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Fungal Genetic Diversity within Decomposing Woody Conifer Roots in Oregon, U.S.A.

Abstract

Internal Transcribed Spacer - Restriction Fragment Length Polymorphism (ITS-RFLP) and sequencing techniques were used to compare similarity of fungi decomposing roots of *Picea sitchensis* (Sitka spruce)*, Pinus contorta* (lodgepole pine), *Pinus ponderosa* (ponderosa pine), *Pseudotsuga menziesii* (Douglas-fir), and *Tsuga heterophylla* (western hemlock). Fungal DNA from hyphae growing on coarse roots, cultures isolated from roots, herbarium specimens, culture collections, and field collected samples was extracted, amplified, and digested with endonucleases. Over two hundred ITS-RFLP patterns from fungal samples were separated. Linearly increasing ITS-RFLP sampling intensity curves indicate high total genetic diversity of fungi. Nei and Li similarity index analysis showed similarities of fungi on roots as measured by ITS-RFLP patterns samples ranged from 0 to 1 (i.e., 0% to 100% similar) depending upon the root species, location, and harvest stand age. Sequences were obtained for the most common ITS-RFLP patterns. Results indicate that zygomycete fungi were most prominent in decomposing woody conifer roots and that the similarity of fungi was low between tree species and locations.

Introduction

Saprotrophic fungi play a key role in the decomposition of woody debris in forest ecosystems. Saprotrophs aid in the breakdown of lignin, cellulose, and hemicellulose into smaller and less complex molecules (Gilbertson 1981, Worrall et al. 1997, Allen et al. 2000). Moreover, sporocarp production changes the nutrient availability in logs over time through temporarily immobilizing nutrients and ultimately transferring nutrients onto the forest floor, and decomposition alters the physical structure of woody debris in the habitat (Harmon et al. 1994).

Previous studies have examined the effects of saprotrophic fungi and the diversity of fungal sporocarps on aboveground coarse woody debris. For example, in Washington State (USA), basidio-mycete diversity was correlated to woody debris conditions including moisture content, decay class (with high species richness in intermediate decay class logs), and seasonal weather variation (Edmonds and Lebo 1998). In New Zealand, fungal species richness was correlated to log volume and nutrient availability regardless of decay class (Allen et al. 2000). In Panama, the diversity of specific species of polypores was correlated with the abundance of the host trees within the wet tropical forest (Gilbert et al. 2002). In Denmark, fungal community composition on coarse woody debris has been shown to be affected not only by the stage of decay of the log, but also by the size, structure, and the log microclimate (Heilman-Clausen and Christensen 2003). Few studies have been conducted on decomposition of woody roots and the diversity of fungi in decomposed woody roots (Harmon et al. 1986, Chen et al. 2001, Chen et al. 2002). Molecular techniques identifying mycorrhizal species associated with conifer trees and estimating fungal genetic diversity of culturable fungi in mixed soil samples provide a promising avenue for the identification of saprotrophic fungi in decomposed woody roots (Viaud et al. 2000, Horton and Bruns 2001).

Chen et al. (2001, 2002) measured decomposition rates of roots from five conifer species

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(*Picea sitchensis* (Bong.) Carrière (Sitka spruce)*, Pinus contorta* Dougl. ex Loud. (lodgepole pine), *Pinus ponderosa* P. Laws. ex C. Laws. (ponderosa pine), *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir), and *Tsuga heterophylla* (Raf.) Sarg (western hemlock)) from three climatic areas within Oregon State (USA): the Coast Range, foothills of the Cascade Range, and the high desert east of the Cascade Range (Nomenclature follows Hitchcock and Cronquist (1976)). More evidence of white-rot fungi was observed on *Pinus ponderosa* roots and this tree species decomposed more rapidly than other conifer species (Chen et al. 2001). Environmental differences among the sites, including moisture, temperature, and wood chemistry of each tree species failed to entirely explain the observed differences in decomposition rates (Chen et al. 2001, Chen et al. 2002).

Based on the results of the studies by Chen et al. (2001, 2002) we hypothesized that differences in saprotrophic fungal communities within tree root systems influence the rate of root decomposition. We also hypothesized, as many studies of mycorrhizal fungi have found, that there would be few dominant fungi common to each site and tree species (Horton and Bruns 2001). The objective of our study was to compare genetic biodiversity and levels of similarity in the root fungal communities on coarse roots of five conifer species from three sites across Oregon with different forest harvest ages. We endeavored to isolate and collect ascomycete, basidiomycete, and zygomycete fungi from coarse roots, genetically identify them with nuclease (ITS-RFLP typing) and sequence techniques and available databases, and compare similarities of fungal isolates from each location and tree species. To capture the greatest fungal biodiversity in coarse roots, multiple sampling and fungi isolation methods were utilized. Based on available resources when the study was begun, ITS-RFLP typing was selected as the best method to genetically separate fungi in the roots, followed by sequencing techniques to further identify the most common fungi present.

Materials and Methods

Study Area and Field Collection of Root Samples

The study sites, Cascade Head Experimental Forest (CHE), H.J. Andrews Experimental Forest (HJA), and Deschutes National Forest (DNF), followed a west-to-east environmental gradient across Oregon (Table 1). CHE is located near the town of Otis on the Siuslaw National Forest and extends from the edge of the Pacific Ocean inland through the Coast Range. HJA is located 80 km east of Eugene on the Willamette National Forest along the western slope of the Cascade Range. DNF is located 57 km south of Bend east of the Cascade Range with a modified continental climate of the central Oregon high desert plateau.

Roots were collected in clear-cut or thinned stands that were burned. Site selection was based on the harvest age (ranging from seven to fifteen years). Coarse roots were collected from (i) stumps of five tree species from roots freshly harvested in 2000 and 2001 and (ii) roots buried in 1995 as part of a long term "reciprocal transplant" study that were retrieved from the field in 2000.

TABLE 1. Climate, precipitation, temperature, and soil characteristics for each of the three study locations.

Forest	Location	Climate	Annual Precipitation	Mean Annual Temperature	Soil
Cascade Head Experimental Forest (CHE), Siuslaw National Forest	45.2° N latitude, 123.58° W longitude	Maritime	3420 mm	10ºC	Well-drained silt and silt clay loams derived from marine silt stones and high concentration of nitrogen and organic matter
H.J. Andrews Experimental Forest (HJA), Willamette National Forest	44.2° N latitude, 122.2° W longitude	Maritime	2300 mm	8.5ºC	Deep, well-drained Dystrochrepts
Deschutes National Forest (DNF)	43.7° N latitude, 121.6° W longitude	Modified continental	525 mm	5.7ºC	Coarse loamy, derived from aerial deposited dacite pumice

In summer of 2000, roots were collected from three stumps with intact roots of each species at each forest stand (Table 2). The first stumps with intact roots encountered while walking in from the road were selected for sampling. Stump species was identified by remaining bark and growth characteristics such as presence of buttresses and thickness of the bark, and stump decay classes were

TABLE 2. Locations of sample collection and stand characteristics for all roots collected in the field in 2000 and 2001.

Location	Stand Year of Harvest	Species (# samples per species)	Year Roots Harvested from Stumps	Lab Processing of Hyphae	Culturing Medium	Forest Composition	Harvest Treatment
CHE	1986 (A)	*P. sitchensis* (9) *T. heterophylla* (9)	2000	IC*, H^	Malt	*P. sitchensis* *T. heterophylla* *P. menziesii*	Clear-cut and burned
CHE	1986 (B)	*P. sitchensis* (9) *T. heterophylla* (9)	2000	IC, H	Malt	*Alnus rubra* Bong. *P. sitchensis* *P. menziesii* *T. heterophylla*	Clear-cut and burned
CHE	1989 (C)	*P. sitchensis* (9) *T. heterophylla* (9)	2000	IC, H	Malt	*P. sitchensis* *P. menziesii* *T. heterophylla*	Clear-cut and burned
CHE	(D)	*P. contorta* (2) *P. ponderosa* (2) *T. heterophylla* (2) *P. menziesii* (2)	1995†	IC, H	Malt		
HJA	1986 (A)	*P. menziesii* (9) *T. heterophylla* (9)	2000	IC, H	Malt	*Alnus rubra* *P. menziesii*	Clear-cut and burned
HJA	1993 (C)	*P. menziesii* (9) *P. heterophylla* (9)	2000	IC	Malt	*Abies* spp. *P. menziesii*	Thinned with 10% leave trees and burned
HJA	1993 (C)	*P. menziesii* (3)	2001	IC	Malt Goldfarb's	*Abies* spp. *P. menziesii*	Thinned with 10% leave trees and burned
HJA	(D)	*P. contorta* (2) *P. ponderosa* (2) *T. heterophylla* (1) *P. menziesii* (2) Unknown (1)	1995	IC, H	Malt		
DNF	1985 (A)	*P. contorta* (9) *P. ponderosa* (9)	2000	IC, H	Malt	*P. contorta* *P. ponderosa*	Clear-cut and burned
DNF	1988 (B)	*P. contorta* (9) *P. ponderosa* (9)	2000	IC, H	Malt	*P. contorta* *P. ponderosa*	Thinned with 30% leave trees and burned
DNF`	1991 (C)	*P. contorta* (9) *P. ponderosa* (9)	2000	IC, H	Malt	*P. contorta* *P. ponderosa*	Thinned with 30% leave trees and burned
DNF	(D)	*P. contorta* (1) *P. ponderosa* (2) *T. heterophylla* (2) *P. menziesii* (1) Unknown (2)	1995	IC, H	Malt		

*IC: Fungi isolated with culturing techniques

^H: Hyphae collected directly from roots

† Roots harvested in 1995 were reciprocally transplanted and retrieved from the field in 2000.

recorded on a scale of one to five (Harmon et al. 1986). Decay class one represents relatively intact and non-decomposed wood, and decay class five is highly decomposed. CHE stands generally had more stumps in later stages of decay, especially decay class three (78% of stumps), than stands of the same harvest age at DNF (0% of stumps) and HJA (33% of stumps).

Two root samples (1-20 cm in diameter and 15-40 cm in length) were dug from the base of each stump. Samples were removed from the ground, labeled, and placed into plastic bags. One hundred eight samples of roots were collected directly from stumps. Additionally, three *P. menziesii* roots were collected from the 1993-harvest stand at the HJA in October 2001.

For the reciprocal transplant study, coarse roots of i) *Pseudotsuga menziesii, Tsuga heterophylla,* and an unidentified species from CHE; ii) *P. menziesii, T. heterophylla,* and an unidentified species from HJA; and iii) *Pinus contorta, P. ponderosa,* and an unidentified species from DNF were harvested from freshly cut stumps in 1995. Samples were placed into a mesh bag and left exposed to the elements. Samples of each species from each location were reciprocally transplanted and buried both at the native site and the other two study sites. In summer of 2000, twenty-four of these reciprocally transplanted roots were collected from each of the three study locations and treated as described above (Table 2).

Laboratory Processing of Root Samples

After scrubbing in deionized water for five to ten minutes to remove adhering soil particles, each root sample was further divided into two groups. For all samples collected in 2000, one group was kept at room temperature for two to four weeks until a growth of fungal hyphae was visible. Mycelium growing on the wood was collected in microcentrifuge tubes with sterile forceps and freeze-dried for DNA extraction. The second group of root subsamples collected in 2000 and all root samples collected in 2001 were stored at 4°C for one to two weeks until fungi could be isolated with culturing techniques. Both methods were employed because certain fungal species do not grow readily from wood pieces placed on media.

Isolation of Fungi

Five wood pieces from each root sample collected from the field in 2000 and 40 from each root sample collected in 2001 were removed with a flame sterilized chisel and forceps, and plated together on a Petri dish with 1.5% malt-agar (MA) medium (1L sterilized MA: 15g malt extract, 10ml benlate (10ppm), 10ml streptomycin (1mg/1ml), 1ml lactic acid, and 10g agar) or Goldfarb's medium (1L Goldfarb's sterilized: 30g malt, 1ppm benlate (1mg/1ml), 1ppm prochloraz (1mg/1ml), 1ppm thiobendazole (83.5mg/100ml), 100mg streptomycin, 20mg rose bengal, and 20g agar) (Goldfarb et al. 1989). Samples were checked weekly for fungal growth for six weeks. Recognizable isolates were then individually transferred to Petri dishes with malt-agar or Goldfarb's medium with or without cellophane for single isolate growth. After two to four weeks fungal hyphae samples were harvested from surface medium and freeze-dried or stored in 2X CTAB (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA). The morphology of each isolate that amplified with PCR was examined with microscopy.

DNA Extraction, PCR, and ITS-RFLP

DNA extraction, PCR amplification, and ITS-RFLP analysis of samples followed methods from Gardes and Bruns (1996). During DNA extraction, a few grains of Proteinase K (Amersco, Solon, Ohio, USA) were placed directly into each sample as a substitute for ß-mercaptoethanol. Using a CTAB extraction, samples were frozen with dry ice and heated to break cell walls, and further broken down by grinding with a micropestle. DNA was separated from proteins with chloroform, precipitated with isoproponal, and washed with 70% ethanol. Samples were stored in 50 µl TE buffer (1mM Tris-HCl, 0.1M EDTA) in a -20°F freezer until PCR amplification. The internal transcriber spacer (ITS) region between the nuclear small rDNA and nuclear large rDNA was amplified using ITS-1F and ITS-4 fungal specific primers (Operon, Alameda, California, USA). The samples collected in 2000 were diluted in a 1:100 mixture with sterile water before being mixed with PCR "cocktail." Samples collected in 2001 were added directly to the PCR "cocktail" with a final dilution concentration of 1:100. An annealing temperature of 52°C was used for optimal amplification.

ITS-RFLP patterns from PCR products were generated using *Hinf* I, *Dpn* II, and *Hae* III enzymes (Promega, Madison, Wisconsin, USA) (Gardes and Bruns 1996). The ITS-RFLP fragment lengths from all samples were visually and numerically analyzed using Gene Profiler (Scanalytics). Samples with matching ITS-RFLP patterns for three enzymes were grouped as distinct fungal ITS-RFLP types (Horton 2002).

ITS-RFLP types were compared with ITS-RFLP patterns from (i) 66 fungal sporocarps collected in November 2000 and April 2001 from our study areas, primarily from stumps and logs, (ii) 28 species of saprotrophic ascomycetes and basidiomycetes from the Oregon State University herbarium, and (iii) 47 saprotrophic basidiomycete cultures from the Oregon State University Wood Science and Engineering Department collection (Table 3).

Sequencing

Thirty-one fungal samples from 28 common ITS-RFLP types, (defined as ITS-RFLP patterns found in more than one stump) and three fungal samples with uncommon ITS-RFLP patterns were sequenced according to the methods used by White et al. (1990). Samples were amplified

using NL1 and NL4 primers (supplied by K. O'Donnell, Peoria, Illinois, USA) from domains one and two on the nuclear large subunit (LSU) or NS1 and NS2 primers (Operon, Alameda, California, USA) for the nuclear small subunit (SSU). A sequence reaction was set up with ZNL2 or ZNL3 internal primers for LSU (supplied by K. O'Donnell, Peoria, Illinois, USA) or NS1 or NS2 primers (Operon, Alameda, California, USA) for SSU (White et al. 1990). Sequences were run on Applied Biosystems 373A DNA Sequencer machine (Foster City, California, USA). Completed sequences were edited and aligned with each other. Sequences were compared for potential alignment matches with other fungal sequences in GenBank (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) 2.0 algorithm. The alignment of an unknown fungus with a known sequence in the GenBank database was considered a strong match with high percentage accuracy, a low error (E<0.05) value, and a large score (bits sizes) (Altschul et al. 1997).

Data Analysis

The Nei and Li (1979) similarity index, designed to equal 1 in cases of complete similarity and 0 if

TABLE 3. Number of samples collected from each location, PCR amplification rates, and total number of ITS-RFLPs recorded.

Sample origination	Number of samples	Percent PCR amplification	Number of collected RFLP patterns
Collected directly from roots placed at room temperature in 2000	326	26%	44
Fungi isolated from roots in 2000	449	55%	142
Collected directly from roots placed at room temperature from samples harvested in 1995, reciprocally transplanted, and retrieved from the field in 2000	34	6%	2
Fungi isolated from roots collected in 2001	74	54%	17
Total collected samples	**944**	**45%**	**230**
Fungi collected from study areas	124	53%	66
Samples from Oregon State herbarium	49	57%	28
Fungi from Wood Science and Engineering Department	51	92%	47
Total known fungi for comparison	**224**	**63%**	**141**

comparisons are dissimilar and have no ITS-RFLP patterns in common, was used to compare fungal ITS-RFLP types common among sites, tree species, stands, and stumps using RAPDPLOT 3.0 (Black 1997). Sites, species, stands, or stumps which did not yield any ITS-RFLP patterns were not included in the analysis.

Similarity analyses were conducted at the level of sites, species, stand, stumps, or individual roots if appropriate, for each of the following categories: (i) all samples collected from HJA in 2001, (ii) all samples collected in 2000, (iii) samples with the 28 common ITS-RFLP types collected in 2000, (iv) samples that were isolated by culture medium in 2000, (v) samples grown at room temperature and collected directly from roots in 2000, (vi) samples grown at room temperature and those isolated on culture medium in 2000 compared separately, and (vii) roots harvested in 1995 that were reciprocally transplanted and retrieved from the field in 2000.

Results

Fungal Samples from Roots

A total of 944 fungal samples were collected through culture isolation or direct collection from roots (Table 3). One hundred eight sample roots were harvested from stumps in the summer of 2000 yielding 326 hyphae samples collected directly from roots placed at room temperature for two weeks and 449 fungi isolated from root pieces placed on media. Reciprocally transplanted roots yielded 34 hyphae samples collected directly from roots placed at room temperature for two weeks and 61 fungi isolated from root pieces placed on media. Seventy-four fungi were isolated from the roots of *P. menziesii* that were intensively sampled in 2001.

ITS-RFLPs

PCR amplification was successful for 45% of all samples from roots and ITS-RFLP patterns were obtained for 421 samples representing 230 individual ITS-RFLP patterns from samples collected in 2000 and 2001 (Table 3). Sixty-three percent of the Oregon State University herbarium and culture collections and field collected sporocarps amplified yielding 76 ITS-RFLP patterns from 141 samples. Twenty-eight ITS-RFLP patterns were common to more than one stump or root sampled

in 2000. When the results from the two media of the samples collected in 2001 were combined, 25 ITS-RFLP patterns were identified. Specifically, 18 ITS-RFLP patterns were isolated with malt-agar medium and 15 with Goldfarb's medium. There were three ITS-RFLP patterns common to samples isolated from both media. Six of the 25 ITS-RFLP patterns collected in 2001 matched with those collected 2000.

Biodiversity Results

A sampling intensity curve, similar to a species-area curve, (Figure 1) shows a continual linear increase in ITS-RFLP patterns for each new stump sampled in 2000. This trend occurred for (i) fungi isolated from stumps (Figure 1), (ii) roots from the reciprocal transplant study collected in 2000, and (iii) samples collected in 2001 from the intensive examination of three HJA *P. menziesii* roots.

Similarity Index of Fungal Diversity in Root Samples at Various Scales

Similarities between the sample ITS-RFLP patterns are summarized in Table 4. In general similarity was low regardless of the level of comparison (i.e., sites, stands, species, source, method of isolation, and media). Similarity of ITS-RFLP patterns between HJA and DNF was 0.062, between CHE and HJA was 0.048, and between CHE and DNF was 0.031 (Table 4). Likewise, similarity analysis of ITS-RFLP patterns for tree species ranged from 0 to 0.20 (Tables 4 and Table 5). The greatest levels of similarity were found between (i) *Picea sitchensis* and *Tsuga heterophylla* from CHE (0.032), (ii) *T. heterophylla* from CHE and *T. heterophylla* from HJA (0.029), (iii) *T. heterophylla* and *Pseudotsuga menziesii* from HJA (0.20), and (iv) *Pinus ponderosa* and *P. contorta* from DNF (0.11) (Table 5). The similarity of ITS-RFLP types compared between stands ranged from 0 to 0.28 with the greatest similarities between the HJA stands cut in 1993 and 1988 (0.28) and the HJA stand cut in 1993 and the DNF stand cut in 1988 (0.15) (Table 4 and Table 6).

For individual stumps the similarity of fungal ITS-RFLP patterns ranged from 0 to 0.67 (Table 4). The highest level of similarity (0.67) was found between a *Tsuga heterophylla* stump (1989 stand) and a *Pseudotsuga menziesii* stump (1986 stand) both from the HJA. The CHE *Picea sitchensis*
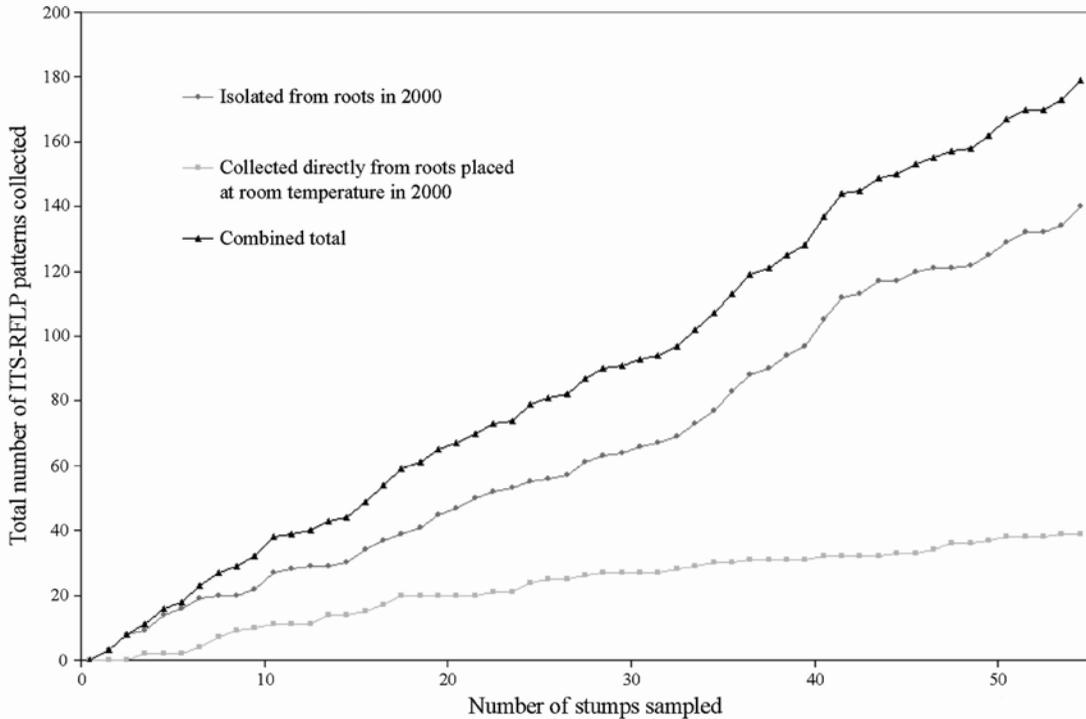
Figure 1. ITS-RFLP sampling intensity curve representing the number of new fungal ITS-RFLP types gained with each new stump sampled in 2000 across Oregon where a linear curve indicates an underestimation of total genetic biodiversity.

stumps had no similarity with stumps from the other two sites. Only two of the *T. heterophylla* stumps sampled from CHE and HJA had a measurable level of similarity (0.18).

The similarity index of the individual roots harvested in 2001 ranged from 0.10 to 0.30, and the index between each root when the Goldfarb and malt agar culture media were compared separately, ranged from 0.18 to 0.33 (Table 4).

Similarity Between Samples with Common ITS-RFLP Patterns

Examining the similarity of the most common ITS-RFLP patterns generally increased the degree of similarity between the various levels examined. For example, using the 28 common ITS-RFLP patterns found in samples collected in 2000, similarity index between HJA and DNF (0.31) was larger than between CHE and HJA (0.24) or between CHE and DNF (0.29) (Table 4). The similarity among all stands ranged from 0 to 0.57 (Table 4). The similarity among tree species for common ITS-RFLP patterns ranged

from 0 to 0.64 with the greatest similarity between *Pseudotsuga menziesii* and *Tsuga heterophylla* from HJA (Table 4). HJA *T. heterophylla* and CHE *T. heterophylla* had a similarity of 0.12. At CHE, *Picea sitchensis* and *T. heterophylla* had a similarity of 0.18. At DNF, *Pinus ponderosa* and *P. contorta* had a similarity of 0.53.

Species Matching and Sequences

Sequences from the LSU of two samples collected at room temperature directly from wood in 2000 matched sequences of *Resinicium bicolor* (Corticiaceae). One sample from a root harvested in 1995 matched sequences of *Hydnum repandum* (Hydnaceae) on the SSU search. BLAST searches on sequences for the other samples matched species in the Zygomycota. Searches in the LSU matched four samples with each *Micromucor ramannianus, Umbelopsis isabellina,* and *U. nana* (all Mortierellaceae). One sample was matched with each of *Mortierella verticillata* (Mortierellaceae), *Zygohynchus heterogamus* (Mucoraceae), and *Elisomyces anomalus* (Thamnidiaceae). Searches

TABLE 4. Range of similarity index values for ITS-RFLP patterns for stump derived collected in 2000 and 2001 and reciprocal transplant roots collected in 2000 in Oregon.

Sample origination	Level of analysis	Range of Similarity Index values
Fungi from roots harvested from HJA in 2001	Individual sample	0.10-0.30
	Two media compared separately	0.18-0.33
All fungi collected from roots harvested in 2000	Site	0.031-0.062
	Species	0-0.20
	Stand	0-0.28
	Stump	0-0.67
Samples collected in 2000 with common ITS-RFLP patterns	Site	0.24-0.31
	Species	0-0.64
	Stand	0-0.57
Fungi isolated from roots harvested in 2000	Site	0.061-0.074
	Species	0-0.24
	Stand	0-0.16
	Stump	0-0.67
Fungi collected at room temperature from roots harvested in 2000	Site	0
	Species	0
	Stand	0
	Stump	0-1.0
Fungi collected at room temperature and isolated from roots harvested in 2000 compared separately	Site	0-0.074
	Species	0-0.24
	Stand	0-0.33
	Stump	0-1.0
Fungi from roots harvested in 1995, reciprocally transplanted, and retrieved from the field in 2000	Site	0-0.14
	Roots	0-0.67

TABLE 5. Similarity index matrix for stump derived samples from 2000 for comparisons of ITS-RFLP patterns found in each species in Oregon. See legend below for key to abbreviations.

	CHE-PISI	CHE-TSHE	HJA-PSME	HJA-TSHE	DNF-PICO	DNF-PIPO
CHE*-PISI#	1					
CHE-TSHE	0.032	1				
HJA-PSME	0.031	0.068	1			
HJA-TSHE	0	0.029	0.20	1		
DNF-PICO	0	0.061	0.060	0.053	1	
DNF-PIPO	0	0.064	0.094	0.082	0.11	1

*CHE=Cascade Head Experimental Forest; HJA=H.J. Andrews Experimental Forest; DNF=Deschutes National Forest

#PICO=*Pinus contorta*; PIPO=*Pinus ponderosa*; TSHE=*Tsuga heterophylla*; PSME=*Pseudotsuga menziesii*; PISI=*Picea sitchensis*

in the SSU matched 13 samples with *U. isabellina,* four with *Mucor hiemalis* (Mortierellaceae), and one each with *Mortierella chlamydospora* (Mortierellaceae) and *Z. heterogamus.* Six samples matched two different species for the LSU and SSU searches. When sequences were compared with both the LSU and SSU for one sample, the LSU detected greater variability and species variation than the more conserved SSU.

One sample isolated from *Pinus contorta* collected from the 1985 harvest stand at DNF matched with a *Phanerochaete sordida* (Phanerochaetaceae)

culture from the Wood Science and Engineering collection. None of the common ITS-RFLP types matched with the known ITS-RFLP patterns for sporocarps and cultures from the Oregon State University collections or field collected sporocarps.

Microscopic Considerations

Ninety percent of the cultures displayed common zygomycete characteristics including aseptate or irregularly septate hyphae, sporangia, sporangiospores, and columellae (Benjamin 1979, O'Donnell et al. 2001, Meyer and Gams 2003). Most samples were aseptate; however, there were some dark septate fungi, and very few fungi that were strictly septate. There was no evidence of clamp connections, a common basidiomycete characteristic.

Discussion

Based on the number of recorded ITS-RFLP patterns, we found higher than expected levels of fungal genetic ITS-RFLP diversity indicating that there was not a core fungal community for sites, tree species, harvest stands, stumps, or roots. ITS-RFLP sampling intensity curves demonstrated a continual linear increase in genetic diversity with each new sample added indicating that additional ITS-RFLP patterns could be recovered from the study areas if all sample collection methods were continued. Comparisons of fungal ITS-RFLP patterns in some instances demonstrate relatively high similarity, but most levels of comparison showed minimal to no genetic similarity. The low similarity index numbers correlate with a correspondingly high level of genetic diversity across the samples

(Table 4, 5, and 6). These results indicate a range of methods is required to maximize the number of ITS-RFLP patterns observed.

Our diversity results based on molecular techniques are similar to those reported for microfungal communities estimated by microscopic techniques (Christensen 1969, Bills and Polishook 1994, Polishook et al. 1996). Microfungi isolated from various habitats in Wisconsin lacked discrete fungal communities with diversity correlated with the diversity of local soil environments and vegetation (Christensen 1969). Additionally, high levels of species diversity and dissimilarity of community composition with a majority of species found infrequently have been recorded in tropical microfungi communities on leaf litter in Central America (Bills and Polishook 1994, Polishook et al. 1996). Total underground fungal diversity is often hypothesized to be greater than what can be sampled with current sampling methods, and new fungi are continuously being discovered with newly available molecular techniques through meticulous study of well defined root and soil habitats (Vandenkoornhuyse et al. 2002, Jumpponen and Johnson 2005). Composition of the plant community has been found to affect the biodiversity of fungal communities measured in a given location (Christensen 1969, Franklin and Dyrness 1971, Franklin and Dyrness 1973). Additionally, stumps may provide habitat for fewer fungal sporocarps than above ground logs, and decay class can affect the number and type of fungi found on coarse woody debris (Heilman-Clausen and Christensen 2003). All of these factors may in turn relate to the fungal communities on decomposing woody roots.

TABLE 6. Similarity index matrix for stump derived samples from 2000 for comparisons of ITS-RFLP patterns found in each stand in Oregon. See legend below for key to abbreviations.

Site	Harvest Year	CHE 1986a	CHE 1986b	CHE 1989	HJA 1986	HJA 1989	HJA 1993	DNF 1985	DNF 1988	DNF 1991
CHE	1986a	1								
CHE	1986b	0	1							
CHE	1989	0.049	0	0						
HJA	1986	0	0.080	0	1					
HJA	1989	0.049	0	0	0.039	1				
HJA	1993	0	0.048	0	0.083	0.28	1			
DNF	1985	0	0.036	0	0.032	0.035	0.15	1		
DNF	1988	0	0	0	0	0	0	0	1	
DNF	1991	0.061	0	0	0	0	0	0	0.059	1

*CHE=Cascade Head Experimental Forest; HJA=H.J. Andrews Experimental Forest; DNF=Deschutes National Forest

Increased sampling over different seasons in multiple years may help to estimate total biodiversity more accurately because species richness data can be influenced by the number of sampling episodes (Polishook et al. 1996, Edmonds and Lebo 1998, Smith et al. 2002). However, the methods currently available for isolation and identification of fungi may be inadequate to measure the total diversity (Bridge and Spooner 2001). Only seventeen percent of known fungi can be grown in culture, and selected media can influence growth as evidenced by the differences in fungi isolated using Goldfarb's and malt agar media in our study (Parkinson and Crouch 1969, Hawksworth 1991). Consequently, it is likely that multiple media recipes and a variety of hyphal collection methods are required, but still might not allow for complete identification of all fungi ITS-RFLP patterns. Therefore the pool of culturable fungi we observed was smaller than the total number of fungi present at the study locations (Hawksworth 1991, Hawksworth 2001). We found a greater similarity between groups of isolated fungi as compared to the fungi samples collected directly from wood, possibly because not all fungi grow in culture. Adding to the low similarities, the DNA from fungi collected directly from wood had disappointingly low amplification rates, potentially due to difficulties in separating fungal hyphae from solid wood.

However, despite the large ITS-RFLP diversity, when sequenced, the majority of the common ITS-RFLP types were identified as closely related zygomycetes. Due to the ubiquitous nature of zygomycetes and the ease with which they can be grown in culture (Kuhlman 1969, O'Donnell et al. 2001), it is not surprising that they were prominent species within this study as demonstrated through microscopic and sequencing observations. The washing method we used to process roots, could change the community of fungi collected from basidiomycete saprotrophic fungi to zygomycetes which are often present on roots even after several washings (Christensen 1969, Kuhlman 1969, Parkinson and Crouch 1969, O'Donnell et al. 2001). These processes would not have influenced the isolates taken from wood chips in the interior of roots. Regardless, the common genus in our study, *Umbelopsis* spp., has been collected and sequenced from such varied locations around the world including *Quercus, Acer,* and *Liriodendron*

roots (living and dead) in West Virginia, USA (Amos and Barnett 1966), *Picea abies* in Germany (as *Mortierella* species) (Holdenrieder and Sieber 1992), in leaf litter in Japan (Sugiyama et al. 2003), and the closely related genus *Mortierella* in woody roots of *Picea sylvestris* in the United Kingdom (Fisher et al. 1991). These and other studies have found that Zygomycota species are widespread in woody roots and found in a variety of habitats. Many of the species within these genera are closely related to each other and genetic research is ongoing to better distinguish between individual species (Meyer and Gams 2003, Lutzoni et al. 2004, Kwasna et al. 2006).

Recently, an *Umbelopsis* that was genetically very closely aligned to samples from our study (92-98%) was identified from living *P. ponderosa* and *P. menziesii* roots in the dry forest regions of Eastern Washington (Hoff 2002, Hoff et al. 2004). Unlike our study where *Umbelopsis* was evenly distributed among all of the tree species, greater numbers of *Umbelopsis* were recovered from *P. ponderosa* roots. If that trend could be further studied, it might account for the faster decomposition of *P. ponderosa* roots in studies by Chen et al. (2001 and 2002).

Zygomycetes are typically found in the earliest and latest stages of decomposition where simple carbohydrates are readily available (Rayner and Boddy 1988), and may therefore play an ecologically important role in decomposition. Physiologically the Umbelopsidaceae and Mucorales (Mortierellaceae) are particularly well suited to nutrient poor environments and disturbed sites (Meyer and Gams 2003) such as the burned at harvest forest stands in this study. Those fungi isolated in this study may have been part of the active wood decomposer community or taking advantage of the simple carbohydrate by-products created through decomposition by other fungi in this nutrient poor habitat. We hypothesized white- and brown-rot basidiomycetes would be prominent groups. However, while some roots showed evidence of decomposition by white- and brown-rot, others did not, possibly indicating our sampling occurred at a successional period when low nutrient availability did not allow basidiomycetes to dominate.

Ectomycorrhizal studies often employ ITS-RFLP methodology to identify samples matching fungi producing sporocarps with ITS-RFLP

databases (Horton and Bruns 2001). Our study indicated, as others have found, that these methods can also be useful in identifying and assessing potential genetic diversity of saprotrophic fungi on coarse roots (Hoff et al. 2004). The aboveground field collected fungi were ascomycete and basidiomycete species and not the same as the zygomycetes cultured from roots in the same areas. Results indicate that basidiomycete and ascomycete hyphae could be recovered from belowground coarse roots using different techniques or may only be sampled from living tissue mycorrhizae.

Future studies identifying saprotrophic fungi and their roles in decomposition should employ methods to better extract fungal DNA from decomposing woody samples, increase the number of roots sampled, and include more concentrated sampling of greater numbers of stumps and roots from one stand, from one tree species, or from along the entire length of a root (Polishook et al. 1996, Vandenkoornhuyse et al. 2002). Such approaches may better characterize the fungal community at a local scale with increased replications at each site. Several methods for collecting fungi from roots create a more complete estimate of fungal diversity. Long-term studies following the change in resource availability, fire regimes, tree species composition and changing fungal communities are needed to determine when zygomycete populations dominate coarse root fungal communities. However, the large number of ITS-RFLP patterns collected from the roots in our study highlight the complexities of microfungi communities found in the decomposition process of coarse roots. This study indicates that similarities of fungi between different sampling scales may be low while the genetic diversity of fungi on decomposing roots is substantial.

Acknowledgements

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