cudoniella sp.	Sacc.	0	100%, M, t
Cylindrocarpon destructans	(Zinssm.) Scholten	1	99%, m, t
Cylindrocarpon macrodidymum	Schroers, Halleen & Crous	0	100%, t
Cylindrocarpon magnusianum	Wollenw.	1	100%, m, t
Drechslera affin. erythrospila	(Drechsler) Shoemaker	0	96%, t
affin. Exophiala	J.W. Carmich.	0	96%
Geomyces asperulatus	Sigler & J.W. Carmich.	0	M
Geomyces pannorum	(Link) Sigler & J.W. Carmich.	0	M
Geotrichopsis *c.f.* mycoparasitica	Tzean & Estey	0	M
affin. Gibberella avenacea	R.J. Cook	0	99%, t
affin. Hydrocina	Scheuer	0	95%
affin. Hymenoscyphus monotropae	Kernan & Finocchio	0	96%, t
affin. Lachnum pygmaeum	(Fr.) Bres.	4	97%, t
Lecanicillium lecanii	(Zimm.) Zare & W. Gams	1	M
Lecythophora *c.f.* hoffmannii	(J.F.H. Beyma) W. Gams & McGinnis	0	M
Leptodontidium orchidicola	Sigler & Currah	1	99%, M, t
affin. Leptosphaeria korrae	J. Walker & A.M. Sm. bis	0	99%
Mortierella alpina	Peyronel	0	M

Species	Authority	Specificity	ID Code
affin. Mycena murina	Murrill	0	99%
Myxotrichum setosum	(Eidam) G.F. Orr & Plunkett	0	M, a
Oidiodendron maius	G.L. Barron	6	M
Paecilomyces carneus	(Duché & R. Heim) A.H.S. Br. & G. Sm.	4	M
Paecilomyces farinosus	(Holmsk.) A.H.S. Br. & G. Sm.	0	M
Penicillium *c.f.* waksmanii	K.M. Zalessky	0	M
Penicillium canescens	Sopp	3	M
Penicillium janczewskii	K.M. Zalessky	0	M
Phialocephala fortinii	C.J.K. Wang & H.E. Wilcox	1	M
Phoma leveillei	Boerema & G.J. Bollen	0	M
affin. Rhizoctonia	DC.	0	93%
Scopulariopsis *c.f.* brumptii	Salv.-Duval	0	M
Tetracladium affin. furcatum	Descals	0	98%, t
Tetracladium affin. maxilliforme	(Rostr.) Ingold	4	100%, t
affin. Tolypocladium cylindrosporum	W. Gams	0	99%, t
affin. Trichocladium minimum	de Hoog & Grinb.	0	97%
Trichosporiella cerebriformis	(G.A. de Vries & Kleine-Natrop) W. Gams	2	M
Umbelopsis autotrophica	(E.H. Evans) W. Gams,	0	M
Umbelopsis isabellina	(Oudem.) W. Gams	0	M
Umbelopsis ramanniana	(A. Møller) W. Gams	0	M
Umbelopsis vinacea	(Dixon-Stew.) Arx,	1	M
Verticillium chlamydosporium var. catenulatum	(Kamyschko ex Onions & G.L. Barron) W. Gams,	1	M
affin. Xylaria	Hill ex Schrank	0	91%
Yeast		0	M

Table 5 Isolation history of all species isolated. The numbers of samples of each type of root or nodule and of each site that hosted each species are shown with total number of isolates for each species, root and nodule type, and site.

	Root or nodule type			Sites				Total
	Alder root	Alder nodule	Birch root	1	2	3	4	
Species: Total samples>	15	15	13	9	9	9	16	43
Acremonium crotocinigenum	1	0	1	1	0	0	1	2
Acremonium kiliense	0	0	1	1	0	0	0	1
Acremonium strictum	1	0	1	1	0	0	1	2
Beauveria bassiana	1	0	0	0	0	0	1	1
Cadophora luteo-olivacea	1	0	1	0	1	0	1	2
Cadophora affin. malorum	2	2	3	3	0	4	0	7
affin. Chalara	1	0	0	1	0	0	0	1
Chalara longipes	0	0	1	0	0	0	1	1
Cladorrhinum *c.f.* foecundissimum	1	0	0	0	1	0	0	1
Cladosporium cladosporioides	0	2	0	2	0	0	0	2
Cladosporium sphaerospermum	1	1	0	0	0	0	2	2
Clonostachys *c.f.* compactiuscula	1	0	0	0	0	1	0	1
Cryptosporiopsis radicicola	3	1	5	2	2	1	4	9
Cudoniella sp.	1	0	0	0	1	0	0	1
Cylindrocarpon destructans	1	1	3	1	0	1	3	5
Cylindrocarpon macrodidymum	1	0	0	0	0	0	1	1
Cylindrocarpon magnusianum	3	2	1	0	1	0	5	6
Drechslera affin. erythrospila	0	1	0	1	0	0	0	1
affin. Exophiala	1	0	0	0	0	0	1	1
Geomyces asperulatus	0	1	0	0	0	0	1	1
Geomyces pannorum	0	1	1	0	0	2	0	2
Geotrichopsis *c.f.* mycoparasitica	1	0	0	1	0	0	0	1
affin. Gibberella avenacea	1	0	0	0	0	0	1	1
affin. Hydrocina	0	1	0	0	0	0	1	1
affin. Hymenoscyphus monotropae	1	0	0	0	1	0	0	1
affin. Lachnum pygmaeum	2	0	2	0	0	3	1	4
Lecanicillium lecanii	1	2	2	0	1	0	4	5
Lecythophora *c.f.* hoffmannii	1	0	0	1	0	0	0	1
Leptodontidium orchidicola	6	2	2	3	5	0	2	10
affin. Leptosphaeria korrae	1	0	1	0	0	0	2	2
Mortierella alpina	0	1	1	0	0	2	0	2
affin. Mycena murina	1	1	0	0	0	2	0	2
Myxotrichum setosum	0	0	1	1	0	0	0	1
Oidiodendron maius	0	0	3	0	0	0	3	3
Paecilomyces carneus	2	0	2	1	0	1	2	4
Paecilomyces farinosus	0	0	1	0	0	0	1	1
Penicillium *c.f.* waksmanii	0	1	1	1	0	0	1	2
Penicillium canescens	0	0	3	1	0	1	1	3
Penicillium janczewskii	0	0	1	0	0	0	1	1

	Root or nodule type			Sites				Total
Species: Total samples>	Alder root 15	Alder nodule 15	Birch root 13	1 9	2 9	3 9	4 16	43
Phialocephala fortinii	11	11	11	7	6	6	14	33
Phoma leveillei	2	0	0	0	0	0	2	2
affin. Rhizoctonia	1	0	0	0	0	0	1	1
Scopulariopsis *c.f.* brumptii	0	1	0	0	1	0	0	1
Tetracladium affin. furcatum	2	0	0	0	0	0	2	2
Tetracladium affin. maxilliforme	3	0	1	1	2	0	1	4
affin. Tolypocladium cylindrosporum	1	0	0	0	0	0	1	1
affin. Trichocladium minimum	2	0	0	0	1	0	1	2
Trichosporiella cerebriformis	3	1	0	1	1	2	0	4
Umbelopsis autotrophica	0	0	1	1	0	0	0	1
Umbelopsis isabellina	0	0	2	2	0	0	0	2
Umbelopsis ramanniana	1	0	0	0	0	1	0	1
Umbelopsis vinacea	1	1	1	1	2	0	0	3
Verticillium chlamydosporium var. catenulatum	2	1	2	2	0	0	3	5
affin. Xylaria	0	0	1	0	0	1	0	1
Yeast	1	0	0	0	0	1	0	1
Totals	67	35	57	37	26	29	67	159

Figure 3-1. Species accumulation curves of alder roots and nodules and birch roots (**a**), of individual sites (**b**), and of all samples pooled. The species accumulation curve for pooled samples is included in both Figs 1a and 1b for comparison. It has been truncated in 1a and 1b for proper scaling. Alder roots: ⋯⋯; alder nodules: ——; birch roots: – –. Site 1: ⋯⋯; site 2: ——; site 3: – – site 4: – ·· . Pooled samples: ▬.

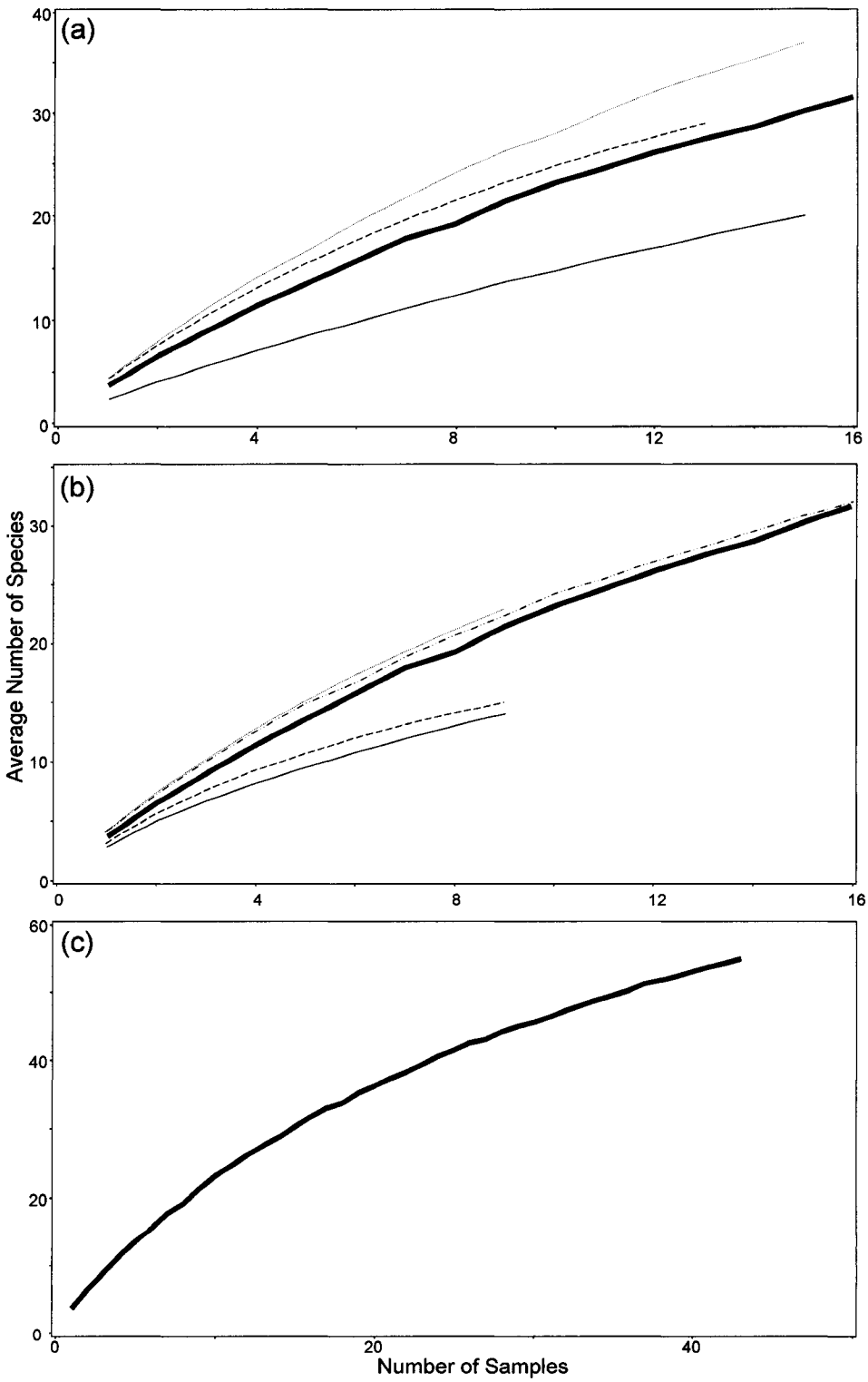


Figure 3-2. Ordinations of taxonomically described samples displaying possible patterns of community structure in response to type of root or nodule (**a**) or to site (**b**). Alder roots: △, ⋯⋯; alder nodules: ▲, ——; birch roots: □, – –. Site 1: △, ⋯⋯; site 2: ▲, ——; site 3: □, – – site 4: ■, –··.

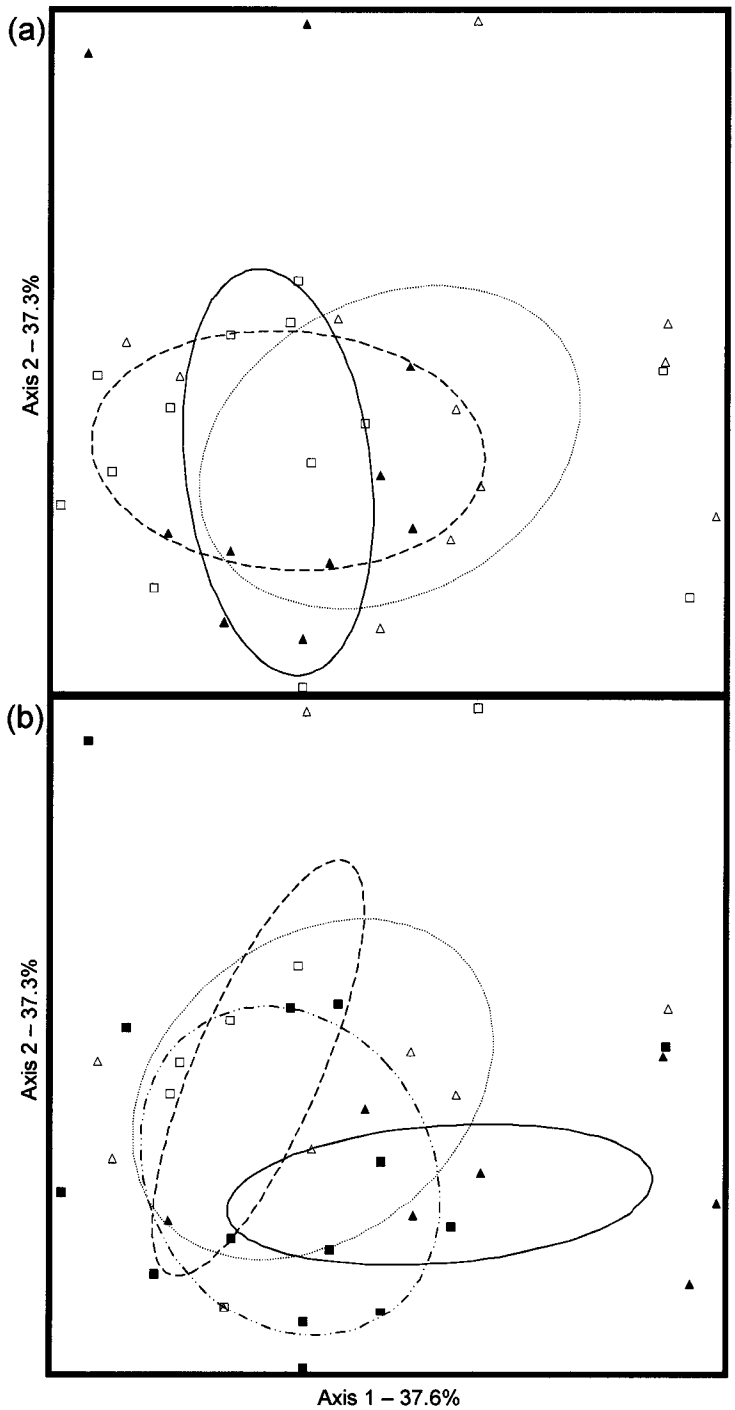
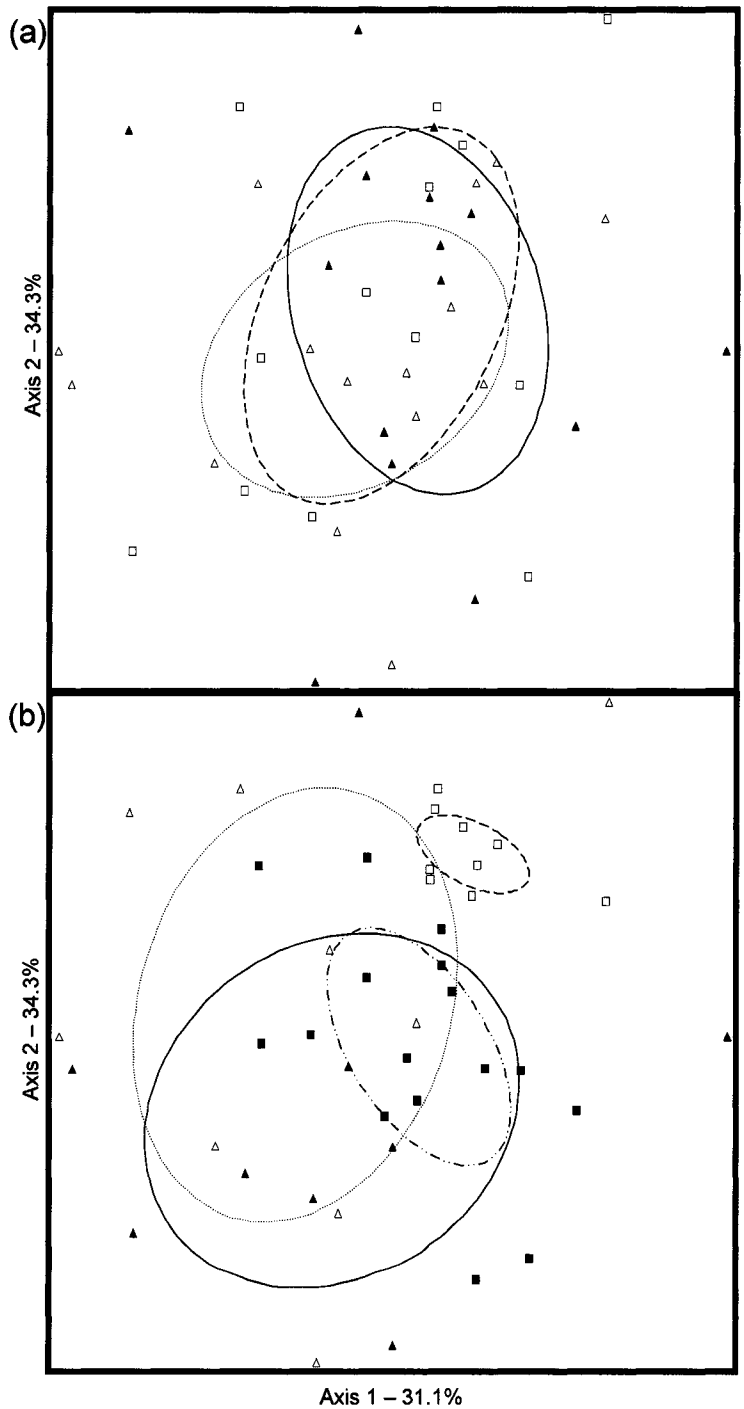


Figure 3-3. Ordinations of physiologically described samples displaying possible patterns of community structure in response to type of root or nodule (**a**) or to site (**b**). Alder roots: △, ⋯⋯; alder nodules: ▲ , ——; birch roots: □, – –. Site 1: △, ⋯⋯; site 2: ▲, ——; site 3: □, – – site 4: ■, –··.



Chapter 4 - Conclusion

Context

Studies of microfungal communities tend to be difficult because microfungi are microscopic, often have poorly defined species concepts, and occur in opaque habitats. Incomplete or inadequate taxonomic keys further add to the challenge of microfungal ecology. Nonetheless, several earlier studies of microfungal communities have investigated the species composition of various habitats, including agricultural soils, rhizospheres, leaves, stems, and roots (e.g., Fisher & Petrini, 1987; Petrini & Fisher, 1988; Fisher & Petrini, 1990; Fisher *et al.*, 1991; Holdenrieder & Sieber, 1992; Kumar & Hyde, 2004). Common to many of these studies were comparisons of species composition among habitats and the isolation of unnamed or unidentifiable species. More recent studies have extended these approaches to include studies of shifts in species composition (using morphological and molecular characters for identification), shifts in community functioning or physiology, and shifts in genetic structure, in response to environmental variables, substrate, disturbance or geographic location (e.g., Sobek & Zak, 2003; Allmér *et al.*, 2006; Arenz *et al.*, 2006).

The studies in this thesis focused on microfungal communities associated with roots and nodules *Alnus incana* subsp. *tenuifolia* and roots of *Betula papyrifera.* Fungi associated with roots or nodules may be categorized as: mycorrhizal, pathogenic, endophytic, saprobic or, depending on timing and conditions, a combination of these. Mycorrhizal fungi were not studied in this

thesis due to methodological limitations, despite the presence of ectomycorrhizae in all the root samples. However, a species that has both endophytic and saprobic characters was described in the second chapter. The third chapter described the use of taxonomic and physiological approaches to look at the relative importance of host and site related factors in influencing communities of non-mycorrhizal root- and nodule-associated fungi.

Mollisia rhizophila, sp. nov.: the teleomorph of *Leptodontidium orchidicola*

The form-species *Leptodontidium orchidicola* was detected at three of the four sites sampled in this study and was isolated from birch roots, alder roots, and alder nodules. This species is characterized by sessile conidia born laterally on undifferentiated hyphae or sympodially from swollen hyphal tips (Currah *et al.*, 1987). It has been frequently isolated from the roots of forbs, sedges, shrubs and trees (Jumpponen & Trappe, 1998), and has a demonstrated endophytic behavior (Fernando & Currah, 1995).

Some isolates of *Leptodontidium orchidicola* isolated from Lily Lake Natural Area near Edmonton, Alberta, formed apothecia in culture. These apothecia, as confirmed by microscopic inspection, are typical of the apothecial genus *Mollisia* in the *Dermateaceae* (*Helotiales*), although their ascus dehiscence mechanism is unusual for the family. Previous studies of *Mollisia* may have overlooked this mechanism due to limitations of their methods.

A review of the available literature on the taxonomy of *Mollisia* revealed no obvious morphological matches with any previously described species. However,

considering that this genus is in need of revision and includes over 500 species, an exhaustive literature search was near impossible. DNA sequence data also suggest that, despite forming their own distinct sub-clade, these apothecium-forming isolates are con-specific with sexually sterile *Leptodontidium orchidicola* isolates from other sites in this study and with other studies further afield. These data do not link *Leptodontidium orchidicola* to any other species of *Mollisia* with comparable sequence data in GenBank. The combination of morphological and molecular evidence was sufficient to justify the naming of the new teleomorphic species *Mollisia rhizophila*, sp. nov.

Leptodontidium orchidicola is a commonly isolated root endophyte, and is one of several species collectively known as Dark Septate Endophytes (DSE) (Jumpponen & Trappe, 1998). This polyphyletic assemblage of superficially similar root endophytes has been the focus of numerous studies (Addy *et al.*, 2005), and has been described in the roots of divergent taxa around the world. Despite this, the association of *L. orchidicola* with a teleomorph has not previously been made. This new anamorph-teleomorph connection is of further taxonomic interest because it is both the first connecting a teleomorph to any species in *Leptodontidium*, and the first linking a non-Ingoldian and non-phialidic anamorph to a *Mollisia*. Of ecological interest, *Leptodontidium orchidicola* is known to demonstrate endophytic characteristics, while *Mollisia* is typically considered saprobic. In a holomorphic sense, this species may therefore be a root-specific example of a branch-pruning fungus hypothesized by Kowalski & Kehr (1992). According to this hypothesis,

these fungi behave as endophytes until a branch senesces, whereupon they assume a more saprobic role. As saprobes, they accelerate branch pruning to the benefit of the plant.

Communities of root and nodule associated fungi

Three types of samples, i.e., roots and nodules of *Alnus incana* subsp. *tenuifolia* and roots of adjacent *Betula papyrifera*, were collected from four sites near Edmonton. The fungal species (taxonomic approach) and fungal substrate utilization profiles (functional approach) associated with these collected organs were compared. Both approaches showed subtle, yet significant, differences in structure among fungal communities distinguished by site. In contrast, neither approach could differentiate the structures of communities associated with each of the roots and nodules. These results imply that the structure of these fungal communities is more influenced by site-specific factors, external to their host roots or nodules, than they are by their specific host organ (e.g., birch root, alder root, or alder nodule). Other researchers of root endophyte communities have reached a similar conclusion (Petrini *et al.*, 1992). The congruency of the two approaches also implies that either may be suitable for comparisons of fungal community structure.

Alder nodules were less species rich and diverse compared to the roots of either species. The cause of this reduced diversity was not tested further; however this pattern is likely attributable to the physiologically and chemically unusual conditions associated with alder nodules and their prokaryotic endophyte, *Frankia*. Additional comparisons showed that while the majority of frequently detected

species were not substrate or site specific, several substrates and some species were indicators of one site.

The methods used to elucidate the composition and structure of root and nodule associated fungi were partially new, and yielded promising results. This study was the first to use Biolog® EcoPlates® to study fungal communities and found that despite their incorporation of a bacterial-specific metabolism indicator dye, EcoPlates® were able to respond to general patterns in fungal community structure. This was also one of the first studies of plant-associated fungi to use bulk processed plant organs. This approach may be an efficient way to detect the presence of constituent fungal species, since the rates of species accumulation presented in the third chapter appear to be higher than comparable studies using more labour-intensive techniques.

Summary

The studies described in this thesis have contributed to the field of microfungal ecology by investigating the relative influences of host organ and geographical location on root and nodule associated fungi. These studies explored new methods, and improved the taxonomic placement of an ecologically interesting species. Specifically, Chapter Two looked at the isolation of *Mollisia rhizophila*, sp. nov. from substrates collected at one field site. Chapter Three used two ecological approaches to show the large site influence on microfungal communities. Both chapters underscore the variability of root and nodule associated fungal communities from site to site, but not necessarily among host species or even

among hosts with different ecological functions. This observation has implications in future fungal species inventories and possibly in the design of conservation strategies. The isolation of a new taxonomically important fungal species highlights the value of culture-based fungal censuses.

Future Research

As stated in the introduction, biodiversity may be considered the synthesis of taxonomic, genetic, and functional diversities within a defined system (Solbrig, 1991; Zak *et al.*, 1994). My research has addressed the taxonomic and, indirectly, the functional aspects of this definition as it relates to some fungal communities associated with plants. Despite using some molecular tools, these studies did not specifically address genetic diversity because these techniques were applied only to individual isolates grown in culture.

A possible extension of these studies is to use purely molecular methods to continue investigating the relative importance of host substrate and site factors to communities of root and nodule associated fungi. There are several molecular procedures that could be employed in such a study. Denaturing gradient gel electrophoresis (DGGE) is able to resolve species based on the sequence differences of DNA amplified from environmental samples (Arenz *et al.*, 2006). Other approaches, based on cloning fungal genes from environmental samples or on terminal restriction fragment length polymorphisms (T-RFLP) of DNA amplified from environmental samples (Allmér *et al.*, 2006), could also be used. Such approaches, unlike those used here, would not be biased towards culturable species,

but may have their own inherent biases. If a molecular approach corroborated the conclusions presented here, the validity of my findings would be greatly strengthened.

Observations of the morphology and development of *Mollisia rhizophila* revealed some unusual features that merit further study, including sub-apical ascus dehiscence. This feature, noted in Chapter Two, may have significant taxonomic implications. In addition, several observations of degenerating apothecia revealed bodies that appeared to be either the result of ascospore germination *in situ* or the result of ascospore fusion (Appendix 3). The exact ontogeny of these bodies and their taxonomic and ecological significance are potential topics for future mycological studies.

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Appendix 1 – Raw taxonomic data from Chapter 3

The following four tables show the presence/absence in each sample of all species detected in the study; there is one table per site.

Site	1								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Acremonium crotocinigenum								X	
Acremonium kiliense							X		
Acremonium strictum							X		
Beauveria bassiana									
Cadophora luteo-olivacea									
Cadophora affin. malorum	X			X		X			
affin. Chalara			X						
Chalara longipes									
Cladorrhinum *c.f.* foecundissimum									
Cladosporium cladosporioides				X	X				
Cladosporium sphaerospermum									
Clonostachys *c.f.* compactiuscula									
Cryptosporiopsis radicicola		X						X	
Cudoniella sp.									
Cylindrocarpon destructans									X
Cylindrocarpon macrodidymum									
Cylindrocarpon magnusianum									
Drechslera affin. erythrospila					X				
affin. Exophiala salmonis									
Geomyces asperulatus									
Geomyces pannorum									
Geotrichopsis *c.f.* mycoparasitica			X						
affin. Gibberella avenacea									
affin. Hydrocina chaetocladia									
affin. Hymenoscyphus monotropae									
affin. Lachnum pygmaeum									
Lecanicillium lecanii									
Lecythophora *c.f.* hoffmannii	X								
Leptodontidium orchidicola	X	X				X			
affin. Leptosphaeria korrae									
Mortierella alpine									
affin. Mycena murina									
Myxotrichum setosum									X
Oidiodendron maius									
Paecilomyces carneus									X
Paecilomyces farinosus									
Penicillium *c.f.* waksmanii				X					
Penicillium canescens							X		
Penicillium janczewskii									
Phialocephala fortinii	X		X		X	X	X	X	X
Phoma leveillei									
affin. Rhizoctonia sp.									
Scopulariopsis *c.f.* brumptii									
Tetracladium affin. furcatum									
Tetracladium affin. maxilliforme	X								
affin. Tolypocladium cylindrosporum									
affin. Trichocladium minimum									
Trichosporiella cerebriformis			X						
Umbelopsis autotrophica							X		
Umbelopsis isabellina							X	X	
Umbelopsis ramanniana									
Umbelopsis vinacea									X
Verticillium chlamydosporium var. catenulatum						X	X		
affin. Xylaria									
Yeast									

Site	2								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Acremonium crotocinigenum									
Acremonium kiliense									
Acremonium strictum									
Beauveria bassiana									
Cadophora luteo-olivacea								X	
Cadophora affin. malorum									
affin. Chalara									
Chalara longipes									
Cladorrhinum *c.f.* foecundissimum		X							
Cladosporium cladosporioides									
Cladosporium sphaerospermum									
Clonostachys *c.f.* compactiuscula									
Cryptosporiopsis radicicola							X	X	
Cudoniella sp.			X						
Cylindrocarpon destructans									
Cylindrocarpon macrodidymum									
Cylindrocarpon magnusianum								X	
Drechslera affin. erythrospila									
affin. Exophiala salmonis									
Geomyces asperulatus									
Geomyces pannorum									
Geotrichopsis *c.f.* mycoparasitica									
affin. Gibberella avenacea									
affin. Hydrocina chaetocladia									
affin. Hymenoscyphus monotropae		X							
affin. Lachnum pygmaeum									
Lecanicillium lecanii									X
Lecythophora *c.f.* hoffmannii									
Leptodontidium orchidicola	X	X			X			X	X
affin. Leptosphaeria korrae									
Mortierella alpina									
affin. Mycena murina									
Myxotrichum setosum									
Oidiodendron maius									
Paecilomyces carneus									
Paecilomyces farinosus									
Penicillium *c.f.* waksmanii									
Penicillium canescens									
Penicillium janczewskii									
Phialocephala fortinii	X		X	X	X	X	X		
Phoma leveillei									
affin. Rhizoctonia sp.									
Scopulariopsis *c.f.* brumptii				X					
Tetracladium affin. furcatum									
Tetracladium affin. maxilliforme	X						X		
affin. Tolypocladium cylindrosporum									
affin. Trichocladium minimum	X								
Trichosporiella cerebriformis		X							
Umbelopsis autotrophica									
Umbelopsis isabellina									
Umbelopsis ramanniana									
Umbelopsis vinacea	X			X					
Verticillium chlamydosporium var. catenulatum									
affin. Xylaria									
Yeast									

Site	3								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Acremonium crotocinigenum									
Acremonium kiliense									
Acremonium strictum									
Beauveria bassiana									
Cadophora luteo-olivacea									
Cadophora affin. malorum			X				X	X	X
affin. Chalara									
Chalara longipes									
Cladorrhinum *c.f.* foecundissimum									
Cladosporium cladosporioides									
Cladosporium sphaerospermum									
Clonostachys *c.f.* compactiuscula			X						
Cryptosporiopsis radicicola									X
Cudoniella sp.									
Cylindrocarpon destructans									X
Cylindrocarpon macrodidymum									
Cylindrocarpon magnusianum									
Drechslera affin. erythrospila									
affin. Exophiala salmonis									
Geomyces asperulatus									
Geomyces pannorum					X		X		
Geotrichopsis *c.f.* mycoparasitica									
affin. Gibberella avenacea									
affin. Hydrocina chaetocladia									
affin. Hymenoscyphus monotropae									
affin. Lachnum pygmaeum	X							X	X
Lecanicillium lecanii									
Lecythophora *c.f.* hoffmannii									
Leptodontidium orchidicola									
affin. Leptosphaeria korrae									
Mortierella alpina				X			X		
affin. Mycena murina		X			X				
Myxotrichum setosum									
Oidiodendron maius									
Paecilomyces carneus			X						
Paecilomyces farinosus									
Penicillium *c.f.* waksmanii									
Penicillium canescens							X		
Penicillium janczewskii									
Phialocephala fortinii		X	X	X			X	X	X
Phoma leveillei									
affin. Rhizoctonia sp.									
Scopulariopsis *c.f.* brumptii									
Tetracladium affin. furcatum									
Tetracladium affin. maxilliforme									
affin. Tolypocladium cylindrosporum									
affin. Trichocladium minimum									
Trichosporiella cerebriformis		X		X					
Umbelopsis autotrophica									
Umbelopsis isabellina									
Umbelopsis ramanniana			X						
Umbelopsis vinacea									
Verticillium chlamydosporium var. catenulatum									
affin. Xylaria							X		
Yeast			X						

Site	4															
Host species	Alder												Birch			
Organ	Root						Nodule						Root			
Sample number	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4
Acremonium crotocinigenum		X														
Acremonium kiliense																
Acremonium strictum	X															
Beauveria bassiana				X												
Cadophora luteo-olivacea		X														
Cadophora affin. malorum																
affin. Chalara																
Chalara longipes														X		
Cladorrhinum *c.f.* foecundissimum																
Cladosporium cladosporioides																
Cladosporium sphaerospermum			X							X						
Clonostachys *c.f.* compactiuscula																
Cryptosporiopsis radicicola				X	X					X					X	
Cudoniella sp.																
Cylindrocarpon destructans						X	X								X	
Cylindrocarpon macrodidymum	X															
Cylindrocarpon magnusianum	X		X	X						X	X					
Drechslera affin. erythrospila																
affin. Exophiala salmonis			X													
Geomyces asperulatus								X								
Geomyces pannorum																
Geotrichopsis *c.f.* mycoparasitica																
affin. Gibberella avenacea	X															
affin. Hydrocina chaetocladia										X						
affin. Hymenoscyphus monotropae																
affin. Lachnum pygmaeum					X											
Lecanicillium lecanii	X							X	X				X			
Lecythophora *c.f.* hoffmannii																
Leptodontidium orchidicola			X	X												
affin. Leptosphaeria korrae		X												X		
Mortierella alpina																
affin. Mycena murina																
Myxotrichum setosum																
Oidiodendron maius													X	X		X
Paecilomyces carneus						X										X
Paecilomyces farinosus													X			
Penicillium *c.f.* waksmanii													X			
Penicillium canescens																X
Penicillium janczewskii													X			
Phialocephala fortinii	X	X	X		X	X		X	X	X	X	X	X	X	X	X
Phoma leveillei		X				X										
affin. Rhizoctonia sp.	X															
Scopulariopsis *c.f.* brumptii																
Tetracladium affin. furcatum			X	X												
Tetracladium affin. maxilliforme				X												
affin. Tolypocladium cylindrosporum		X														
affin. Trichocladium minimum			X													
Trichosporiella cerebriformis																
Umbelopsis autotrophica																
Umbelopsis isabellina																
Umbelopsis ramanniana																
Umbelopsis vinacea																
Verticillium chlamydosporium var. catenulatum					X	X									X	
affin. Xylaria																
Yeast																

Appendix 2 – Raw physiological data from Chapter 3

The following five tables show the unadjusted absorbance at 590 nm for each substrate in Biolog® EcoPlates® for each sample. Observations with an average well colour development (AWCD) closest to 0.7 are presented. Actual AWCD and respective observation times are shown. The first three tables present the data for sites one to three, respectively; the forth and fifth tables present the data for site four.

Site	1								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Incubation time (Hrs)	407	407	502	407	407	189	189.5	314	189
AWCD	0.664	0.722	0.662	0.660	0.618	0.492	0.746	0.776	0.557
Water	0.373	0.167	0.73	0.341	0.241	0.180	0.213	0.236	0.263
B-Methyl-D-Glucoside	0.275	0.377	0.675	0.907	0.922	0.362	0.871	0.562	0.478
D-Galactonic Acid gamma-Lactone	0.166	1.283	0.165	0.368	0.248	0.172	0.693	0.281	0.261
L-Arginine	0.873	0.290	0.505	0.194	0.233	0.532	0.942	0.265	0.544
Pyruvic Acid Methyl Ester	0.465	0.888	0.543	0.750	0.200	1.047	0.836	0.391	0.265
D-Xylose	1.231	1.002	1.529	0.830	0.169	0.592	1.498	1.461	0.868
D-Galacturonic Acid	1.037	0.364	0.400	0.476	0.816	0.330	0.865	0.713	0.314
L-Asparagine	1.465	1.103	0.513	0.209	0.818	0.566	0.793	1.094	0.502
Tween 40	0.667	0.610	1.181	0.745	1.355	0.735	0.713	0.906	0.193
I-Erythritol	1.461	1.641	1.071	0.203	0.345	0.697	0.699	0.671	0.173
2-Hydroxy Benzoic Acid	0.501	0.925	0.858	0.352	0.962	0.37	0.332	1.199	0.609
L-Phenylalanine	0.414	1.136	1.059	0.176	1.456	0.207	0.383	0.943	0.186
Tween 80	0.163	1.387	1.249	1.006	1.004	0.444	1.102	1.104	1.075
D-Mannitol	2.704	1.569	3.252	2.444	0.571	0.642	1.685	0.193	0.169
4-Hydroxy Benzoic Acid	0.984	0.865	0.971	0.988	0.183	0.342	0.32	0.931	0.634
L-Serine	0.383	0.277	0.313	0.337	0.763	0.292	0.577	1.063	0.143
Alpha-Cyclodextrin	0.665	0.260	0.704	0.239	0.262	0.673	0.157	0.231	0.697
N-Acetyl-D-Glucosamine	1.106	1.109	0.877	1.345	1.124	0.990	1.294	2.257	0.229
Gamma-Hydroxybutric Acid	0.686	0.679	0.473	0.658	0.559	0.86	0.690	0.951	1.556
L-Threonine	0.199	0.177	0.186	1.633	0.566	0.518	0.907	0.239	0.944
Glycogen	0.891	1.071	0.297	0.834	0.659	0.807	1.196	1.369	0.578
D-Glucosaminic Acid	0.190	0.344	0.237	0.201	0.201	0.143	0.298	0.610	0.968
Itaconic Acid	0.366	0.253	0.800	0.171	0.193	0.224	0.974	0.827	0.766
Glycyl-L-Glutamic Acid	0.365	0.311	0.167	1.482	0.791	0.145	1.011	0.578	1.213
D-Cellobiose	0.978	1.446	0.486	1.365	0.882	0.628	1.185	0.562	0.912
Glucose-1-Phosphate	0.164	0.201	0.716	0.249	0.225	0.200	0.168	0.605	0.179
Alpha-Ketobutyric Acid	0.557	0.291	0.184	1.283	0.179	0.372	0.236	1.424	0.244
Phenylethylamine	0.300	0.191	0.146	0.216	0.779	0.759	0.670	0.828	0.757
Alpha-D-Lactose	0.245	1.528	0.152	0.360	2.058	0.56	0.737	0.269	0.808
D,L-alpha-Glycerol Phosphate	0.173	0.278	0.242	0.28	0.204	0.164	0.213	0.248	0.245
D-Malic Acid	0.669	0.668	0.268	0.241	0.576	0.623	0.787	0.779	0.790
Putrescine	0.538	0.421	0.233	0.243	0.246	0.579	0.832	1.028	0.261

Site	2								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Incubation time (Hrs)	360	360	360	408	288	408	288	360	240
AWCD	0.705	0.690	0.752	0.567	0.784	0.617	0.668	0.727	0.698
Water	0.252	0.178	0.248	0.176	0.244	0.390	0.224	0.227	0.239
B-Methyl-D-Glucoside	0.893	0.572	0.758	1.403	0.740	1.382	0.584	0.498	0.980
D-Galactonic Acid gamma-Lactone	0.228	0.487	1.138	0.278	0.437	0.454	0.322	0.194	0.250
L-Arginine	0.788	0.484	0.581	1.149	1.087	0.808	0.301	0.209	0.241
Pyruvic Acid Methyl Ester	0.793	0.325	0.681	1.275	0.505	0.987	0.750	0.952	0.659
D-Xylose	1.350	1.292	1.375	1.388	1.642	1.472	0.892	0.965	1.223
D-Galacturonic Acid	0.885	0.154	0.921	0.625	0.875	0.359	0.614	0.739	0.516
L-Asparagine	1.227	0.859	0.405	0.253	1.626	0.391	0.950	1.153	1.295
Tween 40	1.140	1.090	0.973	0.508	0.591	0.368	1.031	1.260	0.783
I-Erythritol	0.63	0.793	0.195	0.186	0.677	0.397	2.249	1.473	0.322
2-Hydroxy Benzoic Acid	0.234	0.562	0.816	1.052	0.958	0.764	0.366	1.902	0.811
L-Phenylalanine	0.740	1.536	0.710	0.233	0.282	0.845	0.506	0.806	0.229
Tween 80	0.963	1.526	1.339	0.449	0.759	0.319	0.743	1.275	1.466
D-Mannitol	1.561	0.166	2.618	0.243	3.138	0.271	2.506	2.008	0.924
4-Hydroxy Benzoic Acid	0.550	1.256	0.201	0.285	0.280	0.395	0.627	0.348	1.499
L-Serine	0.229	0.238	0.255	0.267	0.645	0.285	0.425	0.333	0.286
Alpha-Cyclodextrin	0.992	2.140	2.072	0.325	1.155	1.785	0.251	0.284	2.967
N-Acetyl-D-Glucosamine	1.140	1.525	1.845	1.978	1.125	1.101	1.254	1.107	0.500
Gamma-Hydroxybutric Acid	1.019	0.669	0.258	1.110	0.953	0.982	1.049	0.721	0.746
L-Threonine	0.230	0.277	0.390	0.294	0.628	0.470	0.582	0.359	1.134
Glycogen	1.196	0.991	0.209	0.285	1.314	0.488	0.827	1.337	1.225
D-Glucosaminic Acid	0.970	0.200	0.175	0.356	0.335	0.362	0.203	0.204	0.221
Itaconic Acid	0.208	0.907	0.15	0.279	0.311	0.482	0.213	0.206	0.177
Glycyl-L-Glutamic Acid	0.529	0.189	0.908	0.696	1.025	0.425	0.610	0.457	0.335
D-Cellobiose	0.848	1.503	1.831	1.074	1.191	0.453	1.255	1.161	1.271
Glucose-1-Phosphate	0.196	0.229	0.230	0.295	0.349	0.449	0.258	0.229	0.269
Alpha-Ketobutyric Acid	0.259	0.190	0.318	0.249	0.378	0.351	0.211	0.725	0.336
Phenylethylamine	0.169	0.204	0.211	0.301	0.300	0.411	0.772	0.226	0.291
Alpha-D-Lactose	0.959	0.438	0.470	0.290	0.359	0.586	0.226	0.965	0.579
D,L-alpha-Glycerol Phosphate	0.285	0.227	0.271	0.291	0.274	0.506	0.204	0.279	0.181
D-Malic Acid	0.797	0.699	1.259	0.257	0.592	0.643	0.198	0.368	0.166
Putrescine	0.312	0.167	0.266	0.308	0.303	0.348	0.178	0.278	0.221

Site	3								
Host species	Alder						Birch		
Organ	Root			Nodule			Root		
Sample number	1	2	3	1	2	3	1	2	3
Incubation time (Hrs)	144	264	192	144	144	144	144	144	144
AWCD	0.563	0.741	0.696	0.747	0.769	0.735	0.680	0.684	0.656
Water	0.683	0.743	0.663	0.662	0.261	0.719	0.344	0.597	0.323
B-Methyl-D-Glucoside	0.672	1.557	1.066	0.98	0.892	0.238	0.746	0.239	0.230
D-Galactonic Acid gamma-Lactone	0.619	0.226	0.702	0.75	0.828	0.761	0.568	0.212	0.237
L-Arginine	0.648	0.807	1.403	0.784	0.903	0.530	0.797	0.754	0.519
Pyruvic Acid Methyl Ester	0.727	0.650	0.674	0.700	0.983	0.913	0.798	0.688	0.846
D-Xylose	0.585	2.053	1.300	0.846	0.907	0.134	0.722	0.724	0.745
D-Galacturonic Acid	0.672	0.633	0.706	0.889	1.106	1.115	0.687	0.814	0.957
L-Asparagine	0.745	0.887	0.476	1.067	0.811	1.010	0.308	0.847	0.753
Tween 40	0.325	0.22	0.997	0.612	0.767	0.521	0.608	0.504	0.981
I-Erythritol	0.571	0.681	0.764	0.757	1.125	1.138	0.517	0.829	0.890
2-Hydroxy Benzoic Acid	0.534	0.408	0.650	0.779	0.880	0.702	0.647	0.945	0.877
L-Phenylalanine	0.370	0.221	0.581	1.208	0.702	0.666	0.690	0.736	0.221
Tween 80	0.182	0.528	0.932	0.187	0.582	0.649	0.578	0.239	0.296
D-Mannitol	0.631	1.472	0.859	0.722	0.751	1.801	0.608	0.835	0.820
4-Hydroxy Benzoic Acid	0.458	0.688	0.564	0.336	0.941	0.681	0.743	0.577	0.626
L-Serine	0.677	0.276	0.248	1.098	0.776	0.624	0.804	0.730	0.443
Alpha-Cyclodextrin	0.496	0.755	0.292	0.717	0.501	0.687	0.668	0.696	0.779
N-Acetyl-D-Glucosamine	0.691	0.389	0.873	0.857	0.867	0.872	0.872	0.714	0.424
Gamma-Hydroxybutric Acid	0.610	1.330	0.301	0.639	0.766	0.572	0.507	0.571	0.873
L-Threonine	0.694	0.821	0.669	0.331	0.696	0.767	0.789	0.800	0.252
Glycogen	0.556	0.312	0.63	0.646	0.636	0.677	0.837	0.657	0.762
D-Glucosaminic Acid	0.680	0.578	0.788	0.796	0.626	1.130	0.559	0.871	0.757
Itaconic Acid	0.909	0.894	0.942	0.822	0.636	0.783	0.701	0.866	0.925
Glycyl-L-Glutamic Acid	0.798	0.753	0.534	0.868	0.744	0.765	0.791	0.348	0.907
D-Cellobiose	0.719	1.743	0.758	0.691	0.790	0.711	0.702	0.611	0.790
Glucose-1-Phosphate	0.776	0.730	0.779	0.837	0.853	0.244	0.682	0.692	0.786
Alpha-Ketobutyric Acid	0.483	0.881	0.566	0.241	0.181	0.831	0.574	0.592	0.906
Phenylethylamine	0.327	0.177	0.525	1.046	0.497	0.287	0.745	0.796	0.891
Alpha-D-Lactose	0.584	0.811	0.591	0.729	0.76	0.873	1.226	0.805	0.609
D,L-alpha-Glycerol Phosphate	0.156	0.257	0.613	0.768	0.779	0.808	0.585	0.711	0.233
D-Malic Acid	0.173	0.717	0.171	0.782	1.296	0.645	0.657	0.896	0.650
Putrescine	0.255	0.519	0.641	0.741	0.771	0.668	0.692	0.985	0.685

Site	4								
Host species	Alder								
Organ	Root						Nodule		
Sample number	1	2	3	4	5	6	1	2	3
Incubation time (Hrs)	192	144	144	144	144	144	144	144	336
AWCD	0.648	0.703	0.594	0.660	0.885	0.802	0.805	0.776	0.532
Water	0.108	0.406	0.294	0.240	0.315	0.256	0.648	0.335	0.203
B-Methyl-D-Glucoside	0.982	0.686	0.332	0.782	0.961	0.254	0.774	1.284	0.194
D-Galactonic Acid gamma-Lactone	0.175	0.204	0.289	0.221	0.763	0.699	0.302	0.340	0.444
L-Arginine	0.591	0.574	1.043	1.676	0.874	1.426	1.227	0.642	0.619
Pyruvic Acid Methyl Ester	0.638	0.975	0.836	0.356	0.521	0.892	0.637	0.350	0.417
D-Xylose	0.969	0.797	1.098	1.247	0.961	0.745	0.367	1.585	1.119
D-Galacturonic Acid	0.468	1.122	0.864	0.319	0.532	0.474	0.342	0.490	0.701
L-Asparagine	0.842	1.487	1.370	0.920	0.980	1.080	1.340	1.846	0.633
Tween 40	0.620	0.757	0.586	0.562	0.732	0.724	0.637	0.792	0.595
I-Erythritol	0.718	0.227	0.358	1.333	1.599	1.197	0.288	0.600	0.903
2-Hydroxy Benzoic Acid	0.555	0.654	0.499	0.635	0.424	0.388	0.890	0.227	0.172
L-Phenylalanine	0.481	0.447	0.386	0.619	0.419	0.244	0.719	0.317	0.529
Tween 80	0.818	0.760	1.020	0.703	1.242	0.890	1.157	1.029	0.442
D-Mannitol	1.888	2.02	0.837	0.865	1.968	1.797	0.540	2.393	1.006
4-Hydroxy Benzoic Acid	0.580	0.705	0.417	0.233	0.661	0.661	0.825	0.426	0.475
L-Serine	0.820	0.886	0.602	0.232	1.358	1.115	0.933	0.247	0.609
Alpha-Cyclodextrin	1.227	0.769	0.259	0.969	1.764	1.731	0.523	0.710	0.188
N-Acetyl-D-Glucosamine	1.498	0.501	0.423	1.956	1.538	1.116	1.985	1.727	1.158
Gamma-Hydroxybutric Acid	0.822	0.835	0.978	0.840	0.641	0.572	0.807	1.105	0.722
L-Threonine	0.848	0.640	0.506	0.443	1.075	0.414	0.839	0.599	0.564
Glycogen	0.595	0.933	0.875	0.288	1.036	0.988	0.672	1.176	0.230
D-Glucosaminic Acid	0.215	0.329	0.642	0.276	0.787	0.921	0.431	0.856	0.560
Itaconic Acid	0.073	0.505	0.271	1.324	0.225	0.896	0.814	0.317	0.837
Glycyl-L-Glutamic Acid	0.763	0.404	0.620	0.405	1.099	0.843	1.073	0.767	0.551
D-Cellobiose	0.300	1.698	1.328	0.811	1.847	1.104	1.879	1.028	0.442
Glucose-1-Phosphate	0.243	0.243	0.352	0.288	0.287	0.331	0.344	0.882	0.307
Alpha-Ketobutyric Acid	0.517	0.314	0.224	0.240	0.455	0.208	1.250	0.236	0.376
Phenylethylamine	0.142	0.212	0.213	0.638	0.265	0.664	0.704	0.238	0.726
Alpha-D-Lactose	1.227	1.095	0.360	0.434	0.427	1.359	0.846	0.740	0.263
D,L-alpha-Glycerol Phosphate	0.048	0.229	0.304	0.299	0.951	0.464	0.310	0.278	0.254
D-Malic Acid	0.333	0.469	0.413	0.252	0.796	0.555	0.687	0.282	0.326
Putrescine	0.626	0.614	0.410	0.698	0.827	0.666	0.971	0.990	0.451

Site	4						
Host species	Alder			Birch			
Organ	Nodule			Root			
Sample number	4	5	6	1	2	3	4
Incubation time (Hrs)	336	144	336	144	144	192	144
AWCD	0.648	0.664	0.689	0.603	0.714	0.794	0.820
Water	0.343	0.668	0.34	0.362	0.281	0.373	0.589
B-Methyl-D-Glucoside	0.613	0.620	0.593	1.016	0.364	0.811	1.225
D-Galactonic Acid gamma-Lactone	0.421	0.323	0.980	0.375	1.026	0.368	0.370
L-Arginine	0.279	0.830	0.992	0.850	1.121	0.536	1.185
Pyruvic Acid Methyl Ester	0.765	1.232	0.721	0.557	0.914	1.218	0.497
D-Xylose	0.564	0.649	1.828	0.675	0.219	0.786	1.159
D-Galacturonic Acid	0.636	0.942	0.241	0.371	0.823	0.585	0.586
L-Asparagine	2.258	0.379	1.597	0.736	1.629	1.376	0.781
Tween 40	0.826	0.737	1.303	0.827	0.439	0.920	1.027
I-Erythritol	0.590	0.304	0.170	0.641	0.195	0.312	0.438
2-Hydroxy Benzoic Acid	0.259	0.158	0.190	0.213	0.210	0.284	0.742
L-Phenylalanine	1.051	0.483	0.422	0.276	0.399	1.031	1.740
Tween 80	0.656	0.432	1.284	1.038	1.201	1.196	0.738
D-Mannitol	0.691	1.501	2.127	0.972	2.493	2.157	2.252
4-Hydroxy Benzoic Acid	0.511	0.611	0.460	0.672	0.22	0.748	0.428
L-Serine	0.838	0.663	0.703	0.800	0.835	0.229	0.537
Alpha-Cyclodextrin	0.249	0.684	0.231	0.201	1.292	1.867	1.032
N-Acetyl-D-Glucosamine	0.753	1.388	1.873	0.707	1.510	2.079	1.320
Gamma-Hydroxybutric Acid	0.859	0.901	0.691	0.345	0.258	0.625	0.755
L-Threonine	0.267	0.651	0.174	0.206	0.285	0.224	0.685
Glycogen	0.603	1.127	0.261	1.123	0.313	0.783	0.812
D-Glucosaminic Acid	0.536	0.594	0.569	0.214	0.634	1.295	1.005
Itaconic Acid	0.207	0.594	0.179	0.287	0.232	0.346	0.348
Glycyl-L-Glutamic Acid	1.457	0.622	0.362	0.456	0.856	0.356	1.105
D-Cellobiose	1.788	0.778	1.006	0.962	1.576	0.851	0.800
Glucose-1-Phosphate	0.225	0.500	0.226	0.356	0.272	0.272	0.296
Alpha-Ketobutyric Acid	0.189	0.276	0.218	0.292	0.233	0.262	0.373
Phenylethylamine	0.713	0.746	0.355	1.687	0.649	1.078	0.975
Alpha-D-Lactose	0.555	0.677	0.193	0.384	0.406	1.117	0.571
D,L-alpha-Glycerol Phosphate	0.228	0.288	0.235	0.180	0.232	0.446	0.336
D-Malic Acid	0.593	0.292	0.999	0.614	1.018	0.595	0.678
Putrescine	0.218	0.591	0.529	0.886	0.715	0.272	0.865

Appendix 3 – Photographs of unusual ascospores of *Mollisia rhizophila*

Appendix 1. A-C. Darkly stained bodies resembling ascospores in size and shape that appear to have budded laterally within the hymenium. Isthmuses shown with arrows. **D, E**. Chains of darkly stained bodies resembling ascospores in size and shape that appear to have fused end to end (arrows). Scale bars A, B ~ 2.5 μm; C, D, E = 16 μm.