PRELIMINARY REPORT

Ectomycorrhizal fungi associated with Ozark chinquapin

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Introduction

Ozark chinquapin (*Castanea ozarkensis* Ashe), like all members of the family Fagaceae, is ectomycorrhizal. The primary objective of this project was to generate the first body of data on the ectomycorrhizal fungi associated with Ozark chinquapin in northwest Arkansas. The project represents the first part of a larger research effort with the ultimate goals of (1) comparing the assemblages of ectomycorrhizal fungi associated with Ozark chinquapin with the assemblages associated with American chestnut (*Castanea dentata*) and Allegheny chinquapin (*Castanea pumila*), (2) comparing these data with the assemblages of ectomycorrhizal fungi associated with other ectomycorrhizal-forming trees (e.g., various species of *Quercus*) in the forests of northwest Arkansas and (3) comparing the data from the three species of *Castanea* in the Eastern and Central United States with similar data for European chestnut.

Study Sites

The populations of chinquapin investigated in the present study were 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 characterize 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 Visitors 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 visitors center at Hobbs State Park. Most of the trees associated with Ozark chinquapin sprouts at the collection sites consisted of white oak, post oak, black oak, and mockernut hickory. 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.

Materials and Methods

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 (Figure. 1).

In addition to the samples collected from Ozark chinquapin, root-tip samples were collected from five individuals of American chestnut (*Castanea dentata* [Marsh.] Borkh.) in a study site located near the city of Harrogate in Claiborne County, Tennessee.

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.

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 “Leica DFC495" binocular microscope using black background illumination at various magnifications. Individual ECM root tips were then 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 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 extracted from ectomycorrhizal root-tips was amplified via the polymerase chain reaction (PCR), using the fungal-specific primers ITS1F and ITS4. 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).

In addition to the Ozark chinquapin sprouts sampled for individual root-tip extraction and Sanger sequencing for fungal identification (as describe above), an effort is being made to assess fungal diversity on Ozark chinquapin using Next Generation Sequencing methods. Five Ozark chinquapin sprouts were sampled, and the root-tip material of each sprout was pooled for 10 separate DNA extractions (2 per sprout). Extracted DNA was then quantified via Nano Drop spectrophotometry. Replicate DNA extractions were then pooled, and run through a DNA clean up and concentration kit to a final volume of 50 µl. This DNA suspension was then used in a verification PCR for fungal ITS amplicons (using primers ITS1F and ITS4). Currently, library preparation of ITS2 amplicons for these samples is underway. PCR with special primers containing known barcodes will be carried out and the subsequent products will be placed in an equimolar pool for sequencing on an Ion Torrent PGM machine. Upon completion of sequencing, it is expected that numerous taxa beyond those identified thus far with the use of Sanger sequencing are likely be recorded as associates of Ozark chinquapin. This component of the proposed project should be completed by May 2016.

Results and Discussion

DNA was isolated from a total of 150 individual root-tips obtained from Ozark chinquapin. Thirty-two taxa of fungi were identified from the sequences obtained from these root-tips (Table 1), including 28 ectomycorrhizal fungi, one representative each from an order (Helotiales) and a family (Hyaloscyphaceae) known to include some species that are ectomycorrhizal, and two saprotrophic fungi. Based on the number of root-tips from which they

were recorded, the ectomycorrhizal fungi most commonly associated with *Castanea ozarkensis* in northwest Arkansas belong to the genera *Russula* and *Lactarius.* This includes including five taxa identified to the species level for *Russula* (*Russula amoenolens*, *R. chloroides, R. decipiens, R. pectinatoides* and *R. subemetica*) and four taxa identified to the species level for *Lactarius* (*Lactarius atroviridis, L. camphoratus, L. evosmus* and *L. yazooensis*). Two other species of *Russula* were identified only to the genus level. ITS sequence data revealed the occurrence of other ECM fungi in association with *Castanea ozarkensis*, including species of *Amanita, Clavulina, Cortinarius, Hebeloma, Tricholoma* and *Craterellus*. Different morphotypes of ECM species were found in the different experimental sites (Figure 2). The vast majority of identified ectomycorrhizal fungi, as would have been expected, belong to the phylum Basidiomycota.

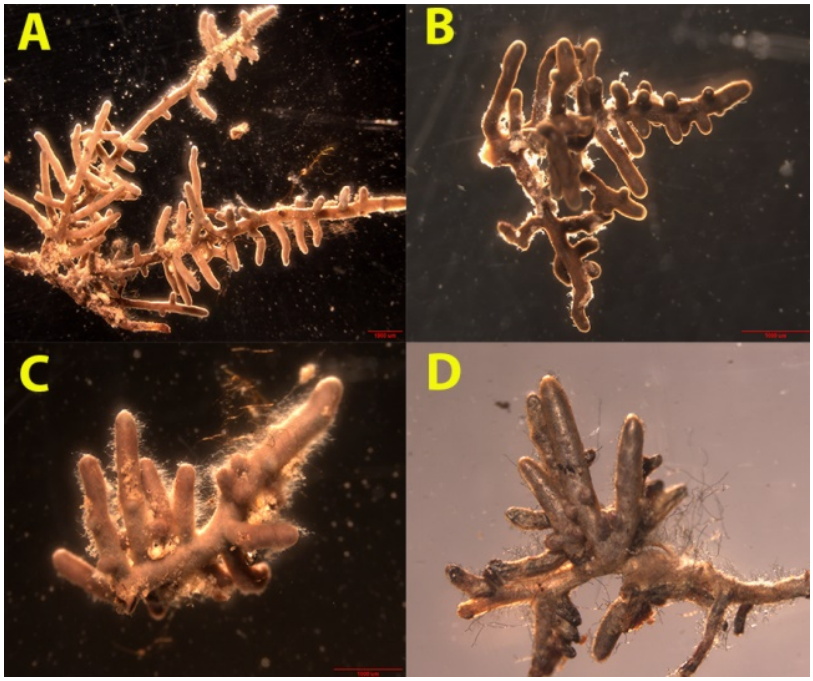

Figure 2. Ectomycorrhizal morphotypes on *Castanea ozarkensis* roots-tips collected in northwest Arkansas. **A** *Clavulicium* sp. **B** *Clavulina* sp. **C** *Russula* sp. **D** *Cenococcum geophilum.*

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

DNA was extracted from fewer root-tips of American chestnut (only 24) than was the case for Ozark chinquapin, so a lower number of associated fungi might have been expected. However, the actual number of species recorded (a total of only six, including five species of ectomycorrhizal fungi) was very low. These data suggest that the assemblage of ectomycorrhizal fungi associated with American chestnut is characterized by a much lower level of biodiversity than is the case for Ozark chinquapin. At the time this preliminary report was compiled, not all of the root-tips from American chestnut had been processed, so the number of species recorded may increase. Interestingly, only a single ectomycorrhizal fungus (*Russula pectinatoides*)

Table 2. List of fungi identified from root-tips of American chestnut. ECM = ectomycorrhizal fungus and SAP = saprotrophic fungus.

Taxon	**Study site**	**Ecology**
Tricholoma sejunctum (Sowerby) Quél.	Tennessee	ECM
Tomentellopsis sp. 1	Tennessee	SAP
Thelephoraceae (unidentified species 1)	Tennessee	ECM
Russula pectinatoides Peck	Tennessee	ECM
Leotia lubrica (Scop.) Pers.	Tennessee	ECM
Lactarius imperceptus Beardslee & Burl.	Tennessee	ECM

identified to the level of species was associated with both American chestnut and Ozark chinquapin.

As a result of another research project that is currently ongoing at the University of Arkansas, data relating to the ectomycorrhizal fungi associated with three species of oak that tend to occur in the same types of forests in which Ozark chinquapin is found in northwest Arkansas. These data were obtained in the same manner as described for Ozark chinquapin, and the root-tip samples were processed in the same laboratory. The three species of oak from which comparable data were available were white oak (*Quercus alba* L.), black oak (*Q. velutina* Lam.) and post oak (*Q. stellata* Wangenh.). These data are presented in Table 3.

Because white oak is the primary focus of this ongoing research project, more root-tips (100) have been sampled from this species than for black oak (75 root-tip samples) and post oak (81 root-tip samples), and the number of fungi recorded from white oak (56) reflects this fact. The numbers recorded for black oak (24) and post oak (23) are fairly comparable to the number (32) recorded for Ozark chinquapin. However, it would seem noteworthy that only 13 of the 32 taxa recorded for the latter were also recorded from at least one of the three species of oak. This

Table 3. Fungi identified from root-tips of Ozark chinquapin (OC), white oak (WO), black oak (BO) and post oak (PO) in northwest Arkansas. Note: * = recorded from root-tips.

Taxon	OC	WO	BO	PO
Albatrellaceae (unidentified species)	*			
Amanita brunnescens G.F. Atk.			*	
Amanita flavoconia G.F. Atk.	*			
Amanita novinupta Tulloss & J. Lindgr.		*		
Amanita orientigemmata Zhu L. Yang & Yoshim.		*		
Amanita rubescens Pers.	*			
Amanita sp. 1			*	
Ascomycota (unidentified species 1)			*	
Ascomycota (unidentified species 2)		*		
Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud		*		
Boletus rubellus Krombh.				*
Byssocorticium sp. 1				*
Cenococcum geophilum Fr.		*		
Chaetothyriales (unidentified species)		*		
Cistella spicicola Huhtinen & Söderh.		*		
Cladosporium cladosporioides (Fresen.) G.A. de Vries	*			
Clavulicium delectabile (H.S. Jacks.) Hjortstam	*			
Clavulina sp. 1	*	*		
Clavulina sp. 2	*			*
Clavulina sp. 3		*		
Cortinarius camptoros Brandrud & Melot	*			
Cortinarius decipiens (Pers.) Fr.	*			
Cortinarius leiocastaneus Niskanen, Liimat. & Soop	*			
Cortinarius malachius (Fr.) Fr.			*	
Cortinarius sp. 1		*		
Craterellus fallax A.H. Sm.	*			
Elaphomycetaceae (unidentified species)				*

Hebeloma brunneifolium Hesler		*		
Hebeloma subconcolor Bruchet	*	*		
Helotiales (unidentified species 1)	*	*	*	*
Helotiales (unidentified species 2)				*
Helvellosebacina helvelloides (Schwein.) Oberw., Garnica & K. Riess		*		
Helvellosebacina sp. 1		*		
Hyaloscyphaceae (unidentified species 1)	*			
Hydnum repandum L.		*		
Hygrophorus russula (Schaeff.) Kauffman		*		
Inocybe cicatricata Ellis & Everh.			*	
Inocybe fuscidula Velen.		*		
Inocybe lanatodisca Kauffman		*		
Inocybe sp. 1		*		
Inocybe sp. 2		*		
Laccaria laccata (Scop.) Cooke			*	
Lactarius argillaceifolius Hesler & A.H. Sm.		*		
Lactarius atroviridis Peck	*			
Lactarius camphoratus (Bull.) Fr.	*			
Lactarius chrysorrheus Fr.			*	
Lactarius deliciosus (L.) Gray			*	
Lactarius evosmus Kühner & Romagn.	*			
Lactarius hygrophoroides Berk. & M.A. Curtis		*		
Lactarius yazooensis Hesler & A.H. Sm.	*	*		*
Lactifluus flammans (Verbeken) Verbeken		*		
Membranomyces spurius (Bourdot) Jülich	*	*		*
Oidiodendron maius G.L. Barron		*		
Oidiodendron sp. 1	*		*	
Peziza sp.1				*
Pezizomycotina (unidentified species 1)				*
Piloderma lanatum (Jülich) J. Erikss. & Hjortstam				*
Piloderma sp. 1				*

Piloderma sp. 2			*	
Pleosporales (unidentified species 1)				*
Russula amoenolens Romagn.	*		*	*
Russula cerolens Shaffer		*		
Russula chameleontina (Lasch) Fr.		*		
Russula chloroides (Krombh.) Bres.	*	*		
Russula compacta Frost		*		
Russula decipiens (Singer) Kühner & Romagn.	*			
Russula decolorans (Fr.) Fr.			*	
Russula pectinatoides Peck	*	*		*
Russula pulverulenta Peck		*		
Russula quercilicis Sarnari		*		
Russula raoultii Quél.		*		
Russula sp. 1	*	*		
Russula sp. 2	*			
Russula sp. 3			*	
Russula sp. 4		*		
Russula sp. 5			*	
Russula sp, 6			*	
Russula subemetica Schulzer	*			
Russula subtilis Burl.		*		
Russulaceae (unidentified species 1)	*		*	
Russulaceae (unidentified species 2)			*	
Russulaceae (unidentified species 3)			*	
Sebacina epigaea (Berk. & Broome) Bourdot & Galzin				*
Sebacina sp. 1		*		*
Sebacina sp. 2		*		*
Sebacina sp. 3		*		
Sebacinaceae (unidentified species 1)	*			
Sebacinaceae (unidentified species 2)		*		*
Sistotrema alboluteum (Bourdot & Galzin) Bondartsev & Singer		*		

Sistotrema sp. 1		*		
Thelephoraceae (unidentified species 1)	*			
Thelephoraceae (unidentified species 2)		*		
Thelephoraceae (unidentified species 3)		*		
Thelephoraceae (unidentified species 4)		*	*	*
Tomentella lateritia Pat.		*		
Tomentella sp. 1	*	*	*	*
Tomentella sp. 2			*	*
Tomentella sp. 3		*	*	
Tomentella sp. 4		*		
Tomentella stuposa (Link) Stalpers		*		
Trichoderma hamatum (Bonord.) Bainier		*		
Tricholoma caligatum (Viv.) Ricken	*			
Tuber sp. 1				*
Uncultured ectomycorrhizal fungus (*Clavulina*)		*		
Uncultured fungus (Clavulinaceae)			*	
Uncultured member of the Pezizales (Helotiales)		*		
Uncultured member of the Pyronemataceae		*		

relatively low degree of overlap would seem to suggest that the assemblage of fungi (particularly ectomycorrhizal fungi) associated with Ozark chinquapin is rather distinctive.

As a general observation, the ectomycorrhizal fungi associated with Ozark chinquapin contains representatives of several of the more widely distributed and common genera found in temperate forests of the Northern Hemisphere. These include *Amanita*, *Cortinarius*, *Lactarius* and *Russula* (Binion et al. 2008, Stephenson 2010). However, the predominance of members of the genus *Russula* is noteworthy. Several of the species recorded (e.g., *Russula amoenolems* and *R. chloroides*) are not particularly common, and it seems likely that this is the first time they have been reported from the state of Arkansas and perhaps from the South Central United States.

Future Studies

As already noted, additional root-tips collected from American chestnut remain to be processed in the laboratory. Moreover, samples obtained from European chestnut (*Castanea sativa* Mill.) during the summer of 2015 also have yet to be processed, and there has as yet been no opportunity to collect any root-tip samples from Allegheny chinquapin (*Castanea pumila* Mill.).

Literature Cited

Binion, D. E., S. L. Stephenson, W. C. Roody, H. H. Burdsall, O. K. Miller, Jr., and L. N. Vasilyeva. 2008. Macrofungi Associated with Oaks of Eastern North America. West Virginia University Press, Morgantown, West Virginia. 476 pp.

Paillet, F. L., and K. C. Cerny. 2012. Reconstructing the development of two Ozark chinquapin (*Castanea ozarkensis*) stands in the pre-blight forests of northwest Arkansas. Journal of the Torrey Botanical Society 139:211-225.

Stephenson, S. L. 2010. The Kingdom Fungi: The Biology of Mushrooms, Molds, and Lichens. Timber Press, Portland, Oregon. 272 pp.

NOTE: Some of the Ozark chinquapin root-tip data were presented at the annual meeting of the Arkansas Academy of Science, held at the University of Arkansas (Fayetteville, Arkansas) on April 1-2, 2016.

Apulu, N. J., M. Ben Hassine Ben Ali, S. L. Stephenson, and B. G. Kluthe. 2016. Morphological diversity of ectomycorrhizae associated with white oak, black oak and chinquapin in Northwest Arkansas. Proceedings of the Arkansas Academy of Science.