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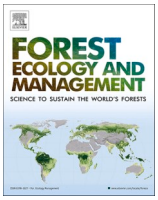

Convergent shifts in soil fungal communities associated with Fagaceae reforestation in the Southern Appalachian Mountains

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ABSTRACT

Forests of the Southern Appalachian Mountains were historically dominated by hardwood species within the family Fagaceae, e.g., American chestnut (*Castanea dentata*) and white oak (*Quercus alba*) among others. Due to numerous biotic and abiotic stressors, including pathogen pressure, populations of many Fagaceae have been greatly reduced, or functionally eliminated as is the case with American chestnut. This has led to reforestation efforts designed to increase American chestnut and white oak populations in this region, but success has been minimal. Since soil fungal communities are crucial to plant health, understanding how reforestation efforts alter soil fungal communities will inform reforestation efforts. Here, using nursery-reared bareroot seedlings of American chestnut, Chinese chestnut, 3rd generation backcross chestnut hybrids (BC_3F_3), and white oaks outplanted at a locally xeric site in the Southern Appalachian Mountains, we investigated if and how these species and backcross families within species differentially impact soil fungal communities. We demonstrate that after three years of growth, plant-associated soil fungal communities change similarly among different Fagaceae and are distinct from pre-planting soil communities. Interestingly, we observed differential shifts in fungal functional guilds among the Fagaceae species, although these were rather minimal. Taken together, the largely convergent shifts in soil communities across Fagaceae species suggest that these tree species may have similar impacts on soils and/or share similar communities that become enriched in closely related fungal species. The similarity in shared mutualist fungal communities suggests that companion planting or reforestation of genetically disease resistant American chestnut adjacent to establishing white oak trees might enhance survival and growth of both species at xeric sites.

1. Introduction

The family Fagaceae includes two historically important and abundant genera of hardwoods trees in the southern Appalachian Mountains (Elias, 1971), the chestnuts and oaks, representing phylogenetic sister subfamilies (Deng et al., 2018; Manos et al., 2001). American chestnut [*Castanea dentata* (Marshall) Borkh.; Fagaceae] was an ecologically and economically important tree species in the eastern deciduous forests that was functionally extirpated in the wild by the chestnut blight fungus (*Cryphonectria parasitica* (Murrill) Barr) during the first half of the 20th century. Prior to the introduction of this pathogen, the American chestnut was a codominant forest species whose range extended from Maine to northern Mississippi (Little, 1977). While various oak species (*Quercus* spp.) long co-existed with American chestnuts, after the elimination of the dominant chestnut populations, these *Quercus* species largely replaced American chestnut in these forests (Korstian and Stickel, 1927; Wang and Hu, 2015; Woods and Shanks, 1959). This co-existence has been generally presumed to have been supported by similarities in plant associated microbial communities, particularly so by ectomycorrhizal fungi (Molina and Horton, 2015), many of which can associate with both species. However, fungal community similarities between co-existing oaks and American chestnuts have only been

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examined using either culturing, or direct sequencing of ectomycorrhizal morphotypes (Dulmer et al., 2014). To our knowledge, investigations into whole-community, plant-associated soil fungi between oaks and chestnuts are lacking. This is a crucial knowledge gap given the common assumption that oaks and chestnuts share many mycorrhizal symbionts (Dulmer et al., 2014). The loss of the dominant American chestnut may have elicited a shift in soil fungi from previously common soil fungi or fungal communities shared with various oaks. Such shifts, if they exist, may impact natural oak regeneration and potential reestablishment of American chestnut in reforestation efforts.

Environmental change in the last century has impacted the stability and growth of hardwood forests. Changes to the disturbance regimes, global climate, and herbivore populations in the Southern Appalachian Mountains have altered *Quercus* forest compositions over historical and recent contemporary time frames (Adams and Rieske, 2001; Andruk et al., 2014; Delcourt and Delcourt, 1997; McEwan et al., 2011; Pinchot et al., 2022; Redick and Jacobs, 2020). More frequent droughts, aging forest stands, and compounding stressors such as root disease (*Armillaria* root rot) and canker pathogens (Oak et al., 2015; Starkey et al., 2004) have resulted in oak decline in the eastern US forests (Yang et al., 2021). These changes in forest vegetation likely result in concomitant changes in soil fungal communities (Beals et al., 2022; Bidartondo et al., 2018; Brown et al., 2019, 2013; Carson et al., 2019; Jansson and Hofmockel, 2020; Pritchard, 2011). Such impacts on forest soil fungal communities may be important in the reestablishment of oaks and disease resistant hybrids of American chestnut (Clark et al., 2016, 2014). Reestablishment of an oak/chestnut forest stands in the Eastern US could be facilitated if these taxa share and are supported by similar root mutualist communities as opposed to mycorrhizal partners with greater host specificity or host preferences (Molina and Horton, 2015). One previous study reported that mycorrhizal fungi that associate with red oak (*Q. rubra* L.) could be used to aid in the establishment of outplanted American chestnut hybrids (Dulmer et al., 2014). Further, another study identified mycorrhizal fungi shared between American chestnut, Chinese chestnut (*C. mollissima*), and various third-generation backcross hybrids bred for resistance (Reazin et al., 2019) grown in a common nursery bed. Representatives of those same lines of American chestnut, Chinese chestnut, and backcross hybrid seedlings from those nurseries were outplanted with white oak (*Quercus alba* L.) seedlings in the Southern Appalachian Mountains here.

The regeneration of oaks has been problematic for many decades, whereby canopy dominant oaks are often replaced by other non-oak species following disturbances in eastern deciduous forests (Abrams, 2003; Lorimer, 1993; Nowacki and Abrams, 2008). Changing forest community dynamics could elicit a realignment in understory vegetation as well as lead to altered soil microbial communities and diversity. Regeneration through planting locally adapted seedlings is an option to restore Fagaceae species but has had limited success with American chestnut (Clark et al., 2016; Pinchot et al., 2022) and some oak species (Clark et al., 2015). However, it remains currently unknown if and how soil fungal communities might shift following planting of *Castanea* and *Quercus* trees. Further, it is uncertain to what extent soil communities may be impacted by genetically distinct nursery stock, especially in the case of plantation of backcross hybrids that have been bred for resistance. Previous studies in different systems have strongly suggested that different host genotypes may fundamentally alter plant associated communities (Brown et al., 2020; Poli et al., 2016; Schweitzer et al., 2008; Wagner et al., 2020, 2016), but this has not been investigated with oak/chestnut in the context of reforestation, although one study reports similar ectomycorrhizal richness and some shared taxa between American chestnut hybrids and understory vegetation (Bauman et al., 2022).

Here, we aimed to investigate how the establishment of American and Chinese chestnut, chestnut hybrids, and white oak alters plant associated soil fungal communities. To test this, we compared soil fungal communities sampled at the time of planting with soil fungal communities sampled three years post-planting using a paired experimental framework. We ask 1) if chestnut and oak associated soil fungal communities are distinct among species and families, and if so, 2) how do specific fungal guilds (e.g., pathogens, mycorrhizae, etc.) differentially shift after plant establishment and do these shifts differ between oaks and chestnuts or across backcross hybrid plant families. Additionally, 3) by comparing our results directly to operational taxonomic units (OTUs) from roots and nursery soils that were co-grown with our outplanted seedlings (Reazin et al., 2019), we also investigated if plant associated fungi from nursery beds may have persisted with the outplanted seedlings three years post-planting. Our goal is to provide important management recommendations to improve reforestation success of oak and chestnuts.

2. Materials and methods

2.1. Study site

Chestnut (1–0; grown for one year in nursery beds) and oak (2–0; grown two years in nursery beds) bare root seedlings were planted in the Nantahala National Forest (Nantahala Ranger District, Macon County, North Carolina, USA; 35.01° N; 83.35° W; 850 m asl). This site has well-drained soils that originated from residuum of granite and gneiss on steep slopes (20–30 %). During the work reported here (2015–2018), the closest (<10 km) US National Weather Service climate station (at Coweeta Experimental Station) had average annual precipitation of 1886 mm per year (range of 1315–2504 mm for 2016 and 2018 respectively) and an average monthly mean temperature ranging from 3.3° C (January) to 23.5° C (July). This site was harvested to basal area less than 3 m^2 per ha of basal area and treated with a prescribed burn in October 2014, the autumn before planting in the spring of 2015. This site has a historic legacy of pitch pine (*Pinus rigida* Mill.) forests with dominant mountain laurel (*Kalmia latifolia* L.) understory. Prior to harvest, white oak and scarlet oak (*Q. coccinea* L.) were the dominant hardwood species. This site, including soils, has been previously characterized for hardwood growth performance (Brown et al., 2022).

2.2. Seedling origin and planting

White oak, American Chestnut, Chinese chestnut, and two backcross chestnut hybrids bred for blight resistance were grown from acorns or nuts collected from open-pollinated orchard or wild trees. White oak acorns were obtained from the University of Tennessee's Tree Improvement Program from two wild tree collections (families AS and ETN) located in the Ridge and Valley province of east Tennessee. Chestnuts were obtained from either The American Chestnut Foundation (TACF) or the Connecticut Agricultural Experimental Station (CAES). The Chinese chestnut was located on a private property with limited pollen contamination (Paul Sisco, TACF, personal communication) (Burnham et al., 1986). The backcross hybrids (BC_3F_3) from TACF (families D22, W3, W4, W5, and W6) are theoretically 94 % *C. dentata* and 6 % *C. mollissima*, and were obtained from orchards located in Meadowview, VA (Hebard, 2006). The CAES hybrids (family 4–75) are theoretically 90 % American chestnut with remaining 10 % a mix of Chinese chestnut, European chestnut (*C. sativa*), and Japanese chestnut (*C. crenata*) and were obtained in New Haven, CT (Anagnostakis, 2012). The chestnut seedlings were grown in nursery beds at the Indiana State Nursery in Vallonia, IN as 1–0 seedlings and the white oak seedlings were grown at the Tennessee State Nursery in Delano, TN as 2–0 seedlings. We will hereafter use 'species' to refer to American chestnut, Chinese chestnut, backcross chestnut hybrids, or white oak.

In total, 225 chestnut and 222 white oak bare root seedlings were planted at the study site on March 24, 2015, at a planting spacing of 3.7 m using KBC bars.

2.3. Soil sampling

For each chestnut species and family (American, Chinese, BC_3F_3, and CAES) and oak family (AS and ETN), we randomly selected four representative trees for soil fungal community analyses. The sampled trees were not in close proximity of each other to reduce potential autocorrelation. Selected plants include American chestnut (families Pryor 182 and Pryor 43), Chinese chestnut (family Princeton), BC_3F_3 (families D22 and W3), CAES (Family 4–75), and white oak (families AS and ETN) for a total of 32 plants. We sampled soils at planting (2015) and from the same trees three years after planting (13 January 2018) for a total of 64 samples. Seedlings at planting had average heights of 133 cm (American), 88 cm (Chinese), 77–123 cm (BC_3F_3), 128 cm (CAES), 118 cm (AS) and 79 cm (ETN) and after three years of growth (2018) average heights were 192 cm (American), 132 cm (Chinese), 140–188 cm (BC_3F_3), 164 cm (CAES), 141 cm (AS) and 108 cm (ETN) (Brown et al., 2022). The soils at the time of planting were collected using 10 soil probe subsamples at the planting location and 2018 samples were collected using 10 soil probe subsamples. The soils were collected at a depth of 10 cm and 10 cm from the root-soil interface as not to damage the young root system. Soil probes were surface sterilized using disinfecting wipes to prevent cross-sample contamination. At collection, soils were placed into a clean zip top plastic bag for each sample, manually homogenized, and transported on ice to Mississippi State University where frozen at −80 °C within 24 h of sampling.

2.4. DNA extraction and metabarcoding

DNA was extracted from soils (0.25 g; no root material was seen in soils) using PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA). Soils for each experimental unit were extracted in triplicate and following the extractions, the DNA subsamples were pooled by experimental unit and quantified (Nanodrop 2000 Spectophotometer; ThermoScientific, Waltham, MA, USA) and DNAs were normalized to a concentration of 2 ng μl^{-1}.

Fungal libraries were generated using a 2-step PCR (*following* Reazin et al., 2019) targeting the Internal Transcribed Spacer Region 2 (ITS2) of the rRNA operon using the primer pair fITS7-ITS4 (Ihrmark et al., 2012; White et al., 1990). Primary PCRs were conducted in duplicate 50 μl reactions with the following conditions: 20 ng of template DNA, 200 μM dNTPs, 1 μM of each primer, 10 μl of Phusion 5x HF buffer (ThermoFisher, Pittsburgh, PA) and molecular grade water up to reaction volume. The primary PCR parameters were 30 s denaturing at 98 °C, followed by 30 cycles of 10 s denaturing at 98 °C, 10 s annealing at 56 °C, 1 min extension at 72 °C, and final 5-minute extension at 72 °C. Duplicate amplicons were combined into one and cleaned using Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (GE Healthcare, Little Chalfont Buckinghamshire, UK). Secondary PCRs were conducted to include unique 12 bp barcodes (synthesized into the 5′-end of the fITS7 and ITS4 primers) for multiplexing using 5-cycle PCR (as above) followed by a second Sera-Mag clean-up.

Amplicons were pooled (200 ng from each sample) and Illumina linkers and adapters ligated to the library using a NEBNext® DNA MasterMix for Illumina kit (New England Biolabs Inc., Ipswich, MA) at the Integrated Genomics Facility at Kansas State University (Manhattan, KS) and was sequenced using Illumina MiSeq (300PE). All data were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under the following accessions: BioProject PRJNA820875 and BioSample Runs SRR18548713-SRR1858776.

2.5. Sequence data analyses

Sequence data were processed using the program mothur (v.1.47.0; Schloss et al., 2009). Paired reads were contiged and full length ITS2 regions were extracted using the program ITSx (v.1.1.3 using a conda environment; Bengtsson-Palme et al., 2013) and reads without complete ITS2 regions were removed. Retained sequences were further processed to remove putatively chimeric reads using VSEARCH (Rognes et al., 2016) and sequences were assigned to best taxonomic identities with a Naïve Bayesian classifier (Wang et al., 2007) against a locally modified UNITE Species Hypothesis database (v.8.3) (Nilsson et al., 2019) enriched to include additional microeukaryotes to help identify non-fungal reads; all non-fungal reads were removed. Operational taxonomic units (OTUs) were demarcated using VSEARCH using abundance based greedy clustering (97 % threshold; Rognes et al., 2016). OTUs with fever than 10 global sequences were culled (Brown et al., 2015; Oliver et al., 2015) and representative sequences (most abundant) of each OTU were identified and used to compare OTUs found in this present study with OTUs found from the same nursery root stock (see below; Reazin et al., 2019).

Using an iterative subsampling approach (1000 iterations of subsampling at a sequence depth of 15,000 per sample), we calculated average Bray-Curtis dissimilarity and estimateed Richness (S_{obs}), Diversity (Simpson's 1-D), and Evenness (Simpson's - E_D). To assess if diversity estimates differed across our experimental design, we used a nested two-way ANOVA model which included family (hybrid type or species within chestnuts and families within oaks) nested within species (chestnut or oak) with the following model components: Family[Species], Year, and Year × Family[Species]. To test if the fungal communities differ among our treatments, we used PerMANOVA tests (Anderson, 2001) with 999 iterations using package *vegan (*Oksanen et al., 2017*)* in the R (R core team; v.4.1.1) environment on the Bray-Curtis dissimilarity matrix with the model Time, Species, Time × Species. Because of relatively few samples that precluded inclusion of Family in the full model, we performed separate PerMANOVAs for the 2018 samples (three years post-planting) testing if families differed in their associated soil fungal communities. Because there were no broad differences in soil fungal communities (see results), which may be attributable to sample size and may produce false negatives, we used Dirichlet-Multinominal Mixture (DMM) analyses (Holmes et al., 2012) to assign each sample into a community type. This approach allowed us to assess if any individual samples differed from each other, a difference that may not otherwise be detected. DMMs were conducted using mothur and optimally partitioned into two community types based on Laplace estimates. We also assigned the obtained OTUs into fungal functional guilds using the database FungalTraits (Põlme et al., 2020), only taxa whose traits were unambiguous were included to not confound these analyses. Where at least 10 OTUs were present within a defined guild, we used these reduced functional guild matrices to generate guild specific Bray-Curtis dissimilarity matrices. The post-planting fungal community data across tree families were compared using PerMANOVA to test if different families or hybrids may differentially drive potential fungal community function.

Using the fungal guild identities, we used paired effect size analyses to test if functional guilds are more or less abundant between sampling years whilst taking into account tree identity (both within species and within families). Retained guilds were: animal parasites (31 OTUs), mycoparasites (29 OTUs), ectomycorrhizae (EcM; 76 OTUs), plant pathogens (79 OTUs), and saprotrophs (332 OTUs); functional guild identities for each OTU are presented in Table A1. To calculate paired effect sizes for the same plants between the time of planting (2015) and three years' post-planting (2018), we determined the differences between the relative abundances of each guild for each year and divided this by the sum of the relative abundances for the same paired sample (Brown et al., 2020). In these analyses, the oaks and chestnuts were analyzed separately. We visualized effect size quartiles and tested if relative abundance of guilds differed between years using Wilcoxon Signed Rank tests. Further, to examine if the median paired effect sizes differed across chestnut species and hybrid types, we conducted Van der Waerden based χ^2 tests on median effects sizes for each guild. Where these tests were significant, we used Steel-Dwass multiple comparison

tests to identify which families or hybrids differed.

We also were interested if any OTUs were biomarkers for species or tree families/hybrid types. These analyses permit identification of OTUs that are disproportionately more abundant in one group than in others. To do this, we used LEfSe (Segata et al., 2011) to identify any such biomarker OTUs from plant associated soils only (2018 samples). Additionally, since the Van der Waerden tests comparing plant pathogens within the chestnuts indicated differences between families (see below), we conducted additional LEfSe analyses on only plant pathogens within chestnuts to identify any plant pathogenic taxa may be biomarkers for families.

Additionally, as members within Fagaceae presumably share similar ectomycorrhizal communities (D'Amico et al., 2015; Dulmer et al., 2014), we wanted to specifically test if any ectomycorrhizal (EcM) taxa were more abundant between plant species and to visualize shared and unique EcM taxa between oak and chestnuts and across species. To do this, all EcM taxa that were greater than 0.5 % in the total relative abundance of all EcM taxa (20 OTUs) were tested using Wilcoxon Signed-Rank tests to examine if the abundances differed across species. Further, all EcM taxa were used to generate size-proportional Euler's diagrams (R package *eulerr*) for shared EcM taxa between oak and chestnuts and across all plant species.

Finally, we were curious if any fungal taxa that had established on roots (rhizosphere) in the tree nursery, where our trees were grown and were likely co-transplanted, might still be present after three years of plant growth. This concept of co-transplanted inocula, which may confer benefits to plantings may be an important, but understudied concept that may aid reforestation efforts. It is important to note that the trees for which we have nursery root fungal data are not the same trees that were transplanted here as those were destructively sampled (Reazin et al., 2019). However, these trees were grown together in the same nursery beds and represent the same exact half-sibling acorns and nuts. Previous work (Reazin et al., 2019) strongly indicated homogenous root communities within families but did suggest some differentiation across families. To explore this, we harvested all representative OTU sequences for chestnuts (Reazin et al., 2019) and for white oaks (BioProject PRJNA820875). We pairwise aligned (Needleman-Wunch) these OTU sequences with the representative OTU sequences from this study and calculated genetic distances without punishing end-gaps to allow for different sequence lengths. Only those sequences that were 100 % identical were considered to represent the same OTUs. The OTUs that are shared between nursery root samples and the soil OTUs in the current analyses were then compared to determine if these OTUs might be common soil taxa (that is, detected in our 2015 soil samples prior to our planting) or taxa possibly introduced with our nursery-grown bare root seedlings and then suspected to have persisted three years post-planting (only if 2015 soils samples had less than 0.001 % relative abundance of those OTUs).

2.6. Statistics

All statistics were conducted using a combination of mothur (v.1.47.0; Schloss et al., 2009), JMP Pro (v.15; SAS Institute, Cary, NC, USA) and R (v.4.1.1; R core team).

3. Results

Initial sequencing yielded 6,624,534 sequences and after quality control and OTU demarcation, our data set included 1,276 OTUs and a total of 5,254,034 sequences. Soil fungal communities were dominated by OTUs within the phyla Ascomycota (740 OTUs) and Basidiomycota (340 OTUs), with lesser but significant representation of Mucoromycota (57 OTUs), Mortierellomycota (43 OTUs), Glomeromycota (30 OTUs), and Chytridiomycota (29 OTUs). The most abundant families were Herpotrichiellaceae (113 OTUs), Mortierellaceae (41 OTUs), and Russulaceae (30 OTUs) and the most abundant genera were saprotrophic *Mortierella* (39 OTUs), ectomycorrhizal *Russula* (18 OTUs), and pleomorphic yeast *Coniochaeta* (17 OTUs). Summary of OTU taxon assignments can be found in Table A2.

Our ANOVA tests indicate that diversity estimates changed little across our treatments. Our model (Year, Family, and Family × Year with family nested within species) indicated that fungal richness was stable across treatments ($F_{9,54} = 1.979$, $P = 0.058$). Simpson's diversity (1-D) changed with our model ($F_{9,54} = 4.719$, $P = 0.0001$) but only across years ($F_{1,54} = 36.88$, $P < 0.001$) and not across families or their interactions. Post-hoc analyses indicated that 2018 samples were more diverse than 2015 (Simpson's 1-D = 0.9292 ± 0.0084 and 1-D = 0.8614 ± 0.0083 for mean ± SE, respectively). Evenness was also responsive ($F_{9,54} = 6.246$, $P < 0.001$) but only between years ($F_{1,54} = 51.00$, $P < 0.001$) with 2018 ($E_D = 0.0658 \pm 0.0026$) samples having slightly greater evenness than 2015 ($E_D = 0.0407 \pm 0.0025$). For any diversity estimates, families did not differ.

PerMANOVA tests indicated that soil fungal communities differed across years ($F_{1,60} = 9.255$, $P = 0.001$, $R^2 = 0.129$) but not between oaks and chestnuts ($F_{1,60} = 1.364$, $P = 0.124$, $R^2 = 0.019$) or year by species interaction ($F_{1,60} = 0.735$, $P = 0.822$, $R^2 = 0.010$). This indicates that communities shift over time, but do not differ among the plant species. Because the soil fungal communities did not differ between the confamilial species of Fagaceae, we conducted a series of additional PerMANOVA tests across plant families (or hybrid types) for 2018 samples, focusing on functional guilds. In these analyses, except for plant pathogens ($F_{5,26} = 1.461$, $P = 0.004$), tests including all fungi, animal parasites, mycorrhizae, saprotrophs, and mycoparasites had indistinguishable communities across plant families (Table 1). The pairwise post-hoc tests comparing each family (with a pairwise Bonferroni error rate of 0.003) indicated that only the American and Chinese chestnuts differed in their plant pathogen communities ($F_{1,9} = 2.362$, $P = 0.003$). Other families, including oaks had otherwise comparable communities.

Community type (CT) analyses using Dirichlet Multinominal mixtures partitioned these communities into two discrete community types (CT1, CT2) with identical weights ($\pi = 0.5$) and similar variances (CT1 $\theta = 108.12$, CT2 $\theta = 122.65$). Community types were identical for all samples within sampling year but differed between years; all 2015 samples belonged to CT2, whereas all 2018 samples belonged to CT1. These results further highlight that communities shift with time, but plant identity or breeding lines are less important in structuring these plant-associated soil communities.

Overall, our analyses of paired fungal functional guilds indicated that guilds differed in their shifts over time in direct comparisons of soils from the same oak and chestnut trees (Fig. 1). For chestnuts, fungal animal parasites ($S = 58$, $P = 0.018$) and ectomycorrhizae ($S = 95$, $P < 0.001$) were more abundant three years post-planting (2018) than at planting (2015), whereas mycoparasites ($S = -57$, $P = 0.020$) and saprotrophs ($S = -87$, $P < 0.001$) were more abundant in at planting 2015. Plant pathogens ($S = -12$, $P = 0.651$) did not differ in their abundance irrespective of time. Overall, abundances of fungal functional guilds did not differ among American chestnut trees, Chinese chestnut trees, or the

Table 1
Results of PerMANOVAs testing if soil fungal communities from 2018 differ among families/hybrids (Oak families, American Chestnut, Chinese Chestnut, and chestnut hybrids) for all fungal taxa and for select functional guilds. Presented are F-statistics and P-values and significant tests are in bold and italics.

Tests	F_{df}, P-value
All Fungi	$F_{5,26} = 1.078$, $P = 0.220$
Animal Parasites	$F_{5,26} = 1.026$, $P = 0.241$
Ectomycorrhizae	$F_{5,26} = 0.980$, $P = 0.527$
Saprotrophs	$F_{5,26} = 1.274$, $P = 0.073$
Mycoparasites	$F_{5,26} = 1.253$, $P = 0.107$
Plant Pathogens	***$F_{5,26} = 1.461$, $P = 0.004$***

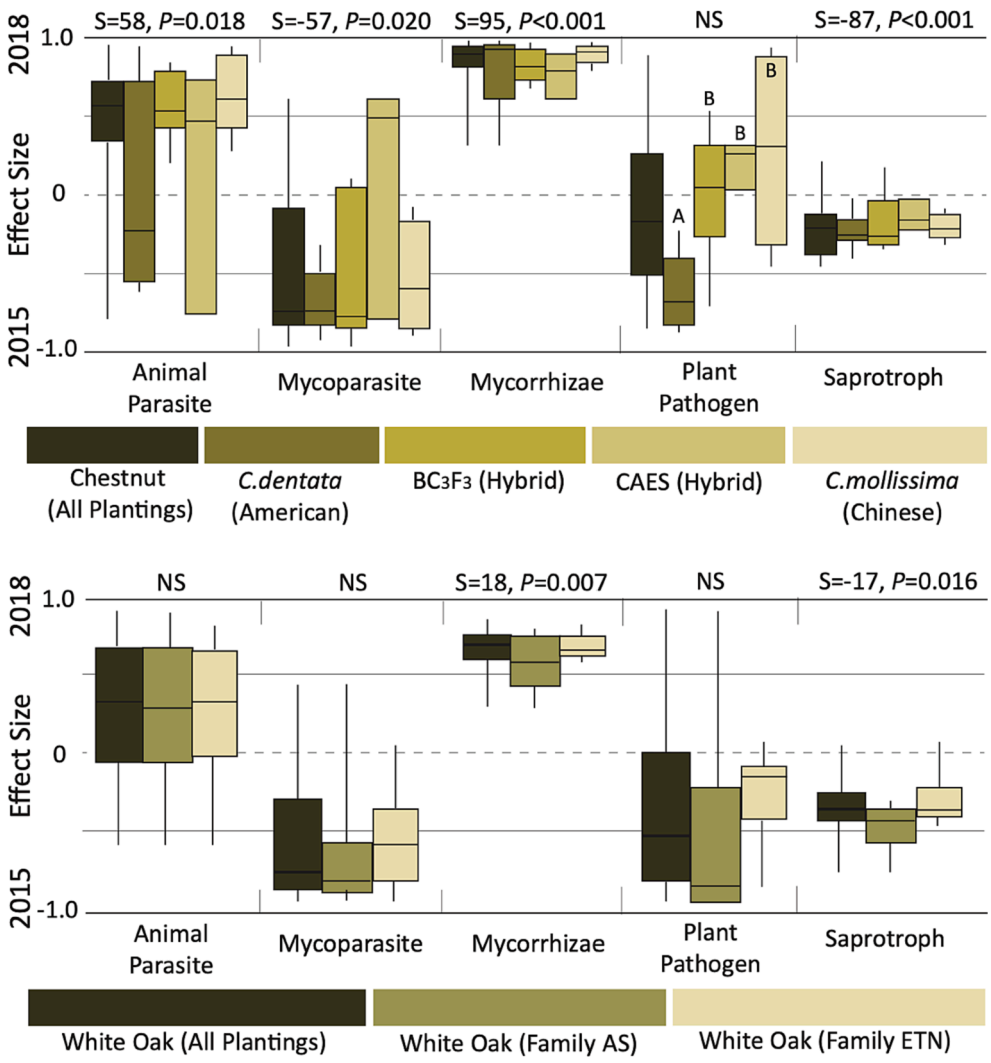

Fig. 1. Paired effect size of fungal guilds comparing the relative abundances (RA) of each guild within soils from the same planting location in 2015 (pre-planting) and 2018 (3 years established plants) for chestnuts (top) and oaks (bottom). Presented are quintiles (maximum, 75 %, median 25 %, minimum) of paired effect size [$RA_{2018} - RA_{2015}/(RA_{2018} + RA_{2015})$] where a value of –1.0 indicates that a guild was only observed in 2015, whereas a value of 1.0 indicates that a guild was only observed in 2018. Presented are guild differences across all plantings; Wilcoxon Signed Rank Test statistics are presented above the graph indicating if there was a difference between years in paired analyses. Further, Van der Waerden tests were conducted to test if medians of guild relative abundances differed with chestnut lines (*C. dentata* (American), *C. mollissima* (Chinese), BC_3F_3 (Hybrid), and CAES (Hybrid)) or oak families; different letters represent statistically different medians.

two backcross hybrids (BC_3F_3 or CAES) (Fig. 1). The exception was plant pathogens that differed among the chestnut types ($\chi^2 = 8.814$, P = 0.0319). Subsequent Steel-Dwass tests indicate that the American chestnuts had lesser plant pathogen abundance in 2018 than the Chinese chestnuts or either hybrid type (as compared to paired 2015 samples from the same tree, Fig. 1). LEfSe analyses of these plant pathogens within chestnuts identified five OTUs (out of 72) that were biomarkers for chestnut families including two for Chinese chestnut (OTU0651 [*Ganoderma lucidum*] and OTU0935 [*Phaeomoniella pinifoliorum*]), two for CAES (OTU0717 [*Strelitziana albiziae*] and OTU0806 [*Ganoderma* sp.]) and one for BC_3F_3 (OTU0334 [*Kabatiella* sp.]) whereas no OTUs were biomarkers for American chestnut. It is important to note that these taxonomic identities are based on best hits to the UNITE species hypothesis dataset with appropriate bootstrap support but identities cannot be verified without an organism present. In contrast, the white oaks only differed in the abundance of EcM (S = 18, P = 0.0078) and saprotrophs (S = -17, P = 0.0156) over time. Similar to chestnuts, EcM fungi were more abundant in 2018 than in 2015 and saprotrophs were more abundant in 2015. None of the other guilds differed among the years (Fig. 1) and none of the functional guild abundances differed between the two white oak families in our Van der Waerden tests (AS and ETN).

The LEfSe biomarker analyses identified a total 34 OTUs across all detected fungi that were overrepresented for a family/hybrid type in the 2018 post-planting samples (Table 2). These included three biomarkers for American chestnut (OTU0011 – *Sagenomella verticillata*, OTU0038 – Saccharomycetales sp., and OTU0042 – *Sagenomella diversispora*), nine biomarkers for Chinese chestnut including OTU0074 (*Cortinarius* sp.) and OTU0078 (Eurotiales sp.), two biomarkers for CAES (OTU0054 – *Paratritirachium curvibasidium* and OTU0105 – Glomeraceae sp.) and six biomarkers for BC_3F_3 hybrids including OTU0025 (*Mortierella humilis*) and OTU0068 (GS11 sp.; a novel Rozellomycotan clade only identified via sequence data (Tedersoo et al., 2017)). We also identified two biomarkers for the white oak AS family and twelve for the ETN family including OTU0033 (GS23 sp.), OTU0049 (Thelephoraceae sp.), and OTU0092 (*Clavaria* sp.). It is of note that although many of the identified biomarkers represent putative saprobes, they also include a number of ectomycorrhizal taxa (Table 2).

Since previous work has suggested that EcM fungal communities are often similar between oaks and chestnuts, we wanted to examine if EcM taxa differed in their abundance and/or presence between oaks and chestnuts and across all tree species. Wilcoxon tests on the most abundant EcM taxa across 'species' indicate that none of these EcM OTUs differed in their abundances (minimum P-value = 0.18). Furthermore, examinations of shared and unique EcM occurrences (Fig. 2) indicate that there are many EcM OTUs that are shared between oak and chestnuts (55 %) and across all 'species' (17 %). Several shared EcM taxa are abundant and include OTU0010 (*Cenococcum* sp.), OTU0012 (*Laccaria* sp.), OTU0015 (*Cenococcum* sp.), and OTU0035 (*Lactifluus* sp.). Although most of the OTUs that are only found within in one tree type

Table 2
Results of LEfSe analyses identifying biomarkers for plant family/hybrids for 2018 samples. The table includes OTU number, relative abundance parenthetically, LDA (Linear Discriminate Analysis) test statistic, P-values, best taxonomic affinity and guild membership where unambiguous at the genus level based on the FungalTraits database. SAP – saprobic, EcM – Ectomycorrhizae, AM – Arbuscular Mycorrhizae, PATH – plant pathogens, MP – mycoparasites, N/A – ambiguous or unknown placement.

OTU ID (RA%)	LDA	P-Value	Best Taxonomic ID	Guild
American Chestnut				
Otu0011 (1.51 %)	3.704	0.0474	*Sagenomella verticillata*	SAP
Otu0038 (0.45 %)	4.441	0.0476	Saccharomycetales sp.	N/A
Otu0042 (0.38 %)	3.168	0.0146	*Sagenomella diversispora*	SAP
Chinese Chestnut				
Otu0074 (0.20 %)	4.295	0.0447	*Cortinarius* sp.	EcM
Otu0078 (0.19 %)	3.726	0.0306	Eurotiales sp.	N/A
Otu0116 (0.11 %)	3.597	0.0069	*Cenococcum* sp.	EcM
Otu0209 (0.05 %)	2.578	0.0286	GS23 sp.	N/A
Otu0240 (0.03 %)	3.401	0.0246	Spizellomycetales sp.	N/A
Otu0291 (0.02 %)	2.842	0.0323	Herpotrichiellaceae sp.	N/A
Otu0448 (0.01 %)	2.228	0.0359	*Mortierella cystojenkinii*	SAP
Otu0708 (<0.01 %)	2.033	0.0289	Sordariomycetes sp.	N/A
Otu0741 (<0.01 %)	2.437	0.0129	Herpotrichiellaceae sp.	N/A
BC_3F_3 Chestnut Hybrid				
Otu0025 (0.60 %)	4.085	0.0370	*Mortierella humilis*	SAP
Otu0068 (0.22 %)	3.328	0.0218	GS11 sp.	N/A
Otu0177 (0.06 %)	3.081	0.0309	Chaetothyriales sp.	N/A
Otu0204 (0.05 %)	3.388	0.0452	Helotiales sp.	N/A
Otu0478 (0.01 %)	2.142	0.0309	Sordariomycetes sp.	N/A
Otu0535 (<0.01 %)	2.918	0.0315	Sebacinales sp.	N/A
CAES Chestnut Hybrid				
Otu0054 (0.26 %)	3.170	0.0417	*Paratritirachium curvibasidium*	SAP
Otu0105 (0.13 %)	3.611	0.0302	Glomeraceae sp.	AM
AS White Oak				
Otu0185 (0.06 %)	2.485	0.0353	Tremellodendropsidales sp.	N/A
Otu0603 (<0.01 %)	2.464	0.0057	*Lactifluus* sp.	EcM
ETN White Oak				
Otu0033 (0.49 %)	4.178	0.0337	GS23 sp.	N/A
Otu0049 (0.31 %)	3.106	0.0483	Thelephoraceae sp.	N/A
Otu0092 (0.16 %)	4.232	0.0220	*Clavaria* sp.	SAP
Otu0228 (0.04 %)	3.096	0.0180	Agaricomycetes sp.	N/A
Otu0279 (0.02 %)	3.059	0.0413	Helotiales sp.	N/A
Otu0282 (0.02 %)	2.365	0.0059	*Didymella exigua*	PATH
Otu0431 (0.01 %)	2.613	0.0475	*Kurtzmanomyces nectairei*	SAP
Otu0452 (<0.01 %)	3.097	0.0129	*Pseudogymnoascus* sp.	SAP
Otu0525 (<0.01 %)	2.802	0.0048	*Cryptococcus* sp.	SAP
Otu0650 (<0.01 %)	2.054	0.0474	*Cystobasidium* sp.	MP
Otu0661 (<0.01 %)	2.146	0.0383	*Cephalotheca* sp.	MP
Otu0985 (<0.01 %)	2.061	0.0460	*Genolevuria* sp.	SAP

are rare, a few common taxa are unique to a tree type including OTU0050 (*Inocybe* sp.) which was only associated with chestnuts (and only with BC_3F_3), OTU0073 (*Thelephora* sp.) which was only found associated with white oak, and OTU0093 (*Sebacina* sp.) which is only found associated with chestnuts (but only with BC_3F_3 and CAES).

Our analyses of shared identical OTUs between nursery bed rhizosphere fungi of chestnut (Reazin et al., 2019) or oak seedlings and those detected here suggest that several OTUs may been co-transplanted and persist. In all, we observed 96 chestnut-associated and 59 oak-associated OTUs with ITS2 reads identical to those also observed in the nursery stock. Most of these were common taxa and abundant either only at the time of planting or both at the time of planting and three years later. However, there were 12 OTUs that were observed in the nursery stock and absent (or functionally absent at < 0.001 % RA) in soils at the time of planting but observed in oak- or chestnut-associated soils three years later. This suggests that these taxa were potentially introduced and persisted in the soils or were enriched by planting the compatible host trees. These OTUs include eight that were associated with both chestnuts and oaks (OTU0039 – Saccharomycetales sp., OTU0080 – Serendipitaceae sp., OTU0239 – Trichomeriaceae sp., OTU0254 – Pleosporales sp., OTU0550 – *Neobulgaria* sp., OTU0659 – Serendipitaceae sp., OTU0729 – *Holtermanniella takashimae*, and OTU0951 – *Paraphaeosphaeria angularis*), one with chestnuts only (OTU1049 – Pleosporales sp.), and three with oaks only (OTU0012 – *Laccaria* sp., OTU0336 – Pleosporales sp., OTU0430 – *Pezoloma ericae*, and OTU0614 – *Didymocyrtis cladoniicola*). Of note among these are *Laccaria,* an ectomycorrhizal fungus commonly observed within the nurseries, two OTUs assigned to Serendipitaceae, a family of basidiomycetes that form mycorrhizal associations with a wide range of hosts, and *Pezoloma ericae*, a member of Helotiales with the potential to form symbioses with ectomycorrhizal plants (Vrålstad et al., 2002).

4. Discussion

Plant-fungal interactions are inextricably coupled components of plant and forest health and could potentially impact reforestation efforts. These interactions may partially contribute to the large variability of success rates often observed in Fagaceae plantings (cf. Clark et al., 2016; Dey et al., 2008). Here, we examined what role reforestation of Fagaceae species through planting American chestnut and white oak plays on plant-associated bulk soil fungal communities. By comparing soils at-planting to those after three years, we demonstrate that these Fagaceae species similarly shifted fungal communities and that – even after three years in the field – communities as a whole remained indistinguishable among species and families. However, relative abundances of putatively plant pathogenic fungi shifted over time for some species: American chestnut had lesser abundances of pathogenic fungi than Chinese chestnut, a difference that was not detected until 3 years after planting. We also demonstrate that some fungal functional guilds were either enriched or suppressed three years after planting. Further, by comparing taxa observed in common nursery bed soils and those associated with soils of out-planted trees three years later, the Fagaceae may have either introduced co-planted fungi that can persist at least three years in the field or enriched several taxa with potential impacts on tree health and reforestation success. These host-fungus associations, their persistence or introduction with the nursery grown seedlings may represent an undervalued component in reforestation dynamics.

After three years in the field, the outplanted seedlings elicited no community-wide shifts in soil fungi community composition, even among the most genetically divergent oak and chestnut species. Our analyses suggest similar fungal communities exist among species and among conspecific families three years after outplanting, as evidenced both by our PerMANOVA tests and our Dirichlet multinomial based community type analyses. This is interesting given chestnuts departed the nurseries with distinct root associated fungal communities (Reazin et al., 2019). It is possible that our small sample size (four trees for each chestnut species and oak family) may be inadequate to distinguish community differences and a larger sample size might have shown differences among communities. However, one could argue that if a community ecological signal is not apparent with fewer samples, then any observed signal with additional sampling is likely to be biologically unimportant. Our results are in contrast with many others reporting that soil fungal communities differ in bulk soils of different plant species, even if sampled in close proximity (Brown and Jumpponen, 2014; Dassen et al., 2017; Mouhamadou et al., 2013; Pivato et al., 2007; Põlme et al., 2013; Zhang et al., 2022). Further, abundant evidence suggests that soil fungal communities can differ even among conspecific genotypes or individuals (Du et al., 2022; Kong et al., 2020; Liu et al., 2022; Schlemper et al., 2018; Tang et al., 2022; Wagner et al., 2016), although this is not always the case as different genotypes/cultivars may host comparable fungal communities (Hannula et al., 2010; Tkacz et al., 2020; Zhang et al., 2018) suggesting the importance of the soil environments as the primary driver of fungal community dynamics in soil. American chestnut and the BC_3F_3 hybrids share on average 83 % of American chestnut genetic background (Westbrook et al., 2019) and are thus likely to recruit and support comparable fungal communities under comparable environmental conditions. Yet, why the soil fungal

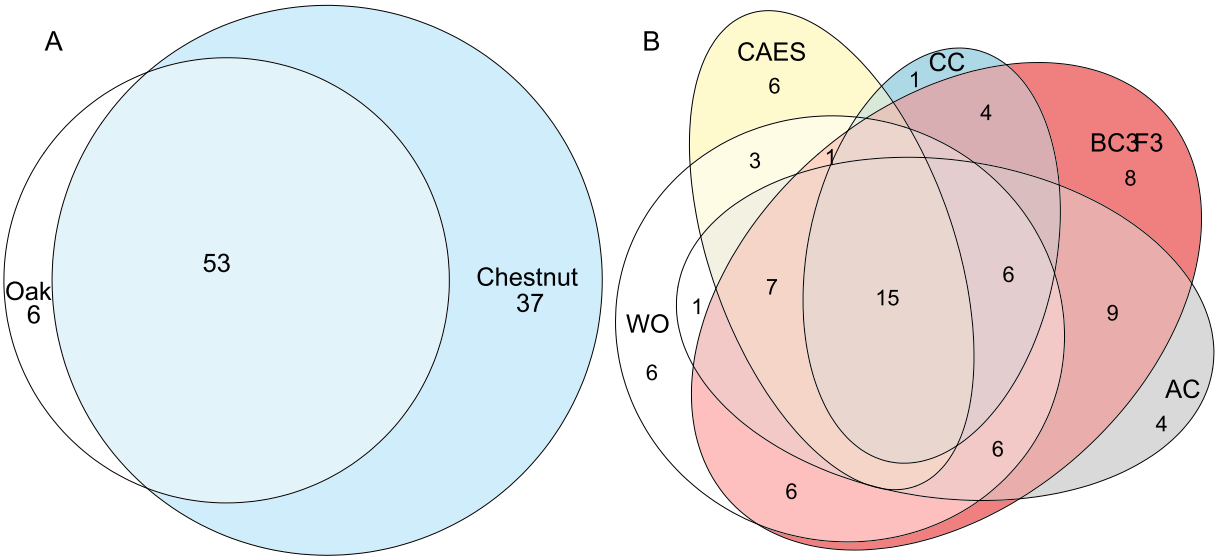

Fig. 2. Size proportional Euler's diagrams of ectomycorrhizal taxa that are shared or unique between (left) oaks and chestnuts and (right) between each species. Only where there was resolvable geometry for the five-way diagram (right) are EcM taxon numbers listed. WO-white oak, AC-American Chestnut, CC-Chinese chestnut and CAES and BC_3F_3 are chestnuts hybrids.

communities are indistinguishable here between American chestnut, Chinese chestnut, and white oaks remains unclear, although previous work suggests that many fungi, particularly ectomycorrhizae, have host specificity acting at the plant taxonomic level of a plant family (Molina and Trappe, 1982). *Castanea* and *Quercus* are close sister genera within Fagaceae (Manos et al., 2001; Yang et al., 2018) and many Fagaceae share similar mycorrhizal communities (D'Amico et al., 2015) potentially explaining the absence of host-specific communities here.

While there were no host-specific community differences, the communities changes with time as plants established and these changes were similar regardless of host. This suggests a strong environmental filter that homogenized fungal communities and minimized potential host effects if any existed. It is possible that the small host trees in this young stand only minimally influenced the soils and communities therein. However, the outplanted seedlings were relatively large compared to similarly aged hardwood seedlings planted in this region (Brown et al., 2022; Dey et al., 2008). As a result, our data likely suggest that communities that include long-distance exploration EcM types and general soil saprobes are structured by similar edaphic or environmental drivers, as soils post-harvest were likely similar. Communities inferred from EcM mycelia in soil are often distinct from those in roots (Kjøller, 2006), even when both have been sampled in close proximity. In temporal studies such as the one presented here, decoupling effects of the host and environment is difficult. In the three years post-planting, the abiotic environment likely shifted due to post-fire effects and subsequent plant and fungal community recovery (Certini et al., 2021; Fox et al., 2022), although those data are not available. While we find it unlikely, this homogenization of fungal communities may also be partially due to pyrogenic carbon generation from the post-harvest prescribed fire or direct fire effects that filters soil fungi toward similar communities, however, given the rather steep slope of this site (~30 %) post-harvest prescribed fires is likely to be of very low intensity (Park, 2001), but intensity and carbon species were not measured here.

Although the fungal communities were not distinct overall, some taxa differed in their abundances in chestnut- and oak-associated soils (Table 2, Fig. 2). This was particularly the case for plant pathogens observed with American chestnut and hybrids or Chinese chestnut (Fig. 1). Soils associated with American chestnut had a lower proportion of plant pathogens (relative to other functional guilds) than those associated with the BC_3F_3 hybrids or Chinese chestnuts. In addition, several biomarker plant pathogens were overrepresented in either the backcross hybrids or the Chinese chestnuts. Yet no plant pathogen biomarkers were associated specifically with American chestnut. This may seem counterintuitive as one might expect that the native American chestnut would be more susceptible to pathogens that have co-evolved in the local pathogen propagule pools. However, the contrary is often the case. Non-native plants and genotypes are often more susceptible to pathogens than native plants (Mangla and Callaway, 2008), presumably because of ecological disequilibrium between non-native trees and native pathogens (Crous et al., 2017). The strength of this disconnect is likely related to plant growth traits (Fahey et al., 2022). Indeed, the Chinese and hybrid chestnut seedlings maintained lower growth rates over the three years at this site (Brown et al. 2022). Why the American chestnut had reduced abundances of pathogenic OTUs in the bulk soil remains uncertain. If this holds true with tissue-associated plant pathogens, rather than bulk soil, remains to be seen. However, our observations warrant further study of local filtering during the chestnut pathogen assembly, as such research may provide crucial insight into reforestation success.

Plant pathogens were the only functional guild that differed in abundance between plant families, and only for chestnuts. In contrast, several guilds differed in their relative abundances between years (Fig. 1). Unsurprisingly, the abundances of ectomycorrhizal fungi were higher in 2018 (three years post planting) for both chestnut and oak. This is coupled with a concomitant decrease in the relative abundance of saprotrophs in 2018, likely due to mycorrhizal recruitment by the establishing plants facilitating a decline in relative abundance of other guilds. In soils associated with chestnuts but not oaks, animal pathogenic fungi also increased in abundance in 2018, whereas mycoparasites declined in 2018, compared to pre-planting in 2015. The increase of animal parasites is possibly an effect of changes in vegetation that may have provided biomass for insect herbivory (Riedel et al., 2013; Schowalter, 1986) and large herbivores (Cox, 2011; Forsyth et al., 2015; Hackworth et al., 2018; Pinchot et al., 2022), facilitating the occurrence of animal pathogenic fungi. The underlying reasons for greater mycoparasite abundance in 2015 shortly after the prescribed fire is uncertain. A recent study (Semenova-Nelsen et al., 2019) reported an increase in mycoparasite loads following prescribed fires in a pine savanna and attributed this to the environmental stress experienced by the surviving fungi that allows for opportunistic pathogenicity. It remains uncertain why the abundances of myco- and animal parasites differed across years in the chestnut but not in the white oak soils. This may be due to the

fewer queried oaks than chestnuts possibly resulting in lower statistical power to detect similar shifts in oaks. Alternatively, these fungi may fundamentally differ in their responses when associated with oaks and chestnuts.

We also compared fungi observed three years post-planting and those inhabiting chestnut and oak rhizospheres in nursery beds (Reazin et al., 2019). These analyses identified several taxa that were ostensibly associated with the nursery-reared seedlings, absent in soil at the time of outplanting, but present in soil three years later. It remains open whether this indicates fungal co-establishment with planted trees or recruitment from the local soil propagule pools by the establishing trees. Many of these fungi were either poorly resolved or general saprobes. However, OTUs assigned to *Laccaria* sp. and *Pezoloma* sp. represent notable exceptions. Species within the genus *Laccaria* are common ectomycorrhizal fungi, whereas *Pezoloma* sp. may be able to colonize both ericaceous (Midgley et al., 2017) and ectomycorrhizal hosts (Vrålstad et al., 2002) or form root endophyte symbioses (Fehrer et al., 2018). What this particular *Pezoloma* species' role in the environment remains uncertain. The co-establishing fungi may be particularly important as root-associated mutualists, an underappreciated component in forest restoration and reforestation efforts (Policelli et al., 2020). Fungi that establish in the nursery-reared seedlings and are subsequently co-transplanted with seedlings may impact the establishment and outplanting success. While many studies indicate that EcM inoculation in nursery beds may improve plant success once planted (Assad et al., 2022; García et al., 2019; Kayama, 2020; Li et al., 2021; Repáč et al., 2022), few thus far demonstrate the temporal stability of the nursery acquired fungi after outplanting (*but see* Selosse et al., 1998 who also identified a persistent *Laccaria* species post-planting). We posit that fungal communities at the nurseries require further attention, as they are an important but understudied component in reforestation.

Taken together, our contribution demonstrates that soil fungal communities associated with outplanted Fagaceae in the Southern Appalachian Mountains changed in similar ways post-planting. We observed no evidence for soil community differentiation between confamilial species or conspecific plant families. This suggests that closely related genera within Fagaceae modulate soil fungi similarly suggesting similar growth requirements and ecological roles in the environment, at least in the short-term during a crucial phase in stand development of regenerating stands. Finally, our comparisons of fungi that may have co-established with the nursery-reared seedlings highlight the potential of maintaining these root-symbioses in the longer-term after outplanting.

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CRediT authorship contribution statement

Shawn P. Brown: Methodology, Formal analysis, Writing – original draft, Visualization. **Stacy L. Clark:** Conceptualization, Methodology, Resources, Formal analysis, Writing – original draft, Visualization. **Emerald Ford:** Investigation, Writing – review & editing. **Nahreen Mirza:** Investigation, Writing – review & editing. **Amerah Odeh:** Investigation, Writing – review & editing. **Scott E. Schlarbaum:** Conceptualization, Methodology, Resources, Writing – review & editing. **Ari Jumpponen:** Investigation, Resources, Writing – review & editing. **Richard Baird:** Methodology, Writing – original draft, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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