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ORIGINAL PAPER

Ectomycorrhizal characterization of an American chestnut (*Castanea dentata*)-dominated community in Western Wisconsin

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Abstract Circa 1900, a farmer from the eastern US planted 11 American chestnut (*Castanea dentata*) seeds on a newly established farm near West Salem in western Wisconsin. These trees were very successful, producing a large stand of over 6,000 trees. Since this area is well outside the natural range of chestnut, these trees remained free from chestnut blight until 1987. In the West Salem stand, chestnuts are the dominant species of a mixed forest community, reminiscent of the chestnut–oak ecosystems of pre-1900 Appalachia. To identify putative mycorrhizal associates of chestnut in this unique forest, our approach was twofold: (1) an extensive fruiting body survey was conducted for four seasons that yielded approximately 100 putative mycorrhizal species and (2) a belowground molecular approach was used to generate DNA sequences of the internal transcribed spacer region from ectomycorrhizae. Unexpectedly, chestnut did not appear to be the dominant underground ectomycorrhizal-forming plant species. This study highlights the need to identify the plant host species when conducting below-ground molecular-based surveys and provides preliminary identification of ectomycorrhizal fungi associated with a disjunct stand of American chestnut.

Keywords American chestnut · *Chromelosporium* · *Cryphonectria parasitica* · Ectomycorrhizae · Eastern hardwood forests

Introduction

American chestnut (*Castanea dentata*) was the dominant tree of the Appalachian mountain range and surrounding areas prior to the accidental introduction of the chestnut blight fungus, *Cryphonectria parasitica*, before the early 1900s. Chestnut blight affected over four billion trees in their native range over a period of approximately 50 years (Anagnostakis and Hillman 1992; Schwadron 1995; Anagnostakis 2001), which represents one of the greatest recorded impacts of an introduced pathogen on a native host species. Around the turn of the twentieth century, a farmer from the eastern US planted 11 American chestnut seeds on a farm in West Salem, WI, USA, located in the "driftless" (unglaciated) area of western Wisconsin. The chestnut trees were very successful, producing approximately 6,000 additional trees that remained blight free until 1987 (J Cummings-Carlson, personal communication).

Like other members of the Fagaceae, *C. dentata* forms ectomycorrhizae (ECM) with members of the Basidiomycota and Ascomycota (Rhoades et al. 2003; Dulmer 2006), although Molina et al. (1992) stated that other *Castanea* species could form arbuscular mycorrhizae (AM). Dulmer (2006) looked at mycorrhizal communities of planted *C. dentata* seedlings and reported that *C. dentata* could tap into

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existing ECM networks. Because American chestnut was virtually eliminated as a forest tree from its native range prior to the 1950s, no modern study has characterized the ECM community of a mature chestnut forest. Although the West Salem chestnuts are well outside the native range of *C. dentata* and have been affected by chestnut blight, the stand remains relatively healthy and provides an excellent opportunity to study the ECM associations of a successful chestnut forest.

The West Salem chestnuts were planted in a geologically unique area located in the central US known as the "driftless" area. The driftless area of southwestern Wisconsin, southeastern Minnesota, northwestern Iowa, and northeastern Illinois remained unglaciated throughout the most recent ice age of the Pleistocene era. Thus, the driftless area is dramatically different from the rest of Wisconsin and is essentially an Appalachian relict, sharing many environmental and geological characteristics with the native range of *C. dentata* (Paillet and Rutter 1989). The plant and fungal species and community structures are more similar to Appalachia than to the rest of Wisconsin despite being more than 600 km removed from their native range (Paillet and Rutter 1989). The West Salem stand is currently dominated by *C. dentata* with an approximate basal area of 37% (BC McCarthy, personal communication) although other species are also present (Table 1), including *Betula papyrifera*, *Carya cordiformis*, *Carya ovata*, *Populus grandidentata*, *Prunus serotina*, *Quercus alba*, *Quercus coccinea*, *Quercus ellipsoidalis*, *Quercus macrocarpa*, *Quercus rubra*, *Quercus velutina*, *Tilia americana*, and *Ulmus americana* (Paillet and Rutter 1989; McEwan et al. 2006). A detailed site description can be found in Paillet and Rutter (1989) and expanded in McEwan et al. (2006).

Table 1 Reported mycorrhizal statuses of the tree vegetation at the West Salem stand

Species	Relative importance value (%)[a]	AM/ECM
Castanea dentata	37.05	ECM
Quercus spp.[b]	19.56	ECM
Ulmus spp.[c]	12.58	AM (?)
Carya spp.[d]	10.73	ECM (?)
Betula papyrifera	6.36	ECM
Prunus serotina	3.44	AM
Populus grandidentata	3.26	ECM
Tilia americana	3.12	AM (?)
Acer spp.[e]	2.26	AM
Other spp.[f]	1.64	–
Totals	100.00	–

AM Arbuscular mycorrhizae or Endomycorhizae, *ECM* Ectomycorrhizae
[a] Average of the relative basal area and relative density (BC McCarthy, personal communication)
[b] *Q. rubra*, *Q. alba*, *Q. velutina*, *Q. coccinea*, *Q. macrocarpa*, and *Q. ellipsoidalis* (BC McCarthy, personal communication)
[c] *U. americana* and *U. rubra*
[d] *C. ovata* and *C. cordiformis*
[e] *Acer rubrum*, *Acer saccharum*, and *Acer negundo*
[f] Others include: *Corylus americana*, *Pinus resinosa*, *Picea rubra*, *Viburnum lentago*, *Vitis* sp., *Amelanchier arborea*, *Fraxinus americana*, and *Malus* sp.

Morphotyping and restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) have emerged as important techniques for identification of ECM root tips (reviewed in Horton and Bruns 2001). However, morphotyping can underestimate ECM diversity while RFLP analysis can overestimate ECM diversity (Horton and Bruns 2001) and sometimes neither technique will resolve closely related species (Kårén et al. 1997; Glen et al. 2001). Although more expensive, direct sequencing of the rDNA-ITS region of ECM root tips usually provides accurate identification to the species level, resolves closely related species (Horton and Bruns 2001), identifies unexpected species that could be missed with other methods (Vandenkoornhuyse et al. 2002; O'Brien et al. 2005), and provides information for future phylogenetic analysis of molecular data (Tedersoo et al. 2003).

Because of the tree diversity in eastern mixed forests, soil samples collected randomly will most likely contain an assemblage of root tips from many tree species. Thus, the identification of the plant symbiont is imperative. A few studies have identified ECM plant hosts based on morphological root tip characteristics (Kernaghan et al. 2003; Richard et al. 2005), while other studies have employed RFLP analysis of chloroplast DNA (Taberlet et al. 1991), which seemed to work well with conifers (Kennedy et al. 2003; Izzo et al. 2005). The rDNA-ITS region has also been targeted with primers designed by White et al. (1990) and Michelangeli et al. (2004). Dulmer (2006) used RFLP analysis on both the rDNA-ITS region and the chloroplast region when dealing with an eastern mixed forest. Horton and Bruns (1998) and Cullings et al. (2000) used RFLP analysis of 28S rDNA to differentiate host species. We used a similar approach by directly sequencing the rDNA-ITS region of host DNA which was sufficient to differentiate species in the West Salem chestnut stand.

Direct sequencing produces an enormous amount of information that can only be effectively managed in digital format. Computer technology and the internet have become crucial when dealing with the amount of data that is generated by direct sequencing of ECM root tips and fruiting bodies. In order to make sequences easily attainable in a digital format, a publicly available web site was created where our sequence data can be accessed (www.chestnutfungi.com). Our site allows for peer review of the sequences while avoiding some of the problems associated

with public DNA databases (Bridge et al. 2003; Vilgalys 2003). However, realizing the importance of public sequence databases, mainly the convenience of being able to search all sequences at one time, sequences obtained herein have also been deposited in GenBank (Table 2 and Supplementary Table 1).

The primary objective of the study was to acquire a preliminary assessment of the ECM community of a disjunct American-chestnut-dominated ecosystem in western Wisconsin. A twofold approach of an aboveground fruiting body survey and a belowground ECM root tip survey was utilized to gain insight into the composition of this potentially unique ECM community.

Materials and methods

Aboveground sampling

Putative mycorrhizal fruiting bodies were collected in the West Salem chestnut stand approximately once every 3–4 weeks from May to October in 2001, 2003, 2004, and 2005. Additionally, the Alexander H. Smith Foray, a meeting of professional and highly skilled amateur mycologists from the Midwest, was conducted at the West Salem chestnut stand in 2001. More recently, the 2005 North American Mycological Society (NAMA) annual foray was held in La Crosse, WI, USA. Participants in the forays collected all fungal fruiting bodies and putative mycorrhizal species were included in our dataset. The ITS region was sequenced for the putative mycorrhizal species collected from the West Salem chestnut stand and voucher specimens were kept in the University of Wisconsin-La Crosse herbarium.

Belowground sampling

The belowground molecular-based portion of the study was conducted from June to October of 2005. Three to four soil samples approximately 25 by 25 by 25 cm were excavated from within 13 preexisting plots (Fig. 2; Cummings-Carlson, Wisconsin DNR, personal communication). Soil–root samples were obtained on the following dates in 2005: June 15, June 19, June 23, July 5, August 26, September 13, October 04, October 21, and October 26. The samples were intentionally collected in areas entirely surrounded by *C. dentata* in an attempt to select for chestnut root tips. Samples were handled independently and ECM root tips were picked out of each sample, separated based on color, size, texture, and branching patterns, photographed (Nikon Coolpix 4500) under a stereo microscope (Nikon SMZ-645), and frozen at −20°C in cell lysis buffer (CLB).

DNA extraction, PCR, and sequencing

The ITS region of rDNA was sequenced for the fruiting body material and ECM root tips. DNA was isolated from approximately 50–100 mg of dried fruiting body material or 8–10 mm ECM root tips. The dried fruiting body material or ECM root tip was placed in a microcentrifuge tube containing 500 μL of CLB. The CLB contained 1.4 M NaCl, 0.1 M Tris–HCl, 20 mM ethylenediaminetetraacetic acid, and 2% hexadecyltrimethylammonium bromide. The sample was ground using a plastic pestle and then an additional 500 μL of CLB was added. Tubes were agitated for 20 s and then heated at 65°C for 1 h. This was followed by centrifugation at 16,000 rcf for 6 min, after which time 700 μL of supernatant was transferred to a new microcentrifuge tube and 750 μL of −20°C isopropanol was added. Tubes were inverted several times and placed at −80 C for 10 min. Samples were then centrifuged for 20 min at 13,000 rcf at 4°C. Supernatants were discarded and the pellets washed with 70% ethanol. Pellets were air-dried for 5 min and then resuspended in 50 μL of water.

The DNA in aqueous solution was cleaned using GeneClean III kits (Qbiogene) with the following modifications. Fifty microliters of aqueous DNA solution was combined with 150 μL of NaI solution and 3 μL of glass milk. Tubes were agitated intermittently for 6 min, followed by centrifugation at 16,000 rcf for 8 s. The supernatant was then discarded and the pellet washed three times using the New Wash solution provided with the kit. After the final wash, pellets were air-dried for 15 min and DNA was eluted in 50 μL of water. The DNA solution was then used directly for polymerase chain reaction (PCR).

The fungal-specific primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) was used for PCR. PCR was performed using 5× Green GoTaq reaction buffer and GoTaq DNA polymerase (Promega, Madison, WI, USA). 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 mM and each deoxynucleotide triphosphate (Promega, Madison, WI, USA) had a final concentration of 200 mM. Template DNA (obtained using the GeneClean III kit) was diluted 1:50 in the final reaction volume. Thermocycler conditions were as follows: initial denaturing at 94°C for 2 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 5 min. The PCR products were cleaned using AmPure magnetic beads (Agencourt) following manufacturer's directions. Sequencing reactions were performed following the BigDye terminator protocol (ABI Prism) using both the ITS1F and ITS4 primers for fruiting bodies and the ITS4 primer for ECM root tips. Sequencing

Table 2 Sequence accession numbers and molecular identification of ECM root tips collected at the West Salem chestnut stand

DCM accession # and sequence length	GenBank accession #	Number of root tips	Closest sporocarp match (% identity)	Source database	Source accession #	Plant host (% identity)
RT00053 (598 bp)	EU819531	1	*Boletus* cf *chrysenteron* (98% 598 bp)	DCM	JMP0007	B (98% 598 bp)
RT00004 (713 bp)	EU819502	3	*B. pulverulentus* (99% 694 bp)	DCM	JMP0012	C (95% 325 bp)
RT00008 (665 bp)	EU819526	3	*Byssocorticium atrovirens* (98% 644 bp)	UNITE	UDB000075	–
RT00021 (628 bp)	EU819508	3	*Chromelosporium* sp. tjv1 (99% 628 bp)	DCM	JMP0016	RO (97% 724 bp)
RT00075 (146 bp)	EU819543	1	*Cortinarius diasemospermus* (96% 100 bp)	GenBank	UDB001230	–
RT00046 (563 bp)	EU819527	1	*C. alboviolaceus* (99% 550 bp)	GenBank	DQ097877	–
RT00010 (597 bp)	EU819499	1	*C. subsertipes* (96% 550 bp)	GenBank	AY669679	–
RT00073 (500 bp)	EU819541	1	*Hebeloma crustuliniforme* (92% 479 bp)	DCM	JMP0100	–
RT00017 (683 bp)	EU819506	1	*Humaria hemisphaerica* (95% 461 bp)	UNITE	UDB000988	C (94% 370 bp)
RT00078 (676 bp)	EU819538	1	*H. hemisphaerica* (95% 611 bp)	UNITE	UDB000988	WO (98% 430 bp)
RT00080 (575 bp)	EU819504	2	*Laccaria amethystina* (94% 582 bp)	DCM	JMP0035	RO (96% 748 bp)
RT00015 (666 bp)	EU819503	1	*L. laccata* var. *pallidifolia* (99% 665 bp)	DCM	JMP0037	C (92% 241 bp)
RT00063 (629 bp)	EU819535	1	*Peziza depressa* (93% 487 bp)	UNITE	UDB000993	P (97% 664 bp)
RT00016 (623 bp)	EU819505	2	*Peziza* sp. tjv1 (100% 621 bp)	DCM	JMP0053	–
RT00024 (537 bp)	EU819510	2	*Piloderma* sp. A18 (96% 484 bp)	GenBank	AJ534902	–
RT00049 (476 bp)	EU819528	1	*Russula aeruginea* (97% 471 bp)	DCM	JMP0057	–
RT00029 (531 bp)	EU819514	1	*R. cremeirosea* (99% 532 bp)	DCM	JMP0061	–
RT00054 (425 bp)	EU819532	1	*R. mustelina* (97% 359 bp)	UNITE	UDB000893	–
RT00011 (670 bp)	EU819500	8	*R. pectinatoides* (99% 670 bp)	DCM	NAMA499	P (94% 511 bp) H (93% 200 bp)
RT00062 (543 bp)	EU819534	1	*R. pectinatoides* (97% 544 bp)	DCM	NAMA499	–
RT00027 (670 bp)	EU819512	5	*Russula* sp. tjv4 (99% 671 bp)	DCM	JMP0068	RO (97% 732 bp) WO (96% 216 bp)
RT00028 (679 bp)	EU819513	2	*Russula* sp. tjv4 (96% 677 bp)	DCM	JMP0068	H (97% 827 bp) H (97% 842 bp)
RT00025 (646 bp)	EU819511	2	*Russula* sp. tjv5 (99% 445 bp)	DCM	JMP0069	–
RT00031 (683 bp)	EU819516	7	*Russula* sp. tjv6 (100% 677 bp)	DCM	JMP0070	C (96% 387 bp) RO (91% 437 bp)
RT00030 (479 bp)	EU819515	2	*Russula* sp. ue53 (97% 478 bp)	GenBank	AF418629	WO (95% 364 bp)
RT00034 (653 bp)	EU819517	5	*Scleroderma bovista* (98% 602 bp)	GenBank	AB099901	–
RT00036 (742 bp)	EU819518	14	*S. areolatum* (99% 678 bp)	DCM	JMP0080	C (95% 416 bp) C (95% 420 bp) C (95% 421 bp) C (96% 414 bp) WO (91% 754 bp) WO (92% 753 bp) RO (96% 749 bp)
RT00038 (623 bp)	EU819519	3	*Sebacina* aff. *epigaea* MW 526 (93% 561 bp)	GenBank	AF490393	H (98% 872 bp) H (96% 283 bp)
RT00039 (364 bp)	EU819520	2	*S. helvelloides* (96% 364 bp)	UNITE	UDB000972	E (98% 405 bp) A (95% 595 bp)
RT00068 (557 bp)	EU819537	2	*S. epigaea* (90% 472 bp)	GenBank	AF490397	C (94% 417 bp) C (93% 421 bp)
RT00051 (624 bp)	EU819530	1	*Sebacinaceae* sp. O56 (93% 619 bp)	GenBank	AJ534907	–
RT00041 (629 bp)	EU819522	1	*Tomentella badia* (94% 524 bp)	UNITE	UDB000961	WO (93% 291 bp)
RT00044 (651 bp)	EU819524	1	*T. ferruginea* (92% 636 bp)	DCM	NAMA246	–

Table 2 (continued)

DCM accession # and sequence length	GenBank accession #	Number of root tips	Closest sporocarp match (% identity)	Source database	Source accession #	Plant host (% identity)
RT00043 (648 bp)	EU819523	1	*T. stuposa* (94% 542 bp)	UNITE	UDB000245	C (93% 444 bp)
RT00079 (607 bp)	EU819501	2	*T. stuposa* (98% 377 bp)	UNITE	UDB000967	–
RT00071 (665 bp)	EU819540	2	*T. sublilacina* (98% 654 bp)	GenBank	U83481	C (93% 426 bp)
RT00045 (520 bp)	EU819525	1	*Tuber scruposum* (96% 461 bp)	GenBank	DQ011848	T (97% 912 bp)
RT00069 (595 bp)	EU819539	1	Unidentified (Ascomycota)	GenBank		H (98% 871 bp)
RT00050 (541 bp)	EU819529	1	Unidentified (Basidiomycota)	GenBank		H (98% 597 bp)
RT00019 (569 bp)	EU819507	2	Unidentified (Pezizaceae)	GenBank		–
RT00022 (613 bp)	EU819509	2	Unidentified (Pezizaceae)	GenBank		WO (99% 319 bp)
RT00074 (162 bp)	EU819542	1	Unidentified (Pezizaceae)	GenBank		C (92% 246 bp)
RT00056 (510 bp)	EU819533	1	Unidentified (Pezizales)	GenBank		C (93% 236 bp)
RT00077 (110 bp)	EU819544	2	Unidentified (Russulaceae)	GenBank		–
RT00040 (150 bp)	EU819521	1	Unidentified (Sebacinaceae)	GenBank		–
RT00032 (460 bp)	EU819536	1	Unidentified (Tricholomataceae)	DCM		–

DCM Database of Chestnut Mycorrhizae, *UNITE* http://unite.ut.ee/index.php, *GenBank* http://www.ncbi.nlm.nih.gov/, *B* B. papyrifera, *C* C. dentata, *RO* Red oak group, *WO* Q. alba, *P* P. grandidentata, *H* Carya spp., *E* U. americana, *T* T. americana, *A* Apiaceae

reactions were cleaned using CleanSeq (Agencourt) according to manufacturer's directions. Sequencing products were analyzed at the University of Wisconsin Biotech Center and final sequences were aligned using Sequencher 4.2 (GeneCodes Corporation). Sequences of ECM root tips were identified using BLAST searches of the fruiting body sequences obtained herein (www.chestnutfungi.com), the GenBank database (National Center for Biotechnology Information), and/or the UNITE database (Kõljalg et al. 2005).

The extracted root tip DNA was also amplified with the plant-specific primers ITS-5 (White et al. 1990) and ITS-24lr (Michelangeli et al. 2004). Thermocycler conditions were as follows: initial denaturing at 94°C for 85 s, 34 cycles of denaturing at 94°C for 35 s, annealing at 50°C for 55 s, and extension at 72°C for 2 min, and a final extension step of 72°C for 10 min (Dulmer 2006). The resulting PCR products were sequenced as above and compared to sequences obtained in the same manner from reference leaf material of putative plant hosts collected from the West Salem stand.

Web site design

The Database of Chestnut Mycorrhizae (www.chestnutfungi.com) utilizes Pre-Hypertext Processer (PHP; www.php.net), a server side scripting language, and MySQL (www.mysql.com), an open source database program. PHP and MySQL run seamlessly to integrate the information stored in the database to a dynamic web site. The site runs on a LINUX platform that includes the BLASTN algorithm (Altschul et al. 1997) that is capable of BLAST searching the entire database of rDNA-ITS sequences and a Boolean search function that searches the database by name. The BLAST results are then cross-linked to individual "species pages" that contain pictures of the specimen (fruiting body and root tips, where available) coupled with an rDNA-ITS sequence.

Results

Aboveground survey

Specimens of putatively mycorrhizal fungal species were collected, identified, dried, and stored in the University of Wisconsin-La Crosse herbarium, which resulted in 99 morphological species. The ITS region was sequenced for 88 of these 99 species (Supplementary Table S1) and sequences were deposited at www.chestnutfungi.com and GenBank (75 complete rDNA-ITS sequence and 13 partial sequences). Eleven fruiting body collections did not yield interpretable sequence data. The major taxonomic groups included members of the Russulaceae (28 taxa), Boletales (16 taxa), Cortinariaceae (12 taxa), Tricholomataceae (nine taxa), Amanitaceae (seven taxa), Pezizales (six taxa), Thelephoraceae (five taxa), Sclerodermataceae (four taxa), and the Cantharellales (four taxa), while the Leotiaceae, Gomphaceae, Sebacinaceae, Hygrophoraceae, and Atheliaceae were represented by two or fewer taxa each (Fig. 1).

Belowground survey

Three to four soil–root samples were acquired from each of the 13 preexisting plots from the chestnut stand over the course of June through October of 2005 (Fig. 2). Approximately five putative ECM root tips were collected from each soil sample, thus generating 233 root tips that were extracted and PCR-amplified at multiple dilutions. Sequenc-

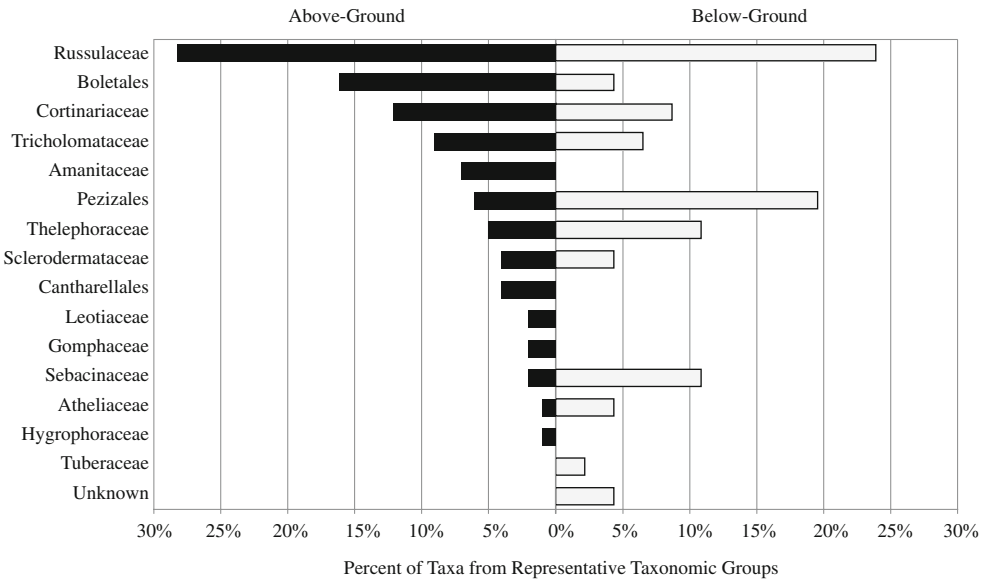

Fig. 1 Species distribution of selected taxonomic groups illustrates the discontinuity between aboveground and belowground sampling methodologies. Percentages were generated by dividing the number of taxa in each family by the total number of putatively mycorrhizal collections for both the aboveground and belowground survey. The Russulaceae group was the most commonly collected in our aboveground survey and the belowground sampling, while other groups such as the Pezizales and Thelephoraceae are more common belowground

ing yielded 100 fungal ITS sequences (43% success rate). These sequences represented 46 "species" (Table 2) in 11 diverse taxonomic groups (Fig. 1). Thirty-seven sequences were identified to the genus level (defined as ≥90% sequence identity), and nine remained unidentified past the family level (Table 2). Twenty-eight of the sequences were identified to the species level (defined as ≥95% sequence identity, a conservative estimate of species). Sixteen of the 46 (34.8%) matched sequences from species found in the aboveground survey (Table 2). *Scleroderma areolatum* (RT00036) was identified 14 times from five different plots and *Russula pectinatoides* (RT00011) was identified eight times from three different plots (Table 3). Of the 46 ITS sequence types, ten were identified from two or more different field plots while the remaining species were identified only from a single plot.

In addition, 50 plant rDNA-ITS sequences were generated from the root tip extractions. *Quercus* spp. (*Q. rubra*, *Q. alba*, *Q. velutina*, *Q. coccinea*, *Q. macrocarpa*, and *Q. ellipsoidalis*) dominated at 38% of the total plant rDNA-ITS sequences, followed by *C. dentata* at 32% (Table 4). Sequences of *C. ovata* or *C. cordiformis*, *P. grandidentata*, *B. papyrifera*, *T. americana*, *U. americana*, and an unknown

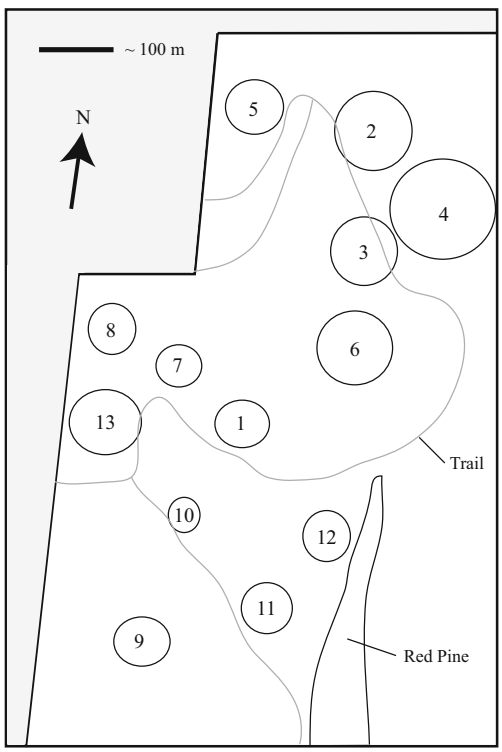

Fig. 2 Diagram of the 13 preexisting plots set up by the Wisconsin DNR (Cummings-Carlson) at the chestnut stand in West Salem, WI, USA (roughly drawn to scale). ECM fruiting bodies were collected from the entire forest for the aboveground survey and three to four soil samples were excavated from each of the 13 plots for the belowground survey

Table 3 Belowground ECM identified from multiple plots

Species	# of root tips	Plots	Date(s) collected (2005)
Scleroderma areolatum	14	1, 7, 9, 10, 12	7/5, 7/12, 9/13, 10/21
Russula pectinatoides	8	9, 10, 11	6/23, 7/5, 9/13
Russula sp. tjv4	5	3, 5, 7	6/19, 7/5
Sebacina aff. *epigaea*	3	3, 12	6/23, 8/26
Russula sp. tjv5	2	3, 5	6/19, 7/5
Tomentella stuposa	2	3, 7	6/15, 6/19
Tomentella sublilacina	2	6, 9	9/13
Pezizaceae	2	2, 3	6/19
Russula sp. ue53	2	2, 11	6/19, 6/23

Sampling was not quantitative

Table 4 Molecular identifications of putative ECM plant root tips based on DNA sequences

Plant host	# of root tips	Percent of community
Quercus spp.[a]	19	38
Castanea dentata	16	32
Carya spp.[b]	9	18
Others[c]	6	12
Totals	50	100

[a] *Q. rubra*, *Q. alba*, *Q. velutina*, *Q. coccinea*, *Q. macrocarpa*, and *Q. ellipsoidalis*
[b] *C. ovata* and *C. cordiformis*
[c] *B. papyrifera*, *T. americana*, *U. americana*, *P. grandidentata*, and a member of the Apiaceae

member of the Apiaceae were also identified from the root tips (Table 4).

Discussion

ECM composition and diversity

An aboveground survey of fruiting bodies is vital for identification of ECM fungi; however, when used alone, it is a poor indicator of community diversity (Gardes and Bruns 1996; Dahlberg 2001; Horton and Bruns 2001; Taylor 2002). With continued sequencing of fruiting bodies from aboveground surveys, there should eventually be a reduction in the number of unidentified root tip sequences in ECM diversity studies (Horton and Bruns 2001; Dahlberg 2001), assuming the fruiting body sequences are made available in DNA databases. Our data provided nine root tip sequences (20%) that could not be identified to species level with confidence using the available sequence databases. An increased sampling and sequencing effort would improve the identification of these sequences. The aboveground survey of the chestnut site is unique because of the large number of experts in various taxonomic groups that have helped to identify the collections. Using a large number of collectors helps to alleviate some of the sampling bias often present in biodiversity surveying. In addition, instead of using a quantitative methodology such as plot-based or transect collecting methods, a general "foraying" approach was used to cover large areas. While an opportunistic methodology does not allow for quantitative analysis of the fungal community relative to sampling effort, it does offer good baseline data of ECM fungi in this particular ecosystem.

Most ECM diversity studies sample belowground root tips in one large sampling effort, which shows community composition only at a given time point (Horton and Bruns 2001; Taylor 2002). The sampling methodology used in this study involved taking small sample sizes (referring to both number and volume) over multiple time points throughout the growing season. Thus, root tips could be processed on a weekly basis, which may have helped ameliorate the effects of DNA degradation in storage. In addition, this may have allowed for identification of ECM that form transiently during one growing season, a phenomenon highlighted by Walker et al. (2008). Consistent with other studies that incorporate both an aboveground and belowground survey (e.g., Gardes and Bruns 1996), our study found a discontinuity in the species composition between the two methods (Fig. 1), with an overlap of approximately 35%. That is, of the 46 sequences identified on root tips, only 16 were represented in the aboveground fruiting body survey. Discrepancies have been explained by the fact that some species rarely or never produce fruiting bodies (Horton and Bruns 2001), some species form inconspicuous fruiting bodies and therefore are undersampled (Gardes and Bruns 1996; Tedersoo et al. 2006), and some species (such as the Cantharellales) do not sequence well (Feibelman et al. 1994). However, given that the sampling effort for the belowground portion of our study was very low compared to the aboveground survey, it is likely that the belowground ECM community is not fully represented in this study. Further belowground sampling is warranted and will provide more conclusive data on the discontinuity of the two sampling methodologies.

Tedersoo et al. (2006) highlighted the importance of pezizalean fungi, a group of ascomycetes that have typically been overlooked in ECM biodiversity studies. Our aboveground biodiversity survey resulted in collections of inconspicuous hyphal mats on the soil. Subsequent microscopic and sequence analysis identified the hyphal mats as *Chromelosporium* species. *Chromelosporium* is an anamorphic genus of pezizalean fungi (teleomorph: *Peziza* or *Ascobolus*) that reproduce by small mycelial mats on the surface of the soil, occasionally producing small apothecia; however, no apothecia were observed in the samples we collected. These fungi are easily overlooked and may be significantly undersampled in biodiversity surveys. We also identified a *Chromelosporium* species from the roots of a *Quercus* species in our stand. Interestingly, the pezizalean group represented almost 20% of the belowground community sampled. As sequence data increase for the pezizalean fungi, identification to species will be less problematic.

Plant host complexity

In a mixed forest ecosystem, identification of host plants is imperative. It cannot be assumed that a dominant aboveground plant species, such as *C. dentata*, is also dominant in the belowground mycorrhizal community. Nor can it be assumed that a soil sample taken underneath a given tree

species will yield root tips of only that species. Although our dataset for belowground sampling is small, in the West Salem chestnut stand, *C. dentata* is the dominant aboveground species (RIV, 37.05%) while *Quercus* spp. occupies 19.56% (RIV) of the stand. When soil samples taken from an area completely surrounded by *C. dentata* (5–10 m) were sorted for ECM root tips, *Quercus* spp. dominated the belowground community (38%) followed by *C. dentata* (32%; Table 4). Although there are no data to show the exact distance between the soil samples and the closest mature *Quercus* tree or seedling, root systems are known to occupy large volumes of soil. Therefore, roots of nearby species may grow farther than anticipated and consequently these roots can be found at some distance from the parent tree. Brown and Woods (1968) reported that root systems of hardwood species could extend up to approximately 16 m.

Another plausible explanation for the reduced number of *C. dentata* roots collected belowground may be an artifact of sampling bias. The data presented here represent a small belowground sample size which has the potential to skew results. Even though soil samples were collected in an attempt to select for *C. dentata* root tips (i.e., in an area completely surrounded by chestnut, approximately 5–10 m in every direction), only root tips that had visible signs of ECM colonization were separated out of the soil samples. Since no previous study has been done on mature chestnut mycorrhizae, there are no reports on what extent *C. dentata* ECM root tips display classical signs of colonization, e.g., branching patterns, mantle, color, or size. From our initial observations, the West Salem chestnut ECM root tips are much smaller in size, exhibit less extracellular hyphal mass, and generally do not branch as much as ECM root tips of *Quercus* spp. or members of Pinaceae. Thus, *Quercus* spp. ECM root tips may have been picked out preferentially over *C. dentata* ECM root tips because they show clearer evidence of ECM colonization. While it is relatively easy to pick out *Quercus* spp. or *Pinus* spp. ECM root tips from a soil sample, root tips of other ECM species, such as chestnut, may be less obvious. Other species, such as *Populus tremuloides* and *P. grandidentata*, do not display clear signs of ECM colonization but do form ECM (Neville et al. 2002).

It is also worth noting that the West Salem chestnuts have been infected with the chestnut blight fungus, *C. parasitica*, since 1987, although we collected in areas largely free from the blight. There are no data on the impact that chestnut blight may have on ECM communities, but in general a diseased tree would be expected to be less productive and therefore have less photosynthate to pass on to its mycobiont. This may in part explain the lack of obvious morphological ECM structures. Although no AM fungi were found in this study, there is some observational evidence that *C. dentata* seedlings can form an AM association (Dulmer 2006) and consequently may be less dependent on ECM associations. It has been documented that other tree species have the ability to switch between ECM associations and AM associations during development (Dickie et al. 2001; Horton et al. 1999), but this phenomenon has not been formally addressed in *C. dentata*.

The plant-based sequence data produced by this study also raised some interesting questions about the mycorrhizal status of three eastern hardwood species: *C. cordiformis* (yellow-bud hickory), *C. ovata* (shagbark hickory), and *T. americana* (basswood). Interestingly, *T. americana* has appeared in the ECM literature before. Giomaro et al. (2002) were able to show that *Tuber brumale* could form an ECM association with *T. americana* in the laboratory. We have also identified a truffle, *Tuber scruposum*, that was associated with *T. americana* in our stand.

The mycorrhizal status of hickory species remains the most ambiguous and literature on these trees is scarce. Other members of the Juglandaceae seem to be variable in terms of their ability to form mycorrhizae. For example, *Juglans nigra* (black walnut) is an AM-forming species (Kormanik et al. 1982) while *Carya illinoensis* (pecan) forms ECM (Marx 1979). The mycorrhizal-forming ability of the hickory species, *C. cordiformis* and *C. ovata*, in the chestnut stand is unknown but they have been widely assumed to be ECM formers because of their frequent occurrence with ECM-forming oak species. There has been no study that has specifically targeted this question. *Russula pectinatoides*, *Russula* sp. tjv4, *Sebacina* aff. *epigaea*, an unidentified member of the Ascomycota, and an unidentified member of the Basidiomycota were identified from the roots of hickory species in the chestnut stand. *Russula* spp. and *Sebacina* spp. are known ectomycorrhizal formers, suggesting that hickory species are in fact ECM formers. Interestingly, the two unidentified fungal species from hickory both produced DNA sequences of sufficient length and quality that identification should have been possible if reference sequences were available. The lack of any near match suggests the fruiting bodies of these fungi may be cryptic or easily overlooked, so extensive collecting in association with hickory is needed to identify these species. Sequencing of additional gene regions, such as the large subunit rDNA, would help to place these sequences in larger fungal phylogenies.

ECM fungi of American chestnut

Only ten putative ECM species were definitively associated with *C. dentata*: *Boletus pulverulentus*, *Humaria hemisphaerica*, *Laccaria laccata* var. *pallidifolia*, *Russula* sp. tjv6, *Scleroderma. areolatum*, *Sebacina epigaea*, *Tomentella stuposa*, *T. sublilacina*, and two unidentified

members of the Pezizales. All of the species on this list, with the exception of *S. areolatum* and *Russula* sp. tjv6, were identified only from the roots of *C. dentata*. *Russula* sp. tjv6 was found on both *C. dentata* and *Q. rubra* in the same soil sample, while *S. areolatum* was found on *C. dentata*, *Q. rubra*, and *Q. alba* but from different soil samples. However, given the small sample size, these data represent a preliminary characterization of ECM associated with *C. dentata*. Dulmer (2006) reported that *Cenococcum geophilum* was found quite abundantly on the roots of American chestnut seedlings in New York. Additionally, *C. geophilum* was found on seedlings in a bioassay done by Rhoades et al. (2003); however, *C. geophilum* was not found in any of our soil samples. Consistent with our data, the study by Dulmer (2006) also found various *Russula* spp., a *Tomentella* sp., and a member of the Pezizales on the roots of chestnut seedlings in the field; *L. laccata* was abundant on roots of American chestnut in a seedling bioassay.

The evidence from this study indicates that mature American chestnut (*C. dentata*) trees in the West Salem stand do form ECM associations with members of the Basidiomycota and Ascomycota despite being far removed from their native range. Even though the chestnut site is referred to as a chestnut forest or chestnut stand throughout this paper, the forest actually displays relatively high tree diversity, similar to other eastern hardwood forests. Due to the unexpectedly small number of chestnut roots sampled, we can only offer a preliminary view of the ECM community of a disjunct stand of American chestnut. Future studies of ECM communities in the native range of American chestnut will provide the ability to draw comparisons between the mycorrhizal communities of Appalachian areas and the driftless area of western Wisconsin.

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