FOREST STAND TYPE AND ECTOMYCORRHIZAL FUNGAL COMMUNITIES OF WESTERN HEMLOCK ON NORTHERN VANCOUVER ISLAND, CANADA

by

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Abstract

This thesis explores the diversity and phylogenetic structure of the ectomycorrhizal fungal community of western hemlock from five forest types with different tree productivity on northern Vancouver Island. Chapter One reviews methods used in the studies of ectomycorrhizal fungal communities and major factors believed to contribute to ectomycorrhizal fungal diversity and species composition. This chapter also provides background on my study sites. In Chapter Two, I describe the results of a new correlative study using recently developed measures of phylogenetic diversity as well as standard measures of diversity and of fungal species composition to relate ectomycorrhizal fungal species to productivity of hemlock trees. I sampled ectomycorrhizal root tips of western hemlock from northern Vancouver Island and amplified, cloned and sequenced the fungal DNA from root extracts. In my analyses, I combined new data from plots of mature western hemlock-amabilis fir stands on Hemlock-Amabilis fir (HA) sites, and from plots of old-growth western red cedar-western hemlock stands on Cedar-Hemlock (CH) sites, with data previously gathered from plots of 24-year-old regenerating hemlock on CH sites. I detected 147 Operational Taxonomic Units (OTUs) among 1435 fungal clone sequences. Phylogenetic diversity indices showed that mature hemlock stands on HA sites had significantly higher ectomycorrhizal fungal diversity than regenerating hemlock stands on CH sites. *Cantharellus tubaeformis* was dominant in the plots of old-growth hemlock stands on CH sites whereas the other forest types had many low-frequency OTUs rather than a single dominant species. *Leucophleps spinispora* was found only on the plots of old-growth stands on CH sites, and *Russula xerampelina* was only on the plots of mature stands on HA sites. None of the fertilization treatments of regenerating plots on CH sites significantly changed fungal species composition according to Non-metric Multidimensional Scaling and Principal Coordinates Analysis results. In an analysis of beta diversity, I found that the species composition of the 24-year-old stands was more similar to the composition of old-growth stands on CH sites than to the species composition of mature stands on HA sites. Fungal species composition was strongly correlated with foliar nitrogen concentration, with only moderate correlations with tree productivity. My phylogenetic analyses of net relatedness of species from forests of

different types provided some of the first insights into how ectomycorrhizal fungal communities are structured. I found phylogenetic clustering in the plots of 24-year-old regenerating hemlock stands that contrasted with a pattern of phylogenetic evenness in the plots of mature and old-growth stands. A possible explanation for the difference between the patterns is that the regenerating hemlock stands were selecting for related, *r*-adapted fungi with similar traits while the older stands had more complex environments and selected for divergent fungi with varied traits. Finally, in Chapter Three I discuss some limitations and strengths of my research study, incorporating ideas on future research and implications.

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To God, my Heavenly Father

Co-authorship statement

Dr. M. Berbee initiated and designed the research project. S. Wright contributed to the sampling design and fieldwork. E. Carruthers assisted with the molecular lab work. I was responsible for lab work, data analyses, and thesis preparation with the supervision of Dr. M. Berbee.

1 Introduction

1.1 Literature review

Ectomycorrhizal fungi are important players connecting plant hosts and soil. Ectomycorrhizal fungi grow on the roots and between the cortical cells of most temperate trees and shrubs, providing water and mineral salts (nitrogen, phosphorus and potassium) and in turn receiving photosynthates and vitamins. Compared with their host plants, ectomycorrhizal fungi have remarkably high species diversity with high phylogenetic diversity. Approximately 6,000 species of ectomycorrhizal fungi have been described (Johnson et al., 2005), or about 40 times more than the number of species of arbuscular mycorrhizal fungi (Schübler et al., 2001). Unlike the arbuscular mycorrhizal fungi, which form a monophyletic group having one common ancestor (Schübler et al., 2001), the symbiosis between ectomycorrhizal fungi and plants is convergently derived (Bruns and Shefferson, 2004). Even communities within a single monoculture forest stand have diverse arrays of ectomycorrhizae (Bruns, 1995). A single host species such as western hemlock (*Tsuga heterophylla*) can associate with at least 150 species of ectomycorrhizal fungi (Kropp and Trappe, 1982). The total inventory of species of fungi that form ectomycorrhizae is still incomplete. Many of the fungal DNAs discovered from molecular sampling of mycorrhizae cannot be matched with species described from sporocarps (Chapter Two). The taxonomy of many species described from sporocarps remains inadequate (Chapter Three). The high species diversity and scant data for most species of ectomycorrhizal fungi, together with the complexity of biotic and abiotic factors in soil ecosystems, make a correlation of species with different habitats difficult.

Correlating fungal species with environmental variables will contribute to interpreting the functional roles of the ectomycorrhizal fungal community. Developing the correlations will require accurate detection of fungal species and appropriate descriptions of fungal communities, as well as comparisons among fungi from different communities. In the next paragraphs, I will discuss:

1. Limitations to ectomycorrhizal research imposed by the available methods of detection by sporocarp and root-tip surveys;

2. The environmental variables including stand age, disturbance, and nutrient availability in the context of studies of response of fungi to these variables;
3. Evidence of ectomycorrhizal fungi influencing aboveground tree productivity; and
4. The utility of phylogenetics in assessing the ectomycorrhizal fungal diversity.

Finally, I will explain my study sites, thesis aims and objectives.

1.2 Sporocarp surveys verses root-tip analyses

Sporocarps such as mushrooms represent only the reproductive portion of the total body of a fungus while the perennial, belowground body of the fungus forms hyphae on roots and extends into the soil. Sporocarps of many ectomycorrhizal fungal species can be ecologically important as dietary items for humans, rodents, other vertebrates, and insects. Small mammals can consume them for over 90% of their food intake (Maser et al., 1978; Claridge et al., 1996; Carey et al., 1999). Northern flying squirrels and California red-backed voles eat truffles, and these hypogeous (belowground) sporocarps of ectomycorrhizal fungi are relevant to the conservation of northern spotted owls, which are predators of the squirrels and voles. Sporocarps can be readily collected and identified and for these reasons, Countess et al. (1998) and Durall et al. (2006) measured diversity by the production of epigeous (aboveground) sporocarps alone.

Sporocarp production is not necessarily easy to monitor, however. Sporocarps of many important ectomycorrhizal fungal species are inconspicuous and difficult to find in surveys, leading to underestimates of the overall ectomycorrhizal fungal diversity and miscalculation of these species' abundances. Ectomycorrhizal fungi that produce truffle-like hypogeous sporocarps (e.g., *Rhizopogon* and *Leucophleps* species) are easily missed in visual surveys. Some common ectomycorrhizal fungal genera such as *Tomentella* produce resupinate (flat) fruiting bodies hidden on the underside of decaying wood. *Cenococcum geophilum* has no known sporocarp. These fungi without sporocarps or with inconspicuous sporocarps often dominate the underground ectomycorrhizal fungal community (Gardes and Bruns, 1996; Dahlberg et al., 1997; Peter et al., 2001).

Another limitation to basing surveys of ectomycorrhizae on sporocarps is that their fruiting season is brief and unpredictable. Abiotic factors that influence fruiting

include moisture, light availability, and temperature. Moisture availability has been studied extensively (Worley and Hacskaylo, 1959; Eveling et al., 1990; O'Dell et al., 1999; Trudell and Edmonds, 2004; Durall et al., 2006). Epigeous sporocarps of ectomycorrhizal fungi were collected from old-growth coniferous forests along a wet to dry precipitation gradient in Olympic National Park, Washington (O'Dell et al., 1999). In the sample plots, sporocarp standing crop and fungal species richness were correlated with mean annual precipitation. These results suggested that old-growth stands contain many species of fungi that infrequently produce epigeous sporocarps if the moisture level is low. In contrast, hypogeous sporocarps fruit more abundantly and constantly with seasonal or stand age variations than epigeous sporocarps (Luoma et al., 1991; O'Dell et al., 1992; North et al., 1997). Hypogeous sporocarps may be difficult to survey, but their production has more uniform seasonal distribution.

For all the reasons outlined above, perfect correspondence between sporocarp and root-tip surveys might not necessarily be expected. An alternative to detecting ectomycorrhizal fungi by sporocarp production is to collect ectomycorrhizal root tips and identify the associated fungi by microscopic analysis of the morphotype (the morphological form of the root tip and associated fungal hyphae) or by DNA sequence analysis. Molecular DNA sequence data prevents numerous misidentifications and misclassifications compared with using morphological characteristics alone. Further, the new molecular and bioinformatics tools have facilitated powerful phylogenetic analyses leading to better understanding of the evolutionary history of fungi (Tedersoo et al., 2009).

Not surprisingly, fungal DNAs from root tips have helped establish the ectomycorrhizal status of many additional fungal species. Dahlberg et al. (1997) reported that the species responsible for 70% of the annual fruiting biomass accounted for less than 30% of the colonized root tips. Peter et al. (2001) found species that produced large sporocarps accounted for only 25% of all sampled root tips. Many other studies have shown little correlation between the abundance of particular ectomycorrhizal types and the abundance of sporocarps (Jansen and de Nie, 1988; Visser, 1995; Baar and ter Braak, 1996; Durall et al., 1999). Porter et al. (2008) found 119 species in regular sporocarp surveys conducted over a year and a half, compared to 53 species detected in pooled

DNA extracts from soil from the same site. Only 13 species were detected both as sporocarps and in soil extracts. While the sporocarp surveys proved useful in characterizing species diversity, the species detected only in the soil extracts included mycorrhizal fungi that probably play important roles in ecosystem function (Porter et al., 2008). Gardes and Bruns (1996) hypothesized that ectomycorrhizal fungal species that are abundant on roots but fruit rarely or are in low abundance may simply invest more in vegetative growth and competition than in reproduction. Overall, root tip analyses are important in revealing fungal species with rare or inconspicuous sporocarps. Because they involve sampling the part of the fungus that interacts directly with the plant, root tip analyses may give something closer to a true picture of ectomycorrhizal fungal diversity.

1.3 Ectomycorrhizal fungal diversity and their habitats

1.3.1 Forest stand age and ectomycorrhizal fungal succession

The earliest uses of the term 'succession' generally referred to primary succession and were applied to model patterns of plant community development that exhibited discrete replacement of species or species groups over time (Chapin et al., 2002). For ectomycorrhizal fungi, succession refers to a pattern of fungal community development that takes place as their host plants age. Many studies showing patterns of succession have been based on epigeous sporocarps alone (O'Dell et al., 1992; Brandrud, 1995; Countess et al., 1998; Kranabetter et al., 2005; Durall et al., 2006) whereas few have been based on root tips (Visser, 1995; Gebhardt et al., 2007; Twieg et al., 2007). In some cases, during ectomycorrhizal fungal succession fungal species accumulate, adding richness to the ectomycorrhizal community until a forest stand becomes mature. Nitrogen-poor conditions could affect the fungal succession favouring those species that are capable of organic nitrogen uptake (Chalot and Brun, 1998; Lilleskov et al., 2002; Lipson and Nasholm, 2001).

A few studies based on analysis of ectomycorrhizal root tips rather than sporocarps were conducted in jack pine (*Pinus banksiana*) stands (Visser, 1995) and Douglas-fir (*Pseudotsuga menziesii*) forests (Twieg et al., 2007). When Twieg et al. (2007) used molecular techniques, ectomycorrhizal fungal diversities on Douglas-fir were lower in five-year-old stands than in older stands of mixed Douglas-fir and paper birch

(*Betula papyrifera*), but were similar among age classes of paper birch. Interestingly, paper birch roots were found to remain intact and healthy following cutting or burning of shoots, still providing a large carbon source and ectomycorrhizae for stump sprouts (Simard et al., 2004). Except for paper birch stands, however, surveys of either root tips or sporocarps agree that as stands aged, ectomycorrhizal fungal species richness increased or that a large number of species were uniquely present in older stands.

Although it is not as yet clear which factors are responsible for fungal succession, older stands may provide more heterogeneous habitat, selecting for fungi with distinct functional roles. As forest stands age, the combination of canopy closure, gradual changes in host physiology, and development of a more heterogeneous soil environment due to accumulation of leaf and root litter may provide the foundation for a more complex and stable ectomycorrhizal fungal community (Visser, 1995). Accumulation of poor quality litter in aging stands may select for different fungal species (Dighton and Mason, 1985; Last et al., 1987; Termorshuizen, 1991). Last et al. (1987) observed a correlation between ectomycorrhizal fungal succession and an increase of recalcitrant tree litter having high concentrations of polyphenols. These biochemical changes in the soil may select for reproduction of microbes that produce enzymes for breaking down recalcitrant litter, further facilitating nutrient mobilization. Alternatively, succession may be explained by accumulation of a larger component of coarse woody debris as stands age, favouring different species of fungi (Goodman and Trofymow, 1998). The increase in ectomycorrhizal species richness with stand age may be controlled in part by host physiology, perhaps age-related changes in photosynthate allocation as suggested by Danielson and Visser (1989) and Deacon and Fleming (1992) although patterns in belowground carbon allocation with stand age are not yet well understood (Ryan et al., 1997). Lastly, soil factors such as nutrient availability in the rooting zone may change with stand age, and leachates from the forest floor may alter the chemistry of the underlying mineral soil (Visser, 1995).

1.3.2 Ectomycorrhizal fungal community in response to disturbance

Forest types vary depending on their level of disturbance. Some forests have been recently disturbed naturally (e.g., wildfire and catastrophic windthrow) or by human

(tree harvesting and fertilization). My study sites included 24-year-old regenerating forests that had been disturbed by clearcutting followed by slashburning, 300-year-old, old-growth cedar-hemlock forests, which represented the climatic climax ecosystems in the area of study, and 100-year-old mature hemlock-amabilis fir forests established following catastrophic windthrow. In spite of gap phase disturbance, windthrow, etc., the older forests were relatively stable. May (1981) noted that in stable or predictable environments, species are controlled to a large extent by the interaction of numerous factors. This same concept can be applied to ectomycorrhizal fungal communities. Spatial variability in roots (age, physiology) and soil resources (concentrations and forms of nutrients, pH, aeration, moisture, temperature, light availability) is enormous. In old-growth forests with minimal disturbance, the interaction of these variables may lead to relatively equal abundance of many different species of ectomycorrhizal fungi (reviewed by Horton and Bruns, 2001).

In contrast to old-growth forests, one or more ectomycorrhizal fungal species commonly dominated recently disturbed environments. These dominant ectomycorrhizal fungi can be species that rapidly colonize roots from spores after disturbance, persist as propagules in the soil, or persist on roots after colonization (Deacon et al., 1983; Miller et al., 1994; Taylor and Bruns, 1999). *Suillus brevipes*, a species that disperses quickly and competes well on disturbed sites dominated six-year-old jack pine stands, which, following wildfire, were otherwise low in species richness (Visser, 1995). Bishop pine (*Pinus muricata*) seedlings that established in the first year after a stand-replacing fire were largely colonized by *Rhizopogon*, *Wilcoxina* and *Tuber* species that survived as resistant propagules in the soil (Baar et al., 1999). *Rhizopogon* species were common colonizers on Douglas-fir roots at 4 months to 28 months following logging in soil field bioassay (Jones et al., 1997). Twieg et al. (2007) found that another species, *Rhizopogon vinicolor*, was far more frequent on Douglas-fir roots in five-year-old stands than in older stands from both soil and seedling samples. As expected from an early successional ectomycorrhizal fungus, *Rhizopogon occidentalis* was able to colonize seedlings earlier than *Tomentella sublilacina* when mycelium of the two species was co-inoculated, but later the *R. occidentalis* root-tip numbers peaked then declined (Lilleskov and Bruns, 2003). In contrast, *T. sublilacina* colonization rates increased steadily over the course of

the experiment as might be expected from a species with greater long-term competitive ability.

Nitrogen deposition as fertilizer can have a variety of effects on mycorrhizae. It did not affect the richness or diversity of belowground ectomycorrhizal fungal species (nitrogen-enriched water in Jonsson et al., 2000), but a reduction of fine-root biomass was observed when ammonium sulphate was applied on a Norway spruce (*Picea abies*) stand in southwestern Sweden (Kårén and Nylund, 1997). For ectomycorrhizal sporocarps, species richness and diversity were lower in nitrogen-treated stands (Brandrud, 1995; Jonsson et al., 2000; Bidartondo, et al. 2001; Lilleskov et al., 2001; Carfrae et al., 2006). Reduced sporocarp production and mycelial growth after nitrogen addition may be due to high carbon cost of ammonium uptake. Nitrogen assimilation by fungi requires large amounts of energy, and the uptake of ammonium would lead to less carbon available for vegetative growth (Wallander, 1995). Bidartondo et al. (2001) tested this hypothesis in microcosm experiments. One ectomycorrhizal fungal species, *Paxillus involutus*, produced the lowest biomass of ectomycorrhizal connections to its host, but it consumed proportionally more carbon per connection and transferred more than twice as much ammonium to the host per mycorrhizal biomass unit than other fungi. This finding supports the hypothesis that the reduced growth of ectomycorrhizal external mycelium induced by high levels of nitrogen addition reflects a shift in carbon allocation within the fungus from vegetative growth and eventually sexual reproduction to the process of nitrogen assimilation. For a more general discussion of host productivity in relation to carbon supply to fungi, see Chapter Two, Section 2.2.1.1.

1.3.3 Ectomycorrhizal fungal species distribution along a nutrient gradient

Where the variation in other factors is small, significant change in the composition and abundance of ectomycorrhizal fungal species along a nutrient gradient can sometimes be detected. Kranabetter et al. (2009) showed that ectomycorrhizal fungal species richness of subalpine fir (*Abies lasiocarpa*) increased with host foliar nitrogen concentrations and with soil nutrients (inorganic nitrogen and organic phosphorus). The authors argued that many of these ectomycorrhizal fungi were well suited to soils with abundant inorganic nitrogen in the boreal ecosystems.

However, not all studies have shown a correlation between ectomycorrhizal fungal species diversity and nutrient gradients. Ectomycorrhizal fungal species richness associated with pinyon pine (*Pinus edulis*) was not correlated with nutrient level in different soil types in a semiarid region of northern Arizona (Gehring et al., 1998), possibly because the fungal mutualists that receive most of their energy from the plants may be buffered against the environmental extremes which are experienced by their plant associates (Villeneuve et al., 1989). No relationship was apparent between total fungal species richness of Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*), and birch (*Betula pubescens*) and nutrient gradients in a boreal forest in northern Sweden (Toljander et al., 2006).

An informative way to analyze ectomycorrhizal fungal composition along nutrient gradient has been to look at abundance of each fungal species at different nutrient levels (Toljander et al., 2006; Kranabetter et al., 2009). Studies by Toljander et al. (2006) and Kranabetter et al. (2009) showed that some ectomycorrhizal fungal species had discrete patterns of response to a nutrient gradient or site series. *Cenococcum geophilum* and *Piloderma fallax* had a parabolic relationship, occurring at maximum frequencies at intermediate levels of foliar nitrogen (Kranabetter et al., 2009). *Russula decolorans*, unknown fungi VI, and dark septate fungi 'MRA' had a negative linear relationship; and *Tylospora asterophora*, *Amphinema byssoides*, and *Laccaria laccata* had a positive exponential relationship to extractable soil ammonium levels or to foliar nitrogen concentration (Toljander et al., 2006; Kranabetter et al., 2009). These results show that consideration of responses of individual ectomycorrhizal fungal species, in addition to the measures of species richness, can reveal the effects of environmental factors such as soil nutrient levels.

1.3.4 Tree productivity may be correlated with the fungal community

1.3.4.1 Explanations on how fungal community controls tree productivity

In species-rich ectomycorrhizal fungal communities related fungal species may have similar functional roles. Some species excel in transporting water, protecting roots against pathogens, colonizing specific types of soils, breaking down recalcitrant litter, taking up organic nitrogen, or reducing damaging salt effects in their host plants

(Langenfeld-Heyser et al., 2007). Evidence of considerable interspecific variation in production of nutrient-mobilizing enzyme systems by these fungi (Leake and Read, 1997) provides strong theoretical support for the hypothesis that high diversity of ectomycorrhizal fungi promotes more effective nutrient acquisition by host plants from different spatial locations and different substrates in the soil (Leake, 2001). If the niche or trait evolution of ectomycorrhizal fungi is phylogenetically conserved (i.e., functional difference among distantly related species), then the aboveground tree productivity may increase with phylogenetically diverse, ectomycorrhizal fungal community.

The unified theory of fungal generalists (Hubbell, 2001) offers a contrasting view of functional roles of fungi by hypothesizing that different ectomycorrhizal fungal species have equivalent roles and responses, regardless of their evolutionary relationships, with differences in distribution being due primarily to dispersal limitations. Some ectomycorrhizal fungal taxa appeared to be functionally similar at a larger spatial scale (Twieg et al., 2009), and the possibility that niche partitioning occurs at finer spatial scales than at the site level was suggested by differences in ectomycorrhizal diversity between soil layers (Dickie et al., 2002). If all fungal species are functionally equivalent, highly productive forests at a larger spatial scale may be maintained just as effectively by an ectomycorrhizal fungal community of high or low diversity.

1.3.4.2 Differences in the fungal community may have an effect on tree productivity

Recently, there has been a growing interest in determining the nature of the relationships between soil microbial diversity and plant productivity (Wardle et al., 2004; van der Heijden et al., 2008). A key question is whether species-rich microbial communities are better adapted to enhance plant growth compared with species-poor microbial communities. Although some studies have found that plant productivity and nutrient acquisition increased with increasing arbuscular mycorrhizal fungal diversity (van der Heijden et al., 1998; Maherali and Klironomos, 2007), other studies found no effects (van der Heijden et al., 2003; van der Heijden et al., 2006). Van der Heijden et al. (2006) suggested fungal species identity and functional roles are more important than overall diversity. For ectomycorrhizae, Jonsson et al. (2001) showed that effects of mycorrhizal fungal diversity on tree seedling productivity might be positive, negative or

neutral depending on tree species and soil fertility. It appears that diverse ectomycorrhizal communities do not always enhance shoot biomass of tree seedlings (Baxter and Dighton, 2001). Previous studies of ectomycorrhizae and tree productivity did not use phylogenetic analyses for determining fungal community structure. Perhaps, the more accurate characterization of fungal community composition and phylogenetic structure will allow a better understanding of the relationship between ectomycorrhizal fungi and aboveground tree productivity.

1.4 Phylogenetic relationships among ectomycorrhizal fungal species

Beyond exploring the species-specific roles of fungi, recent divergence-based methods have been introduced to take into consideration the evolutionary history of each species, providing new insights into microbial community structure and function (Lozupone and Knight, 2008). The preliminary results from studies of phylogenetic diversity are leading to increased interest in these relatively simple approaches.

Phylogenetic diversity is a divergence-based method that calculates the amount or proportion of branch length in a phylogenetic tree that leads to organisms from one community (Faith, 1992). For comparison of phylogenetic diversity among communities, computer programs, Phylocom (Webb et al., 2008) and UniFrac (Lozupone and Knight, 2005), were recently introduced. Because DNA sequences of the fungal community members are readily available, it is easy to apply divergence-based methods. Use of divergence-based methods begins with a phylogeny of all species found in one or more communities. Branches of a phylogenetic tree reflect the evolutionary relationships of ancestral taxa and their descendants. Thus, the branch length usually indicates some form of evolutionary distance between taxa. Phylocom randomizes phylogeny and community structure to determine whether a particular sample of species is more or less related than expected by chance, given the pool of species from all communities under consideration. If the community structure could not be explained by a random draw from the total pool of species in the area, this implies that traits important in reproductive success in local habitats have a phylogenetic component.

With arbuscular mycorrhizal fungi, a hypothesis on phylogenetic diversity influencing ecosystem functioning has been tested by Maherali and Klironomos (2007).

They found that evolutionarily distantly related species coexisted longer in microcosms than did closely related species, presumably because of niche differentiation. Different niches selected for fungi with different traits. Since traits were phylogenetically conserved, this resulted in selection for distantly related fungi. At the same time, the number of closely related fungi that could occupy the same niche was limited by competitive exclusion. The distantly related lineages also enhanced ecosystem function (i.e., aboveground tree productivity) (Maherali and Klironomos, 2007).

Peay et al. (2010) analyzed phylogenetic diversity among fungal species associated with tropical trees in the Dipterocarpaceae. The ectomycorrhizal fungi from Dipterocarpaceae from sites with clay soils were more closely related to one another than expected by chance, suggesting that traits contributing to reproductive success at these sites were shared within phylogenetic groups (Peay et al., 1010). Porter et al. (2008) showed that lineages within the Agaricales, which were highly divergent, accounted for a greater proportion of phylogenetic diversity compared with their contribution to richness and abundance. Whether these divergent lineages had special functional characteristics was untested. By analyzing phylogenetic diversity, Arnold et al. (2007) were able to show spatial heterogeneity in endophyte assemblages among loblolly pine (*Pinus taeda*) trees that was not evident in taxon-based diversity indices, although the ecological reasons for the heterogeneity were unclear.

Assessments of phylogenetic diversity contrast with the traditional taxon-based measures of diversity at the species level that assume all species are equally related. For example, Shannon's index (Shannon and Weaver, 1949) and Simpson's index (Simpson, 1949) are commonly used nonparametric measures of diversity within a single community. For comparing species composition, the Sørensen index (Sørensen, 1948) and the Jaccard index (Jaccard, 1908) are used as distance measures between communities for ordination and multivariate analyses. In this thesis, I apply both traditional taxon-based measures of diversity as well as the newer phylogenetic methods to try to correlate ectomycorrhizal fungal communities with different habitat types.

1.5 Background on study site

The Salal Cedar Hemlock Integrated Research Project (SCHIRP) was initiated in the early 1980s to determine the causes of poor growth of regenerating western red cedar (*Thuja plicata*), western hemlock (*Tsuga heterophylla*), amabilis fir (*Abies amabilis*) and Sitka spruce (*Picea sitchensis*) on cedar-hemlock cutovers invaded by salal (*Gaultheria shallon*) on northern Vancouver Island. The SCHIRP study sites on northern Vancouver Island differ in forest stand type, plant species composition, tree age, etc. (Table 1.1). Through studies of silviculture, soil chemistry, biology and ecology, SCHIRP has expanded basic knowledge of habitats for tree growth on Vancouver Island and has offered recommendations to improve tree productivity (Blevins and van Niejenhuis, 2003).

Although the old-growth cedar-hemlock stands and the mature hemlock-amabilis fir stands were in close proximity, they differed in more than just tree species composition (Table 1.1C). Stand structure differed in the diameter-class distribution (Chapter Two, Section 2.3.1; Keenan, 1993). The Hemlock-Amabilis fir (HA) sites were drier and had medium levels of soil nutrients whereas the Cedar-Hemlock (CH) sites were wetter and poor in nutrients (Klinka, 2006). Prescott and Weetman (1994) reported that many CH sites were on lower slopes, and they were not only wetter, but also cooler, more compacted, less well drained and less well aerated in comparison to HA sites. The moisture levels in CH forest floors reduced decomposition rates and the abundance of soil fauna, and led to incomplete decomposition and mineralization. Prescott and Weetman (1994) found that red cedar litter contained less nitrogen and more recalcitrant material than other tree species, and that the forest floors had low nitrogen availability. The low supply of nitrogen and phosphorus in CH forest floors contributed in turn to nutrient supply problems in regenerating western hemlock stands on cedar-hemlock cutovers (Prescott et al., 1993). In contrast to the CH sites, decomposition and mineralization were more rapid in HA forest floors (deMontigny et al., 1993).

For ectomycorrhizae on the regenerating hemlock on the CH sites, Wright (2006) found a significant difference in fungal species composition in the nitrogen (N) plus phosphorus (P)-fertilized plots compared to the unfertilized or N-fertilized plots. At another SCHIRP regenerating site referred to as the SCHIRP installation, N plus P

fertilization of hemlock on CH sites resulted in a growth response of the trees similar to regenerating unfertilized hemlock on HA sites (Blevins and Prescott, 2002). This raised the question as to whether stands of similar types or with similar fertilization histories would also have similar ectomycorrhizal fungal communities. By comparing these forest types, I wanted to explore whether different fertilization histories in the regenerating plots on CH sites influenced the development of their ectomycorrhizal communities into those more reflective of old-growth hemlock on CH sites or mature hemlock stands on HA sites (Wright, 2006).

1.6 Thesis theme and objectives

No previous study has compared the ectomycorrhizal fungal communities of the five forest types: unfertilized, N-fertilized, or N plus P-fertilized regenerating hemlock on CH sites, old-growth hemlock on CH sites, and mature hemlock on HA sites. Ectomycorrhizal fungal communities in my study sites were influenced by many factors (Section 1.5). The complex differences among the forest types made it very difficult to associate a specific environmental variable with fungal diversity. However, the hypotheses tested included whether ecosystem functioning, specifically tree productivity and foliar nutrient concentration, has any effect on ectomycorrhizal fungal communities and whether forest age or stand type had the greater influence on fungal diversity. I expected this research to improve understanding of habitat specificity of ectomycorrhizal fungi and their roles, and to contribute to the information base for forestry management plans that will protect overall biodiversity across our landscape.

Table 1.1 Comparative studies conducted at the salal cedar hemlock integrated research project sites

A. Regenerating Cedar-Hemlock 'Fertilization Trials'

Topic	Main findings	Reference
Ectomycorrhizal fungi	Species composition in **NP plots differed** from U or N plots 7 yrs after the 2nd fertilization	Wright (2006)
Dominant tree height & stand volume of hemlock	**Increased growth only in NP plots** 5 yrs after the 2nd fertilization	Blevins et al. (2006)
Foliar phosphorus concentration of hemlock	Significantly **higher in NP plots** 10 yrs after the 1st fertilization. Hemlocks in U and N plots were phosphorus deficient.	Blevins et al. (2006)
Foliar nitrogen concentration of hemlock	**No difference** 10 yrs after the 1st fertilization. Hemlocks were all nitrogen deficient.	Blevins et al. (2006)

U, unfertilized; N, nitrogen-fertilized; NP, nitrogen plus phosphorus-fertilized

B. Regenerating and old-growth stands on Cedar-Hemlock sites

Topic	Main findings	Reference
Soluble organic nitrogen in soil	**Lower in clearcuts** (not fertilization trials) than in old-growth	Hannam and Prescott (2003)
Phosphorus availability in soil	**A shift from organic to inorganic forms** with clearcutting and burning	Cade-Menun et al. (2000a)

C. Old-growth cedar-hemlock stands on Cedar-Hemlock (CH) sites and mature hemlock-amabilis fir stands on Hemlock-Amabilis (HA) sites

Topic	Main findings	Reference
Foliar nitrogen concentration of hemlock	**No** statistical difference. Hemlocks on CH or HA were not nitrogen deficient.	Keenan et al. (1995)
Foliar phosphorus concentration of hemlock	**Not** greatly different, but hemlocks on CH were phosphorus deficient.	Keenan et al. (1995)
Phosphorus availability in soil	**No difference**	Cade-Menun et al. (2000b)
Availability of nitrogen (N) and phosphorus (P)	**Smaller concentrations** of total extractable N, mineralized N, and total and extractable P in CH forest floors	Prescott et al. (1993)
Total annual aboveground litterfall	**Higher in HA**. Hemlock had a higher litter N concentration in HA.	Keenan et al. (1995)
Woody debris mass	Total mass in **CH was significantly higher** than HA due to the higher quantity of dead standing trees in CH	Keenan et al. (1993)
Internal nutrient cycling	Decomposition rate of lodgepole pine needles in litter sample bags was **identical** in CH and HA	Keenan et al. (1996).
Soil humus forms	**Higher tannin and higher ratio of carbohydrate to lignin carbon** in CH, indicating less effective decomposition in CH	deMontigny et al. (1993)
Soil moisture content	**High** in forest floor and soil from CH	Prescott and Weetman (1994)
Microbial communities	**Differences in composition**. Fungi more abundant in CH, but bacteria more abundant in HA.	Leckie et al. (2004)
Soil fauna	Similar group diversity, but **higher** abundance and biomass in HA	Battigelli et al. (1994)

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2 Phylogenetic structure of ectomycorrhizal fungal communities of western hemlock changes with forest age and stand type[1]

2.1 Summary

My goal in this study was to relate the belowground ectomycorrhizal fungal species to environmental variables in western hemlock dominated forests on replicate plots on northern Vancouver Island. I compared ectomycorrhizal communities from five forest types with different histories and different levels of tree productivity: 24-year-old regenerating hemlock on Cedar-Hemlock (CH) sites with three different fertilization regimes; 300-year-old old-growth hemlock on CH sites; and 100-year-old mature hemlock on Hemlock-Amabilis fir (HA) sites. I analyzed cloned DNA sequences from 100 ectomycorrhizal root tips per sample, four samples per plot and three plots per forest type. Fungal sequences from the regenerating and old-growth hemlock had been determined prior to my studies, but I performed the sampling and sequencing from the plots in the hemlock-amabilis fir stands, and I analyzed all the data. I detected 147 fungal operational taxonomic units among sequences from a total of 1435 DNA clones. Of the environmental variables, foliar nitrogen concentration correlated most closely with fungal species composition. Foliar nitrogen concentration was lower in regenerating stands and higher in the older stands. Less strongly correlated with fungal species composition was tree productivity measured as site index (SI), which was low in the unfertilized, nitrogen fertilized, and old-growth cedar-hemlock stands (SI = ~20), and higher on the plots in regenerating stands that were fertilized with nitrogen plus phosphorus and on plots on the HA sites (SI = 28-33). Nitrogen plus phosphorus fertilization had a small effect on the ectomycorrhizal communities in the regenerating hemlock stands on CH sites. Fertilization did not increase the similarity between the regenerating hemlock and mature hemlock-amabilis fir stands, even though the site indices for these two forest types were similar. When I considered phylogenetic distances between the observed fungal species,

[1] A version of this chapter will be submitted for publication. Lim, S. and Berbee, M.L. (2010) Phylogenetic structure of ectomycorrhizal fungal communities of western hemlock changes with forest age and stand type.

the regenerating stands, regardless of their fertilization history, had lowest diversity and most closely related species (phylogenetic clustering). The older stands had higher phylogenetic diversity and showed phylogenetic evenness. Environmental filtering with selection for a relatively small number of traits may have been operating to minimize the phylogenetic diversity in the regenerating stands. Phylogenetic evenness may be explained by an increase in the complexity of the forest floor environment as stands age, leading to an increase in the number of niches. The varied niches may have selected for traits found in different lineages of fungi, resulting in the observed pattern, a community of fungi that were more distantly related than expected by chance. Phylogenetic methods for community ecology have been developed only recently, and this is the first time that a transition from phylogenetic clustering in younger forests to phylogenetic evenness in older forests has been described for ectomycorrhizal fungi. I hypothesize that similar transitions will be detected in future studies of ectomycorrhizal fungal succession at other sites and under other environmental conditions.

2.2 Introduction

2.2.1 Factors contributing to ectomycorrhizal fungal diversity and composition

A better understanding of forest ecosystems can be acquired with studies of the ectomycorrhizal fungal community. In boreal and temperate forests, which are usually nitrogen limited, trees depend on their associated ectomycorrhizal fungi to supply them with sufficient amounts of nutrients (Read and Perez-Moreno, 2003). About 75% of annual phosphorus uptake and up to 80% of nitrogen are derived from mycorrhizal fungi (Simard et al. 2002; Hobbie and Hobbie 2006; van der Heijden et al. 2008).

Within the community, overall diversity and composition of ectomycorrhizal fungi are influenced by the complexity of spatial variability in tree roots depending on age and physiology (Danielson and Visser, 1989; Deacon and Fleming, 1992) and soil resources where many abiotic (Dighton and Mason, 1985; Last et al., 1987; Termorshuizen, 1991; Goodman and Trofymow, 1998) and biotic factors are interacting with each other. Johnson et al. (2005) reviewed the relationship between plant productivity and the ectomycorrhizal fungal community, concluding that although host

species diversity, composition, and stand age all affect ectomycorrhizal fungal communities, the mechanisms and details of the interactions remain to be explored.

Some of the soil and plant characteristics likely to have influenced the ectomycorrhizal fungal community at my study sites had previously been characterized as part of the Salal Cedar Hemlock Integrated Research Project (SCHIRP) (Chapter One, Table 1.1; Prescott and Weetman 1994). A long-term collaborative project established in 1985, the goals of the SCHIRP include improving understanding of ecological processes in the cedar-hemlock forests and then applying ecological principles to improve forest productivity.

Two types of sites were identified within the study area: Cedar-Hemlock (CH) and Hemlock-Amabilis fir (HA) (Lewis, 1982). Nutrient supply and productivity on these sites is compared in Chapter One, Section 1.5 and Table 1.1C. CH sites had old-growth (ca. 300 years) stands with an open canopy, dense salal understory, and relatively low annual productivity. The diameter-class structure of the old-growth cedar-hemlock stands suggested they were self-replacing, climax communities (Keenan, 1993). During the 1960's, logging of old-growth cedar-hemlock forests generated substantial areas of cutovers, which regenerated very slowly with western red cedar and western hemlock (Prescott and Weetman, 1994). In 1979, an old-growth cedar-hemlock forest on a CH site was clear-cut followed by slash burning and then planted with either western red cedar or western hemlock in 1980 (Blevins and Prescott, 2002; Blevins et al., 2006). Seven years after harvesting, the sites were dominated by salal, and the conifers that had been planted exhibited poor growth and chlorosis. To test whether fertilization could improve growth rates, 'Plot Fertilization Trials' were established in 1987 (Mehmann et al., 1995; Mallik and Prescott, 2001). Regenerating hemlock on plots on CH sites were fertilized in 1987 and 1997 with 300 kg/ ha of nitrogen (N) (applied as urea), with 300 kg/ha of nitrogen in combination with 100 kg/ha of phosphorus (P) (applied as triple superphosphate), or left unfertilized (Blevins and Prescott, 2002). The second-growth, mature hemlock-amabilis fir forests on HA sites originated naturally from a catastrophic windstorm in 1906. The diameter class distribution of the hemlock-amabilis fir was unimodal, suggesting an even-aged stand, but a sample of tree ages indicated that many trees established some time before or after the 1906 windstorm (Keenan, 1993). The

hemlock-amabilis fir forests had dense canopies, a sparse understory consisting mostly of mosses, and high productivity possibly due to the disturbance and soil mixing from the time of the windthrow.

Site indices, which represent an integrated measure of the ability of trees to grow at a site, were either available or could be estimated (Table 2.1). Discrete measures of variables including tree age and foliar N and P concentrations were available for all sites (Table 2.1; Blevins et al. 2006; Keenan et al. 1995). Annual litterfall and aspects of litter composition of old-growth cedar-hemlock and mature hemlock-amabilis fir stands were also available (Chapter One, Table 1.1C; Keenan et al. 1995). I anticipated that ectomycorrhizal fungal diversity and species composition might be correlated with some of these measured environmental variables.

2.2.1.1 Host productivity may control the ectomycorrhizal fungal community

In terms of host productivity, ectomycorrhizal fungal associations are thought to place a high carbon demand on the host plant and, therefore, plant-fungal interactions may not always be positive, especially when carbon resources are limited (Druebert et al., 2009). The fungal partners may consume 20-30% of the carbon assimilated by the host plant according to microcosm ectomycorrhizal studies and a girdling field study (reviewed by Söderström, 2002). This substantial proportion of the carbon is respired by the fungi to produce energy for cellular maintenance, and part of it is used in the growth of extensive mycelial networks. Thus, ectomycorrhizal fungi are significant sinks for photosynthetically-derived carbon (Smith and Read, 1997).

If costs and benefits to hosts were directly linked to costs and benefits to fungi, alterations in carbon supply might be expected to directly affect root colonization and fungal community structures. Druebert et al. (2009) suggested a strong dependence of ectomycorrhizal colonization on host productivity. Light-driven increases in beech (*Fagus sylvatica*) productivity affected the abundance and diversity of ectomycorrhizal fungi. Other studies indicated that alterations in carbon availability either by defoliation or by exposure to elevated CO_2 affected fungal community structures (Godbold and Berntson 1997; Saikkonen et al. 1999; Parrent et al., 2006). Restriction of carbon flow to ectomycorrhizae by defoliation of the previous year's needles in Scots pine (*Pinus*

sylvestris) had no effect on belowground diversity in terms of the number of morphotypes or morphotype diversity index, but the formation of sporocarps decreased (Kuikka et al. 2003).

2.2.1.2 Fertilization affects the fungal community

While fertilization can have a variety of effects on ectomycorrhizae (Chapter One, Section 1.3.2), it usually increases tree productivity. After a short-term negative affect on the ectomycorrhizal fungal community, the increase in host productivity may continue to affect the fungal community. At the SCHIRP sites, Wright (2006) found a significant difference in ectomycorrhizal fungal species composition in the N plus P-fertilized plots compared to the unfertilized or N-fertilized plots. She found that the presence of DNA sequences of three *Cortinarius* species was correlated with fertilization history. The abundances of *Cortinarius* species #18 and #19 were high in the N plus P-fertilized plots whereas *C. cinnamomeus* was abundant in the N-fertilized plots and unfertilized plots (Wright 2006). This difference in fungal species composition was associated with the increasing height response of dominant western hemlock trees in the N plus P-fertilized plots (Blevins et al., 2006).

2.2.1.3 Other edaphic factors affect the fungal community

Change in the ectomycorrhizal fungal community has also been correlated with host species or various measures of the nutrient status in the host plants including forest floor nutrient levels, moisture levels and variation in litter quality. Ectomycorrhizal fungal species richness per plot increased asymptotically with foliar N concentrations of subalpine fir in a southern boreal forest (Kranabetter et al., 2009). Forest floor organic P was positively related to ectomycorrhizal fungal diversity on Douglas-fir, and mineral soil available P was negatively related to ectomycorrhizal fungal richness (Twieg et al., 2009). However, Twieg et al. (2009) also found that the ectomycorrhizal fungal community structure was more strongly influenced by stand age than specific soil nutrients. In a multivariate analysis of ectomycorrhizal fungal genera of creeping willow (*Salix repens*), soil moisture levels significantly contributed to the explanation of species composition variation (van der Heijden et al., 1999).

Litter quality can play a role in mycorrhizal fungal community abundance and community composition. More ectomycorrhizal tips and more types were found in the litter near tree trunks, possibly because of the presence of both small woody debris and other litter types (Goodman and Trofymow, 1998). A different ectomycorrhizal community development was observed in a mixture of forest litters (pine and oak), compared with litterbags of either pine needles or oak leaves (Conn and Dighton, 2000). Natural conifer seedling roots and associated ectomycorrhizae in old-growth forests had the tendency to concentrate in soil organic components (humus and decayed wood), rather than in the mineral soil (Harvey et al., 1987).

2.2.1.4 Evidence that forest stand age selects for particular fungal species

During ectomycorrhizal fungal succession, fungal species may either turn over or accumulate, adding richness to the ectomycorrhizal community until a forest stand becomes mature (Chapter One, Section 3.1.1). Kranabetter et al. (2005) reported, for example, that 20 year-old stands of western hemlock-lodgepole pine had the lowest total ectomycorrhizal mushroom species richness, with approximately half as many species as the 120 and 225 year-old stands. They also observed that epigeous ectomycorrhizal species were limited to mature stands associated with low mineral soil N availability. In contrast, the cumulative richness of hypogeous and epigeous ectomycorrhizal fungal species was similar among age-classes of Douglas-fir dominated stands in the Cascade Range of Oregon, but a large number of fungal species (19%) were found only rarely in old-growth stands (Smith et al., 2002).

Studies by Mason et al. (1983), Dighton et al. (1986), Mason et al. (1987), and Deacon and Fleming (1992) also showed differences in ectomycorrhizal species composition correlated with forest stand age. Some fungi (e.g., species of *Hebeloma* and *Laccaria*) were *r*-selected species in terms of their vegetative growth and invasiveness, readily colonizing roots of seedlings when their spores or mycelium were added to either sterilized or non-sterilized soil (Mason et al., 1983). In contrast, *K*-selected species such as *Leccinum* species and *Amanita muscaria* appeared to establish with great difficulty by spores; indeed, they always failed to produce ectomycorrhizae on seedlings in sterilized soil (Deacon et al., 1983). These *K*-selected species, however, were very effective

competitors once they are established on the root system of a moderately sized tree (Bruns, 1995). This difference seems to be related to the occurrence of other soil microbes (Last et al., 1983).

It is not as yet clear which factors are responsible for fungal succession and older stands may provide more heterogeneous habitat, selecting for fungi with distinct functional roles. In contrast to Twieg et al. (2009) (discussed above), Termorshuizen (1991) reported that the succession of ectomycorrhizal fungi of Scots pine was primarily influenced by changes in the soil and not by ageing of the trees. Differences in species composition were not only observed between stands of different ages but also between stands with the same tree age. Therefore, ectomycorrhizal fungi species may be unequally distributed among the forest stands because of other edaphic factors beside host tree age.

2.2.2 Characterizing phylogenetic community structure

Methods for comparative studies of net phylogenetic relatedness of members of biological communities are recent in development and increasingly widely applied (Kraft et al., 2007; Kembel, 2009). The phylogenetic approach is exciting due to its potential to provide insight into the nature of functional interactions of species and their communities even in studies like this one where the species involved are highly diverse and little is known about responses of individual species to the environment. If the community structure could not be explained by a random draw from the total pool of species in the area, this implies that traits important in reproductive success in local habitats have a phylogenetic component.

If the species from each community are more closely related to one another than expected based on the phylogeny of species from the whole pool, the community is phylogenetically clustered. Phylogenetic clustering may indicate that the environment is serving as a filter to exclude most of the lineages present in the community pool, allowing establishment only of species from lineages with special traits required for success in the environment. Competition among closely related species with similar traits is less important in structuring the community than possession of the inherited traits. Phylogenetic clustering has been reported in disturbed or unfavourable

environments. Helmus et al. (2007) showed that phylogenetic clustering was evident among fish species in lakes with relatively high acidity, and Dinnage (2009) showed that phylogenetic clustering occurred in the herbaceous plants in fields recently disturbed by plowing.

If the species are more distantly related to one another than expected by chance, the community shows phylogenetic evenness. This pattern suggests that different phylogenetic lineages show different habitat specializations and also that the habitat contains different niches and selects for inhabitants with niche-specific traits. Within a niche, competition limits the number of closely related species that can co-inhabit. Maherali and Klironomos (2007) provided experimental support for competitive exclusion of closely related species in studies of arbuscular mycorrhizal fungi inoculated into pot cultures. When the initial inoculum consisted of closely related species, the number of species remaining at the end of the one-year experiment was low, presumably because these close relatives had similar traits and were in competition for the same niche. Species richness at the end of a one-year experiment was highest when the initial phylogenetic diversity of the inoculum was also highest, presumably because the distantly related fungi had different traits and evaded competition by inhabiting different niches (Maherali and Klironomos 2007).

Aside from the study by Maherali and Klironomos (2007), biological interpretations of patterns of phylogenetic community structure have been tested mainly using computer simulation studies where species traits, phylogenies, and community interactions have been modeled (Helmus et al., 2007; Kembel, 2009; Kraft et al., 2007). In comparative analyses of communities that are based solely on species presence or absence, high species diversity leads to incomplete sampling and difficulties in comparing species composition. Encouragingly, however, in studies of phylogenetic structure, having a large pool of different clades, each containing several or many closely-related fungi, can increase the probability that phylogenetic evenness can be detected (Kraft et al., 2007).

If the species in specific communities represent a random sampling from the total pool of species across all communities, then neutral processes and chance may have determined community structure. Species in stable old-field herbaceous plant

communities, in contrast to species in recently plowed fields, appeared to be a random sampling from the phylogeny of species in the larger community, leading to the interpretation that neutral processes dominated in community assembly (Dinnage, 2009). In another study, ectomycorrhizal fungal communities associated with tropical dipterocarp forests on sandy soil were assembled randomly with respect to phylogenetic distance (contrasting with species from clay soils, which were phylogenetically clustered) (Peay et al., 2010).

2.2.3 Aims and objectives

In this study, I compared ectomycorrhizal fungal community data from western hemlock from five forest types. The types were old-growth cedar-hemlock forests growing on CH sites, mature hemlock-amabilis fir on HA sites, and hemlock regenerating under three different fertilization treatments on CH sites (Wright, 2006) (Table 2.1). I had two main objectives. First, I wanted to determine which measured variables were most closely correlated with belowground ectomycorrhizal fungal diversity and species composition. If tree productivity correlated closely with the fungal community, then the fertilization of the regenerating hemlock stands on CH sites, which increased tree productivity, should also have increased the similarity of their mycorrhizal communities to the communities of the mature hemlock on the highly productive HA sites. If discrete variables such as forest stand age, forest type, or foliar N or P concentration played the main role, then the fungal community may show strong correlation with one or more of these other factors. Secondly, I planned to compare the patterns of phylogenetic diversity across forest types. Are ectomycorrhizal fungal species either more or less closely related in some forest stands than expected by chance alone? Few studies of patterns of phylogenetic diversity have been conducted to date, and this study provided an opportunity to set initial expectations about whether different forest stand types select for communities of ectomycorrhizal fungi with similar or with different patterns of relatedness.

2.3 Materials and methods

2.3.1 Site description

The SCHIRP study sites were located between the towns of Port Hardy (50°43′N, 127°29′W) and Port McNeill (50°34′N, 127°04′W), BC. This locality is considered to be one of the first places along the British Columbian coast that became free of ice after the most recent (Fraser) glaciation, with vegetation becoming established around 14,000 years ago (Hebda, 1983). Western hemlock (*Tsuga heterophylla*), western red cedar (*Thuja plicata*), salal (*Gaultheria shallon*), amabilis fir (*Abies amabilis*), and Sitka spruce (*Picea sitchensis*) comprised forest vegetation. The study sites were in the very wet maritime Coastal Western Hemlock biogeoclimatic subzone (Pojar et al., 1991).

2.3.2 Experimental design

To test for a correlation between forest type characteristics and ectomycorrhizal fungal species, I compared ectomycorrhizal fungal species of western hemlock from five different forest types (Table 2.1). For each forest type, three replicate plots were established. Plots were 25 m × 25 m (0.0625 ha) for the regenerating hemlock stands on CH sites (Wright, 2006) and 50 m × 50 m (0.25 ha) for the older stands. Distances between plots were not consistent due to active logging by Western Forest Products, Inc. Plots of regenerating hemlock on CH sites were randomly located within a radius of 180 m whereas the older plots were at least 730 m apart from the other replicate plots of the same forest type (Fig. 2.1).

Plots in old-growth cedar-hemlock and mature hemlock-amabilis fir stands showed some variation in elevation, moisture content, light availability, and plant species present. Field observations showed that replicate plot A of the old-growth cedar-hemlock was located on the highest elevation at 140 m (compared with B at 129 m and C at 90 m); had the lowest soil moisture; had mostly western hemlock and some amabilis fir trees and much less salal coverage compared with the other two replicate plots (Wright, personal communication). Replicate plot #1 of the mature hemlock on HA sites was located on the highest elevation at 163 m (#2 at 122 m and #3 at 72 m) with the lowest light availability among the three plots of hemlock-amabilis fir, and it had the least understory vegetation (e.g., mosses and ferns) and the fewest fallen logs. The plot-to-plot variation

was consistent with Klinka's (2006) observation that an extraordinarily high microsite variation on CH and HA sites presents a difficulty for site classification.

2.3.3 Tree productivity measured as site index

Site index (SI50) measures the capacity of an area of land to grow trees of a given species at 50 years breast height age, and it is the most widely accepted and practical, quantitative measure of timber site quality in North America (Davis et al., 1987: Avery and Burkhart, 2002). A. van Niejenhuis at Western Forest Products Inc. (Saanichton, BC) provided the stand height of regenerating western hemlock trees on the CH sites (Blevins et al., 2006) and a SI50 estimate for the old-growth CH sites (Table 2.1). D. Munro at Forintek Division of FPInnovations (Vancouver, BC) provided SI50 data of HA sites with mature hemlock-amabilis fir stands, located at the eastern end of Rupert's Inlet west of Port Hardy (50°36′N, 127°30′W), and I used this data for my HA sites with van Niejenhuis' permission.

In order to calculate SI50 of the 24-year-old regenerating western hemlock on the CH sites, the growth intercept model was used (BC Ministry of Forests, 1995). Dominant trees with the largest diameter at breast height per 0.01 hectare were selected for each fertilization treatment. Total age of western hemlock was converted into breast-height age, and this breast-height age and the stand height of the dominant trees were used to calculate SI50 in SiteTools version 3.3 (BC Ministry of Forests, 2004).

2.3.4 Sampling methods

Samples from the regenerating hemlock were collected in October 2004, November 2004, and February 2005, and one replicate plot was sampled from each fertilization treatment during each collecting trip (Wright, 2006). S. Wright collected samples from the old-growth hemlock on CH sites in November 2006 and I, with Wright's assistance, collected samples from the mature hemlock on HA sites in November 2007.

Four composite root samples were taken from each plot, for a total of 12 samples from each of the five forest types, or 60 samples in all. Each composite sample was made up of ectomycorrhizal root tips from four cores. At the canopy edge of each of four

randomly selected western hemlock trees per regenerating plot, four soil cores were taken using an auger (diameter of 4.5 cm to a depth of 10 cm) (Wright, 2006). No other conifers were near the sampled trees on the regenerating plots. For the older stands, four subplots of 1 m × 1 m that were mostly surrounded by western hemlocks were chosen per plot, and then four cores were taken within these subplots and combined. The closest conifers to the subplots were all western hemlock trees. The soil samples were stored at 4^{o}C.

Within 18 days after collection, ectomycorrhizal root tips were examined under dissecting and compound microscopes for colonization. Amabilis fir and Sitka spruce form ectomycorrhizae as well, and their roots were not easily differentiated when viewed in cross-section under a compound microscope. Thus, they might have been included in the root samples (Section 2.3.5). One hundred ectomycorrhizal root tips were selected from each composite sample of four cores from one tree or subplot, and stored at -20^{o}C.

2.3.5 DNA extraction, amplification, and cloning

Genomic DNAs were extracted from a total of 60 samples: 36 samples from regenerating hemlock stands on CH sites by Wright (2006), 12 samples from old-growth stands on CH sites by E. Carruthers (former research technician in M. Berbee's lab), and 12 samples from mature hemlock-amabilis fir stands by myself. The procedures outlined in Wright (2006) were carried out.

To check whether western hemlock predominated in the mixture of DNAs from ectomycorrhizal root tips, I designed plant-specific primers (Table 2.2). Then, I tested DNA extracts of two arbitrarily chosen samples from the regenerating hemlock stands on CH sites, and all samples from old-growth hemlock on CH sites and from mature hemlock on HA sites. The gene for the chloroplast-encoded large subunit of ribulose bisphosphate carboxylase (rbcL) was amplified using illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare Biosciences, Piscataway, NJ, USA) in a GeneAmp® PCR System 9700 thermocycler (PE Biosystems, Foster City, CA, USA). Reaction conditions were as follows: an initial denaturation cycle at 94°C for 5 min, 35 cycles of denaturation (at 94°C for 30 s), annealing (at 45°C for 1 min) and extension (at 72°C for 2 min), and a final extension at 72°C for 10 min. Four microliters of each sample were then

electrophoresed on a 1% agarose gel to confirm that the correct DNA had been amplified. The resulting PCR fragment was sequenced with primer rbcL-R2 using ABI PRISM® BigDye® Terminator version 3.1 Cycle Sequencing Kit and following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The PCR program used for sequencing consisted of an initial denaturation step of 5 min at 96°C, and 25 cycles of denaturation (10 s at 96°C), annealing (5 s at 50°C), and extension (4 min at 60°C) in the PCR machine given above. Sequences were electrophoresed using an Applied Biosystems™ automated sequencer at the UBC Nucleic Acid and Protein Service unit.

The two samples of ectomycorrhizal roots from the regenerating hemlock from CH sites and most of the samples from the older hemlock from CH and HA sites were of western hemlock (Table 2.3). Four out of 12 samples from the old-growth cedar-hemlock stands were of amabilis fir, and one sample from the mature hemlock from the hemlock-amabilis fir stands was of Sitka spruce. I performed the analyses of phylogenetic dispersion and indicator species analysis (in subsequent sections) in two ways, once with all samples and once including only samples where hemlock DNA predominated.

2.3.6 Phylogenetic identification of ectomycorrhizal fungi

Thirty-six samples, 12 from each of three fertilization treatments from the regenerating hemlock plots on CH sites were analyzed for molecular identification by Wright (2006). Carruthers began the identification of 12 samples from old-growth plots from CH sites, but I completed her analysis and, in addition, identified fungal species in the 12 samples from mature hemlock plots from HA sites. A total of 60 samples, each comprising 100 pooled ectomycorrhizal root tips, were analyzed for molecular identification following Wright (2006). Thorough phylogenetic analyses were performed, so 'Hemlock root associated fungus clones' and 'Ectomycorrhizal fungus clones' from GenBank from Wright (2006) were assigned into taxonomic groups.

Sequences were usually considered to represent different Operational Taxonomic Units (OTUs) if they were more than 3% different across their Internal Transcribed Spacer (ITS) 1, 5.8S rDNA gene, and ITS2 regions (Hughes et al., 2009). The OTUs from *Cortinarius* and *Lactarius*, however, were defined in two different ways. For most

analyses, sequences in these genera were assigned to different species-level OTUs if they were more than 1% different. The 1% cutoff tended to bring the species names associated with GenBank sequences into good congruence with OTUs, whereas a 3% cutoff would have grouped sequences from different species into the same OTU. Analysis of net relatedness was repeated with each of two cutoffs, 1% and 3% for *Cortinarius* and *Lactarius*, to help ensure that results were not an artifact of an arbitrary cutoff.

Representative DNA sequences for each OTU were submitted to GenBank. The 61 sequences from the old-growth plots on CH sites are available under accession numbers EU057079 - EU057126 and FJ236843 - FJ236855, and the 63 sequences from plots on HA sites are accessioned under FJ152482-FJ152544.

2.3.7 Species diversity measures

I measured the ectomycorrhizal fungal diversity using the frequencies of DNA sequences recovered from the random clone libraries of the five forest types. Assessment of diversity commonly includes traditional taxon-based measures at the species level that assume all species are equally related (e.g., Shannon's index (Shannon and Weaver, 1949)). I used EstimateS software version 8.0.0 (Colwell, 2005) to calculate Shannon's H' ($-\sum p_i \ln p_i$) diversity index, observed species richness (Colwell et al., 2004), and the Chao1 (Chao, 1984) and Jack1 (Burnham and Overton, 1978) estimated richness. The settings were 100 randomizations; strong hash encryption (a non-repeating random number series that passes the most demanding tests); randomize with replacement; use bias-corrected formula for Chao1 & 2; and 10 upper abundance limit for rare or infrequent species. Fungal diversity of the mature hemlock from HA sites and old-growth hemlock from CH sites was compared to the high diversity found in the regenerating hemlock stands on CH sites (Wright, 2006).

Calculated observed species richness was plotted as the species accumulation curve using the three replicates for each forest type to examine the intensity of sampling effort. The relative log-scale percent abundance of each OTU, plotted against OTUs ranked by abundance, was compared for the five stand types. This rank-abundance plot has been used in studies of ectomycorrhizal fungal communities and is effective for

evaluating the structure of fungal communities (Visser, 1995; Jonsson et al., 1999; Horton and Bruns, 2001).

To take evolutionary relatedness between species into consideration (Chapter One, Section 1.4), I used Phylocom version 4.0.1 beta (Webb et al., 2008) to calculate the distance matrix between communities based on phylogenetic distances, given a phylogenetic tree, using both species abundance and presence/absence data. Another phylogenetic diversity measure, Rao's quadratic entropy (Rao, 1892) was calculated using presence/absence data. Rao's quadratic entropy is the expected phylogenetic distance between two randomly drawn individuals (i.e., DNA clone sequences) from the community.

For statistically comparing the means of the five forest types, the Shapiro-Wilk test for normal distribution, the Levene's and Bartlett's tests for equal variances, the analysis of variance (ANOVA), and the Tukey-Kramer test were performed in JMP® version 7.0.2 (SAS Institute Inc., Cary, NC, 1989-2007). The Shannon and Faith diversities of the five forest types were not normally distributed (Appendix A), so the nonparametric Kruskal-Wallis test was used to compare five forest types (null hypothesis, H_0 = group means are centered at the same location). Nonparametric tests are usually based on the ranks of the data points rather than the actual values of the data. Unfortunately, the Kruskal-Wallis test assumes that the distribution of variables must have the same shape in every population (Whitlock and Schluter, 2009). In addition, nonparametric tests are less likely to reject a false H_0 than the corresponding parametric tests, especially with smaller sample sizes. However, when the H_0 of the Kruskal-Wallis test was rejected and the variance was equal and the H_0 of the ANOVA was rejected, the Tukey-Kramer test was performed.

2.3.8 Species composition analysis

To assess overall variation in ectomycorrhizal fungal species composition, multivariate analyses were performed using PC-ORD statistical software (version 5.10, MjM Software Design, Oregon, USA). I used Non-metric Multidimensional Scaling (NMS) to determine and display the overall structure of species data among root samples. NMS is a non-constrained ordination technique that is ideal for data that is non-normal,

or has arbitrary, discontinuous, or questionable scales (McCune and Grace, 2002). It can also be used as a method for determining the dimensionality of the data set. NMS also detects whether the structure in the response data is stronger than expected by chance (McCune and Grace, 2002). I used Sørensen's distance measure (Sørensen, 1948) with the automatic settings of PC-ORD. The Sørensen index is one of the traditional taxon-based measures at the species level that assume all species are equally related. The NMS included a maximum number of iterations of 500, using a starting number from a random seed, a starting number of axes of 6, and 250 real and 250 randomized runs. The final instability criterion was 0.00001.

The most important advance in the study of ectomycorrhizal fungal communities has been the application of molecular methodologies such as Polymerase Chain Reaction (PCR) for identification; however, biases are known to be introduced during the process (von Wintzingerode et al., 1997; Kanagawa, 2003). For example, some species have DNA sequences that PCR selectively detects and amplifies more frequently than others. As a result, frequency or abundance data will be affected by these biases. Abundance data can alternatively be left as a set of absolute values, or abundances can be transformed into relative percentages or ratios. Therefore, I processed the input species composition data for the NMS analyses in three different ways:

1. Presence or absence of OTUs in an ectomycorrhizal root sample – less prone biases when molecular techniques are used
2. Absolute abundance of OTUs in a sample – number of DNA clone sequences found for each species
3. Relative abundance of OTUs in a sample – standardized number of DNA sequences found in each sample. The total number of DNA sequences per sample ranged from 15 to 34.

These manipulations of my data may affect the ability to demonstrate a change in ectomycorrhizal fungal communities. I wanted to find out which approach to data processing best demonstrates species composition. Overall, species presence/absence, absolute abundance, and relative abundance data gave similar results, and the three data sets worked equally well for the NMS (BOTA 526 class project).

Twelve root samples of each forest type were used in NMS ordinations to detect patterns among forest types. Biplot over-lay was performed using the site index, stand age, and the foliar N and P concentrations of the forest types. Then, the Pearson and Kendall correlations were calculated to detect correction between NMS axes and the four variables (Appendix B). Next, the NMS axes were rotated using the most correlated variable, foliar N concentration, maximizing the spread of points along the first NMS axis and the foliar N concentration. Again, the Pearson and Kendall correlations were calculated (Table 2.4).

To determine whether species composition among forest types were significantly different, a nonparametric statistical method, multi-response permutation procedures, was performed to test the null hypothesis of no differences between groups (McCune and Grace, 2002) in PC-ORD. I used Sørensen's distance measure.

Using the parsimony tree built for calculating phylogenetic diversity, Principal Coordinate Analysis (PCoA) with 1,000 permutations were performed in UniFrac (Lozupone and Knight, 2005) to consider evolutionary relatedness between species.

To assess similarity of fungal species composition, I used Chao-Jaccard abundance-based similarity indices to estimate shared species between a given pair of forest types in EstimateS software version 8.0.0 (Colwell, 2005) with 200 replicates for bootstraps (Chao et al., 2005). The resulting measure of similarity was based on the probability that two randomly chosen DNA sequence types, one from each of two forest types, both belong to DNA sequence type shared by both samples. Then, non-parametric methods were involved in estimating the unseen species (species that are likely to be present in a larger root sample of the forest type, but that are missing from actual sample data). This approach has been shown to reduce substantially the negative bias that would undermine the usefulness of traditional similarity indices where communities are rich but sampling is incomplete (Chao et al, 2005).

2.3.9 Phylogenetic community structure

In Phylocom version 4.0.1 beta (Webb et al., 2008), net relatedness index was calculated for each replicate plot as described in Webb et al. (2002) to determine whether the fungal communities are phylogenetically clustered or are shown evenness (Section

2.2.2). First, I created aligned 28S rDNA data in MacClade version 4.05 (Sinauer Associates, Inc., Sunderland, MA, USA). I added one sequence for each OTU that was defined based on the ITS data from the phylogenetic identification (Section 2.3.6). The parsimony tree based on aligned 28S rDNA data was calculated using heuristic searches implemented by PAUP* version 4.0b10 (Swofford, 2003). The parsimony tree assumes that DNA nucleotide can change into another one with equal cost (Fitch, 1971) and has the fewest evolutionary changes at the DNA level. A bootstrap method with fast stepwise-addition and 1,000 replicates was run. Next, the optimality criterion was set to distance, resulting in the dotted line branches (Fig. 2.2). A clade of nine *Cortinarius* DNA sequences from the regenerating plots had very short branch lengths, so all nine would have been considered to represent the same OTU based on 28S the data alone. However, each of the nine was included as an independent OTU because the prior analysis showed they were 97% or less similar to each other in the more variable ITS regions (Section 2.3.6).

2.3.10 Ectomycorrhizal fungal species that are specific to a forest type

Indicator Species Analyses (ISA) were performed to determine whether particular OTUs were significantly correlated with forest type. ISA may provide insight into the habitat specificity of fungal OTUs. This method combines information on the concentration of species abundance in a given group and on whether a species is always present in that group (McCune and Grace, 2002). Monte Carlo test were run with 4,999 permutations to determine whether indicator values were likely to occur by chance alone. Only root samples with hemlock presence demonstrated by PCR (Table 2.3) were included in the analyses.

2.4 Results

2.4.1 Fungal diversity and tree productivity

Using fungal ribosomal gene fragments – ITS and 5' end of the 28S rDNA gene – that were amplified, 147 Operational Taxonomic Units (OTUs) or sequence types were detected using phylogenetic identification among 1435 fungal DNA clone sequences

from the five forest types (Fig. 2.2 and Appendix C). Frequently found taxonomic groups are listed in Table 2.5.

Ectomycorrhizal fungal diversity did not correlate closely with site index of western hemlock on northern Vancouver Island. Fertilization by N plus P increased estimated site index of the CH sites with the regenerating hemlock to 28 m, compared with the unfertilized or N-fertilized plots that had site indices of 19 m (Table 2.1). In spite of the difference between their site indices, the ectomycorrhizal fungal diversity of the fertilized versus unfertilized plots was approximately the same, whether measured by Shannon's index (Fig. 2.3A), estimated using Chao1 or Jack1 (Fig. 2.3B), or estimated as phylogenetic diversity (Fig. 2.4).

Although the observed richness and richness estimated by Chao1 or Jack1 were slightly higher in the older stands than the regenerating plots (Fig. 2.3B), the differences were not significant. The H_0 (no difference among the groups) was not rejected because the p-values were above 0.05, but they were very close (0.06-0.08) (Appendix A). The old-growth cedar-hemlock plots had a low site index (20 m), but their levels of phylogenetic diversity were intermediate between the mature hemlock-amabilis fir plots with a high site index and the unfertilized or N-fertilized plots with site indices of 19 m (Fig. 2.4). The only significant difference in phylogenetic diversity was between the regenerating stands and the mature hemlock-amabilis fir stands (Fig. 2.4).

Species accumulation curves had not reached an asymptote in any of the forest types, indicating that additional sampling would be needed for a complete inventory of all species present (Fig. 2.5). However, the trends in the accumulation curve were consistent with diversity trends; the slope of the species accumulation curve was highest for the hemlock-amabilis fir plots and lowest for the N-fertilized plots.

2.4.2 Ectomycorrhizal fungal species composition

Based on species abundance, the ectomycorrhizal fungal communities of the five forest stand types had many low-frequency OTUs rather than a single dominant species except for the old-growth cedar-hemlock plots, which were dominated by *Cantharellus tubaeformis*, accounting for 36% of occurrences (Fig. 2.6 and Fig. 2.7). The pie charts in Fig. 2.7 showing the relative abundances of ectomycorrhizal fungal OTUs in the five

forest types demonstrate the species composition differences between the hemlock-amabilis fir plots and the rest of the forest types.

With a site index of 33 m, the HA sites were the most highly productive of all sites studied (Table 2.1). However, the two forest types with the highest site indices–the mature hemlock on HA sites and the N plus P-fertilized regenerating hemlock–were not strongly similar. Instead, as analyzed by the shared species estimator (Table 2.6), the mature hemlock on the HA sites had the least number of shared species with the N plus P-fertilized plots (Table 2.6). The mature hemlock on the HA sites also had significantly higher phylogenetic diversity than the N plus P-fertilized plots (Fig. 2.4). The fungal species from the N plus P-fertilized plots were most similar to those from the N-fertilized plots (Table 2.6). In comparisons with the other forest types, the old-growth hemlock plots on CH sites shared most fungal species with the N plus P-fertilized regenerating plots from CH sites. The old-growth plots shared fewer species with the unfertilized regenerating plots and fewest species with the plots of mature hemlock on the HA sites (Table 2.7).

Multivariate analyses also showed that fungal species composition was more closely correlated with forest type than with site index (Figures 2.8 and 2.9). Plots of regenerating fertilized hemlock on CH sites and plots of old-growth stands on CH sites were more similar to each other than the plots with N plus P-fertilized hemlock on CH sites were to plots of mature hemlock from HA sites (Tables 2.7 and 2.6). An NMS analysis of species presence/absence data showed that by far the strongest correlation was between foliar N concentration and Axis 1 (Table 2.4 and Fig. 2.8). Site index showed moderate correlation with Axis 1 while stand age showed moderate correlation with Axis 3. In the ordinations, the ectomycorrhizal fungal communities were clearly separated by stand type. Strong differences in pairwise comparisons of species assemblages were observed between regenerating hemlock, old-growth cedar-hemlock, and mature hemlock-amabilis fir plots (Appendix D). The separation by stand type was also supported by PCoA analyses of phylogenetic distances (Fig. 2.9).

While 19 of the samples from the plots in the old-growth cedar-hemlock and the mature hemlock-amabilis fir stands contained predominantly western hemlock host DNA, four samples had predominantly amabilis fir and one had a predominance of Sitka spruce

DNA (Table 2.3). In the multivariate ordination, the predominant host species in the sample did not have an obvious effect on the fungal species composition (Figures 2.8 and 2.9).

Indicating relative homogeneity among replicates of the same forest type, multiple pairwise comparisons of species composition in replicate plots of old-growth hemlock on CH sites or of replicates of mature hemlock on HA sites were not significantly different (Appendix E). In contrast, the regenerating plots showed more heterogeneity and some replicate plots from the same fertilizer treatment were significantly different from each other (Appendix E).

2.4.3 Phylogenetic community structure

The net relatedness indices were calculated based on ectomycorrhizal fungal species presence/absence data. They described the difference between average phylogenetic distances in the observed and randomly generated null communities from the five forest types (Fig. 2.10). Under three different fertilization regimes, the regenerating plots had significant phylogenetically clustered fungal communities. Relatively few lineages of Ascomycota and Basidiomycota were present in the regenerating plots, and Fig. 2.2 shows that, for example, closely related species in the genus *Cortinarius* in the basidiomycete, order Agaricales, accounted for many of the OTUs detected in the these plots. The plots with old-growth or mature hemlock showed a contrasting pattern of evenness with more phylogenetic diversity than expected by chance. The plots of old-growth cedar-hemlock and mature hemlock-amabilis fir stands contained species in Agaricales, including *Cortinarius* species, but they also included representative of the Atheliaceae, Russulales, the Boletales, and several other orders of Ascomycota and Basidiomycota, many of which were not present in the regenerating plots.

Although the trends were consistent no matter how the analysis was conducted, the significance of the differences among the net relatedness indices from different forest types depended on how the samples were pooled (Fig. 2.10). When all root samples were included and the four samples from one replicate plot were pooled (n = 3, the number of plots), N-fertilized, N plus P-fertilized, and the old-growth hemlock plots were not

significantly different from each other (Fig. 2.10A). Similar patterns resulted when the net relatedness indices were calculated using species presence/absence data (not shown). However, the old-growth cedar-hemlock plots showed evenness and were significantly different from the regenerating hemlock plots if *n* was the number of root samples (*n* = 12 samples per forest type) (Fig. 2.10B and C), but not if samples were pooled and *n* equaled the number of replicate plots (Fig. 2.10A). Only root samples with hemlock's presence demonstrated by PCR (Table 2.3) were included in Fig. 2.10B, and the alternate definition of species (97% species identity used for all species including *Cortinarius* and *Lactarius* instead of 99% for these two genera) was applied in Fig. 2.10C. The standard errors of the three replicates of the unfertilized and the old-growth cedar-hemlock plots were noticeably larger in Fig. 2.10A, compared with Fig. 2.10B and C where samples were used to calculate the standard error. When I checked the data, the variation among the replicates of the unfertilized and old-growth hemlock plots was high (in the range of 3.4 and 2.7, respectively), consistent with the error bars. Overall, in all three cases the ectomycorrhizal fungi of the mature hemlock plots from the HA sites always showed strong phylogenetic evenness and were significantly different from the fungi from of the regenerating hemlock plots.

2.4.4 Ectomycorrhizal fungal species that are specific to a forest type

Of the 147 OTUs detected, seven occurred on all forest types: 2 OTUs of *Cenococcum geophilum; Lactarius pseudomucidus; Cantharellus tubaeformis; Meliniomyces variabilis; Cortinarius* cf. *azureus*; and a species in Clavulinaceae (Fig. 2.11). The three fertilization treatments of the regenerating hemlock plots shared six ectomycorrhizal fungal species, but *Piloderma fallax* was the only indicator species in the regenerating hemlock plots. Indicator species were supported by statistical randomizations, rather than restriction by treatment type, which could just be chance. Some of low-frequency OTUs especially those in genus *Cortinarius* were indicator species restricted to one fertilization treatment: *C. cinnamomeus* to the unfertilized hemlock plots, and *C*. sp19 and *C*. sp18 to N plus P-fertilized plots (Fig. 2.11). Key indicator species in the older stands were *C. tubaeformis* in the old-growth hemlock on CH sites and *Russula xerampelina* in the mature hemlock on HA sites.

2.5 Discussion

2.5.1 Phylogenetic structure of ectomycorrhizal fungal communities

My study is the first to reveal opposite patterns of net relatedness of ectomycorrhizal fungal species from forest types of different ages. All the replicate plots whether from the regenerating hemlock and old-growth hemlock on CH sites, or from the mature hemlock on HA sites, were located in close proximity to one another, within at most 15 km. Yet the fungal communities of the regenerating hemlock showed phylogenetic clustering while the plots of mature or old-growth stands showed phylogenetic evenness (Fig. 2.10). The phylogenetic evenness in old-growth cedar-hemlock and mature hemlock-amabilis fir stands may reflect trait and niche conservatism among the ectomycorrhizal fungi. Competition for the same niche among related species may have led to competitive exclusion of 'excess' species with similar traits. At the same time, the environment of the old-growth and the mature forests may have offered diverse niches, which were occupied by distantly related species (Webb et al., 2002). Some particular ectomycorrhizal lineages were shown to have phylogenetic conservatism of soil niches based on morphological similarities (Agerer, 2006) or habitat preferences (Tedersoo et al., 2003).

2.5.1.1 Possible reasons for habitat filtering in the regenerating stands

The ectomycorrhizal fungi of the 24-year-old, regenerating hemlock on CH sites were phylogenetically clustered (Fig. 2.10). This implies that the fungal species were more closely related than expected, had the community been made up of a random sample of the 147 different OTUs detected on these sites. The classical explanation for phylogenetic clustering is that the environment serves as a filter (Webb et al., 2002). Species that can pass through the filter have a good chance of becoming established. I can only hypothesize about the nature of the filter in the young, relatively and recently disturbed regenerating stands on northern Vancouver Island. The clearcutting and slashburning 24 years before sampling may have eliminated the ectomycorrhizal fungi that had dominated the stand before logging while also reducing the ectomycorrhizal mycelium available for inoculum of regenerating trees. Ability to survive logging and

burning, ability to disperse to the regenerating trees, and then ability to establish on sites with a much different microclimate than the original forests may have been among the traits shared by the phylogenetically restricted group of fungi found on the regenerating hemlock plots. The seedlings of western hemlock that had been planted on the cutover sites were probably more uniform genetically than the old-growth hemlock or mature hemlock that had regenerated naturally. Growing tree roots may have been more uniform in the regenerating hemlocks, also perhaps selecting for a restricted group of fungi. Classical *r*-selected traits may be most common in a restricted number of clades of fungi. Some of the traits involved may include dispersal ability, ability to degrade protein nitrogen (Abuzinadah and Read, 1986), foraging morphology (Agerer, 2001), drought tolerance (Mexal and Reid, 1973), and host preference (Tedersoo et al., 2008). Host preference may have been an important determinant for ectomycorrhizal fungal species from the cedar-hemlock stands and the hemlock-amabilis fir stands.

Some of the significant species in the plots of regenerating hemlock were *Piloderma fallax* and some *Cortinarius* species. Some fungi (e.g., species of *Hebeloma* and *Laccaria*) are *r*-selected species, readily colonizing roots of seedlings when their spores or mycelium are added to either sterilized or non-sterilized soil (Mason et al., 1983). Unfortunately, we do not know enough about the biology of the species we detected from the plots of regenerating hemlock to classify them as *r*-adapted species, and it is unknown whether they immediately colonized after the slash burning. In contrast, *K*-selected species (*Leccinum* species and *Amanita muscaria*) appeared to establish with great difficulty by spores (Deacon et al., 1983). This difference between *r*-selected and *K*-selected species seems to be related to the occurrence of other soil microbes (Last et al., 1983).

2.5.2 Foliar nitrogen concentration correlates with ectomycorrhizal fungal species composition

Trends in net relatedness index of the different forest types followed foliar nitrogen levels, suggesting that foliar nitrogen may also serve as an indicator of the number of niches available for colonization by phylogenetically diverse ectomycorrhizal fungi. Foliar N increased from 7 mg/g in the regenerating fertilized and unfertilized plots

(Blevins et al. 2006) to 12.6 mg/g in old-growth hemlock on CH sites and 14 mg/g in the mature hemlock-amabilis fir stands (Keenan et al. 1995). Of the variables that have been measured, foliar N concentration correlated most closely with fungal species composition as analyzed using NMS (Fig. 2.8 and Table 2.4). Fungal diversity tended to increase from the regenerating hemlock stands on CH sites through old-growth hemlock stands on CH sites to the hemlock-amabilis fir stands on HA sites (Figures 2.3 and 2.4). Kranabetter et al. (2009) recently reported a pattern strikingly similar to mine, with increasing fungal phylogenetic diversity paralleling increase in foliar N concentration. They also showed a correlation between foliar nitrogen and species composition. This similarity in study results was evident although Kranabetter et al. (2009) studied a different host species, subalpine fir (*Abies lasiocarpa*) and a different biogeoclimatic zone, the subboreal spruce zone of British Columbia, rather than the coastal western hemlock zone of my study.

If the same environmental variables that controlled foliar N concentrations also exercised direct control over fungal species composition, then the hemlock in hemlock-amabilis fir stands and in old-growth cedar-hemlock stands, having similar high levels of foliar N, should show similar fungal species composition. As is evident in Table 2.7, they do not, even though hemlock-amabilis fir stands and old-growth cedar-hemlock stands had some fungal species in common (17 out of 147 OTUs). The Chao-Jaccard estimated shared similarity between plots of old-growth cedar-hemlock and plots of hemlock-amabilis fir stands was only 0.15 (Table 2.7), while the similarity between plots of old-growth cedar-hemlock and fertilized plots of regenerating hemlock was significantly higher at 0.39 (Table 2.7). In the NMS ordination, fungi of the hemlock-amabilis fir plots contributed to the correlation between foliar N and species composition, not because of patterns of shared fungal species, but because the plots had high foliar N concentrations and a distinctive fungal species composition.

Foliar N concentrations may be correlated with other variables such as the amount of litter produced by trees, the amount of carbon stored in the soil, and the complexity of soil organic material. More litter may be correlated with more different kinds of biological activity in the soil, producing niches specific for fungi with different traits, and perhaps giving rise to the phylogenetic evenness detected in the plots of mature hemlock-

amabilis fir and the plots of old-growth cedar-hemlock stands. Compared with the old-growth cedar-hemlock stands, the hemlock-amabilis fir stands had higher litterfall and better litter quality (Chapter One, Table 1.1C; Keenan et al., 1995). The hemlock-amabilis fir forest floors also had higher concentrations of N and P compared with the old-growth cedar-hemlock stands (Chapter One, Table 1.1C; Prescott et al., 1993). Data are not available on litterfall in the plots of regenerating hemlock, but the amount of litter was probably low. Studies of carbon accumulation in western hemlock-Sitka spruce sites in the USA's Pacific Northwest showed that carbon in downed wood and in the forest floor (such as litter) was relatively high at the time of logging and declined to a minimum in 25 – 35-year-old stands, roughly the age of my regenerating plots (Appendix A in Smith et al., 2006). The forest floor carbon at the time of logging was 27.5 tonnes/ha, and at 25 years post logging, it was about 21.2 tonnes/ha. Then, forest floor carbon accumulated, albeit at a declining rate as the stand aged, to a final level of 45.3 tonnes/ha in 125-year-old stands (Appendix A in Smith et al., 2006).

Tree productivity, as indicated by site index, showed only moderate correlation with ectomycorrhizal fungal species composition (Table 2.4). Contrary to expectation if net primary productivity were the main factor controlling fungal competition, fungal species composition of the more productive sites for tree growth, the N plus P-fertilized hemlocks on CH sites and the HA sites, showed low similarity. Looking at the results from the perspective of the influence of the fungi on tree productivity, it is evident that different groups of ectomycorrhizal fungal species were able to support similar tree productivity. Many ectomycorrhizal fungi capable of enhancing or maintaining similar tree growth rates would be consistent with the unified theory of fungal generalists (Hubbell, 2001). However, the expectation of the theory of fungal generalists, that fungi have similar roles, is contradicted by my data on patterns of net relatedness.

This study was not primarily designed to correlate stand age with fungal species composition, and study of cedar-hemlock stands of a range of ages would be more appropriate to characterize fungal succession. Old-growth forests are not homogeneous, and occasional gap disturbance and windthrow may result in localized resetting of the successional clock. I found only moderate correlation between stand age and fungal species composition, possibly because a higher proportion of unique fungal species were

associated with the mature hemlock-amabilis fir stands rather than with the old-growth cedar-hemlock stands.

2.5.3 Indicator species and their ecological niches

Nine *Russula* species were found in only the older stands, mostly in the hemlock-amabilis fir plots. This genus is conspicuous and abundant in forest and arctic-alpine ecosystems, and it has a cosmopolitan distribution. Its species were diverse in their appearance as epigeous sporocarp on seral stages of forest development (Kranabetter et al., 2005). This genus was shown to have a positive linear correlation in species richness along the nitrogen gradient in soil, with many *Russula* species favouring richer soils with elevated concentrations of inorganic nitrogen (Kranabetter et al., 2009). This may explain high abundance of *Russula* species in the hemlock-amabilis fir stands, and also the one species that appeared on the N and P-fertilized plots. *R. xerampelina*, the indicator species of the hemlock-amabilis fir stands, exhibited a possible "U" shaped response to nitrogen availability in epigeous sporocarp survey (Kranabetter et al., 2009). Ability of some *Russula* species to survive in low-nutrient conditions may explain the five *Russula* species in the nutrient-poor old-growth stands.

Of the groups that showed significant habitat associations in my study, there is evidence from temperate systems that *Cortinarius* species are specialized in the acquisition of organically bound nitrogen in low nitrogen settings (Lilleskov et al., 2002). This pattern does not fully match the spatial partitioning that I observed for *Cortinarius* species as different *Cortinarius* species were present in different forest types. Examples were the two *Cortinarius* OTUs (18 and 19) which were indicator species for N and P-fertilized plots and *Cortinarius cinnamomeus*, an indicator for the unfertilized plots. *Cortinarius* species were not most abundant in the old-growth cedar-hemlock stands of lower nutrient concentrations, slower decomposition and increased organic matter.

Three species in Leucogastraceae (a family in the order Boletales, Basidiomycetes) were only present on the old-growth hemlock on low productivity CH sites. Leucogastraceae are potato-like underground fungi that commonly associate with trees in Pinaceae (Fogel, 1979). The hypogeous habit and morphology of species in the Leucogastraceae prevent aerial spore dispersal. Spore dispersal is effected through

mycophagy by arthropods, mollusks, deer, squirrels, chipmunks, and wild pigs (Fogel and Trappe, 1978). Two species commonly found across forest types, *Cenococcum geophilum* and *Lactarius pseudomucidus*, were also prevalent in other studies (Kranabetter et al., 2005; Kranabetter et al., 2009).

2.5.3.1 An interesting indicator fungal species

One of the 11 indicator species, an edible mushroom *C. tubaeformis,* was commonly found in all five forest types and was especially abundant as an indicator species in the old-growth cedar-hemlock stands (Fig. 2.11). It commonly fruits on late-seral stands with an abundance of well-decayed coarse, woody debris (Trappe, 2004), but our analyses showed that its mycelium was common even in the young regenerating stands (Wright, 2006). Western hemlock seedlings often become established on well-decayed woody debris, so the western hemlock and winter chanterelle may just be partners of convenience (M.J. Trappe, personal communication). The old-growth cedar-hemlock stands provided abundant well-decayed woody debris for *C. tubaeformis,* which may explain the high abundance of this edible mushroom species in these sites (Keenan et al., 1993).

Table 2.1 Total stand age, foliar nutrient concentration, and site index of western hemlock from the five forest types on northern Vancouver Island

Western hemlock stand	Site	Stand age	Foliar nitrogen	Foliar phosphorus	Average site index[1]
1. Regenerating: unfertilized	Cedar-Hemlock	24 years	0.70 %	0.08%	19.3 m (0.7 m)
2. Regenerating: fertilized with nitrogen	Cedar-Hemlock	24 years	0.70 %	0.07%	19.2 m (1.1 m)
3. Regenerating: fertilized with nitrogen plus phosphorus	Cedar-Hemlock	24 years	0.70 %	0.14%	28.2 m (0.6 m)
4. Uncut old-growth	Cedar-Hemlock	ca. 300 years	1.26 %	0.09 %	20 m[2]
5. Uncut mature	Hemlock-Amabilis fir	ca. 100 years	1.40 %	0.12 %	32.5 m (0.5 m)

References: Keenan et al. (1995) and Blevins et al. (2006)

Hemlocks in the regenerating plots were all nitrogen deficient (<1.2 %) whereas hemlocks in the unfertilized, nitrogen-fertilized, and old-growth plots were phosphorus deficient (<0.12%).

[1]Standard error of mean in parentheses (n = 12 for the regenerating plots; n = 10 for the hemlock-amabilis fir plots).

[2]This is an estimate.

Table 2.2 Plant-specific primers designed and used for PCR amplification in this study

Primer*	DNA sequence
rbcL-F	TGG CAG CAT TCC GAG TAA CTC
rbcL-R2 (equally specific to amabilis fir, Sitka spruce, and western hemlock)	GGA ATA AGC AGG GGG AAT CCG C

*Located in ribulose bisphosphate large subunit (rbcL) gene; chloroplast gene for chloroplast product. DNA Sequences are written 5'–3'.

Table 2.3 PCR amplification results using plant-specific primers on the ectomycorrhizal root samples

Forest type	Replicate plot number	Root sample number	Host identification
Regenerating hemlock on Cedar-Hemlock sites: unfertilized	21	21-2	*Tsuga heterophylla*
Regenerating hemlock on Cedar-Hemlock sites: fertilized with nitrogen plus phosphorus	20	20-4	*Tsuga heterophylla*
Old-growth hemlock on Cedar-Hemlock sites	A	A1	*Tsuga heterophylla*
		A2	***Abies amabilis***
		A3	*Tsuga heterophylla*
		A5	*Tsuga heterophylla*
	B	B1	*Tsuga heterophylla*
		B3	***Abies amabilis***
		B4	*Tsuga heterophylla*
		B6	***Abies amabilis***
	C	C1	*Tsuga heterophylla*
		C2	*Tsuga heterophylla*
		C3	***Abies amabilis***
		C6	*Tsuga heterophylla*
Mature hemlock on Hemlock-Amabilis fir sites	1	1C	*Tsuga heterophylla*
		1D	*Tsuga heterophylla*
		1E	*Tsuga heterophylla*
		1F	*Tsuga heterophylla*

Forest type	Replicate plot number	Root sample number	Host identification
	2	2A	*Tsuga heterophylla*
		2B	*Tsuga heterophylla*
		2C	*Tsuga heterophylla*
		2E	*Tsuga heterophylla*
	3	3A	*Tsuga heterophylla*
		3B	*Tsuga heterophylla*
		3C	***Picea sitchensis***
		3D	*Tsuga heterophylla*

Table 2.4 Pearson and Kendall correlations for four variables indirectly correlated with the non-multidimensional scaling (NMS) axes after the rotation. Based on the NMS using presence/absence data of ectomycorrhizal fungal communities of western hemlock (Fig. 2.8). **A number in bold** is a strong correlation (n = 60).

Variable	Axis 1	Axis 2	Axis 3
Foliar nitrogen concentration	**0.79**	0.15	-0.01
Site index	0.42	-0.20	-0.12
Stand age	0.38	0.23	0.21
Foliar phosphorus concentration	0.31	-0.16	0.01

Table 2.5 Relative abundance of DNA clone sequences according to their taxonomic group. The clone DNA sequences are from ectomycorrhizae of western hemlock from the five forest types.

Relative abundance (%)	Number of sequence types (OTUs)	Taxonomic group
18.5	41	*Cortinarius*
17.1	7	Cantharellales
10.5	8	*Piloderma*
8.6	10	*Russula*
8.4	5	*Lactarius*
5.6	20	Leotiomycetes including Helotiales
3.8	6	Atheliaceae including *Tylospora*
1.5	4	Thelephoraceae including *Tomentella*
1.2	3	Leucogastraceae
0.6	4	*Hebeloma*

Table 2.6 Estimating shared ectomycorrhizal fungal species from the five forest types. The regenerating hemlock on cedar-hemlock (CH) sites that were fertilized with nitrogen plus phosphorus (N+P) were compared with the other forest types. Species abundance data was used (n = 9).

Forest type	Similarity to N+P plots (Chao-Jaccard estimator with standard error)
Regenerating hemlock on CH sites: fertilized with nitrogen	0.45 [a] ± 0.04
Regenerating hemlock on CH sites: unfertilized	0.39 [a] ± 0.04
Old-growth hemlock on CH sites	0.39 [a] ± 0.05
Mature hemlock on Hemlock-Amabilis fir sites	0.15 [b] ± 0.03

Estimator index varies from 0 to 1 (0, no shared species; 1, all species are shared). Means followed by the same letter are not significantly different based on the Tukey-Kramer test.

Table 2.7 Estimating shared ectomycorrhizal fungal species from the five forest types. The old-growth hemlock on cedar-hemlock (CH) sites were compared with the other forest types. Species abundance data was used (n = 9).

Forest type	Similarity to plots of old-growth hemlock (Chao-Jaccard estimator with standard error)
Regenerating hemlock on CH sites: fertilized with nitrogen plus phosphorus	0.39 [a] ± 0.05
Regenerating hemlock on CH sites: fertilized with nitrogen	0.25 [ab] ± 0.05
Regenerating hemlock on CH sites: unfertilized	0.18 [b] ± 0.03
Mature hemlock on Hemlock-Amabilis fir sites	0.15 [b] ± 0.04

Estimator index varies from 0 to 1 (0, no shared species; 1, all species are shared). Means followed by the same letter are not significantly different based on the Tukey-Kramer test.

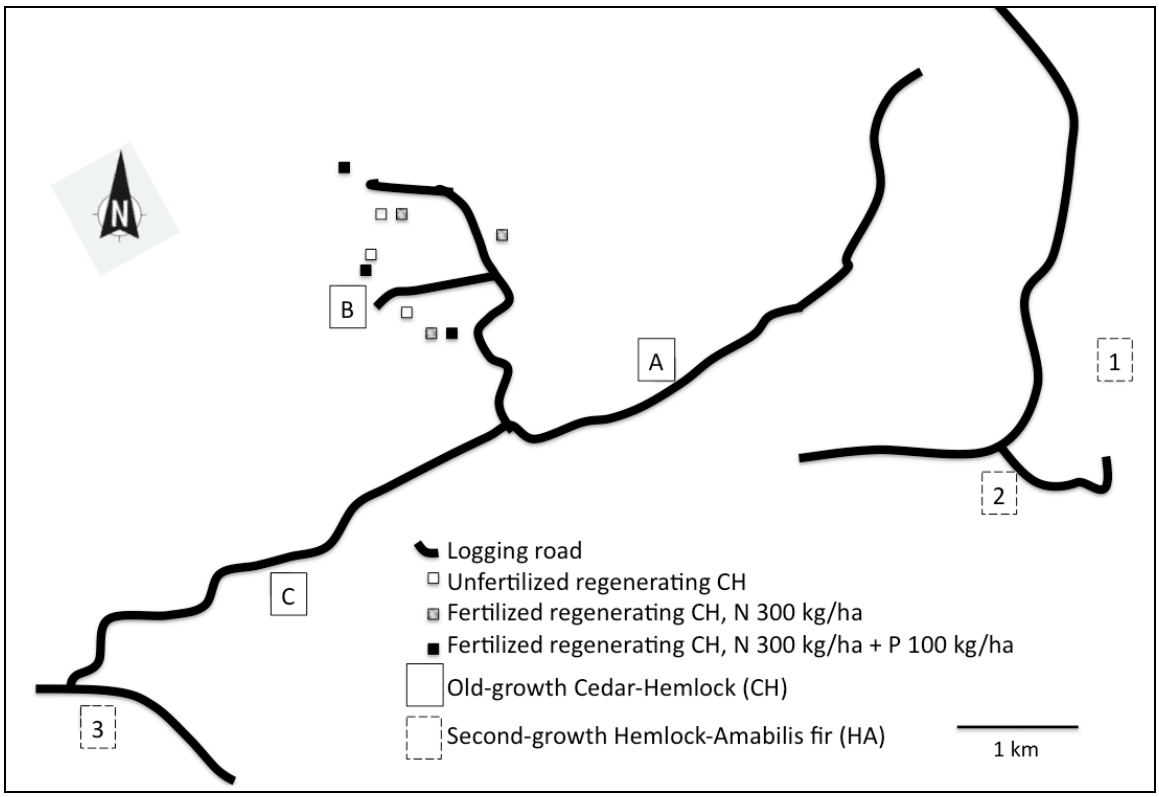
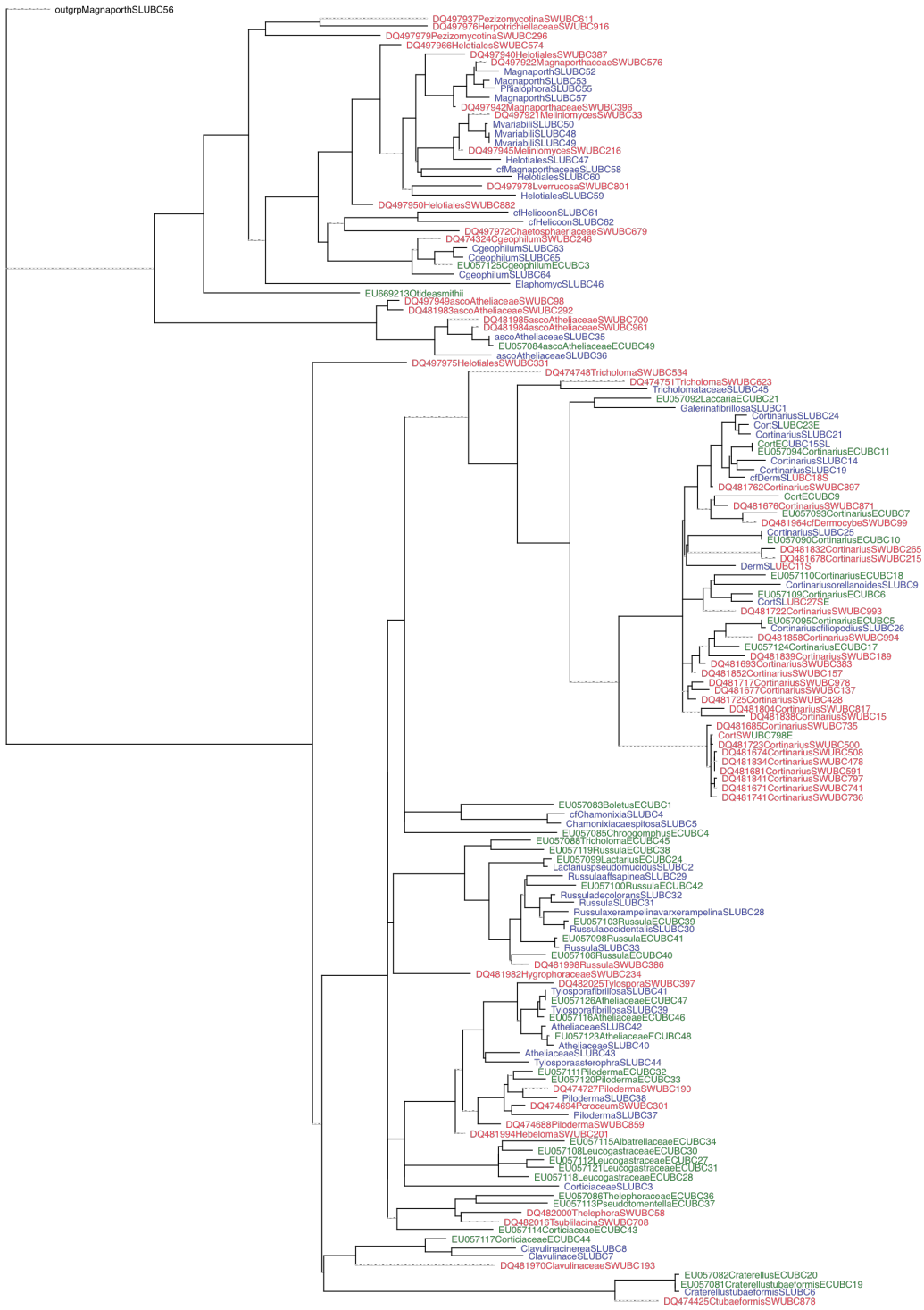


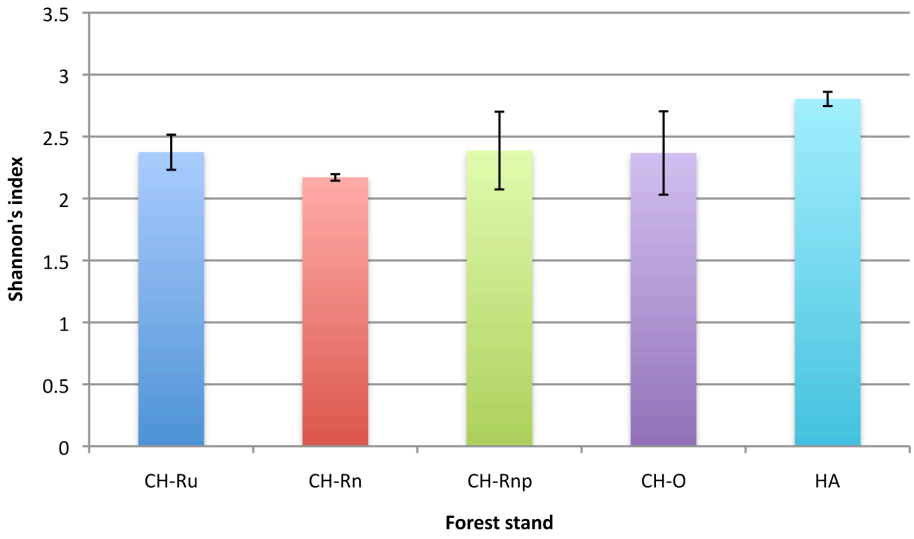
Fig. 2.1 Map of replicate plots of the five forest types. Regenerating plots were 25 m × 25 m, and the older plots were 50 m × 50 m (not drawn to scale for an easier view). N, nitrogen; P, phosphorus.

Fig. 2.2 (next page) Parsimony tree based on aligned 28S rDNA data calculated using heuristic searches. A bootstrap method with fast stepwise-addition and 1000 replicates was run. Optimality criterion was set to distance, which is responsible for the dotted line branches. Representative DNA clone sequences from regenerating hemlock stands are in red, from old-growth cedar-hemlock stands in green, and from mature hemlock-amabilis fir stands in blue.



— 0.005 substitutions/site

(A)



(B)

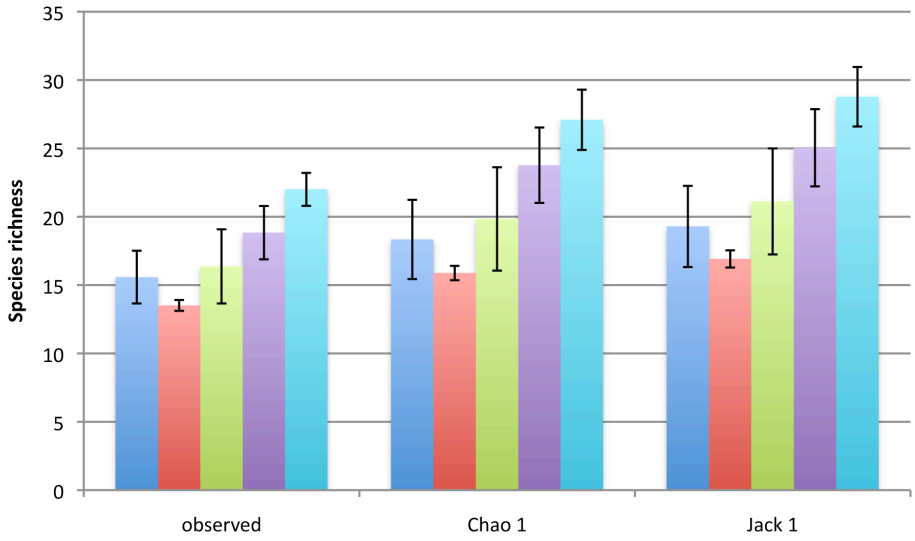
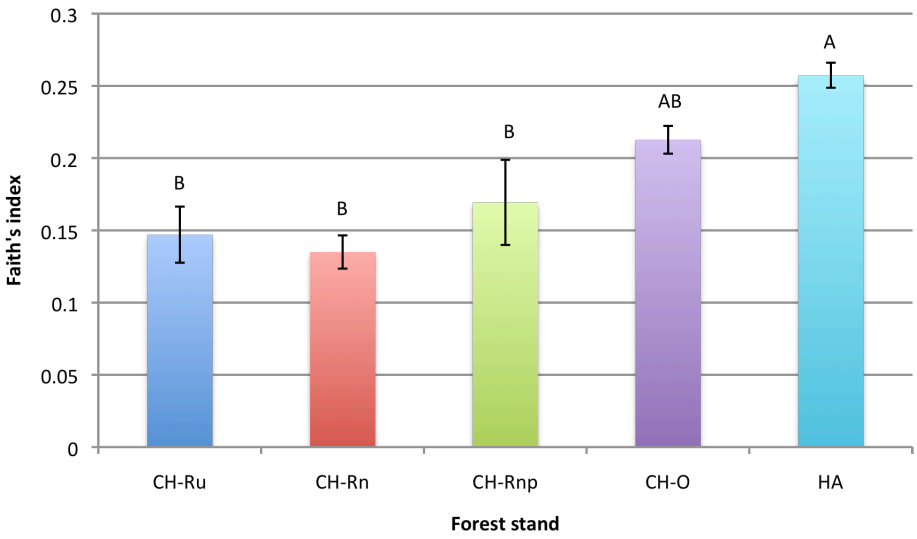


Fig. 2.3 Shannon's diversity index (A), the observed species richness, and the Chao1 and Jack1 estimated richness (B) of the ectomycorrhizal fungal communities from the five forest types using species abundance data were not significantly different across forest type (Appendix A). Colour code is same for (A) and (B). CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus. Error bars represent one standard error of the mean (n = 3).

(A)



(B)

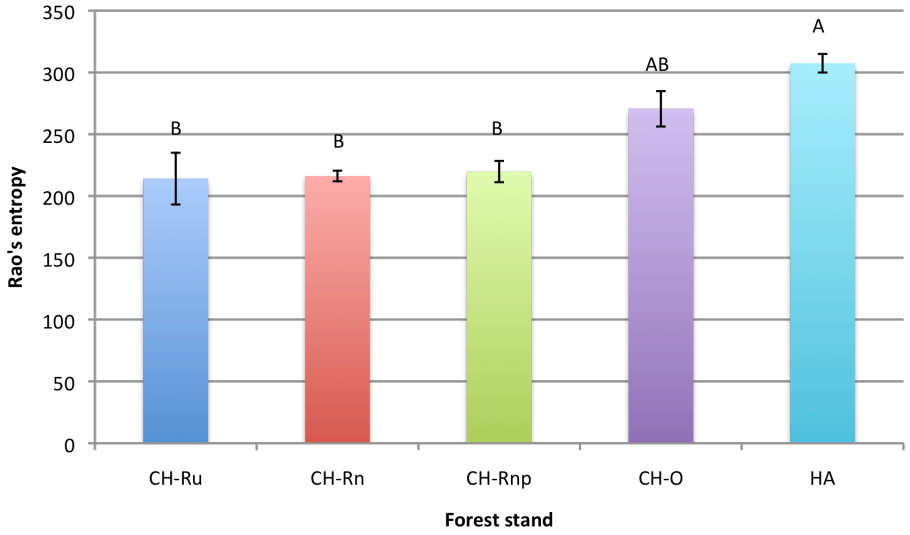


Fig. 2.4 Faith's index (A) and Rao's entropy (B) of phylogenetic diversity of the ectomycorrhizal fungal communities from the five forest types using species abundance data. Bars that share the same letter were not significantly different based on the Tukey-Kramer test (Appendix A). CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus. Error bars represent one standard error of the mean (n = 3).

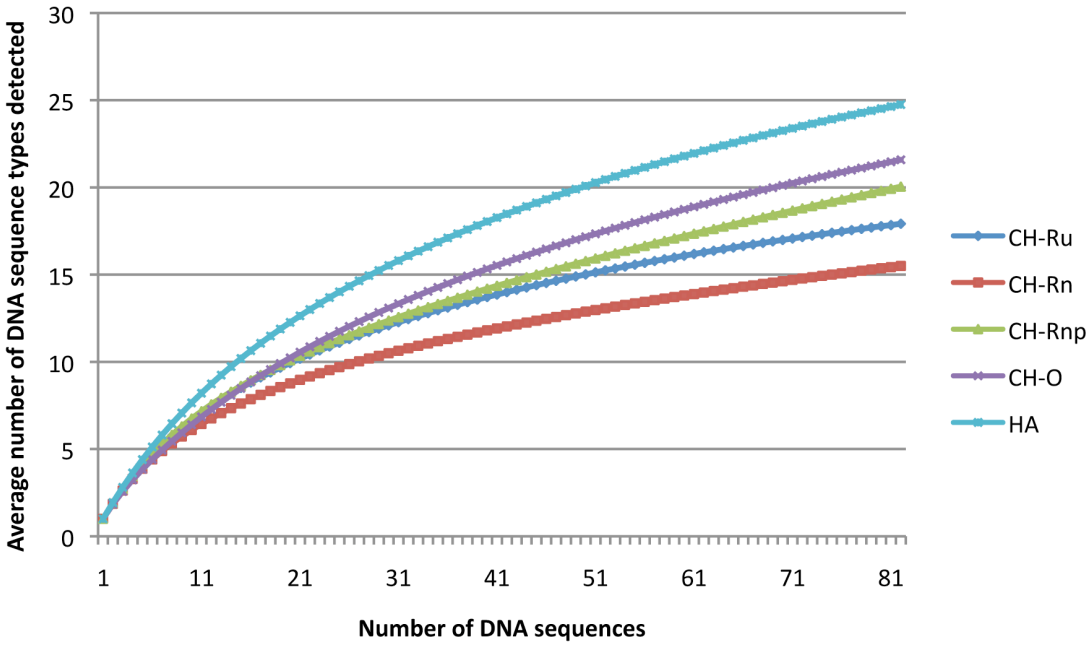


Fig. 2.5 Estimated species accumulation curves of the ectomycorrhizal fungal communities by forest type (n = 3). CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

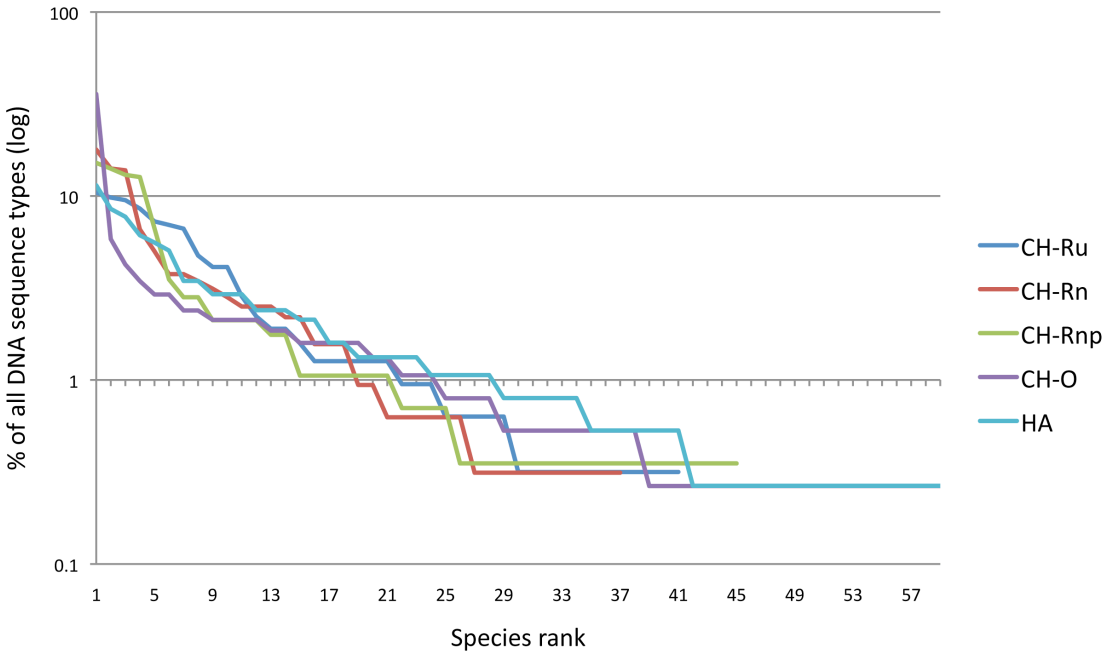


Fig. 2.6 Rank abundance plots of the ectomycorrhizal fungi detected in each of the five forest types. Except for the CH-O plots, they illustrate the typical shape of a well-known species abundance model: log normal. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

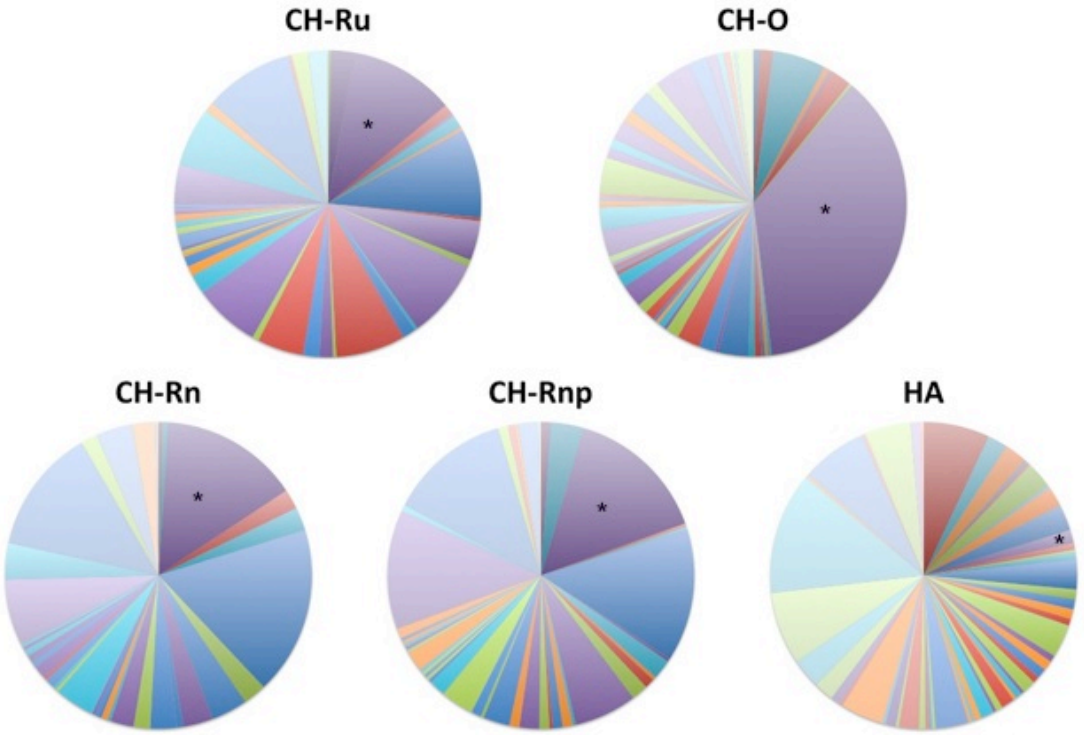


Fig. 2.7 Pie charts showing the relative abundance of the ectomycorrhizal fungal communities by forest type. Colour code is same for all five charts. For example, purple indicates by an asterisk represents *Cantharellus tubaeformis*. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

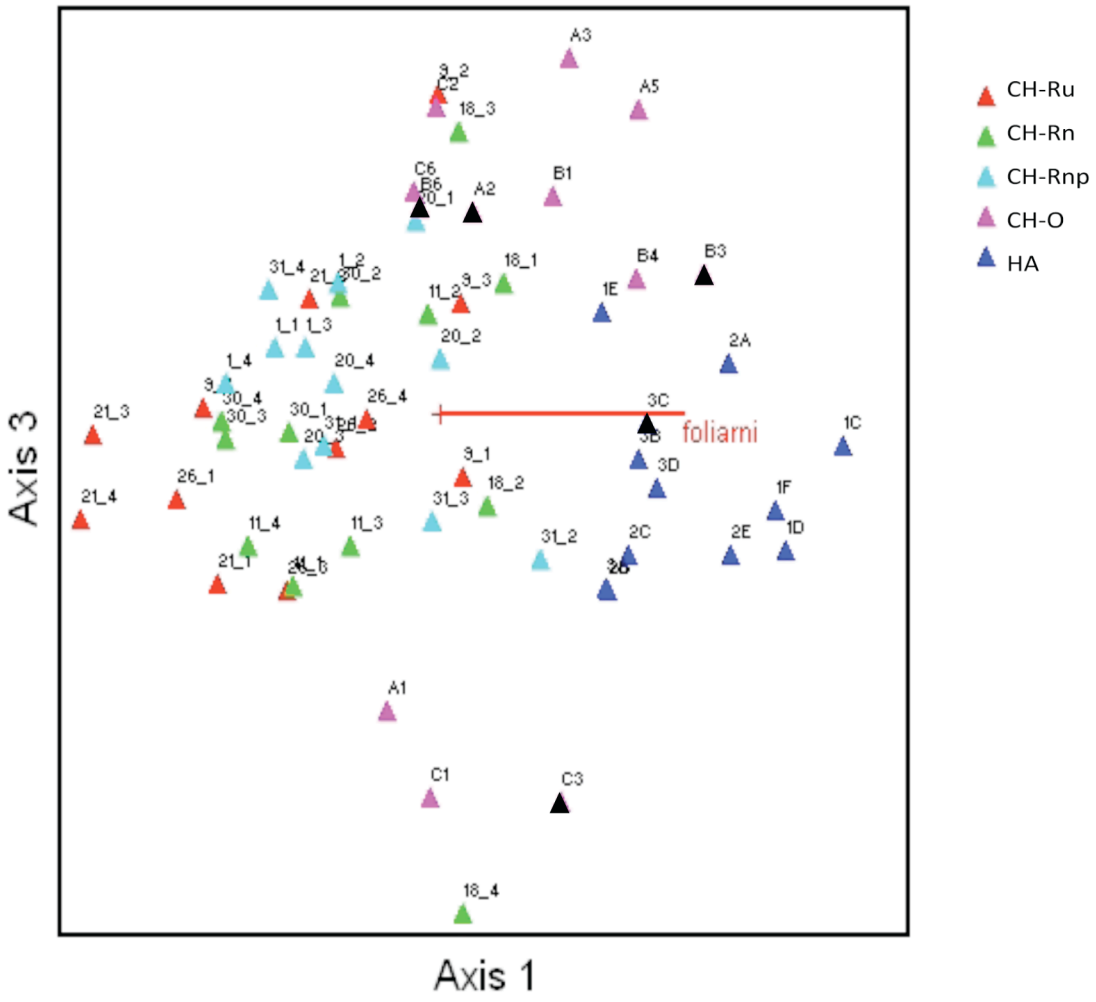


Fig. 2.8 Non-metric multidimensional scaling of the 12 ectomycorrhizal root samples from each forest type based on species presence/absence data. The two axes with the highest R^2 values are shown in Appendix F and were rotated to maximize correlation with foliar nitrogen concentration (foliarni). Pearson and Kendall correlation with Axis 1 and foliar nitrogen after rotation was 0.79 (Table 2.4), and the final stress was 20.2. Five samples that are not of western hemlock (Table 2.3) are indicated with black colour. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

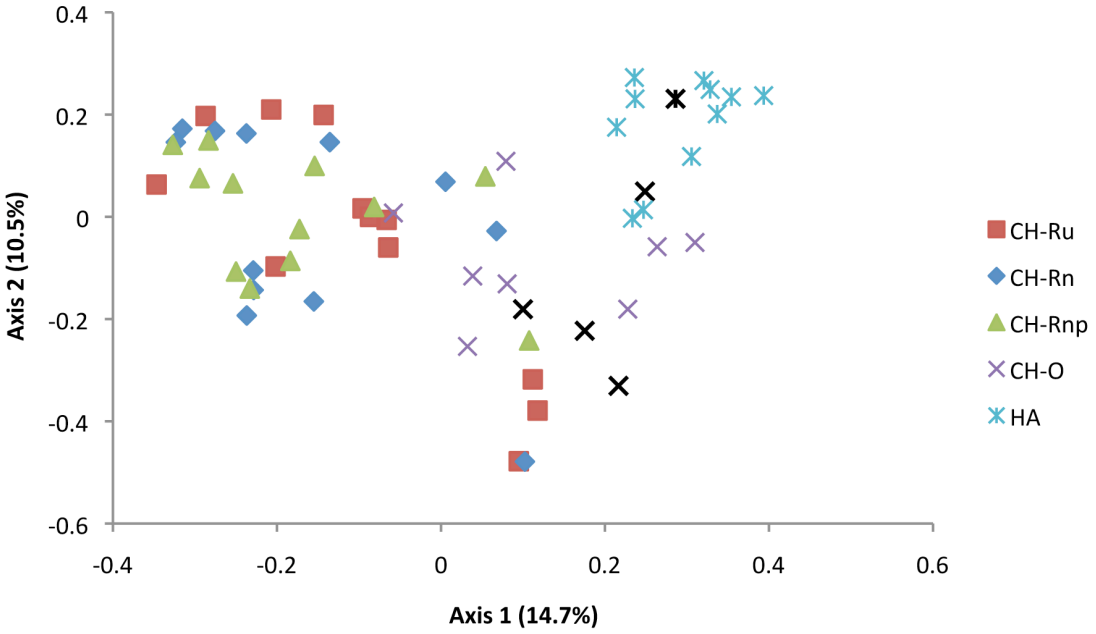
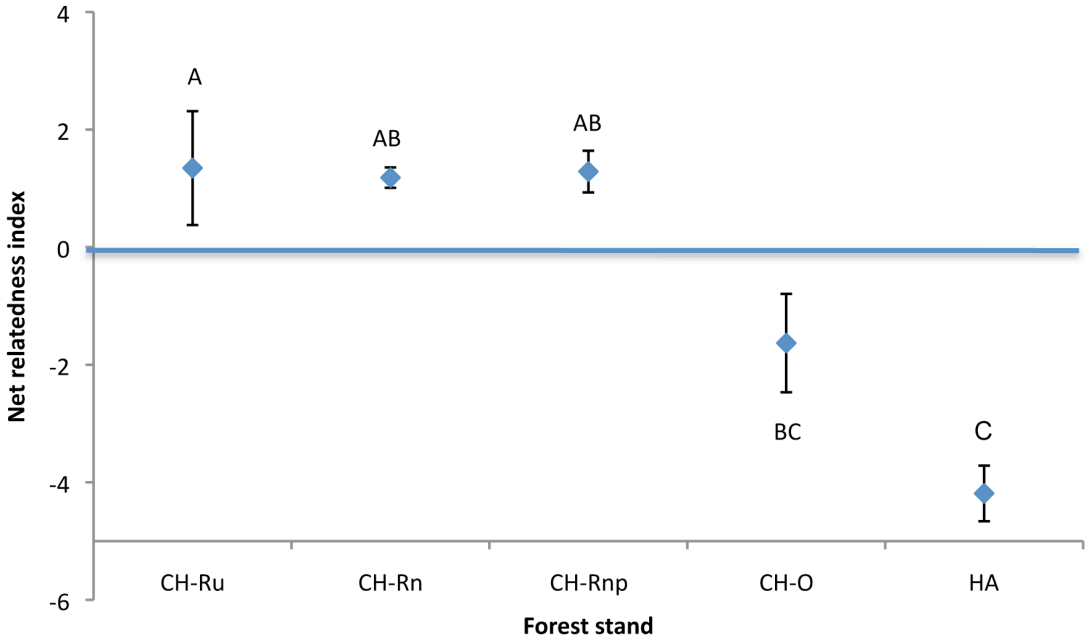
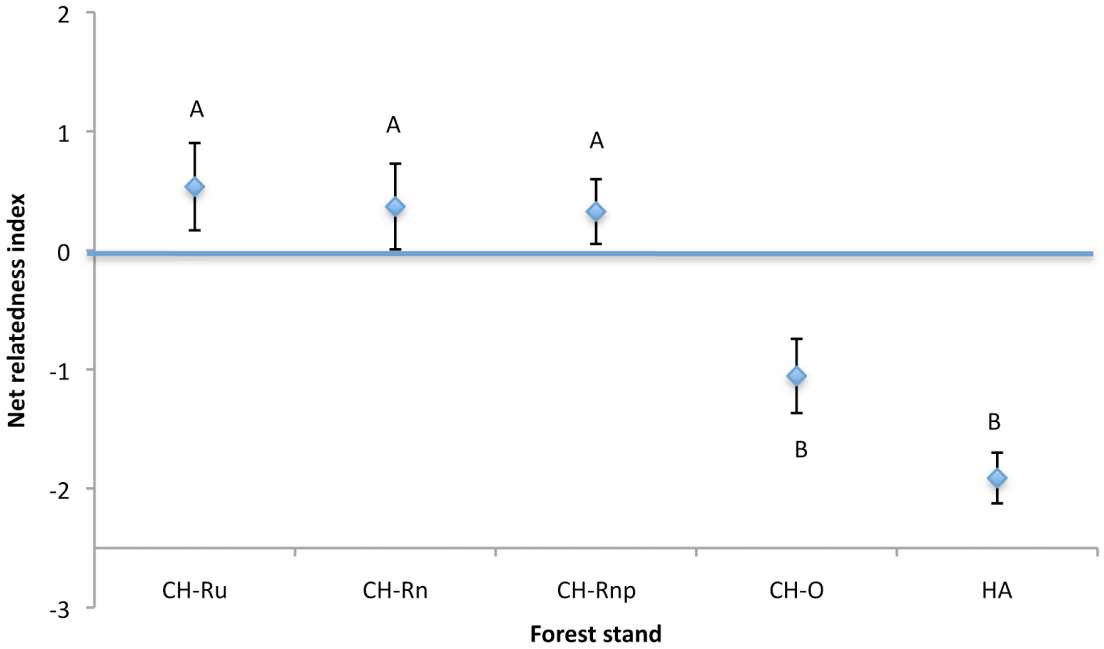


Fig. 2.9 Principal coordinates analysis using phylogenetic distances between pairs of the 12 ectomycorrhizal root samples from each forest type based on species presence/absence data. Five samples that are not of western hemlock (Table 2.3) are indicated with black colour. Percent variation explained by the axes is shown in parentheses. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

(A)


4
2
0
-2
-4
-6
Net relatedness index
A
AB
AB
BC
C
CH-Ru
CH-Rn
CH-Rnp
CH-O
HA
Forest stand

(B)


2
1
0
-1
-2
-3
Net relatedness index
A
A
A
B
B
CH-Ru
CH-Rn
CH-Rnp
CH-O
HA
Forest stand

(C)

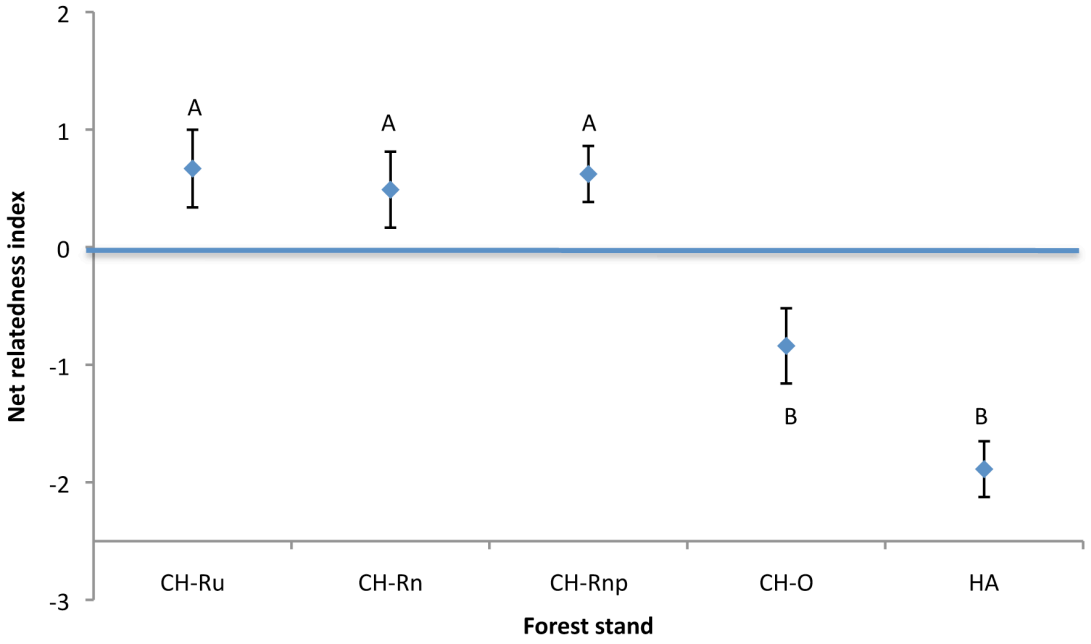
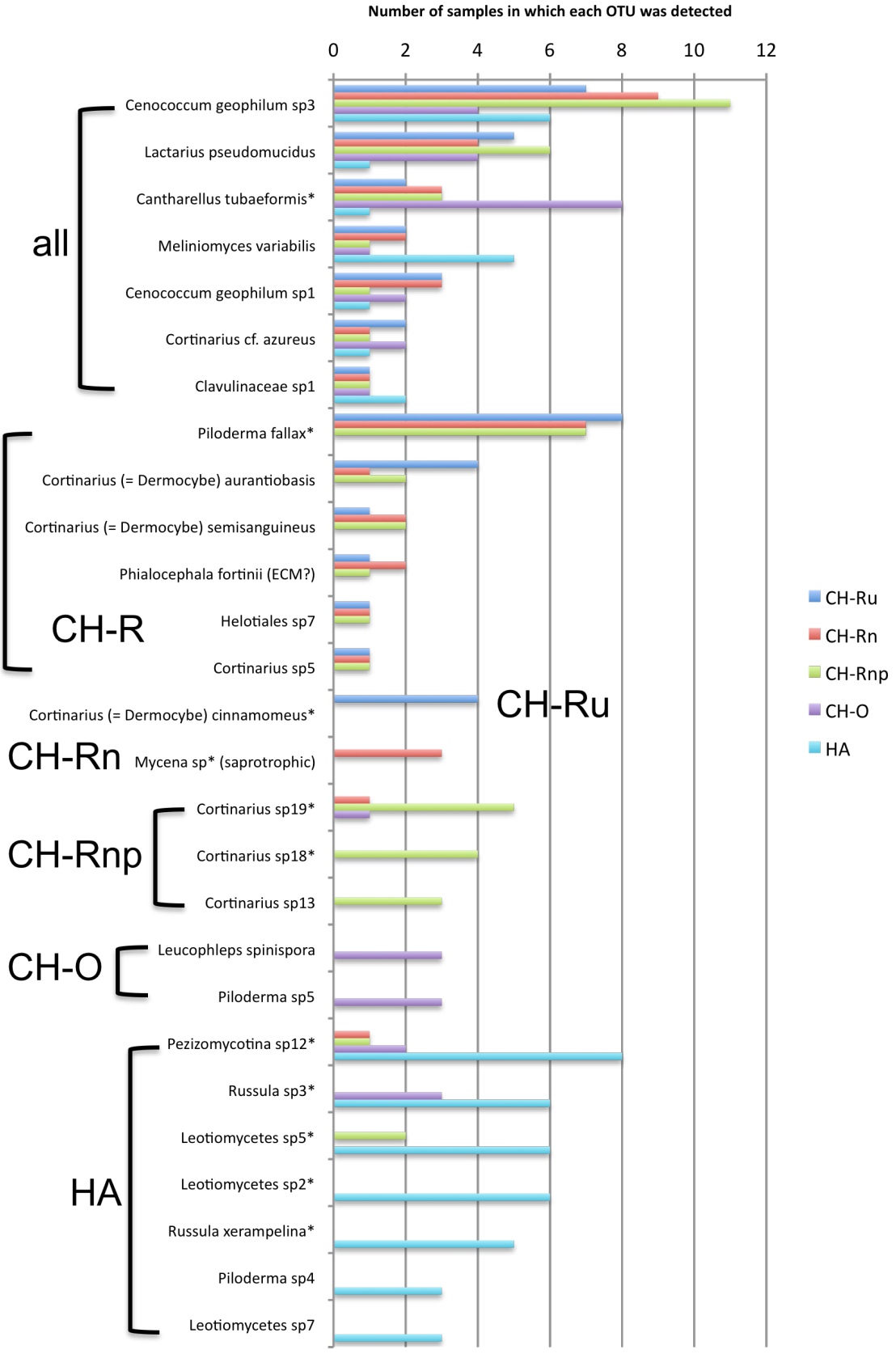


Fig. 2.10 Net relatedness index of the ectomycorrhizal fungal communities from the five forest types using species abundance data. All 12 ectomycorrhizal root samples from each of the three replicate plots (n = 3) were used in (A). Twelve samples from each of the CH-R plots were used (n = 12), and only 8 and 11 samples of western hemlock for the CH-O and HA plots, respectively, were used for analysis (n = 8; n = 11) in (B) and (C). 97% species identity was used for all species including *Cortinarius* and *Lactarius* in (C). Positive values indicate phylogenetically clustered communities; negative values indicate communities of phylogenetic evenness; and zero indicates the communities are random. Points that share the same letter are not significantly different based on the Tukey-Kramer test (Appendix A). The phylogenetic tree used for (C) is illustrated in Fig. 2.2. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus. Error bars represent one standard error of the mean.

Fig. 2.11 (next page) Presence of specific DNA sequence types (OTUs) from the ectomycorrhizal root samples from five forest types. [all] shared species in all types. [CH-R] shared species only in the regenerating hemlock on Cedar-Hemlock sites. Species that are specific to [CH-Ru] unfertilized regenerating hemlock, [CH-Rn] nitrogen-fertilized regenerating hemlock, [CH-Rnp] nitrogen plus phosphorus-fertilized regenerating hemlock, [CH-O] old-growth hemlock on Cedar-Hemlock sites, and mature hemlock [HA] on Hemlock-Amabilis fir sites. An asterisk beside species identification designates indicator species (Appendix G). *Phialocephala fortinii*, found only in the regenerating plots, is not confirmed as ectomycorrhizal (ECM). The only non-ECM indicator species was a *Mycena* species in the regenerating plots fertilized with nitrogen alone.


Number of samples in which each OTU was detected
0
2
4
6
8
10
12
all
Cenococcum geophilum sp3
Lactarius pseudomucidus
Cantharellus tubaeformis*
Meliniomyces variabilis
Cenococcum geophilum sp1
Cortinarius cf. azureus
Clavulinaceae sp1
CH-R
Piloderma fallax*
Cortinarius (= Dermocybe) aurantiobasis
Cortinarius (= Dermocybe) semisanguineus
Phialocephala fortinii (ECM?)
Helotiales sp7
Cortinarius sp5
CH-Ru
Cortinarius (= Dermocybe) cinnamomeus*
CH-Rn
Mycena sp* (saprotrophic)
CH-Rnp
Cortinarius sp19*
Cortinarius sp18*
Cortinarius sp13
CH-O
Leucophleps spinispora
Piloderma sp5
HA
Pezizomycotina sp12*
Russula sp3*
Leotiomycetes sp5*
Leotiomycetes sp2*
Russula xerampelina*
Piloderma sp4
Leotiomycetes sp7
CH-Ru
CH-Rn
CH-Rnp
CH-O
HA

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3 Conclusion

3.1 Summary

To better understand belowground ectomycorrhizal communities and their phylogenetic structure, I compared fungal species from five forest types that differed in tree productivity, stand type and age, and in foliar nutrient concentration. This was a continuation of an earlier research project that compared three fertilization treatments on regenerating hemlock on CH sites (Wright et al., 2009). I showed that although the regenerating hemlock on CH sites fertilized with N plus P were as productive as the second growth, mature hemlock-amabilis fir stands on HA sites, the phylogenetic structure of the fungal communities of these regenerating and the mature stands were not similar. Fungal species composition was most strongly correlated with foliar N concentration and site type, and the regenerating hemlock on CH sites, the old-growth hemlock on CH sites, and the mature hemlock on HA sites each had distinctive fungal communities.

As I will discuss in the next paragraphs, my study had limitations, many of which are common to other recently published studies in the field of ectomycorrhizal community ecology. The strengths of my study included well-supported and interpretable results showing patterns of phylogenetic diversity that correlated with stand type. Finally, I will mention the new and testable hypotheses about change in fungal communities over time that arose from my M.Sc. project.

3.2 Limitations of my study

Limitations of my study were related to:

1. Complex environmental variables in my study sites,
2. Highly diverse fungal species,
3. Abundant rare fungal species,
4. Phylogenetic identification challenges, and
5. Size of fungal genets and possible autocorrelation among samples.

3.2.1 Complex environmental variables in my study sites

My study sites were influenced by a large number of variables including forest stand age, plant species composition, and foliar nutrient concentration. I did show that regenerating hemlock stands on CH sites, old-growth hemlock on CH sites and mature hemlock on HA sites had different fungal communities, and I was able to correlate foliar N concentration of the host trees with ectomycorrhizal fungal communities in spite of the complexity of the differences among sites. As one example of the complexity in the old-growth cedar-hemlock and mature hemlock-amabilis fir stands, roots from different tree species became intermingled in the soil samples. Although we sampled under the drip line of western hemlock trees, molecular analysis of the tree species in root samples showed that western hemlock, amabilis fir and Sitka spruce were tangled together (Table 3 in Chapter 2). In contrast, the two root samples from the regenerating plots were predominately from western hemlock, based on molecular analysis. Not only the age of the trees, but also complexity of the belowground environment was different in the old-growth or mature forests and the regenerating stands.

One of the long-term goals of study of ectomycorrhizal fungi is to test whether different ectomycorrhizal communities affect tree growth differently. My study revealed high phylogenetic diversity and phylogenetic evenness in the fungal communities of both the low productivity old-growth stands on CH sites and the mature hemlock on the highly productive HA sites. However, many other characteristics were different between the CH and HA sites (Chapter One, Section 1.5 and Table 1.1C), any of which could be responsible for the absence of correlation between productivity and the ectomycorrhizal fungal community. To correlate fungal communities with tree productivity, an ideal experimental design for a future study would involve research on sites that were similar to one another with respect to all environmental variables except the ectomycorrhizal fungal community. However, even under conditions where other environmental factors were relatively uniform, diverse ectomycorrhizal communities did not always enhance aboveground tree productivity (Baxter and Dighton, 2001; Jonsson et al., 2001).

Another important limitation for this project was lack of availability of comparable data for some environmental variables across forest types. Although these forest types have been the subjects of numerous studies over the past 20 years (Chapter

One, Table 1.1), measurements are not available for all of the variables that may have been important to ectomycorrhizal fungi. Data for litterfall and composition of litter were available for old-growth cedar-hemlock stands and mature hemlock-amabilis fir stands, but not for regenerating stands (Keenan et al. 1995). Except for the CH sites with regenerating hemlock, the actual values for calculating site index for the older stands could not be obtained. I did not measure tree height and diameter at breast height of the replicate plots of the older stands where the ectomycorrhizal root samples were collected. In addition, many studies showed higher site index in plantations than old-growth stands because of their rapid growth rate, so I might have overestimated site index in the regenerating stands.

My particular plots may be different from the plots where the average site index value was obtained from the forest companies, as there was variation even among my replicate plots of the same forest type (Chapter Two, Section 2.3.2). This was also the case for total stand age and foliar nutrient concentration values. Foliar N concentrations were measured in different studies at different times (Keenan et al., 1995; Blevins et al., 2006) from when the ectomycorrhizal root tips were collected.

3.2.2 High diversity of fungal species leads to insufficient sampling effort

Most commonly, ectomycorrhizal fungal species are so highly diverse that a complete inventory of all species at a site has not been possible (Bruns, 1995; Wright et al., 2009). Achieving adequate samples sizes has always been challenging given limited funding, time and energy. In my study, as in the majority of previous studies, species accumulation curves never reach an asymptote, indicating that number of ectomycorrhizal root tips that could be sampled was insufficient to detect most of the species that were actually present in sampling categories (reviewed in Horton and Bruns, 2001).

Only in a small number of studies of habitats where diversity was exceptionally low, has sampling of the richness of ectomycorrhizal fungal species been anywhere near complete. Sthultz et al. (2009) compared the ectomycorrhizal fungal species associated with moth susceptible and moth resistant pinyon pine in dry soils of volcanic origin, from 1,200 year-old cinder deposits. They found a total of only 21 fungal taxa, and partly

because of the limited fungal diversity, they were able to show that tree genetics influenced the ectomycorrhizal community but herbivory did not. Similarly, Nara et al. (2003) detected only 21 ectomycorrhizal fungal species in their study of an early successional desert site. The cumulative number of ectomycorrhizal fungal species versus the number of sampled root tips leveled off in all their sampling categories. Nara et al. (2003) were not only sampling from a less diverse community than I was, but they also used wider and larger soil cubes (10 × 10 × 10 cm) than the soil auger we used (4.5 cm diameter, 10 cm in depth). Their subsequent subsampling of root tips led to 2,400 root tips for each sampling category, compared with my 1,200 root tips for each forest type. However, although subsampling increases the number of species detected, it can have the less desirable side effect of producing samples showing autocorrelation (Section 3.2.5).

In stable environments, the ectomycorrhizal fungal community in a large spatial scale is composed of a large number of species with functional differences contributing to niche complementarity (Dickie et al., 2002). Abundances of different species are relatively equally distributed. This lack of species dominance along with a large number of rare types of ectomycorrhizal fungal species results in the log-normal patterns in rank-abundance curves seen in ectomycorrhizal studies of various environments (reviewed in Horton and Bruns, 2001). Ectomycorrhizal fungal communities from my study sites, except for the old-growth cedar-hemlock stands, showed similar patterns (Chapter Two, Fig. 2.6). Rather than being dominated by few common DNA sequence types, the DNA clone libraries from the four forest types had many low-frequency DNA sequence types. Out of 147 OTUs, 82 OTUs (56%) occurred only once in a forest type.

Although I found many rare and infrequent species, my species accumulation curves suggested that many more rare species remained to be found (Section 3.2.2). Gibson et al. (1999) called these rare species possible transient "satellite" species, and this is a common feature of ectomycorrhizal fungal communities (Smith et al., 2002; Richard et al., 2004). These rare species may play an intermittent yet important role in responding to ongoing ecosystem processes or possible changes in climate and host species distribution (Kranabetter et al., 2009).

Sampling strategy greatly affects the results of underground ectomycorrhizal

community studies (Taylor, 2002). A simple sampling method using a small soil core without subsampling is usually used for underground ectomycorrhizal fungal community studies (Horton and Bruns, 2001), and the number of root tips usually varies from core to core and between different sampling categories because of root physiological differences (Taylor, 2002). Taylor (2002) suggested that because species richness generally increases with the number of tips studied, this variation in number of tips could cause misunderstanding of the species richness and community structure of ectomycorrhizal fungi. In my study, I avoided this bias by selecting the same number of tips from each core. Four soil cores were extracted around four trees or within four subplots of a replicate plot, and it took me more than two weeks to check for ectomycorrhizal status of the root tips and select 200 for a sample.

When diversity is higher and sampling cannot be exhaustive, statistical approaches allow for comparison among sites. To allow a valid comparison of species richness and diversity (Gotelli and Colwell, 2001), I made sure that the datasets were rarefied to the same number of DNA clone sequences to each other. Also, Monte Carlo randomization tests were performed with every multivariate analysis to obtain the significance of the observed value and evaluate whether the observed pattern was expected by chance (McCune and Grace, 2002).

3.2.3 Rare species generate problems in making statistical comparisons

Even if complete sampling of highly diverse ectomycorrhizal fungal community were possible, the large number of rare species can pose a problem in statistical comparisons. It is difficult to correlate rare species with environmental variables. In my nonmetric multidimensional scaling (NMS) analysis, the large number of species (147, the number of DNA sequence types) compared with the relatively high number of samples (60) resulted in very high stress (20.2) in my analyses (Chapter Two, Fig. 2.8). Stress exceeding 20 is a concern, so we cannot emphasize certain patterns or details from the resulting plots to answer the research questions with the NMS alone (McCune and Grace, 2002). When rare species that occurred only once in the dataset were deleted, the final stress (= 20.9) was quite similar to that in the original dataset. Including only indicator species or the known species that were strongly correlated with the separation

of groups in the original dataset dramatically decreased the number of samples, the DNA sequence types, and the final stress (Wright, 2006).

3.2.4 Phylogenetic and taxonomic identification challenges

Identification of species is important in facilitating communication of results across ecological and physiological studies. Many of the ectomycorrhizal fungi corresponding to DNA sequences detected from a pool of clones cannot be identified as species. Therefore, if we solely examine fungal DNA sequences, we lose information on their ecology and physiology. In order to correct this problem, there is a need to collect ectomycorrhizal fungal sporocarps and obtain their DNA sequences, so that their morphological and ecological observations can be matched with DNA sequences from ectomycorrhizal root tips. Sporocarps at my study sites were not collected due to the logging activities. Instead, the DNA sequences from identified herbarium specimens collected at the Pacific Northwest will contribute to the database of DNA sequences from root tips. I helped determine over 300 sequences from herbarium specimens from sporocarps of the genus *Cortinarius*. These sequences are now publically available in the GenBank database.

Identification can also be based on studies of fungi isolated from mycorrhizae, but this is more difficult than molecular comparisons. Most mycorrhizal fungi are not easily grown or manipulated in natural or lab settings for morphological or functional observations (Horton and Bruns, 2001). An alternative approach to molecular identification is to use morphotyping to identify fungi as species using only their mycorrhizal root tip morphology or their mycelial morphology. For those species that produce no fruiting bodies (e.g., the commonly found *Cenococcum geophilum*), morphotyping and molecular techniques are the only ways for identification. Morphotyping alone, while useful in sorting ectomycorrhizae into related groups, is not always sufficient to identify fungi as species. Indeed, holes in the identification process are very serious problems in studying the biology of ectomycorrhizal fungi.

3.2.5 Are individual genets of ectomycorrhizal fungi large enough to give rise to autocorrelation among samples?

In my study, I assumed that each pooled root sample from a tree or a subplot represented an independent sample from the fungal community. Unless the minimum distance between root samples collected was greater than the size of the individuals being investigated, the same fungal individuals may have been sampled repeatedly, resulting in autocorrelation and lack of independence among samples. Autocorrelation among samples would increase the risk of error in statistical tests. Among the ectomycorrhizal fungi, an individual is a genet consisting of strands of genetically identical cells that together form a thread-like underground mycelium. Table 3.1 provides some estimated genet sizes for ectomycorrhizal fungi. The largest genet found was *Suillus pungens*: 40 m in diameter (Bonello et al., 1998). My older replicate plots were at least 730 m apart from each other (Chapter Two, Fig. 2.1), far enough apart so that autocorrelation related to re-sampling from the same genet was not suspected.

On the other hand, the nine regenerating plots were located within a radius of 180 m. Reassuringly, when the ectomycorrhizal fungal species compositions of the three replicate plots of the same treatment were compared with multiple pair-wise comparisons, some plots were significantly different from others (Appendix E). Clearly, the high heterogeneity among replicate plots of the same treatment was evident in the regenerating cedar-hemlock stands. However, all three different treatments on the regenerating cedar-hemlock stands were similar in their species composition regardless of their fertilization history. Little is known about the genet sizes of the species detected in these plots. I cannot completely exclude the possibility of autocorrelation in the regenerating stands.

Most studies in Table 3.1 determined the genet size in a particular habitat based on the occurrence of sporocarps. Dahlberg and Stenlid (1994) analyzed the size of genets of *Suillus bovinus* from the Scots pine stands differing in forest history and age. With increasing stand age, the size of genets increased and the number of genets per unit area decreased. Genets of *Cortinarius rotundisporus* varied in size in the same environment (Sawyer et al., 1999). The size differences within and between species may be due to the life history traits of the fungi. For example, species forming small genets are thought to

be short-lived, early-successional species dispersed primarily through sexual reproduction (e.g. spores), while large genet sizes are considered to represent late-stage, perennial species spreading primarily through vegetative growth (Dahlberg and Stenlid, 1994; Bergemann and Miller, 2002). Clearly, more studies of the genet size and life strategies of ectomycorrhizal fungi, including the same species under different ecological conditions, are required to avoid autocorrelation.

I was fortunate to have available replicate plots with known histories for my studies. However, further studies increasing the number of replicate plots and ensuring that plots are at least 30 m apart (based on maximum genet size in Table 3.1) may capture more species while reducing some of the possible sampling error from my study sites.

3.3 Strengths of my study and future research

Although interactions among environmental variables undoubtedly occurred in my study sites, the replicate plot structure presented a good opportunity for rigorous comparison of ectomycorrhizal fungal diversity and composition across forest types. My results clearly showed that foliar nitrogen levels correlated more strongly than tree productivity with the belowground species composition of ectomycorrhizal fungi. Patterns of phylogenetic diversity may be related to stand age. In the regenerating stands, fungal species were phylogenetically clustered, with more closely related species than expected by chance. In the older cedar-hemlock or hemlock-amabilis fir stands, fungal structure showed phylogenetic evenness, or less closely related species than expected by chance. This is the first report of a shift in patterns of phylogenetic diversity related to stand age.

Further studies are needed to evaluate whether increase in phylogenetic dispersion in old-growth forests is a general phenomenon, but my work suggests this new and testable hypotheses: As forests age, phylogenetic diversity of ectomycorrhizal fungi increases. Disturbed sites are colonized by *r*-selected fungi that show low phylogenetic diversity and phylogenetic clustering. Old-growth stands may be colonized by *K*-selected fungi (Chapter Two, Section 2.2.1.4) that show high phylogenetic diversity and phylogenetic evenness.

Table 3.1 Previous studies in genet size of ectomycorrhizal fungi

Ectomycorrhizal fungal species	Genet size	Reference
Hebeloma cylindrosporum	a few cm in diameter	Gryta et al., 1997
Laccaria amethystina	a few cm^2	Gherbi et al., 1999
L. bicolor	up to 12.5 m	Baar et al., 1994
Suillus bovinus in younger forests	1.7 m to 5.3 m	Dahlberg and Stenlid, 1994
S. bovinus in older forests	up to 17.5 m	Dahlberg and Stenlid, 1994
S. variegatus	27 m	Dahlberg, 1997
S. pungens	up to 40 m in diameter	Bonello et al., 1998
Rhizopogon vinicolor	up to 2.0 m; or up to 12.1 m	Kretzer et al., 2004; Beiler et al., 2010
R. vesiculosus	13.4 m up to 20.1 m	Kretzer et al., 2004; Beiler et al., 2010
Cortinarius rotundisporus (small)	6 m in diameter	Sawyer et al., 1999
C. rotundisporus (large)	9-30 m in diameter	Sawyer et al., 1999
Pisolithus tinctorius	up to 30 m	Anderson et al., 1998

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Appendices

Appendix A

Summary of mean values and standard errors (SE) for Shannon, Evenness, Simpson, Faith and Rao's diversity indices, observed richness, richness estimates, and net relatedness index.

							Shapiro-Wilk test	Kruskal-Wallis test	Levene's test	Bartlett's test	Welch's ANOVA	ANOVA	
		Forest stand					All normal?	Chi-square	F ratio	F ratio	F ratio	F ratio	Tukey-Kramer
		CH-Ru	CH-Rn	CH-Rnp	CH-O	HA		p-value	p-value	p-value	p-value	p-value	test done?
Diversity													
Shannon *H'* (*n* =3)		2.37	2.17	2.39	2.37	2.80	No	8.25	-	-	-	-	-
	SE	0.03	0.14	0.34	0.31	0.06		**0.08**					
Evenness *J'* (*n* = 3)		0.81	0.77	0.79	0.73	0.84	Yes	-	3.45	1.17	-	4.94	**Yes**
	SE	0.02	0.01	0.01	0.03	0.02			0.05	0.32		0.0185*	
Simpson *D* (*n* = 3)		0.89	0.86	0.89	0.84	0.93	Yes	-	6.84	4.23	70.32	-	-
	SE	0.004	0.004	0.018	0.037	0.001			0.0064*	0.002*	**0.0003**		
Faith *PD* (*n* = 3)		0.15	0.14	0.17	0.21	0.26	No	10.23	1.30	0.93	-	8.25	**Yes**
	SE	0.02	0.01	0.03	0.01	0.01		**0.04***	0.33	0.44		0.0033*	
Rao *Dp* (*n* = 3)		214.05	216.19	219.76	270.50	307.36	Yes	-	1.33	1.11	-	10.98	**Yes**
	SE	20.94	4.30	8.61	14.32	7.52			0.32	0.35		0.0011*	
Richness esimates													
observed (*n* = 3)		15.58	13.51	16.37	18.83	22.00	Yes	-	2.05	1.16	-	3.23	No
	SE	1.92	0.40	2.71	1.95	1.21			0.16	0.33		**0.06**	
Chao1 (*n* = 3)		18.34	15.88	19.84	23.77	27.09	Yes	-	1.35	1.12	-	2.81	No
	SE	2.90	0.52	3.78	2.76	2.20			0.32	0.35		**0.08**	
Jack1 (*n* = 3)		19.29	16.91	21.12	25.04	28.78	Yes	-	1.47	1.01	-	3.01	No
	SE	2.97	0.63	3.88	2.83	2.17			0.28	0.40		**0.07**	
Net related index													
3 replicate plots (*n* = 3)		1.35	1.18	1.29	-1.63	-4.19	Yes	-	1.29	1.24		15.05	**Yes**
	SE	0.97	0.17	0.35	0.84	0.47			0.34	0.29		0.0003*	
only *T. heterophylla*		0.54	0.37	0.33	-1.05	-1.91	Yes		0.79	1.13		11.70	**Yes**
(*n* = 12, 8 & 11 for CH-R, CH-O & HA)	SE	0.37	0.36	0.27	0.31	0.21			0.53	0.34		<.0001*	
only *T. heterophylla* + 97% species identity		0.67	0.49	0.62	-0.84	-1.89	Yes		0.56	0.56		16.08	**Yes**
(*n* = 12, 8 & 11 for CH-R, CH-O & HA)	SE	0.33	0.32	0.24	0.32	0.24			0.69	0.70		<.0001*	
* indicates significance (< 0.05)													

Appendix B

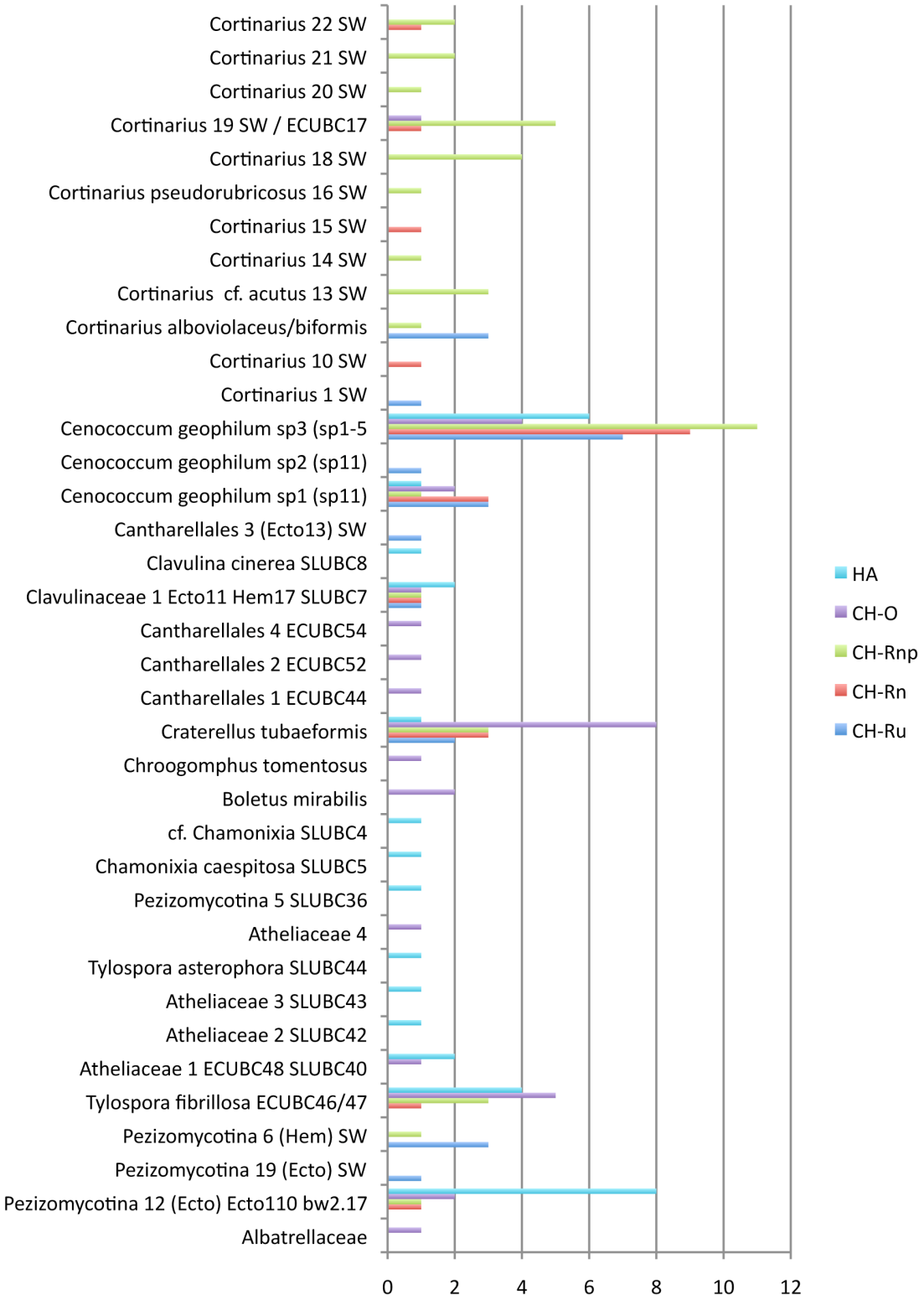
Pearson and Kendall correlations for four variables indirectly correlated with the Non-Multidimensional Scaling (NMS) axes before the rotation. Based on the NMS using presence/absence data of ectomycorrhizal fungal communities of western hemlock. **A number in bold** is a strong correlation (n = 60).

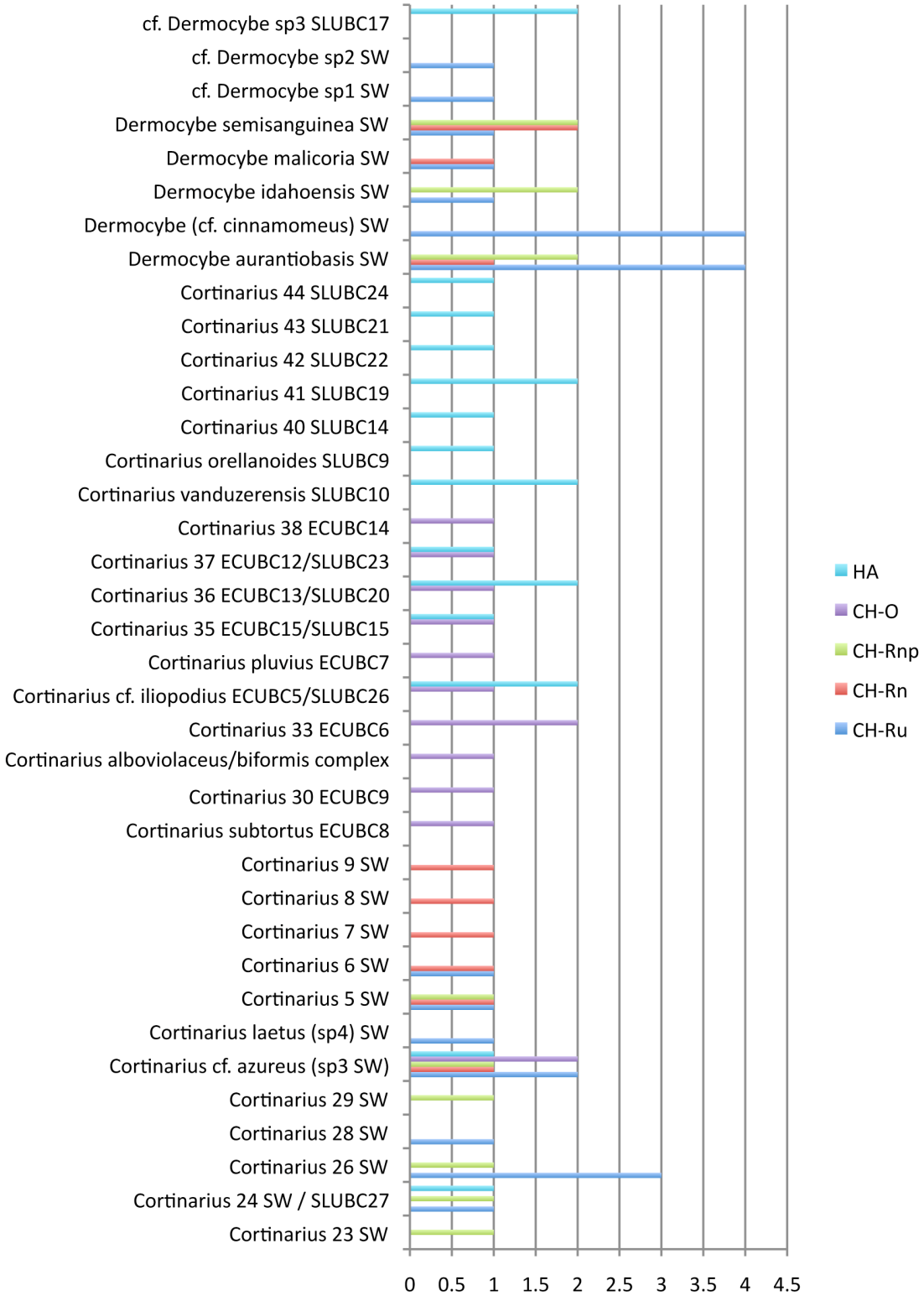
Variable	Axis 1	Axis 2	Axis 3
Foliar nitrogen	**-0.70**	0.15	-0.43
Site index	-0.43	-0.20	-0.14
Stand age	-0.23	0.23	-0.38
Foliar phosphorus	-0.27	-0.16	-0.18

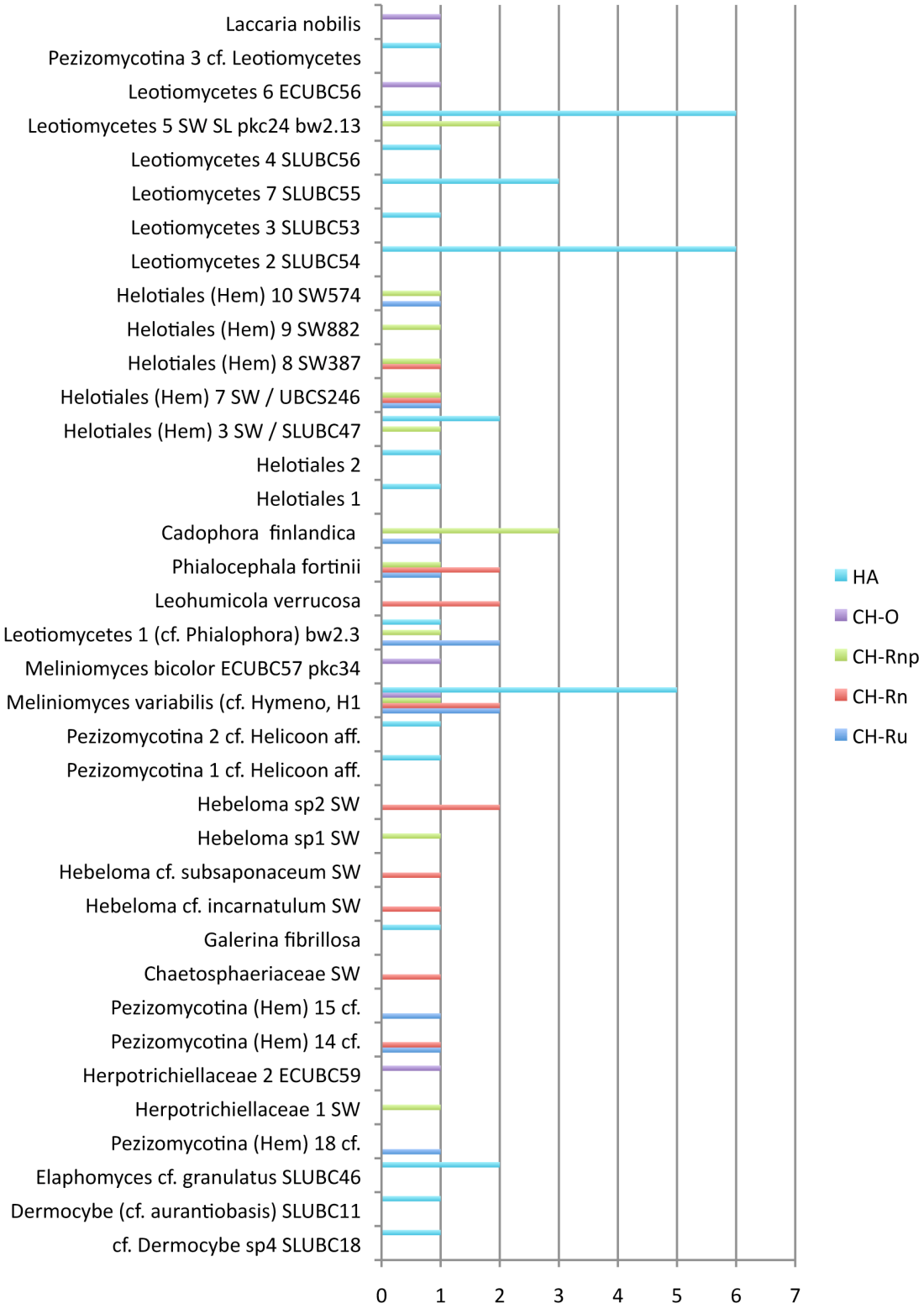
Appendix C

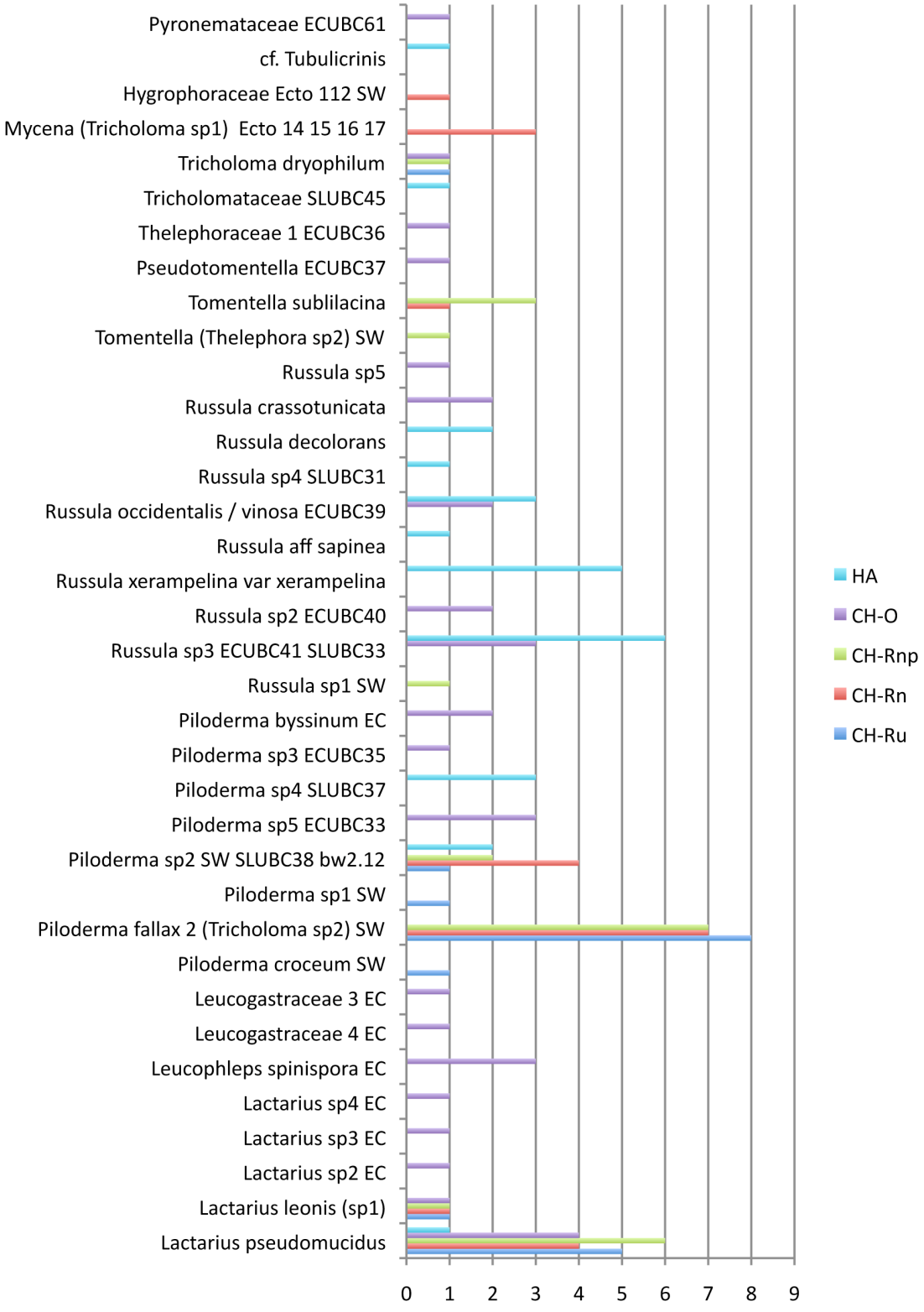
(next four pages)

Presence of 147 DNA sequence types (OTUs) from the 12 ectomycorrhizal root samples from each forest type. The x-axis is the number of root samples in which each OTU was detected. HA, Hemlock-Amabilis fir sites; CH, Cedar-Hemlock sites; O, old-growth hemlock stands; R, 24-year-old regenerating hemlock stands; np, fertilized with nitrogen plus phosphorus, n, fertilized with nitrogen; u, unfertilized.


Cortinarius 22 SW
Cortinarius 21 SW
Cortinarius 20 SW
Cortinarius 19 SW / ECUBC17
Cortinarius 18 SW
Cortinarius pseudorubricosus 16 SW
Cortinarius 15 SW
Cortinarius 14 SW
Cortinarius cf. acutus 13 SW
Cortinarius alboviolaceus/biformis
Cortinarius 10 SW
Cortinarius 1 SW
Cenococcum geophilum sp3 (sp1-5
Cenococcum geophilum sp2 (sp11)
Cenococcum geophilum sp1 (sp11)
Cantharellales 3 (Ecto13) SW
Clavulina cinerea SLUBC8
Clavulinaceae 1 Ecto11 Hem17 SLUBC7
Cantharellales 4 ECUBC54
Cantharellales 2 ECUBC52
Cantharellales 1 ECUBC44
Craterellus tubaeformis
Chroogomphus tomentosus
Boletus mirabilis
cf. Chamonixia SLUBC4
Chamonixia caespitosa SLUBC5
Pezizomycotina 5 SLUBC36
Atheliaceae 4
Tylospora asterophora SLUBC44
Atheliaceae 3 SLUBC43
Atheliaceae 2 SLUBC42
Atheliaceae 1 ECUBC48 SLUBC40
Tylospora fibrillosa ECUBC46/47
Pezizomycotina 6 (Hem) SW
Pezizomycotina 19 (Ecto) SW
Pezizomycotina 12 (Ecto) Ecto110 bw2.17
Albatrellaceae
0
2
4
6
8
10
12
HA
CH-O
CH-Rnp
CH-Rn
CH-Ru


cf. Dermocybe sp3 SLUBC17
cf. Dermocybe sp2 SW
cf. Dermocybe sp1 SW
Dermocybe semisanguinea SW
Dermocybe malicoria SW
Dermocybe idahoensis SW
Dermocybe (cf. cinnamomeus) SW
Dermocybe aurantiobasis SW
Cortinarius 44 SLUBC24
Cortinarius 43 SLUBC21
Cortinarius 42 SLUBC22
Cortinarius 41 SLUBC19
Cortinarius 40 SLUBC14
Cortinarius orellanoides SLUBC9
Cortinarius vanduzerensis SLUBC10
Cortinarius 38 ECUBC14
Cortinarius 37 ECUBC12/SLUBC23
Cortinarius 36 ECUBC13/SLUBC20
Cortinarius 35 ECUBC15/SLUBC15
Cortinarius pluvius ECUBC7
Cortinarius cf. iliopodius ECUBC5/SLUBC26
Cortinarius 33 ECUBC6
Cortinarius alboviolaceus/biformis complex
Cortinarius 30 ECUBC9
Cortinarius subtortus ECUBC8
Cortinarius 9 SW
Cortinarius 8 SW
Cortinarius 7 SW
Cortinarius 6 SW
Cortinarius 5 SW
Cortinarius laetus (sp4) SW
Cortinarius cf. azureus (sp3 SW)
Cortinarius 29 SW
Cortinarius 28 SW
Cortinarius 26 SW
Cortinarius 24 SW / SLUBC27
Cortinarius 23 SW
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
HA
CH-O
CH-Rnp
CH-Rn
CH-Ru


Laccaria nobilis
Pezizomycotina 3 cf. Leotiomycetes
Leotiomycetes 6 ECUBC56
Leotiomycetes 5 SW SL pkc24 bw2.13
Leotiomycetes 4 SLUBC56
Leotiomycetes 7 SLUBC55
Leotiomycetes 3 SLUBC53
Leotiomycetes 2 SLUBC54
Helotiales (Hem) 10 SW574
Helotiales (Hem) 9 SW882
Helotiales (Hem) 8 SW387
Helotiales (Hem) 7 SW / UBCS246
Helotiales (Hem) 3 SW / SLUBC47
Helotiales 2
Helotiales 1
Cadophora finlandica
Phialocephala fortinii
Leohumicola verrucosa
Leotiomycetes 1 (cf. Phialophora) bw2.3
Meliniomyces bicolor ECUBC57 pkc34
Meliniomyces variabilis (cf. Hymeno, H1
Pezizomycotina 2 cf. Helicoon aff.
Pezizomycotina 1 cf. Helicoon aff.
Hebeloma sp2 SW
Hebeloma sp1 SW
Hebeloma cf. subsaponaceum SW
Hebeloma cf. incarnatulum SW
Galerina fibrillosa
Chaetosphaeriaceae SW
Pezizomycotina (Hem) 15 cf.
Pezizomycotina (Hem) 14 cf.
Herpotrichiellaceae 2 ECUBC59
Herpotrichiellaceae 1 SW
Pezizomycotina (Hem) 18 cf.
Elaphomyces cf. granulatus SLUBC46
Dermocybe (cf. aurantiobasis) SLUBC11
cf. Dermocybe sp4 SLUBC18
0
1
2
3
4
5
6
7
HA
CH-O
CH-Rnp
CH-Rn
CH-Ru


Pyronemataceae ECUBC61
cf. Tubulicrinis
Hygrophoraceae Ecto 112 SW
Mycena (Tricholoma sp1) Ecto 14 15 16 17
Tricholoma dryophilum
Tricholomataceae SLUBC45
Thelephoraceae 1 ECUBC36
Pseudotomentella ECUBC37
Tomentella sublilacina
Tomentella (Thelephora sp2) SW
Russula sp5
Russula crassotunicata
Russula decolorans
Russula sp4 SLUBC31
Russula occidentalis / vinosa ECUBC39
Russula aff sapinea
Russula xerampelina var xerampelina
Russula sp2 ECUBC40
Russula sp3 ECUBC41 SLUBC33
Russula sp1 SW
Piloderma byssinum EC
Piloderma sp3 ECUBC35
Piloderma sp4 SLUBC37
Piloderma sp5 ECUBC33
Piloderma sp2 SW SLUBC38 bw2.12
Piloderma sp1 SW
Piloderma fallax 2 (Tricholoma sp2) SW
Piloderma croceum SW
Leucogastraceae 3 EC
Leucogastraceae 4 EC
Leucophleps spinispora EC
Lactarius sp4 EC
Lactarius sp3 EC
Lactarius sp2 EC
Lactarius leonis (sp1)
Lactarius pseudomucidus
0 1 2 3 4 5 6 7 8 9
HA
CH-O
CH-Rnp
CH-Rn
CH-Ru

Appendix D

Multi-response permutation procedures in Sørensen distance (n = 12). Chance-corrected within-group agreement for all forest types was 0.07 (p-value = 0.0).

Forest types compared	Chance-corrected within-group agreement	p-value
CH-Ru vs. **CH-Rn**	0.002	0.3928540
CH-Ru vs. **CH-Rnp**	0.012	0.1096779
CH-Ru vs. CH-O	0.048	0.0001739*
CH-Ru vs. HA	0.084	0.0000007*
CH-Rn vs. **CH-Rnp**	0.002	0.3903212
CH-Rn vs. CH-O	0.042	0.0008419*
CH-Rn vs. HA	0.079	0.0000025*
CH-Rnp vs. CH-O	0.053	0.0000238*
CH-Rnp vs. HA	0.089	0.0000028*
CH-O vs. HA	0.051	0.0000164*

* indicates significance (< 0.05)

Forest types in bold are similar in their ectomycorrhizal fungal community structure. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

Appendix E

Multi-response permutation procedures in Sørensen distance for comparing fungal species composition of three replicate plots in each forest type. Chance-corrected within-group agreement for all plots was 0.11 (*p*-value = 0.0).

Plots compared	Chance-corrected within-group agreement	*p*-value
[CH-Ru]		
21 vs. 26	0.060	0.024*
21 vs. 9	0.066	0.052
26 vs. 9	-0.015	0.679
[CH-Rn]		
11 vs. 18	0.037	0.132
11 vs. 30	-0.001	0.525
18 vs. 30	0.064	0.031*
[CH-Rnp]		
1 vs. 20	0.059	0.049*
1 vs. 31	0.063	0.029*
20 vs. 31	0.024	0.200
[CH-O]		
A vs. B	0.023	0.165
A vs. C	0.016	0.249
B vs. C	0.017	0.235
[HA]		
1 vs. 2	0.011	0.335
1 vs. 3	0.039	0.083
2 vs. 3	0.016	0.261

* indicates significance (< 0.05)

Plots in bold are similar in their ectomycorrhizal fungal community structure. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.

Appendix F

Summary of variance explained of ectomycorrhizal fungal species composition by the Non-metric Multidimensional Scaling axes (Chapter Two, Fig. 2.8). R^2 represents the variation in Sørensen distance among the root samples explained by the axes. **Numbers in bold** are the highest R^2 values.

Axis / R^2	Variance explained	Cumulative
1	**0.22**	0.22
2	0.21	0.43
3	**0.24**	0.67

Appendix G

Observed indicator values (% of perfect indication) for specific DNA sequence types to a particular forest type, based on species presence/absence data of ectomycorrhizal root samples that are of western hemlock (Chapter Two, Table 2.3). Monte Carlo test of significance performed with 4,999 permutations.

DNA sequence type	Indicator value	p-value	Forest type
Cantharellus tubaeformis	34.6	0.0448*	CH-O
Piloderma fallax	70.8	0.0002*	CH-R
***Cortinarius cinnamomeu*s**	50.0	0.0054*	CH-Ru
***Mycena* species**	37.5	0.0306*	CH-Rn
***Cortinarius* species 19**	40.0	0.0144*	CH-Rnp
***Cortinarius* species 18**	50.0	0.0040*	CH-Rnp
Cortinarius species 13	25.0	0.1824	CH-Rnp
Leucophleps spinispora	25.0	0.1848	CH-O
Piloderma species	25.0	0.1766	CH-O
Pezizomycotina species 12	39.1	0.0210*	HA
***Russula* species 3**	33.3	0.0470*	HA
Leotiomycetes species 5	33.3	0.0454*	HA
Leotiomycetes species 2	62.5	0.0004*	HA
Russula xerampelina	37.5	0.0296*	HA
Piloderma species 4	12.5	1.0000	HA
Leotiomycetes species 7	12.5	1.0000	HA

* indicates significance (< 0.05)

DNA sequence types in bold are significant indicator species to a particular forest type. CH, Cedar-Hemlock sites; HA, Hemlock-Amabilis fir sites; R, 24-year-old regenerating hemlock stands; O, old-growth hemlock stands; u, unfertilized; n, fertilized with nitrogen; np, fertilized with nitrogen plus phosphorus.