

T. R. Horton · R. Molina · K. Hood

Douglas-fir ectomycorrhizae in 40- and 400-year-old stands: mycobiont availability to late successional western hemlock

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Abstract We investigated ectomycorrhizal (EM) fungi in forest stands containing both early successional Douglas-fir and late successional western hemlock at two points in the typical stand development by identifying EM fungi from roots of Douglas-fir and western hemlock in mixed stands. In an early seral stage forest, EM roots of western hemlock seedlings and intermingling 40-year-old Douglas-fir were sampled. In a late seral stage forest, EM roots of trees of both species were sampled in a 400-year-old stand. We use molecular approaches to identify the symbionts from field samples in this descriptive study. In the early seral stage study, >95% of the western hemlock root tips by biomass were colonized by fungi also colonizing Douglas-fir roots. This result supports the prediction that western hemlock can associate with fungi in Douglas-fir EM networks. In the same study, fungi specific to Douglas-fir colonized 14% of its EM root tips. In the late seral stage study, 14% of the western hemlock root tips were colonized by fungi also observed in association with Douglas-fir, a result strongly influenced by sampling issues and likely represents a conservative estimate of multiple host fungi in this old growth setting. Fungi specific to Douglas-fir colonized 25% of its root tip biomass in the old growth study, in tight coralloid clusters within five of the 24 soil samples. The trends revealed in this study corroborate

earlier studies suggesting a predominance of multiple host fungi in mixed communities of EM plants. The role of host-specific fungi in these stands remains unclear.

Keywords Fungi · Internal transcribed spacer–restriction fragment length polymorphism · Common mycorrhizal networks · Succession · Specificity

Introduction

Mycorrhizal fungi influence plant community dynamics in a number of ways. While it is tempting to consider species of mycorrhizal fungi as redundant in terms of the benefits they provide plants, the high diversity of mycorrhizal fungi typically observed at a site indicates that the species have unique ecological niches (Bruns 1995; Bever et al. 2002). Evidence from arbuscular mycorrhizal systems suggests that the diversity of plants at a site is influenced by the diversity of mycorrhizal fungi in the soils (van der Heijden et al. 1998). Fungi differ in the kind and level of resource acquisition service they provide host plants (Smith and Read 1997), and some may be less mutualistic than is often considered (Johnson and Smith 1997). Finally, plant competition for soil resources may be influenced by common mycorrhizal networks that distribute soil nutrients among competing hosts (Allen 1984; Newman 1988; Perry et al. 1989b; Simard et al. 1997; Booth 2004). However, even when fungi associate with multiple hosts, the benefit to the different hosts may not be equal (Bever et al. 2002). The first step in sorting out the different roles fungal species play in plant community dynamics is to identify the symbionts from belowground samples. Efforts to sort out which fungi colonize which plants in mixed stands have been hampered because identifying fungal and plant symbionts based on the morphology of mycorrhizal roots is difficult, time-consuming, and rarely yields a species name.

Recent advances using the polymerase chain reaction (PCR) to amplify fungal and plant specific regions of DNA from root tip extractions have allowed researchers to identify symbionts in ectomycorrhizal (EM) networks

T. R. Horton (✉) · K. Hood
Department of Forest Science, Oregon State University,
Corvallis, OR, 97331, USA
e-mail: trhorton@esf.edu
Tel.: +1-315-4706794
Fax: +1-315-4706934

R. Molina
USDA Forest Service, Pacific Northwest Research Station,
Corvallis, OR, 97331, USA

T. R. Horton
Present address: State University of New York,
College of Environmental Science and Forestry,
241 Illick Hall,
Syracuse, NY, 13210-2788, USA

(Horton and Bruns 2001). Using this approach, several studies have now shown that fungi colonizing multiple hosts were the most frequent and abundant types in mixed host stands (Horton and Bruns 1998; Cullings et al. 2000; Kennedy et al. 2003). Other studies have shown that fungi associated with one plant species can provide mycorrhizal inoculum for a second species (Horton et al. 1999; Hagerman et al. 2001; Dickie et al. 2004), suggesting plant community succession may be facilitated by multiple host fungi (Molina and Trappe 1982; Perry et al. 1989a). Consideration of these mutualistic interactions on plant dynamics should be viewed as a modification of the models proposed by Connell and Slatyer (1977) and Pickett et al. (1987).

The *Tsuga heterophylla* zone occupies large areas from California to Alaska and contains Douglas-fir (*Pseudotsuga menziesii* Mirbo. Franco) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) as the seral and climax species, respectively (Franklin 1988). Following disturbance, Douglas-fir often dominates in the establishing forest. Due to its relative shade intolerance, Douglas-fir does not recruit new individuals following canopy closure. Western hemlock can establish immediately after disturbance, but because it is shade tolerant, recruitment of new individuals continues after canopy closure (Schrader 1998). Old growth Douglas-fir forests (>200 years old) are characterized by a proliferation of western hemlock (Franklin et al. 1981; Oliver and Larson 1990; Spiese 1991).

Douglas-fir is estimated to associate with 2,000 species of EM fungi (Trappe and Fogel 1977), of which about 72% are estimated to associate with multiple hosts (Molina et al. 1992). Although a large body of literature is available regarding EM fungi associated with Douglas-fir, relatively little attention has been given to EM fungi associated with western hemlock (Molina et al. 1992). Christy et al. (1982) reported that western hemlock seedlings were capable of surviving in decaying logs and mineral soil for at least a year without EM development. Kranabetter and Wylie (1998) provided evidence that EM fungal species richness on naturally regenerating western hemlock seedlings declined from areas beneath the canopy out into gaps, and that most species were observed at each gap position. A reduction in species richness was also observed by Durall et al. (1999) based on seedlings harvested within varying sized gaps compared to those from the forest edge. Pure culture synthesis experiments and collections of EM sporocarps in pure stands suggest that western hemlock has few host-specific fungi (Kropp and Trappe 1982; Molina and Trappe 1982, 1994; Massicotte et al. 1994; Smith et al. 1995; O'Dell et al. 1999). To our knowledge, no below-ground studies have investigated the belowground community structure in mixed stands of Douglas-fir and western hemlock.

We investigate belowground EM community structure associated with Douglas-fir and western hemlock in an early seral stage stand and in a late seral stage stand. We assess the degree of overlap in EM symbionts of Douglas-fir and western hemlock in the stands. We also revisit hypotheses proposed by Kropp and Trappe (1982) related

to the role of host specificity in plant succession involving Douglas-fir and western hemlock.

Materials and methods

Site descriptions

The early seral stage study was conducted in the Coast Range of Oregon on Mary's Peak, near Corvallis. Average temperatures range from -2 to 28°C . The annual precipitation of about 230 cm falls mostly as rain between October and April. Summers are relatively warm and dry. Soils are derived from sedimentary sandstones and mudstones with basalt intrusions (Franklin and Dyrness 1973). Five western hemlock seedlings growing in a 40- to 50-year-old Douglas-fir forest were found on the west slope of Mary's Peak. The forest represents natural regeneration following logging. The western hemlock seedlings were selected because they were growing in the soil at least 100 m from western hemlock saplings or trees and we used these to assess fungal colonization of hemlock when the only inoculum was associated with Douglas-fir (mycelial networks and spore banks). The forest included an understory dominated by *Gaultheria shallon* Pursh and *Polystichum minutum* (Kaulf.) Presl.

The late seral stage study was conducted at the H. J. Andrews Experimental Forest along the west side of the Cascade Range in Oregon. Average temperatures and rainfall are similar to those at Mary's Peak, but the area is characteristically more moist and cool during the summer season. Soils are inceptisols (Brown and Parsons 1973; Franklin and Dyrness 1973). The stand has never been logged and includes 400-year-old Douglas-fir and various age classes of western hemlock (potentially as old as the Douglas-fir for some individuals). We use the old growth stand to assess the relative dominance of host-specific fungi on Douglas-fir in a well-established forest with a sizeable western hemlock component. The stand includes an understory of *Gaultheria shallon*, and *Rhododendron macrophyllum* D. Don ex G. Don.

EM sampling

In the early seral stage study, five western hemlock seedlings were harvested by cutting out a 15 cm^2 soil block to a depth of approximately 10 cm around each seedling. EM roots of the western hemlock seedlings and the intermingling Douglas-fir roots were processed from each soil block. The samples were collected on 5 July and 7 September 1999, and these data were pooled in the analyses. Our sample size was low because of the lack of available seedlings growing under these conditions (Schrader 1998).

In the late seral stage study, we utilized strip plot 2 in reference stand 15 from Smith et al. (2002). A soil sample was taken at every odd meter down the $2\text{ m} \times 50\text{-m}$ strip plot with each sample taken in the middle of four 1-m^2 quadrats

where sporocarp occurrence had been recorded over the 4 previous years within these quadrats (Smith et al. 2002). A total of twenty-four 2.5-cm-diameter soil cores were driven into the ground to a depth of 30 cm; all samples were taken in November 1997.

Soil samples were stored at 4°C. The soil samples were filtered through a soil sieve with a mesh size of 0.50 mm (no. 35 USA standard testing sieve). Washed roots were collected and stored in 4°C water for up to 1 week until further sorting. Final separation of root material from the soil samples was conducted under a dissecting microscope.

All viable lateral short roots covered by a fungal mantle were classified as EM. Viability of the root tips was based on color and turgidity (Harvey et al. 1976). The presence of a Hartig net was used to confirm mycorrhizal status of root tips that were questionable. Ectomycorrhizae were sorted into morphological types (morphotypes) based on color, size, type of ramification, unique cell types on the mantle (cystidia), and extramatrical hyphae (see Agerer 1994). Morphotypes were determined to be of Douglas-fir or western hemlock based on attachment to hemlock seedlings, branching pattern (pinnate in Douglas-fir, less organized in western hemlock) and coloration of the tissue below the EM mantle (magenta in western hemlock). While these features were not always evident, they were reliable when present. We examined and sorted ectomycorrhizae from each soil sample separately. Ectomycorrhizae of each morphotype from a given soil sample were placed in individual plastic 1.5-ml centrifuge tubes, frozen, and lyophilized. The entire process, from field collection to lyophilization, was performed within 3 weeks of harvest date. After lyophilization, the morphotype samples from each soil sample were weighed separately, and stored at -20°C until molecular typing.

We made no attempt to relate morphotypes between cores until molecular analysis was complete. Indeed, we intentionally split the EM in each core into more categories than was necessary and combined the biomass of types showing intraspecific variation in morphology only after molecular identification was complete. This way we avoided combining different species with similar morphotypes. This was particularly important for morphotypes within a family (i.e., Russulaceae, Thelephoraceae, Cortinariaceae).

Sporocarp sampling

Epigeous sporocarps were collected within 2 m×50-m permanent strip plots of the late seral stage stand over a 4-year period, with their positions mapped within the 1-m² quadrats (Smith et al. 2002). All sporocarps from Smith et al. (2002) were made available to us for comparison to our root tip data. Additional data were obtained from hypogeous species harvested by Smith et al. (2002) in their larger study, and herbarium collections of other species of interest as indicated by root tip morphology or DNA sequence analysis. Sporocarps were also collected from the strip plot and surrounding area during visits to the site in

1997 and from the early seral stage forest. In total, 61 species in 30 genera of EM fungi were included in our sporocarp database and 23 hypogeous or resupinate species in 18 genera were included. Internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) patterns (see below) were generated for at least one sporocarp of each species in the voucher collection. Although a large number of these fungi were collected on the strip plot by Smith et al. (2002) and during the current study, few matched the EM fungi we observed on the root tips.

Molecular techniques

DNA was extracted from one to three root tips per sample as described in Gardes and Bruns (1996). Several tips were extracted together only when part of a single morphologically uniform clump. When available, at least two samples from each morphotype were processed. DNA was also extracted from small pieces of voucher sporocarps and plant leaf material by the same method.

The reagents, protocols, and cycling parameters used in PCRs followed Gardes and Bruns (1996). Identifications of fungal symbionts were based on PCR amplification of the ITS using ITS-1F and ITS-4B as primers (Gardes and Bruns 1993). In cases where this pair did not work, ITS-1F and ITS-4 were used (White et al. 1990). Both primer pairs preferentially amplify specific fragments of fungal DNA from mixtures of plant and fungal DNA. The primer pair ITS-1F and ITS-4B is further specific to basidiomycetes. If a DNA extraction for a sample did not yield a clean PCR product, a second root tip from the sample was processed. This was continued until two clean PCR products were obtained for each morphotype from each core, or until the morphotype sample was used up in processing.

The ITS region was characterized by RFLP analysis, which was used to match ectomycorrhizae to one another and to sporocarps of voucher collections. Identical RFLP matches with digests of three enzymes, *AluI*, *DpnII*, and *HinfI*, determined species-level grouping or identification. This method has proven to be robust for identifying species especially at the local scale where ITS sequences within species tend to be less variable (Kårén et al. 1997; Horton 2002). RFLP data were analyzed using Gene Profiler (Scanalytics). To avoid over-representing diversity, RFLPs were generated with PCR product from ITS-1F and ITS-4 reactions for all types that yielded RFLPs with ITS-1F and ITS-4B, and checked against the ITS-1F and ITS-4 RFLP data.

To check for the accuracy of plant identification at the morphotyping step, molecular identification of the plant host was conducted on two root tips from each morphotype in each soil core. The plant specific primer pair 28KJ and TW14 was used to amplify a portion of the 28S gene in the nuclear rRNA gene repeat from the mycorrhizal root and plant leaf extracts (Cullings 1992; Horton and Bruns 1998; Horton et al. 1999). The two plants can be unambiguously differentiated with RFLP patterns when this region is di-

gested with the restriction enzyme *DpnII* (data not shown). In all cases, the original plant identification was confirmed.

We sequenced a portion of the mitochondrial large subunit rRNA gene (M15/M16 region) or the entire nuclear ITS region of major RFLP types (fungi) that were not matched to a sporocarp. The M15/M16 region was used to identify the family group of basidiomycetes following Bruns et al. (1998). A sequence database of rDNA ITS sequences was used for the genus *Suillus* (Kretzer et al. 1996). The identity of many RFLP types was clarified by subjecting the unknown ITS sequence to a BLAST search in Genbank (<http://www.ncbi.nlm.nih.gov/BLAST/>). Only BLAST search results of $\geq 90\%$ similarity over at least 90% of the ITS sequence were accepted. The primer pairs ML-5 and ML-6, ITS-1f and ITS-4b, or ITS-1f and ITS-4 were used as required for sequencing (in pairs to amplify the target and singly for the sequence reaction). The Central Services Laboratory at Oregon State University determined sequences with an ABI model 377 DNA sequencer (Perkin-Elmer). DNA sequencing Analysis (version 2.01) and Sequence Navigator software were used to process the raw data. Sequences were aligned by visual estimation using a matrix created in PAUP* (Swofford 2002). Identification was based on phylogenetic analysis with PAUP* using the neighbor joining option. The Genbank accession numbers are provided in Table 1.

ITS sequences from the *Byssocorticium* morphotype did not show a clear similarity to any sequences in Genbank, likely a result of insufficient ITS data for related taxa in

the database. However, we identify two RFLP types as *Byssocorticium*-morph 1 and 2 based on their morphology (relatively sparse web of blue hyphae) and their similar ITS sequences. Two *Cenococcum geophilum* RFLP types were also recovered and a BLAST search with their ITS sequences clearly indicated their similarity and identity as variants of *C. geophilum*. *C. geophilum* strains have been shown to contain an intron downstream from the ITS-1f priming site in the 18S rDNA, which can lead to variation in ITS-RFLP patterns (Rogers et al. 1993; Horton and Bruns 1998).

Treatment of data

Biomass data from morphotypes that yielded identical RFLP types were combined for each soil sample, and for the study as a whole depending on the analysis. In three cases (morphotypes from a soil sample), molecular analysis revealed the sample contained multiple RFLP types, and all root tips from these samples were then processed. The biomass in these samples was split according to the frequency of extracts yielding each RFLP type. For example, if six extracts yielded three RFLPs of type A and three RFLPs of type B, then the biomass data for that sample only were split 50:50 between the two genotypes.

We define community structure as the frequency and abundance of EM fungi on roots. Although eight of the soil cores did not contain roots from both hosts, all anal-

Table 1 GenBank accession numbers for sequenced ectomycorrhizal (EM) samples. ITS Internal transcribed spacer of the nuclear rDNA, M15/M16 portion of the mitochondrial rDNA small subunit (Bruns et al. 1998)

Name used in Figures	Morphotype sample no.	Sequence region	GenBank no.
Agaricoid 1	HJA2040	ITS	AY534204
Agaricoid 2	MP3035	ITS	AY534209
Albatrelloid 1	HJA2058	M15/M16	AY534216
Boleteloid 1	MP3019	ITS	AY534211
<i>Byssocorticium</i> -morph 1	HJA2042	ITS	AY534212
<i>Byssocorticium</i> -morph 2	HJA 2011	ITS	AY534203
Cantharelloid 1	HJA2170	M15/M16	AY534215
<i>Cenococcum geophilum</i> 1	HJA2075	ITS	AY534205
<i>Cenococcum geophilum</i> 2	HJA2130	ITS	AY534197
Clavulinoid 1	MP3052	ITS	AY534200
Cortinarioid 1	HJA2206	ITS	AY534207
<i>Lactarius pseudomucidus</i>	JS4948	ITS	AY534201
<i>Piloderma fallax</i>	HJA2138	ITS	AY534198
<i>Russula fragilis</i>	MP3045	ITS	AY534198
<i>Russula heterophylla</i> complex	HJA2001	ITS	AY534202
<i>Russula occidentalis</i>	HJA2163	ITS	AY534206
<i>Russula xerampelina</i>	MP3049	ITS	AY534210
Russuloid 1	HJA2030	M15/M16	AY534219
Russuloid 2	HJA2225	M15/M16	AY534221
Russuloid 3	HJA2258	M15/M16	AY534214
Russuloid 4	MP3018	ITS	AY534195
Sebacinoid 1	MP3010	ITS	AY534208
<i>Suillus punctatipes</i>	HJA2234	ITS	AY534213
Thelephoroid 1	HJA2099	ITS	AY534196
Thelephoroid 2	MP3028	M15/M16	AY534217
Tricholomatoid 1	HJA2013	M15/M16	AY534218

yses were conducted with all data from all soil cores included. After molecular grouping and identification, the absolute frequency (no. of soil samples in which a type occurred divided by the total no. of soil samples) and relative abundance (summed biomass of a type for all cores divided by the total biomass of all types in all cores) for

each EM type was quantified. In the old growth study, the relative frequency and relative abundance for each type were then summed for an importance value (Horton and Bruns 2001), where the relative frequency equals the absolute frequency of individual species divided by the sum of absolute frequencies for all species. Data from all EM

Table 2 Molecular identification of EM types. Only restriction fragment length polymorphism (RFLP) types >0.01 g dry weight are shown for the late seral stage study. Numbers represent an estimation of the number of base pairs for each fragment based on a single gel. Numbers in parentheses represent fragments that were

less intense than the smaller fragments in the lane and were repeatable and diagnostic here (see Gardes and Bruns 1996). -morph Identification based on root tip morphology; for other abbreviations, see Table 1

Representative root tip identification	Seral stage	Fungus/RFLP type ^a	HinfI	AluI	DpnII
HJA2040	Late	Agaricoid 1 ^b	350/260/110	405/110	390/250/205
MP3035	Early	Agaricoid 2	511/220	642	339/311/114
HJA2058	Late	Albatrelloid 1 ^b	343	448/164	230/150
MP3019	Early	Boletoid 1	351/278/130	503/161/142	360/239
HJA2042	Early/late	Byssocorticium-morph 1 ^b	(169)/151	519	385/203
HJA2011	Late	Byssocorticium-morph 2 ^b	(167)/150	519	205
HJA2170	Late	Cantharelloid 1	423/223/133/113	482/361/149/91	435/393/257
HJA2075	Early/late	Cenococcum geophilum 1 ^b	155/117/108/94	361/178	293/136
HJA2130	Late	Cenococcum geophilum 2 ^b	563/152/108/93	524/365/152	423/296/137/115
MP3052	Early	Clavulinoid 1	281/240/225	420/97	301/188/155/112
HJA2206	Late	Cortinarioid 1	405/342/115	298/226/136	536/270
HJA2210	Late	Cortinarius sp. trh404 ^{c,d}	397/239/119	495/143	538/265
HJA2179	Late	Hydnum umbilicatum trh411 ^{b,d}	319/271	297/160/102	393/193
HJA2245	Late	Lactarius pseudomucidus js4948 ^d	388/121	521/270	331/257/140/106
HJA2138	Early/late	Piloderma fallax	337/149	360/212/93	216/154/126/105
HJA2132	Late	Ramaria celerivirens ^d	316/155/144	728	301/193/100
HJA2263	Late	RFLP 1f/4-12 ^b	332/235	378/151	202/158/141/106
HJA2128	Late	RFLP 1f/4-15 ^b	345/149	537	375/209
MP3037	Early	RFLP 1f/4-3.1 ^b	373/341	520/228	465/220
MP3032	Early	RFLP 1f/4-3023 ^b	371/196/148	531/172/122	375/255
MP3062	Early	RFLP 1f/4-5 ^b	331/222/122	396/203/111	339/235/111
HJA2144	Late	RFLP 1f/4b-18	168/152/113	593/573	526/207
HJA2057	Late	RFLP 1f/4b-36	341/167/111	621	538/218
MP3051	Early	RFLP 1f/4b-9	344/188/100	420/95	370/220/200
MP3003	Early	Rhizopogon parksii trh125 ^b	224/118/107	607	235/218/152
HJA2161	Late	Rhizopogon vesiculosus mci-98-bo ^e	221/127/122	734	264/247
MP3045	Early	Russula fragilis trh614 ^f	577/214	502/262	385/334/112
HJA2001	Late	Russula heterophylla complex ^f	359/269/113	504/163	298/254/199
HJA2091	Late	Russula nigricans osc61041 ^d	354/108	483/460/113	300/184/160
HJA2163	Late	Russula occidentalis trh386 ^{d,f}	402/336/112	372/283/116	328/210/158/105
HJA2230	Late	Russula sp. trh401 ^d	328/111	486/289	328/258/170
MP3049	Early	Russula xerampelina trh742 ^f	394/322/110	492/270	309/257/190
HJA2030	Late	Russuloid 1	434/352/111	544/504/291	309/222/166/107
HJA2225	Late	Russuloid 2	163/150/120	597	538/202
HJA2258	Late	Russuloid 3 ^b	330/116	562	230/204/152
MP3018	Early	Russuloid 4 (Martellia sp.) ^f	387/358	513/155	266/200/129/99
MP3067	Early	Russuloid-morph ^b	320/201/180	556/141	395/149/98
MP3010	Early	Sebacinoid ^b	328	647	236/188/156
HJA2234	Late	Suillus punctatipes osc64059	221/140/118	660/102	276/227
HJA2099	Late	Thelephoroid 1	353/115	489/108	543/226
MP3028	Early	Thelephoroid 2	370/218	480/129	348/112
MP3056	Early	Thelephoroid-morph (dark brown)	350/173/116	435/143	223/192/145
HJA2114	Late	Tricholoma portentosum trh400	400/352/111	400/140	542/265
HJA2013	Late	Tricholomatoid 1	390/367/127/117	496/323/157	386/261/176/120
HJA2111	Late	Truncocollumella citrina tdb2001	239/197/113	795	283/265/219

^aNames ending in -oid identified with M15/M16 database (Bruns et al. 1998) or on <90% identity to related sequences in Genbank in a BLAST search with the ITS sequence (see Table 2); species names with a collection number indicate a three enzyme RFLP match with a sporocarp voucher; species names without a collection number indicate ≥98 identity to related sequences in Genbank in a BLAST search with the ITS sequence

^bPolymerase chain reaction (PCR) amplification with primers ITS-1F and ITS-4. All others ITS amplifications conducted with ITS-1F and ITS-4B

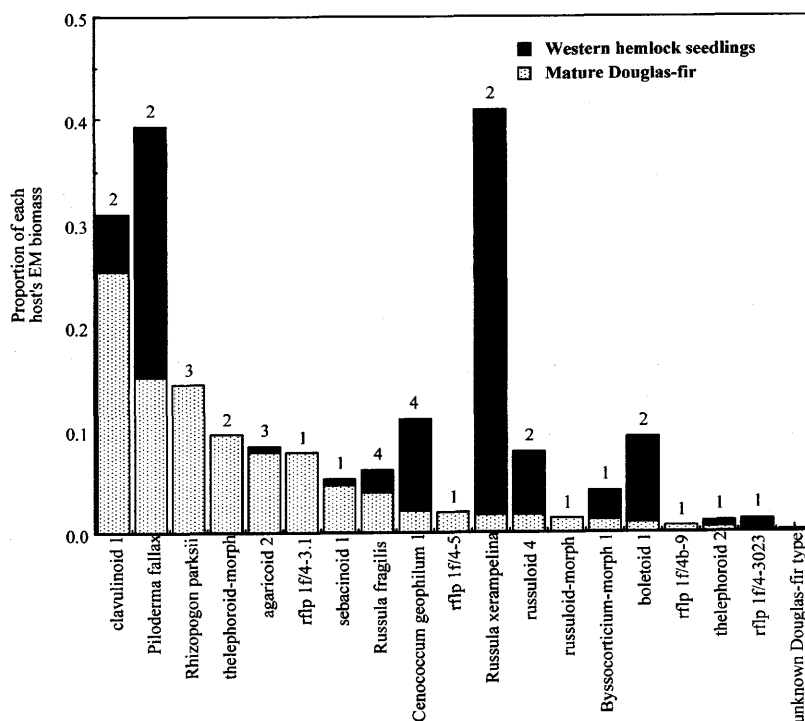
^cVoucher sporocarps collector/location: Smith/OSU (js), Annette Kretzer tuberculate EM sample (mci-98-bo), Oregon State University Herbarium (OSC), Tom Bruns/UC Berkeley (tdb), Horton/SUNY-ESF (trh)

^dVoucher specimen collected from the strip plot from which EM samples were taken

^eThis species has often been identified as *R. vinicolor* (see Kretzer et al. 2003)

^fSteve Miller personal communication, based on ITS sequence

Fig. 1 Host-specificity analysis observed in the early seral stage study. Data are the proportion of ectomycorrhizal (EM) root tip biomass for each tree species. Numbers *above the bars* represent the number of samples in which the type was observed ($n=5$)



root tips found in the young seral stage study are included in Table 2 and Fig. 1. In the old growth study, 30 EM types with ≥ 0.01 g total dry weight (DW) yielded clear ITS-RFLP patterns; data for RFLP types with < 0.01 g total DW are not included in Table 2 or Fig. 2, but are included in Figs. 3 and 4.

Results

Early seral stage study

A total of 0.664 g and 0.181 g DW of EM root tips were harvested from mature Douglas-fir root systems and west-

Fig. 2 EM fungal community structure observed in the late seral stage study. *Importance values* represent a combination of the relative frequency and relative abundance (see Materials and methods). Types with a total EM biomass of < 0.01 g dry weight (DW) and those that were not adequately characterized with morphological or molecular approaches, are not shown

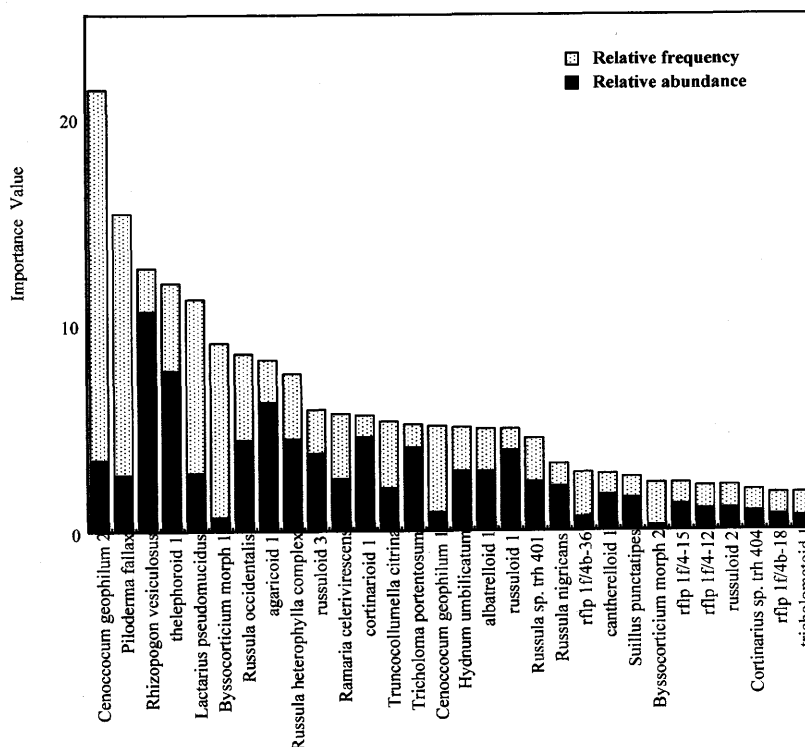
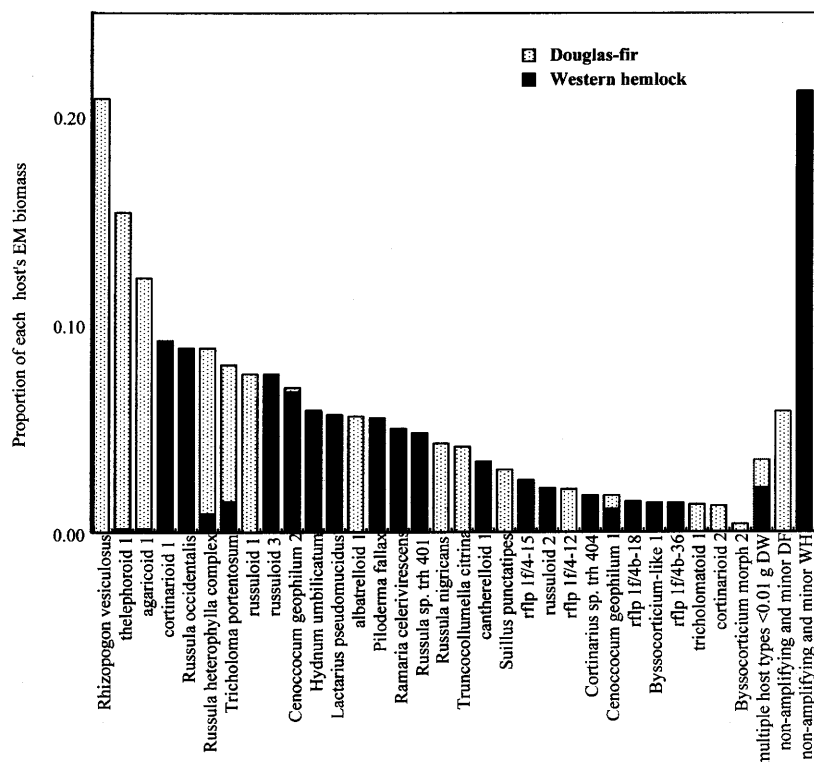


Fig. 3 Host-specificity analysis observed in the late seral stage study. Data are the proportion of EM root tip biomass for each tree species. Although not evident in the figure, *Byssocortici*-morph 1 was observed on both hosts. Data for types with a biomass <0.01 g DW and those that were not adequately characterized with morphological or molecular techniques are included in this figure. *DF* Douglas-fir, *WH* western hemlock



ern hemlock seedlings, respectively. Nineteen types were identified (Fig. 1), with 11 types observed on both hosts and one type on western hemlock alone (Fig. 1, Table 2). The only known host-specific fungus observed, *Rhizopogon parksii*, colonized 21% of the Douglas-fir root tip biomass. Fungi associated with Douglas-fir colonized >95% of the western hemlock root tip biomass.

Late seral stage study

A total of 0.828 g and 0.802 g DW Douglas-fir and western hemlock EM tips, respectively, were harvested in the old growth study. The root tip biomass of 30 RFLP types totaled >0.01 g DW (see Table 2; Figs. 2, 3). Ten of the 30 main RFLP types occurred in three or more soil samples including *C. geophilum* 2 (17 cores), *P. fallax* (12 cores), *Lactarius pseudomucidus* (eight cores), *Byssocortici*-morph 1 (eight cores), *Russula occidentalis* (four cores), theophoroid 1 (four cores), *C. geophilum* 1 (four cores), *Russula heterophylla* complex (three cores), *Ramaria celervirescens* (three cores), and *Truncocollumella citrina* (three cores). The combined biomass of the following 12 EM types made up 61% of the total biomass in the old growth study, given in decreasing order: *Rhizopogon vesiculosus*, theophoroid 1, agaricoid 1, cortinarioid 1, *Russula occidentalis*, *Russula heterophylla* complex, *Tricholoma portentosum*, russuloid 1, theophoroid 2, *C. geophilum* 2, *Hydnum umbilicatum*, *L. pseudomucidus* and albatrelloid 1. Of the 27 species of fungi found fruiting on the strip plot from which the soils samples were taken,

eight were observed on the EM root tips (26% of the main RFLP types).

Twelve types (29% of the total EM biomass) occurred with both tree hosts: theophoroid 1, agaricoid 1, *R. heterophylla* complex, *T. portentosum*, *C. geophilum* 2, *C. geophilum* 1, *Byssocortici*-morph 1 (Fig. 3; data from the following five types occurring at <0.01 g DW are represented in the "multiple host" category: RFLP 2260, RFLP 2286, RFLP 38, RFLP 16, *Tomentella ramosissima*

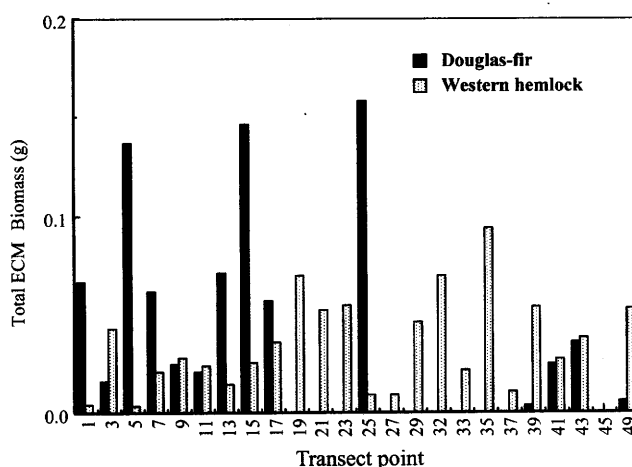


Fig. 4 Total EM root tip biomass at each sampling point in the late seral stage study. Douglas-fir was largely absent from transect point 19–37. Two large *Rhizopogon vesiculosus* tubercles are responsible for the Douglas-fir EM root tip biomass at transect point 25

trh 331). Data for RFLP types that were unique to one host and that either occurred at <0.01 g or could not be unambiguously separated based on morphological or molecular data are included at the far right of Fig. 3; these represented 6% and 21% of the Douglas-fir and hemlock total EM biomass, respectively. Overall, 71% of the EM biomass was colonized by fungi observed with only one host, with eight major types observed on Douglas-fir only and 15 major types observed on western hemlock only. Fungi observed on Douglas-fir colonized 14% of the root tip biomass of western hemlock. Fifty-three percent of the root tip biomass of Douglas-fir was colonized by fungi not observed on western hemlock. Twenty-five percent of the Douglas-fir root tip biomass occurred in five of the 24 soil cores as tight coralloid clusters.

Douglas-fir root tips were nearly absent from transect points 19–37 (Fig. 4). Eight of these soil cores contained only western hemlock roots; one soil core contained only a single Douglas-fir root tip and the soil core taken at transect point 25 contained a massive amount of Douglas-fir EM roots in two tubercles of *R. vesiculosus*. Plant symbionts were confirmed with molecular typing for most root tips from these soil samples to avoid missing Douglas-fir EM roots. Forty-three percent of the EM biomass in the study occurred in this region and five of the 14 EM types that appeared specific to western hemlock occurred only in this western hemlock-dominated portion of the strip plot. Analyzing just the soil cores that had both hosts present, 50% of the Douglas-fir and 40% of the western hemlock root tip biomass was colonized by fungi observed on both hosts.

Discussion

The structure of the EM community

Our results suggest that the belowground structure of these forests is typical for communities of EM fungi found in both conifer and angiosperm stands in that a few species are frequently encountered and/or abundant while the majority of species are rare, and that members of the Russulales or Thelephorales were in the high abundance group (Horton and Bruns 2001; Lilleskov et al. 2002; Dickie et al. 2002; Kennedy et al. 2003). The combined view of relative frequency and relative abundance in the old growth study provides an informative assessment of fungal dominance that biomass or frequency do not reveal independently (Horton and Bruns 2001). For instance, the relatively high importance values of *C. geophilum* 2, *P. fallax*, *L. pseudomucidus* and *Byssocorticium*-morph 1 are driven primarily by their frequency, while the high importance values of *R. vesiculosus* and thelephoroid 1 are driven by their abundance. EM communities are complex and diverse, and sampling them belowground continues to be problematic (Taylor 2002). The sampling strategies we used were not ideal for assessing the overall structure of a belowground EM community, but they did reveal some

insight into the number of fungi associating with both hosts, putatively forming common mycorrhizal networks. While the root tip view is likely different from the soil mycelial view for most species (see Agerer 2001), we feel the root tip data can be used here because the production of extramatrical mycelium is not uniquely represented or prominent in multiple host versus host-specific fungi.

Evidence of common mycorrhizal networks

We use belowground data to assess whether EM fungal networks in these forests are host specific or include both hosts interacting through common associations. In both the young and old growth settings, many fungi were observed on both hosts. While direct evidence that fungal individuals link the two hosts was not obtained, studies to date reveal that individuals of EM fungi are much larger than the soil volumes we sampled with roots of the two host intermingling (Baar et al. 1994; Dahlberg and Stenlid 1994; De La Bastide et al. 1994; Gyrtá et al. 1997; Anderson et al. 1998; Bonello et al. 1998; Selosse et al. 1998a,b; Sawyer et al. 1999; Zhou et al. 1999; 2001; Redecker et al. 2001; Bergemann and Miller 2002; Dunham et al. 2003; Kretzer et al. 2004).

Many fungi associated with Douglas-fir are available to western hemlock (Kropp and Trappe 1982; Molina et al. 1992; Massicotte et al. 1999). In the early seral stage study most types observed on western hemlock seedlings also associated with Douglas-fir. In the late seral stage study many of the types occurred on a single host, but then they only occurred in one soil sample or in samples taken from a zone in which Douglas-fir roots were not available (soil cores 19–37). We suspect root pathogens specific to Douglas-fir may have been in the zone where soil samples 19–37 were taken, as evidenced by an adjacent canopy gap with abundant hemlock saplings. We suggest that many of the fungi observed only on western hemlock likely have the ability to colonize Douglas-fir when roots are available. The EM networks connecting the two host species are probably patchily distributed for any one fungus species, but fairly well developed overall in the later seral stage stand.

Our data are similar to those reported elsewhere where common mycorrhizal networks connect multiple host species in mixed stands (Horton and Bruns 1998; Cullings et al. 2000; Kennedy et al. 2003). Several recent studies have shown that the common mycorrhizal networks can influence succession. For instance, in a chaparral ecosystem, Douglas-fir seedling establishment was facilitated by mycelial networks associated with the shrub *Arctostaphylos* but not with the arbuscular mycorrhizal-dominated shrub *Adenostoma* (Horton et al. 1999). Dickie et al. (2002) observed a similar pattern with increased establishment of *Quercus rubra* seedlings under the canopy of congeners compared to non-EM settings, and in a follow-up study, increased ectomycorrhizal infection of oak seedlings in soils associated with the herbaceous perennial *Helianthe-*

mum bicknellii (Dickie et al. 2004). In the tropics, increased survival of *Paraberlina bifoliata* seedlings was observed under *Brachystegia cynometroides* where the seedlings became associated with the mycelial networks of the trees (Onguene and Kuyper 2002).

Kropp and Trappe (1982) revisited

Kropp and Trappe (1982) predicted that late successional species such as western hemlock would have few host-specific fungi because they establish in plant patches that are inhabited by EM fungal networks of other species. Establishment is facilitated if late successional seedlings can tap into an EM fungal network already established and maintained by an earlier successional species. If late successional plant species must rely on host-specific fungi for establishment, then they can only establish where propagules of their fungi occur on the site, which may be a rare situation when the stand is dominated by another plant species. Even if there is adequate spore inoculum in a stand, the seedling must allocate a substantial portion of its net primary productivity to support fungal metabolic activity as required for growth and the acquisition of soil resources. However, if the mycelial network is supported by the early successional plant species, then seedlings may be able to allocate fewer resources to the fungi while gaining from the associations (Newman 1988).

Our data suggest a low reliance by western hemlock on host-specific fungi when its seedlings establish in soils dominated by Douglas-fir mycorrhizal networks. These data support the hypothesis put forth by Kropp and Trappe (1982) that there is selection pressure away from host specificity in the later successional species. Mutations to resistance in mutualists may be disadvantageous whenever multiple hosts are present because they reduce the number of compatible species available to the symbiont (Vanderplank 1978; Harley and Smith 1983). However, unique functions provided to a plant by a fungus may incur an increased level of fitness, a condition that could make host specificity advantageous even at a high cost to the plant (Molina et al. 1992; Bruns et al. 2002).

Kropp and Trappe (1982) further predicted that it would be selectively advantageous for early successional plant species to associate with host-specific fungi because the fungi provide an inhibitory mechanism for the establishment of late successional plant species. Our data suggest that Douglas-fir specific fungi (primarily *Rhizopogon* and *Truncocollumella*, but also *Suillus*) are relatively common in both young and late successional Douglas-fir forests as ectomycorrhizae and presumably in the mycelial network. Sporocarp surveys, pure culture synthesis experiments and soil bioassays with multiple hosts have demonstrated the ability of some of these fungi to associate with other hosts including western hemlock (Kropp and Trappe 1982; Molina and Trappe 1982, 1994; Massicotte et al. 1999). However, our field data support the prediction that while

these fungi may have the potential to associate with multiple hosts under laboratory conditions, they show ecological specificity under field conditions (Harley and Smith 1983).

In our early seral stage study, Douglas-fir-specific *Rhizopogon parksii* occurred in three of the five soil blocks and made up 14% of the total Douglas-fir EM root tip biomass. In our late seral stage study, Douglas-fir specific *Rhizopogon vesiculosus* and *Truncocollumella citrini* made up 25% of the Douglas-fir EM root tip biomass. However, these two fungi were not evenly distributed belowground, occurring in only five of the 24 cores, and then in tight coralloid clusters of about 1 cm in diameter. In contrast, the combined biomass of the observed multiple host EM fungi in the old growth study made up 32% of the Douglas-fir EM biomass, with species represented in 22 of the 24 soil cores. Other Douglas-fir EM types were too rare to confidently assign as host specific, but based on our current knowledge for most of the species, they likely did not exhibit a high level of host specificity (Molina et al. 1992). The belowground data suggest that host-specific fungi do not dominate the mycelial networks enough in older stands to inhibit succession, a pattern also observed in stands of *Pinus contorta* Dougl. Ex Loud and *Picea engelmannii* Engelm. in Yellowstone National Park by Cullings et al. (2000).

From our study it appears that multiple host fungi associated with relatively pure 40-year-old Douglas-fir stands already share dominance with Douglas-fir-specific fungi, and that Douglas-fir-specific fungi are not dominant enough to effectively inhibit the establishment of western hemlock. An interesting follow-up to the current study would be a manipulation study in which western hemlock is planted in pure stands of Douglas-fir younger than the 40 year-old forest we utilized (e.g., 1, 5, 10, 20 years post-disturbance), to assess if western hemlock establishment is ever inhibited, and link this to the importance of Douglas-fir-specific fungi in the stands. If host-specific fungi are found to dominate Douglas-fir EM networks immediately following disturbance, selection pressure for host specificity may not relate as much to interspecific interactions between trees in later stages of succession as predicted by Kropp and Trappe (1982), but rather to adaptations to marginal habitats (post disturbance) by the plant and its fungal symbionts (Molina et al. 1992).

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