Mycorrhiza (2011) 21:601–612
DOI 10.1007/s00572-011-0368-0

ORIGINAL PAPER

Ectomycorrhizal fungal diversity in orchards of cultivated pecan (*Carya illinoinensis*; Juglandaceae)

Gregory Bonito · Timothy Brenneman · Rytas Vilgalys

Received: 1 December 2010 / Accepted: 17 February 2011 / Published online: 3 March 2011

Abstract *Carya illinoinensis* (pecan) belongs to the Juglandaceae (walnut family) and is a major economic nut crop in the southern USA. Although evidence suggests that some species in the Juglandaceae are ectomycorrhizal, investigations on their ectomycorrhizal fungal symbionts are quite limited. Here we assessed the ectomycorrhizal fungal diversity in cultivated orchards of *C. illinoinensis*. Five pecan orchards in southern Georgia, USA, were studied, three of which were known to fruit the native edible truffle species *Tuber lyonii*. We sequenced rDNA from single ectomycorrhizal root tips sampled from a total of 50 individual trees. Mycorrhizae were identified by ITS and LSU rDNA sequence-based methods. Forty-four distinct ectomycorrhizal taxa were detected. Sequestrate taxa including *Tuber* and *Scleroderma* were particularly abundant. The two most abundant sequence types belonged to *T. lyonii* (17%) and an undescribed *Tuber* species (~20%). Because of our interest in the ecology of *T. lyonii*, we also conducted greenhouse studies to determine whether this species would colonize and form ectomycorrhizae on roots of pecan, oak, or pine species endemic to the region. *T. lyonii* ectomycorrhizae were formed on pecan and oak seedlings, but not pine, when these were inoculated with spores. That oak and pecan seedling roots were receptive to truffle spores indicates that spore slurry inoculation could be a suitable method for commercial use and that, ecologically, *T. lyonii* may function as a pioneer ectomycorrhizal species for these hosts.

Keywords Truffles · *Tuber lyonii* · *Carya illinoinensis* · Ectomycorrhizal fungi · Pecan orchards · Spore inoculations

Electronic supplementary material The online version of this article (doi:10.1007/s00572-011-0368-0) contains supplementary material, which is available to authorized users.

G. Bonito (✉) · R. Vilgalys
Biology Department, Duke University,
Durham, NC 27708, USA
e-mail: Gregory.Bonito@duke.edu

T. Brenneman
Department of Plant Pathology, University of Georgia,
Tifton, GA 31794, USA

Introduction

Carya illinoinensis (pecan) belongs to the Juglandaceae and is one of a few agronomically important nut species native to North America. Pecan cultivation is particularly important in the southern USA. In 2007, pecan production in the USA was valued at over $433 million (USDA 2008). Endemic to the Mississippi basin, *C. illinoinensis* is now cultivated globally (Ruan et al. 1992; Wakeling et al. 2001).

The Juglandaceae (walnut family) is a monophyletic lineage within the Fagales and is comprised of nine genera and 60 species (Li et al. 2004; Manos et al. 2007). Species within the Juglandaceae are generally considered to be ectomycorrhizal (Harley and Harley 1987; Brundrett et al. 1990; Wang and Qiu 2006), aside from *Juglans nigra*, which is reported to form only arbuscular mycorrhizae (Dixon 1988; Bainard et al. 2011). Few studies on the ectomycorrhizae of Juglandaceae (Woodroof 1933; Haug et al. 1991; Rivero et al. 2009) exist in comparison with those of other families in the order such as the Fagaceae and Betulaceae (Durall et al. 2006; Ishida et al. 2007; Twieg et al. 2007).

The first study of pecan mycorrhizae was prompted by the emergence of "Rosette disease," later determined to be caused by zinc deficiency (Woodroof 1933; Alben and

Boggs 1936). Woodroof (1933) documented seven ectomycorrhizal morphotypes on pecan including *Russula* and *Boletus* species. Higher levels of root colonization by ectomycorrhizae were reported to be correlated with fewer symptoms of health decline. We now know that ectomycorrhizal fungi have the capacity to access nutrients in many forms and are functionally important in the mineral nutrition of their hosts (Haselwandter and Bowen 1996; Andersson et al. 1997; Nygren et al. 2008; Treseder et al. 2008). These fungi may also protect against pathogens (Branzanti et al. 1999; Machon et al. 2006). Ectomycorrhizae of *Scleroderma bovista* and *Pisolithus tinctorus* have been formed with *C. illinoinensis* and in some cases appear to improve seedling health (Marx and Bryan 1969; Marx 1979).

In the mid-1980s, a truffle species, *Tuber lyonii* Butters (Trappe) (Fig. 1), was found fruiting in a pecan field in Georgia (Hanlin et al. 1989). Over the following decade, similar truffle species were collected from other pecan orchards across southern Georgia, USA. *T. lyonii* is commonly referred to as the "pecan truffle," because of its association with pecan trees, but this native truffle species also associates and fruits with oaks (Heimsch 1958; Trappe et al. 1996; Jumpponen et al. 2010). *T. lyonii* is generally considered to be an angiosperm-associated ectomycorrhizal fungus species, an ecological characteristic typical of other species in its clade (Rufum clade) (Trappe et al. 1996; Bonito et al. 2010).

Georgia has over 46,000 ha under pecan cultivation and remains one of the top pecan-producing states in the USA (USDA 2009). Although there is no organized effort to collect truffles from pecan fields, truffles are regularly encountered as by-product during the processing of harvested nuts (GB and TB, personal observation). Studies have yet to focus on the ecology of *T. lyonii*, but there is growing interest in whether this truffle species can be cultivated or even co-cropped with pecan or other hosts as a means for boosting rural economies.

The objectives of this study are threefold: (1) to acquire a preliminary assessment of the ectomycorrhizal diversity associated with *C. illinoinensis*, (2) to assess whether *T. lyonii* is present in the ectomycorrhizal fungal community of *C. illinoinensis*, and (3) to determine whether *T. lyonii* ectomycorrhizae can be formed on seedling roots of pecan, oak, or pine species endemic to the region through spore slurry inoculations. We hypothesized that ectomycorrhizal diversity in pecan orchards would be relatively low due to limitations of only a single host plant and regular inputs of biocides and fertilizers in orchards. To address these aims, we sampled roots from multiple pecan orchards and, aided by DNA sequence-based molecular tools, we provide the first regional-scale assessment on the ectomycorrhizal associations of *C. illinoinensis*.

Materials and methods

Site and soil characteristics

Five pecan orchards were chosen as study sites. One site has a 20-year history of consistent *T. lyonii* truffle production (Magnolia+). An adjoining site where truffles had not previously been collected was also sampled (Magnolia−). Two other sites with truffle production in years past but far less production in recent years were sampled (Nilo+, Pine Knoll+). Because considerable research on pecans has been conducted at the UGA Tifton Experimental Station (Ponder-), it was chosen as a reference site. *T. lyonii* has never been reported from this location, which is over 100 km away from the other sites.

Available management records were compiled from each site. These included date established, area planted, varieties planted, spacing between trees, irrigation method, and soil data. These also included soil characters taken from a composite of over ten samples (20–30-cm depth) from which soil texture, classification, pH, and nutrient levels (based on Mehlich I extraction) were determined.

Sampling

During September of 2006, ten pecan trees were randomly sampled within 1-km^2 blocks at five sites (total of 50 trees). Three soil cores were taken from each tree. Since these truffles appear most commonly on the northern side of the trees (TB, personal observation), each tree was sampled approximately 1 m from the tree bole at north, northeast, and northwest (S1). Samples were taken using a 2.5-cm core to a 10-cm depth (NW and NE) and by an auger shovel (N) to ensure that a large cluster of roots was collected from each tree. Samples were placed in polypropylene bags, transported on ice, stored at 4°C, and processed over the following 10 days. Briefly, this entailed soaking the root samples in tap water for 1 h before washing under a gentle stream of tap water over a 1-mm sieve. Cleaned sections of mycorrhizal roots were placed in a Petri dish with tap water and observed with a dissecting microscope. Six random individual mycorrhizal root tips were picked with sterile forceps and each single root tip was placed in its own Eppendorf tube containing 250 μl of CTAB 2× extraction buffer. Digital photos were taken of ectomycorrhizae using a Nikon Coolpix 990 digital camera. Non-mycorrhizal roots were selected as negative controls when no ectomycorrhizae were observed.

Inoculation of pecan seedlings with truffles

To determine whether ectomycorrhizae could be synthesized onto seedlings of *C. illinoinensis* (pecan), *Quercus*

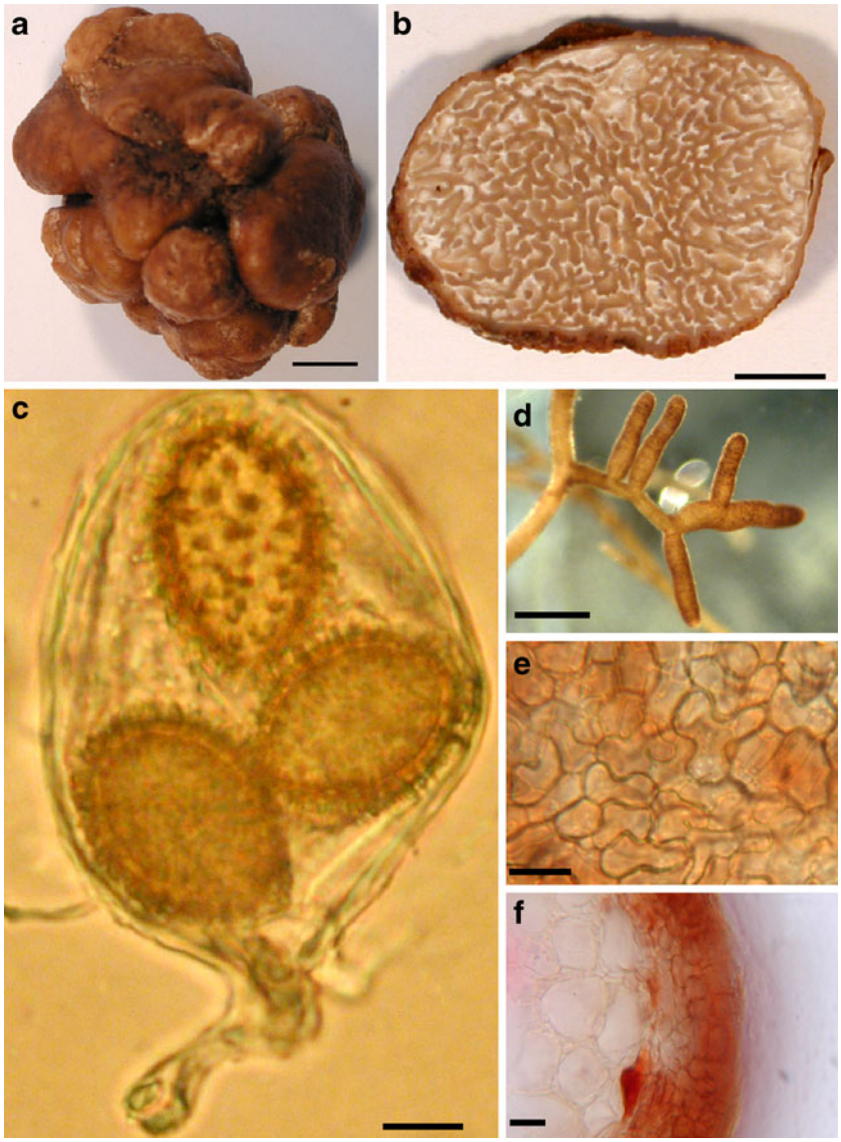

Fig. 1 **a** Lobed outer peridium of a *T. lyonii* fruitbody. **b** Cross-section of a fruitbody showing marbled gleba characteristic of *Tuber*. **c** *T. lyonii* ascus filled with three spores ornamented with spines. **d**–**f**. Morphological–anatomical characteristics of *T. lyonii* ectomycorrhizae synthesized on *C. illinoinensis*. **d** Simple and irregularly pinnate ectomycorrhizal roots. **e** Puzzle-like outer mantle layer with psuedoparenchymatic epidermal cells. **f** Transverse section of ectomycorrhiza showing the outer mantle and inner Hartig net. **a**, **b** *bars*=1 cm; **c** *bar*=10 μm; **d** *bar*=1 mm; **e**, **f** *bars*=10 μm

alba (white oak), and *Pinus taeda* (loblolly pine), seedlings were inoculated with spores of *T. lyonii* in a greenhouse study that included five replicates for each species. *Q. alba* and *P. taeda* were chosen because they occur naturally across this region and are found adjacent to the sampled pecan fields. Acorns were collected from the canopy of *Q. alba* trees in Durham, NC, USA, and were washed and stratified 30 days prior to germination. Pine seeds were obtained through the North Carolina State Cooperative Tree Improvement Program. For stratification, seeds were soaked for 24 h in tap water and were then placed in a zip-lock bag and stored at 4°C for 30 days. A "seed variety" of pecan from North Carolina and "Desirables" from Georgia were collected and stored at 4°C until use. Pecans were soaked in tap water for 10 days prior to germination, with the water changed daily (Adams and Thielges 1978). They were then surface-disinfected in 6% hydrogen peroxide for 10 min and placed in sterile perlite with bottom heat (90°C) for germination. Once the apical meristem emerged, the plants were placed under fluorescent lights for 18 h-days.

Ripe truffles collected from the Magnolia+ orchard in Georgia were used as inoculum after their identification was confirmed by DNA sequence analysis of the ITS rDNA. Voucher collections were deposited in the DUK herbarium (GB26, GB29, GB31, GB108, GB112). The truffles were washed and stored in a freezer until the seedlings were ready for inoculation (approximately 5 months). They were then cleaned again under water and surface-disinfected in a 6% hydrogen peroxide bath for 10 min. After rinsing well in deionized water, they were blended for over 3 min in deionized water amended with crushed ice (to prevent overheating). Trees were inoculated with approximately 1 g of truffle per plant by mixing a

spore slurry (made with 15 g of truffle) into the appropriate volume of sterile soil-less media for planting 15 plants; the soil-less media was composed of peat, vermiculite, and perlite at a ratio of 2:2:1 (Michaels 1982; Hall et al. 2007). Crushed limestone was used as mulch on the top of the potting mixture to keep the soil in place. Seedlings were watered three times/week with a 1:10 Hoagland's solution. After 5 months, the seedlings were checked for mycorrhization. The sub-sections of the whole root system were examined for ectomycorrhiza under the stereoscope, and the fungal sheath and Hartig net of these were observed through a compound microscope. Polymerase chain reaction (PCR) amplification and sequencing were used to verify their identity.

DNA extraction and sequencing

DNA from one of six single mycorrhiza sampled from each core was randomly selected and extracted with 24:1 chloroform–isoamyl alcohol and PCR-amplified using the primer set 5.8SR (forward) and LR3 (reverse) and in a few cases ITS1f and LR5 (Vilgalys and Hester 1990; White et al. 1990). For samples that failed to amplify, a second (or third) sample was extracted until one ectomycorrhizae had been amplified from each core.

For the greenhouse experiment, DNA was extracted from individual ectomycorrhiza (eight per seedling) with the Extract-N-Amp kit (Sigma) following the manufacturer's protocol with the modification that only 20 ul of the extraction and dilution buffer was used per root tip. DNA extracts were also amplified with a *T. lyonii*-specific primer set nested within the ITS rDNA region: (forward primer) T_lyonii_f (GGT CCC TGA ATC CAT CTC CTC A) and (reverse primer) T_lyonii_r (CTA AGT CCA TTG CAG TTG TCA C) using thermocycler conditions that will be described later. To confirm the specificity of the primers, this region was also sequenced for a sub-sample of mycorrhizae.

The PCR protocol employed an initial denaturation at 94°C (3 min), followed by 35 cycles at 94°C (2 min), 50°C annealing (30 s), and a 72°C extension (1.5 min), with a final extension at 72°C (7 min). Each 25 μl PCR reaction consisted of 4.5 μl ddH20, 4 μl dNTPs (1.25 uM), 2.5 μl PCR buffer, 1 μl BSA, 1.25 μl forward primer (10 uM), 1.25 reverse primer (10 uM), 0.15 μl TAQ polymerase (5 U/μl), and 10 μl of DNA extract (~10 ng/μl).

Two microliters of each PCR product was loaded into a 1% agarose gel buffered with TAE buffer and stained with 2 μl SYBR safe (Invitrogen, Carlsbad, CA, SUA) per 80 ml gel. Gel electrophoresis products were viewed on a GelDoc XR imager (BioRad Laboratories, Inc., Hercules, CA, USA). PCR products were cleaned with Qiagen quick-clean columns. One microliter of cleaned PCR product (~20 ng) was used as template in the sequencing reaction. Sanger sequencing was performed in both directions with BigDye Chemistry v3.1 (Applied Biosystems, Foster City, CA, USA) using the forward primer (ITS1) and reverse primer (LR5). DNA sequences were determined on an ABI3700 DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analyses

DNA sequences were manually edited with Sequencher 4.0 (Gene Codes, Ann Arbor, MI, USA) and ambiguous regions at the ends were trimmed. Both the ITS and LSU sequences were queried against the NCBI public database Genbank with the BLASTn algorithm and the top five blast results were recorded. The resulting LSU rDNA sequences were mostly between 510 and 580 bp in length and were aligned to reference taxa manually using MacClade 4.0 (Maddison and Maddison 2002). Ambiguously aligned regions were excluded from the alignment. To determine the phylogenetic affiliations of the recovered fungal sequences, a maximum likelihood analysis based on a GTR +G+I model of nucleotide substitution was conducted on the LSU alignment with PAUP* 4.0b10 (Swofford 2002). To gain a better phylogenetic resolution of the *Tuber* sequences from pecan orchards that did not belong to *T. lyonii*, ITS sequences from these samples and closely related *Tuber* taxa were assessed with maximum likelihood. The sequences produced in this study have been deposited in Genbank with accession numbers GQ379721–GQ379737 and HQ541825–HQ541864.

Statistical analyses

Operational taxonomic units (OTUs) were defined as sequences sharing 99% similarity at the LSU and 96% similarity within the ITS region as calculated by Sequencher 4.0 (Gene Codes, Ann Arbor, MI, USA). The rank abundance of OTUs was determined and plotted. The expected OTU diversity within and across sites was calculated with an incidence-based coverage estimator and the abundance-based species richness estimators Chao1 and ACE by sampling with replacement. Simpson diversity within sites and community dissimilarity between sites were determined in Estimates (Colwell 2005) with incidence-based (Sorensen index) and abundance-based (Bray–Curtis and Chao–Sorensen) indices. To assess the levels of intraspecific variation of *T. lyonii*, haplotype network sequence analyses were constructed under a parsimony framework with the software TCS (Clement et al. 2000).

To assess how edaphic factors were correlated with ectomycorrhizal fungal communities in species space, non-metric multidimensional scaling (NMDS) was implemented

in PC-ord (McCune and Mefford 2006). Ordinations were calculated for both binary and abundance-transformed data.

To determine whether the communities of ectomycorrhizal fungi from pecan orchards were phylogenetically different, we assessed the Unifrac significance for each site, each pair of sites, and all sites combined based on 100 permutations in UniFrac (Lozupone et al. 2006). The Unifrac significance test uses the Unifrac metric, a measure of the branch length unique to one community, for statistical comparisons of the phylogenetic structure between communities in different environments (Lozupone and Knight 2005). These statistics were used to assess whether ectomycorrhizal fungal communities of pecan differ significantly from one another in their phylogenetic composition. An environmental distance matrix based on UniFrac values was determined for all pairs of environments and sites were clustered by phylogenetic similarities. Phylogenetic similarities between communities were calculated under a parsimony framework by using the *P*-test (Martin 2002).

Results

Site and soil characteristics

The general site characteristics for all locations are presented in Table 1. The studied soils were characterized by high levels of fertility, base cations, and phosphorus. The soils ranged in texture from loamy sand to sandy clay loam. The soil pHs ranged from 5.8 to 7.2 between sites and levels of major and minor elements were generally medium to very high, except for the control site (Ponder) which had lower, but still adequate, levels of micronutrients for pecan production (Table 1). This was particularly true of potassium (81 kg/ha versus>251 kg/ha at other sites) and magnesium (46 kg/ha versus>122 kg/ha at other sites).

The cultivar "Desirable" was the most numerous variety of pecan established in sampled orchards, but other varieties including "Cape Fear", "Stuart", "Schley," and "Pawnee" were also sampled (Table 1). Trees ranged in age from 19 to 65 years and were spaced between 9 and 14 m apart within a row and 12–18 m apart between rows. All of the sites studied maintained vegetation-free herbicide strips between tree rows (i.e., under the tree canopy). The Ponder farm was the only site that did not use sprinkler irrigation or have a mixture of grass and clover between rows. Instead, drip irrigation was used at Ponder and rows were separated by grass only (Table 1).

Sequence phylotypes and statistical analyses

A moderate diversity of ectomycorrhizal morphotypes was observed on pecan at each site (Fig. 2), although roots from the Ponder site had <50% colonization by ectomycorrhizal fungi. In total, DNA sequences were generated for 134 of the 150 samples (Table 2). The difference was largely due to poor mycorrhization for some samples (11 of which were from the Ponder site). In total, 44 phylotypes were recovered. Abundance-based species richness estimators place lower bound estimates of 14–62 ectomycorrhizal species within sites and 107–131 species across all sites (S2; Table 2). Ascomycete genera detected in this ectomycorrhizal fungal community survey included *Tuber, Pachyphloeus, Elaphomyces, and Cenococcum* and basidiomycete genera included *Hebeloma, Hymenogaster, Scleroderma, Russula, Inocybe, Thelephora, Xerocomus*, and *Sebacina* (Fig. 3).

Diversity within and across sites was moderate (Table 2). Ponder and Magnolia+ had the highest Shannon diversity index (2.69 and 2.42, respectively), and Nilo had the lowest diversity by all measures (Table 2). The trees at Nilo were older than those at the other sites, and the soil type and texture were also unique compared to those from the other sites in this study. The ectomycorrhizal fungal community at Magnolia− had the highest Sorensen similarity and number of shared OTUs with other sites, particularly with Ponder and Magnolia+ (S4). No significant phylogenetic differences were observed between ectomycorrhizal fungal communities based on UniFrac significance (P=0.640) or *P*-tests (P=0.160). In addition, with the exception of Magnolia+ and Pine Knoll, the sites did not cluster in species space by NMDS ordinations (Fig. 4). Instead, most differences in species space between sites were correlated strongly with soil pH and fertility (particularly to potassium and magnesium).

Sequestrate genera (e.g., *Tuber, Pachyphloeus, Scleroderma, Hymenogaster*) were abundant in the ectomycorrhizal community of fungi sampled in pecan orchards (Fig. 3; S3). The most abundant phylotype belonged to an undescribed species of *Tuber*. We refer to this species as *Tuber* sp.36 hereafter because this is the 36th OTU in the genus *Tuber* that we cannot yet assign to a Latin binomial (Bonito et al. 2010). For *Tuber*, ectomycorrhizae of *T. lyonii* and three undescribed species were detected (Fig. 5). The three undescribed *Tuber* species belong to the Maculatum clade, a group with characteristically small light-colored fruitbodies and alveolate-reticulated (honeycomb-patterned) spores.

T. lyonii was the second most abundant mycorrhizal species sampled and accounted for 17% of the sequences. In total, *T. lyonii* was recovered from 23 samples, 15 trees, and three sites and consisted of ten haplotypes (Fig. 6). By site, *T. lyonii* was detected in nine samples from Magnolia+ and Magnolia− and from five samples from Pine Knoll (Fig. 3). Orchards in which *T. lyonii* was detected had similarly aged trees (30–35 year), soil types (Greenville),

Table 1 Site and soil data for pecan orchards sampled in this study. Nutrient levels are reported in units of kg/ha

Parameter	Magnolia+	Magnolia−	Nilo	Pine knoll	Ponder[a]
Tree					
Cultivar	Desirable and Cape Fear	Desirable and Cape Fear	Desirable, Stuart, and Schley	Desirable and Pawnee	Desirable
Age	35 year	35 year	65 year	30 year	19 year
Spacing	30×60 ft	30×60 ft	47×47 ft	30×60 ft	40×40 ft
Soil					
Type	Greenville	Greenville	Red Bay	Greenville	Tifton
Texture	Sandy clay loam	Sandy clay loam	Loamy sand	Sandy clay loam	Sand
pH	6.4	6.5	6.2	6.7	5.9
P	93	131	100	90	68
K	258	319	458	450	81
Ca	1,177	1,305	1,115	2,111	839
Mg	241	321	122	332	46
Fe	–	–	20	9	–
Cu	1.0	1.4	2.1	1.6	–
Zn	27.8	46.7	11.1	8.9	–

[a] With the exclusion of Ponder, all sites had an herbicide strip, sprinkler irrigation, and a mix of grass and clover between rows. Ponder had drip irrigation and only grass between rows

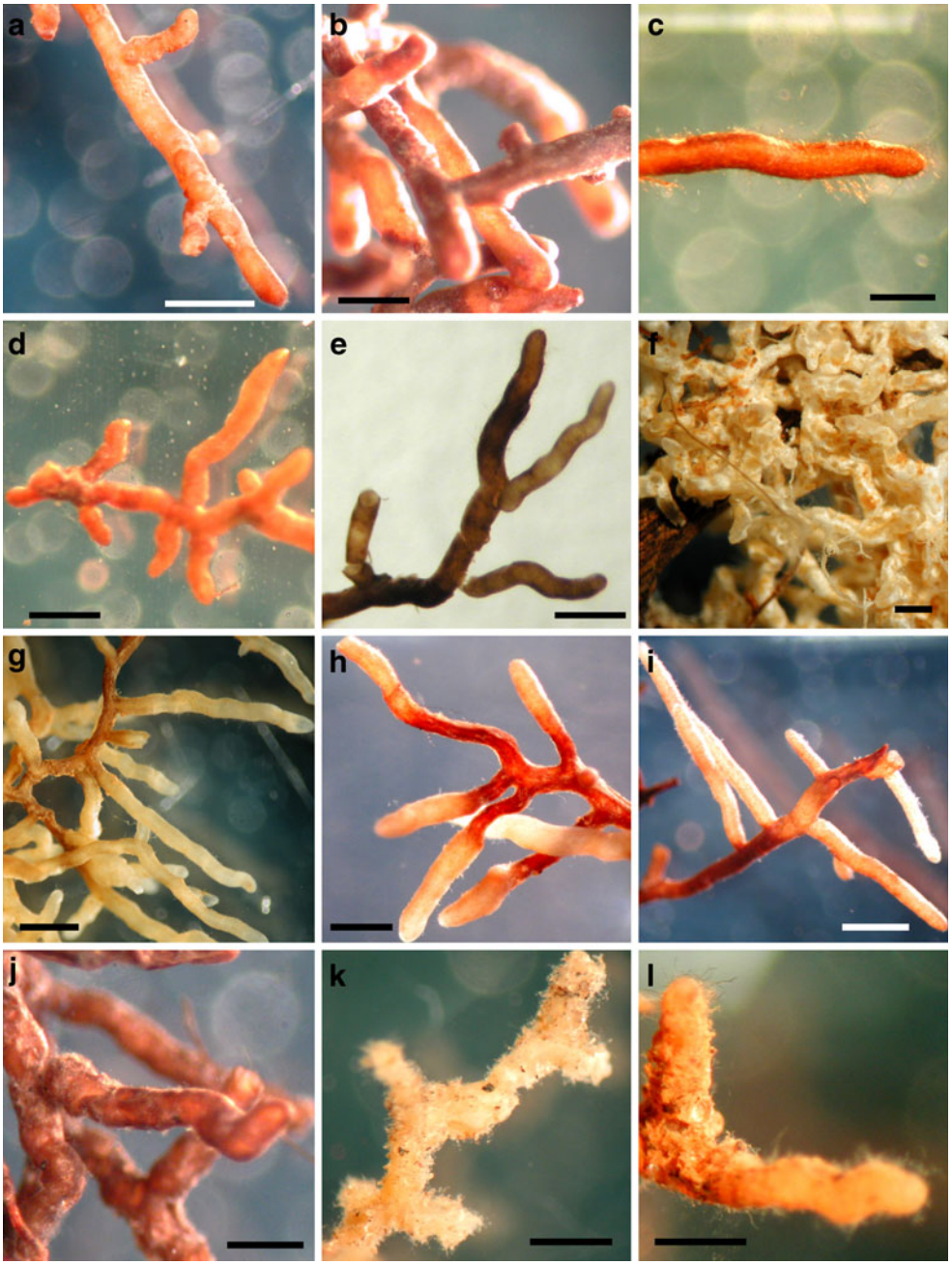

Fig. 2 Morphological diversity of *C. illinoinensis* ectomycorrhizae. **a** Tuberaceae, **b** Tuberaceae, **c** Tricholomataceae, **d** *Tomentella*, **e** Thelephoraceae, **f** *Scleroderma*, **g** Pezizaceae, **h** Pezizaceae, **i** Pezizaceae, **j** *Inocybe*, **k** *Hymenogaster*, **l** Elaphomycetaceae. *Bars*=0.5 mm

soil textures (sandy-loam-clay), pecan variety (Desirable), tree spacing (30′×60′), irrigation (sprinkler), and understory (grass/clover with herbicide strips) (Table 1). We detected the most (six) haplotype diversity at the site that has historically had the highest production of truffles (Magnolia+). The most common haplotype was recovered from all three sites where *T. lyonii* was detected.

T. lyonii ectomycorrhizae were well formed on pecan and oak seedlings 6 months after they had been inoculated with *T. lyonii* spores. Their ectomycorrhiza are characterized by a thin and smooth mantle and well-formed Hartig net (Fig. 1). In contrast, pine seedlings had not formed ectomycorrhizae by 6 or even 9 months post-inoculation (data not shown).

Discussion

Pecan trees associate with moderate ectomycorrhizal fungal diversity

An earlier study of *C. illinoinensis* ectomycorrhizae found that they were present throughout the year and seven morphotypes were distinguished, two of which

Table 2 Ectomycorrhizal community diversity statistics for sampled pecan orchards

Sites	Number of sequences	Number of OTU[a]	Number of terminal taxa[b]	Ace[c]	Shannon index
Magnolia+	28	16	15	27	2.42
Magnolia−	30	13	10	32	2.08
Nilo	29	10	9	13.8	1.63
Pine knoll	28	13	12	31	2.17
Ponder	19	16	13	61.5	2.69
Total[d]	134	44	59	107	3.14

[a] Defined as 96% ITS rDNA sequence similarity

[b] UniFrac environmental counts

[c] Abundance-based coverage estimator

[d] Across all sites

were intimately associated with *Boletus* and *Russula* fruitbodies (Woodroof 1933). We also detected *Boletus* and *Russula* on pecan roots in our study. From 137 sequenced root tips, 44 phylotypes were recovered across five managed tree plantations. For comparison, Parrent et al. (2006) found 72 phylotypes out of 411 sequenced root tips in a *P. taeda*-mixed forest in North Carolina, USA. Smith et al. (2007) screened 9,400 root tips from 94 soil cores and detected 92 ectomycorrhizal species associated with *Quercus douglasii* in an oak–savannah forest in California, USA. Thus, even with our limited sampling, we show that a single host growing in soils of high

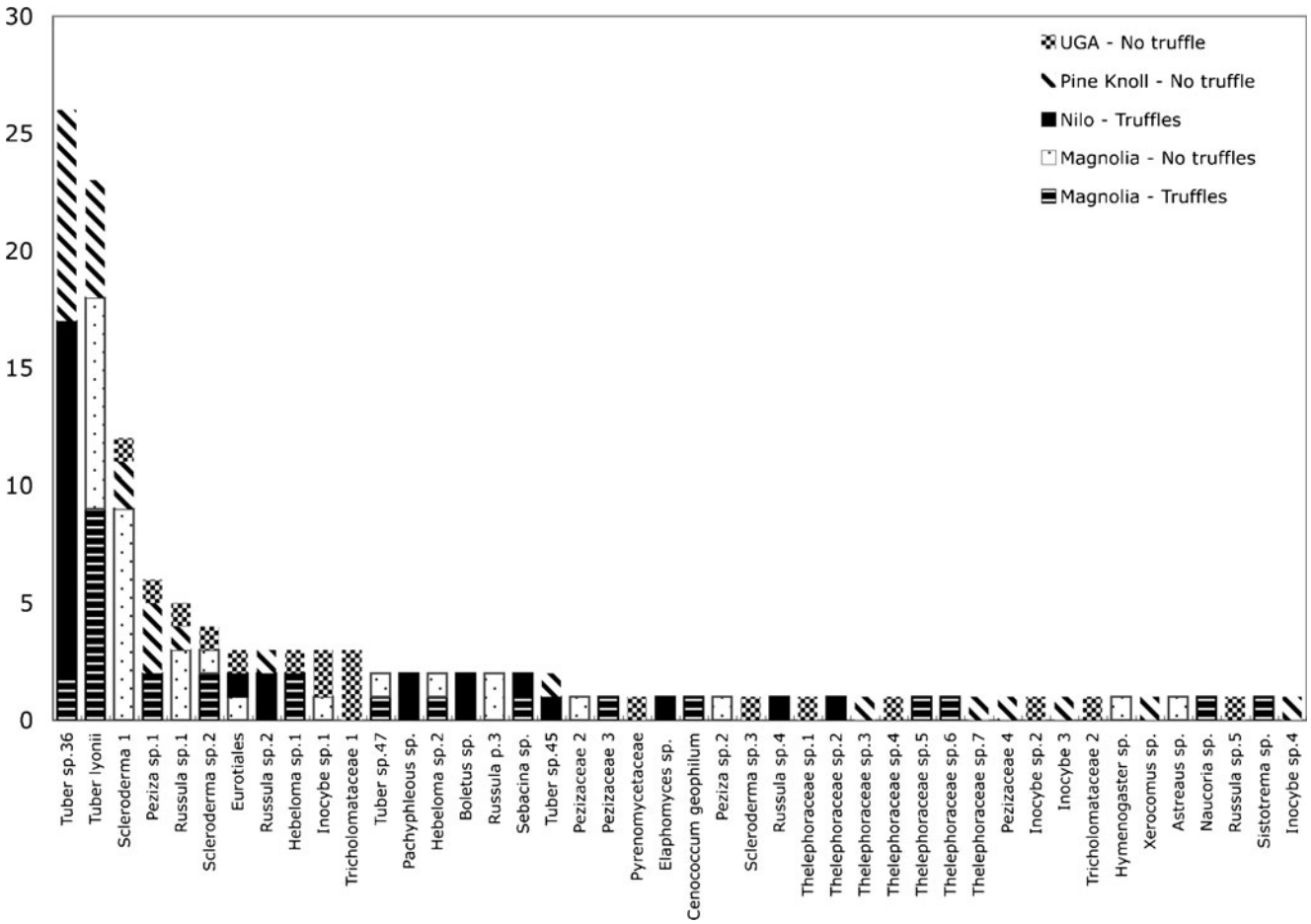

Fig. 3 Rank abundance of ectomycorrhizal taxa detected on *C. illinoinensis*

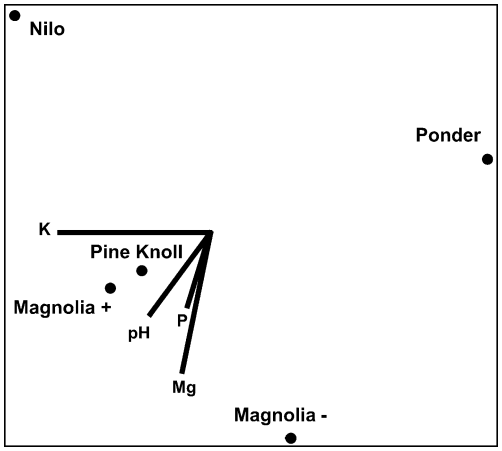

Fig. 4 Non-metric multidimensional scaling ordination of ectomycorrhizal communities of fungi sampled from cultivated pecan fields based on binary data. *Axes 1 and 3* explained 0.801 of the variation in species space. Soil pH, K^{+}, P^{+}, and Mg^{+2} were correlated to these axes

fertility can support moderate levels of ectomycorrhizal diversity.

Although some sites showed higher levels of diversity and similarity, and ectomycorrhizal fungal community composition was correlated with soil nutrient levels, there were no significant differences in the phylogenetic structure of ectomycorrhizal fungal communities recovered from the five sampled pecan orchards. This could be due to selection by the host plant for particular groups of ectomycorrhizal fungi or due to broader-scale patterns in the distribution of ectomycorrhizal fungal diversity. Alternatively, this may be an artifact of limited sampling. Although the two non-truffle-producing sites (Ponder and Magnolia−) had high community similarity values and shared taxa, none of the shared taxa were *Tuber* species. Additional sampling may provide better resolution on the structuring of ectomycorrhizal fungus communities within and across these sites.

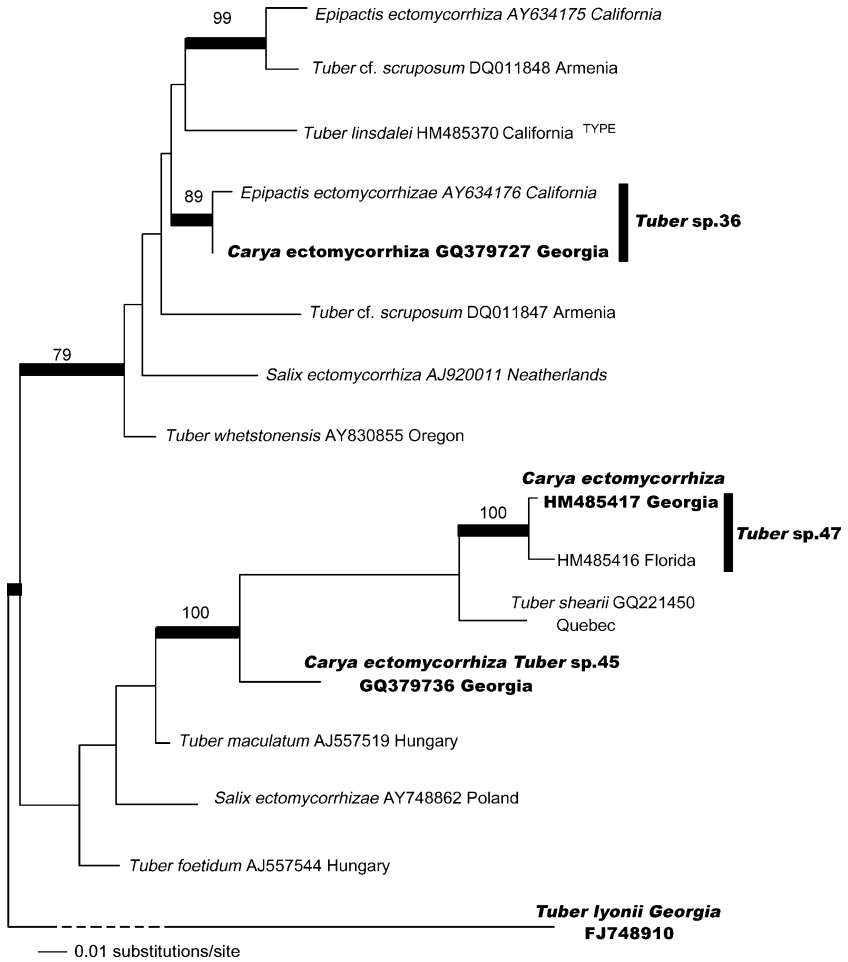

Fig. 5 The most likely ITS phylogeny for the Maculatum clade of *Tuber*. The phylogenetic placement of the three undescribed species of *Tuber* detected in this study is shown in *bold*. The analysis included 16 taxa and 411 characters (82 parsimony-informative) and a GTR +G +I model of nucleotide substitution. *Thickened nodes* indicate nodes supported by high ML bootstrap values based on 1,000 replicates. *T. lyonii* of the Rufum clade represents an outgroup

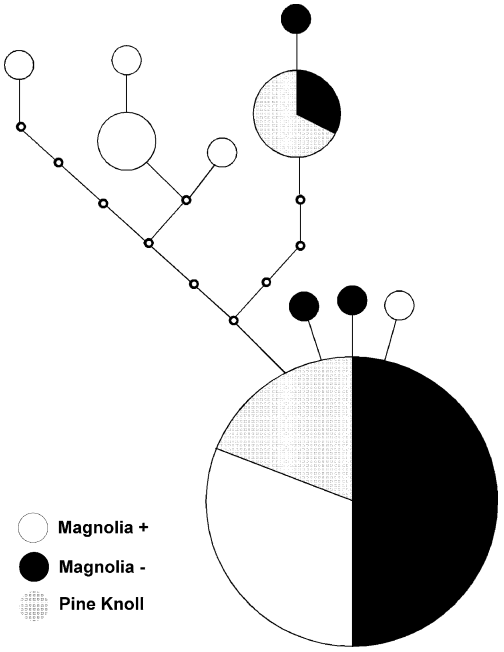

Fig. 6 Haplotype network of *T. lyonii* ectomycorrhizae from cultivated pecan fields based on 800 bp of ITS and 28 S rDNA. A total of ten haplotypes of *T. lyonii* were detected. The size of the circles is proportional to the abundance of each haplotype and each node represents one nucleotide substitution. The distribution of haplotypes among sampled pecan orchards is shown with *shading*

Tuber was abundant belowground in pecan orchards

The two most abundant ectomycorrhizal fungal species associated with pecan belonged to the genus *Tuber*. *Tuber* ectomycorrhizae were sequenced from all sites except Ponder. In particular, *T. lyonii* ranked as the second most abundant taxa recovered and was even detected in orchards where truffles have not been collected. Other studies have also shown that the presence of *Tuber* ectomycorrhizae is not always correlated with truffle production levels (Baciarelli-Falini et al. 2006).

Interestingly, the site distinguished by the highest level of *T. lyonii* productivity (Magnolia+) also had the highest level of *T. lyonii* rDNA haplotype diversity. The high level of haplotype diversity for *T. lyonii* between and within sites and the fact that dominant haplotypes are shared between sites indicate that this species is dispersing across and between surrounding habitats. *T. lyonii* has also been found as a dominant member on the roots of *Quercus* spp. (Jumpponen et al. 2010).

Pioneer ectomycorrhizal fungal species often exhibit the ecological trait of having high spore germination and infectivity on seedling roots (Ishida et al. 2008). Our success in inoculating pecan and oak seedlings using a slurry of *T. lyonii* spores suggests that *T. lyonii* is adapted as a pioneer ectomycorrhizal species and opens up commercial possibilities concerning its cultivation. We are aware of at least five instances where *T. lyonii* ectomycorrhizae or fruitbodies have been found within truffle orchards in eastern North America established with *Corylus* (hazelnut) and oak seedlings inoculated with the European black truffle species *Tuber melanosporum*. *T. lyonii* appears to respond favorably to the elevated soil pH (~7.9) and management regimes recommended for *T. melanosporum* cultivation (Hall et al. 2007). Similar to other *Tuber* species, *T. lyonii* appears to be adapted to savannah-like habitats characterized by low tree densities (Smith et al. 2007). However, the most prolific fruiting of *T. lyonii* observed in pecan orchards has been in dense plantings of younger trees (TB, personal observation). A standard production practice for pecans is to reduce the tree numbers as they grow, which appears to reduce truffle fruiting but increase pecan yields. We are interested in management approaches that could be applicable for both truffle and pecan production.

We detected four species of *Tuber* in this study, revealing a higher diversity of *Tuber* in Georgia, USA, than was previously recognized. Three of these *Tuber* taxa are unnamed species (*Tuber* sp 36, *Tuber* sp.45, *Tuber* sp.47; see Fig. 5). *Tuber* sp.36 ranked as the most abundant taxon recovered in our study. Although this truffle species has been detected in other published studies (Bidartondo et al. 2004; Pruett et al. 2008; Jumpponen et al. 2010), as far as we know this species is only known by DNA sequences from soils and roots. Phylogenetically, this species belongs to the Maculatum clade, a group of smaller, light-colored, and taxonomically challenging truffle species.

Truffle production may respond to pecan orchard management

Currently, pecan orchards are managed solely for the production of pecans, but it may be possible to manage them in a way that is conducive to both truffle and pecan production. *T. lyonii* fruits annually in many pecan orchards in the southeastern USA. However, truffles are not easy to locate and there is little organized attempt to harvest truffles from these orchards. Reliable estimates of truffle production and yield are therefore not possible at this time. *T. lyonii* has been used commercially in several restaurants with great success, but markets are not well developed due to a lack of consistent supply. One possibility for getting more reliable estimates of truffle occurrence and production levels would be to monitor pecan orchards with dogs trained in finding truffles.

Sequestrate taxa in the genera *Tuber* and *Scleroderma* were particularly abundant in the mycorrhizal fungal communities of pecan orchards (Fig. 3; S3). It may be that the orchard environment, with wide spacing between trees, or the management regime of high fertilizer inputs and wet/

dry cycles due to irrigation is selective for fungi with this growth form. Alternatively, it could be that the below-ground community is responding to the biocide applications (Trappe et al. 1984). Studies have shown that treatments of a nematocide (1,2-dibromo-3-chloropropane) and fungicides (e.g., Captan, Demosan, Dexon, Terrazole) can elevate the levels of ectomycorrhizae on pecan roots and stimulate the fruiting of the false-truffle *S. bovista* in pecan orchards (Powell et al. 1968). Although *S. bovista* mycorrhizae are thought to increase the absorbing capacity of the root system and act as a biological deterrent of root pathogens through antibiotic production (Marx and Bryan 1969), this basidiomycete is a natural competitor of *Tuber* (Zambonelli and Iotti 2001). The effects of fungicides on most species of ectomycorrhizal fungi are not well understood.

The sites that currently or previously produced truffles (*T. lyonii*) had soil nutrient levels that were uniformly very high. This was particularly true of calcium, which was present in these soils at levels much higher than is needed for pecan production. This is certainly the result of frequent applications of lime to maintain the pH of these naturally acidic soils at levels favorable for pecan production (Sparks 1976). Soil from the reference site (Ponder-) was sandier in texture and had lower levels of calcium and other nutrients. In the course of this study, we noted several pecan orchards with elevated levels of soil nutrients that do not appear to produce truffles. Previous observations indicate that *T. lyonii* fruitbodies are more common on more heavily textured soils (Tim Brenneman, personal observation). Consistent with this, we found no *T. lyonii* in the two sites with low amounts of clay. Further studies are needed to determine the influence of soil texture on the growth of *T. lyonii*.

T. lyonii has a wide distribution and occurs from Quebec, Canada, south to Monterrey, Mexico, and as far west as New Mexico (Trappe et al. 1996). It is unknown how common or widespread the *T. lyonii*–pecan phenomenon is, but there are reports of this truffle species being collected from pecan orchards in Missouri and New Mexico (Trappe et al. 1996; Bruhn 2007). Currently, attempts are being made to establish an industry in North America based on cultivation of the European black truffle *T. melanosporum*. However, the native truffle *T. lyonii* may be a promising alternative truffle species to crop on this continent given its broad native range and the receptivity of oak and pecan seedlings to spore slurry inoculation. The spore slurry method for inoculating seedlings with *T. lyonii* may be viable for commercial use. The fact that *T. lyonii* ectomycorrhizae were not formed on *P. taeda* in our inoculation trials is consistent with our understanding of *T. lyonii*'s preference for angiosperm hosts based on comments by collectors who associate this truffle species with *Quercus*, *Carya*, *Corylus*, and *Tilia* (Trappe et al. 1996; Bruhn 2007). A coupling of truffle and pecan production could improve the economic viability of both industries, yet further research is needed on specific strategies for managing pecan orchards for both truffles and pecans.

Acknowledgements The authors thank the willingness and cooperation of the Sunshine, Magnolia, Ducker, Nilo pecan farms to participate in this study. We thank Bernadette O'Reilley, Allen Lowrance for field assistance, and Gonzalo Guevara, Alessandra Zambonelli, Rosanne Healy, Johann Bruhn for herbarium material. Thanks to Connie Robertson for curating truffle collections for the DUK herbarium. Many thanks to Charles Lefevre for instigating this study and Bill Bunn for providing pecans. The manuscript was improved by comments provided by three anonymous reviewers. This study was funded through an NSF award and DBI-0710213 to RV and GB. The authors declare that they have no conflicts of interest.

References

Adams JC, Thielges BA (1978) Seed treatment for optimum pecan germination. Tree Plant Notes 35:13–14

Alben AO, Boggs HM (1936) Zinc content of soils in relation to pecan rosette. Soil Sci 41:329–332

Andersson S, Ek H, Soderstrom B (1997) Effects of liming on the uptake of organic and inorganic nitrogen by mycorrhizal (*Paxillus involutus*) and non-mycorrhizal *Pinus sylvestris* plants. New Phytol 135:763–771

Baciarelli-Falini L, Rubini A, Riccioni C, Paolocci F (2006) Morphological and molecular analyses of ectomycorrhizal diversity in a man-made *T. melanosporum* plantation: description of novel truffle-like morphotypes. Mycorrhiza 16:475–484

Bainard LD, Klironomos JN, Gordon AM (2011) The mycorrhizal status and colonization of 26 tree species growing in urban and rural environments. Mycorrhiza 21:91–96

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. P Roy Soc Lond B Bio 271:1799–1806

Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R (2010) A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol Ecol 19:4994–5008

Branzanti MB, Rocca E, Pisi A (1999) Effect of ectomycorrhizal fungi on chestnut ink disease. Mycorrhiza 9:103–109

Bruhn JN (2007) How widespread is the pecan truffle (Texas truffle)? Pecan Grower 18:50–51

Brundrett M, Murase G, Kendrick B (1990) Comparative anatomy of roots and mycorrhizae of common Ontario trees. Can J Bot 68:551–578

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659

Colwell RK (2005) EstimateS: statistical estimation of species richness and shared species from samples, version 7.5. User's guide and application published at: http://purl.oclc.org/estimates

Dixon RK (1988) Seed source and vesicular–arbuscular mycorrhizal symbiont affects growth of *Juglans nigra* seedlings. New For 2:203–211

Durall DM, Gamiet S, Simard SW, Kudrna L, Sakakibara SM (2006) Effects of clearcut logging and tree species composition on the diversity and community composition of epigeous fruit bodies formed by ectomycorrhizal fungi. Can J Bot 84:966–980

Hall IR, Brown GT, Zambonelli A (2007) Taming the truffle—the history, lore and science of the ultimate mushroom. Timber, Portland

Hanlin RT, Wu M, Brenneman TB (1989) The occurrence of *Tuber texense* in Georgia. Mycotaxon 34:387–394

Harley JL, Harley EL (1987) A checklist of mycorrhiza in the British flora—addenda, errata and index. New Phytol 107:741–749

Haselwandter K, Bowen GD (1996) Mycorrhizal relations in trees for agroforestry and land rehabilitation. For Ecol Manag 81:1–17

Haug I, Weber R, Oberwinkler F, Tschen J (1991) Tuberculate mycorrhizas of *Castanopsis borneensis* King and *Engelhardtia roxburghiana* Wall. New Phytol 117:25–35

Heimsch C (1958) The first recorded truffle from Texas. Mycologia 50:657–660

Ishida TA, Nara K, Hogetsu T (2007) Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. New Phytol 174:430–440

Ishida TA, Nara K, Tanaka M, Kinoshita A, Hogetsu T (2008) Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession. New Phytol 180:491–500

Jumpponen A, Jones KL, Mattox D, Yaege C (2010) Massively parallel 454-sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. Mol Ecol 19:41–53

Li RQ, Chen ZD, Lu AM, Soltis DE, Soltis PS, Manos PS (2004) Phylogenetic relationships in Fagales based on DNA sequences from three genomes. Int J Plant Sci 165:311–324

Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–8235

Lozupone C, Hamady M, Knight R (2006) UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 7:371

Machon P, Santamaria O, Pajares JA, Alves-Santos FM, Diez JJ (2006) Influence of the ectomycorrhizal fungus *Laccaria laccata* on pre-emergence, post-emergence and late damping-off by *Fusarium moniliforme* and *F. oxysporum* on Scots pine seedlings. Symbiosis 42:153–160

Maddison D, Maddison W (2002) MacClade: analysis of phylogeny and character evolution, 40th edn. Sinauer, Sunderland

Manos PS et al (2007) Phylogeny of extant and fossil Juglandaceae inferred from the integration of molecular and morphological data sets. Syst Biol 56:412–430

Martin AP (2002) Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol 68:3673–3682

Marx D (1979) Synthesis of *Pisolithus* ectomycorrhizae on pecan seedlings in fumigated soil. In: USDA FSRNS- (ed), pp 1–4

Marx DH, Bryan WC (1969) *Scleroderma bovista*, an ectotrophic mycorrhizal fungus of pecan. Phytopathology 59:1128–1132

McCune B, Mefford MJ (2006) PC-ORD. Multivariate analysis of ecological data, version 5. MjM Software, Gleneden Beach

Michaels T (1982) In vitro culture and growth modeling of *Tuber* spp. and inoculation of hardwoods with *T. melanosporum* ascospores. In: Botany and plant pathology. Oregon State University, Corvallis, p 117

Nygren CMR, Eberhardt U, Karlsson M, Parrent JL, Lindahl BD, Taylor AFS (2008) Growth on nitrate and occurrence of nitrate reductase-encoding genes in a phylogenetically diverse range of ectomycorrhizal fungi. New Phytol 180:875–889

Parrent JL, Morris WF, Vilgalys R (2006) CO_2-enrichment and nutrient availability alter ectomycorrhizal fungal communities. Ecology 87:2278–2287

Powell WM, Hendrix FF, Marx DH (1968) Chemical control of feeder root necrosis of pecans caused by *Pythium* species and nematodes. Plant Dis Rep 52:577–578

Pruett G, Bruhn J, Mihail J (2008) Temporal dynamics of ectomycorrhizal community composition on root systems of oak seedlings infected with Burgundy truffle. Mycol Res 112:1344–1354

Rivero SHT, Moorillon VGN, Borunda EO (2009) Growth, yield, and nutrient status of pecans fertilized with biosolids and inoculated with rizosphere fungi. Bioresour Technol 100:1992–1998

Ruan YL, Wood BW, Payne JA (1992) Chinese hickory—an emerging nut–tree crop. Fruit Var J 46:16–22

Smith ME, Douhan GW, Rizzo DM (2007) Ectomycorrhizal community structure in a xeric *Quercus* woodland based on rDNA sequence analysis of sporocarps and pooled roots. New Phytol 174:847–863

Sparks D (1976) Soil pH and the pecan. 67th annual report of the Northern Nut Growers Association, pp 93–99

Swofford DL (2002) PAUP* phylogenetic analysis using parsimony (*and other methods), 40th edn. Sinauer, Sunderland

Trappe JM, Molina R, Castellano M (1984) Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Annu Rev Phytopathol 22:331–359

Trappe JM, Jumpponen AM, Cázares E (1996) NATS truffle and truffle-like fungi. 5. *Tuber lyonii* (= *T. texense*), with a key to the spiny-spored *Tuber* species groups. Mycotaxon 60:365–372

Treseder KK, Czimczik CI, Trumbore SE, Allison SD (2008) Uptake of an amino acid by ectomycorrhizal fungi in a boreal forest. Soil Biol Biochem 40:1964–1966

Twieg BD, Durall DM, Simard SW (2007) Ectomycorrhizal fungal succession in mixed temperate forests. New Phytol 176:437–447

USDA (2008) National Agricultural Statistics Service. Noncitrus fruits and nuts: Fr Nt 1-3 (09). United States Department of Agriculture. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1

USDA (2009) 2007 census of agriculture, Georgia. State and county data. United States Department of Agriculture. http://www.agcensus.usda.gov/Publications/2007/Full_Report/Census_by_State/Georgia/index.asp

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

Wakeling LT, Mason RL, D'Arcy BR, Caffin NA (2001) Composition of pecan cultivars Wichita and Western Schley [*Carya illinoinensis* (Wangenh.) K. Koh] grown in Australia. J Agric Food Chem 49:1277–1281

Wang B, Qiu YL (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16:299–363

White TM, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–321

Woodroof N (1933) Pecan mycorrhizae. Ga Exp Stat Bull 178:1–26

Zambonelli A, Iotti M (2001) Effects of fungicides on *Tuber borchii* and *Hebeloma sinapizans* ectomycorrhizas. Mycol Res 105:611–614