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ORIGINAL ARTICLE

Chestnuts bred for blight resistance depart nursery with distinct fungal rhizobiomes

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Abstract

Restoration of the American chestnut (*Castanea dentata*) is underway using backcross breeding that confers chestnut blight disease resistance from Asian chestnuts (most often *Castanea mollissima*) to the susceptible host. Successful restoration will depend on blight resistance and performance of hybrid seedlings, which can be impacted by below-ground fungal communities. We compared fungal communities in roots and rhizospheres (rhizobiomes) of nursery-grown, 1-year-old chestnut seedlings from different genetic families of American chestnut, Chinese chestnut, and hybrids from backcross breeding generations as well as those present in the nursery soil. We specifically focused on the ectomycorrhizal (EcM) fungi that may facilitate host performance in the nursery and aid in seedling establishment after outplanting. Seedling rhizobiomes and nursery soil communities were distinct and seedlings recruited heterogeneous communities from shared nursery soil. The rhizobiomes included EcM fungi as well as endophytes, putative pathogens, and likely saprobes, but their relative proportions varied widely within and among the chestnut families. Notably, hybrid seedlings that hosted few EcM fungi hosted a large proportion of potential pathogens and endophytes, with possible consequences in outplanting success. Our data show that chestnut seedlings recruit divergent rhizobiomes and depart nurseries with communities that may facilitate or compromise the seedling performance in the field.

Keywords Illumina MiSeq · Forest nursery · Chestnut · Hybrid

Introduction

Non-native forest pests and invasive pathogens have had catastrophic impacts on tree species around the globe (Boyd et al. 2013; Santini et al. 2013; Lovett et al. 2016). In the USA, one such pathogen is the chestnut blight fungus, ascomycete *Cryphonectria parasitica* (*Murr.*) *Barr*, that caused a disease that rapidly eliminated the American chestnut (*Castanea dentata* (Marsh.) Borkh.) as an upper canopy dominant throughout its native range in the first half of the twentieth century (Anagnostakis 1987). Chestnut blight causes necrotic cankers on the branch and trunk surfaces leading to girdling and eventual mortality in susceptible trees. To restore the chestnut to eastern North American forests, a backcross breeding approach has been developed in an attempt to confer blight resistant genes from Asian chestnut species, most often the Chinese chestnut (*Castanea mollisima* Blume), into the American chestnut using conventional breeding (Burnham et al. 1986; Anagnostakis 2012). Such breeding programs aim to generate progeny that exhibit American chestnut phenotypic form and growth characteristics as well as maintain durable blight resistance (Hebard 2001; Diskin et al. 2006; Sniezko 2006; Anagnostakis 2012).

These breeding programs currently produce material that is being field tested under various forest management conditions and is exhibiting low to intermediate resistance (Clark et al. 2014a; Clark et al. 2016; Steiner et al. 2016). Despite the importance of resistance breeding programs for restoring pathogen-decimated tree populations and species, sparse research is available to better understand factors, other than disease resistance, that will affect outplanting success of

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hybrid populations (Thompson et al. 2006; Seddon 2010; Jacobs 2013; Clark et al. 2014a, 2014b; Pinchot et al. 2017). Like many other temperate forest trees, American chestnut forms important ectomycorrhizal (EcM) mutualisms (Palmer et al. 2008; Bauman et al. 2017; Newhouse et al. 2018) that facilitate host nutrient uptake, improve host growth and performance, and may improve pathogen resistance/tolerance (Smith and Read 1997). Emerging evidence suggests that different host genotypes may recruit distinct fungal communities (e.g., Lamit et al. 2016; Perez-Izquierdo et al. 2017), resulting in potential breeding-associated effects on non-target fungal communities that may be consequential to the establishment and survival of juvenile plants.

Nursery-reared seedlings recruit diverse communities of rhizosphere fungi (fungal rhizobiomes, or fungi associated with roots and adhering soils) including pathogens, endophytes, and putative mutualists (Menkis et al. 2005, 2016; Stenström et al. 2014). These nursery-borne fungi may affect the seedling performance under the nursery conditions (Sinclair et al. 1982; Menkis et al. 2007) and can be particularly important for seedling establishment once outplanted to the field (Kropp and Langlois 1990; Lilja and Rikala 2000). Heavy pathogen loads at the nursery can remain asymptomatic—and therefore undetected—yet compromising the outplanting success (Lilja and Rikala 2000). Alternatively, mycorrhizal mutualists that establish in the nursery may aid in survival after outplanting (Menkis et al. 2007). Furthermore, understanding the communities that may have been introduced into soil with the outplanted nursery stocks can later serve as a baseline for assessment of host performance and rhizobiome composition after outplanting.

Only a few studies have targeted the root-associated fungi of American chestnuts (Dulmer 2006; Bauman et al. 2013, 2017; D'Amico et al. 2015; Stephenson et al. 2017), although associating with compatible mutualists may be essential for restoration of threatened plants (Perry et al. 1987). As a result, restoration strategies already consider the importance of mycorrhizal inocula (Jacobs et al. 2013). Mycorrhizal seedlings generally outperform their nonmycorrhizal counterparts (Kropp and Langlois 1990; Quoreshi and Timmer 2000; Menkis et al. 2007), even though the early, nursery-recruited symbionts may be short-lived and rapidly outcompeted by naturally occurring fungi after outplanting (Menkis et al. 2007).

Fungi that colonize American chestnut include a number of common EcM taxa (Palmer et al. 2008; Dulmer et al. 2014; Stephenson et al. 2017) shared with other EcM hosts. The establishing seedlings likely benefit from sharing mycorrhizal partners with older overstory trees (Horton and van der Heijden 2008), as existing EcM networks are extensive and can expedite colonization (Dickie et al. 2002; Nara 2006a, 2006b), improve access to resources (Dickie et al. 2002, 2007), and minimize negative effects of root competition (Booth 2004). However, it remains largely unknown if chestnut blight breeding programs have adverse non-target effects on EcM colonization and diversity, *i.e.*, does selection of blight resistant progeny alter the host compatibility with mycorrhizal partners (but see D'Amico et al. 2015).

In this study, we examined 1-year-old chestnut nursery seedlings for fungal rhizobiomes prior to outplanting to the field. We compared and contrasted six backcross hybrid, two American chestnut, and one Chinese chestnut families, and analyzed the nursery soil—that we considered the potential source inoculum. Similarly to D'Amico et al. (2015), we focused on EcM communities but surveyed them using high throughput sequencing to deeply dissect the rhizobiomes recruited from the nursery. We specifically aimed to address following research questions: (i) Does the nursery soil differ from the seedling host rhizobiomes in fungal richness, diversity and community composition? (ii) Do backcross hybrids and American and Chinese chestnut families differ in their rhizobiomes while growing in a presumably homogeneous nursery substrate? (iii) Finally, should the host rhizobiomes be distinct among species, breeding generations, or families, we also aimed to identify the distinguishing fungal taxa. The resultant data indicate substantial heterogeneity in the communities that are associated with the nursery-reared seedlings, raising thus questions about the need for mycorrhizal inoculation of high-value nursery stock prior to outplanting.

Materials and methods

Experimental material

We obtained nuts from two American Chestnuts trees (Pryor 043, Pryor182), one Chinese Chestnut tree (Princeton), five backcross hybrid trees from The American Chestnut Foundation (TACF), and one backcross hybrid tree from the Connecticut Agricultural Experimental Station (CAES) (Table 1). Hereafter, a "family" refers to progeny from a single open-pollinated orchard tree with a distinct lineage and limited pollen contamination from outside sources (Hebard 2006). The Chinese chestnut family was located on private property with limited pollen contamination (Paul Sisco, TACF, Asheville, NC, USA, personal communication) (Burnham et al. 1986). The TACF hybrids (D22, W3, W4, W5, and W6) are theoretically 94% *C. dentata* and 6% *C. mollissima* (Hebard 2006), whereas the CAES hybrid (4-75) is theoretically 90% American chestnut with remaining 10% a mix of Chinese chestnut, European chestnut (*Castanea sativa*), and Japanese chestnut (*Castanea crenata*) (Anagnostakis 2012).

We sampled roots and soils within an operational nursery at the Indiana State Nursery in Vallonia, Indiana. The nursery beds were methyl bromide (CH_3Br) fumigated before the nuts

Table 1 Breeding and source information for each genetic family

Species/generation	Family abbreviated name	Orchard identification	Source
American chestnut	Pryor 1-182	Pryor 1-82	TACF
American chestnut	Pryor 1-43	Pryor 0-43	TACF
Chinese chestnut	Princeton	Not Available	TACF
BC3F3	D22	D 3-28-57	TACF
BC3F3	W3	W 6-31-33	TACF
BC3F3	W4	W 6-22-97	TACF
BC3F3	W5	W 3-32-49	TACF
BC3F3	W6	W 1-31-60	TACF
BC2 × BC3	4-75	4-75	CAES

Further detail and descriptions of breeding generations can be found in Hebard (2006) for the TACF families and Anagnostakis (2012) for the CAES family

TACF The American Chestnut Foundation, *CAES* Connecticut Agricultural Experiment Station

were sown by family in seed lots at the nursery on November 19, 2013. Likely as a result of the fumigation, we observed no fruiting bodies in the nursery beds at the time of sowing or at the time of seedling collection. At the nursery, each family seed lot was sown at a density of 65 nuts per m^2, measured approximately 0.75 × 0.2 m, and was separated by 0.5 m of empty bed space. After sowing, the beds were open and arrival of inoculum was not controlled during the experiment. The seedlings were fertilized according to standard prescriptions to produce large, high-quality seedlings and irrigated as needed (cf. Kormanik et al. 1994). The fertilization regime ranged from roughly 5 g-N m^{-2} to an excess of 13 g-N m^{-2} applied every 2 weeks as NH_4NO_3 (Nov. 19, 5.2 g-N m^{-2}; Dec. 3, 4.3 g-N m^{-2}; Dec. 17, 4.5 g-N m^{-2}; Dec. 31, 5.8 g-N m^{-2}; Jan. 14, 8.2 g-N m^{-2}; Jan. 28, 13.6 g-N m^{-2}; Feb. 11, 13.6 g-N m^{-2}) for a total of 55 g-N m^{-2} over the first 3 months in the nursery. Seedlings ranged from 13 to 213 cm in height averaging 99 cm at the time of preparation for outplanting. A machine lifter was used to undercut seedlings (25–30 cm) and loosen soil around the roots. Seedlings were manually removed from the nursery beds, roots packed in sphagnum moss as per the nursery standard operating protocol to minimize seedling desiccation during transport, and placed in poly-coated paper tree bags in cold storage until root sampling. We did not collect any root colonization data prior to outplanting. While we did not control for fungal inoculum in the sphagnum moss, we expect it to be uniform across the material, exposure short in duration, and an unlikely factor to explain divergence among the analyzed genetic families.

Our experiment included a total of four replicates of each of the nine chestnut families for a total of 36 root samples for the rhizobiome analyses. Seedlings were removed from the bags, rinsed free of sphagnum moss, and roots sampled with a pruner that removed approximately 20 g of secondary and feeder roots from each seedling. The pruner was sterilized in 20% solution of domestic bleach (0.534% sodium hypochlorite) between family samples. Tap roots were not sampled to avoid problems at planting to the field. The sampled roots were placed in Ziplock bags stored at 4 °C until shipped to Mississippi State University within 48 h.

To also assess the background soil inoculum in the nursery, we sampled four replicate bulk soil samples from Vallonia nursery immediately after lifting seedlings. Approximately 500 g of soil was collected for each sample within a 1 m by 1 m area by combining ten subsamples collected to a depth of 13 cm using a trowel. We sampled in the center of the beds to minimize potential edge effects. Sampling was conducted at four relatively equidistant locations to ensure adequate coverage of the nursery beds. The samples were refrigerated at 4 °C and shipped overnight on ice to Mississippi State University, where stored at − 80 °C until further processing. In total, our experimental design consisted of 36 root and 4 soil samples for a total of 40 samples.

DNA isolation and PCR

For each root and soil sample, genomic DNA was extracted from three subsamples of either roots or soil (~ 0.25 g fresh weight) using the PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) as per the manufacturer's protocol. The three replicate subsamples were pooled into one, DNA quantified with Nanodrop 2000 Spectophotometer (Thermo Scientific Waltham, MA), and DNA adjusted to 2 ng/μl. We also included a negative control, in which the tissue or soil was omitted. The control yielded minimal DNA; the elute was used as a negative control in the PCRs and included in the subsequent sequencing library.

For fungal community analyses, we targeted the Internal Transcribed Spacer region 2 (ITS2) that has been proposed as the universal metabarcode marker (Schoch et al. 2012). We amplified the ITS2 region in a 2-step PCR with the forward primer fITS7 (5′ - GTGARTCATCGAATCTTTG - 3′; Ihrmark et al. 2012) and the reverse primer ITS4 (5′ - TCCTCCGCTTATTGATATGC - 3′; White et al. 1990). All

PCR reactions were carried out in duplicate 50 μl volumes with the following concentrations and volumes: 20 ng of template DNA (10 μl), 200 μM dNTPs (5 μl of 2 mM dNTP stock), 1 μM of forward and reverse primer (5 μl of each 10 μM primer stock), 10 μl of Phusion 5× HF buffer containing 7.5 mM $MgCl_2$ for a final 1.5 mM $MgCl_2$ concentration, 14.5 μl of molecular grade DEPC-treated water, and 1 unit (0.5 μl) of Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, Pittsburgh, USA). The cycle conditions for the primary PCRs included an initial 30s denaturing at 98 °C, followed by 30 cycles of 10s denaturing at 98 °C, 10s annealing at 56 °C, 1 min extension at 72 °C, and final 5-min extension at 72 °C. Resulting duplicate amplicons were combined and purified using Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (GE Healthcare, Little Chalfont Buckinghamshire, UK) in a 96-well SPRI plate format to remove excess primers and residual contaminants from the samples. We utilized a clean-up protocol identical to that provided by AgentCourt AMPure XP (Backman Coulter, Indianapolis, IN) but replaced the magnetic bead solution with Sera-Mag SpeedBead solution. To avoid the potential erroneous assignment of samples to experimental units (Carlsen et al. 2012), a secondary 5-cycle PCR included unique 12 bp barcodes appended to both forward and reverse primers under the same conditions as above, followed by a second magnetic Sera-Mag SpeedBead clean-up.

Illumina MiSeq library preparation

Purified amplicons were quantified using the ND2000 and 200 ng of each sample was pooled for sequencing. The negative extraction control did not yield comparable DNA concentration and the entire volume of the cleaned amplicon was included into the sequencing pool. Illumina specific primers and adapters were ligated to the amplicons using a NEBNext® DNA MasterMix for Illumina kit (New England Biolabs Inc., Ipswich, MA, USA) at the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA). The library was sequenced using paired-end MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) with 2 × 300 cycles. The resulting raw paired-end sequence data are available at the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA432081, BioSamples SRS2903649-2903688.

Sequence data analyses

The sequence data were analyzed using the bioinformatics software mothur (v. 1.38, Schloss et al. 2009). After contig construction, the library contained 4,500,599 sequences. Contigs were screened to remove any that contained ambiguous bases, a disagreement in primer or barcode sequence, or a homopolymer of 8 bp or longer. The remaining 4,470,285 sequences were truncated to 236 bp to facilitate pre-clustering (Huse et al. 2008) and subsequent clustering using VSEARCH (Rognes et al. 2016) both of which require aligned sequences or sequences of equal length. Near identical sequences (up to 2 nucleotide differences) were pre-clustered to reduce sequencing bias (Huse et al. 2008) and screened for potential chimeras (UCHIME; Edgar et al. 2011). After the removal of the presumed chimeric sequences, the sequence data were clustered into operational taxonomic units (OTUs) at 97% similarity using VSEARCH (Rognes et al. 2016). Rare OTUs represented by 10 or fewer sequences were removed, as they may represent PCR and/or MiSeq artifacts (Brown et al. 2015; Oliver et al. 2015). The negative control yielded a small number of sequences, which were culled during the data processing. OTUs were assigned to taxon affinities using the Naïve Bayesian Classifier (Wang et al. 2007) and the UNITE taxonomy reference (http://unite.ut.ee/repository.php). The final data set contained 872 OTUs across the 40 samples representing chestnut roots and nursery soils. We subsampled the data to an equal 10,000 sequences per sample and iteratively calculated Good's coverage, observed (S_{Obs}) and extrapolated (Chao1, Boneh) richness, diversity (Shannon's H'), and evenness (Shannon's E_H) using mothur (v. 1.38, Schloss et al. 2009).

Statistical analyses

Estimators for coverage, richness (S_{obs}), extrapolative richness (ChaoI, Boneh), diversity (H'), and evenness (E_H) data were non-normal and heteroscedastic. These data were accordingly natural log transformed (ln; S_{obs}, ChaoI, Boneh, H') or arc sine square root-transformed (E_H). To test for differences in richness (S_{obs}), extrapolative richness (ChaoI, Boneh), diversity (H′), and evenness (E_H) between the roots and soils, we compared means using Dunnett's test (Dunnett 1955). In this test, each richness, diversity, and evenness estimator for each family was contrasted against those in the nursery bulk soil using JMP (version 10.0.0). To compare richness, diversity, and evenness among the chestnut families, we used one-way ANOVAs followed by Tukey's HSD to test for differences among all possible comparisons. These analyses excluded the soil samples to better focus on the differences among the families.

To visualize the fungal community composition, we calculated pairwise Bray–Curtis distance matrices and visualized the community composition using non-metric multidimensional scaling (NMS) in PC-ORD (version 6.19). Consistently with the analyses of the richness and diversity estimators, we conducted these analyses in two steps. First, we analyzed NMS ordination that included the nursery soil samples as well as the samples representing the nine chestnut families. In these analyses, a three-dimensional ordination ($k = 3$) provided an optimal solution and represented 86.4%

of the variation with a stress of 0.13, separating the soils and a majority of the chestnut family rhizobiomes; these analyses are provided as a supplement (Supplemental Fig. S1a–c). Second, we reanalyzed the data after omitting the soil samples to focus only on the nine chestnut families. In these latter analyses, a three-dimensional ordination ($k = 3$) again provided an optimal solution and represented 85.6% of the variation with a stress of 0.14. The community data across the different treatments were compared using permutation-based MANOVA (PerMANOVA; Anderson 2001). In addition to these analyses that included the entire data matrix for the nine families, we analyzed a dataset that included only the 90 core OTUs present in at least half of the samples (see Unterseher et al. 2011 for core taxon analyses) and compared these communities using PerMANOVA. Similarly to the broader data matrix, a three-dimensional solution was optimal, representing 83.0% of the variation with a stress of 0.14.

To identify OTUs that were disproportionally enriched under one treatment condition over others, we performed Indicator Taxon Analyses (Dufrene and Legendre 1997) in PC-ORD. These analyses identified a large number of potential indicators ($P < 0.05$)—171 indicators in total, 26 for chestnut family rhizobiomes and 145 for soils (Supplemental Table S1). Indicator analyses that excluded the soils identified a total of 86 indicators ($P < 0.05$) across the nine families (Supplemental Table S2). As a result of the large number of potential indicator taxa and to better focus on the commonly occurring indicators, we present and discuss the indicator analyses only for the reduced core taxa that occurred in at least half of the 36 rhizobiome samples. However, we present the full indicator taxon lists for the complete datasets as supplements (Supplemental Tables S1 and S2).

To assign the detected OTUs to potential ecological functions and ecological guilds, we used FUNGuild (Nguyen et al. 2016) as described in Veach et al. (2017). We were specifically interested in the EcM fungi and those that were present when EcM fungi were in low abundance in our root samples. We used Dunnett's test to compare the mean abundance of EcM and putative plant pathogens in the roots and soil. We also compared the FUNGuild-assigned functional roles across the nine breeding lines using one-way ANOVA followed by Tukey's HSD post hoc tests. These analyses were first conducted using only the high probability FUNGuild assignments, followed by a separate analysis considering all assignments.

Results

General community description

Of the 872 OTUs in total, 330 (37.8%) OTUs occurred in soil, 617 (70.8%) in chestnut rhizobiomes, 177 (20.3%) in both soil and chestnut family rhizobiomes, and 153 (17.5%) OTUs were unique to soil and 440 (50.5%) were unique to chestnut rhizobiomes. The large proportion of OTUs observed only in the rhizobiomes is likely a result of nine times greater sampling of the rhizobiomes than of the nursery soils. Similarly to the small proportion of OTUs that were observed in both soils and rhizobiomes (20.3%), only 56 (9.1%) of the OTUs that occurred in rhizobiomes were present in all sampled chestnut families and 303 (49.1%) were shared by two or more sampled families. Taken together, these data indicate a great heterogeneity in the fungal communities in the nursery soils and in chestnut family rhizobiomes after 1 year in the nursery.

Overall, Ascomycota (242,468 sequences, 60.6%; 419 OTUs) and Basidiomycota (140,163 sequences, 35.0%; 223 OTUs) dominated the fungal communities, with only a small proportion of the data representing basal taxa formerly assigned to Zygomycota (10,744 sequences, 2.7%; 43 OTUs) or Chytridiomycota (570 sequences, 0.14%; 25 OTUs). Additionally, a small proportion of the data remained unclassified beyond kingdom Fungi (5981 sequences, 1.5%; 69 OTUs). Interestingly, approximately 95% of these unclassified sequences were found in the nursery soils (5707 sequences), whereas only few occurred in the rhizobiomes (274 sequences, 4.5%). The soil and rhizobiome communities were distinct (Supplemental Fig. S1a–c). Of the taxa that were assigned to a genus level, soils were dominated by *Podospora* (2311 sequences, 5.8%), *Chaetomium* (1962 sequences, 4.9%), and *Mortierella* (1342 sequences, 3.4%), whereas the rhizobiomes were dominated by *Guehomyces* (23,455 sequence, 6.5%), *Aureobasidium* (17,534 sequences, 4.9%), and *Fusarium* (12,480 sequences, 3.5%).

Of the 872 OTUs, 462 (52.9%) received a FUNGuild assignment (Fig. 1), representing 155,942 sequences (39.0% of total). Only a total of 72 OTUs (37,744 sequences, 9.4% of all sequences and 8.3% of all OTUs) received an assignment with a "high probability." Among all OTUs that were assigned to a guild, "undefined saprotroph" OTUs were overwhelmingly most common (168, 36.4% of OTUs with a guild assignment; 16.3% of sequences representing those OTUs), followed by "fungal parasite-undefined saprotroph" OTUs (39, 8.4%; 1.9%) and "plant pathogen" OTUs (38, 8.2%; 7.8%). Although only relatively few OTUs were assigned to "ectomycorrhizal" guild, these OTUs (12, 2.6%) represented the second largest sequence count among the assigned sequences (12.2%) after the "saprotroph" OTUs (Fig. 1). These EcM included OTUs assigned to *Ceratobasidium* (1 OTU), *Chloridium* (1 OTU), *Hebeloma* (1 OTU), *Laccaria* (2 OTU), *Pisolithus* (2 OTUs), *Tomentella* (1 OTU), *Scleroderma* (3 OTUs), and *Sphaerosporella* (1 OTU). The relative abundances of EcM fungi were variable in soils (4.1 ± 3.3%) and ranged widely among the chestnut family rhizobiomes (from 0.04 ± 0.03% in Princeton to 17.4 ± 10.3% in D22; Fig. 1). Yet, none of

the families differed in the sequence abundance of EcM fungi from the nursery soil (Dunnett's test; $P \geq 0.05$), likely due to large variability. When the rhizobiomes were compared, some chestnut families differed from one another (one-way ANOVA: $F_{8,27} = 3.33$, $P = 0.0089$): the family with the greatest proportion of EcM fungi (D22, 17.4 ± 10.3%) had a greater abundance of EcM fungi than the Chinese chestnut (Princeton, 0.04 ± 0.03%) and two of the other hybrids (W3, 0.08 ± 0.09% and W6, 0.12 ± 0.18%) with low EcM abundances (Tukey's HSD, $P < 0.05$). Interestingly, in the near absence of EcM fungi, the Chinese chestnut family had a high abundance of putative plant pathogens (9.8% ± 11.3%; Fig. 1) representing a variety of taxa assigned to Xylariales (*e.g.*, genus *Monographella*), Ophiostomatales (*e.g.*, genus *Ophiostoma*), Pleosporales (*e.g.*, genus *Leptosphaeria*), and Hypocreales (*e.g.*, *Clonostachys* and *Fusarium* teleomorph *Gibberella*) among others. In contrast, the W3 hybrid had a large proportion of sequences assigned to a combined guild that contained plant pathogens, soil, and wood saprotrophs (13.7 ± 25.9%; Mixed 2 in Fig. 1) representing exclusively OTUs assigned to genus *Fusarium*, whereas hybrid W6 had a large proportion of putative fungal endophytes (14.7 ± 28.0%; Fig. 1) representing common root-associated taxa (e.g., *Cadophora*, *Capronia*, *Leptodontidium*, *Phialocephala*, and *Trichoderma*). These analyses highlight (1) the low and variable presence of EcM taxa in 1-year old seedlings in the nursery and (2) the heterogeneity among the fungal functional groups associated with their roots.

Richness and diversity

Our coverage estimators (Supplemental Fig. S2a) indicate rather complete sampling of the fungal communities in both roots (99.5 ± 0.1%) and nursery soils (99.2 ± 0.1%). Although generally high, coverage was higher in all rhizobiomes than in the sampled nursery soils (Dunnett's test, $P < 0.05$). The fungal community richness differed between the nursery soil and some chestnut roots (Supplemental Fig. S2b), four of the nine chestnut families had lower richness than soil (W3, D22, 4-75, and Pryor 043; Dunnett's test, $P < 0.05$). Extrapolative richness estimators corroborated: ChaoI estimates were commonly lower in the rhizobiomes than in the soil (Dunnett's test, $P < 0.05$) except for the Chinese chestnut family (Princeton; Dunnett's test, $P = 0.08$) and one hybrid (W5; Dunnett's test, $P = 0.07$) that did not differ (Supplemental Fig. S2c); and, Boneh estimators for the potential number of additional OTUs that would have been detected if sampling had been complete were consistently lower (Dunnett's test, $P < 0.05$) except for the Chinese chestnut family that did not differ from the nursery soil (Princeton; Dunnett's test, $P = 0.07$) (Supplemental Fig. S2d). In contrast to richness, diversity and evenness estimators between the chestnut rhizobiomes and the nursery soil did not differ (Supplemental Figs. S2e, f; Dunnett's test; $P > 0.34$). Further analyses that omitted the soils and compared only the nine families, indicated no differences in coverage (one-way ANOVA: $F_{8,27} = 1.16$, $P = 0.36$), richness (one-way ANOVA: $F_{8,27} = 1.34$, $P = 0.27$), extrapolative richness–ChaoI (one-way ANOVA: $F_{8,27} = 1.17$, $P = 0.35$) and Boneh (one-way ANOVA: $F_{8,27} = 1.94$, $P = 0.10$),

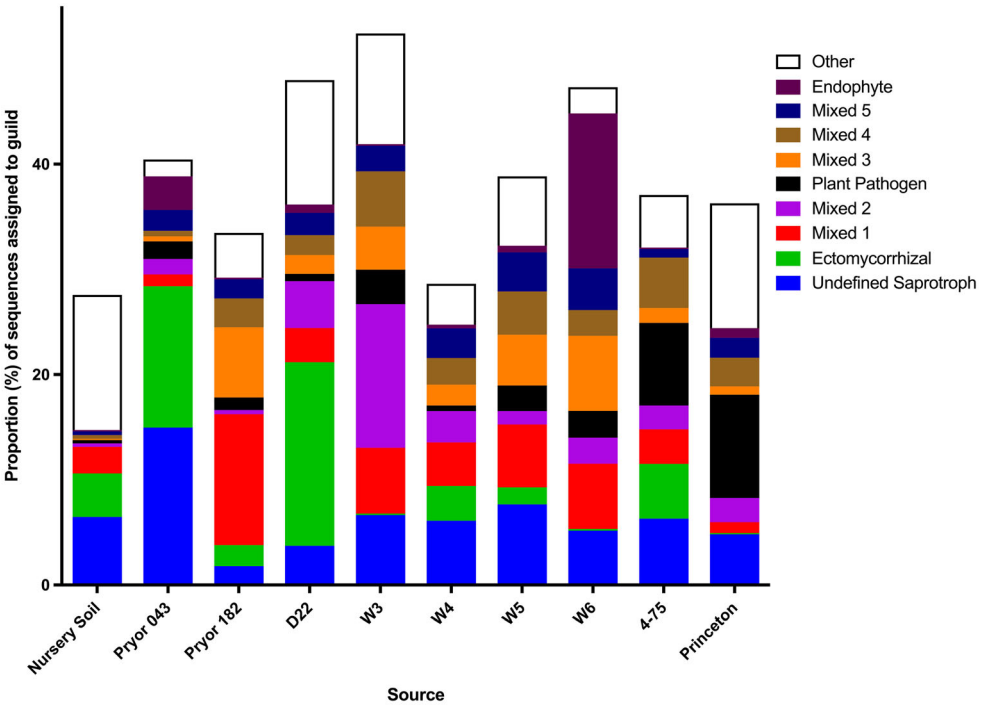

Fig. 1 Assignment of the sequence data to the most commonly encountered fungal ecological guilds using FUNGuild (Nguyen et al. 2016). OTUs with no assignment were omitted for better visualization. Guilds listed here are as follows: 1, undefined saprotroph; 2, ectomycorrhizal; 3, mixed 1: animal pathogen-endophyte-epiphyte-plant pathogen; 4, mixed 2: plant pathogen-soil saprotroph-wood saprotroph; 5, plant pathogen; 6, mixed 3: animal endosymbiont-undefined saprotroph; 7, mixed 4: animal pathogen-endophyte-epiphyte-undefined saprotroph; 8, mixed 5: animal pathogen-endophyte-plant pathogen-wood saprotroph; 9, endophyte; 10, other guilds

diversity (one-way ANOVA: $F_{8,27} = 0.82$, $P = 0.59$), or evenness (one-way ANOVA: $F_{8,27} = 0.78$, $P = 0.63$) (Supplemental Fig. S2).

Community composition

We first visualized and tested for differences in fungal community composition using the complete dataset including both the nursery soil and all rhizobiome samples (Supplemental Fig. S1a–c). These analyses indicated distinct rhizobiome and nursery soil communities (PerMANOVA; $F_{9,30} = 3.08$, $P = 0.001$). Further analyses that excluded soils distinguished fungal communities among the chestnut family rhizobiomes (Supplemental Fig. S3a–c; $F_{8,27} = 2.98$, $P = 0.001$). Subsequent pairwise comparisons indicated that fungal communities in the roots of most chestnut families differed from each other, with only a few exceptions (Table 2): only six of the possible 36 pairwise comparisons suggested non-distinct communities. The NMS ordinations suggest that the American and Chinese chestnut families—as well as their hybrids—recruited distinct fungal communities from nursery soil (Supplemental Fig. S3a–c).

We performed ordination analyses using only core OTUs (Fig. 2). Again, despite the limited replication and great within-treatment variability, the communities differed among the chestnut families (PerMANOVA; $F_{8,27} = 2.93$, $P = 0.001$). Pairwise comparisons show that these communities differed in 31 of the 36 possible comparisons (Supplemental Table S3). These analyses suggest that it is not only the peripheral members that distinguish the fungal communities but that core components are also recruited differently.

Indicator taxa

To identify taxa that were disproportionately represented, we used indicator taxon analyses (Supplemental Tables S1 and S2). The analyses that focused on the core rhizobiomes identified a total of 30 indicator OTUs (Table 3). Among these, OTUs representing common soil-inhabiting genera—*Cryptococcus* (5 OTUs), *Rhodotorula* (4 OTUs), and *Mortierella* (2 OTUs)—were most abundant, but included putative pathogens exemplified by an OTU assigned to genus *Ophiostoma* and mycorrhizal symbionts exemplified by an OTU assigned to genus *Tomentella*. Interestingly, the three pure species families accounted for more than half of the indicator OTUs (Pryor 182, 11; Pryor 43, 2; and Princeton, 4), whereas—in general—the hybrid families had few indicator OTUs (e.g., D22, W3, W4, and W5 had only one indicator each; Table 3).

Discussion

We analyzed chestnut hybrid and parent family rhizobiomes from an operational nursery to evaluate their rhizobiomes prior to outplanting. As a result of the use of high-value nursery stock from an operational nursery, our within-treatment replication was low and variation high. Yet, these data show (i) that chestnut seedling rhizobiomes are distinct from those in nursery inoculum; (ii) that chestnuts of different species and hybrid families recruit distinct rhizobiomes from the nursery inoculum with no discernable pattern conforming to the backcross breeding program; and (iii) that the rhizobiomes include fungi ranging from saprobes and mycorrhizal mutualists to

Table 2 Pairwise comparisons of the fungal communities associated with the rhizospheres of nine chestnut families

	Pryor 182	Pryor 43	D22	W3	W4	W5	W6	4-75
Pryor 182	1							
Pryor 43	$t = 1.69$; $P = 0.028$	1						
D22	$t = 2.39$; $P = 0.035$	$t = 1.95$; $P = 0.025$	1					
W3	$t = 1.83$; $P = 0.032$	$t = 1.86$; $P = 0.032$	$t = 1.67$; $P = 0.030$	1				
W4	$t = 1.88$; $P = 0.027$	*$t = 1.57$; $P = 0.063$*	$t = 1.86$; $P = 0.029$	$t = 1.64$; $P = 0.035$	1			
W5	$t = 1.61$; $P = 0.038$	$t = 1.75$; $P = 0.029$	$t = 1.82$; $P = 0.031$	$t = 1.29$; $P = 0.036$	*$t = 1.56$; $P = 0.062$*	1		
W6	$t = 1.44$; $P = 0.033$	$t = 1.67$; $P = 0.026$	$t = 1.89$; $P = 0.022$	$t = 1.45$; $P = 0.032$	$t = 1.77$; $P = 0.027$	*$t = 1.26$; $P = 0.055$*	1	
4-75	$t = 1.92$; $P = 0.035$	*$t = 1.23$; $P = 0.118$*	$t = 2.07$; $P = 0.029$	*$t = 1.50$; $P = 0.065$*	$t = 1.66$; $P = 0.032$	$t = 1.60$; $P = 0.036$	$t = 1.72$; $P = 0.026$	1
Princeton	$t = 2.25$; $P = 0.024$	$t = 1.82$; $P = 0.031$	$t = 1.77$; $P = 0.037$	$t = 1.36$; $P = 0.027$	$t = 1.85$; $P = 0.025$	*$t = 1.73$; $P = 0.050$*	$t = 1.76$; $P = 0.031$	$t = 1.82$; $P = 0.036$

PerMANOVA analyses that included a full OTU data matrix but excluded the soils distinguished fungal communities among the chestnut family rhizobiomes (Supplemental Fig. 3; $F_{8,27} = 2.98$, $P = 0.001$). These pairwise comparisons indicate that fungal communities of most breeding lines differ from each other. The *t* test variables and associated *P* values are shown, and those that do not differ are highlighted in italic

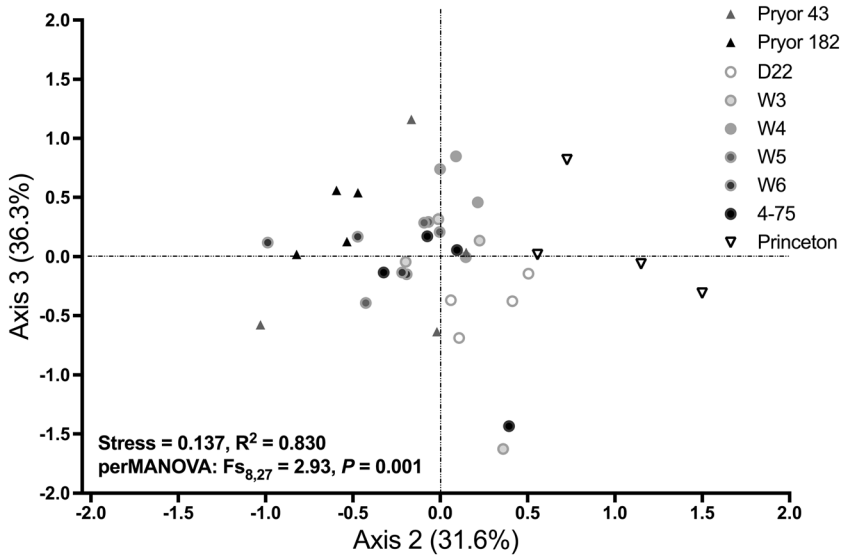

Fig. 2 Non-metric multidimensional scaling (NMS) ordination of the core fungal communities that include only those 90 OTUs that occurred at least in half of the 36 chestnut rhizobiome samples. The ordination was optimally resolved on three axes that represent 15.1%, 31.6%, and 36.3% of the variability, for a total of 83.0% with stress 0.14. The permutation-based MANOVA indicated that fungal communities differ among the nine analyzed chestnut rhizobiomes ($F_{8,27} = 2.93$, $P < 0.001$). Shown are Axis 2 and Axis 3 that represent 67.9% of the variation. The ordination distinguishes most chestnut families (Supplemental Table S3)

root-associated endophytes and plant pathogens. Importantly, although our data poorly permit decoupling effects of the genotypic background and the possible founder/priority effects (see Kennedy et al. 2009; Fukami et al. 2010) or spatial heterogeneity in the nursery soils, they highlight the distinct and heterogeneous rhizobiomes and distinguish the loads of putative pathogens and benign endophytes when a substantial EcM component is absent. Taken together, these findings indicate diverse fungal rhizobiomes that have the potential to either burden (see Lilja and Rikala 2000) or aid (see Menkis et al. 2007) post-transplanting performance and successful establishment.

We broadly dissected fungal rhizobiomes present within an operational nursery. The data—for OTUs with functional assignments—included a continuum of potential functions but were dominated by diverse OTUs assigned to putative saprotrophic guilds, or guilds that combined saprotrophs and plant pathogens. Although OTUs assigned to plant pathogens (38 OTUs) were more diverse than those assigned to EcM (12 OTUs), the latter were more abundant based on sequence counts. The EcM fungi can positively impact tree seedling growth in forest nurseries (Sinclair et al. 1982; Menkis et al. 2007) and improve transplanting success (Perry et al. 1987; Kropp and Langlois 1990; Ortega et al. 2004; Menkis et al. 2007). In contrast, in their absence, the nursery-grown seedlings may be more susceptible to environmental stressors, such as root-borne pathogens, drought, and/or toxic metals (Morin et al. 1999; Lilja and Rikala 2000; van Tichelen et al. 2001; Ortega et al. 2004). Our data indicate that, although the EcM inhabit both nursery soil and the chestnut rhizobiomes therein, the EcM communities include only a limited number of taxa likely as a result of the fumigation and fertilization treatments as per standard operating protocols. Our estimates of low EcM richness agree with others focusing on EcM in forest nurseries. For example, Menkis et al. (2005), studying Lithuanian forest nurseries, observed 21 EcM taxa in *Pinus sylvestris* and 13 in *Picea abies*.

Most EcM fungi have broad host ranges (Molina et al. 1992), although host ranges may vary from a few hosts to wide generality (Molina and Horton 2012). Recent studies have concluded that genotypic differences among conspecific hosts may select for distinct EcM (Korkama et al. 2006; Velmala et al. 2013; Lamit et al. 2016) or fungal rhizobiomes (Perez-Izquierdo et al. 2017). Although our data suggest differences among the chestnut families, we observed no clear trends supporting that breeding for pathogen resistance in American chestnut may alter its compatibility with EcM fungi. Our results contrast those of D'Amico et al. (2015), who bioassayed root tips to compare EcM colonization and communities of a *C. dentata* wild-type, transgenic resistance line, American-Chinese hybrids, or other Fagaceae. However, the hybrids that were included in our experiment did not consistently possess less diverse or less abundant EcM than their parents suggesting no strong negative selection against EcM partners as a result of the breeding focusing on disease resistance.

The EcM communities that we observed are not unique compared to other nurseries or studies that target American chestnut. We observed EcM taxa previously detected in nursery soils or even considered adapted to nursery conditions (*Laccaria* and *Hebeloma* (Mikola 1970; Stenström and Ek 1990; Henrion et al. 1994; Menkis et al. 2005, 2016; Menkis

Table 3 Indicator taxon analyses (mean ± st. dev. and associated *P* values) of the core OTUs that were present in half of the analyzed rhizosphere samples. The listed 30 are significantly more abundant in the source family compared to others

Core OTU ID	Family	Indicator value mean ± st. dev.	*P* value	Taxon
Otu0005	Pryor 182	18.1 ± 2.54	0.001	*Aureobasidium* sp.
Otu0042	Pryor 182	20.3 ± 3.45	0.001	*Auriculibuller* sp.
Otu0094	W5	21.6 ± 6.78	0.001	*Trichosporon* sp.
Otu0108	Pryor 182	27.1 ± 7.14	0.001	*Endoconidioma* sp.
Otu0068	Princeton	36.5 ± 5.53	0.001	*Cryptococcus* sp.
Otu0112	Pryor 182	24.4 ± 7.02	0.001	*Sydowia* sp.
Otu0024	4-75	33 ± 7.66	0.001	*Ophiostoma* sp.
Otu0113	Pryor 182	24.6 ± 8.86	0.001	*Fibulobasidium* sp.
Otu0065	W3	31.8 ± 9.92	0.001	*Pleosporales* sp.
Otu0095	Pryor 182	20.5 ± 4.1	0.002	*Rhodotorula* sp.
Otu0128	Pryor 182	20 ± 5.31	0.002	Dothideomycetes unclassified
Otu0036	4-75	36.9 ± 6.6	0.002	*Mortierella* sp.
Otu0004	W4	22.1 ± 3.89	0.005	Sebacinales Group B
Otu0191	Pryor 182	20.2 ± 6.4	0.006	*Cryptococcus* sp.
Otu0090	Pryor 182	24.9 ± 7.15	0.008	*Rhodotorula* sp.
Otu0014	W6	76.8 ± 16.58	0.008	*Trichoderma* sp.
Otu0075	W6	28.7 ± 7.32	0.011	*Mortierella* sp.
Otu0022	D22	39.8 ± 6.67	0.014	*Hypocrea* sp.
Otu0157	Princeton	23.6 ± 8.5	0.017	*Cryptococcus* sp.
Otu0008	W6	18.9 ± 2.67	0.018	*Rhodotorula* sp.
Otu0074	4-75	22.7 ± 5.1	0.021	*Cryptococcus* sp.
Otu0127	Pryor 182	18.5 ± 3.1	0.024	*Cryptococcus* sp.
Otu0003	Princeton	20 ± 2.96	0.031	*Guehomyces* sp.
Otu0021	Pryor 43	30.2 ± 7.4	0.04	*Tomentella* sp.
Otu0020	Princeton	42.7 ± 2.84	0.04	*Leucosporidium* sp.
Otu0073	W6	23.6 ± 5.57	0.041	*Rhodosporidium* sp.
Otu0061	Pryor 43	71.1 ± 11.09	0.044	*Mastigobasidium* sp.
Otu0119	Pryor 182	26.9 ± 9.8	0.048	Helotiales unclassified

and Vasaitis 2011), *Pisolithus* (Marx 1977), *Tomentella* (Menkis et al. 2005)). Similarly, previous studies on American chestnut using either laboratory or field bioassays (Palmer et al. 2008; Dulmer et al. 2014; Stephenson et al. 2017; Bauman et al. 2018) reported *Laccaria*, *Tomentella*, and *Scleroderma* that we also observed in our nursery-reared seedlings. Another study (Bauman et al. 2013), focusing on EcM of backcrossed bare-root hybrid and seed-initiated seedlings (inoculated with *Pisolithus tinctorius*) in a coal mine restoration project using select soil preparation practices in central Ohio, observed genera detected in the current study including *Hebeloma*, *Laccaria*, *Pisolithus*, *Scleroderma*, and *Tomentella*. In sum, our data indicate that nursery soil inocula include EcM taxa that nursery-reared seedlings likely encounter at their outplanting sites.

In addition to the low EcM richness, our data were characterized by the highly variable EcM occurrence as indicated by the large within and between treatment variability. It is of note that we did not estimate colonization rates microscopically, but rely exclusively on sequence yields and relative abundances. Some sampled chestnut rhizobiomes yielded very few EcM sequences. These data agree with previous observations from forest nurseries. Menkis et al. (2016), analyzing container-grown conifer forest nursery seedlings in Sweden, concluded that the communities "were largely composed of saprotrophic, mycorrhizal and endophytic fungi, while pathogens were relatively rare." Although OTUs assigned to "plant pathogens" using FUNGuild (Nguyen et al. 2016) were definitely present in our data, they were not abundant across all soil and many rhizobiome samples. Even so, the sparse presence of EcM fungi seemed to permit pathogen establishment as indicated by the Chinese parent line Princeton. This may have substantial consequences for the seedling establishment success. Saunders et al. (1992) estimated that a quarter of post-planting seedling mortality could be a result of pathogens that originated from the nursery, and Lilja and Rikala (2000) concluded that a majority of *Rhizoctonia*-inoculated seedlings failed to survive in the field. Although the pathogens can negatively impact stand regeneration, they occurred heterogeneously in our experiment: even

when pathogens were present, their occurrence was highly variable. In sum, our data highlight the continuum of fungi ranging from pathogens to mycorrhizal symbionts that occupy the rhizobiomes as well as the stochasticity in their occurrence.

Similarly to previous reports (Kernaghan et al. 2003; Menkis et al. 2005, 2016; Stenström et al. 2014), our data show that putative endophytes commonly occupy nursery-grown seedling rhizobiomes—particularly when EcM OTUs are few. In our study, the hybrid line W6 serves as an example: with its low EcM abundance, endophytes were a large component in the rhizobiome. Interestingly, OTUs from chestnut rhizobiomes represented nursery-borne endophytes commonly detected in both Europe (Menkis et al. 2005; Stenström et al. 2014) and North America (Kernaghan et al. 2003): *Cadophora*, *Leptodontidium*, *Meliniomyces*, and *Phialocephala*. There is a considerable debate about the function of these root-associated fungi. Meta-analyses disagree and suggest that the endophyte effects on the hosts are either primarily negative (Alberton et al. 2010; Mayerhofer et al. 2013) or, if positive, may depend on availability or form of nitrogen (Newsham 2011). Mandyam and Jumpponen (2015) combined and analyzed a large number of small endophyte inoculation experiments. They highlighted context dependencies perhaps best explained by interactions specific to host genotypes and fungal strains. Our data focusing on chestnut families highlight that the endophytes can be abundant in the rhizobiomes and may thus impact the seedling outplanting performance, albeit perhaps in an unpredictable manner.

Many comparisons between the chestnut families and nursery soil were consistent with expectations. Soils often had greater fungal richness than the rhizobiomes, whereas these estimates did not differ among the chestnut families. Similarly to the richness estimates, the soil and rhizobiome communities were compositionally distinct. Interestingly, our ordination analyses highlighted also distinct fungal communities in the chestnut rhizobiomes—even when only the so-called core OTUs were included. Our subsequent indicator taxon analyses identified a total of 30 indicator OTUs—many of which assigned to common soil-inhabiting taxa: *Cryptococcus*, *Rhodotorula*, and *Mortierella*. Half of the observed indicator taxa were associated with two of the three parental lines, whereas the hybrid lines had few indicator OTUs. It remains unclear whether or not such parent-hybrid line distinctions suggest a limited ability of the hybrid families to recruit rhizobiomes. This is particularly the case since our data provided no evidence for less diverse fungal communities in these hybrids bred for greater disease resistance.

We dissected the rhizobiomes of chestnut families bred for resistance against the devastating pathogen to evaluate the potential non-target effects. Our data unequivocally show that chestnut families depart the nurseries with distinct fungal loads that may be consequential for their outplanting success. While some seedlings and families may host a large EcM component, others may be burdened by large proportion of potential antagonists or endophytes whose host interactions are unpredictable. Follow-up studies of the seedlings whose rhizobiomes have been pre-screened may provide a means to evaluate whether or not the nursery-recruited rhizobiomes differently determine the establishment success after transplanting.

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