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ARTICLE

Host preference of ectomycorrhizal fungi in mixed pine–oak woodlands

Ann L. Rasmussen, Ryan R. Busby, and Jason D. Hoeksema

Abstract: Many ectomycorrhizal fungi (EMF) are generalists, but most plant genera that form ectomycorrhizas have at least some fungal partners that are specific to that host genus. Because shared mycorrhizal fungi mediate plant community interactions, host preference has implications for plant succession and competition. We studied the EMF of oaks (*Quercus* spp.) and pines (*Pinus* spp.) in a forest in northern Florida, USA, focusing on symbionts shared with longleaf pine (*Pinus palustris* Mill.). Longleaf pine is an important species in the southeastern USA, both for timber plantations and for restoring savanna and woodland habitat. However, we found no research on the composition of naturally occurring EMF on longleaf pine roots. A lower proportion of EMF operational taxonomic units (OTUs) were found colonizing both oaks and pines than expected, providing evidence of host preference within the community. Although most EMF were detected only on either oaks or pines, the OTUs found on both tended to be frequently occurring and abundant. *Cenococcum* OTUs were found to be significantly associated with oaks, an unexpected finding as this genus is widespread, with a broad host range. These results suggest that host preference of EMF may structure EMF communities and therefore influence ecosystem effects of mycorrhizal networks.

Key words: host specificity, host preference, ectomycorrhizal fungi, mycorrhizal networks, *Pinus palustris*.

Résumé : Plusieurs champignons ectomycorhiziens (CEM) sont des généralistes mais la plupart des genres de végétaux qui forment des ectomycorhizes comptent au moins un certain nombre de partenaires fongiques spécifiques à un genre hôte donné. Parce que les champignons mycorhiziens partagés sont à la base des interactions dans les communautés végétales, la préférence pour un hôte a des répercussions sur la compétition et la succession chez les plantes. Nous avons étudié les CEM des chênes (*Quercus* spp.) et des pins (*Pinus* spp.) dans une forêt du nord de la Floride, aux États-Unis. Nous avons mis l'accent sur les symbiotes partagés avec le pin des marais (*Pinus palustris* Mill.). Le pin des marais est une espèce importante dans le sud-est des États-Unis pour la production de bois en plantation et la restauration des savanes et des habitats boisés. Cependant, nous n'avons trouvé aucune étude portant sur la composition des CEM naturellement présents sur les racines du pin des marais. La proportion d'unités taxonomiques opérationnelles (OTU) de CEM qui colonisaient autant les chênes que les pins était plus faible que ce qui avait été anticipé, ce qui constitue un indice d'hôte préférentiel au sein de la communauté. Bien que la plupart des CEM aient été détectés exclusivement soit sur les chênes, soit sur les pins, les OTU trouvées sur les deux avaient tendance à être présentes fréquemment et de nombreuses OTU de *Cenococcum* étaient associées de façon significative aux chênes, un résultat inattendu étant donné que ce genre est largement répandu et possède une vaste gamme d'hôtes. Ces résultats indiquent que la préférence des CEM pour un hôte peut structurer les communautés de CEM et par conséquent influencer les effets des réseaux mycorhiziens sur l'écosystème. [Traduit par la Rédaction]

Mots-clés : spécificité de l'hôte, préférence pour un hôte, champignons ectomycorhiziens, réseaux mycorhiziens, *Pinus palustris*.

Introduction

Specificity in species' interactions is important in understanding community ecology, coevolution, and even predicting extinction risk (Molina et al. 1992; Bruns et al. 2002; Thompson 2009; Devictor et al. 2010). Ectomycorrhizal fungi (EMF), which are typically beneficial root symbionts of trees, show a range of specificity in which plant hosts they colonize. Host specificity refers to the breadth of plants with which a fungus can form mycorrhizas (Molina and Horton 2015). Although many EMF, often referred to as generalists, can colonize a broad range of hosts, some EMF show specificity to ectomycorrhizal host genera (Molina et al. 1992; Toju et al. 2013). Members of Pinaceae, in particular, have many family- and genus-restricted EMF partners (Molina et al. 1992; Bruns et al. 2002). EMF also exhibit host preference, which refers to mycorrhizae forming between plant and fungal species more or less frequently than expected by chance in an experimental setting or more frequently on one host than a different neighboring host species in field studies, despite a lack of limitations on compatibility among symbionts (Molina and Horton 2015).

Host associations of EMF are especially compelling because interspecies mycorrhizal interaction is a mediator of plant community ecology. Shared EMF allow the potential formation of common mycorrhizal networks among plant species (Kennedy et al. 2003; Twieg et al. 2007; Molina and Horton 2015), which can significantly alter the outcomes of plant–plant interactions by transferring water, nutrients, hormones, and allelochemicals between plants (Newmann 1988; Simard et al. 2012; Horton 2015). For example, EMF networks associated with canopy *Pinus radiata* D. Don increased drought tolerance of conspecific seedlings and offset the

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negative effect of root competition on the seedlings, likely by transferring water from adults to seedlings (Booth and Hoeksema 2010). Stressed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) transferred photosynthetic carbon to ponderosa pine (*Pinus ponderosa* Douglas ex P. Lawson & C. Lawson) via mycorrhizal networks (Song et al. 2015). The availability of compatible fungi can also drive plant succession and guild formation (Molina et al. 1992). For example, *Arctostaphylos* chaparral has been shown to provide EMF inoculum that drives *Pseudotsuga* succession in California central coast chaparal (Horton et al. 1999). In contrast, selective pressure on ruderal EMF such as some *Rhizopogon* spp. may explain why many of them are specialized to early successional trees — the need to locate and colonize a host when only a few seedlings are available may conflict with the ability to colonize a broad host range (Bruns et al. 2002). Understanding whether specialization is a benefit or a detriment to the symbiotic partners will help drive applied decisions about types of inoculum to use in nurseries, as well as resolve theoretical questions about the evolution of specificity.

Although host specificity in some EMF taxa is documented, research is increasingly turning to host preference to examine possible effects of EMF host associations on plant communities. Molina and Horton (2015) define host preference as occurring when "consistent patterns of nonrandom assemblages between plant and fungal species are observed more or less frequently than expected by chance, despite an absence of compatibility limitations between the symbionts." As a result of host preference, variation in host plant composition can drive dissimilarity in EMF communities (Ishida et al. 2007). For example, the frequency of most of the common fungi in an Australian wet sclerophyll forest was found to partially depend on host plant species (Tedersoo et al. 2008). Smith et al. (2009) found that EMF communities were structured by host in a mixed *Quercus* and *Pinus* stand, even when the fungi known to show host specificity were excluded. From a plant-focused perspective, host preferences by EMF could contribute to plant–soil feedbacks, which can promote or discourage plant species coexistence, depending on whether EMF that prefer particular plants are relatively better or worse at promoting growth of those plants (Bever 2003; Bever et al. 2010).

Longleaf pine, *Pinus palustris* Mill., is important commercially and ecologically in the southeastern USA. Longleaf pine is resistant to many diseases that affect other pines grown commercially in the area (Otrosina et al. 1999) and can grow on poor soils that often make the most common commercial species, *Pinus taeda* L. (loblolly pine), weakened and more susceptible to disease (Eckhardt et al. 2010; Coyle et al. 2015). Longleaf pine is also more resistant to windfall than loblolly, an increasing concern as hurricane frequency and severity increase near the Gulf of Mexico (Gresham et al. 1991; Johnsen et al. 2009). Finally, longleaf pine is a keystone species in longleaf pine savannas, a critically endangered habitat that supports extremely high species diversity (Frost 1993; Mitchell et al. 2006).

Although research has examined the amount of EMF mycelium found in longleaf pine stands (Runion et al. 1997; Hendricks et al. 2006; Sims et al. 2007; McCormack et al. 2010), we were unable to find surveys of the EMF taxa present beyond observation of a single *Thelephora terrestris* sporocarp. *Pisolithus tinctorius* has been trialed as a possible inoculum on longleaf, with varying results (Kais et al. 1981; Cram et al. 1999). EMF are expected to be important to longleaf pine success, as their typical habitat is sandy, fire-maintained communities, where acquisition of water is important and the minimal organic layer may make nutrient acquisition difficult (Hendricks et al. 2006).

This research examines host preference of EMF in oaks and pines, with a focus on *P. palustris*. Specifically, we set out to answer the question of whether EMF with broad host range or narrow host range are more prevalent in longleaf-dominant pine–oak forests by sampling the roots of longleaf pine trees and paired nearby oak or pine trees. We hypothesized that EMF with broad host range would be more commonly detected and constitute a higher proportion of colonized root tips. We further hypothesized that due to the dominant nature of multi-host EMF, the proportion of taxa colonizing oaks, pines, or both would be consistent with an assumption of no host specificity.

Methods

Site description

Samples were collected from Eglin Air Force Base near Niceville, FL, USA (30.5247, −86.4921). The area includes pine plantations, as well as areas with more varied vegetation. Sites within the base were selected in consultation with base staff to include all pine species occurring locally and a variety of oak species. Unfortunately, the difficulty in finding appropriate trees for sampling meant that sites chosen often contained a variety of soil types, making soil texture and soil organic matter more useful measures than site. Soils ranged from very sandy and well-drained to saturated soils with high organic matter content to soils with high clay content. The predominant soil type in the area is Lakeland sand (NRCS Soil Survey Staff 2016). All sampling sites were located within approximately 30 km of each other. *Pinus palustris* is common at the study location, as is *P. taeda*. *Quercus laevis* Walter is the most frequent oak species, although others are also common, including *Quercus geminata* Small and *Quercus incana* W. Bartram. Apparent oak hybrids were also common and were excluded from sampling, although hybridization in oaks is not always apparent from phenotype. Soil pH ranged from about 4 to 6, with 5 being a typical value.

Sample collection and processing

Sampling was conducted 12–14 May 2014. To maximize the likelihood of finding fungal species shared by different hosts, samples were taken between pairs of mature trees. Because of the site management's interest in increasing the use of *P. palustris* for plantations, each pair of trees was composed of a *P. palustris* and another tree. Other tree species included the other pine species present at Eglin (*Pinus clausa* (Chapm. ex Engelm.) Vasey ex Sarg. ($n = 8$), *Pinus elliottii* Engelm. ($n = 8$), and *P. taeda* ($n = 8$)) and a variety of red and white oaks chosen due to on-site abundance (red oaks: *Quercus arkansana* Sarg. ($n = 5$), *Quercus hemisphaerica* Bartram ex Willd. ($n = 5$), *Quercus incana* W. Bartram ($n = 6$), and *Quercus laevis* Walter ($n = 7$); white oaks: *Q. geminata* Small ($n = 7$) and *Q. margaretta* (Ashe) Small ($n = 6$)). Between each pair of trees, four 7 cm diameter by 15 cm deep cores were taken in the root zone of each tree and compounded. Sixty pairs of trees were sampled, for a total of 120 samples. Sixty samples were from *P. palustris*, 24 samples were from other *Pinus* species, and 36 samples were from *Quercus* species. When possible, trees were selected such that their root zones as estimated by canopy dripline were within 2–3 m of each other, a typical distance for EMF spatial autocorrelation (Lilleskov et al. 2004). However, finding appropriate tree species pairs led to sampling trees with trunks up to 10 m apart. Where they could be reached, leaves were also collected from sampled trees to provide a reference for analysis of plant DNA in roots. Additional pine needles sampled from trees in other locations along the Gulf Coast were also used to create reference sequences. Soil was kept in coolers in the field to prevent samples heating in the sun and refrigerated at the end of each sampling day. Upon return to the lab, samples that could not be processed within 2 weeks of harvest were frozen at 0 °C until processing. Soil was sieved using a 2 mm mesh, debris was removed, and roots were washed with tap water and placed in a Petri dish. Samples with large quantities of roots were subsampled. Colonized root tips were classified into morphotypes based on colour, surface texture, and branching pattern under a dissecting scope, and the number of root tips corresponding to each morphotype was counted. Three tips from each morphotype in each sample were saved for molecular identification. Sieved soil from each sample was saved for soil texture and soil organic matter assays. Soil

Table 1. Ectomycorrhizal fungal operational taxonomic units from most to fewest occurrences, with occurrences on oak, occurrences on pine, total number of root tips, and accession used for identification.

Fungal OTU	Total occurrences	Oaks	Pines	Total root tips	Accession used for identification
Russula 7	11	3	4	486	UDB014194
Cenococcum geophilum	9	3	4	557	KC967410
Russula 2	7	1	5	680	AB507025
Hebeloma 1	5	1	3	352	GU328547
Lactifluus piperatus	5	1	3	202	KF220050
Lactarius corrugis	4	—	—	454	JQ753822
Lactarius imperceptus	4	—	3	257	JQ272401
Russula 1	4	—	2	263	FJ803979
Amanita brunnescens	3	—	2	76	KC855217
Cortinarius quarciticus	3	—	2	154	UDB000748
Lactarius 3	3	—	2	497	AJ633589
Rhizopogon 1	3	—	3	206	AJ810040
Rhizopogonaceae 1	3	—	2	347	DQ351512
Russulaceae 2	3	—	1	123	AJ633583
Amanitaceae 1	2	—	1	346	UDB015627
Amanita recutita	2	1	2	88	JX844736
Bankeraceae 1	2	—	1	90	UDB015699
Cortinarius 2	2	—	—	104	JQ991693
Gloniaceae 1	2	1	—	91	JN943886
Gloniaceae 2	2	1	1	39	JQ711879
Gomphaceae 1	2	—	1	199	FJ196943
Laccaria trichodermophora	2	—	2	53	KC152146
Lactarius 1	2	—	2	70	KF220050
Lactarius 2	2	1	1	113	AY456344
Lactarius 4	2	—	2	98	AJ633589
Pulveroboletus 1	2	—	2	192	UDB011961
Rhizopogon 3	2	—	2	431	AJ810034
Russula 10	2	—	1	91	JQ396469
Russula 17	2	—	1	262	JX457011
Russula 4	2	1	1	82	FJ196947
Russula 8	2	1	—	73	KF810121
Russulaceae 1	2	—	1	109	AY281091
Russulaceae 17	2	1	—	186	UDB014058
Russulaceae 3	2	1	—	124	KF220092
Russulaceae 4	2	—	2	106	JQ753908
Suillaceae 2	2	—	1	231	L54088
Suillus decipiens	2	—	1	153	AF166508
Tomentellopsis zygodesmoides	2	—	2	48	UDB011640
Tricholoma flavovirens	2	—	2	130	JF899574
Tuber 1	2	—	1	176	GQ379737
Amanita 1	1	—	1	26	KC424527
Amanita 2	1	—	—	39	JX029931
Amanita 3	1	—	1	36	KF359589
Amanita 4	1	—	1	9	HE820439
Amanita 5	1	—	1	21	FM999626
Amanita 6	1	—	1	55	KC855218
Amanita 7	1	—	—	89	JX029931
Amanita 8	1	—	1	32	KC855224
Amanita 9	1	—	—	41	KC855217
Amanitaceae 2	1	—	1	58	KJ638264
Amanitaceae 3	1	—	—	22	EU819463
Amanitaceae 4	1	—	—	316	KJ638264
Boletaceae 1	1	1	—	55	DQ273368
Cantharellaceae 1	1	—	1	17	AB445116
Cantharellaceae 2	1	—	1	27	AB211251
Cenococcum 1	1	1	—	54	AY818585
Cenococcum 2	1	—	—	128	JN943886
Cenococcum 3	1	1	—	36	JN943890
Cenococcum 4	1	—	—	68	JX316439
Cenococcum 5	1	—	—	19	EF619647
Cenococcum 6	1	—	—	32	EF619647
Cenococcum 7	1	—	—	32	KJ701295
Clavulina 1	1	—	1	46	JN247429
Clavulina 2	1	1	—	23	FM999678
Clavulinaceae 1	1	—	1	4	AY456373
Cortinariaceae 1	1	—	—	18	GQ159913
Cortinariaceae 2	1	—	—	46	UDB011758
Cortinariaceae 3	1	—	1	209	GQ159913
Cortinarius 1	1	—	1	195	FJ157077
Cortinarius 10	1	—	1	89	UDB018654
Cortinarius 3	1	—	—	87	FJ157077
Cortinarius 4	1	—	—	104	UDB018664
Cortinarius 5	1	—	1	15	GU328603
Cortinarius 6	1	1	—	114	JN197989
Cortinarius 7	1	—	—	36	KJ705113
Cortinarius 8	1	1	—	9	KJ705138
Cortinarius 9	1	—	1	28	JX029949
Gloniaceae 5	1	1	—	77	KF879454
Gloniaceae 3	1	1	—	43	AY818585
Gloniaceae 4	1	1	—	24	JN943889
Hydnaceae 1	1	1	1	102	UDB012035
Hydnellum caeruleum	1	1	—	118	EU622335
Hydnum 1	1	—	1	38	KC686877
Hydnum 2	1	1	—	29	HE820661
Hygrophorus 1	1	—	—	512	EU292531
Inocybaceae 1	1	—	1	125	HQ604561
Laccaria 1	1	—	—	91	JX030197
Lactarius 10	1	—	1	7	AJ633589
Lactarius 11	1	—	—	107	AJ633589
Lactarius 12	1	—	1	46	AY456347
Lactarius 13	1	—	—	27	AY456347
Lactarius 5	1	—	1	87	AJ633589
Lactarius 6	1	1	—	81	AJ633589
Lactarius 7	1	—	—	69	UDB000836
Lactarius 8	1	1	—	43	KF937340
Lactarius 9	1	—	1	29	AY456344
Lactarius subserifluus	1	—	1	16	EU819482
Lactifluus 1	1	—	—	56	KF220048
Lactifluus 2	1	1	—	43	KF220050
Lactifluus 3	1	—	—	56	KF220015
Lactifluus 4	1	—	1	31	KF220017
Lactifluus 5	1	—	—	56	KF220050
Lactifluus 6	1	—	—	48	KF220050
Lactifluus 7	1	—	—	94	JQ753830
Ramaria 1	1	—	1	62	HM234140
Rhizopogon 2	1	—	1	212	JX017263
Rhizopogon 4	1	—	1	18	JX017263
Rhizopogon 5	1	—	—	121	JX017263
Rhizopogonaceae 2	1	—	1	8	DQ822821
Rhizopogonaceae 3	1	—	—	49	AB507025
Russula 11	1	—	1	36	FJ196947
Russula 12	1	—	1	16	FJ196947
Russula 13	1	—	1	39	KM576559
Russula 14	1	—	—	46	JX457011
Russula 15	1	—	—	92	DQ778004
Russula 16	1	—	—	64	UDB016029
Russula 18	1	—	1	102	AB507025
Russula 3	1	1	—	18	JQ396496
Russula 5	1	—	1	39	AB507025
Russula 6	1	—	—	293	UDB014194
Russula 9	1	—	1	86	HE820652
Russulaceae 10	1	—	—	8	HE820682
Russulaceae 11	1	—	1	25	AB218078
Russulaceae 12	1	—	1	32	AB507025
Russulaceae 13	1	—	1	39	AB507025

Table 1. (*concluded*).

Fungal OTU	Total occurrences	Oaks	Pines	Total root tips	Accession used for identification
Russulaceae 14	1	—	1	36	GU328540
Russulaceae 15	1	—	—	41	FR852027
Russulaceae 16	1	—	—	93	AB769910
Russulaceae 6	1	—	1	18	KJ769279
Russulaceae 7	1	—	1	103	KF220050
Russulaceae 8	1	—	—	116	JQ272401
Russulaceae 9	1	—	1	54	AJ633583
Russula cyanoxantha	1	—	1	66	EU598196
Russula flavisiccans	1	—	1	6	EU598162
Sarcodon scabrosus	1	1	—	68	KC571778
Scleroderma polyrhizum	1	—	1	63	EU718123
Sistotrema 1	1	—	—	67	FR838002
Suillaceae 1	1	1	—	144	L54088
Suillaceae 3	1	—	1	26	L54088
Suillus 1	1	—	—	114	AF166510
Thelephoraceae 1	1	—	1	47	KM402988
Thelephoraceae 2	1	—	1	83	UDB002646
Thelephoraceae 3	1	—	—	31	GQ219947
Thelephoraceae 4	1	—	1	9	FR731298
Tomentella 1	1	—	1	80	FM999528
Tomentella 2	1	1	—	21	UDB018519
Tomentella 3	1	—	1	34	UDB018688
Tomentella 4	1	—	1	19	UDB018457
Tomentella 5	1	1	—	29	HM370471
Tomentella 6	1	—	1	39	DQ482015
Tomentella 7	1	—	1	56	GU907787
Tomentella 8	1	1	—	32	UDB018519
Tomentella 9	1	1	—	181	UDB018441
Tomentellopsis 1	1	—	1	42	UDB018503
Tricholoma 1	1	—	—	124	EU563482
Tricholoma 2	1	—	1	15	UDB019449
Tricholoma 3	1	—	1	26	HQ285404
Tricholoma 4	1	—	1	62	AF309522
Tricholoma 5	1	—	—	66	AF309522
Tricholoma 6	1	—	1	4	KC152249
Tricholoma 7	1	—	—	31	FJ596911
Tricholoma 8	1	—	—	64	HQ285404
Tricholoma 9	1	—	1	99	FJ596910
Tylospora 1	1	—	1	47	AY969614

Note: Total occurrences is the number of samples in which the operational taxonomic unit (OTU) was found. Occurrences on oaks and pines may not sum to total occurrences due to hosts that were neither oak nor pine, inability to identify host, or finding an OTU on different hosts within the same sample.

texture was measured using a LaMotte soil texture test (LaMotte Company, Chestertown, MD, USA). Soil organic matter content was measured using a loss-on-ignition method. Soil was dried to a steady weight at 100 °C, then a subsample was placed in a tin of known weight, weighed, heated in a muffle furnace for 2 h at 360 °C, and reweighed when cool enough to handle (Davies 1974).

DNA was extracted from all sampled root tips on the day the soil sample was processed. Components of Extract-N-Amp extraction kits (Sigma-Aldrich, St. Louis, MO, USA) were used as described by Rúa et al. (2015) with the exception that extracts were diluted with 160 μL PCR-grade water and were stored at –20 °C. To facilitate Sanger sequencing of EMF species sampled, the internal transcribed spacer (ITS) region of fungal nuclear DNA was amplified using forward primer ITS1-F and reverse primer ITS4 (Gardes and Bruns 1993). Amplification reactions for each sample contained 2.2 μL PCR-grade water, 4 μL of 2X RedTaq Premix (Apex Bioresearch Products, Inc., San Diego, CA, USA), 0.4 μL of each primer at 10 μmol·L^{-1} concentration, and 1 μL of DNA extract. Reactions occurred in sterile 96-well PCR plates sealed with a sterile silicone sealing mat, briefly vortexed and centrifuged, and amplified as follows: initial denaturation at 94 °C for 3 min; 40 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 53 °C, extension for 72 s at 72 °C; and a final extension for 10 min at 72 °C.

To identify the plant host, PCR amplified the chloroplast DNA locus bounded by psbA and trnH primers using a touchdown PCR program due to difficulties in finding an optimum annealing temperature (Sang et al. 1997). The thermocycling parameters started with 3 min at 94 °C, then 15 cycles were run, over which the annealing temperature was decreased from 55 °C to 51 °C in 0.2 °C increments, followed by 25 cycles with an annealing temp of 51 °C. The cycles were denatured for 40 s at 94 °C, 40 s at the annealing temp, and 45 s at 72 °C. The cycling was followed by a final extension of 10 min at 72 °C. Representative sequences have been accessioned to GenBank (MF945989–MF945997). The psbA-trnH locus was selected because of its relatively high variability, particularly in oaks (Simeone et al. 2013), and common use as a plant barcoding locus (Hollingsworth et al. 2011). The trnL-trnF locus (Taberlet et al. 1991) was also tested and did not provide additional resolution in identifying plant species.

Success of PCR was evaluated on a 1% agarose gel cast with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). Successful PCR reactions had excess primer and mononucleotides removed enzymatically, with each reaction containing 0.05 μL ExoI (New England Biolabs, Ipswitch, MA, USA), 0.2 μL Antarctic Phosphatase (New England Biolabs), 4.75 μL PCR-grade water, and 5 μL of amplified DNA. Reactions were incubated at 37 °C for 30 min, then 80 °C for 20 min, followed by at least 5 min at 4 °C. Purified fungal DNA was sequenced using the forward primer ITS5 (White et al. 1990), and purified plant DNA was sequenced using the psbA forward primer. All sequencing used the BigDye Terminator Sequencing Kit (v3.1, Invitrogen Corp., Grand Island, NY, USA), with each sequencing reaction containing 0.4 μL BigDye Reaction Premix, 1.8 μL BigDye 5X Sequencing Buffer, 0.5 μL primer at 10 μmol·L^{-1} concentration, 6.3 μL PCR-grade water, and 1 μL purified DNA. Sequencing reactions were incubated thus: initial denaturation at 96 °C for 1 min; 45 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, and extension at 60 °C for 4 min. A ramp speed of no more than 1 °C·s^{-1} was used. Reactions were dried and shipped overnight to the DNA Lab at Arizona State University, Tempe, AZ, where the BigDye reactions were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The fungal sequences obtained were edited, assembled into operational taxonomic units (OTUs) at 97% similarity, and identified by comparison to sequences in public databases. The methods used were as described in Rúa et al. (2015), with the exception that matches >99% similarity were assigned a species epithet (or genus if no closely-matching sequence was identified to species), 95%–99% similarity to closest match assigned to the same genus as the match, and 90%–95% similarity to closest match assigned to the taxonomic family of the match. This enabled us to assign identities to many more sequences without overstating their similarity to the reference sequences. Fungal sequence lengths ranged from 200 to 828 bases. Plant DNA sequences were aligned and compared to sequences from collected leaves and compared with sequences on the GenBank database using the BLAST utility. Because of the limited range of hosts, some short plant sequences were usable, and sequence lengths ranged from 49 to 721 bases.

Data analysis

Although the sampling strategy was planned to collect fine roots from particular host trees, the identities obtained through Sanger sequencing frequently did not match the intended host. Because the host identities did not support analyzing the samples separately, samples from the same pair were pooled for analysis.

The locus sampled could only resolve the plant hosts into three groups: red oaks (*Quercus* section *Lobatae*), white oaks (*Quercus* section *Quercus*), and pines (genus *Pinus*). Due to a low number of root tips identified as belonging to white oaks, only three fungal OTUs

were identified on white oaks (*Russula* 7, *Cenococcum geophilum*, and Gloniaceae 3). Therefore, red and white oaks were pooled for analysis. Given these constraints in identifying hosts, we effectively sampled 84 pines and 36 oaks. Data were analyzed using R version 3.2.5 (R Core Team 2016). A list of fungal species associated with each identifiable plant group was compiled and compared to determine the amount of overlap within and among groups. A χ^2goodness-of-fit test was conducted to determine if the occurrence of OTUs associated with oaks, pines, both, or an unidentified host was consistent with a null hypothesis of no specificity. The χ^2 test was conducted with and without OTUs that occurred only once. As data were non-normal, Spearman's rank correlation was used to relate occurrence (presence or absence) and abundance (count of colonized root tips) of common taxa with soil organic matter and soil texture, using the cor.test() function. Proportion tests were run using prop.test() to test if the proportion of common OTUs, genera, and families found on pine varied significantly from the proportion of pine root tips to oak root tips, suggesting host preference.

Results

The 292 EMF morphotypes were identified and sequences were accessioned to GenBank (MF945998–MF946289). These were categorized as164 EMF OTUs, which represented 16 290 ectomycorrhizal root tips. See Table 1 for a list of detected OTUs, total number of occurrences, number of occurrences on oaks and pines, and number of root tips associated with each (Supplementary material[1]). The OTUs occurring in the most samples were *Russula* 7 (11 samples), *Cenococcum geophilum* (in 9 samples), *Russula* 2 (7 samples), *Hebeloma* 1 (5 samples), and *Lactifluus piperatus* (5 samples).

At least one oak or pine host was identified for 120 of the fungal OTUs. Of OTUs with an identified host, 85 OTUs were found only with pines, 25 OTUs were found only with oaks, and 10 OTUs were detected on both hosts. A χ^2 test found that this distribution of OTUs is significantly different from the expected distribution (χ^2_1 = 14.239, $p < 0.001$), with most of the χ^2 value coming from fewer OTUs than expected found on both oak and pines (χ^2 = 5.114) and more OTUs than expected found on oaks but not pines (χ^2 = 6.940). When the analysis was restricted to the 40 OTUs that occurred in at least two samples, 25 OTUs were found only with pines, 4 OTUs were found only with oaks, and 9 OTUs were detected on both hosts. Because of the low number of expected observations in some categories, Yates' continuity correction was applied. This distribution was not significantly different from the expected distribution (χ^2_1 = 2.147, p = 0.143).

However, the OTUs shared by oaks and pines included dominant fungi that were the most commonly detected OTUs and substantial proportions of the total number of root tips, including *Russula* 2 (4.2% of root tips), *Cenococcum geophilum* (3.4% of total root tips), *Russula* 7 (3.0% of root tips), *Hebeloma* 1 (2.1% of root tips), and *Lactifluus piperatus* (1.2% of root tips). Common EMF found only on pines were *Russula* 1 (detected in 4 cores, 1.6% of root tips), *Lactarius imperceptus* (detected in 4 cores, 1.6% of root tips), *Lactarius* 3 (detected in 3 cores, 3.1% of root tips), Rhizopogonaceae 1 (detected in 3 cores, 2.1% of root tips), *Rhizopogon* 3 (detected in 2 cores, 2.6% of root tips), and Amanitaceae 1 (detected in 2 cores, 2.1% of root tips). A few other taxa also represented similarly high percentages of total root tips but host identity could not be determined: *Hygrophorus* 1 (3.1% of root tips) and *Lactarius corrugis* (2.8% of root tips).

Comparing taxa at the family level, Russulaceae dominated the EMF community, colonizing 41% of identified root tips and occurring in 49 out of 60 samples. Gloniaceae, the family that includes *C. geophilum*, was detected in 21 samples, representing 7.4% of total root tips. Rhizopogonaceae was found in only 11 samples but colonized the second-highest number of root tips, 8.5%.

Russula 2 was negatively correlated with the percent silt in the soil (occurrence ρ = –0.303, p = 0.19; abundance ρ = –0.310, p = 0.016). Russulaceae occurrence was negatively correlated with soil organic matter, although the relationship with Russulaceae abundance was not significant (occurrence ρ = –0.289, p = 0.026; abundance ρ = –0.155, p = 0.236). Russulaceae occurrence was also positively correlated with percentage sand in the soil, and the relationship between abundance and sand showed a similar trend (occurrence ρ = 0.316, p = 0.014; abundance ρ = 0.221, p = 0.089). No other common OTUs or families were significantly correlated with soil organic matter or soil texture.

Of OTU occurrences with both host plant and EMF identified, 145 were from pines and 42 were from oaks. Proportion tests were run on EMF taxa with the null hypothesis of 78% of OTU occurrences belonging to pines and 22% belonging to oaks. No common OTUs were significantly associated with one host or the other. When aggregated to genus level, *Cenoccocum* was significantly associated with oaks (5/10 occurrences, p = 0.037). The effect was stronger at the family level, with 9/15 occurrences of Gloniaceae occurring on oaks ($p < 0.001$). No other taxa were significant. Rhizopogonaceae, which is a family known to have host specificity for Pinaceae, did occur exclusively on pines but with 13 occurrences and heavy sampling of pines the effect was only near significant (p = 0.052).

Discussion

Finding *Rhizopogon* and Rhizopogonaceae species only on pines is consistent with what is already known about host specificity in EMF (Molina et al. 1992; Horton and Bruns 1998). The distribution of OTUs among oaks and pines also demonstrates that host specificity affects EMF community structure. However, most commonly occurring taxa and abundant taxa were found on both oak and pine, and this dominance of multihost fungi is typical of EMF communities (Kennedy et al. 2003; Richard et al. 2005; Roy et al. 2008; Toju et al. 2013). One possible explanation for this is that the need for both partners to quickly form a symbiosis to successfully compete for resources drives the lack of specificity found in dominant EMF (den Bakker et al. 2004). Alternately, the diverse community of compatible EMF may swamp the host plant's ability to preferentially reward the most beneficial fungus and start the process of evolving specificity (Thompson 2009). The EMF guild may also be coevolving relatively uniformly with their host plants in diffuse coevolution (Hoeksema 2010).

The results of the χ^2 tests suggest an interaction between whether EMF associate with oaks and whether they associate with pines. There were fewer taxa than expected associating with both oaks and pines, suggesting that host preference is common among fungi. Also, despite the presence of pine-specific fungi in the Rhizopogonaceae and no known oak-specific fungi detected, there were more OTUs than expected found on oaks but not on pines, which is further evidence that host preference is important in these forests. Although taxa found in only one sample cannot be said to exhibit host preference per se, including these taxa can still provide useful information about host preference in the community as a whole. Excluding fungi found in only one sample is also a substantial loss in power, and the nonsignificance of this test may be due to this limited sample size.

The patchiness of mycorrhizal occurrence leading to uncommon OTUs representing a large number of root tips is also consistent with typical EMF community structure (Horton and Bruns 2001). Typifying this pattern was the family Rhizopogonaceae, which was found in only 11 out of 60 samples but was the second

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2017-0227.

most abundant family in terms of roots tips colonized. There is also evidence that environment plays a role in defining niche for EMF, as Russulaceae occurred more often in sandier soils. Both of these observations have implications for our understanding of species coexistence in communities of EMF. For example, a patch occupancy model has been hypothesized to explain coexistence among EMF species (Hoeksema and Kummel 2003), but it assumes that EMF are randomly distributed among all available host root tips. Patchy colonization and distinct soil niches for particular species clearly violate this assumption.

The finding that *Cenococcum* was significantly associated with oak was surprising, as it is an ubiquitous genus with a broad host range. In a previous study at Eglin, *Cenococcum geophilum* was the most common EMF in longleaf pine plantations (Busby, unpublished data). These conflicting findings demonstrate that the patchiness of ectomycorrhizal root tip occurrence also produces difficulty in achieving adequate sampling and in interpreting results. The inability to show significant host preference of Rhizopogonaceae despite the family being fairly common and host specific suggests that more intensive sampling may have uncovered additional patterns.

The dominance of generalist EMF also suggests that common mycorrhizal networks (CMNs) may form between pines and oaks, despite the likelihood that some fungi exhibit host preference. Environmental factors could also affect the degree of sharing, with physical disturbance such as burrowing or rooting animals leading to disconnection of potentially multihost fungi. Factors affecting the amount of ectomycorrhizal biomass, e.g., water and nutrient availability and distribution, could also affect genet size of EMF and thus their ability to colonize multiple hosts.

Lack of plant DNA barcodes with resolution at fine taxonomic levels is a barrier to investigating host specificity of plant symbionts at the plant species level (Shaw et al. 2005). The plant taxa used for this study were particularly difficult to resolve because oaks have both narrowly defined species and hybridize extensively, and pines also hybridize, leading to difficulties in using chloroplast loci (Hollingsworth et al. 2011; Piredda et al. 2011; Simeone et al. 2013). Improving the available loci for identifying plants from environmental samples will make identification of root tips to plant species more accessible to projects with large numbers of samples.

Overall, however, the decreasing cost of sequencing is facilitating studies of specificity and coevolution. Although ectomycorrhizal root tips are still important as functional units of symbiosis, high-throughput sequencing of soil allows for detection of many more organisms than other sampling methods, enhancing ability to detect specificity (Öpik et al. 2009). Advances in sequencing also make it easier to detect cryptic species and construct phylogenies to investigate patterns of specialization (Roy et al. 2008; Rochet et al. 2011).

Understanding preference in mycorrhizal associations is a promising path to increased understanding of symbiosis, coevolution, and plant community ecology. This study was limited in its applicability by the lack of appropriately spaced trees and heterogenous soil types — future studies with stands of only two tree species and more consistent soils could help to tease apart patterns at the host–species level and effects of environment on host preference. Although many mycorrhizal fungi have an apparently broad host range, host preference may structure EMF communities and should be included when considering the possible ecosystem effects of mycorrhizal networks.

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