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4 **Isolating a functionally relevant guild of fungi from the root microbiome of**
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6 ***Populus***
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Abstract

Plant roots interact with a bewilderingly complex community of microbes, including root-associated fungi that are essential for maintaining plant health. To improve understanding of the diversity of fungi in the rhizobiome of *Populus deltoides*, *P. trichocarpa* and co-occurring plant hosts *Quercus alba* and *Pinus taeda*, we conducted field and greenhouse studies and sampled, isolated, and characterized the diversity of culturable root-associated fungi on these hosts. Using both general and selective isolation media we obtained more than 1800 fungal isolates from individual surface sterilized root tips. Sequences from the ITS and/or D1 – D2 regions of the LSU rDNA were obtained from 1042 of the >1800 pure culture isolates and were compared to accessions in the NCBI nucleotide database and analyzed through phylogenetics for preliminary taxonomic identification. Sequences from these isolates were also compared to 454 sequence datasets obtained directly from the *Populus* rhizosphere. Although most of the ectomycorrhizal taxa known to associate with *Populus* evaded isolation, many of the abundant sequence types from rhizosphere and endosphere 454 datasets were isolated, including novel species belonging to the Atractiellales. Isolation and identification of key endorhizal fungi will enable more targeted study of plant-fungal interactions. Genome sequencing is currently underway for a subset of our culture library with the aim of understanding the mechanisms involved in host-endophyte establishment and function. This diverse culture library of fungal root associates is a valuable resource for metagenomic research, experimentation and further studies on plant-fungal interactions.

Keywords; *Populus deltoides*, *Populus trichocarpa*, *endophytes*, *ectomycorrhizal fungi*, *ecological interactions*, *rhizobiome*.

Introduction

Plant roots are known to harbor a diverse community of fungi that perform a variety of roles in the rhizosphere as saprotrophs, symbiotic mutualists - including arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), and pathogens (Smith and

Read 2008). Through environmental sequencing and metagenomic approaches we have been able to detect and classify much of the fungal diversity in plant roots, however the impact of these living fungi on plant health or ecosystem functions remains obscure because these fungi are only known from DNA sequences (Gottel et al. 2011). To address this limitation of metagenomic approaches we aimed to isolate a functionally relevant guild of fungi from the *Populus* rhizobiome.

Populus is an economically important genus of woodland and riparian trees and is distinctive in its rapid growth and ability to form both arbuscular and ectomycorrhizas (Lodge 1989). *Populus* also associates with a high diversity of root endophytic fungi and bacteria, many of which are likely to play important roles in the rhizosphere and in soils (Shakya et al. 2013). While there is growing interest in the use of *Populus* feedstock for energy production, its associated belowground fungal diversity remains largely uncharacterized and poorly understood. A better understanding of these fungi and their biology is relevant to plant growth and health.

Belowground ectomycorrhizal interactions in *Populus* were recently reviewed (Podila et al. 2009). These authors reiterated the need for further functional genomics and ecological metabolomic studies to address the biology and ecology of the plant-fungal-soil interface. Soil water tension has been reported as one factor that affects mycorrhizal colonization levels in *Populus deltoides* (Lodge 1989), with greater ectomycorrhizal colonization in soils with median levels of moisture. Ectomycorrhizal associates of *Populus* include species of *Laccaria*, *Inocybe*, *Hebeloma*, *Cortinarius* and *Tuber* (Baum and Makeschin 2000; Bois et al. 2005; Podila et al. 2009; Gottel et al. 2011; Guevara et al. 2013). Soil nutrient levels and plant genotype have also been reported to influence rhizosphere community structure and function (Baum and Makschin 2000; Beauchamp et al. 2005; Karlinski et al. 2010; Courty et al. 2011). A better knowledge of the structure, development and regulation of the fungal associates of *Populus* has broad relevance to plant productivity (Baum et al. 2002; Judith 2009; Liang 2010), plant tolerance to toxic levels of chemicals in soils (Krpata 2008; Khosla et

al. 2009), drought stress (Luo et al. 2009) and plant immunity to pathogenic microorganisms (Shaul et al. 1999).

Many of the pre-molecular studies on belowground fungal associates of *Populus* are descriptive in nature (Kaldorf et al. 2005; Zhang et al. 2010; Mueller et al. 2012) and ecological in scope (Helm et al. 1996; Klironomos et al. 1997). These studies indicate that diversity of both EMF and AMF varies across *Populus* species, genotypes, and environments (Lodge 1989; Zhong 1995). However, our recent studies of root associated microbial communities using directed ITS, 28S and 16S rDNA high throughput sequencing approaches have shown that traditional AMF and EMF taxa are present but not dominant in natural *Populus deltoides* ecosystems (Gottel et al. 2011; Shakya et al. 2013), or in *P. trichocarpa* in plantation systems (Schadt et al. unpublished). Rather, *Populus* appears to associate with a high diversity of root endophytic fungi and bacteria, which currently have unknown or poorly understood roles in rhizosphere and root function. Using direct rRNA and more traditional microscopic and isolation approaches other researchers have also confirmed *Populus* spp. harbor a high diversity of fungi in their roots, including both traditional mycorrhizal and non-mycorrhizal taxa (Cripps and Miller 1995; Visser et al. 1998; Khasa et al. 2002; Jabeen et al. 2012; Gottel et al. 2011; Karlinski et al. 2013; Shakya et al. 2013; Bonito et al. 2014). Much of this belowground fungal diversity is uncharacterized, phylogenetically and functionally, and remains underexploited.

To begin to disentangle the complexity in structure and function of fungi associated with the *Populus* rhizobiome we carried out intense efforts to isolate endophytic and mycorrhizal fungi from healthy *Populus* roots. To accomplish this goal, fungi were isolated from fresh surface sterilized *Populus* roots collected from three sources: (1) natural stands of *P. deltoides* in eastern North America; (2) a common garden experiment established with genotypes of *P. trichocarpa* from across its geographic range in western North America (below); (3) *Populus* cuttings planted in field soil and reared under controlled greenhouse conditions (i.e. trap-plants). This sampling included

multiple *Populus* species and genotypes and roots from co-occurring *Quercus alba* (white oak) and *Pinus taeda* (loblolly pine) species.

Materials and Methods

Sampling roots of *Populus*

Root samples of *P. deltoides* were collected from both natural populations of mature *P. deltoides* along the Yadkin river in NC (see Shakya et al. 2013) and diverse *P. trichocarpa* genotypes planted in common garden plots in CA, OR and British Columbia (see Slavov et al. 2012). To obtain field samples from *P. deltoides* and *P. trichocarpa*, roots from adult trees were traced out from the trunk with hand tools until clusters of fine roots could be sampled, as previously described (Shakya et al. 2013). Encountered fine roots (20-50g) were harvested and then immediately placed in zip-lock bags and under ice. These samples were stored at 4°C until isolations were made (within 3 d). Roots of *P. trichocarpa* were collected in a similar manner from common garden field sites in Placerville CA, Corvallis OR and Clatskanie WA that had been established with natural genotypes of *P. trichocarpa* (Slavov et al. 2012). At least five individual genotypes were sampled from each common garden site. Additionally we obtained isolates from *Populus* cuttings rooted in soil from several of these sites (see Bonito et al. 2014). Soil and *Populus* cuttings were stored at 4°C. Prior to use these 30 cm long cuttings were soaked overnight in tap water and then surface sterilized in a 10 min bath of 3% H₂O₂. Cuttings were triple-rinsed with tap water and potted in a mixture containing 30-50% freshly collected field soil mixed into sterilized sand. Soils for trap-plant experiments were collected from *P. trichocarpa* common garden experiment sites established with natural genotypes of (Slavov et al. 2012), and from natural *Populus* stands along the Yadkin river (Shakya et al. 2013; Bonito et al. 2014). Trap-plants were grown for 5 months in the Duke Phytotron under controlled environmental conditions of 18 h days, 6 h nights, with 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and a day temp of 21.1°C, night temp of 15.6°C, and relative humidity of 60% during the day and 85% at night as described in Bonito et al. (2014). In addition to sampling *P. deltoides* (n=34) and *P. trichocarpa* (n=152), isolations were made from a smaller number of root samples from *Pinus taeda* (n=8) and *Quercus alba* (n=8) trap-plants rooted in the soils above. We include these as

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4 outgroup taxa that could be contrasted with *Populus*. In total we sampled roots from 202
5 individual plants.
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9 *Isolating fungi from plant roots*

10 Both native and trap-plant root samples were harvested and thoroughly washed with tap
11 water and a solution of 0.1% Tween20 to remove soil debris and adhering soil particles.
12 Sections of healthy looking roots (2-3 cm from root-tip and beyond) were selected under
13 a dissecting microscope. We included roots that both appeared to be colonized by
14 ectomycorrhizal fungi or uncolonized. Root sections were wrapped in pre-sterilized
15 squares of synthetic fiber cloth (6-8 cm²) and surface sterilized by submerging in 3%
16 H₂O₂ for 1 min, followed by two washings with sterilized distilled water. Sterility of the
17 surface the roots was verified by streaking root segments across non-antibiotic media.
18 1-3 mm pieces of surface sterilized roots were plated onto modified Melin Norkrans
19 medium (MMN) supplemented with 100 mg L⁻¹ chloramphenicol and streptomycin. To
20 target basidiomycetes, we also employed MMN or 1.5% malt extract agar (MEA) plates
21 supplemented with Benomyl (4mg L⁻¹) (Thorn et al. 1996).
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34 Cultured material was observed daily. Fast growing fungi were aseptically removed to
35 facilitate growth of slower growing fungi and increase the diversity of isolates obtained.
36 Fungal growth from the root tips was examined under magnification and leading edges
37 of the hyphae were sub-cultured onto the same media until a pure culture was obtained.
38 Pure culture isolates were stored on agar slants and small discs cut from actively
39 growing cultures and submerged in sterile distilled water for future use. A subset of
40 representative strains have been deposited at the CBS culture collection (Utrecht,
41 Netherlands).
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51 *Molecular diagnosis*

52 To identify fungal isolates, DNA was extracted from mycelium by placing a small
53 amount of tissue in 20 µl of extraction solution (Sigma) and heating at 95 °C for 10 min.
54 We added 60 µl of bovine serum albumin (BSA 3%) to the lystate and directly amplified
55 the ITS and LSU rDNA with the primers ITS1f/ITS4 and LROR/LR3 (Vilgalys and Hester
56 1990; Gardes et al. 1991) to obtain DNA barcodes for these isolates (Schoch et al.
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2012). The PCR protocol was as follows: reaction mixtures contained 25 ng of DNA, 50 ng of each primer, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl₂, and 0.1 mg ml⁻¹ bovine serum albumin. Amplification conditions on a 2720 thermocycler (Applied Biosystems, Foster City, CA) included an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 50°C for 15 s and 72°C for 1.25 min and concluded with a 7 min extension at 72°C. Amplicons were Sanger sequenced bidirectionally on an ABI3730XL automated sequencer (Applied Biosystems, Carlsbad, CA). Sequences were identified by comparing them to accessions in the NCBI nucleotide database with BLAST (Altschul et al. 1990) (<http://www.ncbi.nlm.nih.gov/genbank/>) and were aligned to closely related taxa and analyzed using phylogenetic methods. Sequenced isolates were clustered into OTUs (operational taxonomic units) using 97% sequence similarity (for ITS) or 99% (for LSU). ITS extractor was used to selectively extract the ITS1 and ITS2 regions from each of the sequences (Nilsson et al. 2010). By removing the conserved 5.8S domain and LSU and SSU sequence at the ends of the read, more reliable clustering of OTUs can be obtained because clusters are based solely upon the variable regions, rather than regions that are conserved across a high diversity of fungi and therefore taxonomically uninformative. The USEARCH algorithm (Edgar 2010) was used to cluster ITS1 & ITS2 sequences into OTUs. LSU sequences were aligned in MUSCLE (Edgar 2004) and unalignable characters were excluded in Mesquite (Maddison and Maddison 2009). Most parsimonious trees were inferred in PAUP* (Swofford 2002). The interactive tree of life software (ITOL – <http://itol.embl.de>) was used to visualize relationships amongst isolates, geographic and host origins. Sequences obtained in this study have been submitted to GenBank (JX243744-JX244069; KC007130-KC007340; KF428214-KF428718) – see Supplemental table 2.

Comparison of fungal diversity in Populus roots obtained through culture and high throughput culture-independent techniques

To determine the correspondence between molecular and culture-based assessments of fungal diversity, we used sequences of ITS and LSU rDNA obtained from fungal isolates through Sanger sequencing (above) and compared these to complementary

sequence datasets obtained through 454 pyrosequencing of *Populus* endosphere and rhizosphere samples, available in the NCBI Sequence Read Archive (SRA) under the accession number SRP034435 and BioProject accession PRJNA209455 (Shakya et al. 2013; Bonito et al. 2014). Specifically, sequence datasets were merged and then OTUs were picked from this merged dataset and classified taxonomically following the UPARSE pipeline as published in Bonito et al. (2014), clustering OTUs at 97% sequence similarity and grouping them by whether they were detected by culture-independent metagenomic techniques, by culturing, or both, and were sorted by rank abundance. Fungal taxonomy was assigned to fungal isolates at the lowest taxonomic level that we could be confident in. This was done using multiple approaches that included BLAST searches against the UNITE 7.0 ITS reference database, queries with the RDP Bayesian Classifier, and manual BLAST searches to NCBI combined with phylogenetic analyses.

Statistical analyses

To determine if the diversity of fungal isolates differed across soil types and host genera, two-sided tests of proportions were performed using R 3.0.1 to test whether well sampled isolates were over or under-represented in particular soils or hosts. To mark the presence of statistical evidence, a 5% significance level was chosen; to mark the presence of strong statistical evidence, a 1% significance level was chosen. Chi-squared tests were carried out to determine whether differences in the isolation of specific taxa between soils and hosts were significant.

Results

Diversity, identity and distribution of fungal isolates

Over 1800 fungal isolates were obtained from surface sterilized roots of *Populus*, *Quercus*, and *Pinus*. These isolates were grouped by morphotype resulting in over 120 distinguishable colony types (Fig 1). While an abundance of *Ilyonectria*, *Fusarium*, *Mortierella*, *Trichoderma* and other taxa were distinguishable based on colony growth, color and sporulation, many isolates were of sterile mycelium or asexual ascomycetes

and species delimitations within these genera are nearly impossible to make based on morphology alone. Thus, DNA barcode sequences were determined for a subset of 1042 of these isolates including multiple replicates from each morphotype when available. Stringent OTU picking with the USEARCH algorithm resulted in 97 OTUs based on the ITS1 and 98 OTUs based on the ITS2 region (excluding singletons; including singletons OTU counts were 220 and 200, respectively). Although many of the OTUs had high identity blast hits, in some cases the taxonomic identity of the isolates could not be confirmed because top hits were from environmental sequences. Using both ITS and LSU, the most abundantly isolated fungal OTUs from *Populus* could be identified as *Leptodontidium* (83 isolates), *Cylindrocarpon pauciseptatum* (69 isolates), *Atractiella* sp. (51 isolates), *Fusarium oxysporum* (46 isolates), and three OTUs of *Ilyonectria* (45, 44, 40 isolates each) (Fig 2). Other fungi isolated from *Populus* roots (at lower abundance) included species of Sordariomycetes, *Melinomyces*, *Paraphoma*, *Phomopsis*, *Exophiala*, *Rhizoctonia*, Helotiales, *Cadophora*, Corticiaceae, *Lachnum*, *Mortierella*, and *Flagelloscypha* (Fig 3; Table S2).

Of the sequenced isolates 710 were from *P. trichocarpa*, 206 from *P. deltoides*, 71 from *Pinus taeda* and 55 from *Quercus alba*. These accounted for 55, 26, 12 and 7 OTUs, respectively (excluding singletons), for each of these host species. Most of the dominant taxa could be isolated from both eastern US and western US soils, with no clear pattern of biogeographical provenance. Many of the dominant taxa could be isolated from both *P. deltoides* and *P. trichocarpa*, and in some cases *Quercus* and *Pinus* too, suggesting broad host range. Clear exceptions to this included *Phialocephala* and *Oidiodendron* spp., which were commonly isolated from *Pinus* (Figs 2 & 3) but not other plant hosts. Similarly, *Melinomyces* were primarily isolated from *P. trichocarpa* grown in soils from the west coast (WA & OR) (Fig 3). We observed differences in the isolation frequency of some of the more abundant taxa with different soils and hosts. For instance, *Atractiella* sp. was isolated from both NC and CA soils, but was statistically more commonly isolated from CA ($P < 0.001$). Chi square tests indicate that the isolation frequency of *Ilyonectria* from *Populus* was significantly greater than expected compared to their isolation from *Pinus* ($P = 0.011$) (Fig S1). Similarly, an unidentified Sordariomycete OTU

was isolated from *Quercus* at a significantly greater frequency ($P=0.001$) than from *Populus*. Finally, several frequently isolated fungi including *Leptodontidium*, *Phomopsis* and *Exophiala* were only isolated from *Populus* roots, and thus appear to be limited to that host. While we were able to isolate many (but not all) fungi detected through metagenomic studies using next generation sequencing, a lot of fungi including mycorrhizal species evaded isolation (Supplementary table 3). Overall, a higher diversity of fungi was detected from high-throughput (454) sequencing than from culturing, with 99 OTUs overlapping between the two methods. These included five of the top ten most abundant fungi detected through culture-independent approaches (Fig 4).

Discussion

Cultivating the core Populus mycobiome

With over 1800 isolates and 1042 DNA barcoded isolates generated, this research makes a significant contribution to establishing baseline resources for understanding the core members of the endorhizal guild of fungi able to live inside of *Populus* roots. A large number of our >200 isolates represent previously uncultured fungi, based upon BLAST results, half of which were singletons. The majority of our sequenced (88%) isolates originated from *Populus trichocarpa* and *P. deltoides*. Few differences in the community of isolated endorhizal fungi were evident between these hosts. Exceptions to this were the greater than expected isolation frequency of *Atractiella*, *Phomopsis* and *Melinomyces* isolates from *P. trichocarpa*, and the greater than expected frequency in the isolation of fungi belonging to the *Chaetosphaeriaceae* and *Ophiostomaceae* on *P. deltoides*. However, it is difficult to determine whether these results are due to host biases, methodological biases, or other factors that are soil, environmental or sample dependent. For both *Populus* hosts a single OTU *Leptodontidium* species was the most common fungal taxon isolated from roots. *Fusarium*, *Ilyonectria*, and other fungal species belonging to the Helotiales were also commonly isolated. The incidence of fungi belonging to groups of fungi traditionally thought of more as plant pathogens (such as those in the Nectriaceae and Pleosporaceae) is interesting, particularly since isolates were made from fresh healthy root tissue. Future studies with representative isolates

are planned to better characterize the ecological roles of these isolates and their interactions with plants.

While *Quercus alba* and *Pinus taeda* were sampled less intensively, even with limited sampling a greater than expected incidence of a particular sordariomycete OTU from *Quercus* was evident, as was the isolation of *Phialocephala* and *Oidiodendron* OTUs only from *Pinus*. The inter- and intra- cellular root colonization of *Phialocephala* on *Pinus* has been previously documented and is likely a widespread phenomenon that may be involved in plant phosphorous and nitrogen uptake (Odell et al. 1993; Jumpponen et al. 1998).

Culturable versus uncultured diversity in fungi

As with all fungal isolation studies it is hard to determine which species are present on the roots but not detected because culture conditions are not suitable, or whether cultured fungi are active on or in the plant roots. To assess whether we were sampling a biologically relevant fraction of fungi we compared our isolates to sequences from previous 454 amplicon pyrosequencing studies of the *Populus* rhizosphere (Gottel et al. 2011; Shakya et al. 2013; Bonito et al. 2014). Indeed, many of our isolates are represented in some of the top OTU clusters and groups identified in these studies as part of the core microbiome in the *Populus* rhizosphere (Supplement 3, Figs 3 & 4). These include *Atractiella* sp., *Cylindrocarpon pauciseptatum*, *Ilyonectria* spp., *Fusarium oxysporum*, *Phoma* sp., *Bionectria ochroleuca*, *Flagelloscypha* sp., *Leptodontidium* sp., *Exophiala* sp. and *Mortierella* spp.. Some fungal isolates were not detected by 454 sequencing, likely due to the fact that fungal isolates were made from different physical samples (culturing is inherently a destructive process) and from a wider diversity of soils and locations than those of published 454 sequence datasets available for comparison. While the function of these root-associated fungi remains to be determined, preliminary results from ongoing re-inoculation studies are showing these taxa have distinct niches in plant roots and interact with their hosts in complex, ecologically important and sometimes beneficial ways.

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4 Methods based on culturing are known to favor rapidly growing fungi, saprotrophs, and
5 otherwise. Symbiotic biotrophic fungi such as ectomycorrhizal species are notoriously
6 difficult to isolate. We clearly were not able to isolate all the fungi from the sampled
7 plants and many of the EcM species that have been detected on *Populus* (e.g. species
8 of *Hebeloma*, *Inocybe*, *Laccaria*, *Tomentella*, *Tuber*, etc.) were absent from our culture
9 collection (Supplementary table 3). While we made great effort to isolate
10 ectomycorrhizal fungi (EcM) we were only successful in isolating a few putative EcM
11 species from *Populus* (*Tuber mexiusanum*) (Guevara et al. 2013) and from *Pinus*
12 (Thelephoraceae sp. and *Clavulina* sp.). Sequence comparisons to GenBank indicate
13 that the Thelephoraceae species is a common uncultured environmental sequence from
14 roots, while the *Clavulina* sequence is unique (closest Blast match of 96% similarity).
15 We also obtained an isolate of *Laccaria* cf. *proxima* from fruit bodies produced by trap-
16 plants in the growth chamber.
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30 Many unique and rarely-cultured fungi were isolated from *Populus*. For instance, two
31 ITS OTUs were isolated belonging to the genus *Atractiella* (Basidiomycota:
32 Puccinomycotina). One species was only isolated once, while the other was isolated
33 from CA and NC soils and from both *Populus* species. Both are quite distinct from
34 taxonomically identified sequences in GenBank although related to other environmental
35 sequences. Our field studies indicate that this second *Atractiella* isolate is often in the
36 top 5 fungal OTUs in rhizosphere and endosphere samples of *P. deltoides* (Gottel et al.
37 2011). Likewise, isolated OTUs belonging to the Chaetosphaeriales, Sordariomycetes,
38 and Helotiales were difficult to identify due to either few sequences in GenBank that
39 were close matches, or a lack of curated reference sequences for phylogenetic
40 comparisons. Many of the best BLAST matches of these sequences were from
41 insufficiently identified environmental sequences of uncultured fungi.
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54 The growth rates of the fungi isolated in this study varies widely. Some isolates such as
55 those belonging to *Mortierella*, *Fusarium*, *Umbelopsis*, *Atractiella* and *Flagelloscypha*
56 grow readily in standard malt extract agar (MEA) filling a 15 cm petri dish within 1-2
57 weeks. In contrast, many of the isolates belonging to the Sordariomycetes, Helotiales,
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4 Corticiales and Thelephoraceae grew more slowly on the culture media that we used
5 (MEA, MNN), requiring many months to fill a 15 cm petri dish. Growth on artificial
6 substrates may be a proxy for the function of endorhizal fungi in nature. We expect that
7 endorhizal fungi are involved in a range of strategies for acquiring and maintaining
8 necessary resources for their life cycle, including the decomposition of dead organic
9 matter.
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17 The fast growing woody plant species *Populus trichocarpa* was the first woody plant to
18 have a sequenced and annotated genome and it has emerged as a model species in
19 plant biology (Tuskan et al. 2006). The vision of *Populus* as a model system was
20 expanded through the Poplar Mesocosm Sequencing project initiated by the Joint
21 Genome Institute (JGI) (Martin et al. 2004). This project resulted in genome sequences
22 of three *Populus*-associated fungi: the ectomycorrhizal basidiomycete *Laccaria bicolor*,
23 the arbuscular mycorrhizal fungus *Glomus intraradices* (*Rhizophagus irregularis*) and
24 the poplar leaf rust *Melampsora larici-populina*. Important discoveries continue to
25 emerge from this project including identification of quantitative trait loci important to
26 ectomycorrhizal colonization (Labbe et al. 2011), the identification of effector molecules
27 produced by *L. bicolor* involved in the initiation of ectomycorrhizal formation (Plett et al.
28 2011), and the expansion of mating type genes in the Glomeromycota (Tisserant et al.
29 2013; Riley et al. 2014). While fungal root endophytes have been studied for over a
30 century and are known to have important contributions to plant physiology and health
31 (Trowbridge and Jumpponen 2004; Summerbell 2005; Porras-Alfaro and Bayman
32 2011), there is renewed interest in studying functions of endorhizal fungi and their
33 interactions with each other, EcM fungi and plants in the context of the plant
34 microbiome. Indeed, many of the endorhizal fungal isolates obtained in this study are of
35 particular interest given their habit and abundance in the *Populus* root microbiome. In
36 addition, a large proportion of the isolates are likely to be undescribed species and their
37 ecology within the *Populus* rhizosphere remains poorly understood. For these reasons a
38 number of genomes from these isolates are being sequenced through the Joint
39 Genome Institute as part of the “Metatranscriptomics of Forest Soil Ecosystems” and
40 “1000 Fungal Genomes” community sequencing projects (Spatafora et al. 2013). These
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4 include isolates of the novel *Atractiella* sp. (PMI 95), *Mortierella elongate* (AG77),
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6 *Ilyonectria europaea* (PMI 82), *Leptodontidium* sp. (PMI 412), *Flagelloscypha* sp. (PMI
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8 526), *Lechythophora* sp. (PMI 546), *Thozetella* sp. (PMI 491), *Lachnum* sp. (PMI 789)
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10 and *Clavulina* sp. (PMI 390). Doing so will expand upon the Poplar Mesocosm
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12 Sequencing project that began a decade ago. This culture library is a valuable
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14 community resource for studying the functional roles of endophytic root diversity on the
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16 growth and health of host plants.
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41 42 **References**

- 43
44 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of*
45
46 *Molecular Biology*. 215:403-410.
47
48 Baum C, Makeschin F, 2000. Effects of nitrogen and phosphorus fertilization on mycorrhizal formation of
49
50 two poplar clones (*Populus trichocarpa* and *P.tremula x tremuloides*). *Journal of Plant Nutrition*
51
52 *and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 163 (5):491-497
53
54 Beauchamp VB, Stromberg JC, Stutz JC, 2005. Interactions between *Tamarix ramosissima* (saltcedar),
55
56 *Populus fremontii* (cottonwood), and mycorrhizal fungi: Effects on seedling growth and plant
57
58 species coexistence. *Plant and Soil* 275 (1-2):221-231
59
60 Bois G, Piche Y, Fung MYP, Khalsa DP, 2005 Mycorrhizal inoculum potentials of pure reclamation
61
62 materials and revegetated tailing sands from the Canadian oil sand industry. *Mycorrhiza* 15
63
64 (3):149-158
65
66 Bonito G, Reynolds H, Robeson MS, Nelson J, Hodkinson BP, Tuskan G, Schadt CW, Vilgalys R, 2014. Plant
67
68 host and soil origin influence fungal and bacterial assemblages in the roots of woody plants.
69
70 *Molecular Ecology* 23 (13):3356-3370. doi:Doi 10.1111/Mec.12821

- Courty PE, Labbe J, Kohler A, Marcais B, Bastien C, Churin JL, Garbaye J, Le Tacon F, 2011. Effect of poplar genotypes on mycorrhizal infection and secreted enzyme activities in mycorrhizal and non-mycorrhizal roots. *Journal of Experimental Botany* 62 (1):249-260
- Cripps CL, Miller OK, 1995. Ectomycorrhizae Formed in-Vitro by Quaking Aspen - Including *Inocybe lacera* and *Amanita pantherina*. *Mycorrhiza* 5 (5):357-370. doi:Doi 10.1007/Bf00207408
- Edgar RC, 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *Bmc Bioinformatics* 5:1-19
- Edgar RC, 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26 (19):2460-2461. doi:Doi 10.1093/Bioinformatics/Btq461
- Gardes M, White TJ, Fortin JA, Bruns TD, Taylor JW, 1991. Identification of Indigenous and Introduced Symbiotic Fungi in Ectomycorrhizae by Amplification of Nuclear and Mitochondrial Ribosomal DNA. *Canadian Journal of Botany-Revue Canadienne De Botanique* 69 (1):180-190
- Gottel NR, Castro HF, Kerley M, Yang ZM, Pelletier DA, Podar M, Karpinets T, Uberbacher E, Tuskan GA, Vilgalys R, Doktycz MJ, Schadt CW, 2011. Distinct Microbial Communities within the Endosphere and Rhizosphere of *Populus deltoides* Roots across Contrasting Soil Types. *Applied and Environmental Microbiology* 77 (17):5934-5944
- Guevara G, Bonito G, Trappe JM, Cazares E, Williams G, Healy RA, Schadt C, Vilgalys R, 2013. New North American truffles (*Tuber* spp.) and their ectomycorrhizal associations. *Mycologia* 105 (1):194-209. doi:Doi 10.3852/12-087
- Helm DJ, Allen EB, Trappe JM, 1996. Mycorrhizal chronosequence near Exit Glacier, Alaska. *Canadian Journal of Botany-Revue Canadienne De Botanique* 74 (9):1496-1506
- Jabeen S, Ilyas S, Niazi ARK, Khalid AN, 2012. Diversity of Ectomycorrhizae Associated with *Populus* Spp. Growing in Two Different Ecological Zones of Pakistan. *Int J Agric Biol* 14 (5):681-688
- Jumpponen A, Mattson KG, Trappe JM, 1998. Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza* 7 (5):261-265
- Kaldorf M, Koch B, Rexer KH, Kost G, Varma A, 2005. Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: A transition from mutualism to antagonism. *Plant Biology* 7 (2):210-218
- Karliński L, Rudawska M, Kieliszewska-Rokicka B, Leski T, 2010. Relationship between genotype and soil environment during colonization of poplar roots by mycorrhizal and endophytic fungi. *Mycorrhiza* 20 (5):315-324
- Karlinski L, Rudawska M, Leski T, 2013. The influence of host genotype and soil conditions on ectomycorrhizal community of poplar clones. *European Journal of Soil Biology* 58:51-58. doi:Doi 10.1016/J.Ejsobi.2013.05.007
- Khasa PD, Chakravarty P, Robertson A, Thomas BR, Dancik BP, 2002. The mycorrhizal status of selected poplar clones introduced in Alberta. *Biomass Bioenerg* 22 (2):99-104. doi:Pii S0961-9534(01)00072-1
- Klironomos JN, Rillig MC, Allen MF, Zak DR, Kubiske M, Pregitzer KS, 1997. Soil fungal-arthropod responses to *Populus tremuloides* grown under enriched atmospheric CO₂ under field conditions. *Global Change Biology* 3 (6):473-478
- Labbe J, Jorge V, Kohler A, Vion P, Marcais B, Bastien C, Tuskan GA, Martin F, Le Tacon F, 2011. Identification of quantitative trait loci affecting ectomycorrhizal symbiosis in an interspecific F(1) poplar cross and differential expression of genes in ectomycorrhizas of the two parents: *Populus deltoides* and *Populus trichocarpa*. *Tree Genetics & Genomes* 7 (3):617-627
- Lodge DJ, 1989. The Influence of Soil-Moisture and Flooding on Formation of Va-Endomycorrhizae and Ectomycorrhizae in *Populus* and *Salix*. *Plant and Soil* 117 (2):243-253. doi:Doi 10.1007/Bf02220718

- Maddison WP, Maddison DR, 2009. Mesquite: a modular system for evolutionary analysis. Version 2.6 edn. <http://mesquiteproject.org>,
- Martin F, Tuskan GA, DiFazio SP, Lammers P, Newcombe G, Podila GK, 2004. Symbiotic sequencing for the *Populus* mesocosm. *New Phytologist* 161 (2):330-335
- Mueller KE, Hobbie SE, Oleksyn J, Reich PB, Eissenstat DM, 2012. Do evergreen and deciduous trees have different effects on net N mineralization in soil? *Ecology* 93 (6):1463-1472
- Nilsson RH, Veldre V, Hartmann M, Unterseher M, Amend A, Bergsten J, Kristiansson E, Ryberg M, Jumpponen A, Abarenkov K, 2010. An open source software package for automated extraction of ITS1 and ITS2 from fungal ITS sequences for use in high-throughput community assays and molecular ecology. *Fungal Ecology* 3 (4):284-287
- Odell TE, Massicotte HB, Trappe JM, 1993. Root Colonization of *Lupinus latifolius* Agardh and *Pinus contorta* Dougl by *Phialocephala fortinii* Wang and Wilcox. *New Phytologist* 124 (1):93-100
- Plett JM, Kempainen M, Kale SD, Kohler A, Legue V, Brun A, Tyler BM, Pardo AG, Martin F, 2011. A Secreted Effector Protein of *Laccaria bicolor* Is Required for Symbiosis Development. *Current Biology* 21 (14):1197-1203. doi:Doi 10.1016/J.Cub.2011.05.033
- Podila GK, Sreedasyam A, Muratet MA, 2009. *Populus* Rhizosphere and the Ectomycorrhizal Interactome. *Critical Reviews in Plant Sciences* 28 (5):359-367
- Porras-Alfaro A, Bayman P, 2011. Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. *Annu Rev Phytopathol* 49:291-315. doi:Doi 10.1146/Annurev-Phyto-080508-081831
- Riley R, Charron P, Idnurm A, Farinelli L, Dalpe Y, Martin F, Corradi N, 2014. Extreme diversification of the mating type-high-mobility group (MATA-HMG) gene family in a plant-associated arbuscular mycorrhizal fungus. *New Phytologist* 201 (1):254-268. doi:Doi 10.1111/Nph.12462
- Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, Miller AN, Wingfield MJ, Aime MC, An KD, Bai FY, Barreto RW, Begerow D, Bergeron MJ, Blackwell M, Boekhout T, Bogale M, Boonyuen N, Burgaz AR, Buyck B, Cai L, Cai Q, Cardinali G, Chaverri P, Coppins BJ, Crespo A, Cubas P, Cummings C, Damm U, de Beer ZW, de Hoog GS, Del-Prado R, B D, Dieguez-Urbeondo J, Divakar PK, Douglas B, Duenas M, Duong TA, Eberhardt U, Edwards JE, Elshahed MS, Fliegerova K, Furtado M, Garcia MA, Ge ZW, Griffith GW, Griffiths K, Groenewald JZ, Groenewald M, Grube M, Gryzenhout M, Guo LD, Hagen F, Hambleton S, Hamelin RC, Hansen K, Harrold P, Heller G, Herrera G, Hirayama K, Hirooka Y, Ho HM, Hoffmann K, Hofstetter V, Hognabba F, Hollingsworth PM, Hong SB, Hosaka K, Houbaken J, Hughes K, Huhtinen S, Hyde KD, James T, Johnson EM, Johnson JE, Johnston PR, Jones EB, Kelly LJ, Kirk PM, Knapp DG, Kõljalg U, GM K, Kurtzman CP, Landvik S, Leavitt SD, Liggenstoffer AS, Liimatainen K, Lombard L, Luangsa-Ard JJ, Lumbsch HT, Maganti H, Maharachchikumbura SS, Martin MP, May TW, McTaggart AR, Methven AS, Meyer W, Moncalvo JM, Mongkolsamrit S, Nagy LG, Nilsson RH, Niskanen T, Nyilasi I, Okada G, Okane I, Olariaga I, Otte J, Papp T, Park D, Petkovits T, Pino-Bodas R, Quaedvlieg W, Raja HA, Redecker D, T R, Ruibal C, Sarmiento-Ramirez JM, Schmitt I, Schussler A, Shearer C, Sotome K, Stefani FO, Stenroos S, Stielow B, Stockinger H, Suetrong S, Suh SO, Sung GH, Suzuki M, Tanaka K, Tedersoo L, Telleria MT, Tretter E, Untereiner WA, Urbina H, Vagvolgyi C, Vialle A, Vu TD, Walther G, Wang QM, Wang Y, Weir BS, Weiss M, White MM, Xu J, Yahr R, Yang ZL, Yurkov A, Zamora JC, Zhang N, Zhuang WY, Schindel D, Fungal Barcoding Consortium, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America* 109 (16):6241-6246
- Shakya M, Gottel N, Castro H, Yang Z, Gunter L, Labbé J, Muchero W, Bonito G, Vilgalys R, Tuskan G, Podar M, Schadt CW, 2013. A Multifactor Analysis of Fungal and Bacterial Community Structure in the Root Microbiome of Mature *Populus deltoides* Trees. *PLOS one* 8 (10):e76382. doi:10.1371/journal.pone.0076382

- Slavov GT, DiFazio SP, Martin J, Schackwitz W, Muchero W, Rodgers-Melnick E, Lipphardt MF, Pennacchio CP, Hellsten U, Pennacchio LA, Gunter LE, Ranjan P, Vining K, Pomraning KR, Wilhelm LJ, Pellegrini M, Mockler TC, Freitag M, Gerald A, El-Kassaby YA, Mansfield SD, Cronk QCB, Douglas CJ, Strauss SH, Rokhsar D, Tuskan GA, 2012. Genome resequencing reveals multiscale geographic structure and extensive linkage disequilibrium in the forest tree *Populus trichocarpa*. *New Phytologist* 196 (3):713-725. doi:10.1111/J.1469-8137.2012.04258.X
- Smith SE, Read DJ, 2008. *Mycorrhizal Symbiosis*. 3rd edition edn. Academic Press,
- Spatafora J, Stajich J, Grigoriev I, 2013. 1000 Fungal Genomes project. *Phytopathology* 103 (6):137-137
- Summerbell RC, 2005. From Lamarckian fertilizers to fungal castles: recapturing the pre-1985 literature on endophytic and saprotrophic fungi associated with ectomycorrhizal root systems. *Studies in Mycology* (53):191-256
- Swofford DL, 2002. *PAUP* Phylogenetic analysis using parsimony (*and other methods)*. 4.0 edn. Sinauer Associates, Sunderland, Massachusetts
- Thorn RG, Reddy CA, Harris D, Paul EA, 1996. Isolation of saprophytic basidiomycetes from soil. *Applied and Environmental Microbiology* 62 (11):4288-4292
- Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Frey NFD, Gianinazzi-Pearson V, Gilbert LB, Handa Y, Herr JR, Hijri M, Koul R, Kawaguchi M, Krajinski F, Lammers PJ, Masclauxm FG, Murat C, Morin E, Ndikumana S, Pagni M, Petitpierre D, Requena N, Rosikiewicz P, Riley R, Saito K, Clemente HS, Shapiro H, Van Tuinen D, Becard G, Bonfante P, Paszkowski U, Shachar-Hill YY, Tuskan GA, Young PW, Sanders IR, Henrissat B, Rensing SA, Grigoriev IV, Corradi N, Roux C, Martin F, 2013. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 110 (50):20117-20122. doi:10.1073/Pnas.1313452110
- Trowbridge J, Jumpponen A, 2004. Fungal colonization of shrub willow roots at the forefront of a receding glacier. *Mycorrhiza* 14 (5):283-293
- Vilgalys R, Hester M, 1990. Rapid Genetic Identification and Mapping of Enzymatically Amplified Ribosomal DNA from Several *Cryptococcus* Species. *J Bacteriol* 172 (8):4238-4246
- Visser S, Maynard D, Danielson RM, 1998. Response of ecto- and arbuscular mycorrhizal fungi to clear-cutting and the application of chipped aspen wood in a mixedwood site in Alberta, Canada. *Applied Soil Ecology* 7 (3):257-269. doi:10.1016/S0929-1393(97)00060-7
- Zhang XY, Liang J, Sun ZQ, Qu ZW, Zhang Y, Lu Q, 2010. Long-term effect of an ectomycorrhizal inoculum and other treatments on survival and growth of *Populus hopeiensis* Hu et Chow. *Forest Ecology and Management* 259 (12):2223-2232
- Zhong Z, 1995. Research on mycorrhizal associations of poplar. *Mycorrhizas for Plantation Forestry in Asia* (62):62-66

Figure 1 – Culture macro-morphology of 12 fungal isolates that were commonly detected in culture independent analyses of *Populus* roots. A. *Atractiella rhizophilia* nom. prov. PMI 95; B. *Bionectria ochroleuca* PMI 827; C. *Exophiala* sp. PMI 424; D. *Flagelloscypha* sp. PMI 526; E. *Fusarium* PMI 1211; F. *Ilyonectria* sp. PMI 1211; G. *Leptodontidium* sp. PMI 1412; H. *Mortierella elongata* PMI 93; I. *Neonectria* sp. PMI 83; J. *Neonectria* sp. PMI 1677; K. *Phomopsis* sp. PMI 847; L. Sordariales PMI 418.

Figure 2 – Rank abundance plots of fungal isolates from specific plant hosts. A. *Populus trichocarpa*. B. *Populus deltoides*. C. *Quercus alba*. D. *Pinus taeda*.

Figure 3 – Phylogenetic affinities of fungal isolates based on 28S rDNA analysis. Plant host from which root-associated fungi were isolated from is color coded on the outer ring (*Populus trichocarpa* – yellow; *Populus deltoides* – dark green; *Pinus taeda* – light green; *Quercus alba* – orange). Region from where the isolates originate is color coded in middle ring (West coast of North America – shades of blue; Eastern North America – green). Taxonomic affinity of isolates are color coded on the phylogenetic tree as indicated inner key.

Figure 4 – Correspondance between fungi isolated from the *Populus* rhizosphere and those detected by 454 amplicon pyrosequencing. A. Venn diagram showing the overlap between cultured fungal isolates and those generated from 454 amplicon pyrosequencing as reported by Bonito et al. (2014). B. Top ten ranked sequenced fungi from the *Populus* rhizosphere and their corresponding representation in cultured isolates.

Figure1

Figure 2

Figure3

Figure 4