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Effects of cloning and root-tip size on observations of fungal ITS sequences from *Picea glauca* roots

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Abstract: To better understand the effects of cloning on observations of fungal ITS sequences from *Picea glauca* (white spruce) roots two techniques were compared: (i) direct sequencing of fungal ITS regions from individual root tips without cloning and (ii) cloning and sequencing of fungal ITS regions from individual root tips. Effect of root tip size was investigated by selecting 20 small root tips (SRT, 1.0–2.0 mm long) and 20 large root tips (LRT, 5.0–6.0 mm long). DNA was isolated from each tip and PCR-amplified with fungal-specific primers. PCR reactions were divided into two portions, one of which was sequenced directly and one of which was cloned first followed by sequencing of 12 random clones. With direct sequencing all 20 SRT produced an identifiable sequence, while only 13 of 20 LRT (65%) yielded an identifiable sequence. With cloning and sequencing all 40 tips produced identifiable fungal ITS sequences regardless of size. Failure of direct sequencing in LRT was associated with the presence of multispecies assemblages. Cloning identified 18 taxa overall while direct sequencing identified four. Cloning was not affected by tip size and identified more taxa relative to direct sequencing, although cost and probability of observing lab-based contaminants (e.g. airborne or reagent-based) were higher. We suggest that standardized controls be run whenever clones are sequenced from environmental samples, including positive controls derived from pure cultures and negative controls that cover the entire extraction, amplification and cloning process. Additional studies on larger root segments and bulked samples are needed to determine whether cloning can detect fungi accurately and cost-effectively in complex environmental samples.

Key words: ectomycorrhizal fungi, endophyte, environmental samples, mycorrhiza, species identification

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INTRODUCTION

DNA-based technologies have revolutionized the study of root-associated fungi (Horton and Bruns 2001) and these techniques are rapidly becoming available to a wider range of researchers (Martin 2007). Although these techniques have opened up many research possibilities, the specific methods used to amplify and identify fungal DNA have the potential to affect observations of the fungal community in roots (e.g. Burke et al 2005, Douhan et al 2005, Renker et al 2006, Dickie and FitzJohn 2007, Jumpponen 2007). Information regarding the biases associated with a particular observational technique, molecular or otherwise, is crucial to understanding and interpreting the ecological significance of fungal observations.

The production and sequencing of clone libraries from environmental samples is a technique that is being used more frequently to study fungal communities associated with roots (Douhan et al 2005, Renker et al 2006, Smith et al 2007a, Smith et al 2007b, Morris et al 2008a, Morris et al 2008b) as well as other substrates such as soil (Anderson et al 2003; Landeweert et al 2003; Jumpponen 2003, 2007). Although the generation of clone libraries represents a powerful, culture-independent tool for observing fungal communities, many potential problems exist, including primer bias and the production of chimeric sequences (Patel et al 1996, Speksnijder et al 2001, Jumpponen 2007); nonspecificity of primers to target organisms (Anderson et al 2003, Douhan et al 2005); and differential amplification of target organisms (Reysenbach et al 1992, Renker et al 2006). More information is needed regarding the ability of clone libraries to accurately reflect the diversity of fungi associated with roots, especially in complex environmental samples.

In this study we compare the generation of clone libraries to direct sequencing for the identification of fungal ITS sequences associated with individual *Picae glauca* (white spruce) root tips. Root tips of varying size (1.0–2.0 mm vs. 5.0–6.0 mm long) were sampled to determine whether the likelihood of encountering multispecies assemblages differed at relatively small spatial scales. Although additional techniques are available for studying fungi in root samples, including DGGE, RFLP and T-RFLP (see Dickie and FitzJohn 2007), the scope of the current study was restricted to a detailed comparison of direct sequencing versus

cloning and sequencing. This study is a starting point for further investigations of the effectiveness of cloning on larger environmental samples and bulked or pooled samples. In such situations some techniques (e.g. RFLP, T-RFLP, micro-arrays, etc.) may perform poorly due to the large numbers (hundreds to thousands) of co-occurring ITS sequences and the high probability of encountering novel sequences. Sequencing of clone libraries, along with techniques such as high throughput pyrosequencing, are powerful techniques that may come closer to revealing the full diversity of fungal sequences in complex environmental samples. However the limitations and biases of these techniques require further examination.

MATERIALS AND METHODS

Collection of root segments.—Root material was collected from a 12 y old *Picea glauca* in a tree farm in Dane County, Wisconsin. Large root sections (30–50 cm) including many root tips were excavated and large soil particles were washed from roots with a water spray. Forty root tips were excised with dissecting microscope; 20 small root tips (SRT) were approximately 0.4 mm diam and 1.0–2.0 mm long, and 20 large roots tips (LRT) were approximately 0.8 mm diam and 5.0–6.0 mm long. Root tip length was measured starting directly at the root apex. Healthy tips were chosen independently of whether they appeared ectomycorrhizal. LRT occasionally had small lateral branches < 1 mm long, while SRT rarely had lateral branches. Root tips were placed individually in 200 μL microcentrifuge tubes and washed by agitating in 200 μL sterile water for 5 s, aspirating wash water and repeating twice. Each root tip was placed in a 1.5 mL microcentrifuge tube containing 50 μL filter-sterilized cell lysis solution (CLS) containing 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM EDTA, and 2% hexadecyltrimethylammonium bromide (CTAB) and frozen at −80 C.

DNA extraction and PCR.—Root tips were thawed at room temperature and ground in 50 μL CLS in 1.5 mL tubes with a sterile plastic pestle. A small amount of *Laetiporus cincinnatus* fruit body was treated similarly to the root tips and served as a positive extraction control; CLS without addition of fungal material served as a negative control. After grinding an additional 200 μL of CLS was added and each tube was agitated briefly. Tubes were incubated at 65 C for 2 h. After incubation the tubes were centrifuged at 16 110 rcf for 5 min and the supernatants transferred to clean 1.5 mL microcentrifuge tubes. Five hundred μL of −20 C 2-propanol (isopropanol) was added to each supernatant, tubes were inverted, incubated at −80 C for 15 min and centrifuged at 10 621 rcf for 20 min at 0 C. Supernatants were discarded, 500 μL of 75% ethanol (v/v) was added and tubes were centrifuged at 16 110 rcf for 5 min at room temperature. Supernatants were removed, pellets air dried at room temperature 10 min and pellets resuspended in 50 μL sterile water.

DNA in aqueous solution was cleaned with GeneClean III kits (Qbiogene) following the manufacturer's protocol with the following modifications. Fifty μL of aqueous DNA solution was combined with 150 μL of NaI solution and 5 μL of glassmilk provided with kit. Tubes were agitated continuously for 5 min followed by centrifugation at 16 110 rcf for 8 s. The supernatant was discarded and the pellet washed once with 1 mL of New Wash solution provided with the kit. After removal of New Wash pellets were air dried 15 min and template DNA eluted in 50 μL of water.

The fungal-specific primer pair ITS1F and ITS4 (Gardes and Bruns 1993) was used for the initial PCR from root material. PCR was performed with 5× green GoTaq reaction buffer and GoTaq DNA polymerase (Promega, Madison, Wisconsin). GoTaq reaction buffer was diluted to a 1× working concentration and 0.025 units of GoTaq DNA polymerase were added per microliter of reaction volume. Each primer had a final concentration of 0.2 μM, and each dNTP (Promega, Madison, Wisconsin) had a final concentration of 200 μM. Template DNA was diluted 1:50 in the final reaction volume. Thermocycler conditions were: initial denaturing at 94 C for 3 min; 30 cycles of denaturing at 94 C for 1 min, annealing at 53 C for 1 min; extension at 72 C for 3 min; and a final extension step of 72 C for 10 min. A relatively long extension time of 3 min per cycle was used to avoid the production of chimeric sequences (Jumpponen 2007).

After amplification 3 μL of product was run on a 1.5% agarose gel stained with ethidium bromide to verify the presence of amplification products. Regardless of whether amplification products were observed, 0.75 μL was removed from each reaction, including positive and negative extraction controls, for use in cloning. In preparation for direct sequencing the remainder of the amplification product was treated with Exonuclease I (EXO) and shrimp alkaline phosphatase (SAP) (USB Corp., Cleveland, Ohio) as follows: for 15 μL PCR reactions a solution containing 3.12 μL water, 0.80 μL SAP and 0.08 μL EXO was added to each reaction; the reactions with EXO/SAP were heated to 37 C for 15 min and then heated to 80 C for 20 min; after cooling, 35 μL of water was added to each reaction. After this procedure PCR products were ready for direct sequencing.

Cloning.—Cloning of PCR products was accomplished with pGEM-T Vector System II kits and JM109 competent cells from Promega (Madison, Wisconsin). Ligations were performed by mixing 0.75 μL of PCR product to be cloned with 1.25 μL of 2× rapid ligation buffer, 0.25 μL of pGEM-T Vector and 0.25 μL of T4 DNA Ligase provided with kits. Ligation reactions were incubated approximately 12 h at 4 C. Bacterial transformations were performed by adding 0.5 μL of ligation product to 12.5 μL of JM109 competent cells, mixing by gently flicking tubes, incubating on ice for 20 min and then heat shocking at 42 C for 47 s. Tubes were incubated on ice for 2 min, after which 237.5 μL liquid SOC medium was added. Tubes were incubated at 37 C while shaking 1 h 45 min, and 125 μL was spread on each of two LB-agar plates amended with 100 mg/L ampicillin (Sigma), 80 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) and 120 mg/L IPTG (isopropyl β-D-1-thiogalactopyranoside) (Promega). Plates were incubated at 37 C for 16 h.

Bacterial cells carrying vector with successfully ligated PCR product result in white colonies under the conditions described above. ITS regions from 12 randomly selected white colonies were sequenced for each of the 40 root tip samples, while 80 white colonies were sequenced from the positive controls. All white colonies produced by negative controls were sequenced. To amplify the cloned ITS regions from bacterial colonies a PCR reaction was prepared as previously described with the exception that template DNA was added by placing a small amount of a transformed bacterial colony into the reaction with a sterile 200 μL pipette tip. Thermocycler conditions were: initial denaturing at 94 C for 10 min; 30 cycles of denaturing at 94 C for 40 s, annealing at 53 C for 40 s, and extension at 72 C for 90 s; and a final extension step of 72 C for 10 min. After PCR the reactions were checked for product and treated with EXO/SAP as previously described.

Sequencing and identification of sequences.—Sequencing reactions were performed following the BigDye terminator protocol (ABI Prism) with primer ITS4. Sequencing products were cleaned with CleanSeq (Agencourt) magnetic beads following the manufacturer's protocol. Sequencing products were analyzed at the University of Wisconsin Biotech Center, and final sequences were aligned with Sequencher 4.2 (GeneCodes Corp.). Chimeric sequences were detected by manually comparing ITS1 and ITS2 regions independently against GenBank data and noting conflicting phylogenetic placement within sequences. Sequences were assigned a putative identification base on BLAST comparisons to GenBank (NCBI) sequences. Sequences varying less than 2% in base pair identity were considered the same and were designated as ''taxa''. Taxon accumulation curves were generated with Sanders' (1968) rarefaction equations as modified by Hurlbert (1971). These equations allow for the exact calculation of the mean taxon accumulation curve over all possible permutations of sampling order.

RESULTS

PCR amplification from 20 of 20 SRT and 17 of 20 (85%) LRT yielded products that could be viewed in agarose gels stained with ethidium bromide (TABLE I); root tips 34, 35 and 38 did not amplify sufficiently to produce PCR products that could be viewed in agarose gels. Direct sequencing of PCR products from 20 of 20 SRT and 13 of 20 (65%) LRT yielded identifiable ITS sequences (TABLE I) and sequences were identified as one of four taxa (TABLE II, FIG. 1). The seven LRT that did not yield identifiable ITS sequences with direct sequencing all produced chromatograms with strong signal and many overlapping peaks; base calling was impossible for these chromatograms. This is in contrast to the negative controls, which produced chromatograms with low signal and completely random peaks when directly sequenced (negative controls are discussed in detail below).

Cloning was successful for all 40 root tips and yielded at least 100 white bacterial colonies, with the exception of three samples that yielded 50, 20 and 40 white colonies (tips 34, 35 and 38 respectively). A total of 480 white colonies that originated from the root samples were sequenced (12 colonies from each of the 40 root tips) and 460 (96%) yielded identifiable sequences, none of which were identified as chimeric. On average each SRT yielded 1.40 unique taxa ($n = 20$, standard error = 0.13), while each LRT yielded 1.85 taxa ($n = 20$, standard error = 0.23). Number of taxa per root tip did not differ significantly ($P = 0.182$) between SRT and LRT with a Mann-Whitney test (Analyse-it Software 2.03). Cloning of PCR products from all 40 root tips resulted in the identification of 18 taxa (TABLE I), seven of which were Basidiomycota and 11 of which were Ascomycota (TABLE II). The 20 SRT yielded nine taxa with cloning, while the 20 LRT yielded 15 taxa (FIG. 1).

Two of the three positive extraction controls using a *Laetiporus cincinnatus* fruiting body produced visible PCR bands and were confirmed to be *Laetiporus cincinnatus* with direct sequencing. The two positive controls with visible bands yielded > 100 white colonies when cloned, while the positive control with no visible band yielded 50 colonies. Thirty-two white colonies were sequenced from the two positive controls that produced visible bands; 31 yielded *L. cincinnatus* sequences and one yielded a *Candida* species. Forty-eight white colonies were sequenced from the single *L. cincinnatus* extraction that produced no visible PCR band; 30 yielded *L. cincinnatus* sequences, 15 yielded the same *Candida* species mentioned above, two yielded a *Cladosporium* species and one an *Epicoccum* species.

None of the three negative controls yielded visible PCR bands. As mentioned previously the negative controls produced chromatograms with low signal and random peaks when directly sequenced. When cloned the three negative extraction controls yielded a total of 19 white colonies, all of which were sequenced. Seventeen of the 19 colonies yielded identifiable sequences representing seven distinct taxa. The closest GenBank matches for the seven taxa were *Alternaria alternata*, *Bullera pseudoalba*, *Flammulina velutipes*, *Graphium basitruncatum*, *Leptosphaeria* sp., *Trametes versicolor* and *Typhula phacorrhiza*. Only one of these, *Alternaria alternata*, also was present among the sequences identified from root tip clones, where it occurred as a single clone in a single LRT (Tip No. 38). This particular root tip did not produce visible PCR products and yielded only 40 white bacterial colonies when cloned.

TABLE I. Putative identifications of fungal ITS sequences from 20 small and 20 large *Picea glauca* root tips

Root Tip Size*	Tip Number	Visible PCR Products?	Direct Sequencing Results**	Cloning Results***
SRT	1	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (12)
SRT	2	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
SRT	3	yes	*Sebacina incrustans*	*Sebacina incrustans* (10), *Wilcoxina* sp. (2)
SRT	4	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (12)
SRT	5	yes	Pezizales sp. A	Pezizales sp. A (9), *Sebacina incrustans* (3)
SRT	6	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
SRT	7	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (11), no sequence (1)
SRT	8	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), *Epicoccum nigrum* (1)
SRT	9	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
SRT	10	yes	Pezizales sp. A	Pezizales sp. A (12)
SRT	11	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
SRT	12	yes	*Sebacina incrustans*	*Sebacina incrustans* (10), no sequence (2)
SRT	13	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (9), *Zalerion varia* (1), *Flagelloscypha minutissima* (1), no sequence (1)
SRT	14	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), *Davidiella tassiana* (1)
SRT	15	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), *Zalerion varia* (1)
SRT	16	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (9), no sequence (3)
SRT	17	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (11), no sequence (1)
SRT	18	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), no sequence (1)
SRT	19	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (12)
SRT	20	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (10), Pezizales sp. B (1), no sequence (1)
LRT	21	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (10), no sequence (2)
LRT	22	yes	Uninterpretable chromatogram	*Sebacina incrustans* (7), *Wilcoxina rehmii* (5)
LRT	23	yes	Uninterpretable chromatogram	*Wilcoxina* sp. (8), *Clitocybula oculus* (4)
LRT	24	yes	Uninterpretable chromatogram	*Clitocybula oculus* (7), *Gymnomyces fallax* (3), *Zalerion varia* (2)
LRT	25	yes	Uninterpretable chromatogram	*Wilcoxina* sp. (7), *Clitocybula oculus* (4), *Gymnomyces fallax* (1)
LRT	26	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), *Davidiella tassiana* (1)
LRT	27	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (11), no sequence (1)
LRT	28	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
LRT	29	yes	Pezizales sp. A	Pezizales sp. A (11), *Sebacina incrustans* (1)
LRT	30	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (12)
LRT	31	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), no sequence (1)
LRT	32	yes	*Gymnomyces fallax*	*Gymnomyces fallax* (12)
LRT	33	yes	*Wilcoxina* sp.	*Wilcoxina sp.* (11), no sequence (1)
LRT	34	no	Uninterpretable chromatogram	*Sebacina incrustans* (7), *Davidiella tassiana* (2), *Malassezia restricta* (1), *Armillaria gallica* (1), no sequence (1)
LRT	35	no	Uninterpretable chromatogram	*Sebacina incrustans* (7), *Aureobasidium pullulans* (1), *Cortinarius alboviolaceus* (1), no sequence (3)
LRT	36	yes	*Sebacina incrustans*	*Sebacina incrustans* (12)
LRT	37	yes	*Sebacina incrustans*	*Sebacina incrustans* (11), no sequence (1)
LRT	38	no	Uninterpretable chromatogram	*Gymnomyces fallax* (9), *Ascomycete* sp. (1), *Alternaria alternata* (1), *Phaeosphaeria* sp. (1)
LRT	39	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (10), no sequence (2)
LRT	40	yes	*Wilcoxina* sp.	*Wilcoxina* sp. (12)

* SRT denotes small root tips (1.0–2.0 mm) and LRT denotes large root tips (5.0–6.0 mm).

** Fungal names are based on the nearest BLAST match in GenBank as presented in Table II. ''Uninterpretable chromatogram'' refers to chromatograms with high signal but many overlapping peaks.

*** For each root tip, 12 randomly selected clones were sequenced. Numbers in parentheses represent the number of clones out of 12 that corresponded to a particular fungal sequence. ''No sequence'' designates clones that produced no or low signal in the sequencing chromatogram.

TABLE II. Closest GenBank BLAST match for ITS rDNA sequences from *Picea glauca* root tips

GenBank accession numbers for ITS sequences observed in this study	Closest GenBank match & accession number	Taxonomic placement	Base pair matches to GenBank sequence		Number of root tips in which sequence was observed (40 total)
			Number	Percent	
FJ266735	*Sebacina incrustans*(AY143340)	Basidiomycota, Sebacinales	512/541	95	20
FJ266730	*Gymnomyces fallax*(AY239349)	Basidiomycota, Russulales	603/660	91	11
FJ266736	*Wilcoxina* sp. (DQ069002)	Ascomycota, Pezizales	539/543	99	10
FJ266725	*Clitocybula oculus*(DQ192178)	Basidiomycota, Agaricales	466/649	72	3
FJ266727	*Davidiella tassiana*(AY463366)	Ascomycota, Capnodiales	506/514	98	3
FJ266732	Pezizales sp. A (AJ893242)	Ascomycota, Pezizales	434/505	86	3
FJ266738	*Zalerion varia*(AJ608987)	Ascomycota	492/509	97	3
FJ266721	*Alternaria alternata*(AY433814)	Ascomycota, Pleosporales	531/533	99	1*
FJ266722	*Armillaria gallica*(AY213571)	Basidiomycota, Agaricales	809/828	98	1*
FJ266723	Ascomycete sp. (DQ092529)	Ascomycota	440/440	100	1*
FJ266724	*Aureobasidium pullulans*(AF455533)	Ascomycota, Dothideomycetes	520/520	100	1*
FJ266726	*Cortinarius alboviolaceus*(AY669657)	Basidiomycota, Agaricales	551/560	98	1*
FJ266728	*Epicoccum nigrum*(DQ026007)	Ascomycota, Pleosporales	496/509	97	1
FJ266729	*Flagelloscypha minutissima*(AY571040)	Basidiomycota, Agaricales	639/698	92	1
FJ266731	*Malassezia restricta* (AJ437695)	Basidiomycota, Ustilaginomycotina	690/692	99	1*
FJ266733	Pezizales sp. B (DQ273333)	Ascomycota, Pezizales	506/531	95	1
FJ266734	*Phaeosphaeria* sp. (DQ092527)	Ascomycota, Pleosporales	453/468	97	1*
FJ266737	*Wilcoxina rehmii*(DQ069001)	Ascomycota, Pezizales	546/552	99	1

*Asterisks denote sequences that occurred once in the dataset and were derived from samples that produced no visible PCR bands.

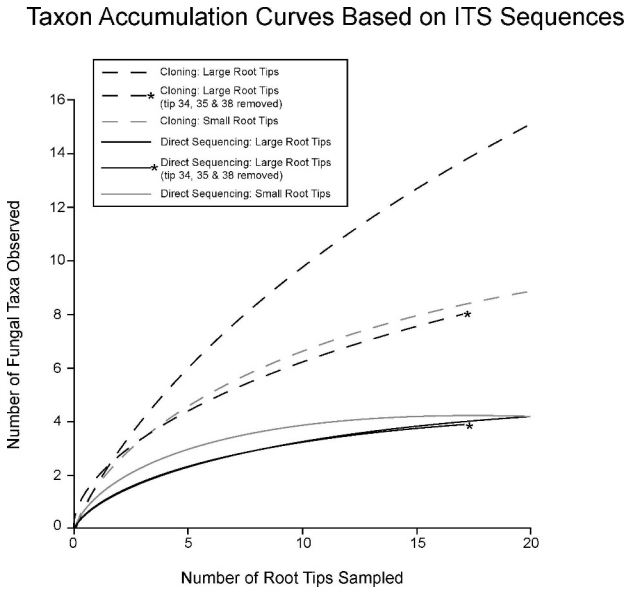

FIG. 1. Taxon accumulation curves are based on identifications of fungal ITS sequences from *Picea glauca* root tips. Taxa were designated based on 98% sequence identity and curves were generated with rarefaction equations that allow for the exact calculation of the mean accumulation curve over all possible permutations of sampling order. Root tips 34, 35 and 38 were excluded from some analyses because they did not produce PCR products that could be viewed with ethidium bromide. The two lowest curves fall directly on top of one another but were staggered slightly for ease of viewing.

DISCUSSION

Although direct sequencing is a cost-effective and relatively simple method for identifying fungal associates of roots (Horton and Bruns 2001), direct sequencing displayed high success rates in this study only when small *Picea glauca* root tips (1.0–2.0 mm) were used. Root tips of this size are difficult to excise from root systems, difficult to clean and process in individual tubes and due to their size may contain only a small amount of template DNA. The *Picea* root systems examined in this study unfortunately were composed primarily of small root tips, with larger tips being rare and difficult to sample due to high levels of branching.

Sampling techniques based on root tip pooling may be necessary in tree species that produce highly divided root systems (e.g. *Castanea*, *Quercus*, *Picea*, *Populus*, *Salix*, etc.). Without techniques that can adequately cover the entire area of finely divided root systems, sampling schemes will be limited to plant species with large and obvious mycorrhizal root tips or will be biased toward the few large root tips that occur within highly branched systems. In fine, highly divided root systems such as were observed in this study individual root tips may be in the early stages of development. By focusing sampling on developing root ends a view of the fungal community may be obtained that is biased toward fungi associated with the early stages of mycorrhizal development. For future sampling it would be desirable to sample larger root sections or pooled samples, a strategy already employed in a limited number of studies (e.g. Renker et al 2006, Smith et al 2007a, 2007b; and apparently Kernaghan et al 2003, where fine *Picea glauca* root tips appear to have been pooled, although sampling details are not entirely clear).

Direct sequencing displayed a poor success rate when larger root tips (5.0–6.0 mm) were used and clearly would not work with bulked or pooled samples. In this study seven of 20 (35%) large root tips failed to produce identifiable sequence with direct sequencing. The chromatograms from all seven root tips displayed strong signal but many overlapping peaks, making base calling impossible. This suggests that failure to produce an identifiable sequence was due to the presence of multiple overlapping sequences instead of failure of the extraction, amplification or sequencing procedures. Cloning results confirmed that the failure of direct sequencing always occurred in roots with multiple fungal ITS sequences. Although more sampling is needed to confirm these results it appears the presence of multispecies assemblages might account for a large portion of the failure rates commonly seen when root tips are subjected to direct sequencing. Data regarding the number of root tips that fail to produce sequence unfortunately are seldom reported, potentially biasing species lists created with direct sequencing. Although failure of root tips to produce an identifiable sequence is often attributed to the presence of inhibitors in the PCR reaction, poor quality template DNA, an outright lack of fungal DNA or failure of the sequencing reaction, the presence of multispecies assemblages also might help to explain the failure rate of direct sequencing.

Cloning was not affected by root tip size and therefore appears to avoid many of the problems associated with direct sequencing. All 40 root tips produced fungal ITS data when cloned regardless of size, and 18 taxa were identified with cloning, compared to only four taxa with direct sequencing. Of note, cloning identified multiple taxa in nine root tips where direct sequencing produced a readable sequence (see TABLE I); the direct sequencing chromatograms for these root tips displayed some evidence of smaller, ''underlying'' peaks, a phenomenon we have observed in other root tip data (unpubl data). This indicates that fungal species might remain unobserved even in root tips that successfully sequence with direct sequencing, suggesting that

multispecies assemblages may be more common than might be believed based on direct sequencing data.

The common occurrence of multiple fungal species in relatively small root tips, a result also observed by Morris et al (2008a) in *Quercus* species, is a strong argument for the use of observational techniques that are not adversely affected by co-occurring species. Situations where a root tip is occupied by a single fungal species may be rare in nature but seem common based on the biases of techniques such as direct sequencing and RFLP, where failed sequencing attempts are seldom reported and messy data often are discarded. Many species, including mycoparasites, may occur only in association with other fungi, making their detection difficult or impossible with direct sequencing.

Although more expensive than direct sequencing, sequencing of clone libraries is a promising tool for exploring the complexity of the associations between fungi and plant roots. However practical problems still need to be resolved before cloning is regularly used on larger or pooled root samples. Two common problems with cloning are: (i) the extreme sensitivity of cloning to airborne or reagent-based contamination and (ii) the lack of clearly defined and widely accepted standards that can be used as positive and negative controls. These problems are interrelated because it is the sensitivity of cloning that makes appropriate controls necessary every time samples are cloned.

The sensitivity of cloning to lab-based contamination was demonstrated by the three independent negative controls run in this study, which consisted of blank samples that were subjected to the entire DNA extraction, amplification and cloning process. Each negative control produced a small number of white bacterial colonies (nine, six and four colonies for the three controls). When these were sequenced, seven taxa were observed, one of which, *Alternaria alternata*, also occurred in the root tip data. When our PCR negative controls were cloned no white colonies were observed, indicating that the PCR and cloning reagents contained no detectable DNA. The extraction process, which includes the longest amount of time where samples are exposed to the air, is therefore the step where contamination was introduced via either reagents or the air. While it is tempting to use PCR negative controls (containing only PCR reagents and water) to test for lab-based contamination, such samples have not been subjected to the entire process of DNA extraction, amplification and cloning.

Although the negative controls employed in this study indicated some level of contamination in the absence of sample DNA, it is important to remember that these controls were relatively rigorous compared to what is commonly used (e.g. cloning of negative PCR controls or no cloning controls) and that all anomalies were reported fully. Until controls such as these are commonly run and reported it will be difficult to determine what constitutes typical levels of contamination in laboratories that conduct ecological research. The results of the positive and negative controls in this study, taken together with the consistent occurrence of the dominant species in the direct sequencing and cloning data, suggest that cloning produced a dataset that is generally trustworthy. Airborne or reagent-based contamination apparently was observed only when cloning was employed in the absence of sample DNA; under normal circumstances sample DNA presumably swamps trace amounts of background DNA.

Even when a sample did not produce a visible PCR band (as was the case for root tips 34, 35 and 38 and one of the positive controls), cloning produced primarily the expected sequences: mycorrhizal fungi in the root tips and *Laetiporus cincinnatus* in the positive control. However samples that do not produce visible bands present a dilemma; should such samples be included with the rest of the dataset or excluded because they amplified differently relative to the other samples? The cloning data indicate that the dominant species in such samples are consistent with the rest of the dataset; however it is impossible to know whether the rare species in such samples are truly derived from the sample or are lab-based contaminants. In such situations it might be best to take a cautious approach; report the data as observed (rather than excluding it), but mark samples in such as way that they can be removed from the dataset if so desired. For the current dataset we present the taxon accumulation curves (FIG. 1) both with and without such samples and taxa (TABLE II) were marked with an asterisk if they were derived from a sample that did not produce a visible band and were observed only once.

For our *L. cincinnatus* positive control that lacked a visible PCR band and exhibited sequence types other than *L. cincinnatus* when cloned, the sample was taken from the context of a *L. cincinnatus* fruiting body. Microscopic examination confirmed that the fruiting body context and adjacent pileus surface harbored darkly pigmented, mitosporic fungi. The two other *L. cincinnatus* positive controls were derived from hymenial tissue, which lacked such fungi; these two controls both produced visible PCR bands and *L. cincinnatus* sequences almost exclusively when cloned.

While fruiting bodies often are thought of as being good sources for positive control DNA, fruiting bodies

can be miniature ecosystems containing a multitude of mitosporic fungi, yeasts, bacteria and insect larvae. Subsequent work with *L. cincinnatus* cultures and fruiting bodies has demonstrated that cultures consistently produce *L. cincinnatus* ITS sequences without exception (> 96 clones have been sequenced from multiple cultures), while various fungal sequences can be recovered from fruiting body tissue (unpubl data). This confirms that culture-based positive controls produce the expected results and emphasize the need for standardized cloning controls, preferably based on pure cultures of fungi. For such controls it is advantageous to select a species that is unlikely to occur naturally in the samples being examined. In this study a wood-decay species was used, while a mycorrhizal species could be used in studies focusing on wood-decay fungi.

With careful selection and use of positive and negative controls cloning has the potential to greatly expand our understanding of root-associated fungal communities. Cloning ideally would be applied to larger root segments (> 6 mm long) or pooled root populations to reduce the number of individual samples that need to be processed. An increase in sampling size is greatly needed in ecological studies of root-associated fungi, where variability often makes it impossible to demonstrate statistical significance. However before cloning is applied commonly to larger samples the inherent biases associated with the entire cloning process need to be investigated in greater detail. Complex environmental samples will have large numbers of co-occurring species as well as a multitude of chemical inhibitors that might vary greatly from one sample to the next. Such factors might exacerbate the intrinsic biases associated with DNA extraction, amplification and cloning. Each step of the cloning process ideally should be tested independently with known samples to demonstrate that cloning of DNA from environmental samples produces an accurate and reproducible view of the fungal community, with species appearing in the expected proportions.

Although more work is needed methods such as the sequencing of large clone libraries and high throughput pyrosequencing represent the future of the exploration of fungal diversity in environmental samples. When applied to root samples these techniques might reveal a large number of ecological guilds of fungi, including saprobes, plant pathogens and mycoparasites in addition to mycorrhizal species. From the perspective of mycorrhizal researchers this additional information might seem unnecessary; however the ecological roles of most fungi remain unknown and it is likely that the function of the mycorrhizal community is affected by a wide range of fungal species. Until more is known about the specific roles played by the entire diversity of root-associated fungi, a more holistic approach to describing the fungal community associated with plant roots will be beneficial.

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