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Diversity of ectomycorrhizal fungi associated with *Quercus alba* in northwest Arkansas

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Abstract

Species of oak (*Quercus* spp.) have considerable ecological and economic importance in the forests of northwest Arkansas, and their growth and survival ultimately depend upon the mutualistic associations they establish with a variety of ectomycorrhizal fungi in the soil. The species diversity of these fungi is known to be quite high in oak forests, but no previous study has examined the assemblages of fungi associated with white oak in the forests of northwest Arkansas. The study reported herein included a below-ground approach to assess fungal communities, making use of molecular identification of ectomycorrhizal root-tips through sequencing of the internal transcribed spacer (ITS) ribosomal DNA region. DNA extracted from root-tips collected from five different white oak trees yielded sequences of at least 32 taxa. Ectomycorrhizal taxa associated with white oak in the general study area appear to be dominated by members of the Basidiomycota. The most common ectomycorrhizal fungi associated with white oak roots belong to the genus *Russula*, including seven taxa identified to the species level and three to the genus level.

Key words – Basidiomycota – ectomycorrhizae – ITS ribosomal DNA region – roots

Introduction

The Ozark Mountains of northwest Arkansas and adjacent areas of southern Missouri are among the oldest mountains in North America, having originated in the Paleozoic Era. The Ozark National Forest encompasses a total of about 14.2 million hectares. Using data from the USDA Forest Service Continuous Inventory of Stand Conditions (CISC) data system, it is estimated that in 2001 only about 6% of the hardwood forests on the Ozark National Forest were less than 40 years old, whereas approximately 72% of these forests were between the ages of 40 and 90 years old and 21% were more than 90 years old. Various species of oak (*Quercus* spp.) have dominated many of the forests of eastern United States for nearly 10,000 years, although their abundance and distribution have varied during this period due to several different types of forest disturbances. White oak (*Quercus alba* L.) is an economically valuable commercial hardwood species with

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extensive use in the furniture industry. Moreover, the acorns of this tree are an important food resource for wildlife. White oak is recognized for its ability to form symbiotic associations with a wide range of ectomycorrhizal (ECM) fungi. Sequencing of the Internal Transcribed Spacer (ITS) region of the fungal ribosomal RNA gene has made the DNA-based identification of species of fungi possible (Schoch et al. 2012). The main objective of the present study was to carry out a preliminary DNA-based assessment of the assemblages of ectomycorrhizal fungi associated with the roots of white oak, using the forests of the Ozark Mountains as a model site for possible future studies.

Materials & Methods

Study sites

The present study was carried out in Pea Ridge National Military Park (36°27'15" N, 94°02'05" W). The park, which has a total area of approximately 17 km^2, was established in 1956 to protect the site of the Civil War Battle of Pea Ridge, which was fought on 7–8 March 1862. The forested areas of the park are dominated by a mixture of several species of oak (*Quercus alba* L., *Q. velutina* Lam., *Q. stellata* Wangenh., and *Q. rubra* L.) and hickory (*Carya ovata* [Mill.] K. Koch, *C. texana* Buckley, and *C. tomentosa* Sarg.).

Belowground sampling strategy

The roots of five oak trees were sampled. Trees were opportunistically sampled at least 10 meters apart to avoid resampling the same fungal genets. Root-tips were collected from different sides of the tree at 90° intervals (north, south, east and west). The distance of sampling from the trunk of the tree was between 0.5 and 2.0 meters. Roots were uncovered using a trowel, then feeder roots were traced back to the sample tree and growing colonized root-tips were collected. Because of variability in colonized root tip length, width, and numbers, a total volume of approximately 20 ml was collected for each sampled tree. Root-tip samples were placed in 50 ml screw cap tubes with 2% CTAB solution. Samples were kept refrigerated for further morphological and molecular analyses.

DNA extraction

Before microscopic examination and subsequent DNA extraction, the roots were carefully washed with distilled water, and soil residues were removed. The cleaned roots were transferred into a polystyrene Petri dish. Digital pictures of ECM morphologies were taken with a Leica DFC495 binocular microscope using a black background illumination at various magnifications. Individual ECM root-tips (ca. 1– 5 mm) were then homogenized and transferred into a clean sterile 1.5 ml microfuge tube. Samples were homogenized using a Geno/Grinder 2010 with 3 mm glass beads (10 min, 1620 rpm). DNA extraction from homogenized tissue was carried out using the NucleoSpin Plant II kit (Macherey-Nagel, Bethlehem, PA). Protocol steps were modified from the manufacturer's original protocol to carry out optimal DNA extraction. Modifications included dividing the volumes of PL1 Buffer solution, Rnase A, and PC Buffer solution PC by half, and performing one wash with 350 ml PW1 Buffer solution. DNA samples were eluted in 25 µl of PE Buffer solution.

PCR and sequencing

DNA extracted from ectomycorrhizal root-tips was amplified via polymerase chain reaction (PCR) using the fungal-specific primers ITS1F and ITS4 (White et al. 1990, Gardes & Bruns 1993). PCR amplifications were performed in a thermocycler. The PCR program was as follows: initial denaturation at 95 °C for 5 min, followed by 37 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 30 s, and amplification at 72 °C for 1.30 min, and a final extension at 72 °C for 7 min. PCR products were verified via electrophoresis in a 1.5% agarose gel in 0.5× TAE buffer, stained with SYBR safe. MassRuler Express Forward DNA ladder Mix (Thermo Scientific,

MA) was used as a size standard. DNA was sent for single-pass Sanger sequencing to Beckman-Coulter Genomics (Danvers, MA). Sequences were edited using the software SeqMan-program version 7.1.0 (44.1) and manually corrected before alignment to obtain a consensus sequence. For a DNA-based identification all sequences were *in silico* compared with the results of a nucleotide search using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). At ca 95% identity, sequences were considered identified to species where there was a match with an existing sequence; at a lower % identity, sequences were considered identified to genus.

Results

Diversity of ectomycorrhizal fungi

DNA isolated from 100 individual root-tips collected from five trees in the Pea Ridge National Military Park yielded 32 taxa of ECM fungi (Table 1). Most morphotypes were found only once (Fig. 1). The majority of the ectomycorrhizal fungi identified from DNA sequences belong to the phylum Basidiomycota; however, several taxa (including *Cenococcum geophilum*, *Cistella spicicola,* and unidentified members of the Chaetothyriales and Helotiales) are members of the Ascomycota. The ECM fungi most commonly associated with *Quercus alba* belong to the genus *Russula*, and almost a third of all sequences could be assigned to this genus.

Table 1 Fungi identified with ITS sequence data from root-tips of *Quercus alba*. Note: Percent ID = % of closest matching sequence in GenBank.

Taxon	GenBank Accession no.	Percent ID	Sequence in GenBank
Amanita novinupta Tulloss & J. Lindgr.	KX358019	98%	GQ250403
Amanita sp.	KX358018	93%	KP313583
Ascomycota (unidentified species)	KX358020	98%	KM576302
Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud	KX358021	100%	KM507833
Cenococcum geophilum Fr.	KX358022	99%	LC095204
Chaetothyriales (unidentified species)	KX358923	98%	DO421069
Cistella spicicola Huhtinen & Söderh.	KX358024	98%	GU727553
Clavulina sp.	KX358025	87%	KR019838
Hebeloma brunneifolium Hester	KX358028	100%	NR119892
Helotiales (unidentified species)	KX358030	97%	JX243905
Helvellosebacina sp. 1	KX358031	99%	KF000461
Helvellosebacina sp. 2	KX358032	99%	KF000449
Hydnum sp.	KX358033	96%	DQ218306
Hygrophorus russula (Schaeff.) Kauffman	KX358034	95%	KF291216
Inocybe sp. 1	KX358035	92%	HQ604215
Inocybe sp. 2	KX358037	91%	AB848505
Lactarius argillaceifolius Hesler & A.H. Smith	KX358038	95%	KJ705210
Lactifluus sp.	KX358041	83%	HG426469
Russula cerolens Shaffer	KX358043	99%	JX434674
Russula chamaeleontina (Lasch) Fr.	KX358044	96%	JF834357
Russula compacta Frost	KX358046	99%	KT933952
Russula pectinatoides Peck	KX358047	99%	KJ530759
Russula quercilicis Sarnari	KX358049	97%	JF908700
Russula raoultii Quel.	KX358050	97%	KR082875
Russula subtilis Burt.	KX358053	95%	GQ166871

Table 1 Continued.

Taxon	GenBank Accession no.	Percent ID	Sequence in GenBank
Russula sp. 1	KX358051	100%	JX030256
Russula sp. 2	KX358052	90%	EU569266
Russula sp. 3	KX358048	94%	EU598186
Sebacina sp.	KX358054	98%	HG796956
Sistotrema alboluteum (Bourdot & Galzin) Bondartsev & Singer	KX358055	96%	AJ606042
Trichoderma hamatum (Bonord.) Bainier	KX358057	100%	KT827285
Tomentella sp.	KX358056	97%	KT182921

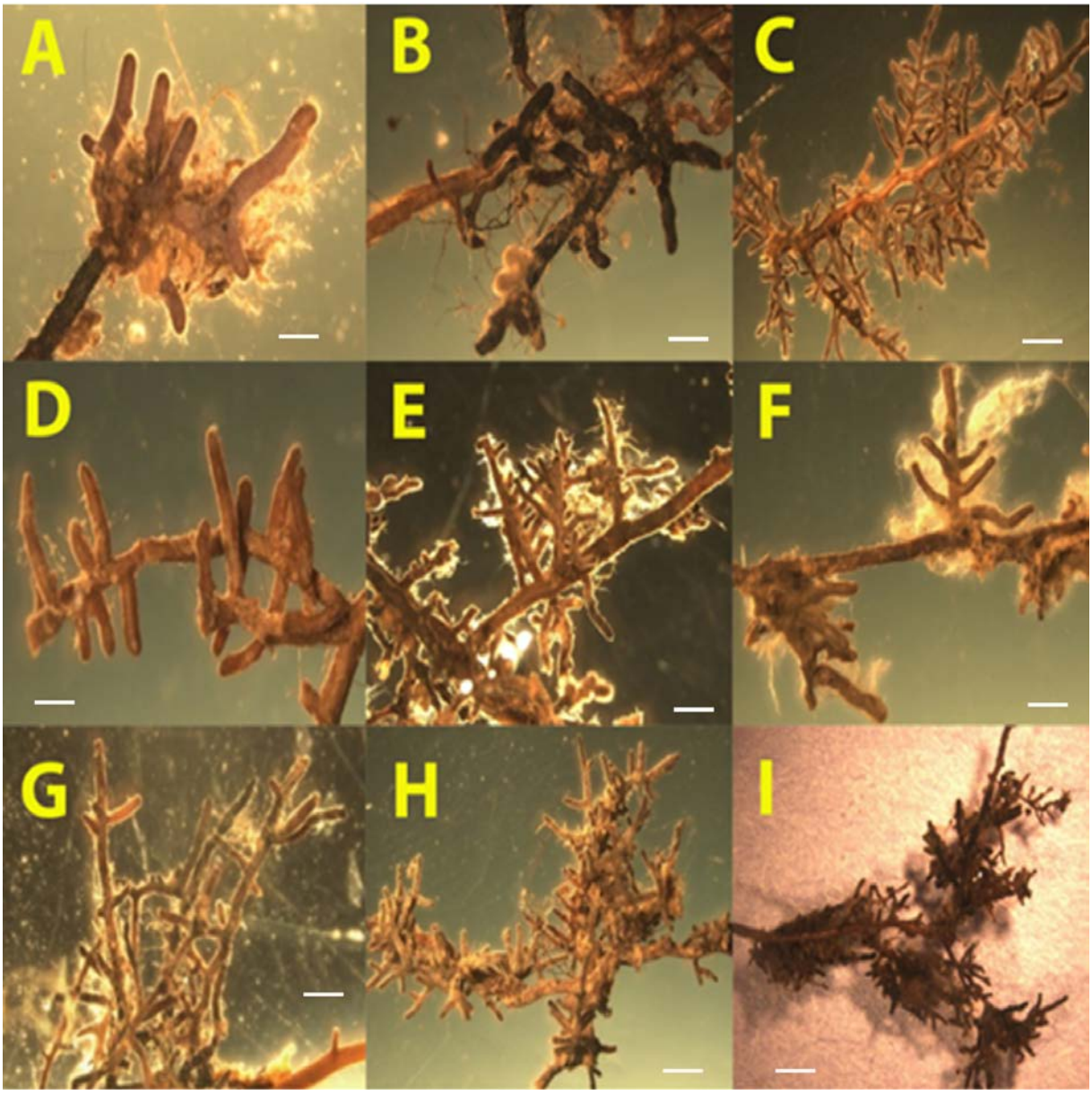

Fig. 1 – Ectomycorrhizal morphotypes on *Quercus alba* roots collected in Pea Ridge National Military Park in northwest Arkansas, A *Amanita* sp. B *Cenococcum geophilum.* C *Helvellosebacina* sp. D *Russula chameleontina.* E *Inocybe* sp. F *Hydnum* sp. G *Hebeloma brunneifolium.* H *Clavulina* sp. I *Russula cerolens.* Scale bar in 1A, 1B and 1D = 0.1 mm, whereas all other scale bars = 1.0 mm.

Discussion

Prior to the advent of the techniques of modern molecular biology, determining the ectomycorrhizal fungi associated with particular trees was exceedingly difficult (Straatsma et al. 2001). Since the majority of ectomycorrhizal fungi are basidiomycetes (Rinaldi et al. 2008), they typically produce macroscopic fruiting bodies that are clearly visible for a brief period of time, usually in late summer and early fall in temperate regions of the world. The proximity of fruiting bodies to a tree provides some evidence that the mycelium which produced these fruiting bodies is associated with the roots of the tree. However, the former does not represent definitive proof that the two organisms are involved in an ectomycorrhizal association (Tóth & Barta 2010, Stephenson 2010). Moreover, since most fruiting bodies don't persist for more than a few days, they are not likely to be observed unless the locality where they occur is closely monitored. In many instances, such monitoring is simply not practical. Northwest Arkansas is characterized by relatively dry summers, and in some years, there is very little evidence of the fruiting bodies of ectomycorrhizal fungi (Stephenson, per. observation).

The past three decades have witnessed an unprecedented increase in the number of publications on ectomycorrhizal fungi, and many of these papers have reported on efforts to assess the diversity of the assemblages of fungi associated with individual species of ectomycorrhizal-forming trees and shrubs or entire forest communities (Taylor 2002). The impetus for this increase has been the application of molecular techniques which involve extracting DNA from the roots of a tree or shrub, amplifying the Internal Transcribed Spacer (ITS) region of ribosomal DNA and ultimately obtaining a sequence which can be compared with the numerous sequences available in major databases such as UNITE (https://unite.ut.ee/) (Kõljalg et al. 2005) or GENBANK (https://www.ncbi.nlm.nih.gov/genbank/) (Schoch et al. 2012).

Although numerous studies of the type described above have been carried out in various localities throughout the world (e.g. Sims et al. 1997, Tóth & Barta 2010, Spake et al. 2016, Nautiyal et al. 2016, Kluthe et al. 2016), the one described herein apparently represents the first such effort in the forests of northwest Arkansas. Indeed, we are not aware of any comparable studies anywhere in the Ozark physiographic province, which encompasses northern Arkansas and the southern half of Missouri while also extending westward into northeast Oklahoma and southeast Kansas. Consequently, one important aspect of the data reported herein is to provide a starting point for future more comprehensive studies.

The total number of taxa (32) identified from just five white oak trees in the present study certainly indicates that a high level of biodiversity exists for ectomycorrhizal fungi in the forests of northwest Arkansas. The fact that half of the sequences recovered did not match any taxon in the UNITE and GENBANK sequence databases at least suggests that this assemblage contains some species that are either rare (i.e. have been described but not yet sequenced) or are possibly new to science. The latter is a possibility, since few species of ectomycorrhizal fungi have been described from material collected in Arkansas (e.g. Rosen 1926).

As noted in Table 1, members of the genus *Russula* appear to be the predominant fungi associated with white oak in the general study area. This would not seem surprising, since this very large genus (approximately 750 species are recognized worldwide) has been one of the more common ectomycorrhizal fungi reported in other surveys. Most species of *Russula* produce relatively large and often brightly colored fruiting bodies, which makes them easy to recognize. However, in the experience of the first author, the fruiting bodies of *Russula* do not appear to be particularly common in the white oak-dominated forests that occur at some localities in the general study area. However, this might not be surprising. Wang et al. (2015), who carried out an intensive study of members of this genus, indicated that the production of fruiting bodies depends upon many factors and varies from year to year so that comprehensive sampling of the species present at a given locality would have to extend over many years. The same is true for several other widespread and important genera of ectomycorrhizal fungi, including *Amanita* and *Lactarius*/*Lactifluus*. Each genus was represented by just two species in the present study, and these data would seem to support the observations noted above. However, the data for *Russula* (ten species identified from

sequences) appear to indicate that the predominance of members of this genus below ground (i.e. associated with roots) is not reflected in their occurrence as fruiting bodies above ground. As such, there are clearly apparent advantages of using a molecular-based approach to characterize the assemblages of ectomycorrhizal fungi associated with a particular type of tree or a particular forest community.

It should be noted that the fungi of northwest Arkansas and adjacent areas of the Ozarks are not especially well-documented, since the appropriate surveys have never been carried out. However, based on an examination of the limited body of data currently available (e.g. Discover Life [www.discoverlife.org], GBIF [www.gbif.org], Swartz 1933), there are no previous records from the region for most of the species identified from the sequences obtained in the present study. It is anticipated that future more comprehensive studies will yield numerous additional records.

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