Ectomycorrhizal fungi associated with Ozark chinquapin (*Castanea ozarkensis*)

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Abstract

Ozark chinquapin (*Castanea ozarkensis*) is a small tree endemic to the Ozark Plateau region of Oklahoma, Arkansas and Missouri in the eastern central United States. Like other North American members of the genus *Castanea* (including American chestnut, the best known example), Ozark chinquapin is susceptible to the chestnut blight fungus (*Cryphonectria parasitica*, Ascomycota), which was inadvertently introduced into North America at the end of the 19th century. Populations of Ozark chinquapin have undergone a major decline since the arrival of the blight in the region where the species is found. As is the case for other members of the family Fagaceae, Ozark chinquapin forms ectomycorrhizal (ECM) associations with various

fungi, but the taxa involved are not known. In the present study, the taxa of ECM fungi associated with Ozark chinquapin in three different study sites in northwest Arkansas were investigated. Root-tips were obtained from 18 different trees, and 42 taxa of fungi were identified from DNA sequences. Forty of these fungi known or suspected to form ECM relationships. The majority of ECM fungi identified belong to the Basidiomycota, with members of the families Russulaceae, Clavulinaceae, Thelephoraceae and Cortinariaceae particularly prominent. The fact that no fungal taxon was recorded from more than a single study site suggests that the total biodiversity of the assemblage of ECM fungi associated with Ozark chinquapin is exceedingly high.

Key words: DNA extraction, forest trees, Fagaceae, Northwest Arkansas, root-tips

Introduction

Ozark chinquapin (*Castanea ozarkensis* Ashe) is a small tree endemic to the Ozark Plateau region of Oklahoma, Arkansas and Missouri in the eastern central United States (Tucker 1975, Paillet 1993, Paillet & Cerny 2012). Like other North American members of the genus *Castanea* (including American chestnut [*Castanea dentata* (Marsh.) Borkh.], the best known example) Ozark chinquapin is susceptible to the chestnut blight fungus (*Cryphonectria parasitica* (Murrill) Barr, Ascomycota), which was inadvertently introduced into North America and Europe at the end of the 19th century (Stephenson 2013). As is widely known, the chestnut blight fungus devastated American chestnut, essentially eliminating the species from the forest canopy,

although root sprouts still persist in some of the forests where it was once dominant (Stephenson et al 1991, Agrawal & Stephenson 1995). Much less publicized is the impact of the fungus on Ozark chinquapin, populations of which have undergone a major decline in the arrival of the blight in the region where the species is found. This took place several decades after the initial introduction of the blight in eastern North America.

Ozark chinquapin was described originally as *Castanea arkansana* (Ashe 1923) but later renamed as *C. ozarkensis* by Moore (1992). Some authorities have recognized this taxon as the variety *arkanensis* (Ashe) G. E. Tucker of the Allegheny chinquapin *Castanea pumila* (L.) Mill., but recent molecular evidence indicates that the two are not this closely related. Indeed, all three North American species form a distinct clade, with Ozark chinquapin as the basal lineage, sister to the group consisting of Allegheny chinquapin and American chestnut (Dane et al. 2003). Like all members of the Fagaceae, Ozark chinquapin forms ectomycorrhizal (ECM) associations with various fungi, mostly basidiomycetes (phylum Basidiomycota) but also including some ascomycetes (phylum Ascomycota). Just what fungal taxa are involved these associations is unknown, simply because the appropriate studies have never been carried out. As such, the primary objective of the project described herein was to generate the first body of data on the ECM fungi associated with Ozark chinquapin in northwest Arkansas.

Materials and Methods

Study Sites

The populations of chinquapin investigated in the present study are located in northwestern Arkansas at (1) the Wedington block of the Ozark National Forest, (2) the Buffalo National River and (3) Hobbs State Park. All three of these study sites are described in detail by Paillet and Cerny

(2012) in their study of the distribution of Ozark chinquapin in northwest Arkansas. Tree-ring data showed that chestnut blight arrived in this area in 1957, so that 1958 was the first year in which oak trees growing next to large Ozark chinquapin trees showed significant release related to the death of the adjacent tree. All collection sites were located near the top of a ridge in deeply dissected terrain underlain by Mississippian limestone of the Boone Formation. Although limestone is often associated with a relatively high soil pH, these sites are characterized by a thick regolith of residual chert where soil pH varies from 4.5 to 5.5, and the shrub layer consists of various species of *Vaccinium* (blueberry) known to be characteristic of relatively acidic soils. Samples of root-tips were collected from two ridgetops about 2 km apart at Wedington. For the Buffalo National River, samples of root-tips were collected near the Turner Bend Visitor's Center, with one additional sample taken from another locality about 30 km east of the Visitors Center. The latter locality was unique in that the substrate was developed on soils derived from sandstone. Samples of root-tips were collected from a single locality about 1 km northwest of the visitor's center at Hobbs State Park. Most of the trees associated with Ozark chinquapin sprouts at the collection sites consisted of white oak (*Quercus alba* L.), post oak (*Q. stellata* Wangenh., black oak (*Q. velutina* Lam), and mockernut hickory (*Carya tomentosa* Sarg.). All three study sites had abundant indications of large original pre-blight Ozark chinquapin trees, although root-tips were collected from sprouts which appeared to be old seedlings that had never attained canopy dominant status.

Belowground sampling strategy

Root-tips were collected from a total of 18 different Ozark chinquapins at the three study sites. Individuals selected for sampling at a particular site were at least 10 meters apart to avoid resampling the same fungal genets. Root-tips were collected from different sides of each Ozark

chinquapin at 90° intervals (north, south, east, and west). In each instance, the distance of sampling from the stem of the Ozark chinquapin was between 0.5 and 2 meters. Roots were uncovered using a trowel, feeder roots traced back to the sample tree, and colonized root-tips were collected (Fig. 1).

All root-tip samples were placed in 50 ml screw cap tubes with 2% CTAB solution and returned to the laboratory. Samples were kept refrigerated for further morphological and molecular analyses. Before microscopic examination and subsequent DNA extraction, the roots were carefully washed and soil residues were removed. The cleaned roots were transferred to a polystyrene Petri dish. Digital pictures of ECM morphologies were taken with a Leica DFC495 binocular microscope using black background illumination at various magnifications. Individual ECM root tips were then transferred to a clean sterile 1.5 ml microfuge tube. Samples were homogenized using a Geno/Grinder 2010 with 3.0 mm glass beads (10 min, 1620 rpm). DNA extraction of homogenized tissue was done 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.

DNA extraction, PCR and sequencing

DNA extracted from ectomycorrhizal root-tips was amplified via the polymerase chain reaction (PCR), using the fungal-specific primers ITS1F and ITS4 (Toju et al. 2012, Bruns et al. 1998). 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 by SYBR safe. MassRuler Express Forward DNA ladder Mix (Thermo Scientific) 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).

Results

DNA was isolated from a total of 150 individual root-tips obtained from Ozark chinquapin. Forty-two taxa of fungi were identified from the ITS sequences obtained from these root-tips (Table 1), including 40 ECM fungi, including one representative each from an order (Helotiales) and a family (Hyaloscyphaceae) known to include some species that are ECM, and two saprotrophic fungi. Based on the number of root-tips from which they were recorded, the ECM fungi most commonly associated with Ozark chinquapin in northwest Arkansas belong to the genera *Russula* and *Lactarius,* both of which are members of the family Russulaceae. This total includes includes five taxa identified to the level of species for *Russula* (*R. amoenolens, R. chloroides, R. decipiens, R. pectinatoides* and *R. subemetica*) and four taxa identified to the level of species for *Lactarius* (*L. atroviridis, L. camphoratus, L. evosmus* and *L. yazooensis*). Four other species of *Russula* could be identified only to the level of genus.

The sequence data revealed the occurrence of a number of other ECM fungi associated with Ozark chinquapin, including species of *Amanita, Clavulina, Cortinarius, Hebeloma, Tricholoma*

and *Craterellus*. All of these are common and widespread ECM fungi. Images of the root-tips of Ozark chinquapin indicated that the ECM fungi display a wide range of different morphotypes (Fig. 2). As indicated in the data presented in Table 1, the vast majority of tge ECM fungi identified in the present study, as might have been expected, belong to the phylum Basidiomycota.

All of the DNA sequences generated in this study were added to the GenBank database, with the assession numbers indicated in Table 1.

Discussion

The present study represents the first effort of which we are aware to characterize, using molecular techniques, the assemblages of ECM fungi associated with native Ozark chinquapin in northwest Arkansas. Although molecular techniques have been widely used elsewhere in the world (Tedersoo et al. 2006, Smith et al. 2011, Lim and Berbee 2013), this is not the case for the region of North American where the study reported herein was carried out. The results obtained clearly indicate that a high level of diversity exists for the ECM fungi associated with Ozark chinquapin and that the fungi present include members of some of the major families of basidiomycetes known to form ECM. These include the Russulaceae, Clavulinaceae, Thelephoraceae and Cortinariaceae. Various taxa representing the Russulaceae were particularly prominent. Palmer et al (2008) also reported the Russulaceae as the taxa most commonly group associated with *Castanea dentata*.

The most surprising result of the present study is that no fungus identified to the level of species was recorded from more than a single study site. This suggests an exceedingly high level of diversity for the assemblage of ECM fungi associated with Ozark chinquapin. This is also reflected in the wide range of fungal morphotypes on the root-tips of chinquapin. Numbers of taxa recorded from the three study sites ranged from 6 to 19, with Hobbs appreciably lower than either

the Buffalo River (17) or Wedington (19). Just what the taxa identified only to genus (or in some cases family or order) represent is unknown, although it is possible that some of these are undescribed species whose sequences are not among those on GenBank.

Interestingly, an exceedingly common ECM fungi (*Cenococcum geophilum* Fr.), which is easily recognized, occurs on a wide range of host tree species and has a broad geographical distribution (Molina and Trappe 1982), was not recorded from any of our root-tip samples. However, it is a common ECM associate of oaks (*Quercus* spp.) in the general study area, where it is the most frequently recorded ECM for the ascomycetes (Ali, unpub. data).

In summary, the data presented herein provide what might be considered as a preliminary assessment of the ECM fungi associated with Ozark chinquapin in one portion of its range. Clearly, additional studies that would consider samples collected from other study sites, including those in the adjacent states of Missouri and Oklahoma, would provide a more complete picture of the ECM fungi associated with this tree species.

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Table 1. List of fungi identified from root-tips of Ozark chinquapin. ECM = ectomycorrhizal fungus and SAP = saprotrophic fungus.

Taxon	**Study site**	**Ecology**
Albatrellaceae (unidentified species)	Buffalo National River	ECM
Amanita flavoconia G.F. Atk.	Lake Wedington	ECM
Amanita rubescens Pers.	Lake Wedington	ECM
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Lake Wedington	SAP
Clavulicium delectabile (H.S. Jacks.) Hjortstam	Lake Wedington	ECM
Clavulina sp. 1	Lake Wedington	ECM
Clavulina sp. 2	Lake Wedington	ECM
Cortinarius camptoros Brandrud & Melot	Buffalo National River	ECM
Cortinarius decipiens (Pers.) Fr.	Hobbs State Park	ECM
Cortinarius leiocastaneus Niskanen, Liimat. & Soop	Hobbs State Park	ECM
Craterellus fallax A.H. Sm.	Hobbs State Park	ECM
Hebeloma subconcolor Bruchet	Buffalo National River	ECM
Helotiales (unidentified species)	Buffalo National River	?ECM
Hyaloscyphaceae (unidentified species)	Lake Wedington	?ECM
Lactarius atroviridis Peck	Hobbs State Park	ECM
Lactarius camphoratus (Bull.) Fr.	Lake Wedington	ECM
Lactarius evosmus Kühner & Romagn.	Buffalo National River	ECM
Lactarius yazooensis Hesler & A.H. Sm.	Buffalo National River	ECM
Membranomyces spurius (Bourdot) Jülich	Lake Wedington	ECM

Oidiodendron sp. 1	Lake Wedington	SAP
Russula amoenolens Romagn.	Lake Wedington	ECM
Russula chloroides (Krombh.) Bres.	Buffalo National River	ECM
Russula decipiens (Singer) Kühner & Romagn.	Buffalo National River	ECM
Russula pectinatoides Peck	Lake Wedington	ECM
Russula sp. 1	Buffalo National River	ECM
Russula sp. 2	Lake Wedington	ECM
Russula subemetica Schulzer	Lake Wedington	ECM
Russulaceae (unidentified species)	Buffalo National River	ECM
Sebacinaceae (unidentified species)	Buffalo National River	ECM
Thelephoraceae (unidentified species)	Buffalo National River	ECM
Tomentella sp. 1	Lake Wedington	ECM
Tricholoma caligatum (Viv.) Ricken	Buffalo National River	ECM

Figure 1. ECM root-tip collecting procedure: Left, Ozark chinquapin sprout. Center, digging up the soil to expose the roots of the sprout. Right, soil mass from which root-tips were extracted.

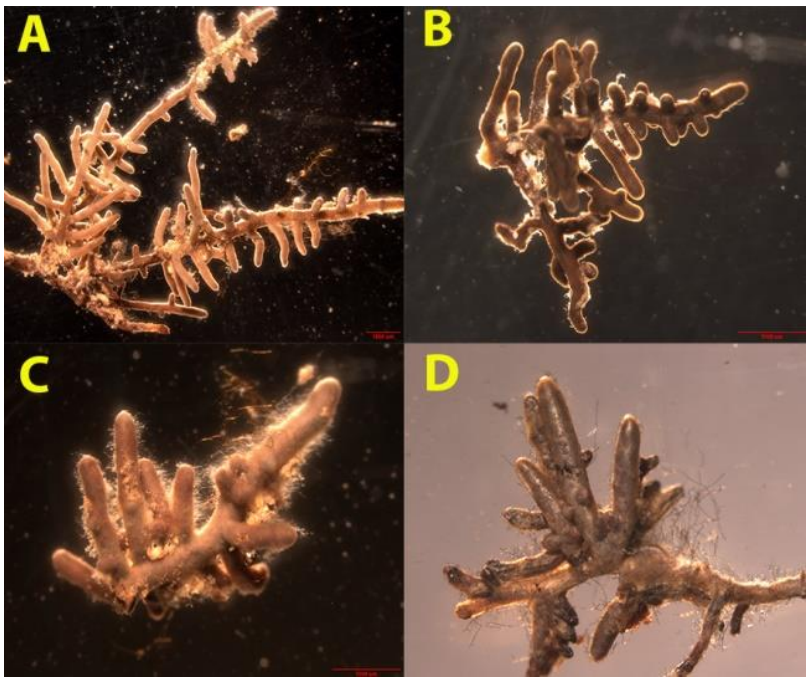

Figure 2. Ectomycorrhizal morphotypes on *Castanea ozarkensis* roots-tips collected in northwest Arkansas. **A** *Clavulicium* sp. **B** *Clavulina* sp. **C** *Russula* sp. **D** *Cenococcum geophilum.*