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The effect of fertilization on the below-ground diversity and community composition of ectomycorrhizal fungi associated with western hemlock (*Tsuga heterophylla*)

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Abstract Fertilization typically reduces ectomycorrhizal diversity shortly after its application but less is known about its longer-term influence on fungal species. Long-term effects are important in forests where fertilizer is rarely applied. We compared fungal species composition in western hemlock control plots with plots last fertilized 7 years ago with nitrogen (N) or nitrogen plus phosphorus (N + P). The N + P fertilization had a significant lingering effect, increasing the tree size and foliar P content of the western hemlocks. From ectomycorrhizal roots of 24-year-old trees from northern Vancouver Island, Canada, we identified fungi from 12 samples per treatment, by amplifying, cloning, and sequencing fungal ribosomal DNA fragments, placing sequences with 97% or more identity in the same operational taxonomic unit (OTU). Diversity was high across treatments; we detected 77 fungal OTUs, 52 from ectomycorrhizal genera, among 922 clone sequences. The five most frequent OTUs were similar in abundance across treatments. Only 19 OTUs matched any of the 197 previously reported ectomycorrhizal species of western hemlock. Species composition but not diversity in nitrogen plus phosphorus plots differed significantly from control or nitrogen plots. Two *Cortinarius* OTUs were indicator species for nitrogen plus phosphorus plots and presence of *Cortinarius cinnamomeus* was correlated with control or nitrogen plots. After 7 years, fertilization history had made no detectable difference in ectomycorrhizal fungal diversity, but long-lasting changes in environment resulting from fertilization had a lingering effect on fungal ectomycorrhizal species composition.

Keywords Ectomycorrhizal diversity · Clone library · Species definition · Species composition · Fertilization

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Introduction

Most studies of the response of the ectomycorrhizal fungal community to fertilizer have focused on the effects of fertilizer within a year or two of application, or the effects of regular and repeated fertilizer application. We were interested in finding out whether fertilizer applied only early in forest stand regeneration altered the diversity or community structure of ectomycorrhizal fungi several years after application. Forest rotation periods are on the order of a hundred years, and forests rarely receive more than an initial fertilization. Fungal populations have many years to recover from initial fertilizer effects.

Part of the explanation for the lack of studies on long-term effects lies in the difficulty involved in maintaining forest research trials over many years. We took advantage of the controlled, replicated plots of the Salal Cedar Hemlock Integration Research Project (SCHIRP), which had been established in 1987 to test whether nitrogen and phosphorus fertilizer would mitigate growth check of regenerating western hemlock (*Tsuga heterophylla*) on northern Vancouver Island (Mehmann et al. 1995; Mallik and Prescott 2001). Plots had been fertilized in 1987 and

1997 with 300 kg/ha nitrogen (N), with 300 kg/ha nitrogen plus 100 kg/ha phosphorus (N + P), or left unfertilized as controls. The fertilization had a dramatic and lasting effect on tree growth, and hemlock volume was ten times as great in N + P plots as in control plots 5 years after the second fertilizer application (Blevins et al. 2006). Plots fertilized with N alone showed an initial increase in growth compared with control plots, but the difference disappeared over the years. Hemlock foliar P levels, after 15 years, were 0.14% in N + P plots but only 0.07% in control plots, a significant difference, while foliar N levels were not significantly changed (Blevins et al. 2006). Last fertilized 7 years before the beginning of our study, these plots offered an excellent opportunity to evaluate whether fertilization also had a long-term effect on the ectomycorrhizal fungal community.

Other studies have shown that within a year or two of application, fertilizer may reduce mycorrhizal populations and alter diversity. The numbers of ectomycorrhizae were, for example, reduced in western hemlock during the first 9 months after an application of urea fertilization, and 18 months after fertilization, the relative abundance of species, but not the number of mycorrhizal tips, had been altered compared with control plots (Gill and Lavender 1983). Also in hemlock, within 18 months of fertilization, Kernaghan et al. (1995) noted a trend towards reduction in the proportion of *Cenococcum geophilum* and mycorrhizal types lacking a mantle. Several earlier studies, including investigations of mycorrhizal communities of Norway spruce, *Picea abies* (Kårén and Nylund 1997; Fransson et al. 2000) and of oak (Avis et al. 2003) showed that annual inputs of nitrogen fertilization changed species composition, as assessed by morphotypes and/or molecular identification of root tips. Berch et al. (2006) detected lower ectomycorrhizal diversity and changes in community composition of lodgepole pine (*Pinus contorta*) in sites receiving annual N + P fertilization. In a red pine (*Pinus resinosa*) forest, ectomycorrhizal diversity was significantly lower in N-fertilized plots; changes in community structure in N plots included a loss of *Lactarius theiogalus* (a dominant species in control plots) and increase in *Piloderma* sp. (making it the dominant species of the N fertilized plots; Frey et al. 2004).

The purpose of this research project was to study the effect of earlier N and P fertilization on the ectomycorrhizal fungal community of western hemlock, by comparing fungal species detected as DNA sequences. Our predictive hypothesis was that fertilization had a long-term legacy of reduced diversity and altered species composition of mycorrhizal fungi, detectable 7 years after the last fertilizer application. In the process, we hoped to contribute basic information about which species were frequent, and therefore potentially important, fungal partners of western hemlock and to test whether fertilization history changed the ectomycorrhizal fungal community.

Materials and methods

Site description

This study took place on the "Plot Fertilization Trials" of the SCHIRP research site on northern Vancouver Island, located between Port McNeill and Port Hardy, British Columbia, Canada (50°38′ N, 127°24′ W). The site is in the Coastal Western Hemlock very wet maritime biogeoclimatic subzone (Pojar et al. 1991). Elevation is 50 m (Blevins et al. 2006). Soils at our study site are moderately to imperfectly drained humo-ferric podzols with more humus, and mineral soil textures ranged from silty clay loams to fine sand with course fragment content (Blevins et al. 2006; Lewis 1982). The pH of soils in neighboring old growth cedar/hemlock plots ranged from 3.4 to 4.8 (Prescott et al. 1993). Site features and soil characteristics match a 01 Site Series, as classified using the Biogeoclimatic Ecosystem Classification (Green and Klinka 1994; McWilliams et al. 2007). This area receives 1,900 mm of precipitation annually; most is in the form of rainfall. The average temperature maximum of just over 17°C occurs during July and August, and the minimum of just below 0°C occurs during October to February. In addition to western hemlock, the major species comprising forest vegetation are *Thuja plicata*, *Gaultheria shallon*, *Vaccinium parvifolium*, *Vaccinium ovalifolium*, *Rubus spectabilis*, *Cornus canadensis*, *Anaphalis margaritaceae*, *Blechnum spicant*, *Dryopteris expansa*, and in some areas, *Pinus contorta*.

Western Forest Products Ltd. operates this site, which they classified as a "S1 Cedar Hemlock phase" (Lewis 1982), as part of Tree License 25 Block 4. In 1979, our sample sites were clearcut and slashburned, and then planted with western hemlock plugs at 1,030 stems per hectare (Blevins et al. 2006). In 1987, when regenerating trees were 7 years old, plots 25×25 m in size, in replicates of three, and with a 2.5-m buffer zone were established in a randomized block design to study the effects of fertilizer treatments (Fig. 1; Blevins and Prescott 2002). Broadcast treatments were re-applied in 1997 as per the original prescriptions for each plot (Blevins et al. 2006).

Sampling design

A total of nine plots were sampled at the SCHIRP site: three plots unfertilized, three plots fertilized with 300 kg/ha nitrogen (applied as urea), and three plots fertilized with 300 kg/ha nitrogen in combination with 100 kg/ha phosphorus (applied as triple superphosphate). Using a soil auger (4.5 cm diameter, 10 cm in depth), a core was extracted from each of the four cardinal directions at the crown edge of each of the four randomly selected trees per plot. Samples included the organic layer of the soil and sometimes included the mineral layer where the organic

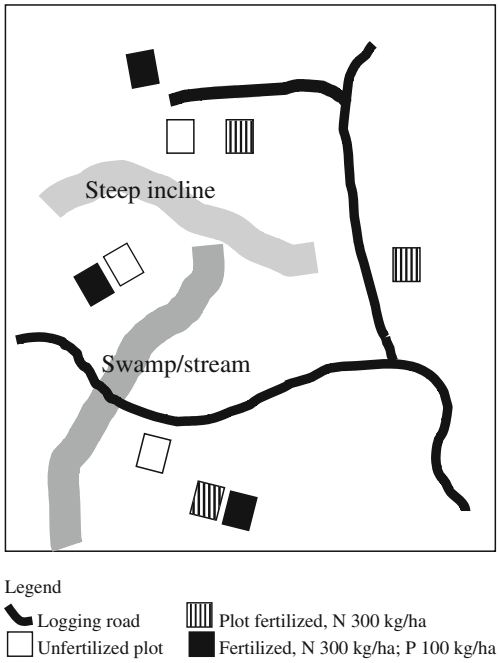

Fig. 1 Map of sample plots, showing random block design of treatments. Plots were 25 m in diameter

layer was thin. When stumps and logs covered the target sample spots, the accessible surface along the crown edge was divided into four sections and a core was collected from each. Diameter at breast height (DBH) and distance to crown edge were measured for each sample tree; distances among trees sampled were also measured.

Samples were collected in October and November 2004, and February 2005. Other studies suggested that changes in ectomycorrhizal colonization over the winter would be minimal (Majdi et al. 2001; Cheng and Bledsoe 2002; de Román and de Miguel 2005). One plot was sampled from each treatment during each collecting trip. Samples were stored at 4°C. Within a week after collection, the four core samples from each tree were washed over a 0.5-mm sieve. Using a dissecting microscope at ×20–40, 25 mycorrhizal root tips were selected. Roots from individual cores were distributed evenly across a grid with 1 cm×1 cm squares. Using a random number table, 25 ectomycorrhizal root tips were harvested from grid locations corresponding to the random numbers. Only tips that looked rounded and healthy, as opposed to shrunken and wrinkled, were included. Typically, this process consumed half or more of the ectomycorrhizae in the sample. The four 25-tip collections from one tree were pooled into 100-tip samples, frozen in liquid nitrogen, lyophilized, weighed, and stored at −20°C. The ectomycorrhizae varied in appearance under the dissection microscope, and tips representing some of the different forms were sectioned and examined at ×400 to verify that a Hartig net and healthy cortical cells were present. Almost 100% of root tips were mycorrhizal.

Amplification and cloning of fungal ribosomal DNA

DNA was extracted from each 100-tip sample using the DNeasy Plant Maxi DNA kit (model 68163, QIAGEN, Mississauga Ontario 2004) following the manufacturer's protocol. The ribosomal internal transcriber spacer (ITS) regions ITS1 and ITS2, the 5.8S ribosomal unit and the 5′ end of the large ribosomal subunit were amplified using 0.5 μM concentrations of primers TW13 and fungal-specific ITS1F (Bruns, http://plantbio.berkeley.edu/~bruns/primers.html), 12.5 μl of a 1:20 dilution of the DNA extract, a PuReTaq Ready-To-Go PCR Bead (Amersham Biosciences Lmt.) following manufacturer's protocol, and a total reaction volume of 25 μl. Cycling parameters were: initial denaturation (5 min, 94°C), followed by 35 cycles (94°C, 10 s; 55°C, 20 s; 72°C for 30 s plus 4 additional seconds per cycle), and then a final extension at 72°C for 7 min. After purification using EZNA Cycle-Pure Kit (Omega Bio-tek, Inc, Doraville, USA) following the manufacturer's protocol, PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions. This resulted in a total of 36 clone libraries (one library per sample; one sample of the DNA extract from 100 pooled root tips per tree, four trees per plot; three replicate plots per treatments; three treatments). Cloned inserts were PCR-amplified using primers M13R and M13F. From each library, 28 clones were selected and sequenced (by Macrogen Inc. South Korea) using the primer ITS1F (28 clones per library, 36 libraries, resulting a total of 1,008 clones sequenced).

Sequence and phylogenetic analyses

Chromatograms were examined and corrected manually using the Autoassembler DNA software (Applied BioSystems Inc, Perkin Elmer Corporation). Preliminary BLAST searches assigned sequences to genera. Putative chimeras were excluded after having been detected by strongly conflicting BLAST species matches of adjacent regions of the same clone. Congeneric sequences were aligned in ClustalX and the alignments were adjusted manually. Phylogenies were inferred using 500 fast parsimony bootstrap replicates (without branch swapping) in PAUP* version 4.0b10 (Swofford 2003). Sequences giving long branch lengths in genus phylogenies or with unusual substitution patterns in alignments were checked by BLAST analysis and again, putative chimeras were excluded from further analysis. Sequences that were 97% or more identical in the ITS1, 5.8S, and ITS2 regions were generally assigned to same operational taxonomic unit (OTU;

O'Brien et al. 2005). Sequences in *Cortinarius* subsection *Dermocybe* were only assigned to the same OTU when they were 99% or more identical (see "Discussion" for explanation of difference in percent identity). OTUs detected, with an exemplar sequence designated by a GenBank accession number, are given in Supplementary Table S.1. Sequences are available in GenBank (accession numbers DQ474311–DQ474385, DQ474392–DQ474757, and DQ481670–DQ482029).

Diversity measures and statistical analyses of clone abundance

To examine how treatments affected ectomycorrhizal fungi, we estimated species diversity and richness at two hierarchical levels, per clone library and per plot. The numbers of sequences, which could be counted, were used instead of numbers of fungal individuals, which cannot readily be counted. Sampling effort was tested within individual clone libraries and within plots using Coleman collector curves generated by EstimateS software version 7.5.0 (Colwell, University of Connecticut, USA). The relative abundance and species dominance between treatments was examined using rank abundance curves. Slopes of the curves were compared using ANCOVA (Zar 1999). Shannon's H', Simpson's D, and abundance measures based on Shannon's index were calculated (McCune et al. 2002) and then averaged for each treatment. Richness was estimated using Chao1 and Jack1 using EstimateS and 100 randomizations for each test. Diversity indices and estimates for each treatment were not normally distributed based on the Shapiro–Wilk test, and Bartlett's test showed that variance was not equal, so non-parametric Kruskal–Wallis tests were used to compare treatment effects.

Multivariate analyses of OTU presence and absence

Non-parametric multivariate analyses using PCORD software (version 4.34, MjM Software Design, OR, USA) were applied to study the effects of fertilizer treatment on species composition. Each clone library was treated as one sample and the presence/absence but not the frequency of each OTU was used. Non-metric multidimensional scaling (NMS) served to display the overall structure of OTU presence/absence, to determine the dimensionality of the data set and to detect whether the structure in the response data was stronger than expected by chance. Sorenson's distance measure was used with the automatic PCORD settings including a maximum number of iterations of 400, beginning from a random seed and starting with six axes, and with 40 real runs and 50 randomized runs (McCune and Mefford 1999). The final instability criterion was 0.00001. Biplot overlays of measured tree response variables on the NMS ordinations included: DBH, distance to crown edge, and root-tip biomass.

Multi-response permutation procedures (MRPP), with Sorenson's distance measures, were applied as nonparametric tests of the null hypothesis that fungal species composition did not significantly differ across fertilizer treatments (McCune et al. 2002). Indicator species analyses and Monte Carlo tests evaluated whether particular fungal OTUs were significantly correlated with treatment.

Results

Patterns of fungal diversity among clone libraries

We identified 77 OTUs from among 922 unambiguous and non-chimeric sequences of ectomycorrhizal fungal species and of fungal root associates of western hemlock. Of the 77 OTUs, 17 corresponded to identified species' sequences in GenBank. An additional 41 OTUs clustered with identified genera (Supplementary Tables S.1 and S.2). The vast majority, or 91.6% of sequences representing 52 OTUs, were from ectomycorrhizal genera (Fig. 2). Five OTUs were responsible for 51% of clones: *Piloderma fallax*, *Lactarius pseudomucidus*, *Lactarius* sp. 1, *Cantharellus tubaeformis*, and *Cenococcum* sp. 3. Each of these five OTUs was responsible for 4–13% of the clone sequences (Fig. 2). *P. fallax*, *Cenococcum* sp. 3, and *L. pseudomucidus* occurred in high frequency among all clone libraries of all treatments. Numbers of clones of a species were not always uniform across samples (Fig. 3). Clones of *C. tubaeformis* and *Lactarius* sp. 1 occurred in few clone libraries (Fig. 3), but when they were present in a library they were extremely abundant (Fig. 2). In contrast to these five, most OTUs were detected infrequently and 49% of the total clones sequenced represented 72 OTUs, each of which accounted for less than 4% of the total number of clones sequenced. Of these OTUs, 39 occurred as singletons.

Of the clones sequenced, 8.4% comprised 25 OTUs of uncertain or unlikely ectomycorrhizal status (Supplementary Table S.2). Of these OTUs, six were 97–99% similar to GenBank sequences of ectomycorrhizal root tips from environmental samples, five were most closely related to dark septate endophytes, one matched a saprophytic ascomycete, *Chaetosphaeria* sp., and 13 did not match any other GenBank sequences identified to genus.

In all plots from all fertilizer treatments, fungi were diverse and heterogeneous. Rank abundance curves showed little evidence of species dominance (Fig. 4a) and their slopes were not significantly different across fertilizer treatments, as determined by ANCOVA (F ratio 1.60, P value 0.21). Observed and estimated species richness did not differ significantly among treatments (Fig. 4b). For all

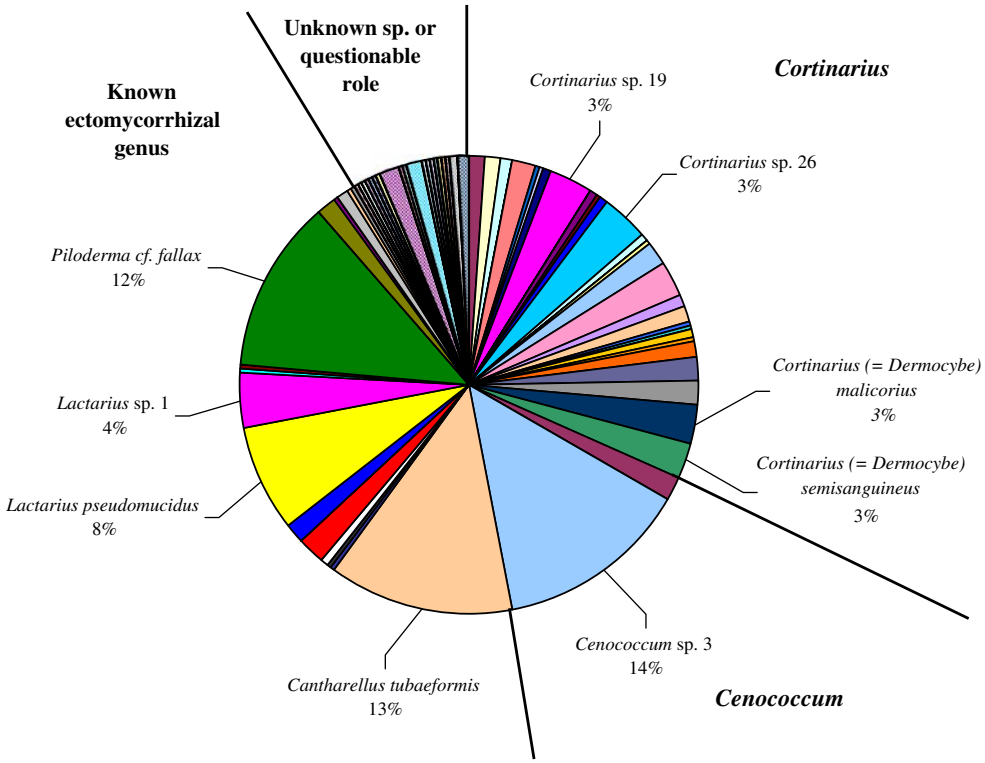

Fig. 2 Pie chart showing the relative abundance of clones by operational taxonomic unit (OTU) group: 32% of the clones (32 OTUs) were from *Cortinarius* including *Cortinarius* subgenus *Dermocybe*; 15% of the clones were from the three OTUs of the *Cenococcum geophilum* species complex; 44% of the clones (17 OTUs) represented other known ectomycorrhizal fungal genera; 8.4% of the clones (comprised of 25 OTUs) were unknowns, from saprotrophs, or closest to ericoid mycorrhizal fungal sequences

treatment groups, the estimated richness was almost double the observed richness at both the clone library and plot level (Fig. 4b). Most of the Coleman collector curves did not plateau for either clone libraries or plots, indicating that sampling effort was insufficient to achieve saturation at either level (Fig. 5). The few individual curves for clone libraries that did reach saturation were typically dominated by *C. tubaeformis* or *Lactarius* sp. 1. All treatment groups had relatively high diversity and evenness (Fig. S1). Shannon's *H'*, Simpson's *D*, and evenness did not differ significantly among treatments at either the mean clone library or plot levels (Fig. S1).

Multivariate analyses of species composition of fungi associated with western hemlock roots from different fertilization histories.

The MRPP analysis indicated a significant difference in species composition among fertilizer treatments (*P* value 0.03). Multiple pair-wise comparisons between treatments indicated that species composition in N + P plots were significantly different from control (*P* value 0.02) and N plots (*P* value 0.01), but those in control and N plots were not significantly different from each other (*P* value 0.70). We detected differences in species composition among treatments only when individual OTUs were the units being analyzed. When congeneric OTUs were pooled, differences in species composition among treatments were no longer significant in MRPP. Indicator species analysis showed three OTUs to be significantly correlated with a particular treatment (*P* value<0.05). The presence of *Cortinarius cinnamomeus* was correlated with control and N plots, whereas the presence of *Cortinarius* sp. 18 and sp. 19 was correlated with N + P plots (Fig. 3). Because MRPP results indicated that N and control plots did not significantly differ in species composition, control and N plots were pooled in the indicator species analysis.

Discussion

Our study showed that differences in mycorrhizal fungal species composition followed a history of different fertilization treatments in western hemlock. Even though plots had not been fertilized since 1997, species composition in N + P plots was significantly different from the control or N plots. Differences in ectomycorrhizal fungal composition between N + P communities compared to control and N plots were driven in part by the presence and absence of three indicator species that were directly correlated with plot type, based on Monte Carlo tests. Other fungal species may also have responded to fertilization, but as Kranabetter et al. (2005) point out, statistically significant patterns are difficult to capture, given that most species were present in high diversity and at low frequencies.

Phosphorus and N fertilization had complex effects on these sites and we do not know if improved tree vigor or site quality caused by the fertilization were driving the changes in the fungal community or if the fertilization directly affected some mycorrhizal species. In our plots,

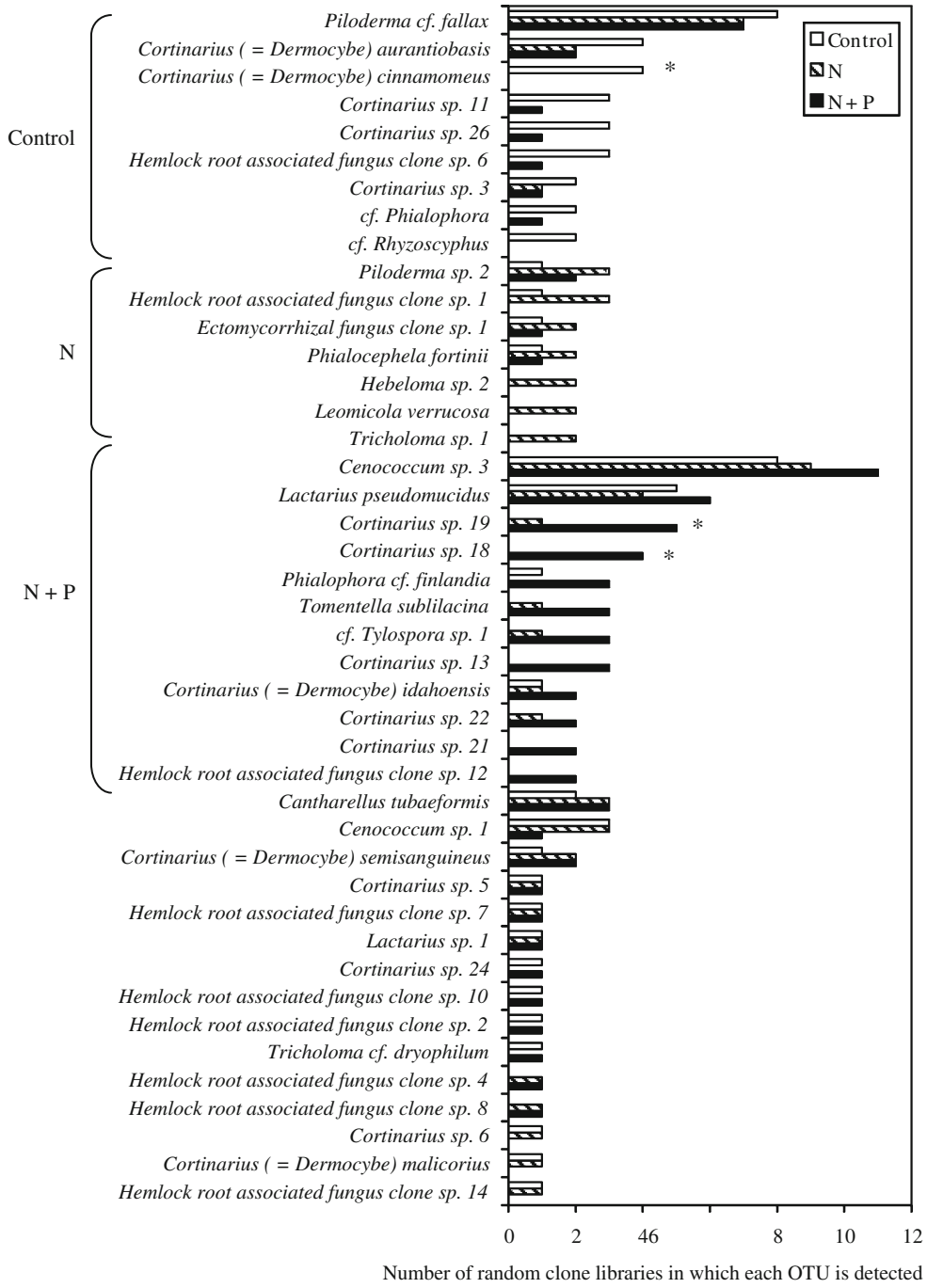

Fig. 3 Presence and absence of sequence types (operational taxonomic units, or 'OTUs') from the 12 mycorrhizal root samples from each fertilizer treatment. *Each bar* represents the number of samples that contained sequences of the OTU listed to the left. An *open bar* gives the number of samples from control plots containing the OTU; a *hatched bar* gives the number from N fertilized plots; and a *solid bar* gives the number from N + P-fertilized plots. *Brackets* indicate the treatment in which an OTU was most consistently present. Some sequence types, such as *Piloderma* cf. *fallax* were present in roughly the same proportion of samples from all three plot types. On the other hand, an *asterisk by a bar* designates an indicator species, defined as an OTU that was unequally distributed among treatments and was present in more samples from at least one treatment than expected by chance

fertilization corrected a severe nutrient deficiency so that western hemlocks had adequate foliar nutrient concentrations and their biomass increased (Blevins and Prescott 2002; Negrave 2004). Bennett et al. (2003) suggested that phosphorus addition increased rates of nitrogen mineralization on similar sites. With improved nutrient status, trees may over time have contributed more photosynthates to their mycorrhizal symbionts (Lynch and Whipps 1990; Wardle et al. 2004). Suggesting that many ectomycorrhizal fungal species recovered from any initial damage from fertilizer in the course of the long life spans of host trees, the five most common fungal species appeared in a similar proportion of clone libraries from all treatments and observed fungal diversity was almost equally high in all treatments. If the fertilization led to increased root growth and density, this may have provided increased niche availability for the establishment of mycorrhizal fungi (Bruns 1995; King et al. 2002), possibly leading to differences in fungal community structure that we observed.

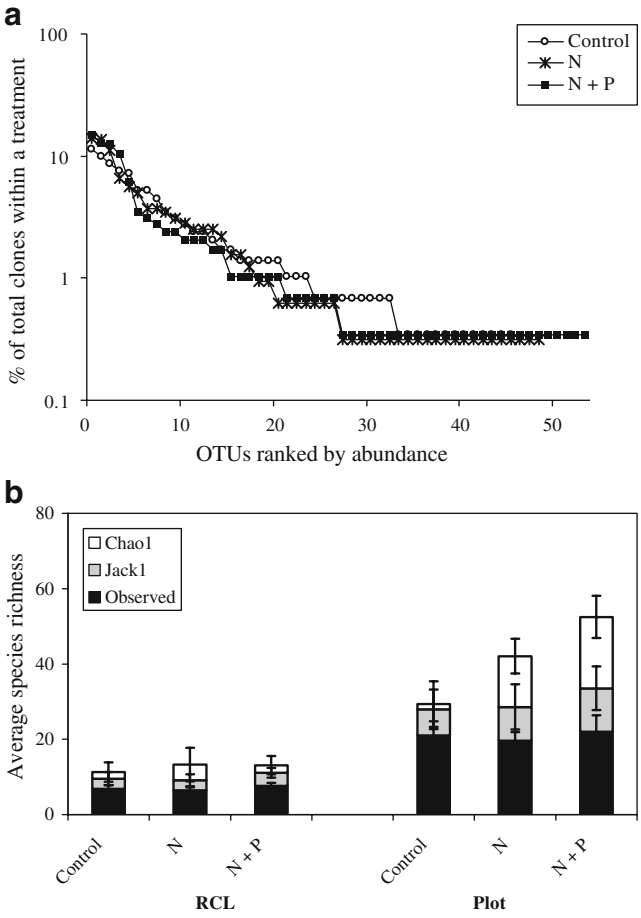

Fig. 4 **a** Rank abundance curves of ectomycorrhizal fungi detected in control (*open circles*), N fertilized (*stars*), and N + P-fertilized (*closed squares*) plots. Rather than being dominated by few common sequence types, the clone libraries from all three treatments had many low-frequency operational taxonomic units. **b** Species richness of ectomycorrhizal fungi was not significantly different across fertilizer treatments. Average richness is plotted for each fertilizer treatment, first for samples of mycorrhizal root tip (*RCL*, random clone libraries; $n=12$), and second for plots ($n=3$). For each bar, the observed richness and the Jack1 and Chao1 estimated richness are given. Error bars are 1 SE for each mean value of observed and estimated richness values

Mycorrhizal communities could also be influenced by other environmental conditions, including soil properties (Bruns 1995; Cairney 1999; Hagerberg et al. 2003; Lilleskov and Bruns 2003). Silvicultural trials have shown that long-term improvements in nutritional site quality in CH ecosystems occur with N + P fertilization. Humus from plots fertilized with N + P had increased mineral N, reduced salal leaf tannin concentrations, and increased microbial activity 10–13 years after a single fertilizer application (Bradley et al. 2000; Bennett et al. 2003). Plots fertilized with N + P also have increased dry mass in the L and F layer of the forest floor (Leckie et al. 2004). Since fungal species differ both in their ability to use various forms of N and in their response to increased N availability (Ek 1997; Bidartondo et al. 2001; Lilleskov et al. 2002b; Lilleskov and Bruns 2003), increases in N may alter the dominance structure of the ectomycorrhizal fungal community because differential sensitivities influence competitive interactions among fungal species (Wallenda and Kottke 1998; Taylor 2002; Edwards et al. 2004). This could have selected for indicator mycorrhizal fungi with different substrate preference (Harvey et al. 1978; Tedersoo et al. 2003). Future studies using multivariate approaches could help identify environmental correlations of fungal community structure with differences in soil properties and different fertilization histories.

Regardless of fertilization treatment, on the western hemlock plots, fungal diversity was high and communities were heterogeneous. High heterogeneity, with only a few common species and many rare species, may be the usual

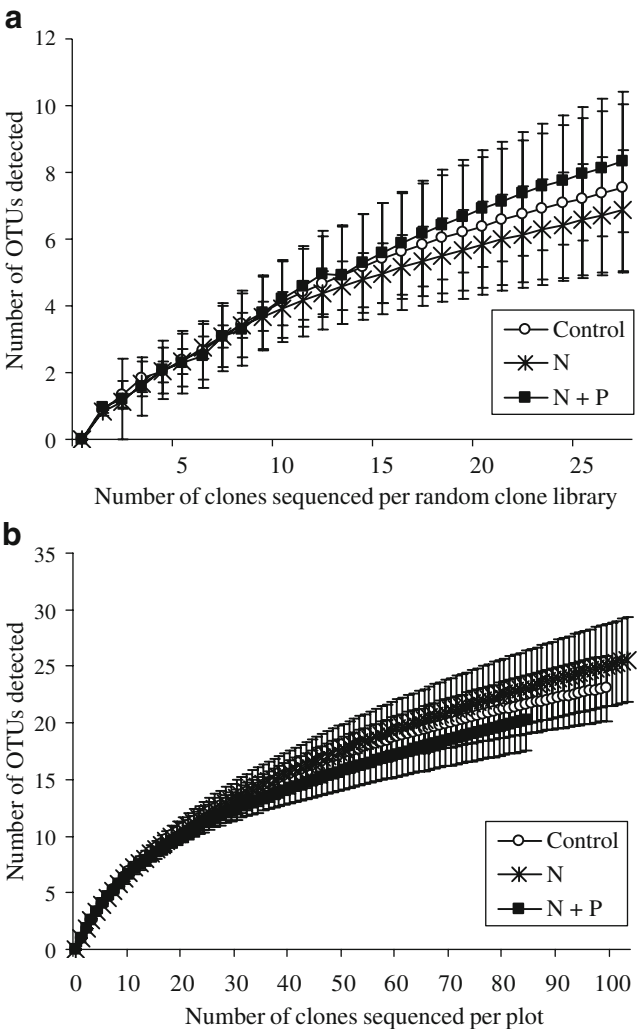

Fig. 5 Mean Coleman collector curves for each fertilization treatment. The number of ectomycorrhizal fungal OTUs detected with increasing the number of clones sequenced do not reach saturation, either for **a** random clone libraries or **b** plots. *Bars* represent 1 SE

structure of ectomycorrhizal communities (Taylor 2002). Our sequences also revealed previously unreported species diversity in association with western hemlock. For comparison of clone library detection with other detection methods, we put together a list of 197 fungal species that were suspected or confirmed ectomycorrhizal partners of western hemlock from the west coast of North America (Trappe 1962, 2004; Molina 1980; Kropp 1981a, b; Kropp and Trappe 1982; Molina and Trappe 1982; Durall et al. 1999; O'Dell et al. 1999; Kranabetter and Kroeger 2001; Kranabetter et al. 2005). Of the 52 OTUs representing likely ectomycorrhizal species that we detected, at most 19 had already been included in the list of 197 known hemlock associates (Supplementary Fig. S2, Table S.1). Based on collector curves and richness estimates, the actual number of OTUs that could have been detected was double the number observed within clone libraries and among samples of a given plot. Even from individual clone libraries, only seven of the 36 collector curves showed signs of reaching sampling saturation and none of the individual curves for plots showed saturation. For several reasons, we believe that the total number of fungal species associated with the roots was probably higher than our estimates. We only sampled from the crown edge on our plots and a different suite of fungi may have been present in other microhabitats beyond or within the crown edge (Kranabetter and Wylie 1998; Nara et al. 2003). The 97% cutoff for OTU identification was likely conservative. DNA extraction, PCR amplification, and cloning biases probably reduced the chance of detecting some species. We sampled only regenerating hemlock and this could explain why we did not find many of the taxa commonly associated with western hemlock in other studies of more mature stands (Supplementary Fig. S2, Table S.1.)

Cortinarius was represented by 32 OTUs, more than any other genus in this study, and it was the most frequently encountered genus in this study, appearing among clones from every library. Unfortunately, we found that few *Cortinarius* OTUs matched GenBank sequences. As members of one of the largest genera of mushrooms, *Cortinarius* species are notoriously difficult to identify. The genus includes many undescribed species, further compounding the identification problem (Peintner et al. 2004). Kranabetter and Kroeger (2001) identified 12 species of *Cortinarius* from hemlock plots but estimated that at least 30 more, unidentifiable species were present. Some *Cortinarius* species are difficult to define even with combined morphological data and sequence data. A likely example of low interspecific variation, *Dermocybe* (now *Cortinarius*) species that were distinguishable based on morphological or biochemical characters showed more than 97% identity in their ITS sequences (Liu et al. 1997). The only five *Cortinarius* OTUs that could be matched to GenBank species identifications were from former *Dermocybe* species. We placed sequences of these former *Dermocybe* in different OTUs unless they were 99% identical, which brought the OTUs into congruence with named species listed in Supplementary Table S.1.

Difficult to delimit though they may be, different species of *Cortinarius* in particular respond differently to fertilizer and to N levels (Baar 1995; Brandrud 1995; Baum and Makeschin 2000; Lilleskov et al. 2001; Peter et al. 2001; Lilleskov et al. 2002a; Avis et al. 2003). Congeneric OTUs in our study responded differently; indicator species *Cortinarius* spp. 18 and 19 were present in a higher than expected proportion of N + P clone libraries while *C. cinnamomeus* was present in a high proportion of control plots. Similarly, 3 years after the end of an 11-year regime of annual fertilization in a poplar plantation, two *Cortinarius* species were significant indicators for different treatments—*Cortinarius uliginosus* occurred only in control plots, whereas *Cortinarius croceocaeruleus* occurred only in N plots (Baum and Makeschin 2000). Studies based on morphotyping may miss significant differences among treatments if the species that respond differently look the same on a root.

In conclusion, any initial negative effects of fertilization were no longer evident after 7 years as fungal diversity, richness, and evenness were similar in fertilized and unfertilized plots and the five most abundant fungal OTUs showed no obvious preference for treatment type. Some fungi did, however, respond differently to fertilization history and most of these were unidentified to species. In fact, most of the fungi detected as sequence OTUs matched neither previously known western hemlock ectomycorrhizal species nor sequences in GenBank. The different responses of the difficult-to-identify fungi may stem from different ecological roles, and these fungi should be considered in studies of the reciprocal effects of fungi on growing trees.

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