MORPHOLOGICAL AND MOLECULAR ASSESSMENT OF ECTOMYCORRHIZAL COMMUNITIES ASSOCIATING WITH BLACK SPRUCE (*PICEA MARIANA* (MILL.) BSP) IN WETLAND AND UPLAND FORESTS IN CENTRAL BRITISH COLUMBIA

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ABSTRACT

Ectomycorrhizal (ECM) symbioses form one of the primary systems for nutrient and energy exchange in northern coniferous forests. ECM communities consist of a diverse array of fungal species that exhibit variable patterns of distribution and abundance, depending on host plant species, soil properties, and environmental factors. Using morphological (microscopy) and molecular (PCR-RFLP) techniques, this study describes ECM community composition and diversity of black spruce across its habitat range in BC's central interior. Habitats included pure black spruce and mixed spruce – tamarack wetlands and black spruce – pine upland forests. Black spruce was found to form ECM with a diverse community of fungal species, several of which were limited to one or two habitats. Although ECM community composition varied between habitats, the total number of types (morphological and molecular) did not. This study emphasizes the importance of sampling across a habitat range to describe ECM communities associated with a particular host species.

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Introduction

Biodiversity is the term adopted to explain all aspects of biological diversity, including species richness (taxonomic diversity), ecosystem complexity (functional diversity) and genetic variation (genetic diversity) (Zak and Visser 1996). The United Nations Conference on Environment and Development at Rio de Janeiro (1992) recognized the planet's dependence on gene, species, population and ecosystem diversity and the serious threats that biodiversity declines pose to human development (Andrén and Balandreau 1999). High diversity has been related to healthy ecosystem function and may provide the means for ecosystems to adapt to changes in environmental conditions such as global climate change, fire, outbreaks of insect or pathogen attacks, or forestry practices.

Soil probably harbors most of the undiscovered biodiversity on earth and the microbial communities within the rhizosphere (zone surrounding plant roots) account for much of the diversity of northern coniferous forests (Amaranthus 1998; Tiedje *et al.* 1999). In these forests, most plants belonging to the families Pinaceae, Fagaceae and Betulaceae normally form mutualistic associations with diverse communities of filamentous fungi known as ectomycorrhizal fungi (Kendrick 1992). The nature of these symbioses is the transfer of poorly accessible, inorganic nutrients (nitrogen and phosphorus) from fungi to plants in exchange for photosynthetically derived, energy-rich carbon compounds to fuel fungal metabolic processes and growth (Harley and Smith 1983; Allen 1991). Ectomycorrhizae (ECM) are essential components of forest ecosystems because of their central role in plant growth and survival, biogeochemical cycling, soil structure, forest food webs, and buffering capacity against environmental stress (Colpaert and van Tichelen 1994; Amaranthus 1998).

Because of their ubiquitous nature and vital importance to plant and soil biology, the focus of recent research has been to describe ECM communities at the taxonomic and genetic levels

to attempt to gain some understanding of functional diversity. There are two major reasons why it is important to understand which fungi are able to form ECM with which plants. First, fungi vary in their ability to form ECM or enhance plant nutrient uptake when grown with different plant species and under different environmental conditions (Molina *et al.* 1992b; Gehring *et al.* 1998). Second, species diversity of fungi appears to be high when host plant species diversity is relatively low (Bruns 1995; Gehring *et al.* 1998). These contributions have advanced our understanding of how ECM communities can vary across different habitats and geographic locations, as well as change in response to environmental changes at scales varying from large disturbance events to local differences in soil characteristics (Haug and Oberwinkler 1987). Applications of such information might include improvements to forestry practices, such as avoiding soil compaction or avoiding the removal of all major refugia for fungal inoculum (Jones *et al.* 2003). Ecosystem recovery programs (such as silviculture or phytoremediation) may also benefit by planting seedlings inoculated with ecologically adapted ECM fungi.

ECM communities can be described by the number of species present (richness), the relative abundance (evenness) of each species, and the physiological role that each species plays in the environment and in interaction(s) with other species (Tiedje *et al.* 1999). Several recent studies have used a combination of morphological and molecular techniques to describe ECM communities in natural ecosystems. The use of light microscopy to directly examine ECM roots allows certain morphological features to be used to taxonomically group ECM into families, genera or species (Agerer 1987-2002; Ingleby *et al.* 1990; Goodman *et al.* 1996); microscopy has the advantage of being relatively inexpensive and enables one to assess large numbers of roots in a short period of time (Taylor *et al.* 2000). Molecular (PCR-RFLP) analysis has the advantage of being largely independent of environmental effects and of being able to improve upon the resolution of ECM identification through

comparisons to reference databases (Egger 1995; Horton and Bruns 2001). In a comparison of methods for assessing diversity of hybrid spruce ECM, Mah *et al.* (2001) found that morphotyping may have underestimated ECM diversity, but identified rare types that did not amplify properly during the PCR analysis. Studies that incorporate both morphological and molecular data provide better descriptions and interpretations of diversity than those that focus on just one approach (Moritz and Hillis 1996).

This study describes the community structure and diversity of ECM associated with naturally regenerating black spruce (*Picea mariana* (Mill.) BSP) across its habitat range in the sub-boreal spruce (SBS) zone of BC's central interior using both morphological (light microscopy) and molecular (PCR-RFLP) techniques. The specific objectives of this study were:

1. To describe the ECM associated with naturally regenerating black spruce seedlings in the Sub-Boreal Spruce (SBS) zone of central BC using morphological techniques (light microscopy).

2. To compare the structure and diversity of black spruce ECM communities between three habitats, including both black spruce-dominated and black spruce – tamarack (*Larix laricina* (Du Roi) K. Koch) wetland forests, as well as black spruce – lodgepole pine (*Pinus contorta* Dougl. *ex* Loud. var. *latifolia*) upland forests.

3. To describe and compare the molecular diversity (using PCR-RFLP analysis) of ECM communities associating with naturally regenerating black spruce seedlings across the three habitats and within each characterized fungal morphotype.

4. To confirm the characterization and improve on the resolution of identified ECM morphotypes by comparing restriction fragment profiles generated from a representative sub-sample of roots from the same morphotype. To also compare these fragment patterns to those in reference databases and descriptions published in the literature.

Literature Review

ECTOMYCORRHIZAE

Definition, classification and structure

Mycorrhizae are mutualistic symbioses (based on a bidirectional exchange of nutrients) between fungi and plant roots (Smith and Read 1997). There are seven currently recognized groups of mycorrhizae, including the vesicular-arbuscular, ectomycorrhizal, ectendomycorrhizal, ericoid, arbutoid, monotropoid and orchid types. These groups are distinguished based on the fungal and plant taxa involved, as well as on differences in structure and physiology of the associations (Allen 1991; Molina *et al.* 1992b; Smith and Read 1997; Ursic and Peterson 1997). All but the ectomycorrhizae exhibit intracellular colonization of the root cortical cells to some extent and, except for the intermediate ectendomycorrhizal type, were formerly classified as endomycorrhizae.

In ectomycorrhizae (ECM), fungal hyphae (the main fungal structures) form a sheath (mantle) around the absorptive areas of growing root tips, which changes fine root morphology (increased thickness, root bifurcation and clustering) for more efficient nutrient transfer between fungus and plant host (Read 1997; Smith and Read 1997). From the mantle, inwardly growing hyphae penetrate the root and grow between the epidermal and cortical cells, producing an intricate netlike structure (the interface of nutrient exchange) known as the Hartig net (Smith and Read 1997). In basidiomycetes, hyphal strands contiguous with the mantle extend into the surrounding soil where they join with the extensive networks of soil mycelia and form essential pathways for nutrient and water transport and connections with fungal fruiting bodies (sporocarps) (Smith and Read 1997). ECM are characteristic of forest trees and shrubs growing in areas where nitrogen (N) and phosphorus (P) availability is seasonal or intermittent and tend to inhabit the acidic litter layers near the soil surface (Allen 1991; Brundrett 1991; Isaac 1992; Kendrick 1992). ECM

fungal colonization has been related to soil moisture, nutrients, organic matter, and pH, with ECM fungi preferring moist but well-drained soils with high organic matter content, low N and P availability, and acidic pH (McAfee and Fortin 1989; Thormann *et al.* 1999).

Functions and benefits of symbioses

ECM symbioses between plants and fungi play some central roles in important ecosystem processes and functions (Brundrett 1991; Read 1991; Cornelissen *et al.* 2001). The structure of ECM not only increases the root absorbing area for greater efficiency of carbon and nutrient exchange between symbionts, but also increases the root area in contact with the soil (Rygiewicz and Andersen 1994). Nitrogen (and to a lesser degree P) is the most important determinant of plant growth and productivity in northern forests (Smith and Read 1997). Ammonium (NH_4^+) is the predominant form of N available to plants, but when soils are relatively acidic, cold, or poorly aerated, the rate of ammonification is generally low, so most N is present in organically-bound forms (Smith and Read 1997). Through increased root absorptive area and soil volume explored, ECM plants have greater access to previously inaccessible sources of inorganic N (NH_4^+) and P in the soil. Colonized roots may also have different uptake properties (such as a lower Michaelis constant [Km]), as uncolonized roots do not absorb P as efficiently (Tinker 1984).

Recent molecular studies have revealed that some fungal species previously regarded (in evolutionary terms) as decomposers of woody debris are both frequent and abundant components of ECM communities (Hibbett *et al.* 2000; Kõljalg *et al.* 2000). These fungi possess proteolytic enzymes involved in the decomposition of organic material and may provide host plants with access to both simple and complex organic forms of N and P (Read 1991; Smith and Read 1997; Read and Perez-Moreno 2003). Future research is required to

assess the roles of ECM fungi capable of degrading recalcitrant compounds in nutrient cycling in forest ecosystems.

The benefits of ECM symbioses to plant hosts have been reported in numerous studies. ECM plants have been shown to exhibit increased growth and survival (Gagnon *et al.* 1988), enhanced rooting (Stein *et al.* 1990), and greater resistance to soil-borne diseases (Morin *et al.* 1999) when compared to non-mycorrhizal plants. Furthermore, the plants require less fertilizer and appear to be better able to withstand environmental pollution, stress, and transplant shock than non-mycorrhizal plants (Anderson 1988; Kendrick 1992; Colpaert and van Tichelen 1994). Some plants are unable to become established or grow normally without an appropriate fungal partner (Harley and Smith 1983). Different species of ECM fungi exhibit phenotypic variation with respect to nutrient transfer and uptake, storage capacity, and promotion of host growth (Haug and Oberwinkler 1987; Bruns 1995). Therefore, it is likely that, for a plant to receive the maximum benefits of ECM symbiosis, it must be associated with the best fungal partner for the environmental conditions present (Haug and Oberwinkler 1987). From bioassay experiments using *Pinus sylvestris* L. and *Betula pendula* Roth, Jonsson *et al.* (2001) recently reported that the effects of ECM symbiosis on host productivity depend on context-specific factors including plant host species, fungal species richness and composition, and soil nutrient regime.

Growing root tips release considerable amounts of photosynthetically-derived carbon (C) into the soil, which plays an important role in soil aggregation, nutrient availability and uptake, and microorganism nutrition (Darrah 1991). ECM fungi require C for sustaining existing fungal biomass (on root tips and in the soil) and for producing new fungal biomass, and acquire most of their C from their plant partners (Harley and Smith 1983; Söderström 1992). Cornelissen *et al.* (2001) suggest that ECM strategies are linked with low ecosystem

C turnover based on intermediate seedling relative growth rates, high foliar N and P content, and intermediate to poor litter decomposition rates as compared to different types of plants harboring ericoid and arbuscular mycorrhizae. Rygiewicz and Andersen (1994) revealed a reduction in overall C retention due to increased C in the roots and higher rates of belowground respiration in a mycorrhizal coniferous seedling (ponderosa pine and *Hebeloma crustuliniforme*) microcosm experiment. The key role of ECM in C cycling (particularly in the positive feedback loop between plant growth rate, leaf and litter quality, and decomposition rate) may have important repercussions for the C gains and losses of ecosystems and thus for the C budget at regional and global scales (Read 1991; Cornelissen *et al.* 2001).

Nutrients are not only exchanged in one plant - one fungus systems, but have been shown to be translocated through soil mycelial networks between the same and different species of plants and fungi (Finlay and Read 1986a, b; Simard *et al.* 1997). In growth chamber experiments, Finlay and Read (1986b) found that P accumulated in mycorrhizal roots of one plant before being transported to shoots of other plants connected through the mycelial network. The distance of translocation was limited only by the size of the growth chamber. It was evident from this study that the plant's investment of C as fuel for fungal metabolic processes provided the potential for exploitation of the soil P resources (Finlay and Read 1986b). Radiolabelled C-transfer has been demonstrated between Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and pine species (*Pinus contorta* Dougl. *ex* Loud. and *P. sylvestris*) in microcosm experiments (Finlay and Read 1986a) and between paper birch (*Betula papyrifera* Marsh.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franc.) in the field (Simard *et al.* 1997). Similarities in ECM communities of lodgepole pine (*Pinus contorta* var. *latifolia*), white spruce (*Picea glauca* (Moench) Voss.) and subalpine fir (*Abies lasiocarpa*

(Hook.) Nutt.) may also indicate potential for hyphal linkages between different plant species (Kranabetter *et al.* 1999).

ECM FUNGAL COMMUNITIES

Composition and diversity

Communities are assemblages of species within a defined area and are described in terms of the species present (composition and richness) and their relative abundance (Egger 1995). At least 5000-6000 species of fungi are involved in ectomycorrhizal associations, representing about 10% of all known soil fungi (Molina *et al.* 1992b; Smith and Read 1997). Most ECM fungi are members of the Basidiomycota, with some representation within the Ascomycota and Zygomycota (Smith and Read 1997). Molecular dating places the basidiomycetes at about 125 million years old, which is about four times younger than the zygomycetes (that form vesicular-arbuscular mycorrhizae) that appear to have originated with land plants (Hibbett *et al.* 2000). Phylogenetic analyses reveal that ECM fungi are not a monophyletic group (i.e. originate from several independent lineages), and symbiosis with plants has been convergently derived (and perhaps lost) many times over millions of years (Bruns 1995; Hibbett *et al.* 2000). Some taxa are closely related to wood-decaying saprobes, some are thought to have wood-rotting ancestors, and some are related to other saprobic taxa (Tanesaka *et al.* 1993; Hibbett *et al.* 2000). Evolutionarily, this variation in ability to degrade wood could have made possible speciation to avoid competition between closely related species that would otherwise use the same resources and occupy the same niche (Tanesaka *et al.* 1993).

Community diversity is the variation in species assemblages within a community, and can be high (many species with relatively even abundance) or low (few species with relatively uneven abundance) (Bruns 1995; Miller 1995). Diversity results from resource partitioning,

disturbance, competition, or interactions with other organisms. It is important to understand factors influencing diversity on a local scale because niche and guild structure among different fungal species may provide information furthering the understanding of the functional significance of ECM diversity (Bruns 1995). ECM fungal communities appear to exhibit high diversity, even within small areas where factors that tend to alter diversity (e.g. host specificity, regional soil differences, climatic differences, and large-scale disturbances) are fairly constant (Bruns 1995; Gehring *et al.* 1998). This high ECM fungal diversity may provide individual trees and entire forests with a range of strategies for efficient functioning in an array of plant-soil systems (Amaranthus 1998). There is still much remaining to be investigated in this area of research.

In field surveys involving various coniferous hosts, species richness (number of ECM types described) has been reported to vary from less than 10 to greater than 50 (Danielson and Pruden 1989; Bradbury 1998; Bradbury *et al.* 1998; Flynn *et al.* 1998; Arocena *et al.* 1999; Hagerman *et al.* 1999a; Kranabetter *et al.* 1999; Mah *et al.* 2001). Bruns (1995) found a range of 20-35 fungal species for several small sites with homogeneous environmental conditions and occupied by a single host species. Studies generally report ECM community structures consisting of few very abundant fungal species and many less abundant or rare species (Kranabetter *et al.* 1999; Taylor *et al.* 2000; Kranabetter and Friesen 2002).

Factors that alter diversity in ECM communities

Host specificity and receptivity

The composition of ECM communities may include several types of ECM fungi, depending on the specificity of the host and the fungus (Egger 1995). ECM fungi form associations with about 30 families of plants, including many important timber species within the Pinaceae, Betulaceae, Fagaceae and Dipterocarpaceae (Smith and Read 1997). Often,

specific host characteristics determine the presence and abundance of fungal species (Samson and Fortin 1986). Host specificity refers to the range of plants with which fungal species can form functional ECM, and varies from narrow (genus- or family-specific) to broad (several different orders or classes) (Molina *et al.* 1992a, b). Many fungal genera show relatively even distribution along the host range spectrum. Host receptivity refers to the range of fungi with which a host plant can form functional ECM, varying from those plants receptive to few to those receptive to many fungal symbionts (Molina *et al.* 1992b). Host receptivity is difficult to measure, although measurements of the fungal diversity associated with a particular host may indirectly reflect this receptivity.

Host-specific fungi provide biological mechanisms to partition soil resources and provide nutrients to specific plant species, but are restricted by the ecological tolerances of their hosts (Brundrett 1991; Molina *et al.* 1992b). For example, *Suillus grevillei* is consistently associated with tamarack over diverse environmental conditions ranging from bogs to well-drained sandy sites (Samson and Fortin 1986). Although host-specific fungi show strongly specialized relationships with specific plants in nature, they have been induced to associate with other genera in pure culture (Molina *et al.* 1992b). Samson and Fortin (1986) grew tamarack seeds in sterile vermiculite and inoculated plantlets with fungi cultured from sporocarps collected beneath tamarack or black spruce stands. ECM fungi previously identified as sporocarp-specific to *Larix* species under field conditions showed faster and better ECM development with this host, indicating some degree of host specialization. Eight of the 12 fungi collected in the vicinity of black spruce also formed ECM with tamarack. Massicotte *et al.* (1994) reported that some *Rhizopogon* species that were restricted to one host in monoculture were able to extend their host range to a nearby (companion) plant in dual culture. This study shows, at least in pure culture and pot experiments, that the presence of companion plants appears to influence the ability of some less host-specific

fungi to colonize neighboring plants by first developing on a primary host, and then spreading onto a secondary host (Walker 1987; Massicotte *et al.* 1994; Massicotte *et al.* 1999). Walker (1987) suggested that pines and larches may be more easily colonized than spruces, and that whereas some fungi may be unable to establish symbioses with spruces alone, they may succeed if pines or larches are present to provide a source of inoculum (the spruce mixture effect). In natural systems, ecological specificity may be more important than host specificity, as companion plants may influence the ability of ECM fungi to colonize neighboring plants (Massicotte *et al.* 1999). Fungal mycelia extending from ectomycorrhizal fine roots of mature trees are thought to be an important source of inoculum available to regenerating or outplanted seedlings (Finlay and Read 1986a; Hagerman *et al.* 1999). The inoculum potential of a fungal mycelium may depend on whether it is linked to a compatible host (Massicotte *et al.* 1994).

Overlaps in host compatibility (i.e. lack of host specificity) permit fungi to connect inter- and intra-specific combinations of host plants through a common mycelial network (Finlay and Read 1986a; Molina *et al.* 1992a, b; Read 1997; Simard *et al.* 1997). These mycorrhizal guilds are groups of plants and fungi sharing compatibilities and forming a functional unit in space and time (Massicotte *et al.* 1999). Linkages play a key role in mycorrhizal community structure and dynamics as they are important for nutrient flow via mycorrhizal mycelia. It has been suggested that hosts such as red alder (*Alnus rubra* Bong.) and Douglas-fir, which often form pure stands early in their succession, tend to form specific associations with ECM fungi, while species such as western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), which grows in the shaded understory of other trees, tend to form non-specific interactions (Kropp and Trappe 1982).

Environmental gradients

Host plant community composition may not accurately reflect fungal community composition, especially across sites with heterogeneous soil conditions (Gehring *et al.* 1998). Environmental gradients help explain patterns of fungal species distribution across landscapes (O'Dell *et al.* 1999). For example, the growth responses of different ECM fungi vary with soil moisture content, and both drought and waterlogging can be limiting factors to ECM formation (Stenström 1991). In waterlogged soils, oxygen deficiency is expected to slow or prevent ECM formation due to inhibition of fungal aerobic metabolic processes (Tinker 1984; Walker 1987; Stenström 1991). The accompanying changes in soil chemistry and redox potential also may favor the accumulation of compounds toxic to fungi (Isaac 1992). *In vitro* experiments to examine the effect of brief flooding on mycorrhizal formation were conducted by Stenström (1991). She found that different groups of ECM fungi varied in their susceptibility to flooding, and also that waterlogging was not associated with fungal oxygen deficiency, possibly because many forest fungi have hydrophobic mycelia, which may provide air pockets in the soil. The effect of dry soil conditions on ECM fungi is not well understood. In greenhouse and field experiments with Norway spruce (*Picea abies* (L.) Karst.), Feil *et al.* (1988) found that drought conditions did not completely inhibit ECM growth, but resulted in an increased branching density of very fine roots, thereby providing contact with greater volumes of soil. This may be an adaptation to water stress that allows enhanced uptake of water from dry soils via ECM.

Nitrogen availability may also be a major factor in structuring ECM fungal communities. Lilleskov *et al.* (2002) recently described a shift in ECM fungal community composition from taxa specialized for N uptake under low N conditions, to taxa specialized for high overall nutrient uptake, and finally to taxa specialized for P uptake under high N, low P, and acidified conditions. Taylor *et al.* (2000) also reported shifts in species composition of ECM

communities in response to increased N input in European spruce and beech forests. The consequences of any changes in ECM fungal community structure for ecosystem function and plant nutrition depend on how community function changes as community structure changes.

Few (if any) studies have attempted to relate ECM fungal distribution to environmental gradients independent of plant hosts (O'Dell *et al.* 1999). Thus, the effects of environmental gradients on ECM fungal communities are difficult to assess in natural soils because gradients may limit the distribution of the host (Gehring *et al.* 1998; O'Dell *et al.* 1999). In ECM fungal sporocarp surveys, Nantel and Neumann (1992) found mushrooms associated with a particular tree species were only found with that species over part of its range. Similarly, O'Dell *et al.* (1999) found some ECM fungal sporocarps under hemlock were restricted to the dry end of the soil moisture gradient (e.g. *Cortinarius olympianus* and *Russula brevipes*), while other fungi were restricted to the wet end of the gradient (e.g. *Amanita constricta* and *Boletus mirabilis*). The maximum number of species was found midway along the soil moisture gradient. Although sporocarp abundance does not directly represent the abundance of ECM belowground (Egger 1995; Mehmann *et al.* 1995; Gardes and Bruns 1996), both studies demonstrate the differences in soil moisture tolerances of different fungal species. In response to long-term atmospheric N deposition in Alaska, Lilleskov *et al.* (2002) found a drastic decline in ECM fungal abundance and diversity. Taylor *et al.* (2000) also reported decreased ECM community diversity with increased N input in Europe. This response occurred rapidly in sporocarp communities and more slowly in belowground ECM communities. Through direct examination of ECM root tips, Walker (1987) described differences in Sitka spruce ECM community diversity that corresponded to changes in soil conditions and nutrient availability. Similarly, Gehring *et al.* (1998) found variable patterns of ECM fungal species occurrence and abundance on Pinyon pine (*Pinus*

edulis Engelm.) roots across environmental gradients ranging from very dry, nutrient-poor soils to sandy loam soils in Arizona, but no difference in fungal species richness. They also found that each tree was dominated by a single fungal species, but that the same species did not dominate on all trees. From the results presented in these studies, it appears that ECM fungal community composition depends on the individual tolerances of individual species for specific site conditions (Doudrick *et al.* 1990). Plants with access to a broad diversity of ECM fungi are probably colonized by those best suited to the range of soil conditions present and may be more capable of adapting to changes in the environment (Amaranthus 1998; Durall *et al.* 1999).

Disturbance and other factors

The role of disturbance in maintaining ECM fungal diversity is to open up patches for colonization (Bruns 1995). Changes associated with disturbance events may decrease C input to fungi, modify the age and species of plants present on a site, remove or displace the forest floor, lose large overstory trees that can change the physical environment of the soil, and alter the composition of soil microflora and fauna communities (Jones *et al.* 2003). These changes might select for different communities of ECM fungi. There are many recent studies that focus on comparing ECM fungal community diversity between sites differing in some form of disturbance (Bradbury 1998; Bradbury *et al.* 1998; Baldwin 1999; Durall *et al.* 1999; Hagerman *et al.* 1999; Kranabetter *et al.* 1999; Stendell *et al.* 1999; Byrd *et al.* 2000; Mah *et al.* 2001; Kranabetter and Friesen 2002; Jones *et al.* 2003). These studies usually found decreased fungal species richness and a shift in abundance for many species following disturbance events such as fire or forestry practices. Very recently, Jones *et al.* (2003) concluded that the major impact of forestry practices on ECM fungal communities is a shift in species composition rather than a reduction in the percentage of roots colonized.

Numerous other biotic and abiotic factors may influence ECM fungal community composition and diversity on very localized scales. This is due to the high heterogeneity of the soil environment, which can be partitioned into multiple niches, each with its own unique combination of nutrient source, moisture level, physical and chemical properties, and particle size distribution (Egger 1995; Gehring *et al.* 1998). Local climate, topography, and aspect may also alter fungal communities. Some authors suggest that changes in community composition and diversity occur as forest stands mature, and there is evidence for replacement competition in ECM fungi (Bruns 1995). Fungal community composition and diversity appear to shift throughout forest succession, until fungi specialized to the climax host and conditions dominate the community (Doudrick *et al.* 1990; Brundrett 1991).

METHODS OF STUDYING ECM COMMUNITIES

Morphological techniques (microscopy)

It is generally agreed that attempts to isolate and culture ECM directly from root tips are of limited value to community studies because many ECM fungi will not grow or may exhibit altered characteristics under experimental conditions in the laboratory. Instead, the use of light microscopy to directly examine ECM roots allows certain morphological features to be used to taxonomically group ECM into families, genera, or species (Agerer 1987-2002; Ingleby *et al.* 1990; Goodman *et al.* 1996). Morphological characterization (morphotyping) of ECM has the advantage that it is relatively inexpensive and enables large numbers of roots to be assessed in a short period of time (Taylor *et al.* 2000). Although some morphological features such as color and ramification may vary under different environmental conditions, other features such as mantle characteristics and hyphal associations do not change considerably (Haug and Oberwinkler 1987; Bruns and Gardes 1993; Egger 1995). However, the taxonomic distinction between similar morphotypes is

sometimes difficult, and whereas some fungi may be identified to species, others may be identified only to genus, family, or larger grouping.

Molecular techniques (PCR-RFLP)

The most important methodological advance in ECM community studies is probably the use of the polymerase chain reaction (PCR) to amplify target sequences of DNA (Horton and Bruns 2001). The DNA most commonly targeted in these studies is the nuclear-encoded ribosomal DNA (rDNA), which is present in high copy number and consists of highly conserved coding regions (the ribosomal small subunit (18S) and large subunit (28S) genes) as well as noncoding (internal transcribed spacer (ITS)) regions between genes (Bruns and Gardes 1993; Egger 1995; Horton and Bruns 2001). The conserved sequences are suitable targets for universal and fungal-specific oligonucleotide primer pairs that are used to selectively amplify specific regions of the more variable ITS regions (Egger 1995; Horton and Bruns 2001).

Restriction fragment length polymorphism (RFLP), the use of restriction endonucleases to digest the amplified rDNA at specific target sequences, is often used in conjunction with PCR (Egger 1995). Sequence differences in ITS DNA are usually the result of insertions or deletions causing length variation (Horton and Bruns 2001). Thus, enzymatic digestion of ITS DNA with just a few restriction endonucleases produces DNA fragments of varying sizes (providing there is no overlap in the specific DNA sequences where cleavage occurs) that may be used to distinguish between closely related species (Egger 1995; Kraigher *et al.* 1995; Mehmann *et al.* 1995). Comparisons of ECM and sporocarp restriction fragments may facilitate fungal identification as sporocarps are generally much easier to identify than root-associated ECM fungi. The RFLP patterns from ECM do not always agree with sporocarp patterns (Gardes and Bruns 1996; Dahlberg *et al.* 1997), but closely related fungi

can often be grouped together based on the presence or absence of ITS fragments (Kårén *et al.* 1997). Providing the same protocols, primers and restriction endonucleases are used, databases of restriction fragment patterns allow comparisons of ECM fungal communities across different studies (Mah *et al.* 2001).

Molecular techniques have the advantages that they are largely independent of environmental effects and improve the resolution of ECM identification. Whereas microscopic examination of ECM is suitable for sorting fungi into families and genera based on differences in morphological characteristics, molecular analyses (PCR-RFLP) potentially allow identification of fungal species, although identification is limited by the availability of reference databases (Egger 1995; Horton and Bruns 2001). Recent ECM community studies have employed a combination of morphological and molecular methods. In a comparison of methods for assessing diversity of hybrid spruce ECM, Mah *et al.* (2001) found that morphotyping may have underestimated ECM diversity, but identified rare types that did not amplify properly during the PCR. According to Moritz and Hillis (1996), studies that incorporate both morphological and molecular data provide better descriptions and interpretations of diversity than those that focus on just one approach for estimating phylogeny. Phylogenetic analysis of the ITS-RFLPs within morphotypes revealed that morphological classification was useful for grouping ECM formed by the same fungal genera and families for many, but not all, fungi (Sakakibara *et al.* 2002).

Measuring ECM community structure and diversity

Community structure can be compared between habitats through analysis of species abundance patterns (β-diversity) within communities (Taylor *et al.* 2000). Rank-abundance curves (plot of arithmetic rank of species versus log abundance) of ECM types are generally straight lines, indicating the dominance of a few fungal types (Taylor *et al.* 2000). Less

diverse and more uneven distributions of morphotypes (broken stick distribution) have also been sometimes observed (Kranabetter and Friesen 2002). These curves are thought to result from situations where availability of a single prevailing ecological resource equally constrains all species present in the community (Taylor *et al.* 2000).

Species richness measures can be expressed as simple counts of individuals, proportions of total populations, or averages per sampling unit. Community diversity (species richness and/or abundance) is often compared between different treatments or studies by calculating non-parametric diversity indices and comparing the means by analysis of variance (ANOVA) (Magurran 1988; Krebs 1989). Information theory indices (e.g. Margalef, Shannon, Shannon Evenness indices) attempt to measure the amount of order in a system by assuming that all individuals are randomly sampled from an infinitely large population, and that all species are represented in a sample (Krebs 1989). The Margalef Index is a measure of species richness and is calculated by dividing one less than the number of species (S) by the natural log of the number of individuals (n), or (S-1 / ln n) (Magurran 1988). The Shannon Index equally emphasizes both richness and abundance (evenness) by determining the relative contribution of each species to the community (Taylor *et al.* 2000). It is calculated from $-\Sigma\, p_i\, (\ln p_i)$, where p_i is the proportion of individuals in the i^{th} species (Magurran 1988). Dividing the Shannon Index by the natural log of the number of species (S) gives the ratio of observed to maximum diversity, and is a measure of species evenness (Shannon Evenness Index) (Magurran 1988). Because the Shannon and Shannon Evenness indices are based on proportional abundances of all morphotypes present (heterogeneity indices), they are sensitive to the presence of rare ECM types. For each of these measures, the diversity index increases with an increase in number of morphotypes. Dominance measures (e.g. Simpson Index) are heterogeneity measures that are weighted towards the more abundant species in a sample. The Simpson Index, which suggests that

diversity is inversely related to the probability that two samples picked at random belong to the same morphotype, is calculated from Σp_i^2, and is usually expressed as its reciprocal value (Magurran 1988).

Genetic diversity of various populations and communities of fungi may also be compared across different treatments and studies, as long as the same molecular methods were used. Any of the previously described non-parametric diversity indices may be calculated for molecularly-derived data by replacing proportional abundance of morphotypes with proportional abundance of genotypes (defined by similarities in restriction fragment patterns or sequences). Some suggest that these analytical approaches to fungal diversity may be of limited value in community studies as they employ sampling approaches and diversity indices that were designed for unitary organisms (Zak and Visser 1996). In particular, the abundance component of diversity may be lost due to loss of samples during the various phases of the molecular procedures.

The Phi (Φ) Index was recently derived to attempt to resolve issues regarding the use of proportional abundance in calculating ECM diversity measures (Mah *et al.* 2001). This index is based on pairwise distances (obtained from the Dice index distance matrices) of ECM restriction fragment patterns and ranges from zero (identical fragment patterns between pairs of samples) to 1 (no fragments shared between pairs of samples).
In a data matrix with i rows and j columns, the pairwise distances (d) for individual root tips are squared, summed, and divided by one less than the total number of samples (n-1). To calculate the Phi Index, the sum of these average squared distances for each column is divided by the total number of samples (n):

$$\Phi = \sum_{j=1}^{n} [\sum_{l=1}^{n} d^2_{ij} / n\text{-}1] / n \qquad \text{where } i = j = n$$

As with the non-parametric diversity index values, an increase in Phi values implies greater diversity.

BLACK SPRUCE AS AN ECM HOST

Distribution

Black spruce is the most widely distributed tree species of the Canadian boreal forest. Its range extends from Alaska through all the provinces and territories of Canada, and reaches south into the United States below eastern Manitoba (Farrar 1995). In British Columbia, its range is limited to the northern regions of the province where it is common north of the Fraser River, and occurs in patches southward to just north of Quesnel (Brayshaw 1996). Its western limit is Seely Lake, between Smithers and Terrace, and it extends past McBride to the east. It is found in the Boreal White and Black Spruce (BWBS), Spruce-Willow-Birch (SWB), Sub-Boreal Spruce (SBS), Sub-Boreal Pine - Spruce (SBPS), Interior Cedar-Hemlock (ICH) and Engelmann spruce – subalpine fir (ESSF) biogeoclimatic zones (Meidinger *et al.* 1991; Brayshaw 1996; C. DeLong, pers. comm.).

In the central interior of BC, black spruce occurs in the SBS zone, which includes the Nechako and Fraser plateaus and Fraser Basin from valley bottoms to 1100-1300 m elevation (Meidinger *et al.* 1991). The "typical" SBS subzone is SBSmk (moist, cold), which ranges from Prince George to Fort St. James to Nation Lakes to the Williston Reservoir (Meidinger *et al.* 1991). The climate of the SBS zone is characterized by severe, snowy winters, warm, moist and short summers, and moderate annual precipitation. Forests are broadly transitional between the Douglas-fir and pine - spruce forests to the south and southwest, and the boreal forests to the north (Meidinger *et al.* 1991).

The commercial range of black spruce is considerably less than its geographic range (Viereck and Johnston 1990). It is one of the major timber crop species in eastern Canada

as its long wood fibres make it ideal for pulp and paper production. In BC, black spruce is considered undesirable and is not selectively harvested (Krestov *et al.* 2000).

Habitat preferences

Black spruce is typically found in cold, poorly drained, nutrient-poor habitats, and generally increases in abundance with increasing latitude and decreasing soil drainage (Argus *et al.* 1992; Krestov *et al.* 2000). The most productive growth of black spruce occurs in subhygric (where water is removed slowly so that soil remains moist for a significant portion of the growing season) and submesic (where water is removed rapidly in relation to supply and is available for only short times following precipitation) soils (Krajina *et al.* 1982; Meidinger *et al.* 1991). Substrates are usually wet organic soils, but black spruce also grows on deep humus, clays, loam, sand, coarse till, boulder pavements and shallow soil mantles over bedrock (Viereck and Johnston 1990; Meidinger *et al.* 1991; Krestov *et al.* 2000). It commonly occurs in acidic boreal bogs and fens, as well as in neutral or alkaline swamps, where it tends to establish on hummock-like mounds of decaying wood material. This remarkable ability to grow in a range of soil conditions makes black spruce an ideal host species for studying ECM community variation across an ecological gradient.

In central BC, black spruce typically grows in *Sphagnum* –dominated wetlands, in pure stands, or with tamarack (*Larix laricina*), a species at the southwestern edge of its range in the central interior (Krajina *et al.* 1982; Meidinger *et al.* 1991; Farrar 1995; Krestov *et al.* 2000). Tamarack is a rare species in the SBS that occurs in only a few fens and swamps of the Nechako, Chilako and Blackwater drainages (Krajina *et al.* 1982; Meidinger *et al.* 1991; Krestov *et al.* 2000). Wetland soils are in the Organic order and vegetation communities include sedge marshes, shrub fens, black spruce and hybrid white spruce fens and swamps, and black spruce – *Sphagnum* bogs (Meidinger *et al.* 1991; Soil Classification Working

Group 1998). Associated vegetation commonly includes scrub birch (*Betula glandulosa* var. *glandulosa* Michx.), Labrador tea (*Ledum groenlandicum* Oeder), and willow (*Salix*) species (Krestov *et al.* 2000).

Black spruce is also found in mid-seral upland habitats, associated with lodgepole pine (*Pinus contorta* var. *latifolia*), interior hybrid spruce (*Picea glauca* (Moench) Voss x *engelmannii* Parry ex Engelm.) and trembling aspen (*Populus tremuloides* Michx.) (Krajina *et al.* 1982; Meidinger *et al.* 1991; Farrar 1995; Krestov *et al.* 2000). Upland forest soils are primarily Luvisols, Podzols, Brunisols and Gleysols (Meidinger *et al.* 1991), and sites are dominated by conifers, mosses, lichens and dwarf woody plants, with reindeer lichen (*Cladina* species), lingonberry (*Vaccinium vitis-idaea* L.), highbush cranberry (*Viburnum edule* (Michx.) Raf.), soopolallie (*Shepherdia canadensis* (L.) Nutt.), and common mitrewort (*Mitella nuda* L.) representing dominant plant associations (Krestov *et al.* 2000). These plant associations generally indicate low to medium productivity communities developed on nutrient-poor, water-deficient sites. However, soil moisture conditions may actually vary from water surplus (after snowmelt and summer precipitation events) to water deficit (in late spring), depending on local topography, soil texture and structure, and drainage constraints posed by the presence of a root-restricting clay soil horizon near the soil surface (Krestov *et al.* 2000).

Morphology and growth

Black spruce is usually a small tree with a characteristic narrow crown (which is often deformed near the top) of branches that turn down and out (Brayshaw 1996). It is a shade-tolerant species with modest spatial requirements (Krajina *et al.* 1982). The needles are dull gray-green, 8-15 cm long, straight and blunt, and are densely set along dark orange or brown twigs that have many short brown hairs. The seed cones are ovoid, 2-3 cm long and

purple-brown at maturity (Farrar 1995). It has a shallow rooting system, an adaptation to permafrost, as it can grow on ground solidly frozen for 5-8 months of the year (Krajina *et al.* 1982). Some roots may penetrate to a depth of 60 cm, but most spread laterally at the moss-humus interface so that the bulk of root biomass is in the top 20 cm of soil. With the rapid accumulation of the organic layer each year, younger adventitious roots grow from the main stem (Viereck and Johnston 1990). Roots function as an anchor for aerial shoots as well as storage organs for nutrients or water.

Black spruce reaches its final developmental stage at 10-15 years, and possesses the ability to restore its structure and integrity as an adaptive response to increased physiological needs or as a reaction to environmental stress (Bégin and Filion 1999). This ability accounts for the high plasticity of the species, which can maintain reproductive sustainability in the absence of cone or seed production and grow in spite of harsh climatic conditions. Over its range and in different habitats, black spruce varies in height growth rate and other phenological traits. For example, in northwestern Ontario, it was observed that black spruce trees in bogs were slow-growing and stunted, while better drained soils of upland sites supported trees that were more robust (Parker *et al.* 1983). To determine whether local site differences corresponded to morphological or chemical differentiation in mature black spruce, Parker *et al.* (1983) compared 17 morphological characters of cones, needles and twigs from the crowns of trees from three sites differing in level of soil drainage. Significant differences in mean measurements were detected for only one characteristic (cone scale concavity), leading them to conclude that ecotypic differentiation is not well developed in black spruce in response to soil moisture level. This was surprising considering the variety of moisture regimes, soil types, levels and types of competition, and gross differences in black spruce phenotypes corresponding to the three habitat types. Although differences were not detected between treatments, there was high variation in characteristics within

sites with poor drainage (Parker *et al.* 1983). In a comparison of water relations in black spruce seedlings of upland versus wetland origin, Zine El Abidine *et al.* (1994, 1995) found no consistent differences, again indicating a lack of ecotypic variation for this species. Though black spruce is very sensitive to drought, Zine El Abidine *et al.* (1994) demonstrated that seedlings can be preconditioned for increased drought resistance. However, the physiological effects of the preconditioning are variable, and response is not cumulative with successive drought episodes (Zine El Abidine *et al.* 1994, 1995).

In wetlands, plant growth is extremely slow due to high water tables, poor aeration, cold substrates and low nutrient availability (Lieffers and Rothwell 1987a; Macdonald and Yin 1999; Mugasha *et al.* 1999). High water tables decrease root growth and nutrient availability (N assimilation) in the rhizosphere (root zone), and lower oxygen levels can result in root asphyxiation and rotting (Roy *et al.* 1999). Anaerobic conditions may also favor microbial activities that produce nitrites and other compounds toxic to the plant (Isaac 1992). Whereas these conditions tend to limit root growth and survival of most woody plant species, black spruce is fairly tolerant of flooding and poor aeration (Lieffers and Rothwell 1987b; Steele *et al.* 1997; Roy *et al.* 1999). It tends to avoid total submergence by establishing on hummocks of decaying wood material and *Sphagnum* and producing small volumes of shallow roots that spread laterally (in the top 5-20 cm) at the moss-humus interface (Lieffers and Rothwell 1987b; Conlin and Lieffers 1993; Steele *et al.* 1997; Roy *et al.* 1999). The lateral roots of mature trees may extend as far as 10 m from the taproot, and interweave with the roots of neighboring trees for anchorage (Krajina *et al.* 1982). Both growth rate and rooting depth have been strongly correlated with depth of the water table and soil temperature, with deeper penetration (to 60 cm) and increased growth observed in drier and warmer peatlands (Lieffers and Rothwell 1987b; Conlin and Lieffers 1993). Cold and wet conditions also decrease the rate of microbial decomposition, which leads to rapid

accumulation of organic material around slow-growing seedlings. Vegetative layering (the rooting of attached branches) is often the primary means of regeneration in organic soils, where accumulation of mosses cover the lower branches of slow growing seedings or saplings (Viereck and Johnston 1990). Where conditions are favorable for layering and unfavorable for seed germination and survival, layering can result in an extending clump of trees all derived from one seedling (Farrar 1995).

Growth on upland sites is comparable to white spruce and on nutrient-rich soils, the two species compete (Krajina *et al.* 1982; Farrar 1995). Black spruce is well adapted to acidic mor humus forms (containing unincorporated organic material) in forests with a high accumulation of spruce litter (Krajina *et al.* 1982). The supply of calcium and magnesium is low in these habitats, indicating it may not require as high a supply as white or Engelmann spruces. Greater quantities of calcium, magnesium and nitrates appear to function adversely on the growth of black spruce, as it exhibits stunted growth in krummholz form on limestone and dolomitic substrata (where white spruce grows well). It has a greater dependency on the NH_4^+ form of N as evidenced by poor growth with even the presence of a small quantity of nitrate (Krajina *et al.* 1982).

ECM fungal associates

Although there have been few attempts to describe the ECM fungal community associated with black spruce, studies examining the mycorrhizal status of this host have reported that the majority of field-collected black spruce roots are indeed mycorrhizal (Malloch and Malloch 1981; McAfee and Fortin 1986; Browning *et al.* 1991; Baldwin 1999; Thormann *et al.* 1999). Field surveys in Ontario, Quebec, and Alberta have identified *Cenococcum geophilum* as a common ECM symbiont, but further characterization of other black spruce ECM has not been attempted (Malloch and Malloch 1981; McAfee and Fortin 1986;

Thormann *et al.* 1999). Summerbell (1989) cultured several ECM fungi from black spruce roots collected from different habitats in Ontario and found the most abundant to be the common, broad host range fungus, *Mycelium radicis atrovirens* (*MRA*). Thormann *et al.* (1999) also found *MRA* on black spruce roots from northern Alberta fens.

Scales and Peterson (1991) described non-mycorrhizal black spruce (growing in pouches) first order lateral roots as slender, with rounded apices and many long, straight root hairs. Ectomycorrhizal roots exhibited at least one continuous layer of mantle tissue and one or more discontinuous layers of vacuolate hyphae surrounding the root. Several fungi have been reported to form ECM with black spruce under laboratory or greenhouse conditions. In pot and growth pouch experiments, ECM were formed with *Hebeloma cylindrosporum*, *Laccaria bicolor*, *L. proxima*, *Pisolithus tinctorius*, *Tricholoma pessundatum*, and *Wilcoxina* species (E-strain fungi) (Thomson *et al.* 1989; Browning and Whitney 1991; Scales and Peterson 1991). In aseptic culture, Doudrick *et al.* (1990) found *Cenococcum*, *L. bicolor*, *L. laccata*, *Rhizopogon* sp. and *Suillus cavipes* cultured from sporocarps formed ECM with black spruce. From this study, the authors concluded that fungal associations with young black spruce on mineral soils appear to have a broad host range and thus a local inoculum source (i.e. other hosts). Browning and Whitney (1991) described black spruce ECM formed upon inoculation with *L. bicolor*, *L. proxima* and *P. tinctorius*, but found that *C. geophilum* and *Suillus granulatus* formed very few ECM. Recently, Piercy *et al.* (2002) described the occurrence of intracellular hyphae of the inoperculate discomycetes *Hymenoscyphus ericae* and Variable White Taxon (which usually form ericoid mycorrhizae) in aseptic culture with black spruce seedlings. In bioassays, Gagnon *et al.* (1988) demonstrated increased growth and survival of black spruce seedlings grown in containers with low N after inoculation with *L. bicolor*, and Stein *et al.* (1990) documented enhanced rooting (increased rooting percent, number of adventitious roots per cutting, and mean root length) of cuttings after inoculation

with *L. bicolor* or *S. cavipes*. Morin *et al.* (1999) found the ECM fungi *Paxillus involutus*, *Hebeloma cylindrosporum* and *Tricholoma* species inhibited *Cylindrocladium floridanum*, the pathogen causing root rot and severe mortality in many conifers including black spruce. There are no known studies that have attempted to describe ECM community structure and diversity on this host, nor to compare black spruce ECM communities across different habitats.

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Morphological Characterization of Ectomycorrhizai Communities Associating with Black Spruce (*Picea mariana*) in Wetland and Upland Forests in the Centrai Interior of BC

ABSTRACT

To compare ectomycorrhizal (ECM) community composition and diversity across black spruce habitats in central BC, 15 naturally regenerating black spruce seedlings were randomly sampled from three sites within each of three habitats, including black spruce-dominated wetland forests, mixed black spruce – tamarack wetland forests, and mixed black spruce – lodgepole pine upland forests. Two hundred root tips per seedling were characterized using morphological techniques (light microscopy). A total of 33 distinct ECM morphotypes were described and 14 morphotypes were found on three or more seedlings. Some morphotypes (e.g. *Cenococcum*, *MRA*, *Amphinema*, Russulaceae 2 and Cortinariaceae 1 and 2) were found in all habitats, whereas others (e.g. *Piloderma*, *Tomentella* and Russulaceae 1) were associated with specific habitats. Seedlings from the black spruce-dominated wetlands had a significantly greater proportion of non-mycorrhizal root tips as compared to the mixed species habitats. Morphotype abundance varied significantly between habitats for six of the 14 most frequently occurring morphotypes, but the total number of ECM types described per habitat did not. In general, ECM community diversity was greatest in the black spruce – pine uplands and lowest in the black spruce-dominated wetlands, with significant differences between habitats detected for only those measures emphasizing species richness (i.e. Margalef and Shannon indices). Variation in ECM community composition and diversity may be due to differences in soil moisture and nutrient availability (between the wetland and upland habitats) or alternate inoculum sources (between the pure and mixed species stands).

INTRODUCTION

In northern coniferous forests, the roots of most woody plants are colonized by a diverse group of filamentous fungi that form mutualistic symbioses known as ectomycorrhizae (ECM) (Harley and Smith 1983). The nature of this association is such that inaccessible pools of nutrients (N and P) and water are supplied to the plant through their fungal partners, while energy-rich carbon compounds that support fungal metabolism are supplied to the fungi via their plant hosts (Harley and Smith 1983). These symbioses are vitally important to forest health and ecosystem function as they mediate nutrient and water uptake, protect roots from pathogens and environmental stress, and maintain soil structure and food webs (Brundrett 1991; Colpaert and van Tichelen 1994; Amaranthus 1998; Morin *et al.* 1999). ECM communities often exhibit high diversity (species richness) even when plant species diversity is low (Bruns 1995). The variation in ECM community diversity across landscapes has been widely reported, and depends on preferences (or tolerances) of individual fungal species for the plant hosts present, soil conditions (e.g. moisture and nutrient regimes, organic content, temperature, etc.), disturbance patterns, and other site-specific ecological and environmental factors (Doudrick *et al.* 1990; Brundrett 1991; Bruns 1995). It has been proposed that plants with access to a diverse array of fungi are most likely colonized by those best suited to the range of conditions present, and that these plants may be better able to adapt to changes in environmental conditions (Haug and Oberwinkler 1987; Amaranthus 1998; Durall *et al.* 1999). Because of their ubiquitous nature and importance to plant and soil biology, there has been growing interest in characterizing ECM in natural systems in order to try to understand the functional significance of individual fungal species.

The focus of many recent studies has been to document variation in ECM communities (species composition and diversity) between disturbed (e.g. by fire, forestry practices, etc.)

and control sites within similar forest types (Bradbury 1998; Bradbury *et al.* 1998; Byrd *et al.* 1999; Hagerman *et al.* 1999; Kranabetter *et al.* 1999; Stendell *et al.* 1999; Mah *et al.* 2001). Fewer studies have examined ECM communities in undisturbed forests, where patterns of both plant and fungal species distribution across landscapes are at least partly determined by environmental (particularly moisture) gradients (Gehring *et al.* 1998; O'Dell *et al.* 1999). Rarely do studies attempt to relate ECM distribution to environmental gradients independent of hosts (O'Dell *et al.* 1999). For example, Gehring *et al.* (1998) reported a change in ECM community composition associated with pinyon pine (*Pinus edulis*) across a moisture gradient (xeric to mesic), but no significant decrease in the overall number of species (species richness) found at different sites. Black spruce (*Picea mariana* (Mill.) BSP), a species with broad tolerances for soil moisture conditions (subhygric to submesic), offers an excellent opportunity to examine variation in ECM communities over an environmental gradient.

For most woody plant species, growth and survival are limited in water-saturated soils due to poor aeration, decreased nutrient availability (N assimilation), and changes in soil chemistry and microbial metabolic processes that mobilize potentially toxic compounds in the rhizosphere (Tinker 1984; Isaac 1992; Roy *et al.* 1999). Wetland species such as black spruce are well adapted to these conditions, establishing on hummocks of woody debris and producing small volumes of shallow roots that spread laterally (in the top 5-20 cm) at the moss-humus interface (Lieffers and Rothwell 1987; Conlin and Lieffers 1993; Steele *et al.* 1997; Roy *et al.* 1999). Rooting depth is limited by the depth of the water table and soil temperature, with deeper penetration (to 60 cm) and increased growth observed in drier and warmer peatlands (Lieffers and Rothwell 1987; Conlin and Lieffers 1993). In these organic soils, low dissolved oxygen content and differences in soil chemistry may inhibit ECM formation by limiting fungal aerobic metabolic processes (Tinker 1984; Walker 1987;

Stenström 1991). However, studies conducted *in vitro* on inoculated jack pine (*Pinus sylvestris*) seedlings showed that different species of ECM fungi varied in their susceptibility to periodic flooding (Stenström 1991). *Hebeloma*, *Laccaria* and *Thelephora* were tolerant of water saturated soil conditions, while *Suillus* species were very sensitive to even brief periods of flooding conditions. Despite challenges presented to ECM formation in wetland habitats, all woody plant species surveyed in bogs and fens in northern Alberta were found to be mycorrhizal (Thormann *et al.* 1999). Little more is known of ECM communities in northern wetland habitats.

Plant communities associated with black spruce in mid-seral upland forests in BC are indicators of nutrient-poor, water-deficient sites (Krestov *et al.* 2000). These habitats generally have sandy loam soils, often with a root-restricting clay horizon near the soil surface that perches the water table during wet periods of the year, leading to seasonal cycles of moisture surplus and deficit (Krestov *et al.* 2000). Several studies have reported a lack of ecotypic variation in black spruce morphological features or water relations due to improved edaphic conditions in upland forests, even though upland trees often appear more robust than their wetland counterparts (Parker *et al.* 1983; Zine El Abidine *et al.* 1994, 1995). Although black spruce tolerates periods of flooding, it is sensitive to drought conditions. Preconditioning of containerized black spruce seedlings for increased drought resistance (higher photosynthetic and gas exchange activities) was achieved by Zine El Abidine *et al.* (1994), but the response was not cumulative with successive drought episodes. In greenhouse and field experiments with Norway spruce (*Picea abies*), Feil *et al.* (1988) noted an increased branching density of very fine roots initiated by drought, an adaptation to water stress allowing uptake of water from dry soil via ECM.

Few studies have been undertaken to investigate ECM symbioses of black spruce. Through field surveys, the ectomycorrhizal status of naturally regenerating black spruce has been confirmed in northeastern Ontario (Malloch and Malloch 1981) and northern Alberta (Thormann *et al.* 1999), and of outplanted black spruce seedlings in Quebec (McAfee and Fortin 1989). Studies, in which containerized black spruce seedlings were inoculated with ECM fungi, have reported increased growth and survival of ECM seedlings grown with low nitrogen (Gagnon *et al.* 1988), enhanced rooting of cuttings (Stein *et al.* 1990), and inhibition of root rot pathogen infections that are responsible for severe mortality in many conifers (Morin *et al.* 1999). There are no known studies that have attempted to describe the ECM associated with black spruce or to compare ECM communities in different habitats.

This study's first objective was to use morphological techniques (light microscopy) to describe ECM associating with naturally regenerating black spruce seedlings across its habitat range in central BC. In this region, black spruce habitats include both black spruce-dominated and black spruce – tamarack wetland forests, as well as black spruce – lodgepole pine upland forests. The second objective of this study was to compare the structure and diversity of ECM communities between the three habitats and to attempt to relate differences to specific habitat characteristics.

METHODS AND MATERIALS

Site descriptions

The study area was located in the Sub-Boreal Spruce (SBS) biogeoclimatic zone in the central interior of British Columbia. This area ranges from 51° 30' to 59° N latitude and 660 to 1140 m in elevation (Meidinger *et al.* 1991; DeLong and Fahlman 1996). The climate of the SBS is characterized by severe and snowy winters, warm, moist and short summers, and moderate annual precipitation (Meidinger *et al.* 1991). Potential field sites were

identified in the Prince George Forest District by studying Forest Cover (1:20,000) maps (1993) and consulting Forest Service personnel. Subsequently, following site reconnaissance, selection resulted in three ("replicate") sites within each of three forest habitats (black spruce-dominated wetlands, mixed black spruce – tamarack wetlands and mixed black spruce – lodgepole pine upland forests) (Figure 2.1). "Replicate" sites were similar in composition of the dominant vegetation and soil properties. All sites contained mature black spruce trees and an abundance of naturally regenerating black spruce seedlings ranging from approximately 15-30 cm in height (estimated to be 10-15 years in age) and lacked visible indications of recent disturbance (such as fire, logging, windthrow and roads). The location and general characteristics of these sites are presented in Table 2.1. Photographs showing examples of each black spruce habitat are presented in Figure 2.2.

Table 2.1. Locations, biogeoclimatic ecological classification (BEC) (DeLong *et al.* 1994) and site characteristics of the 9 study sites (T = black spruce - tamarack wetland sites; W = black spruce-dominated wetland sites; U = black spruce – pine upland forest sites).

Site	Location	FC * Mapsheet	UTM * East	UTM * North	BEC	Site Series	Stand Age	# Flagged Seedlings
T1	Norman Lake Rd (6 km)	93G 084	476580	5965300	SBSdw3	10	~50 y	36
T2	Norman Lake Rd (8.4 km)	93G 084	477890	5963700	SBSdw3	10	~75 y	64
T3	Hwy 16 W (across from Tamarack Lake)	93G 084	476740	5968800	SBSdw3	10	90-95 y	90
W1	Teardrop Rd (km 204)	93J 025	497460	6008200	SBSmk1	10	~60 y	74
W2	Teardrop Rd (km 203)	93J 025	497580	5997200	SBSmk1	10	~60 y	21
W3	Teardrop Rd (1.5 km W from km 207)	93J 025	494260	5999900	SBSmk1	10	80-90 y	37
U1	Teardrop Rd (km 211)	93J 025	496230	6003800	SBSmk1	6	~60 y	21
U2	Teardrop Rd (km 401)	93J 025	494540	6008200	SBSmk1	6	~60 y	21
U3	Teardrop Rd (km 202)	93J 025	497870	5996300	SBSmk1	6	~70 y	46

* geographic locations by Forest Cover mapsheet and universal transverse mercator (UTM) coordinates (approximate) taken from within each plot with a Garmin eTREX GPS unit.

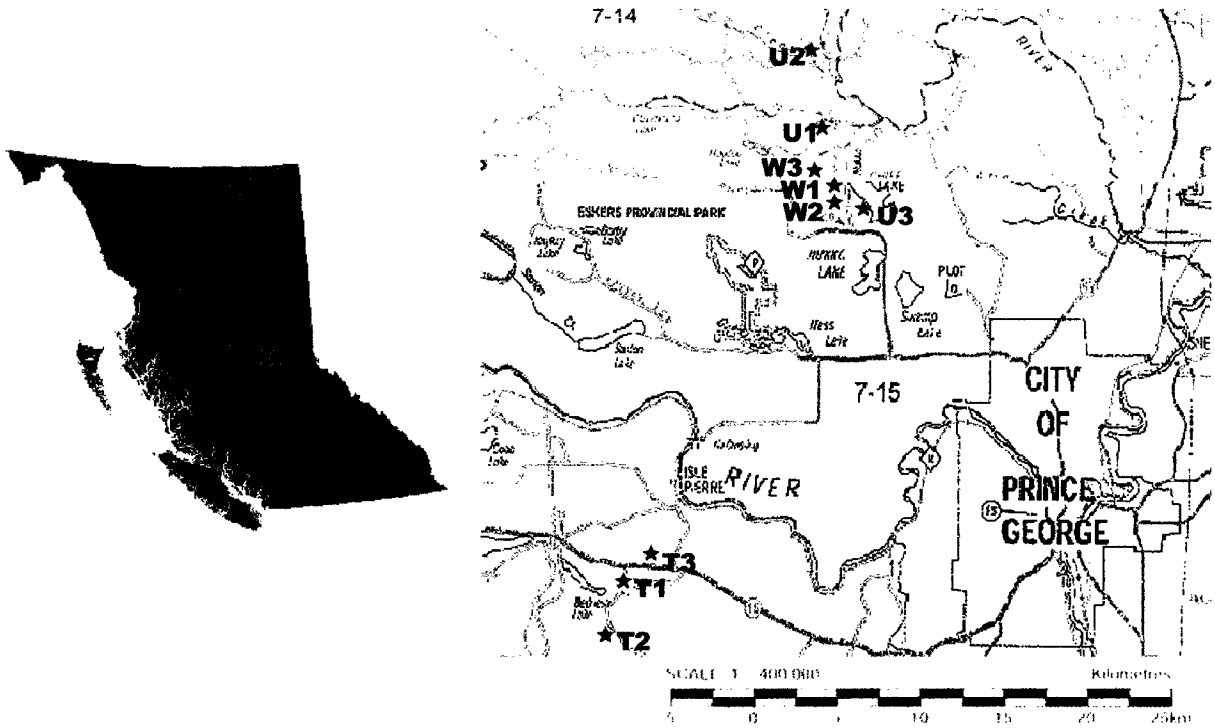

Figure 2.1. Map (left) of British Columbia showing the study area (orange square) in the SBS biogeoclimatic zone in central BC (shading indicates the provincial range of black spruce). Map (right) showing approximate locations of 9 black spruce study sites (T = black spruce - tamarack wetland sites; W = black spruce-dominated wetland sites; U = black spruce – pine upland forest sites) in the Prince George Forest District.

The black spruce - tamarack wetland (T) sites were located near Highway 16, about 40 km west of Prince George. They occurred in fens dominated by black spruce and tamarack in the dry, warm (SBSdw3) subzone variant of the SBS. The shrub understory consisted mainly of scrub birch, with common occurrences of willow (*Salix* species), labrador tea and dwarf nagoonberry (*Rubus arcticus* L.) on some sites. Herbs present included pink wintergreen (*Pyrola asarifolia* Michx.), marsh cinquefoil (*Potentilla palustris* (L.) Scop.), common mitrewort, palmate coltsfoot (*Petasites palmatus* (Ait) A. Gray), buckbean (*Menyanthes trifoliata* L.), and round-leaved sundew (*Drosera rotundifolia* L.). The microtopography of the T sites consisted of raised mounds (hummocks) of partially decomposed moss covering woody debris amongst depressions (hollows) that were often below the level of the water table. *Sphagnum* moss covered most of the open areas, with

other mosses and lichens (*Cladina* and *Peltigera* species) observed on drier hummocks and woody debris. Grasses and sedges were present on sites T2 and T3, and *Equisetum* species were observed in the wetter areas of all T sites. Soils were Typic Humisols, a sub-group of the Organic soils composed of organic material (derived mainly from mosses) in an advanced stage of decomposition to a depth of greater than 160 cm (Soil Classification Working Group 1998).

The black spruce-dominated wetland (W) sites were located along the Teardrop Forest Road (about 60 km northwest of Prince George) in the moist, cool (SBSmk1) subzone variant. These fens were dominated by mature black spruce stands with an understory of scrub birch, willow, labrador tea, bog cranberry (*Vaccinium oxycoccos* L. MacM.) and bog rosemary (*Andromeda polifolia* L.). Herb species included pink wintergreen, white bog orchid (*Platanthera dilatata* (Pursh) Lindl. *ex* Beck), single delight (*Moneses uniflora*), northern twayblade (*Listera borealis* Morong.), marsh cinquefoil, common mitrewort, palmate coltsfoot, and buckbean. Like the T sites, the W sites were characterized by rolling hummock-hollow microtopography covered with *Sphagnum* moss (and other species of mosses and lichens on the warmer, drier hummocks) and organic soils that were also classified as Typic Humisols.

The upland forest (U) sites were also located in the moist, cool (SBSmk1) subzone variant along the Teardrop Forest Road. These sites were dominated by mature black spruce and lodgepole pine, sometimes with a small component of hybrid white spruce, subalpine fir, or sitka alder (*Alnus crispa* var. *sinuata* (Regel.) A. & D. Love). A wide diversity of shrubs and herbs formed the understory of the U-sites: species included prickly rose (*Rosa acicularis* Lindl.), pink spirea (*Spiraea douglasii* ssp. *menziesii* Hook.), black twinberry (*Lonicera involucrata* (Richards.) Banks *ex* Spreng.), highbush cranberry (*Viburnum edule* (Michx.)

Raf.), twinflower (*Linnaea borealis* L.), dwarf blueberry (*Vaccinium caespitosum* Michx.), trailing raspberry (*Rubus pubescens* Raf.), kinnikinnick (*Arctostaphylos uva-ursi* L. Spreng.), round-leaved rein-orchid (*Platanthera orbiculata* (Pursh) Lindl.), rattlesnake plantain (*Goodyera oblongifolia* Raf.), one-sided wintergreen (*Orthilia secunda* (L.) House), pink wintergreen, and bunchberry (*Cornus canadensis* L.). Mosses (including red-stemmed feathermoss (*Pleurozium schreberi*), step moss (*Hylocomium splendens*), knight's plume (*Ptilium crista-castrensis*), and electrified cat's tail moss (*Rhytidiadelphus triquetrus*)) and lichens (such as *Peltigera*, *Cladonia*, and *Cladina* species) formed the forest floor. Upland soils were Orthic Gray Luvisols, which characteristically develop in well to imperfectly drained sites with sandy loam to clay soils, under boreal or mixed forests, in mild to very cold climates (Soil Classification Working Group 1998).

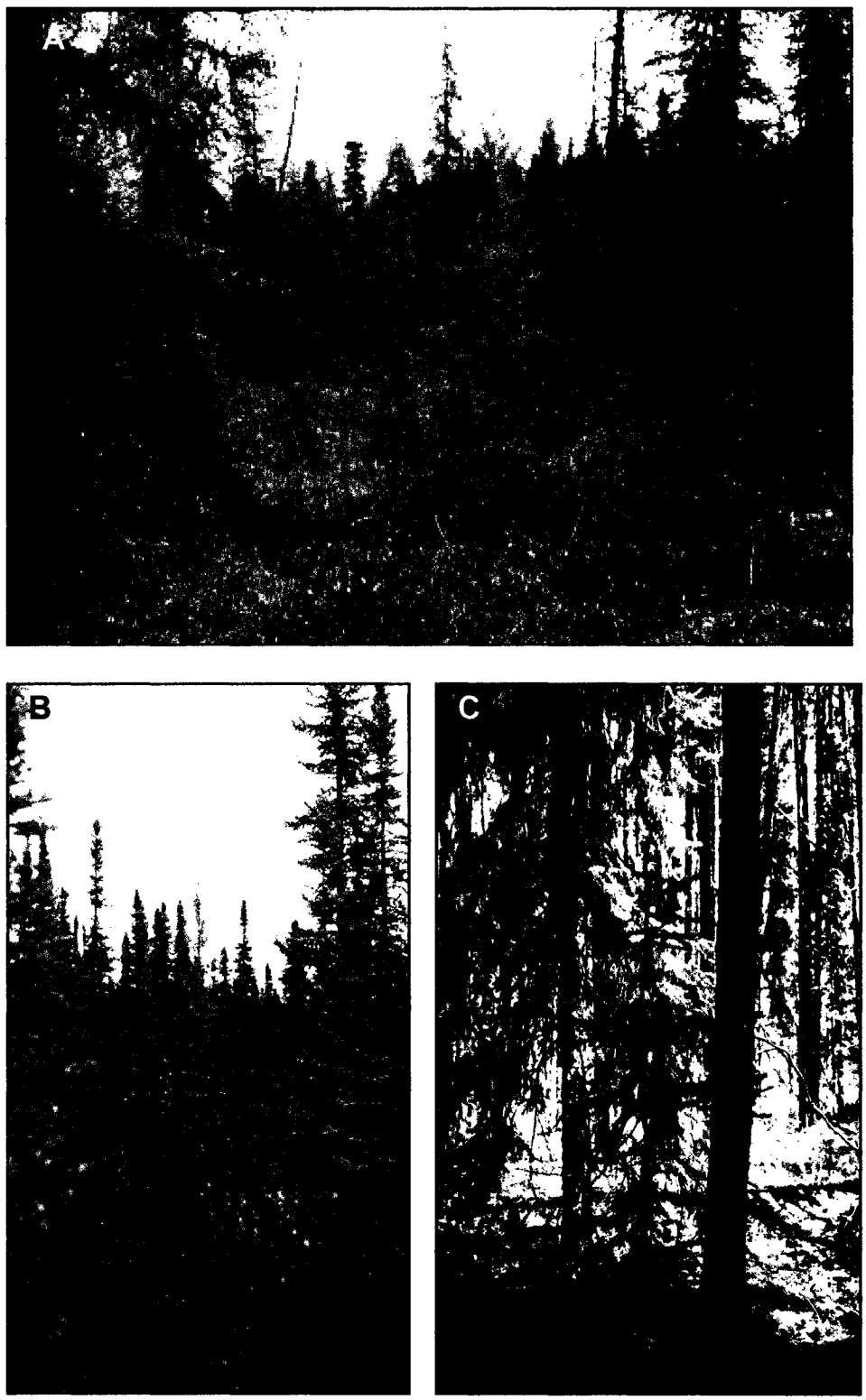

Figure 2.2. Photographs showing examples of three black spruce habitats in central BC: **A**, mixed black spruce – tamarack wetland (T) habitat; **B**, black spruce-dominated wetland (W) habitat; **C**, black spruce – lodgepole pine upland forest (U) habitat.

Seedling sampling and vegetation and soil analyses

Plots (50 x 50 m^2) were established at each site (except for one smaller upland (U1) site on which a 30 x 35 m^2 plot was laid out). Plots were located at least 10 m inside site boundaries to decrease edge effects. All regenerating (no obvious signs of vegetative layering) black spruce seedlings ranging from 15-30 cm in height were located within each plot. These seedlings were flagged, numbered and approximately plotted on site maps. When necessary, cross sections of needles were examined with a hand lens to distinguish black spruce seedlings from hybrid spruce seedlings, based on differences in needle morphology (Weng and Jackson 2000).

Seedlings were selected for harvest following a simple random sampling design, which assumed that aii flagged seedlings were equal in terms of environmental influences, and that each had an equal chance to be sampled. Using a random number table, five seedlings were randomly selected from each site, for a total of 45 seedlings. Details of the specific location (e.g. hummock - hollow microtopography) and rooting substrate were recorded for each selected seedling. In addition, lists of vegetation were compiled for all nine sites (Appendix I), as well as vegetation occurring within a radius of 0.5 m^2 of each selected seedling (45).

Organic soil samples were collected from several locations within each wetland plot and combined in plastic bags for classification according to the Soil Classification Working Group (1998). Soil pits (2-3 per site) were excavated on the upland sites, and the forest floor and mineral horizons were described and classified according to Green *et al.* (1993) and the Soil Classification Working Group (1998). Forest floor and mineral soil (collected from each A and B horizon) samples were analyzed for texture (clay, silt and sand content), as well as

total carbon and nitrogen content, extractable P (Bray P1 method), and pH (Kalra and Maynard 1991). All dried soil samples were stored in plastic bags at 22°C until analyses.

Measuring the depth of rust formation on steel rods has been used to determine the depth of the water table (or depth of the aerobic zone) over an extended period of time (Carnell and Anderson 1986; Bridgham *et al.* 1991; Thormann *et al.* 2000). The method is based on the theory that iron will rust rapidly in aerated zones, but not in the saturated, non-aerated or reduced zone in poorly drained soils (McKee 1978). Steel welding rods (100 cm x 6 mm) were sanded to remove the copper coating and sets of three rods were pounded into the soil near each of three selected seedlings in each plot. Rod placement mimicked the topographical position (i.e. hummock, hollow, level ground, etc.) of the nearby selected seedling. All rods were placed in the soil July 17-19, 2001, and pulled sequentially at approximately monthly intervals (August 22-23, September 17-18, and October 18-19) over three months. Just prior to removal, a mark was etched into each rod to indicate the level of soil surface. Rods were gently cleaned of soil and *Sphagnum*, and the depth of rusting (determined as the lowest point of the obvious, heaviest rust zone) was measured from the substrate surface line. All rods were measured immediately upon removal from the soil substrate.

Fungal sporocarp sampling

During July and August of 2000 and 2001, fungal sporocarps were collected from all study sites. The mushrooms were described (data not included) and samples were taken from the spore-producing tissues (i.e. gills, pores, teeth, etc.) of each sporocarp and stored in microcentrifuge tubes at -20°C for DNA analysis. The sporocarps were then dried and stored at room temperature.

Seedling harvest and ECM characterization

Seedlings were harvested between July 26 to August 2, 2001, when optimal fine root growth and ECM development was expected to occur. On the wetland sites, a saw was used to facilitate cutting through the peat surrounding the entire root systems. Seedlings were placed into 7 L plant pots, double-bagged in heavy-duty garbage bags, and stored at 4°C until processing.

Prior to each examination, the root system was gently washed free of soil (and *Sphagnum*) with tap water. The location of the root collar (the boundary between root and stem tissue) was determined by hand sectioning the taproot or stem just below root emergence points, and examining these sections under the dissecting microscope. Stem tissue was recognized by the presence of distinct vascular bundles or secondary xylem and central pith, while the presence of central xylem indicated root tissue (Raven *et al.* 1986). Seedling height was measured from the root collar and the age of each seedling was determined by counting growth rings using a cross section of stem just above the root collar. At this time, seedlings were identified as either true (regenerating from seed) or layered (regenerating from adventitious roots) seedlings. The presence of stem tissue in cross sections below the root collar was accepted as evidence of a layered seedling. Root quality was described as either good (most roots appeared healthy and robust) or poor (many roots appeared black and withered) and the abundance (high, medium or low) of roots was also noted.

Cleaned roots were floated in a large tray of water overlaying a grid of 1 cm^2 cells. Using a random number table to select grid cell numbers, 1 cm lengths of roots were removed from the tray until approximately 200 root tips had been selected. Root tips (200 per seedling) were described using bright field light microscopy following standard techniques of Agerer (1987-2002), Ingleby *et al.* (1990) and Goodman *et al.* (1996). One unbranched root tip was

considered to be one mycorrhiza and only root tips that appeared healthy and robust were examined. ECM were initially described using a dissecting microscope (9-40 x magnification) and classified according to color, texture, lustre, dimensions, tip shape, branching pattern, and presence or absence of rhizomorphs (mycelial strands). Root squash mounts were prepared and examined under a compound microscope (100-1000 x magnification). Descriptions of mantle features, emanating hyphae, rhizomorphs, and other distinguishing features were recorded and used to further categorize the different ECM morphotypes. Root tips that appeared uncolonized or partially colonized (due to the lack of a well-developed mantle) were categorized as non-mycorrhizal. When possible, preliminary identifications to families or genera were assigned based on similarities in features to published descriptions (Agerer 1987-2002; Ingleby *et al.* 1990; Goodman *et al.* 1996), but when identification was not possible, a descriptive name was assigned. Some morphotypes were photographed using an automatic exposure (PM-10AK) camera attached to a dissecting (Olympus BX-50) or compound (Olympus SZ-40) microscope, using Ektachrome 160T tungsten professional color reversal film.

Analysis of morphological data

Several seedling features (e.g. seedling height, root collar diameter, age, root quality, root abundance, etc.) and site characteristics (e.g. depth of the aerobic zone, microtopography, ECM vegetation, etc.) were compared qualitatively. Soil nutrient content (C, N and P) and pH were compared between habitats (and between different soil horizons in the uplands) using a one-way ANOVA (SYSTAT version 8.0, 1998, SPSS Inc.) to determine significant differences (α=0.05). Mean comparisons were tested using the Fisher's Least Significant Difference (LSD) test (α=0.05).

Prior to data analysis, several morphotypes were merged based on descriptions that were very similar. The ECM frequency (proportion of seedlings with each morphotype) and abundance (proportion of the entire community represented by each morphotype) were calculated from the total sample and within each habitat type. The relative abundance of each morphotype was plotted against species rank order to visualize the overall community structure and to compare community structure between habitats (Taylor *et al.* 2000). Mean ECM abundance values (calculated per seedling and averaged within each habitat) were compared between habitats using a one-way ANOVA to determine significant differences (α=0.05). Mean comparisons were tested using the Fisher's LSD test (α=0.05).

ECM community diversity was measured by means of several nonparametric heterogeneity indices, including the Margalef (measure of species richness), Shannon (measure of species richness and relative abundance), Shannon Evenness (measure of relative abundance) and Simpson (measure of relative abundance weighted towards more the abundant types) indices (Magurran 1988; Krebs 1989). For each index, an increase in value indicates greater diversity. Diversity indices were calculated for each seedling and averaged within habitats. Habitat means were compared using a one-way ANOVA to determine significant differences (α=0.05) and the post-hoc Fisher's LSD test (α=0.05).

RESULTS

Seedling and site characteristics

Seedling height ranged from 15 to 54.2 cm, with a mean value of 31.5 cm. Seedling age ranged from seven to 30 years with an average of 14.3 years. Seedling height and diameter at the root collar generally increased with seedling age, but this relationship was not always consistent. No relationship was found between seedling height or root collar diameter and the extent or diversity of ECM colonization or habitat of origin.

Rooting substrate(s) included moss and silty loam soils (upland sites), moss and peat (wetland sites), and coarse woody debris (CWD), which was present on all sites. Based on the qualitative appearance of root systems (good or poor), root quality was always described as good when seedlings were rooted in CWD, but there was no relationship with root quality for the other substrate types. Root quality did not appear to be related to storage time as poor root quality was observed on seedlings processed early just as frequently as on seedlings stored for up to four months. No relationship was identified between root quality and the number of morphotypes found on each seedling. Root quality appeared to be related to the topographical position of the seedling's location on the site. In general, those seedlings growing in hollow depressions or on level ground to low rises had a greater proportion of poor quality roots (54%) than seedlings harvested from hummocks of any size (20%).

The depth of the aerobic zone generally increased during the period of root growth and ECM establishment, but these results were not consistently observed at every location of rod placement. The level of the water table with respect to the roots of any seedling depended on the microtopographical position of that seedling (i.e. relative water table depth was greater for seedlings established on hummocks as compared to level ground or depressions). On most sites, the water table depth generally fluctuated between 10 and 30 cm; depth was greater on site U3, where fluctuations between 75 and 90 cm were observed. No relationships were found between relative depth of the water table and the number of ECM morphotypes described on a seedling.

Soil nutrient content (C, N and P) varied significantly between the wetland (both T and W) and upland habitats (Table 2.2). Total C and N content was greater ($p=0.000$ and 0.001, respectively) in the wetlands than the upland habitats. Within upland sites, C and N levels in

the forest floor (LFH) were approximately twice the levels in the A horizon; both nutrients were at least 10 times greater in the A horizon than in the B horizons. The C:N ratio was greater (p=0.036) in the upland forest floors than in the wetland soils. The content of available P was also greater (p=0.025) in forest floor soils than in wetland soils, as well as when compared to mineral soil horizons. All soil samples were acidic (pH ranged from 3.8 to 6.9) and there were no significant differences between the wetland and forest floor soils. Acidity decreased with depth in the upland soil profile; the B horizon was significantly more alkaline than all other horizons but the T sites.

Table 2.2. ANOVA comparisons of mean % carbon, % nitrogen, C:N ratio, available phosphorus and pH between habitats based on combined samples from each site (n=3).

Soil Analysis	Treatment Effect		Tamarack – Spruce	Black Spruce	Pine – Spruce
	F	P	Wetland	Wetland	Upland
% Total Carbon	88.067	**0.000**	44.58 (0.70)*a*	48.05 (1.87)*a*	24.80 (1.16)*b*
% Total Nitrogen	23.882	**0.001**	2.60 (0.26)*a*	2.24 (0.13)*a*	0.80 (0.16)*b*
C:N ratio	6.088	**0.036**	17.46 (1.58)*a*	21.51 (0.46)*a*	33.31 (5.54)*b*
Phosphorus (ppm)	7.268	**0.025**	0.61 (0.60)*a*	1.37 (1.36)*a*	191.63 (70.70)*b*
pH	2.709	0.145	5.67 (0.12)	4.92 (0.46)	4.58 (0.34)

* Upland values are from analysis of the forest floor soils only (not A or B horizons).

The F statistic is the ratio of variance in sample means to variance within groups. The P value is the significance level (α=0.05). Values for nutrient content and pH are means with standard error in parentheses. Means followed by the same letter are not significantly different.

Even though only seedlings thought to be naturally regenerating (true seedlings) were selected for harvest, almost half (44.4%) of the harvested seedlings were found to originate from branches of trees that had developed underground adventitious roots and formed new seedlings (layered seedlings). The qualitative attributes of root abundance (high, medium or low) and root quality (good or poor) were compared between layered and true seedlings. A greater proportion of true seedlings seemed to exhibit medium or high root abundance (92%) when compared to layered seedlings (70%). No relationship was found between root quality and regeneration type. However, a comparison between the mean number of

morphotypes found on 25 true seedlings (5.16 ± 0.24) and the number occurring on 20 layered seedlings (4.35 ± 0.29) indicated a significantly greater number of morphotypes on the true seedlings, regardless of habitat (p=0.037).

An average of 4.8 (range of two to eight) ECM morphotypes was identified per seedling. The mean number of morphotypes varied significantly (p=0.005) between habitats, with a greater number of morphotypes occurring in the two mixed species habitats (mean of 5.2 morphotypes in each of the T and U habitats) compared to the black spruce-dominated habitat (mean of 4.0 morphotypes). The number of potential ECM host plants present in the vicinity (within 1 m^2) of harvested black spruce seedlings ranged from zero to four, with an average of 1.82 per seedling. No significant differences were found in the number of potential ECM hosts between sites or habitats. Figure 2.3 shows the lack of relationship between the mean number of morphotypes and potential ECM host plants on each site.

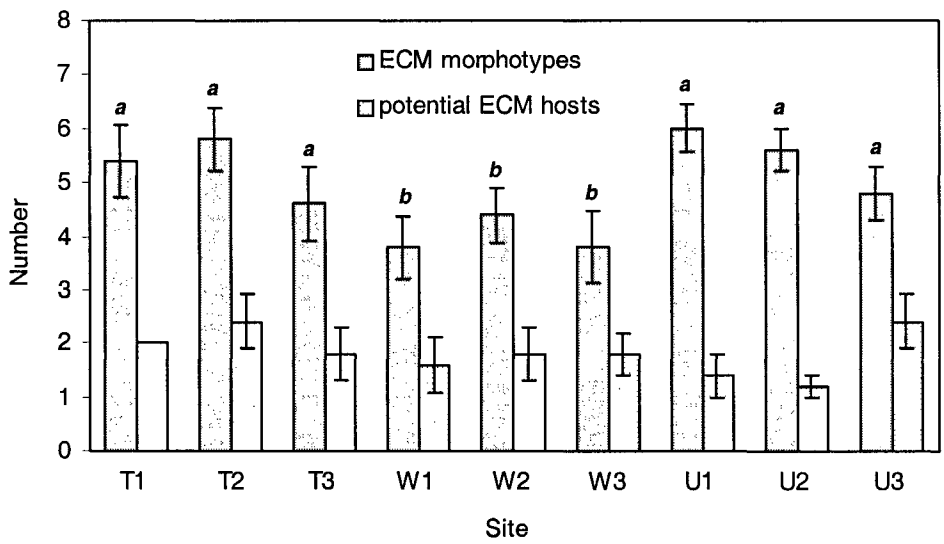

Figure 2.3. Bar graph showing mean (±SE) number of ECM morphotypes compared to mean (±SE) number of potential ECM host vegetation (within 0.5 m of seedlings) for each site (black spruce – tamarack wetlands [T sites], black spruce-dominated wetlands [W sites] and black spruce – pine upland forests [U sites]). ECM morphotype bars labelled with the same letter are not significantly different.

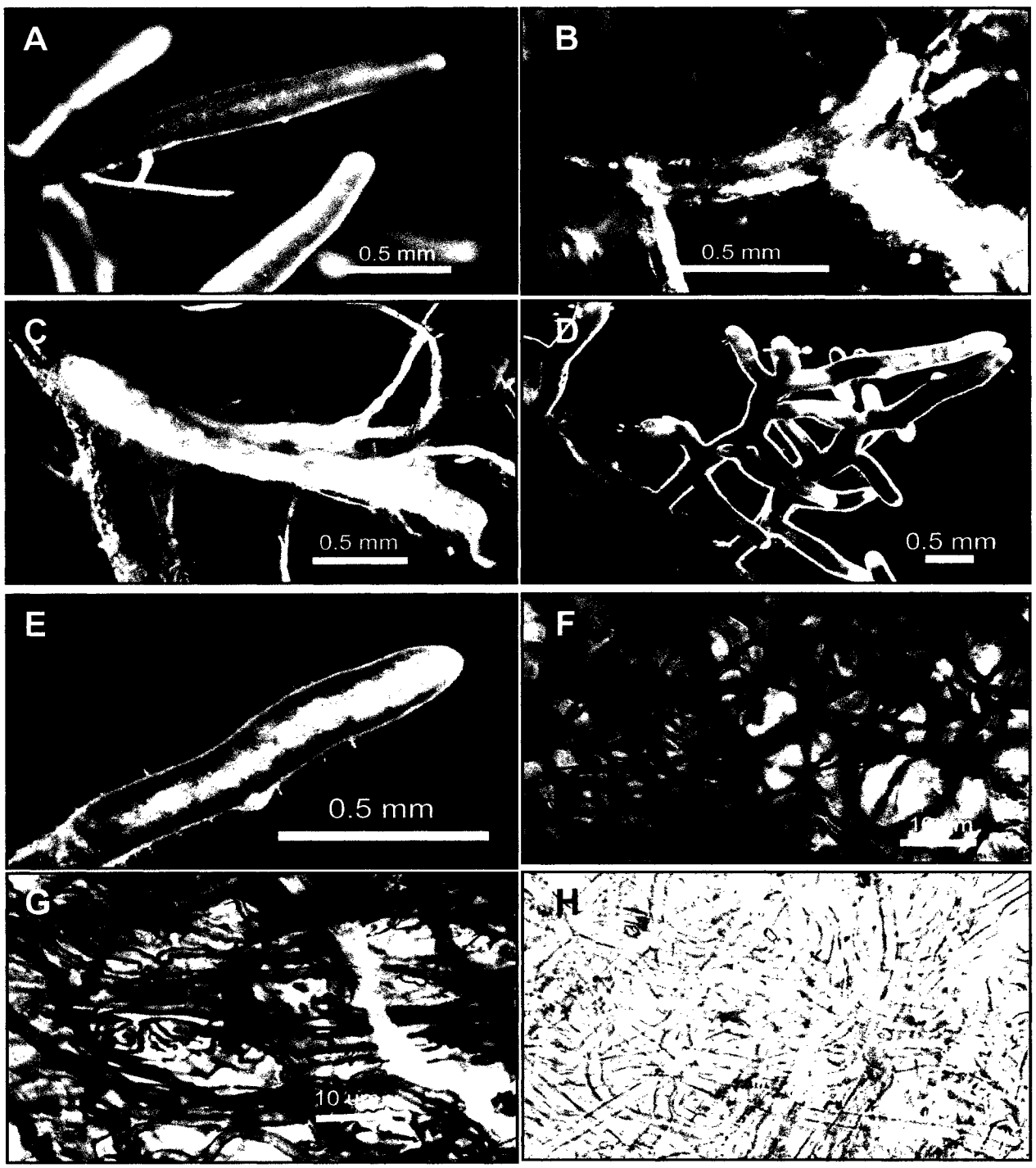

Figure 2.4. ECM morphotypes described on black spruce from three habitats in central BC. A - *Lactarius* 1; B – Cortinariaceae 1; C - *Piloderma*; D – Thelephoraceae-like 4; E - *Tomentella*; F – *Tomentella*-like 1 (outer mantle); G - *MRA* 1 (outer mantle); H - *Amphinema* (outer mantle with emanating hyphae).

ECM morphotype occurrence, frequency and abundance

A total of 33 ECM morphotypes were described from 8858 root tips. Photographs of some morphotypes are presented in Figure 2.4, and complete morphological descriptions are included in Appendix II. Based on morphology, 27 ECM morphotypes were most likely basidiomycetes, three ECM types were probably ascomycetes, and three remain uncertain. Twenty-seven morphotypes were assigned to families including Thelephoraceae, Cortinariaceae and Russulaceae, or genera such as *Cenococcum*, *MRA*, *Amphinema*, *Lactarius*, *Tomentella*, *Piloderma* and *Hebeloma*. Six morphotypes could not be assigned with confidence to any taxonomic group. Overall, 85% of all root tips examined were ectomycorrhizal. Those tips (n=1337) that appeared uncolonized or only partially colonized (lacked developed mantle) were classified into a separate non-mycorrhizal group. Non-mycorrhizal tips were found on 66.7% of seedlings, including seedlings from each habitat and from all sites. The frequency of seedlings with non-mycorrhizal root tips was greater in the W sites (93.3%) than the T (60.0%) or U sites (46.7%) (Table 2.3).

Nineteen morphotypes were found on three or fewer seedlings (<7%) and were defined as rare. Of the 14 more commonly identified morphotypes, elght were found in all three black spruce habitats, and accounted for 51% of all root tips. Overall, *Cenococcum* occurred the most often (68.9% of all seedlings) of any morphotype (Table 2.3). It was found on 80% of seedlings from both the W and U sites, but on only 46.7% of seedlings from the T sites. *Cenococcum* was completely absent from one site (T1), but occurred on 90% of seedlings from T2 and T3. The second most commonly encountered morphotype overall was Cortinariaceae 2 (55.6%), which was almost twice as frequently identified in the U sites (80%) than in either of the wetland habitats. Russulaceae 2, *MRA* 1, *Amphinema* and Cortinariaceae 1 (31.1 to 37.8%) were all found most often on seedlings from the U sites. Thelephoraceae-like 1 and *Tomentella*-like 1 types were more frequently found on seedlings

from the T sites than on seedlings from the W or U sites. Interestingly, Thelephoraceae-like 1 was found on 80% of the seedlings from T1, the site lacking *Cenococcum*.

Six of the most commonly identified morphotypes appeared to be absent from one or more habitats. *Lactarius* 1 ECM were found on seedlings from both wetland habitats but not from the upland habitats and occurred much more often in the T sites (31.1% of seedlings). Three morphotypes were found on seedlings from both the W and U habitats: Thelephoraceae 1 and orange 1 were more frequently described from the U sites and Thelephoraceae-like 4 was more frequently described from W sites. Of the morphotypes described from a single habitat (total of 14), most were considered rare and only two were found on more than three seedlings. *Tomentella* (13.3% of all seedlings) was found only in the T habitat ($p=0.011$) and *Piloderma* (15.6% of all seedlings) was found in only the U habitats ($p=0.001$). Several morphotypes exhibited <6% overall frequency, but were fairly common within a single habitat. These included *Lactarius* 3, Russulaceae 1 and cottony halo types, which occurred on 20% of seedlings from the U, T, and W sites, respectively.

The average community similarity, based on ECM morphotype composition, was 27% between all three black spruce habitats. The T sites shared 36% of ECM morphotypes with each of the W and U sites; 42% of morphotypes were described from both the W and U sites.

Table 2.3. Treatment effects, percent abundance (mean ± SE) and frequency of occurrence (%) for ECM morphotypes of black spruce growing in three habitats. ECM morphotypes are presented in order of decreasing overall frequency rank.

	Treatment Effect		Black Spruce - Tamarack Wetland (T)		Black Spruce Wetland (W)		Black Spruce - Pine Upland Forest (U)	
ECM Morphotype	**F**	**P**	**Abundance**	**Freq**	**Abundance**	**Freq**	**Abundance**	**Freq**
Cenococcum	0.966	0.389	10.2 (4.6)	46.7	18.2 (5.9)	80.0	10.7 (2.3)	80.0
Cortinariaceae 2	1.515	0.232	4.9 (3.3)	40.0	3.9 (1.6)	46.7	10.9 (3.8)	80.0
Russulaceae 2	4.295	**0.020**	4.3 (7.1)*a*	33.3	3.0 (1.8)*a*	26.7	21.8 (6.8)*b*	53.3
MRA 1	1.599	0.214	2.2 (1.4)	33.3	2.2 (1.2)	33.3	6.1 (2.5)	53.3
Amphinema	1.130	0.333	10.1 (7.9)	33.3	4.0 (2.4)	20.0	12.1 (4.2)	46.7
Lactarius 1	3.293	**0.047**	15.7 (6.6)*a*	66.7	6.0 (3.7)*ab*	26.7	0.0 (0.0)*b*	0.0
Cortinariaceae 1	0.413	0.664	5.6 (3.3)	20.0	2.4 (1.9)	33.3	4.2 (2.0)	40.0
Thelephoraceae-like 1	3.165	0.052	7.0 (3.4)	46.7	1.1 (1.1)	13.3	0.3 (0.3)	6.7
Tomentella-like 1	3.567	**0.037**	5.4 (2.6)*a*	33.3	0.5 (0.5)*b*	6.7	0.3 (0.3)*b*	13.3
Piloderma	9.099	**0.001**	0.0 (0.0)*a*	0.0	0.0 (0.0)*a*	0.0	7.0 (2.3)*b*	46.7
Thelephoraceae-like 4	4.013	**0.025**	0.0 (0.0)*a*	0.0	11.7 (5.8)*b*	33.3	0.1 (0.1)*a*	6.7
Tomentella	4.987	**0.011**	7.9 (3.5)*a*	40.0	0.0 (0.0)*b*	0.0	0.0 (0.0)*b*	0.0
cottony gold-brown	1.591	0.216	2.6 (1.8)	13.3	0.7 (0.5)	13.3	0.0 (0.0)	0.0
Thelephoraceae 2	1.292	0.285	0.0 (0.0)	0.0	0.7 (0.7)	6.7	0.7 (0.7)	20.0
Lactarius 3	1.594	0.215	0.0 (0.0)	0.0	0.0 (0.0)	0.0	6.6 (5.2)	20.0
cottony halo	3.343	**0.045**	0.0 (0.0)*a*	0.0	6.0 (3.3)*b*	20.0	0.0 (0.0)*a*	0.0
Russulaceae 1			4.1 (2.7)	20.0	0.0 (0.0)	0.0	0.0 (0.0)	0.0
Thelephoraceae-like 2			2.2 (1.5)	13.3	0.0 (0.0)	0.0	1.6 (1.6)	6.7
Russulaceae 4			0.0 (0.0)	0.0	0.8 (0.5)	13.3	1.7 (1.7)	6.7
Tomentella-like 3			1.6 (0.9)	20.0	0.0 (0.0)	0.0	0.0 (0.0)	0.0
Thelephoraceae-like 3			0.5 (0.4)	13.3	0.0 (0.0)	0.0	1.1 (1.1)	6.7
brown 1			0.3 (0.3)	6.7	0.0 (0.0)	6.7	1.0 (1.0)	6.7
Thelephoraceae 3			0.3 (0.3)	6.7	3.7 (3.7)	6.7	0.0 (0.0)	0.0
Lactarius 2			3.1 (2.8)	13.3	0.0 (0.0)	0.0	0.0 (0.0)	0.0
creamy rhizomorphic clamp			2.0 (2.0)	6.7	0.0 (0.0)	0.0	0.8 (0.8)	6.7
orange 1			0.0 (0.0)	0.0	1.5 (1.5)	6.7	0.6 (0.6)	6.7
Cortinariaceae 3			0.0 (0.0)	0.0	2.8 (2.8)	6.7	0.0 (0.0)	0.0
Russulaceae 3			2.2 (2.2)	6.7	0.0 (0.0)	0.0	0.0 (0.0)	0.0
MRA 2			0.0 (0.0)	0.0	0.0 (0.0)	0.0	0.8 (0.8)	6.7
brown 3			0.0 (0.0)	0.0	0.0 (0.0)	0.0	0.8 (0.8)	6.7
Hebeloma			0.0 (0.0)	0.0	0.7 (0.7)	6.7	0.0 (0.0)	0.0
Tomentella-like 2			0.0 (0.0)	0.0	0.3 (0.3)	6.7	0.0 (0.0)	0.0
Thelephoraceae 1			0.0 (0.0)	6.7	0.0 (0.0)	0.0	0.0 (0.0)	0.0
non-mycorrhizal	9.785	**0.000**	5.2 (1.7)*a*	60.0	30.0 (5.7)*b*	93.3	10.0 (4.1)*a*	46.7

The F statistic is the ratio of variance in sample means to variance within groups. The P value is the significance level (α=0.05). Mean abundance values (calculated per seedling [n=15]) were tested using a one-way ANOVA for each habitat. Post-hoc Fisher's LSD tests were used to determine where significant differences occurred. Means followed by the same letter within rows are not significantly different.

In general, morphotypes that occurred most frequently were also more abundant on root systems (greater proportion of total root tips). *Cenococcum* and Russulaceae 2 each accounted for greater than 10% overall ECM abundance (Table 2.3), whereas nine morphotypes each represented between 2-5% of the overall ECM community and 19 morphotypes accounted for less than 2% each. The rank-abundance curve is shown as a straight line (Figure 2.5).

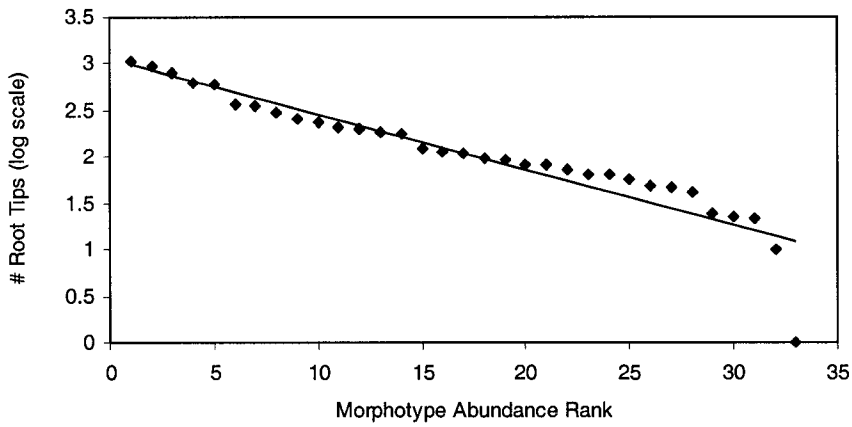

Figure 2.5. Log-transformed rank-abundance plot of the overall ECM fungal community of black spruce. The morphotype abundance rank order, beginning with *Cenococcum*, corresponds to the order on the y-axis of Figure 2.7.

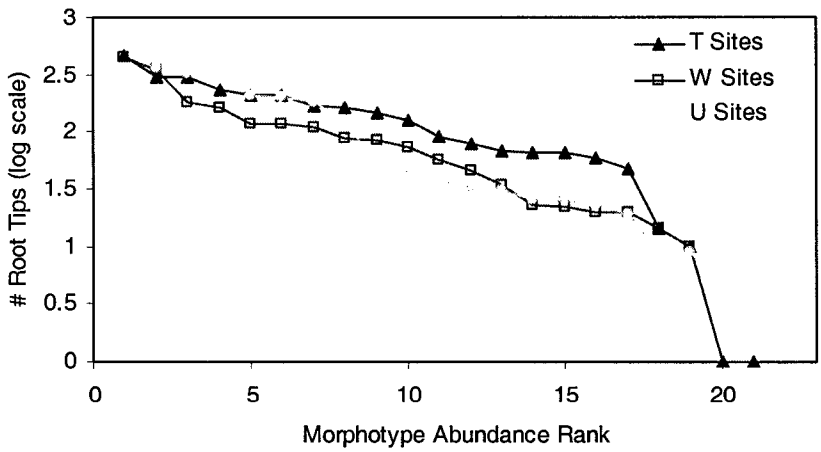

Figure 2.6. Comparison of rank-abundance of ECM morphotype communities of black spruce in three habitats (black spruce – tamarack wetlands [T sites], black spruce-dominated wetlands [W sites] and black spruce – pine upland forests [U sites]), using log abundance plotted against ranked morphotype abundance in each habitat.

The relative abundance of ECM morphotypes is shown for all three habitats in Figure 2.5 and compared between habitats in Figure 2.6. For six of the 14 most frequently described morphotypes, the mean morphotype abundance (calculated for each seedling and averaged for each habitat type) varied significantly between habitats (Table 2.3). Russulaceae 2 was significantly more abundant ($p=0.020$) on roots from the U seedlings than from the W seedlings. This morphotype was found in all three U sites and was exceptionally abundant on Site U3 (ranging from 48.5 to 73.1% on four of five seedlings). The abundance of Russulaceae 2 was much lower in the T sites compared to the U sites, but not significantly so. The ECM abundance for *Tomentella*-like 1 was also significantly different between habitats ($p=0.037$); it was more abundant in the tamarack wetlands than in either of the other habitats. Similarly, *Lactarius* 1 abundance was significantly greater ($p=0.047$) in the tamarack wetlands than in the upland habitat (where it was not detected) and greater (but not significant) than in the spruce-dominated wetlands. Thelephoraceae-like 4 abundance was higher in the spruce-dominated wetlands than either of the mixed species habitats ($p=0.025$). Interestingly, the non-mycorrhizal root tips showed the same trend in the W habitat ($p=0.000$). The cottony halo ECM, a rare morphotype, was described only from W sites ($p=0.045$). Two frequently occurring morphotypes (*Tomentella* and *Piloderma*) were each found in single habitat types ($p=0.011$ and $p=0.001$, respectively). Within habitat sites, *Tomentella* was more broadly distributed than *Piloderma*.

Although ECM frequency varied between habitat types for some of the more commonly occurring morphotypes such as *Cenococcum*, Cortinariaceae 1, Cortinariaceae 2, *Amphinema* and *MRA* 1, mean abundance values did not vary significantly. Although *Cenococcum* appeared to be more abundant in the W sites than the other two habitats, it was locally abundant (60%) on one or more seedlings from each different habitat. *Amphinema* was less abundant in the spruce-dominated wetlands, but had higher within-

habitat variability (especially in the T sites). Cortinariaceae 2 and *MRA* 1 were both more abundant in U sites than either wetland site, however exceptions of high local abundance did occur for both of these ECM on several seedlings on T sites. Cortinariaceae 1 was quite evenly abundant across habitats.

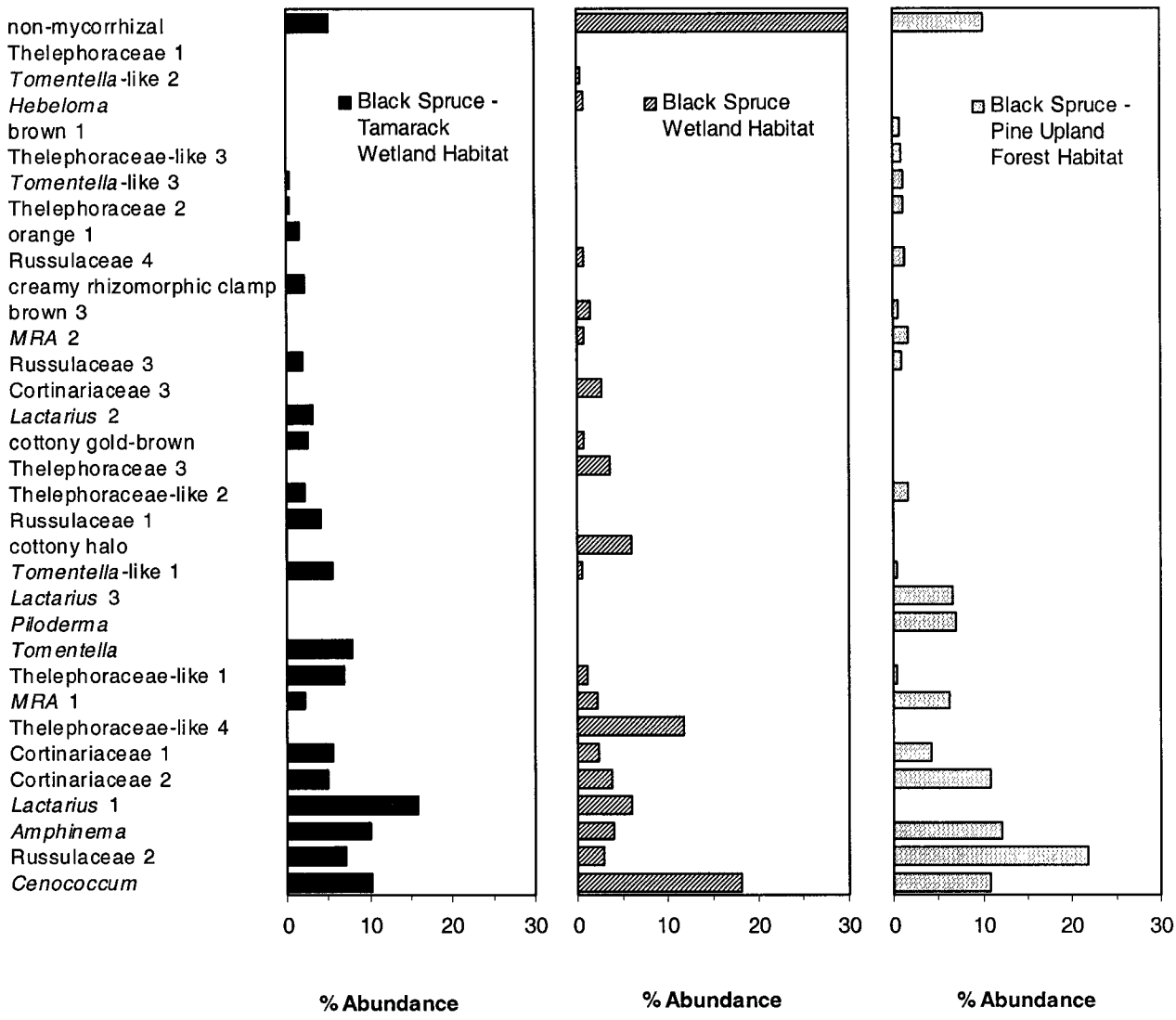

Figure 2.7. Comparison of ECM morphotype abundance (proportion of the community represented by each morphotype per habitat) between three black spruce habitats. ECM morphotypes are ranked in order of overall decreasing abundance from bottom to top (excluding the non-mycorrhizal type).

ECM community diversity

In general, ECM community diversity was greatest in the black spruce - pine upland forests and least in the spruce-dominated wetland, with intermediate diversity in the spruce - tamarack wetland habitat. Species richness (Margalef Index) varied significantly (p=0.038) between the black spruce-dominated wetlands and both mixed habitats, but not between the black spruce – tamarack wetland and the black spruce – pine upland habitats. Mean Shannon diversity values were significantly different (p=0.028) between the black spruce – pine upland and black spruce-dominated wetland habitats only. Shannon evenness and Simpson indices did not detect significant differences in diversity between habitats, but supported the same trends.

Table 2.4. ANOVA comparisons of diversity indices (Margalef, Shannon, Shannon evenness and Simpson) between habitats based on calculations for each seedling (n=15).

Diversity Index	Treatment Effect		Tamarack–Spruce Wetland	Black Spruce Wetland	Pine – Spruce Upland
	F	P			
Margalef	3.527	**0.038**	0.802 (0.071)*a*	0.616 (0.059)*b*	0.814 (0.045)*a*
Shannon	3.899	**0.028**	1.188 (0.085)*ab*	0.950 (0.110)*a*	1.296 (0.070)*b*
Shannon Evenness	1.740	0.188	0.727 (0.038)	0.677 (0.055)	0.795 (0.040)
Simpson	2.949	0.063	2.903 (0.217)	2.456 (0.268)	3.292 (0.243)

Values for indices are means with standard error in parentheses. Means followed by the same letter are not significantly different.

DISCUSSION

ECM morphotype occurrence and abundance

Overall, 33 ECM morphotypes were characterized from the roots of naturally regenerating black spruce (*Picea mariana*) seedlings. Although others have previously confirmed the ectomycorrhizal status of black spruce in natural habitats (Malloch and Malloch 1981; Thormann *et al.* 1999), this is the first study to report on the composition and diversity of ECM fungal communities associating with this host. In similar studies involving other plant hosts, the number of ECM morphotypes (species richness) varies widely. For example, in

central BC, Mah *et al.* (2001) described 24 ECM morphotypes on naturally regenerating hybrid spruce (*Picea glauca* x *engelmannii*) seedlings, while Kranabetter *et al.* (1999) reported an average of 52 morphotypes (total of 74 morphotypes) on white spruce (*Picea glauca*), subalpine fir (*Abies lasiocarpa*), and lodgepole pine (*Pinus contorta* var. *latifolia*) seedlings two years after outplanting. Flynn *et al.* (1998) described 13 morphotypes on naturally regenerating Sitka spruce seedlings (*Picea sitchensis*) harvested from Scottish plantation forests, and in southwestern Alberta cutblocks, Bradbury (1998) described 14 ECM morphotypes on two year-old regenerating lodgepole pine seedlings. On mature hosts, Danielson and Pruden (1989) described eight morphotypes on the roots of urban blue and white spruce (*Picea glauca* and *P. pungens* Engelm., respectively) in Calgary. Bradbury *et al.* (1998) found 20 morphotypes on lodgepole pine in southwestern Alberta and Arocena *et al.* (1999) found 18 morphotypes on subalpine fir in central BC. The morphotype richness reported here is within the range of 20-35 fungal species reported by Bruns (1995) for several small sites occupied by single host tree species and with homogeneous environmental conditions. The variation in reported values between studies may be attributable to differences in host receptivity, inoculum potential at different sites, soil and environmental factors, sampling intensity, as well as in differences in individual techniques of those doing the descriptive analysis. Overall, 85% of all sampled root tips were ectomycorrhizal, which agrees with results from a similar study in Scotland that found about 80% of naturally regenerating Sitka spruce (*Picea sitchensis*) root tips were mycorrhizal (Flynn *et al.* 1998).

The relatively high number of morphotypes found in this study may be partly explained by differences in the composition of ECM communities in the three habitats. The total number of ECM morphotypes present in each habitat was almost equal: 19 morphotypes were found in the spruce-dominated wetlands (including 12 of the 14 most frequently occurring types)

compared to 20 types found in each of the mixed species habitats (including 11 of the 14 most frequently occurring types in each). Based on ECM morphotype composition, the average community similarity was 27% between all three black spruce habitats. Gehring *et al.* (1998) reported a change in ECM community composition associated with pinyon pine (*Pinus edulis*) across a moisture gradient (xeric to mesic), but no significant decrease in the overall number of species found at different sites. These findings emphasize the importance of sampling across the ecological range of a host species in order to completely describe its ECM fungal associates.

The majority of ECM morphotypes described in our study were basidiomycetes (~30) with only a few ascomycetes identified. However, ascomycete morphotype abundance (predominantly *Cenococcum* and *MRA* 1 ECM) accounted for between 17 and 19% of the entire community. In contrast, Danielson and Pruden (1989) found 31% of urban blue or white spruce ECM were basidiomycetes and 47% were ascomycetes. One reason for this difference may be that soils in the urban spruce study were dry and alkaline, as compared to the moist, acidic soils found on our sites. Gehring *et al.* (1998) also reported a greater proportion of ascomycetes to basidiomycetes associated with pinyon pine roots in xeric sites in Arizona. Strand-forming fungi (basidiomycetes) accounted for 60% of the ECM morphotypes described on lodgepole pine, white spruce and subalpine fir in mesic forests in northwestern BC (Kranabetter *et al.* 1999).

When all ECM morphotypes were compared, the occurrence, frequency and abundance of *Cenococcum* were higher than for any other ECM morphotype described. This morphotype was easily recognized by its black color, stellate outer mantle pattern, and large-diameter, unclamped emanating hyphae 5-6 µm in width (Agerer 1987-2002). *Cenococcum* is often considered a cosmopolitan fungus that has a wide host range and broad environmental

tolerances. It does not appear to be negatively affected by high water levels and is also able to survive in dry soils (Goodman and Trofymow 1998; Thormann *et al.* 1999). Malloch and Malloch (1981) found that *Cenococcum* was frequently associated with black spruce roots (16 of 30 root segments) collected from upland forest sites in northeastern Ontario; in northern Alberta, Thormann *et al.* (1999) also found *Cenococcum* on most black spruce roots from bogs and fens. In other studies, *Cenococcum* was the most frequently occurring ECM fungus found on lodgepole pine, white spruce and subalpine fir (Kranabetter *et al.* 1999) and western hemlock (Kranabetter and Friesen 2002) in mixed forests in northwestern BC. It has been found on roots of tamarack and willow in fens of varying nutrient status (Thormann *et al.* 1999). Van der Heijden *et al.* (1999) also describe *Cenococcum* on willow roots in calcareous-wet, acidic-dry and acidic-wet sites, but not from calcareous dry sites in dune ecosystems in Holland. Several authors suggest that *Cenococcum* seems to prefer moist, acidic soils of forests and wetlands, where it occupies the upper organic soil layers (Arocena *et al.* 1999; Taylor 2002).

In our study, *Cenococcum* abundance was higher in the black spruce-dominated wetlands than in either of the other habitats, but this difference was not significant. It was most frequently found in both the black spruce-dominated wetland and upland habitats compared to the black spruce - tamarack wetlands. Interestingly, several other studies did not detect *Cenococcum* or found it in low abundance. For example, *Cenococcum* accounted for only 2% of the overall ECM fungal community of urban blue and white spruce in Calgary, Alberta (Danielson and Pruden 1989), and it was not found on Sitka spruce roots in Scottish plantations (Flynn *et al.* 1998). In our study, it was not detected on seedlings from one site in the T habitat despite similarities in vegetation, soil and stand age compared to the other T sites. In fact, a subsequent study on tamarack and scrub birch roots growing on this same site has described *Cenococcum* on 50% of seedlings of each species, although overall

abundance was low (J. Catherall, pers. comm.). One reason that it may not have been detected on black spruce may have been due to the small number of seedlings sampled (5 from this site). Alternatively, there may be some competitive interactions between *Cenococcum* and other ECM fungi associating with black spruce on this site (e.g. members of the Thelephoraceae that appeared to dominate this site).

Mycelium Radicis Atrovirens (*MRA*) 1, the other abundant ascomycete characterized in our study, has been described as a common, broad host range, root-associated fungus by some (Summerbell 1989), but as a pathogenic fungus by others (De la Bastide and Kendrick 1990). It has been previously described on black spruce growing in northern Alberta fens (Thormann *et al.* 1999), as well as on numerous other hosts including lodgepole pine (Bradbury 1998; Durall *et al.* 1999; Kranabetter *et al.* 1999), hybrid spruce (Mah *et al.* 2001), white spruce (Kranabetter *et al.* 1999), Sitka and Norway spruce (Flynn *et al.* 1998), subalpine fir (Kranabetter *et al.* 1999), and Douglas-fir (Sakakibara *et al.* 2002). Here, *MRA* 1 was found on one third of all seedlings from the wetland habitats (although at low abundance), and on over half of the seedlings from the upland sites (at approximately three times the abundance level). This ECM type appears to be a habitat-generalist fungus, perhaps with a greater ability to compete with other fungal species for roots in upland as compared to wetland soils.

The family Russulaceae was represented by at least five morphotypes, including three in the genus *Lactarius*. These five morphotypes were yellow-orange to orange-brown in color, with mainly smooth textures and outer mantles of net to interlocking synenchyma. Hyphae of the other two Russulaceae types were usually unclamped and were often arranged in parallel sheets on the mantle surface. The Russulaceae 2 type was the second most abundant morphotype overall and was described from all three habitats. The three

Lactarius types had clampless hyphae, including larger laticifers, and hyphae filled with granular contents.

The Russulaceae include both narrow and broad host range ECM fungal species, including several that associate with members of the Pinaceae (including the genera *Picea*, *Larix* and *Pinus*) (Molina *et al.* 1992). Members of the Russulaceae have not been previously described associating with black spruce, although *Lactarius* and *Russula* species have been found on lodgepole pine (Bradbury 1998; Bradbury *et al.* 1998), ponderosa pine (Massicotte *et al.* 1999; Stendell *et al.* 1999), white spruce (Lilleskov *et al.* 2002), Sitka spruce (Flynn *et al.* 1998), Norway spruce (Agerer 1987-2002), Douglas fir (Massicotte *et al.* 1999), kinnikinnick (Sakakibara *et al.* 2002), and willow (Van der Heijden *et al.* 1999). In an *in vitro* study using black spruce seedlings, Doudrick *et al.* (1990) noted *Lactarius deliciosus*, *Lactarius rufus* and a third *Lactarius* species formed only weak hyphal mantles and no Hartig nets on seedling roots when grown in culture. In contrast, we found that the *Lactarius* morphotypes formed robust mantles and labyrinthine Hartig nets on black spruce roots from field sites. It has been suggested that ECM morphology following lab syntheses may differ from those ECM of hosts growing in soil where environmental influences and multiple organisms interact (Massicotte *et al.* 1999). *Lactarius* sporocarps were collected from our field sites, including *Lactarius deliciosus*, which has been described as having wide environmental tolerances and a lack of substrate specificity (Goodman and Trofymow 1998). *Lactarius rubrilacteus*, although not identified from our sporocarp survey, has also been found frequently in both mineral and organic soils (Goodman and Trofymow 1998).

The three *Lactarius* morphotypes identified in our study appear to exhibit habitat preferences. *Lactarius* 1, the most frequently occurring morphotype of the three, was significantly more abundant in the spruce - tamarack wetlands than in the upland habitats

(where it was not found). It also occurred in the spruce-dominated wetlands, but at low abundance. *Lactarius* 3 and *Lactarius* 2 were found in single habitats (upland and spruce-dominated wetland, respectively), but in relatively low abundance. Differences in dominant ECM host plants present in the mixed species habitats and soil moisture and nutrient content may account for the habitat specificity observed in these morphotypes. The *Lactarius* species identified from white spruce along a soil nitrogen gradient in Alaska occurred at low abundance at low N sites, but substantially increased in abundance on high N sites (Lilleskov *et al.* 2002). In our study, it is likely that differences in soil moisture played a greater role than nutrient availability in the occurrence of *Lactarius* 1, as it occurred in both wetland habitats. The occurrence of *Lactarius* 3 in only upland sites may be a result of fungal preference for drier habitats with a lower content of organic matter, and possibly increased nutrient levels. Alternatively, *Lactarius* 3 may be a broad host ranging fungus that forms symbiotic associations with other upland species such as lodgepole pine, which may provide a source of inoculum to regenerating black spruce seedlings.

Eleven morphotypes were classified as Thelephoraceae, including *Tomentella* and other unidentified genera. According to Agerer *et al.* (1995), mantles of irregular to regular synenchyma (pseudoparenchyma) and the consistent presence of hyphal clamps are known only from *Tomentella*. Here, in cases when clamp connections were not observed, morphotypes were classified as Thelephoraceae-like. The *Tomentella* morphotype described in our study closely matches that of an uncommon variant of the "*Tomentella*-like with cystidia" morphotype described by Danielson and Pruden (1989) on urban blue and white spruce in Calgary. The color, mantle features and presence of cystidia of the *Tomentella*-like 1 type are consistent with dark-mantled types of *Tomentella* (Agerer 1987-2002; Lilleskov *et al.* 2002), which have been described from black spruce peatlands in northern Alberta (Thormann *et al.* 1999). However, the lack of clamp connections prevented

classification of this ECM type into the *Tomentella* genus. Except for the lack of clamp connections, Thelephoraceae-like 1 resembles the "*Piceirhiza nigra*" morphotype described by Agerer *et al.* (1995).

The Thelephoraceae are known mycobionts of conifers and may be a widespread and important component of ECM communities (Danielson and Pruden 1989; Bradbury 1998; Bradbury *et al.* 1998; Durall *et al.* 1999; Kõljalg *et al.* 2000; Mah *et al.* 2001). Several species of *Thelephora* have been found to form ECM with little host restriction (Molina *et al.* 1992). Species from several genera of Thelephoraceae have been shown to form ECM with Norway spruce, including *Hydnellum peckii* (Agerer *et al.* 1995). Interestingly, the sporocarp for this species was collected from upland sites in our study. In addition, Thormann *et al.* (1999) described a *Tomentella* species associated with willow roots from Alberta peatlands.

Three frequently occurring Thelephoraceae and Thelephoraceae-like morphotypes appeared to exhibit habitat specificity, occurring predominantly in the wetland habitats compared to the upland sites. A *Tomentella* species was identified only in the spruce - tamarack wetlands; the *Tomentella*-like types also occurred most abundantly in the wetland habitats. Thelephoraceae-like 4 morphotype was found both more often and more abundantly in the spruce-dominated wetlands. Thelephoraceae-like 1 was most often described from the spruce - tamarack wetlands, but did occur in both of the other habitats. Many tomentelloid fungi form inconspicuous resupinate fruiting bodies on the underside of dead plants, wood and soil debris, making them difficult to find during sporocarp surveys (Agerer 1995; Kõljalg 1996; Agerer and Bougher 2001). This fruiting habit also led many to functionally classify this group of fungi as decomposers (Read and Perez-Moreno 2003). Hibbett *et al.* (2000) suggest the occurrence of occasional reversals of ECM fungi to a free-living saprotrophic lifestyle, particularly in the thelephoroid group. Perhaps this ability gives

them a competitive advantage over non-degrading ECM fungal morphotypes in nutrient-poor habitats.

The Cortinariaceae are a large family of woodland fungi, many of which are mycorrhizal (Arora 1986). Genera of this family contain species exhibiting the entire spectrum of narrow to broad host specificity (Molina *et al.* 1992). ECM of *Cortinarius* have been described on lodgepole pine (Bradbury 1998; Kranabetter *et al.* 1999), white spruce (Kranabetter *et al.* 1999; Lilleskov *et al.* 2002), Sitka spruce (Flynn *et al.* 1998), subalpine fir (Kranabetter *et al.* 1999), and willow (van der Heijden *et al.* 1999). *Hebeloma* has been described on hybrid spruce by Mah *et al.* (2001) and white spruce by Lilleskov *et al.* (2002), as well as other host species. In our study, four morphotypes were characterized as belonging to the Cortinariaceae family. Both the Cortinariaceae 1 and 2 types were among the most abundant ECM morphotypes and occurred frequently in all three habitats. The Cortinariaceae 3 and *Hebeloma* types occurred only in the spruce-dominated wetland habitat, but with very low abundance. Although our study shows a lack of habitat specificity by the Cortinariaceae types, Lilleskov *et al.* (2002) found that *Cortinarius* species associating with white spruce roots were confined to sites at the low end of a nitrogen deposition gradient in Alaska. In contrast, *Hebeloma* was found on sites with an intermediate supply of inorganic N. Like *Piloderma*, the Cortinariaceae are mat-forming fungi that may be specialized for nitrogen uptake under N-limiting conditions. Because all habitats in our study were presumed to be N-limiting, no habitat specificity was observed here.

Piloderma is a well-known ECM fungus with worldwide distribution and a broad host range (Harley and Smith 1983; Molina *et al.* 1992). In central BC, it has been described on hybrid spruce and subalpine fir (Arocena *et al.* 1999; Arocena *et al.* 2001). Smith *et al.* (2000)

reported that the presence of *Piloderma* is associated with forest stand age, based on their more frequent observations in old-growth than in younger stands of Douglas-fir. The occurrence of *Piloderma* has been strongly related to the percent cover of coarse woody debris (CWD) in decay class 5 (Goodman and Trofymow 1998; Smith *et al.* 2000), but it has also been described from acidic humus and at the boundary between the forest floor and mineral soils (Agerer 1987-2002; Arocena *et al.* 1999). In our study, the *Piloderma* morphotype was identified only from the upland sites, on all seedlings from site U3 (which were all rooted in CWD) and on one seedling from site U2. This species resembled *Piloderma fallax* (=*croceum*), which has been characterized by having pale to bright yellow, clampless emanating hyphae (often with needle-like crystals on the surface) and being rhizomorphic (Agerer 1987-2002; Arocena *et al.* 2001).

The habitat specificity of *Piloderma* observed in this study (and others) may provide some insight into its functional role in ecosystem processes such as nutrient cycling. Lilleskov *et al.* (2002) hypothesized that *Piloderma* (as well as *Cortinarius* and *Russula*) species are adapted to relatively acidic, N-poor soil conditions and appear to be specialized in efficient uptake of N. Some have suggested that *Piloderma* and other mat-forming fungi may increase nutrient availability to host trees partly by enzymatic degradation of the forest floor and soil organic matter (Griffiths and Caldwell 1992). Arocena *et al.* (1999) described higher exchangeable Ca^{2+}, Mg^{2+} and K^{+} values in soils dominated by *Piloderma* as compared to *MRA*, suggesting differences in biological activities of these two fungi. Soils with high amounts of exchangeable cations are considered fertile soils, which are able to supply trees with essential nutrients (Arocena *et al.* 1999).

The ECM fungus *Amphinema* was the third most abundant morphotype detected in our study and occurred frequently (20-47%) in each of the three habitats. *Amphinema*

(*byssoides*) is known to form ECM with many hosts (Molina *et al.* 1992) and has been reported as a major colonizer of urban blue and white spruce (Danielson and Pruden 1989), white spruce (Kranabetter *et al.* 1999; Lilleskov *et al.* 2002), hybrid spruce (Mah *et al.* 2001), lodgepole pine (Durall *et al.* 1999; Kranabetter *et al.* 1999), subalpine fir (Kranabetter *et al.* 1999), and Douglas-fir (Sakakibara *et al.* 2002). Our results agree with others in support of a lack of habitat specificity exhibited by this species. Despite the widespread distribution and broad host range of this fungus, little is known of its biology. In their N deposition gradient study, Lilleskov *et al.* (2002) found that *Amphinema byssoides* was present on sites where soil N content varied from low to intermediate levels. They suggest that *Amphinema* occurrence and abundance may be limited by soil acidification or nutrient imbalances rather than by the N content of the soil. This may reflect a role of this fungus in increasing the availability of nutrients other than N for plants (Lilleskov *et al.* 2002).

Several morphotypes remained unidentified following morphological characterization of black spruce ECM. The cottony halo type was the most abundant of the unidentified morphotypes and was described from the W habitat only. This type was distinguished by the organized arrangement of emanating hyphae in an interwoven halo loosely encircling the ECM root tip. No match could be located in published descriptions of ECM (Agerer 1987-2002; Ingleby *et al.* 1990; Goodman *et al.* 1996) to suggest the identity of this fungus. Other unidentified fungi occurred quite infrequently and it is possible that complete morphological descriptions (including an appreciation for the variation within a morphotype) may not have been obtained.

ECM community structure and diversity

From comparisons using several diversity indices, ECM community diversity for black spruce was generall greater in the black spruce – pine upland forests than in the mixed

black spruce - tamarack wetlands, which was, in turn, greater than in the black spruce-dominated wetlands. Both the Shannon and Margalef diversity indices are measures of species richness within communities, but the Shannon index places equal emphasis on the richness and relative abundance of community members (Magurran 1988; Krebs 1989). Using these two measures, differences detected were significant between the upland and spruce-dominated wetland habitats. The same community diversity trends were supported when diversity was assessed using the Simpson index, which places emphasis on the ECM types occurring in greater abundance. Neither the Simpson nor the Shannon Evenness measures were able to detect significant differences between habitats. With respect to black spruce, rare ECM types (those with low abundance) probably accounted for much of the diversity detected.

Greater community similarity (based on composition of different ECM morphotypes) was observed between the black spruce-dominated wetland and black spruce – pine upland habitats than between either of these habitats when compared to the black spruce – tamarack habitat. This may suggest that the composition of ECM fungal communities is influenced by the presence of tamarack, a potential host known to form ECM with several narrow to broad host ranging fungal genera (Molina *et al.* 1992). The importance of alternate ECM hosts as sources of inoculum is further supported by our observation that higher proportions of colonized roots were described from both the mixed species habitats (T and U) as compared to the black spruce-dominated habitat (W). Alternate ECM hosts (companion plants) can influence the ability of ECM fungi to colonize neighboring plants and may also influence the proportion of ECM that develop (Molina *et al.* 1992; Massicotte *et al.* 1999). Walker (1987) hypothesized that some ECM fungi that are unable to form symbioses with spruce alone may succeed in mixed spruce forests where pine or larch species are

present. In our study, both tamarack and lodgepole pine may have contributed to black spruce ECM community composition as sources of inoculum for newly growing roots.

ECM morphotype richness (calculated per seedling and averaged per habitat) was also greater in habitats that had a greater number of dominant host species (T and U). There were 11 potential ectomycorrhizal host plants near seedlings harvested for this study. No differences in ECM morphotype diversity were related to differences in number of host species present on a site, nor within the 0.5 m radius plots surrounding seedlings selected for harvest. Any influence of companion piants on ECM diversity may have been due to the biomass of underground roots interacting with the seedling roots. Thus, it may not be the number of potential ECM host species present, but the density of colonized roots acting as inoculum potential. A large proportion of these may have come from other large host species on the sites (i.e. tamarack and pine). Similarities in species composition of ECM colonizing naturally regenerating pine seedlings and mature trees found in Swedish boreal forests by Jonsson *et al.* (1999) may reflect the importance of large root systems as a source of inoculum for regenerating seedlings. The presence of companion species may explain at least part of the greater ECM community diversity found in the mixed species sites compared to the black spruce-dominated sites.

The trend showing greater diversity (significantly greater when compared to the spruce-dominated wetland habitat) in the upland habitat than in either wetland habitat might also be partly explained by differences in soil conditions on upland sites. In water-saturated, organic soils (such as those present on both the T and W sites), oxygen deficiency is expected to slow or prevent ECM formation due to inhibition of fungal aerobic metabolic processes and inability to meet the oxygen demand of the soil mycelial network (Tinker 1984; Walker 1987; Stenström 1991). We observed a significantly greater proportion of non-mycorrhizal roots

from W sites compared to U sites, and intermediate colonization levels from the T sites (which were very similar to W sites with respect to soil characteristics). *In vitro* experiments conducted by Stenström (1991) revealed variation in the susceptibility to flooding between different ECM fungi, and also that waterlogging was not associated with fungal oxygen deficiency. This could be because many forest fungi have hydrophobic mycelium, which may provide air pockets in the soil (Stenström 1991). Our observations of a generally increasing depth of the aerobic zone during the period of root growth and ECM establishment, as well as the propensity of black spruce seedlings to establish on hummocks in the wetland habitats, indicate that oxygen deficiency was unlikely to be a major factor in the composition of ECM communities on wetland black spruce.

Differences in soil nutrient availability may also influence the structure and diversity of ECM communities in different habitats. The methods used in our study to determine C and N content did not distinguish between inorganic and organic sources of these nutrients. Although greater amounts of C and N were found in wetland soils compared to upland soils, much of this may have been in inaccessible, organic forms due to low rates of decomposition in these cold, wet habitats (Thormann *et al.* 1999). All habitats were likely N-limited, as the highest N content found on any site was 2.6%. In habitats where mineralization rates are slow, natural selection is thought to favor associations with fungal symbionts that are physiologically equipped to capture nutrients from organic sources (Read and Perez-Moreno 2003). *Cortinarius*, *Hebeloma*, *Piloderma*, *Russula*, *Lactarius*, *Amphinema*, and *Tomentella* species have all been associated with relatively acidic, N-poor soil conditions (Lilleskov *et al.* 2002). Very low levels of available P were found in wetland soils as compared to upland soils. The availability of P may be less important than availability of N in ectomycorrhizal systems (Smith and Read 1997), but it is expected that

ECM fungi in P-limited habitats would also be equipped to maximize assimilation of P for their plant partners.

In our study, the relative abundance of ECM morphotypes showed the expected dominance of a few ECM types, with many other morphotypes found in progressively lower abundances (Krebs 1989; Horton and Bruns 2001; Taylor 2002). Overall, five morphotypes (*Cenococcum*, Russulaceae 2, *Amphinema*, *Lactarius* 1 and Cortinariaceae 2) were found with greater than 5% abundance, and nine additional morphotypes were found with greater than 2% abundance. All but *Lactarius* 1 were identified in all three habitats. The ability of certain ECM fungal species to dominate the root systems of host trees appears to be a widespread phenomenon, possibly due to competitive interactions between different species of fungi (Flynn *et al.* 1998; Kranabetter *et al.* 1999; Stendell *et al.* 1999; Taylor *et al.* 2000). Some suggest that the total number of species comprising a community may not be as important as the dominant species present because of perceived high levels of functional redundancy that may exist in soil microbial communities (Andrén and Balandreau 1999). When the relative abundance of ECM fungi comprising communities within each of the three habitats (β-diversity) was examined, we again observed the same pattern of dominance by a few ECM types. These results indicate that differences in relative abundance of ECM fungal species do not account for differences in community diversity between habitats.

With respect to our ability to measure richness and abundance, there is a relationship between sampling effort (number of tips sampled), probability of detection, and the relative abundance of species (percentage of root tips colonized) present (Taylor 2002). Compared to other similar assessments, the number of root tips examined in this study is believed to have been high enough to detect most ECM types. In a recent review, Horton and Bruns (2001) emphasized that the distribution of many ECM species is clustered, and that most

species occur in <10% of samples. This may explain why numbers of morphotypes described on individual seedlings are much lower than the overall number of morphotypes described on a site or within a specific habitat. One explanation for this clustered distribution of individual species may be due to the patchy distribution of suitable habitat features, such as the presence of CWD consistently associated with *Piloderma* (Bradbury *et al.* 1998; Smith *et al.* 2000). The correlation between specific habitat features and the occurrence and abundance of ECM fungi is a critical step towards developing functional hypotheses of the importance of individual species to ecosystem dynamics (Smith *et al.* 2000).

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Molecular Characterization of Ectomycorrhizal Communities Associating with Black Spruce (*Picea mariana*) in Wetland and Upland Forests in the Central Interior of BC

ABSTRACT

Ectomycorrhizal (ECM) fungal communities are composed of many genetic individuals, between which exist variations in their ability to colonize different host plants, to promote plant growth and to adapt to abiotic environmental factors. To examine the genetic diversity of ECM communities associating with naturally regenerating black spruce seedlings across three distinct habitats in central BC and to assess differences within characterized fungal morphotypes, a subsample of 888 root tips were subjected to molecular (PCR-RFLP) analysis. A total of 65 genotypes were delimited from 29 ECM morphotypes, with approximately 28 genotypes found in each of the three habitats. Within previously described ECM morphotypes, low diversity (one or two genotypes) was observed for *Piloderma*, *Lactarius* 3 and *Cenococcum*; higher diversity was observed for *Amphinema*, Russulaceae 1, Cortinariaceae 2, *MRA* 1, *Tomentella*-like 1 and Thelephoraceae-like 4. Molecular diversity of ECM communities did not vary between habitats; however, analysis revealed habitat-specificity of some genotypes within morphotypes described from all three habitats. Whereas ECM morphotypes such as *Cenococcum*, *MRA* 1 and Cortinariaceae 1 consisted of genotypes that appeared to be evenly distributed across the habitats, genotypes for *Amphinema*, Cortinariaceae 2, and Russulaceae 1 and 2 had more uneven patterns of distribution, occurring variously in one, two or all three of the habitats. Results may indicate ecotypic adaptation of ECM in response to heterogeneous environmental conditions.

INTRODUCTION

The ubiquitous, mutualistic symbioses between the roots of woody plants and ectomycorrhizal fungi are essential components of northern forest ecosystems (Smith and Read 1997; Amaranthus 1998). Ectomycorrhizae (ECM) mediate nutrient and water uptake in their plant hosts in return for energy-rich carbon compounds that support fungal metabolism and exchange resources with other members of the plant community through a common mycelial network (Smith and Read 1997; Simard *et al.* 1997; Amaranthus 1998). ECM fungal species exist as populations of many genetic individuals, between which there exists phenotypic variation in the ability to colonize different genotypes of host plant, promote plant growth and adapt to abiotic environmental factors (Bruns 1995; Jonsson *et al.* 2001). These fungi form diverse communities, even in forest stands with relatively homogenous soil conditions and dominated by a single host species (Bruns 1995; Horton and Bruns 2001). The functional role of high ECM diversity is still unclear, but it has been suggested that plants with access to a greater diversity of fungi may be better able to adapt to environmental changes and stress (Haug and Oberwinkler 1987; Amaranthus 1998; Durall *et al.* 1999). High ECM diversity may be especially important when considering the ability of entire ecosystems to respond to changing environmental conditions at regional and global scales (Prosser 2002).

Research questions related to ECM community structure and diversity rely on the ability to distinguish individual fungal species (Egger 1995). The use of the polymerase chain reaction (PCR) to amplify target sequences of rDNA is probably the most important methodological advance in the study of ECM communities (Horton and Bruns 2001). Subsequent enzymatic digestion of the internal transcribed spacer (ITS) region of rDNA with just a few restriction endonucleases produces sufficiently variable restriction fragment length polymorphism (RFLP) patterns that can distinguish between closely related species (Egger

1995; Kraigher *et al.* 1995; Mehmann *et al.* 1995). Based on differences in morphological characteristics, microscopic examination of ECM is suitable for sorting fungi into families genera, and in some cases, species; molecular analysis (PCR-RFLP) potentially allows identification of most fungal species through comparisons to reference databases of restriction fragment patterns (Egger 1995; Horton and Bruns 2001; Mah *et al.* 2001; Sakakibara *et al.* 2002).

The composition and diversity of ECM communities have been compared at the molecular level in several recent studies. Mah *et al.* (2001) found that the genetic composition of ECM communities of hybrid spruce (*Picea glauca x engelmannii*) seedlings varied between undisturbed, clear-cut, and cut and burned sites, but that diversity did not. Baldwin (1999) also found no difference in ECM molecular diversity for regenerating black spruce (*Picea mariana*) seedlings from clear-cut and cut and burned sites in paper birch (*Betula papyrifera*) – black spruce forests. In contrast, Byrd *et al.* (2000) reported lower ECM fungal species richness from soil core samples collected from clear-cut as compared to undisturbed sites. Across gradients of N deposition in Alaska and Europe, changes in ECM community composition of white spruce, Norway spruce (*Picea abies* (L.) Karst.) and beech (*Fagus sylvatica* L.) were accompanied by a strong decline in species richness as soil N availability increased (Taylor *et al.* 2000; Lilleskov *et al.* 2002). To our knowledge, molecular studies to compare ECM communities associated with a single host species across soil moisture gradients have not been conducted.

The primary objective of this study was to describe and compare the molecular diversity (using PCR-RFLP) of ECM communities associating with naturally regenerating black spruce seedlings across habitats and within characterized fungal morphotypes. The habitats examined consisted of two wetland forest types (black spruce-dominated and mixed

black spruce – tamarack sites) and an upland forest type (black spruce – lodgepole pine sites) located in the Sub-Boreal Spruce (SBS) biogeoclimatic zone of the central interior of BC (Meidinger *et al.* 1991). Soils were generally cold and nutrient-poor, with moisture content varying from saturated (subhygric) to seasonally dry (Roy *et al.* 1999; Krestov *et al.* 2000).

The second objective of this study was to confirm the characterization of ECM morphotypes by comparing restriction fragment profiles generated from representative sub-samples of roots from the same morphotypes previously described from these habitats. Whereas morphological features such as color, texture, and branching pattern of ECM may vary with changing environmental conditions, molecular approaches to describing ECM communities are largely independent of environmental effects (Egger 1995). It was anticipated that the molecular analysis would improve the resolution of ECM identification to species through comparisons to sporocarp and root tip databases as well as to descriptions published in the literature.

METHODS AND MATERIALS

ECM sample collection for molecular analysis and DNA extraction

Black spruce seedlings were randomly sampled from nine sites within three habitat types in the SBS zone near Prince George, BC as described in the previous chapter (see previous chapter for the detailed sampling protocol and site descriptions). Following morphological assessment of 200 root tips per seedling, a proportionally representative sample of 20 root tips (10% of each morphotype) was saved from each seedling for molecular analysis. Individual samples were placed in 1.5 mL Eppendorf tubes and stored at –20°C until processed for comparative molecular analysis.

DNA was extracted from ectomycorrhizal root tips using a modified Zolan and Pukkila (1986) protocol. Approximately 2 mm of the apical end of each frozen root tip was placed into a cold glass micromortar and crushed with the corresponding micropestel. To each micromortar, 350 µL of 2X hexadecyl trimethyl ammonium bromide (CTAB) buffer with 0.2% β-mercaptoethanol (Sigma Chemical Co.) was added, and the tips were further crushed until tissue was evenly suspended in the solution. The content of each micromortar was then transferred to a 1.5 mL Eppendorf tube, and incubated in a heat block (VWR Scientific) at 60°C for 45-60 min. After heating, 350 µL of chloroform: isoamyl alcohol (24:1 solution) was added to each tube and the tubes were centrifuged at 13000 x *g* for 10 min. at room temperature. The aqueous layer was removed to a new Eppendorf tube to which 350 µL of cold (-20°C) absolute isopropanol was added. The tubes were gently inverted repeatedly for 1 min. and then placed in the -10°C freezer for 5-10 min. After cooling, the tubes were inverted several more times, then centrifuged at 13000 x *g* for 10 min. at room temperature. The aqueous phase was poured off and the pellet was washed twice with 175 µL of cold 70% ethanol followed by centrifugation at 13000 x *g* for 3 min. The DNA pellet was dried overnight in a desiccator and resuspended the following morning in 50 µL Tris-EDTA (ethylenediaminetetraacetic acid) buffer. DNA extracts were stored in the freezer at -40°C.

DNA amplification and restriction endonuclease digestion

Enzymatic amplification of the fungal ITS region of fungal rDNA was conducted using the universal oligonucleotide primer ITS1 (TCC GTA GGT GAA CCT GCG G) (White *et al.* 1991) and the fungal-specific primer NL6Bmun (CAA GCG TTT CCC TTT CAA CA) (Egger 1995). A general PCR master mix was prepared that contained 17.2 µL millipore water, 3 µL 10X PCR buffer, 3 µL 2X dNTP's (2 mM each of dATP, dTTP, dCTP, and cGTP), 2.4 µL $MgCl_2$ (25µM), 1.2µL ITS1 (10 µM), 1.2 µL NL6Bmun (10 µM), and 0.14 µL Platinum Taq

DNA polymerase (Invitrogen). An aliquot of 27 µL PCR master mix was added each 0.2 µL reaction tube along with 3 µL DNA extract. DNA extracts were gently thawed in a 37°C oven for 15-20 min. prior to use. Reaction tubes were then placed in a PTC-100™ programmable thermal controller (MJ Research, Inc.), programmed to the following settings: denaturation at 94°C for 30 s and 93°C for 35 s, annealing at 50-52°C for 53 s, and extension at 72°C, beginning at 30 s and increasing by 5 s per cycle for 35 cycles. Following DNA amplification, 5 µL aliqots of PCR products were added to 3 µL aliquots of 6X loading buffer and loaded into wells of a 0.7% agarose gel (0.7 g agarose in 100 mL of 10X Tris-borate (TBE) buffer) submerged in a gel box of TBE buffer. Ethidium bromide fluorescent stain was added to each gel (20 µL for new gels; 6µL for reused gels) for DNA visualization. The DNA ladder (*Hind* III) was loaded into the first well of each row and the gel was run at 90-110 mV for approximately 45 min. Subsequently, gels were removed to a UV box to view the amplified DNA bands and to photograph using the Gel Print 2000I photographic system (BioPhotonic Corp.). Photographs were printed on Mitsubishi thermal paper (K65H Mitsubishi Electronic Corp.).

Three restriction endonucleases, *Alu* I (AGCT), *Hinf* I (GANTC), and *Rsa* I (GTAC), were used in this study. To 7.4 µL of enzyme solution (5 µL millipore water, 2 µL buffer, and 0.4 µL of one of *Alu* I, *Hinf* I, or *Rsa* I) in 0.2 mL microcentrifuge tubes, 7.4 µL PCR product was added. The tubes were incubated in a 37°C oven for a minimum of 5 h. Following incubation, the tubes were quickly centrifuged to 800 x *g* to remove condensation from the caps, and then 4 µL of 6X loading buffer was added to each tube. Digested DNA was run with 1 kb DNA ladder (Invitrogen) on high resolution 2.5% agarose (1 g agarose (Sigma Chemical Co.) plus 1.5 g low melting point agarose (Invitrogen) in 100 mL 10X TBE buffer) gels containing ethidium bromide for visualization. The gels were run at ~115 mV for approximately 3 h. Gels were removed to a UV box to view the DNA fragment patterns and

to photograph. Digital images were saved on disks using the Gel Print 2000I photographic system (BioPhotonic Corp.).

Analysis of molecular data

RFLP gel images were analyzed using RFLPscan, Version 3.12 (Scanalytics). Individual DNA fragments were marked and their sizes (base pairs) calibrated against the 1 kb standards (1018, 514, 356, 344, 298, 220, 201, 154, 134, and 75 bp fragments) using the Desmile calibration method with log piecewise linear curve-fitting. Fragments less than 80 base pairs were excluded from analysis to reduce the possibility of primer dimer products being included. Fragment patterns for individual samples were imported into both individual morphotype and seedling databases in RFLPscan Database, Version 3.12 (Scanalytics). Fragment patterns obtained from digestion of sporocarp samples (collected from field sites as described in the previous chapter) were imported into a separate database for comparisons.

Within each RFLPscan database, pairwise comparisons of all band patterns (matched at a 5% threshhold) were compiled for each of the three enzymes. Values ranged from 0 to 1 for each enzyme, and from 0 to 3 for each sample, with 3 indicating identical samples. Cluster analysis using the neighbor joining method of the similarity matrix was performed using PHYLIP (Phylogeny Inference Package), Version 3.573c (Felsenstein, J., University of Washington). Resulting neighbor joining trees were viewed in TreeView, Version Win 3.2 (1998 Roderick DM Page). Individual databases were merged into one large database consisting of all fragment patterns in order to compare patterns for the entire ECM community. Where fragment patterns matched and when ECM morphotypes had similar characteristics, two or more groups were merged into a single genotype or morphotype. The database of all fragment patterns was compared with a reference database (K. Egger,

unpublished) as well as the sporocarp database. From these comparisons, we attempted to confirm the morphological classification and taxonomic naming of ECM morphotypes, as well as to identify unknowns based on clustering with known types on the neighbor joining tree. Pairwise matching of all samples were also conducted using Dice's index ([2 x the number of common bands] / [2 x the number of common bands plus the number of polymorphic bands]) and converted to distance matrices (1 – Dice's index) to calculate the Phi (Φ) Index (Mah *et al.* 2001). An increase in Phi values implies greater diversity.

The fragment pattern data were imported into a Microsoft Excel 97 (version SR-1) spreadsheet. Using the neighbor joining tree as a guide, samples were first sorted into morphotypes, then into genotypes. Genotypes were defined as sets of fragment patterns that, when compared to each other, were within the 5% threshold of similarity for all three enzyme digests. To detect root tips that were possibly mis-classified during morphological assessment, contaminants that may have been amplified during PCR, or incomplete digestions, morphotype and genotype groups were cross-referenced by seedling, site and habitat. Samples determined as mis-classified were renamed. Partially digested or potentially contaminated samples were removed from the total sample. Average fragment sizes were calculated for each genotype.

Shannon, Simpson and Phi indices were used to assess genetic diversity between the different habitats (data pooled for sites) as well as morphotype variation. The Shannon and Simpson indices, which were calculated using proportional abundance of each ECM genotype, excluded all partially digested or potentially contaminated samples. The Phi Index, which was calculated from Dice distance matrices, included all successfully digested root tips. All three indices were compared between habitats using one-way ANOVA

(SYSTAT version 8.0, 1998, SPSS Inc.) to determine significant differences (α=0.05). Mean comparisons were tested using Fisher's Least Significant Difference (LSD) test (α=0.05).

RESULTS

DNA extraction, amplification and restriction endonuclease digestion

Based on the original sample of the 888 root tips, 454 (51%) yielded fragment patterns for the molecular analysis. The success rate from the 755 colonized tips was 60%. Non-mycorrhizal tips accounted for 133 of the original sample, of which 32 (24%) actually amplified and produced DNA fragment patterns. The remaining samples included those that were either lost or contaminated during the DNA extraction procedure, did not amplify properly to yield visible bands, produced doublet fragment patterns during the PCR procedure, or produced very faint bands or extra fragments after restriction endonuclease digestion.

The success rate of producing fragment patterns appeared to vary with morphotype. In general, >75% of *Amphinema* and *Piloderma* types produced patterns compared to only 37-48% for the Cortinariaceae and Thelephoraceae –like types (Table 3.1). Both *Cenococcum* and *MRA* 1 had moderate success rates (50% and 74%, respectively). Of non-mycorrhizal tips that produced patterns, over 80% matched patterns of *Cenococcum* (genotypes 1 and 2), *MRA* 1 (genotypes 2 and 3), *Amphinema* (genotype 3), Russulaceae 1 (genotype 1), Russulaceae 2 (genotypes 1 and 6) and Thelephoraceae-like 2 (genotype 2). In each case, the corresponding morphotype was previously described on the seedling or site with which the non-mycorrhizal tip was associated.

Cluster analysis of fragment patterns for ECM morphotypes

Cluster analysis of fragment patterns for all ECM sampled for molecular assessment generally confirmed the morphological classification. ECM classified as the same morphotype tended to cluster together on branches of the neighbor joining tree (Appendix III). Comparisons of these ECM patterns with reference databases containing patterns from ECM root tips from previous studies often confirmed the taxonomic naming of morphotypes (phylograms not included). For example, reference samples of *Amphinema*, *Piloderma*, *Cenococcum* and *MRA* 1 were consistently grouped within clades representing those morphotypes described in our study. In one case, a group of non-mycorrhizal samples clustered with a reference sample of E-strain, allowing tentative identification of these ECM as a morphotype not described in this study.

Comparison of patterns from ECM root tips with patterns generated from sporocarps collected from field sites generally did not aid in identification of morphotypes. The sporocarp patterns usually grouped on their own branches rather than within morphotype clades. However, there were two exceptions to this. *Lactarius deliciosus* (Fries) S.F. Gray grouped within the *Lactarius* 1 genotype 1 clade and *Lactarius torminosus* (Schaeff. ex Fr.) Gray grouped within the *Lactarius* 3 genotype 1 clade.

Most morphotypes consisted of several genotypes; the average fragment lengths for all genotypes are presented in Table 3.1. ECM morphotypes having the most genotypes included *Amphinema* (six), Cortinariaceae 2 (six), and Russulaceae 2 (seven). *Cenococcum* (two), Thelephoraceae-like 1 (one), *Piloderma* (one) and *Lactarius* 3 (one) had the fewest number of genotypes. In some cases, delimitation of genotypes was based on fragment size variation in only one of the three enzyme digests. For example, *Amphinema* genotypes 1, 2 and 3 varied by only the *Alu* I and genotype 4 differed from genotype 1 in

patterns generated by only the *Rsa* I enzyme. The single genotype of *Lactarius* 3 arguably varied from *Lactarius* 1 genotype 1 in only the *Hinf* I pattern, as both profiles showed fragments that were not digested by the *Rsa* I enzyme, even though length variation was observed. For some ECM genotypes, differences were observed in two of the enzyme profiles. For example, with respect to Russulaceae 1, genotypes 1 and 2 showed very similar fragment patterns with the *Hinf* I enzyme, but different patterns with the *Alu* I and *Rsa* I enzymes. Most genotypes differed from one another in all three enzyme profiles. This was especially true when genotypes were compared between morphotypes.

In several instances, the similarity in fragment patterns allowed genotypes and, sometimes, entire morphotypes to be merged. Examples include a Russulaceae 2 genotype that was found to match *Lactarius* 1 genotype 3, and the single cottony gold-brown genotype, which matched *Amphinema* genotype 6. In each case, there were also similarities in the morphological descriptions and habitat occurrence, which supported merging the genotypes.

Interestingly, when multiple genotypes occurred within a morphotype, a single genotype often appeared to dominate. For example, *Cenococcum* genotype 1 accounted for 83% of all *Cenococcum* samples (total of two genotypes), *MRA* 1 genotype 2 represented 59% (total of three genotypes), and Russulaceae 2 genotype 1 made up 46% (total of seven genotypes) of all samples. Within other morphotypes such as *Amphinema* (total of six genotypes), a more even abundance of genotypes was observed.

Table 3.1. Habitat (black spruce – tamarack (T) wetlands, black spruce-dominated (W) wetlands, and black spruce – pine (U) upland forests) and approximate fragment sizes (bp) of the amplified ITS region for black spruce ECM morphotypes and genotypes.

ECM Morphotypes	Habitat			No.	Undigested	Approximate fragment sizes (bp)													
and Genotypes	T	W	U	samples	size (bp)	*Alu* I				*Hinf* I					*Rsa* I				
Cenococcum																			
genotype 1	+	+	+	45	790	440	150	110	80	275	165	130	100		920				
genotype 2		+		8	915	400	240	150	115	435	290	165			950				
MRA 1																			
genotype 1			+	3	870	640	145	120		385	210	145			790	180			
genotype 2	+	+	+	14	855	650	150	110		445	250	160			630	170			
genotype 3	+	+	+	6	935	655	150	115		440	185	165	120		790	190			
Russulaceae 1																			
genotype 1	+	+	+	7	770	365	175	160	110	280	165	150			730	175			
genotype 2	+			2	860	445	230	190	110	285	165	155			835	175			
genotype 3	+			5	920	425	190	130	115	350	165	150	125	105	1000				
Russulaceae 2																			
genotype 1		+	+	26	950	690	190	110		335	290	165	150		555	195	175		
genotype 2			+	4	1000	740	190	115		335	295	170	155		610	205	185		
genotype 3			+	4	980	710	190	115		335	290	165	150		775	200			
genotype 4	+			12	980	530	310	150	110	295	200	165	140	100	465	200	165	105	
genotype 5			+	1	955	600	260	150		365	185	165	145		995				
genotype 6			+	5	1015	620	185	150	110	360	345	160	145		975				
genotype 7			+	3	860	370	195	110		320	290	165	150		980				
Russulaceae 3																			
genotype 1	+			1	1040	590	190	170	110	360	285	170	155		615	300	175		
Russulaceae 4																			
genotype 1			+	4	1040	465	285	190	115	415	310	170	155		1015				
Lactarius 1																			
genotype 1	+	+		21	1070	520	290	190	110	415	350	165	150		1015				
genotype 2	+			3	950	430	245	190	115	325	215	165	145		1020				
genotype 3		+		11	1000	470	280	185	105	335	285	170	155		555	465			

ECM Morphotypes and Genotypes	Habitat T	W	U	No. samples	Undigested size (bp)	Approximate fragment sizes (bp) *Alu* I					*Hinf* I					*Rsa* I		
Lactarius 2																		
genotype 1	+			3	950	510	190	110			350	330	170	155		935	100	
genotype 2	+			3	980	520	190	115	85		340	320	165	155		1055		
Lactarius 3																		
genotype 1			+	16	1070	515	285	185	110		350	315	165	150	100	1040		
Thelephoraceae 3																		
genotype 1		+		4	980	405	230	220	120		340	185	130	90		625	410	175
Thelephoraceae-like 1																		
genotype 1	+			8	950	420	185	150	110	95	320	225	165	150		855	175	
genotype 2		+		4	950	430	185	150	95		350	300	165	150		1025		
Thelephoraceae-like 2																		
genotype 1			+	4	1010	560	190	155	110		320	260	170	150	85	840	185	
genotype 2	+			3	1000	525	235	180	110		330	255	165	150		830	210	
Thelephoraceae-like 3																		
genotype 1	+			1	965	425	185	150	120		360	320	165	150		1025		
Thelephoraceae-like 4																		
genotype 1		+		7	1000	475	280	185	125		325	280	170	150		545	455	
genotype 2		+		3	900	395	260	185	110		320	215	155	145		580	180	160
genotype 3		+		3	965	465	245	185	110		315	295	160	145		975		
Tomentella 1																		
genotype 1	+			6	1000	370	190	125	105	90	325	265	165	155	110	945	180	
genotype 2	+			2	1000	480	380	185			315	285	235	120		1025		
genotype 3	+			1	990	430	190	150	115	95	360	320	165	155		790	205	
Tomentella-like 1																		
genotype 1	+			6	965	435	230	185	120	110	315	190	165	150		990		
genotype 2	+			1	1010	525	250	190	160	115	365	170	155			910	185	
Tomentella-like 3																		
genotype 1	+			1	875	415	185	120	110	90	220	190	165	150		980		

ECM Morphotypes and Genotypes	Habitat T	W	U	No. samples	Undigested size (bp)	Approximate fragment sizes (bp) *Alu* I					*Hinf* I					*Rsa* I				
Cortinariaceae 1																				
genotype 1		+	+	6	1060	670	185	145	110		370	340	140	120		935	175			
genotype 2	+	+	+	9	1040	620	185	145	110		360	345	165	155		850	175			
genotype 3	+			2	1040	605	185	145	110		355	345	165	150		1060				
Cortinariaceae 2																				
genotype 1	+	+		7	955	430	185	145	115	80	335	290	170	155		785	175			
genotype 2		+	+	11	940	355	235	185	150	130	360	170	150			910	175			
genotype 3	+			2	1045	735	225				370	260	165	150		835	225	165		
genotype 4			+	5	1075	440	330	220	185		340	300	170	155		1090				
genotype 5			+	3	1035	440	190	150	110	90	370	350	165	150		905	180			
genotype 6			+	2	985	450	185	145	110	85	335	305	160	150		1030				
Cortinariaceae 3																				
genotype 1		+		10	930	665	165	115			320	290	165	155		520	395			
Hebeloma																				
genotype 1		+		2	895	360	240	180	135		335	275	130			840	195			
Piloderma																				
genotype 1			+	17	920	365	260	190	110		315	180	165	155		850	175			
Amphinema																				
genotype 1			+	16	900	365	190	140	110		325	295	165	155		780	175			
genotype 2	+			14	950	575	185	110	85		325	290	165	155		790	175			
genotype 3		+		10	940	365	235	150	125	100	335	285	165	110		775	175			
genotype 4		+	+	5	950	460	363	160			315	285	165	150		945				
genotype 5			+	10	940	365	190	140	115		320	285	160	145		920	180			
genotype 6	+			6	920	275	240	185	175	110	370	170	155			1085				
cottony halo																				
genotype 1		+		6	1040	425	250	190	115	105	345	330	165	155		1035				
genotype 2		+		8	1055	430	255	190	130	95	355	330	165	150		885	175			
genotype 3		+		2	1000	395	260	185	110	100	320	220	165	155	85	625	200	175		

ECM Morphotypes and Genotypes	Habitat T	W	U	No. samples	Undigested size (bp)	Approximate fragment sizes (bp) *Alu* I						*Hinf* I						*Rsa* I
creamy rhizomorphic clamped																		
genotype 1	+			5	940	595	190	110				295	240	165	155	85	985	
genotype 2			+	4	950	430	185	145	110			295	230	165	145	115	1025	
orange 1																		
genotype 1			+	2	940	430	185	150	125	115	95	220	170	140	115		1070	
brown 1																		
genotype 1	+			2	900	590	420					215	180	165	150		985	
brown 3																		
genotype 1			+	2	1040	365	295	185	125	90		370	335	165	150		1030	
E-strain																		
genotype 1	+	+	+	6	830	360	255	180	105			325	175	140			950	

DNA fragments (bp) from amplification of fungal rDNA using the ITS1 and NL6Bmun primers and digestion with the restriction endonucleases *Alu* I, *Hinf* I and *Rsa* I.

Molecular diversity within ECM morphotypes

Table 3.2 shows Shannon, Simpson and Phi diversity index values for 14 commonly occurring ECM morphotypes producing fragment patterns in this study. All three diversity indices identified *Piloderma*, *Lactarius* 3 and *Cenococcum* as the morphotypes exhibiting the lowest molecular diversity. Both *Piloderma* and *Lactarius* 3 samples (described from the upland sites only) generated only one fragment pattern each; *Cenococcum* had two genotypes. The Phi index value was also low for the cottony halo morphotype. *Amphinema*, Cortinariaceae 2 and Russulaceae 2 (Shannon and Simpson) and *Tomentella*-like 1, Thelephoraceae-like 4 and *MRA* 1 (Phi) resulted in the highest index values. All three diversity indices suggested that *Lactarius* 1, Cortinariaceae 1 and *Tomentella* exhibited intermediate diversity values.

Table 3.2. Diversity values (Shannon, Simpson and Phi) for 14 ECM commonly occurring on regenerating black spruce in three habitats.

ECM Morphotype	No. of genotypes	Habitat T	W	U	% success	n	Shannon	Simpson	Phi
Cenococcum	2	+	+	+	50.5	54	0.42	1.35	0.121
MRA 1	3	+	+	+	74.2	32	0.92	2.32	0.367
Russulaceae 2	7	+	+	+	65.1	56	1.70	4.33	0.247
Lactarius 1	3	+	+		50.0	37	0.73	1.73	0.239
Lactarius 3	1			+	80.0	16	0.00	1.00	0.140
Thelephoraceae-like 1	2	+	+		48.0	12	0.64	1.94	0.177
Thelephoraceae-like 4	3		+		37.1	13	1.01	2.89	0.380
Tomentella 1	3	+			37.5	9	0.85	2.25	0.214
Tomentella-like 1	2	+			36.8	7	0.41	1.40	0.413
Cortinariaceae 1	3	+	+	+	44.7	18	0.96	2.62	0.214
Cortinariaceae 2	6	+	+	+	48.4	34	1.60	4.78	0.211
Piloderma	1			+	77.3	18	0.00	1.00	0.048
Amphinema	6	+	+	+	80.3	61	1.71	5.61	0.217
cottony halo	3		+		94.1	16	0.97	2.73	0.112

Habitat effects on ECM genotype distribution and diversity

With respect to molecular diversity, there were no significant differences between mixed black spruce – tamarack wetland, spruce-dominated wetland, and mixed black spruce – lodgepole pine upland habitats, as measured by the Shannon, Simpson and Phi indices (Table 3.3). The

Simpson index suggested possibly greater diversity in the W sites; in contrast, Phi values were highest for the T and U habitats. Shannon values were similar across all habitats.

Table 3.3. Mean diversity values (Shannon, Simpson and Phi indices) for molecular genotypes of ECM from three black spruce habitats.

Diversity Index	Treatment Effect		Tamarack–Spruce Wetland	Black Spruce Wetland	Pine – Spruce Upland
	F	P			
Shannon	0.075	0.928	2.300 (0.070)	2.297 (0.130)	2.247 (0.117)
Simpson	1.727	0.256	9.513 (0.877)	11.300 (1.857)	7.670 (1.227)
Phi	1.436	0.309	0.363 (0.035)	0.307 (0.027)	0.378 (0.031)

Values for indices are means with standard error in parentheses. Means were tested using a one-way ANOVA (α=0.05) for each habitat.

Genotype occurrence across habitats (Table 3.1) and between sites revealed distribution patterns that were not obvious following morphotype characterization. Some ECM morphotypes such as *Cenococcum*, *MRA* 1 and Cortinariaceae 1 were described morphologically as occurring in all three habitats. Genotypes identified for each of these ECM also appeared to have a fairly even distribution across the habitats. In contrast, genotypes for other ECM that also occurred in all three habitats (morphotypes such as *Amphinema*, Cortinariaceae 2, and Russulaceae 1 and 2) had more uneven patterns of distribution. *Amphinema* genotypes were limited to one (for five genotypes) or two (for one genotype) habitats, with approximately two genotypes in each habitat. Cortinariaceae 2 had two genotypes in two of three habitats, with the remainder (four) in single habitats. One Russulaceae 1 genotype was in all three habitats; two genotypes only occurred in T sites. With respect to Russulaceae 2, six of eight genotypes only occurred in U sites, and one genotype each was in each wetland habitat.

Other habitat distribution patterns were observed when ECM genotypes were considered within larger taxonomic groups. For the Cortinariaceae group, the number of genotypes occurring in all habitats was fairly even. In contrast, more *Lactarius* genotypes were in the T habitat (four)

then in either of the W (two) or U (one) habitats. Interestingly, one half of Russulaceae genotypes occurred in the U habitat (eight), with the remainder divided fairly evenly between the wetland habitats. The majority (88%) of genotypes in the group comprised of Thelephoraceae, *Tomentella* and brown types were in the wetland habitats, with twice as many in the T sites.

DISCUSSION

Molecular diversity: genotype and ECM community variation across habitats

Sixty-five individual genotypes were delimited from the 29 ECM morphotypes successfully amplified and digested in our study. This order of magnitude is in line with findings of others using similar methods. In BC, Mah *et al.* (2001) detected 22 genotypes for the eight most common ECM morphotypes described on hybrid spruce and Sakakibara *et al.* (2002) documented 26 genotypes for 11 morphotypes of Douglas-fir. Mehmann *et al.* (1995) found 23 genotypes within 18 morphotypes on Norway spruce in Switzerland. Not all fungal genotypes were detected in all habitats sampled and this has also been previously corroborated by several authors (Gehring *et al.* 1998; Jonsson *et al.* 1999; Mah *et al.* 2001; Sakakibara *et al.* 2002). Six genotypes were found in all three black spruce habitats, six were identified in two habitats, and 53 occurred in single habitats only. Approximately 28 genotypes were found in each of the three habitats.

Our results suggest that, although some genotypes (within morphotypes) were habitat-generalists, several genotypes were habitat-specialized. Dominant genotypes for *Cenococcum*, *MRA* 1, and Cortinariaceae 1 were found in all three habitats; however, one genotype for each of these ECM occurred in low numbers in only one habitat. All genotypes for *Amphinema*, Cortinariaceae 2, and all Russulaceae (with one exception) occurred in only one or two habitats. Mah *et al.* (2001) also detected site-specific and seedling type-specific genotypes for *MRA*,

Amphinema and *Tuber* on hybrid spruce roots from naturally regenerating or planted seedlings from mature, clear-cut, or cut and burned sites. Sakakibara *et al.* (2002) provide evidence for a habitat-specific genotype of *Cenococcum* on Douglas-fir seedlings in addition to a habitat-generalist *Cenococcum* genotype found in both forest types from which they sampled. Based on RFLP profiles, Byrd *et al.* (2000) identified 106 ECM species for lodgepole pine, of which only 10 species were found in both clear-cut and undisturbed sites. Kõljalg *et al.* (2000) also described a range of site-specificities for tomentelloid genotypes in Swedish boreal forests based on ITS sequences. Few studies have directly examined the spatial distribution of ECM genotypes in different habitats, and incidental distribution information is often lost in studies where the number of samples subjected to molecular analysis is low. For example, Kernaghan (2001) described *Amphinema byssoides*, *Cenococcum geophilum*, and *Tomentella* species as being ubiquitous in temperate ectotrophic plant communities, and as forming symbioses with a wide variety of hosts in two high-elevation sites in the Alberta Rockies. Their small molecular sample size, though suitable for confirming the identity of several ECM through comparisons to a database of sporocarp RFLPs, was not large enough to detect variation in genotypes between sites.

Based on comparisons of diversity index values (Shannon, Simpson and Phi), there was no significant difference in molecular diversity between mixed black spruce – tamarack wetland, black spruce-dominated wetland and black spruce – lodgepole pine upland forest habitats. Similar to Gehring *et al.* (1998), our study found that species (genotype) richness of the ECM fungal community remained fairly constant across environmental gradients ranging from extreme (i.e. very wet in our study) to fairly moderate. One explanation may be that fungal symbionts receive most of their energy from their plant hosts, and therefore may be buffered against the environmental extremes that their plant associates experience (Gehring *et al.* 1998).

Although our original sampling for molecular analysis was proportional to ECM morphotype occurrence, variable amplification and digestion success rates most likely affected the resulting relative abundance of genotypes and subsequent analysis.

The Shannon index values indicated close similarities in genotype richness and evenness between habitats. The Simpson index (which emphasizes dominant genotypes) showed a trend of decreasing diversity from the black spruce-dominated wetlands, to the black spruce – tamarack wetlands, to the black spruce – pine uplands; this trend was opposite to the results for morphotype diversity measurements. The dependence of the Shannon and Simpson indices on defining genotype proportional abundance presents an inherent problem with using these indices to measure the molecular diversity of the ECM community. The Phi Index, which does not rely on proportional abundance for genotypes (Mah *et al.* 2001), resulted in diversity values that exhibited similar trends compared to those observed for morphological diversity (i.e. greater diversity in uplands and mixed wetlands compared to black spruce-dominated wetlands).

Because ECM fungal species exist as populations of many genetic individuals, there is, theoretically, some phenotypic variation in the ability of these species to colonize host plants, promote plant growth, and adapt to changing environmental factors (Molina *et al.* 1992; Gehring *et al.* 1998). Relatively little is known about the levels of phenotypic variation under different environmental conditions (Egger 1995). On large geographic scales, the ITS region is expected to show intraspecific variation because it evolves rapidly and populations of a species can become reproductively isolated (Horton 2002). However, based on a small sporocarp sample, Kårén *et al.* (1997) reported that the intraspecific variation in the ITS region was negligible at local scales. If some intraspecific variation represents ecotypic adaptation to environmental pressures, it seems probable that variation in the ITS region could occur at different geographic

scales ranging from regional landscapes to localized heterogeneity in edaphic conditions (Cairney 1999). In a study that assessed pinyon pine ECM along soil moisture and nutrient gradients in Arizona, Gehring *et al.* (1998) demonstrated a link between ECM community composition (shifts in fungal genera and species between sites) and soil type in abiotically stressed sites.

Abiotically stressed habitats, as defined by Gehring *et al.* (1998), are those at the extreme ends of environmental gradients of soil moisture, nutrients, temperature, etc. In our study, water-saturated soils may present challenges to at least some ECM fungi in their ability to thrive and form functional symbioses (Stenström 1991; Roy *et al.* 1999). In addition, saturated soils may exert selective pressure on the ITS region, resulting in genotypic differences. Localized heterogeneity in other soil characteristics (e.g. nutrient availability, temperature, pH, etc.) and microtopography, as well as relative position to other major ECM vegetation, may account for genotype differences within sites, although no correlations with specific habitat attributes were detected in the present study. Assessing the distribution of genotypes and how this might relate to the ecological roles of ECM fungi would be interesting to explore in future studies; this would require extensive multi-isolate screening to determine the extent of ecotypic adaptation in ECM fungi (Cairney 1999).

Molecular diversity within ECM morphotypes

Variation in the number of fragment patterns within morphotypes ranged from low for types such as *Piloderma*, *Lactarius* 3, and *Cenococcum* to high for *Amphinema*, *MRA* 1, Cortinariaceae 2 and Russulaceae 2. The number of genotypes varied greatly, depending on morphotype. Mah *et al.* (2001) also found large differences in patterns within some morphotypes; in their study, *Cenococcum* exhibited a single genotype compared to six genotypes for *Amphinema*. In

contrast, Sakakibara *et al.* (2002) attempted to sample ECM to increase chances of detecting variation but found very little in the ITS region for the morphotypes they examined. One possible reason for this difference is that there may be little variation in the ITS region of some Douglas-fir ECM. Minor differences in analysis techniques, as well as decisions made during morphotyping (e.g. whether to classify morphotypes together or to separate them) and assigning fragment patterns to genotypes, may all account for differences identified within morphotypes. In addition, genotypes were defined by similarities in fragment patterns within a 5% tolerance limit in our study. Others have used a 6% tolerance limit using the same analysis software (Scanalytics) as used in our study (Mah *et al.* 2001), or set an approximate tolerance limit based on visual pattern comparisons as the method of analysis. Viaud *et al.* (2000) suggest that each step in a molecular analysis (i.e. sampling, DNA extraction, PCR and RFLP) can be a source of bias which may lead to a distorted view of what is happening in the natural system.

Some of the genotypic diversity within morphotypes in our study likely resulted from the relatively large number of samples processed. Sakakibara *et al.* (2002) found that 8 of 11 morphotypes each had one dominant set of fragment patterns; these dominant patterns represented 80-100% of all 227 root tips generating patterns. Situations in which one genotype dominated within a morphotype were also found in our study as well as in those of Gehring *et al.* (1998) and Mah *et al.* (2001); however, within some morphotypes, a more even abundance of several genotypes was observed. Horton (2002) examined several sporocarps for each fungal species from a 10 km^2 area and reported that some species had multiple genotypes with one dominating, while some had multiple genotypes without any genotype dominating (e.g. *Lactarius deliciosus*). In this example, most of the fragment patterns varied in only one of three enzymes, suggesting limited variations in ITS sequence.

The Shannon and Simpson indices assessed ECM molecular diversity as highest for *Amphinema*, Cortinariaceae 2 and Russulaceae 2 morphotypes as compared to Phi index values that were also high but less than values for *MRA* 1, Thelephoraceae-like 4 and *Tomentella*-like 1. With respect to Thelephoraceae-like 4 and *Tomentella*-like 1, relatively low sample sizes and very low amplification and digestion success rates may have contributed to the higher Phi values. For *MRA* 1, the neighbor joining tree shows many small variations (within the 5% tolerance) in fragment patterns grouped under three genotypes of *MRA* 1. These differences might increase the pairwise distances resulting in greater Phi diversity as compared to Shannon and Simpson diversity, where the number of genotypes and their relative abundance were emphasized in the calculations. All diversity indices showed agreement for the middle values, suggesting some level of congruence among indices. Intermediate diversity was observed for *Tomentella*, *Lactarius* 1 and Cortinariaceae 1.

The lowest diversity, indicated by all three diversity indices, was in *Piloderma* and *Lactarius* 3, two habitat-specific morphotypes found only in upland habitats, and *Cenococcum*, a generalist found in all three habitats with one genotype found only in black spruce-dominated wetlands. Fragment patterns of *Piloderma* ECM (found strictly associated with coarse woody debris in this study) were similar to those of *Piloderma* genotype 2 (Mah *et al.* 2001), *Piloderma* III and *P. byssinum* IV (Sakakibara *et al.* 2002). Multiple genotypes for *Piloderma* have been described in the other studies, but not ours. *Lactarius* 3 was tentatively identified as *L. torminosis* through comparison to a sporocarp reference database. *Lactarius* species have been shown to exhibit intraspecific variation in other habitats (Horton 2002).

Most investigators who have examined the ITS region have detected intraspecific variation in some ECM morphotypes or species. Assessing fragment patterns from cultured *Cenococcum geophilum* isolates from geographically-diverse origins, LoBuglio *et al.* (1991) reported a high level of rDNA variation in 71 isolates; Farmer and Sylvia (1998) also detected intraspecific genetic variation in the fungi *Cenococcum geophilum* and *Pisolithus arhizus*. From ITS sequences of cultured organisms, Aanen *et al.* (2001) described variation in *Hebeloma velutipes* and Manian *et al.* (2001) found heterogeneity among common European *Suillus* isolates. Further evidence for multiple genotypic species within morphologically-defined fungal species have been shown for *Cortinarius rotundisporus* and *Hebeloma* species (Sawyer *et al.* 1999) as well as *Lactarius deliciosus* and *Inocybe lacera* (Horton 2002). In our study, the highest molecular variation was observed in the less well-defined morphotypes (i.e. Cortinariaceae and Russulaceae); these grouped in polyphyletic clusters of genotypes on the neighbor joining tree and may actually represent several species. In taxa such as *Laccaria*, *Inocybe*, and Cortinariaceae, there appears to be a higher probability of observing intraspecific genetic variation (Kårén *et al.* 1997; Horton 2002). This was true for the Cortinariaceae types described in our study.

Interestingly, the genetic heterogeneity of *Cenococcum* rDNA reported from culture studies by LoBuglio *et al.* (1991) and Farmer and Sylvia (1998) was not observed in the field-based studies described by Mah *et al.* (2001), Sakakibara *et al.* (2002) and ours. Besides the use of cultured isolates versus field-collected root tips and different PCR primers, an important distinction between these studies was the geographic origin of fungal rDNA. Fungi used in the studies by LoBuglio *et al.* (1991) and Farmer and Sylvia (1998) originated from broad geographic regions in the eastern USA and Europe, while the three latter studies were conducted in temperate forests in the interior of BC. Comparison between fragment patterns of the five *Cenococcum*

genotypes from BC suggests potential overlap in two patterns: genotype 1 (our study) and *Cenococcum*-like I (Sakakibara *et al.* 2002). This similarity is based on patterns from two restriction enzymes (*Alu* I and *Hinf* I) that also produced nearly identical patterns in *Cenococcum* genotype 1 (Mah *et al.* 2001); the latter genotype differs from our dominant *Cenococcum* genotype in the *Rsa* I enzyme pattern. Gehring *et al.* (1998) suggest that restriction endonuclease digestion with two enzymes may produce fragment patterns that distinguish among genera, and frequently species within a genus. Different species were also distinguished by Gardes and Bruns (1996) using two restriction enzymes. Whether or not the *Cenococcum* genotypes described in Mah *et al.* (2001), Sakakibara *et al.* (2002) and our study are of the same species, there appears to be some variation in the *Cenococcum* ITS region across three different host species within relatively similar forest types of interior BC.

Identification of ECM Species

Comparisons between fragment patterns for ECM described in our study and reference databases compiled from previously described ECM root tips and sporocarps generally supported the morphological classification of ECM to fungal genera and families. In most cases, these comparisons did not provide higher resolution for identification of fungal species and, with the possible exception of two *Lactarius* species, failed to definitively identify unidentified ECM morphotypes. Others have reported poor correlation between sporocarp and ECM fragment patterns (Gardes and Bruns 1996; Kårén *et al.* 1997; Horton and Bruns 2001; Horton 2002), but closely related fungi can be grouped together on the basis of the presence or absence of ITS fragments (Kårén *et al.* 1997).

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Conclusions

Our study was the first to describe the structure and diversity of ECM communities associating with black spruce, an important tree species of the Canadian boreal forest. Across its habitat range in the SBS zone of central BC, this species hosted a diverse community of root-associated ECM fungi consisting of a total of 33 morphotypes and 65 genotypes (from 29 morphotypes). The composition of ECM communities varied in the different habitats (overall similarity of 27%), but the total number of species described in each (richness) did not. The number of morphotypes detected in any one of the habitats (black spruce-dominated wetlands, mixed black spruce – tamarack wetlands, and black spruce – lodgepole pine upland forests) represented approximately two thirds of the overall ECM community; approximately 43% of all fungal genotypes were found in each habitat. Results emphasize the importance of sampling across an ecological range to more completely describe ECM communities associated with a particular host species.

The communities described in our study consisted of many ECM taxa, including *Cenococcum*, *MRA*, Russulaceae, *Lactarius*, Thelephoraceae, *Tomentella*, Cortinariaceae, *Hebeloma*, *Piloderma* and *Amphinema*. ECM morphotypes, and in some cases, fungal genotypes, resembled those described on other hosts in similar studies. Many of these taxa have previously been associated with nitrogen-limited, acidic forest soils. The molecular analysis revealed varying degrees of genetic diversity (in the ITS region of fungal rDNA) within the previously described ECM morphotypes. *Piloderma*, *Cenococcum*, and *Lactarius* 3 types showed low intraspecific diversity whereas *Amphinema*, *MRA*, Russulaceae 2, Cortinariaceae 2, Thelephoraceae-like 4 and *Tomentella*-like 1 types exhibited high diversity. These latter types grouped in polyphyletic clusters of genotypes on the neighbor joining tree and may actually represent several species.

Evidence supporting habitat specificity by certain taxa was obtained from both the morphological and molecular analyses. The ECM morphotypes *Tomentella* and *Russulaceae* 1 were exclusively described from the mixed black spruce – tamarack wetlands; both *Piloderma* and *Lactarius* 3 were identified from only the upland forest sites. In fact, *Piloderma* exhibited even greater habitat specificity, as it was consistently associated with seedlings growing in coarse woody debris. In general, fungi within the Thelephoraceae tended to be associated with the wetland habitats whereas the different Russulaceae morphotypes were often associated with either wetland or upland habitats. The molecular analysis provided greater resolution of habitat specificity trends, particularly among the habitat-generalist morphotypes. The distribution of *Cenococcum*, *MRA* and Cortinariaceae 1 morphotypes and genotypes was fairly consistant across all three habitats; however, genotypes of *Amphinema*, Cortinariaceae 2 and Russulaceae 2 showed uneven patterns of distribution between habitats. This may reflect context-specific selective pressure leading to variation in the fungal ITS region due to local differences in soil characteristics such as moisture, nutrient and organic content. To determine specific ecological functions of different fungi forming ECM symbioses with various tree hosts, further studies should be conducted over ecological gradients to identify specific habitat attributes that are consistently associated with certain types of ECM fungi.

ECM community diversity, as assessed by morphological analysis, was highest in the black spruce – pine upland forest habitat and lowest in the black spruce-dominated wetland habitat, with intermediate diversity in the black spruce – tamarack wetland habitat. Higher diversity in both the mixed forest habitats is most likely due to the presence of large, dominant companion plants that may provide a source of fungal inoculum for growing black spruce roots. Through the spruce mixture effect, alternate hosts such as larch and pine species may facilitate fungal colonization of black spruce roots by first allowing establishment of some fungal species on their

root systems. The higher ECM diversity observed in the upland forest habitat as compared to the wetland habitats might be due to soil conditions that are less water-saturated for most of the growing season.

Habitat diversity differences (using ECM morphotype data) were significant when measures emphasizing species richness (Margalef and Shannon indices) were used. No significant differences in ECM diversity between habitats were found following the molecular analysis. These results may indicate that differences in community diversity between habitats were mainly attributable to the presence of rare fungal types occurring at low frequency and abundance. These rare types comprised a small proportion of the molecular sample; in addition, losses during the molecular procedures may have under-represented these morphotypes in the molecular diversity calculations. Similar community structure (dominance by a few ECM morphotypes) was observed in all three habitats, reinforcing that differences in diversity appear to be mainly attributable to differences in morphotype richness (particularly the contribution of the less frequently occurring types) between habitats rather than to their relative abundance. Future studies should be designed to examine the contributions of these rare species to ecosystem processes since they are a widely reported component of ECM fungal communities and appear to account for the differences in community diversity described in our study.

Although all indices used to measure diversity between habitats in the morphology study supported the same trend, this was not the case for the molecular diversity values. Only the Phi Index supported the trends (highest diversity in the upland forests; lowest diversity in the spruce-dominated wetlands) observed in the morphological analysis. Because the Shannon and Simpson indices depend on proportional abundance of genotypes, calculation errors may

be introduced due to low sample sizes or losses during the experimental procedures. Heterogeneity indices as measures of molecular diversity should be used with caution.

Our understanding of the community structure and diversity of black spruce ECM benefited by using a combination of morphological and molecular techniques. Whereas the morphological study captured contributions to diversity by less abundant ECM types, the molecular study revealed patterns of genetic diversity and habitat use at greater resolutions than the morphological study. Although fungal identification (as related to taxonomic placement) was generally not improved by the molecular analysis, verification of morphological classification was achieved in most cases.

Appendix I. Types of vegetation compared between sites in spruce – tamarack wetland (T), spruce-dominated wetland (W) and spruce – pine upland forest (U) habitats in central BC.

COMMON NAME	LATIN NAME	T1	T2	T3	W1	W2	W3	U1	U2	U3
black spruce *	*Picea mariana* (P. Mill.) B.S.P.	X	X	X	X	X	X	X	X	X
tamarack *	*Larix laricina* (Du Roi) K. Koch	X	X	X						
lodgepole pine *	*Pinus contorta* Dougl. *ex* Loud. var. *latifolia*				X			X	X	X
subalpine fir *	*Abies lasiocarpa* (Hook) Nutt.									X
scrub birch *	*Betula glandulosa var. glandulosa* Michx.	X	X	X	X	X	X			
willow *	*Salix* sp.		X		X	X	X		X	
Sitka alder *	*Alnus crispa* var. *sinuata* Regel.									X
western mountain ash	*Sorbus scopulina* Greene							X		
prickly rose	*Rosa acicularis* Lindl.			X				X	X	X
northern black currant	*Ribes hudsonianum* Richard. in Frank.			X						
black gooseberry	*Ribes lacustre* (Persoon) Poiret in Larm.									X
highbush-cranberry	*Viburnum edule* (Michx.) Raf.								X	X
thimbleberry	*Rubus parviflorus* Nutt.							X		X
bog-laurel	*Kalmia polifolia* (Wang.) var. *microphylla*				X					
Labrador tea	*Ledum groenlandicum* Oeder	X		X	X	X	X			
black huckleberry	*Vaccinium membranaceum* Doug. Ex Hook.	X								
hardhack	*Spiraea douglasii* ssp. *menziesii* Hook.					X		X	X	X
saskatoon	*Amelanchier alnifolia* Nutt.							X		X
black twinberry	*Lonicera involucrata* (Richards.) Banks ex. Spreng							X	X	X
kinnikinnick *	*Arctostaphylos uva-ursi* (L.) Spreng.								X	
twinflower	*Linnaea borealis* L.							X	X	X
bog cranberry	*Vaccinium oxycoccos* (L.) MacM.				X	X	X			
dwarf blueberry	*Vaccinium caespitosum* Michx.							X	X	X
lingonberry	*Vaccinium vitis-idaea* L.	X								
bog-rosemary	*Andromeda polifolia* L.	X			X	X				
prince's-pine	*Chimaphila umbellata* (L.) Bart. ssp. *occidentalis* (Rydb.) Hult.									X
dwarf nagoonberry	*Rubus arcticus* L.	X	X	X	X					
trailing raspberry	*Rubus pubescens* Raf.	X		X				X	X	X
rosy pussytoes	*Antennaria rosea* Greene							X	X	
yarrow	*Achillea millefolium* L.							X	X	
heart-leaved arnica	*Arnica cordifolia* Hook.							X		X
palmate coltsfoot	*Petasites palmatus* (Ait.) A. Gray		X			X		X	X	
clasping twistedstalk	*Streptopus amplexifolius* (L.) DC.									X
queen's cup	*Clintonia uniflora* (Menzies *ex* J.A. & J.H. Shult.) Kunth									X
yellow coralroot	*Corallorhiza trifida* Châtelain									X
round-leaved rein-orchid	*Platanthera orbiculata* (Pursh) Lindl.							X		X
white bog-orchid	*Platanthera dilatata* (Pursh) Lindl. *ex* Beck						X			
northern twayblade	*Listera borealis* Morong						X			
rattlesnake-plantain	*Goodyera oblongifolia* Raf.								X	X

COMMON NAME	LATIN NAME	T1	T2	T3	W1	W2	W3	U1	U2	U3
common mitrewort	*Mitella nuda* L.		X	X			X			X
three-leaved foamflower	*Tiarella trifoliata* L. var. *trifoliata*									X
marsh cinquefoil	*Potentilla palustris* (L.) Scop.	X		X		X	X			
wild strawberry	*Fragaria virginiana* Duchesne							X	X	X
single delight *	*Moneses uniflora* (L.) A. Gray						X			X
one-sided wintergreen *	*Orthilia secunda* (L.) House						X		X	X
pink wintergreen *	*Pyrola asarifolia* Michx.		X	X		X	X	X	X	X
common red paintbrush	*Castilleja miniata* Dougl. *ex* Hook								X	
cow-wheat	*Melampyrum lineare* Desr. var. *lineare*								X	
false toad-flax	*Geocaulon lividum* (Richards.) Fern.							X	X	
fireweed	*Epiloblum angustifolium* L.			X				X		
bunchberry	*Cornus canadensis* L.			X					X	X
wild sarsaparilla	*Aralia nudicaulis* L.									X
buckbean	*Menyanthes trifoliata* L.	X			X					
horsetail	*Equisetum* sp.	X	X	X		X	X		X	
sedge	*Carex* sp.			X		X	X			
grass	*Poa* sp.		X	X	X				X	
ground-pine	*Lycopodium obscurum*		X							
ground-cedar	*Lycopodium complanatum*								X	
red-stemmed feathermoss	*Pleurozium schreberi*				X	X	X	X	X	
step moss	*Hylocomium splendens*							X	X	
knight's plume moss	*Ptilium crista-castrensis*					X	X	X		X
electrified cat's-tail moss	*Rhytidiadelphus triquetrus*							X	X	X
juniper haircap moss	*Polytrichum juniperinum*				X	X	X	X	X	
common leafy moss	*Plagiomnium medium*		X							X
glow moss	*Aulacomnium palustre*									X
golden fuzzy fen moss	*Tomenthypnum nitens*	X	X	X	X	X				
golden ragged moss	*Brachythecium salebrosum*				X	X	X	X	X	
common red sphagnum	*Sphagnum capillaceum*	X	X	X	X	X	X			
lungwort	*Lobaria pulmonaria*									X
dog lichen	*Peltigera canina*									X
freckled lichen	*Peltigera aphthosa*		X		X	X		X	X	
pixie cup lichen	*Cladonia* sp.				X					X
reindeer lichen	*Cladina* sp.								X	X

* Plants that are potential ECM hosts.

Appendix II. Descriptions of black spruce ECM morphotypes from black spruce – tamarack wetland (T), black spruce-dominated wetland (W) and black spruce – pine upland forest (U) habitats in central BC.

ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
Cenococcum	black, grainy to woolly, sometimes slightly reflective, unbranched tips (0.5-2 mm)	outer mantle (OM) /inner mantle (IM) net synenchyma (20-25 µm thick); stellate pattern visible at 100 x on less woolly types; cells 3-6 µm wide	dark brown to black, thick-walled, septate emanating hyphae (EH) (5-6 µm wide), sometimes verrucose; no clamps	none
MRA 1	brown-black, velvety, unbranched tip; often whitish or hyaline at tip	OM felt prosenchyma; IM net synenchyma	dark brown, verrucose, thick-walled EH (2-3 µm wide); no clamps; septate H-anastomosis without clamps	none
MRA 2	brown-black, smooth, slightly reflective, unbranched tip	OM felt prosenchyma; IM net synenchyma; mantle stains pink with KOH	EH rarely observed, or hyaline or with dark pigmentation, verrucose, thick-walled EH (2-3 µm wide); no clamps; septate H-anastomosis without clamps	none
Russulaceae 1	creamy or pale grey to orange, smooth to slightly cottony; straight, unbranched or monopodial pinnate tip (~1-2 mm)	OM net to irregular (interlocking) synenchyma; IM net synenchyma; sometimes parallel cells on mantle surface of variable widths (2-7 µm)	hyaline to dark grey, very branched EH (2-3 µm wide); septa appear close together (4-14 µm wide); no clamps; sometimes yellow granular cell contents	none
Russulaceae 2	pale yellow-orange, smooth, velvety or cottony, usually unbranched or monopodial pinnate tip (0.5-2 mm); sometimes dark mottling on basal half;	OM felt prosenchyma towards net synenchyma; IM net synenchyma; yellow deposits on OM stain green or pink with KOH; perhaps half of surface mantle cells with granular contents; mantle ~10-16 µm wide	few to many branched, hyaline, verrucose EH (1-3 µm wide); clamps present on some, but not all samples	yellow, smooth, undifferentiated rhizomorphs (~20 µm wide) sometimes present (not always)
Russulaceae 3	white to pale orange, smooth to slightly cottony, unbranched, club-shaped tip (~0.5 mm)	very thin mantle of net prosenchyma	variable width (2-3 and -5 µm), hyaline, branched EH with clamps	uncertain

ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
Russulaceae 4	yellow-orange to orange-brown, smooth to velvety, straight to bent, unbranched tip (~1 mm)	OM/IM net synenchyma	few, indeterminant, hyaline EH (2-3 µm wide) with large clamps; septate H-anastomosis with clamps	yellow to brown, differentiated (hyphae 2-5 µm wide) rhizomorphs (~40 µm wide) sometimes present (not always)
Lactarius 1	frosty orange-brown, smooth, straight, monopodial pinnate tip, sometimes darker at the base; rhizomorphs present	OM net to irregular (interlocking) synenchyma; IM irregular synenchyma	few short, thick (5-8 µm wide), branched, hyaline (pale yellow), septate EH; no clamps; laticifer hyphae (7-8 µm wide) with pale yellow cell contents	sometimes rhizomorphs (~70 µm wide) consisting of loose, undifferentiated hyphae; attached at a restricted point
Lactarius 2	yellow-orange to pale orange, smooth or velvety to slightly cottony, straight to club-shaped, unbranched tip (<1-4 mm)	OM/IM irregular (interlocking) synenchyma; thin mantle (~14-18 µm thick)	many branched, hyaline EH (2-3 µm wide); clamps uncertain; wide laticifer hyphae on mantle surface (5-6 µm wide); H-anatomosis; sometimes dolipores appear visible on either side of septa	none
Lactarius 3	golden-beige, smooth, tortuous tip (3-4 mm)	OM net to irregular (interlocking) synenchyma; IM net synenchyma; cell widths (3-6 µm) with some elongated cells (6-8 µm)	very few, branched, hyaline EH of variable widths (2-5 µm); no clamps; granular cell contents; H-anatomosis	none

ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
Thelophoraceae 1	orange-brown, velvety, club-shaped, unbranched tip (~1.5 mm)	OM regular synenchyma; IM net synenchyma; angular cells 10-15 µm wide	occasionally branched, hyaline, septate EH (3-4 µm wide) with clamps; awl-shaped, sometimes branched cystidia (45-65 µm long), sometimes with basal clamp	none
Thelephoraceae 2	pale orange to orange-brown (rusty-tawny), smooth to cottony, monopodial pinnate to pyramidal tip	OM net prosenchyma to wards net synenchyma; IM net synenchyma; very thin mantle (~5-6 µm)	hyaline, bent, finely verrucose EH (3-4 µm wide) with granular cell contents; no clamps; septate H-anastomosis; sometimes dolipores appear visible on either side of septa	none
Thelephoraceae 3	pale greenish-yellow, yellow or orange-brown, velvety to slightly cottony, club-shaped, unbranched tip (~1-2 mm)	OM irregular (non-interlocking) to regular synenchyma; IM net synenchyma; sometimes olive-green deposits on mantle surface	indeterminant, branched, hyaline EH (2-3 µm wide) with clamps; aseptate H-anastomosis; yellow granular cell contents	none
Thelephoraceae-like 1	black, grainy to felty, straight to club-shaped tip (0.5-3 mm)	dark black OM with irregular (non-interlocking) synenchyma (cells 6-8 µm wide; 16-22 µm long); IM net synenchyma; with uneven black mottling on mantle surface that stains greenish-black with KOH	hyaline to pale yellow, bent, branched, septate EH (4-6 µm wide); no clamps	none
Thelephoraceae-like 2	dark brown to brown-black, smooth to slightly woolly or stringy, unbranched, bent tip (~2 mm); rhizomorphs present	OM irregular (non-interlocking) synenchyma; IM net synenchyma; ~12 µm thick; uneven black mottling on mantle surface that stains greenish-black with KOH	hyaline to brown, branched, sometimes verrucose, variable width (2-3 and 4-5 µm) EH; no clamps; sometimes bottle-shaped or awl-shaped cystidia (2-3 µm wide)	brown rhizomorphs with verrucose hyphae; consists of differentiated hyphae (25-35 µm thick)

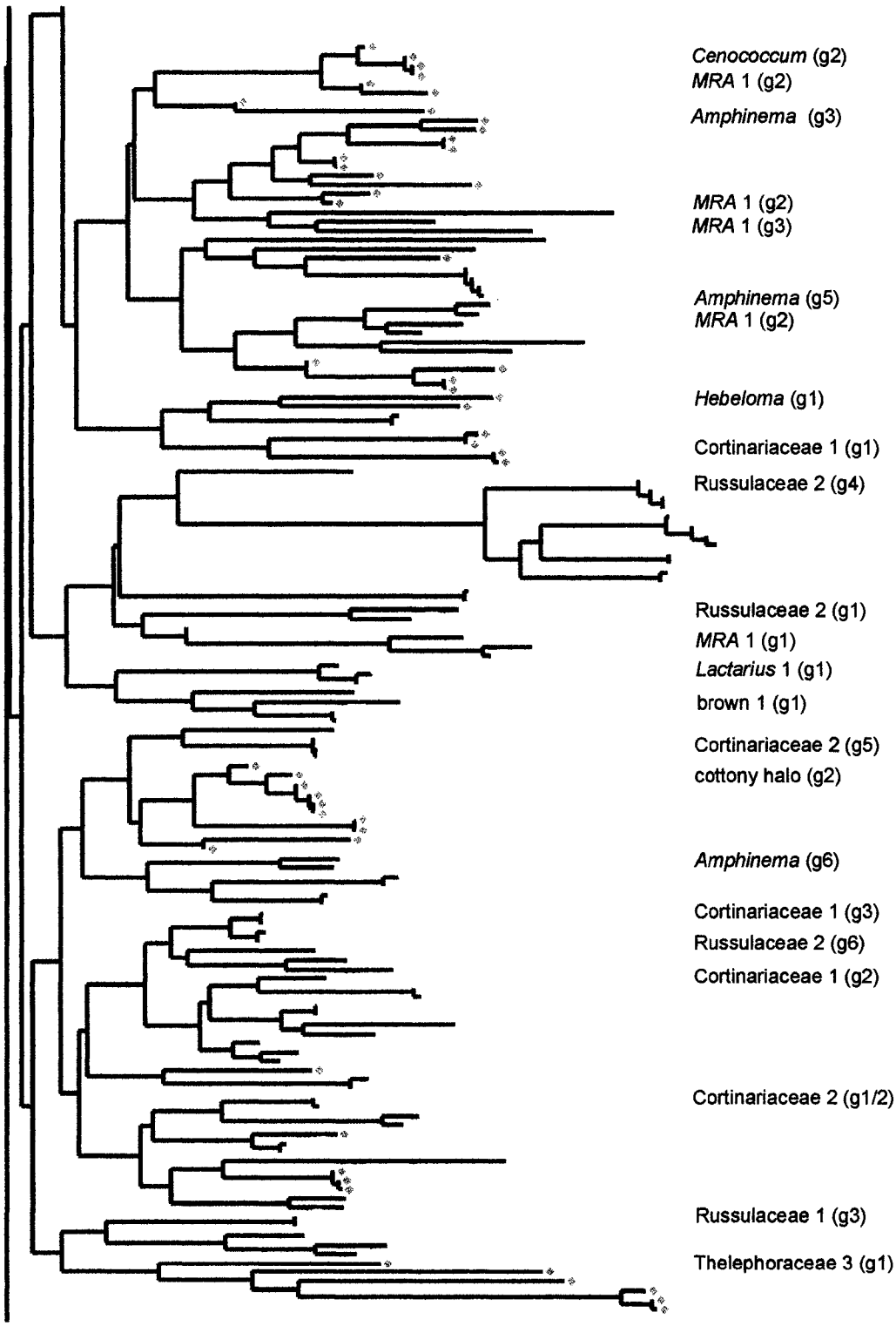
ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
Thelephoraceae-like 3	yellow-orange-brown, smooth, straight, monopodial pinnate tip (0.5-2 mm)	OM irregular (non-interlocking) to regular synenchyma (cells 5-10µ wide; 10-15µ long); IM net synenchyma; mantle ~15-20 µm thick	rare to few branched, thick-walled, hyaline EH (2-3 µm wide), sometimes with clamps (not at every septa); awl-shaped, sometimes branched cystidia (50-60 µm long) with variable basal cell shape and retraction septa 2-3 µm wide; sometimes a basal clamp	none
Thelephoraceae-like 4	yellow-orange, smooth, straight to club-shaped, unbranched tip (~1-3 mm)	OM regular synenchyma; IM net synenchyma; perhaps half of surface mantle cells with granular contents	few hyaline EH (2-4 µm wide); no clamps; awl-shaped, unbranched cystidia 2-4 µm wide and 20-100 µm long	none
Tomentella	golden-yellow to golden-brown, smooth to grainy, unbranched or monopodial pinnate tip (~1-2 mm)	OM regular to irregular (non-interlocking) synenchyma; IM net synenchyma; clumps of round deposits on mantle surface; mantle ~14 µm thick	few branched, hyaline EH (3.5-4 µm wide) with large clamps; possibly tapering cystidia (uncertain)	none
Tomentella-like 1	rusty-dark brown, grainy, slightly reflective, straight, unbranched tip (~1 mm); often with dark (black) mottling	OM regular to irregular (non-interlocking) synenchyma; IM net synenchyma; OM cell widths 2-5 µm	few to many, hyaline to brown, branched, sometimes verrucose EH (2-3 µm wide); no clamps; possibly tapering cystidia (uncertain)	none
Tomentella-like 2	yellowish-brown to brown-black, velvety to cottony, unbranched, club-shaped tip (~1 mm)	OM net to irregular (possibly interlocking) synenchyma; IM net synenchyma	bent, branched, hyaline EH (4-6 µm wide); variable lengths with clamps	none
Tomentella-like 3	olive-brown-black; smooth to velvety, straight to bent, monopodial pinnate tip (1-5 mm); usually hyaline at tip	OM irregular (possibly non-interlocking) synenchyma; IM net synenchyma; thin mantle (~8 µm) with uneven dark brown mottling	few, short (50-80 µm wide), highly branched, hyaline to brown EH (2-4µ wide) with clamps	none

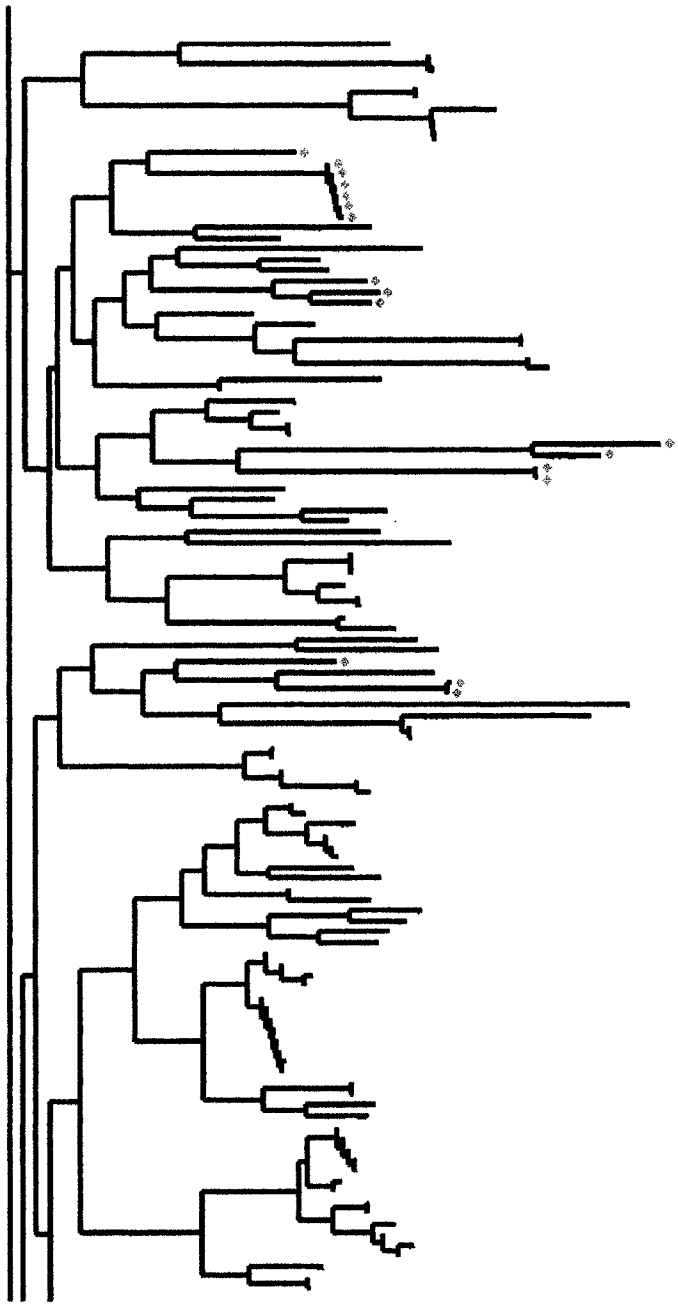
ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
brown 1	chocolate-brown, smooth to velvety, straight, unbranched tip (~1-2.5 mm)	OM/IM net to irregular (interlocking) synenchyma; thin mantle (~8 µm) with uneven dark brown mottling	very few, finely verrucose, hyaline EH (1-2 µm wide); no clamps	none
brown 3	chocolate-brown, smooth to cottony, straight to bent, unbranched tip (2-4 mm)	OM felt to net prosenchyma; IM net synenchyma; OM cells 1-2 µm wide; thin mantle (~14 µm)	hyaline to pale brown EH (1-2 µm wide) without clamps; some <100 µm long	sometimes thick rope-like rhizomorphs
orange 1	frosty coppery-brown, smooth, straight to club-shaped, usually unbranched tip	OM net to irregular synenchyma; mounds of brown, rounded cells on OM; IM irregular synenchyma	few branched, hyaline EH (1-3 µm wide) with clamps	none
Cortinariaceae 2	white, reflective, irregularly-branched, tortuous, cottony tip; rhizomorphs present	very thin or incomplete mantle of net prosenchyma (~10 µm thick)	variable width (1.5-5 µm), hyaline, kinked, branching EH; large clamps; septate H-anastomosls without clamps	often white rhizomorphs (~40 µm wide) consisting of loose, undifferentiated hyphae
Cortinariaceae *3*	yellow-orange to orange-brown, smooth to velvety, straight, unbranched tip (~1 mm)	OM net to irregular (interlocking) synenchyma; IM net synenchyma; mantle ~10 µm thick	few, kinked, branched, verrucose hyaline (sometimes yellow) EH (2-3 µm wide); possibly with clamps; H-anastomosis with clamps, but not all septa	none
Hebeloma	white, short (~1 mm), club-shaped, smooth, velvety or cottony tip	OM net prosenchyma; IM net synenchyma	thin (1-3 µm), hyaline, branching EH	none

ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
Piloderma	creamy pale yellow to bright lemon yellow, unbranched, often bent to tortuous, woolly tip (1-5 mm); rhizomorphs present	OM/IM of net prosenchyma towards net synenchyma; granular material on mantle surface stain purple with KOH	profuse hyaline, highly verrucose, branching EH (~3 µm) without clamps; septate H-anastomosis without clamps; verrucose and resinous deposits (yellow) and needle-like crystals on EH	broadly attached, thick (50-130 µm), yellow (sometimes white-purple) rhizomorphs consisting of loose to smooth, undifferentiated hyphae
Amphinema	pale yellow to yellow-orange, cottony, straight, unbranched or monopodial pinnate tip; rhizomorphs present	OM/IM net prosenchyma towards net synenchyma, ~14 µm thick; difficult to see mantle through EH	profuse hyaline (yellow tinge), branched, curved, verrucose EH (2-4 µm wide) with clamps; granular cell contents	sometimes golden-yellow rhizomorphs (50-100 µm wide) consisting of loose, undifferentiated or slightly differentiated hyphae
cottony halo	creamy-white, yellowish or orange, straight, usually unbranched tip with cottony; short EH form a "halo" around tip	OM irregular (possibly non-interlocking) synenchyma; IM net synenchyma; thin mantle (~10 µm thick)	profuse, long, curved, hyaline (yellowish tinge), highly verrucose EH (1-3 µm wide) with clamps (not all septa) and granular cell contents; septate H-anastomosis with clamps	none
cream rhizomorphic clamped	creamy to pale yellow, monopodial pinnate, straight, cottony tip; sometimes reflective; rhizomorphs present	thin mantle of net to irregular synenchyma (cells 5-6 µm wide)	variable (2-4 µm), hyaline, branching EH with clamps; aseptate H-anastomosis	rhizomorphs consisting of loose, undifferentiated hyphae (not always seen)

ECM Morphotype	Morphological Features	Mantle Features	Emanating Hyphal Features	Rhizomorphs
cottony gold-brown	light golden-yellow to coppery-brown, smooth to cottony, straight or slightly bent, unbranched or monopodial pinnate tip (0.5-2.5 mm)	OM/IM net synenchyma; thin mantle (8-10 µm thick); possibly with oval crystals on mantle surface; mantle and EH stain pink with KOH	indeterminent, branched, bent, verrucose EH; hyaline (yellowish tinge) EH (2-3 µm wide) with clamps; septate H-anastomosis with clamps	none

Appendix III. Unrooted neighbor joining tree generated from restriction fragment patterns of black spruce ECM morphotypes. The tree shows the relationships between ECM morphotypes, genotypes and habitat of origin (spruce – tamarack wetland [T], spruce-dominated wetland [W] and spruce – pine upland forest [U] habitats) in central BC.


Cenococcum (g2)
MRA 1 (g2)
Amphinema (g3)
MRA 1 (g2)
MRA 1 (g3)
Amphinema (g5)
MRA 1 (g2)
Hebeloma (g1)
Cortinariaceae 1 (g1)
Russulaceae 2 (g4)
Russulaceae 2 (g1)
MRA 1 (g1)
Lactarius 1 (g1)
brown 1 (g1)
Cortinariaceae 2 (g5)
cottony halo (g2)
Amphinema (g6)
Cortinariaceae 1 (g3)
Russulaceae 2 (g6)
Cortinariaceae 1 (g2)
Cortinariaceae 2 (g1/2)
Russulaceae 1 (g3)
Thelephoraceae 3 (g1)



Thelephoraceae-like 2 (g1)

Tomentella (g1)

Cottony halo (g1)

Tomentella (g3)

Russulaceae 2 (g6)

Thelephoraceae-like 1 (g2))

Cream rhizomorphic clamp (g2)

Thelephoraceae-like 4 (g1)

Lactarius 1 (g2)

Cottony halo (g3)

Thelephoraceae-like 4 (g2)

Tomentella-like (g1)

Russulaceae 3 (g1)

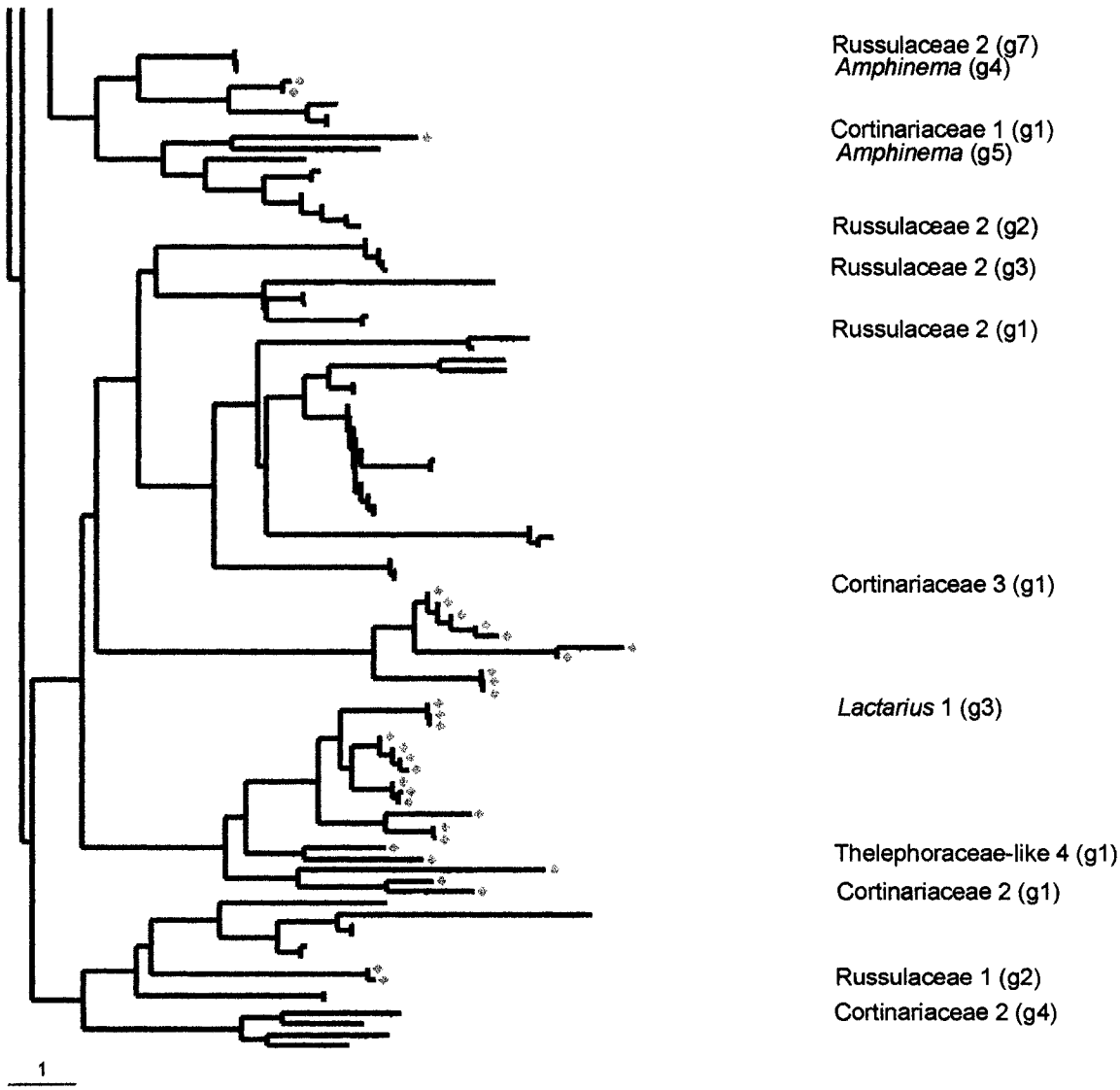
Russulaceae 1 (g1)

Cream rhizomorphic clamp (g1)

Amphinema (g2)

Amphinema (g1)

Piloderma (g1)


Russulaceae 2 (g7)
Amphinema (g4)
Cortinariaceae 1 (g1)
Amphinema (g5)
Russulaceae 2 (g2)
Russulaceae 2 (g3)
Russulaceae 2 (g1)
Cortinariaceae 3 (g1)
Lactarius 1 (g3)
Thelephoraceae-like 4 (g1)
Cortinariaceae 2 (g1)
Russulaceae 1 (g2)
Cortinariaceae 2 (g4)
1
Black spruce - tamarack wetland habitat
Black spruce-dominanted wetland habitat
Black spruce - lodgepole pine upland forest habitat