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TUBERCULATE ECTOMYCORRHIZAE OF ANGIOSPERMS: THE INTERACTION BETWEEN *BOLETUS RUBROPUNCTUS* (BOLETACEAE) AND *QUERCUS* SPECIES (FAGACEAE) IN THE UNITED STATES AND MEXICO[1]

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Tuberculate ectomycorrhizae (TECM) are unique structures in which aggregates of ectomycorrhizal roots are encased in a covering of fungal hyphae. The function of TECM is unknown, but they probably enhance the nitrogen nutrition and disease resistance of host plants. Trees in the Pinaceae form TECM with species of *Rhizopogon* and *Suillus* (Suillineae, Boletales). Similar tubercules are found with diverse angiosperms, but their mycobionts have not been phylogenetically characterized. We collected TECM in Mexico and the USA that were similar to TECM in previous reports. We describe these TECM and identify both the plant and fungal symbionts. Plant DNA confirms that TECM hosts are *Quercus* species. ITS sequences from tubercules and sclerotia (hyphal aggregations that serve as survival structures) matched sporocarps of *Boletus rubropunctus*. Phylogenetic analyses confirm that this fungus belongs to the suborder Boletineae (Boletales). This is the first published report of TECM formation in the Boletineae and of sclerotia formation by a *Boletus* species. Our data suggest that the TECM morphology is an adaptive feature that has evolved separately in two suborders of Boletales (Suillineae and Boletineae) and that TECM formation is controlled by the mycobiont because TECM are found on distantly related angiosperm and gymnosperm host plants.

Key words: Boletales; ectomycorrhizal fungi; *Quercus*; rhizomorph; sclerotia; soil ecology; symbiosis; tuberculate ectomycorrhizae.

The ectomycorrhizal symbiosis (ECM) represents a phylogenetically widespread interaction between diverse vascular plants and fungi from the Basidiomycota, Ascomycota, and Zygomycota (Endogonales) (Agerer, 2006; Tedersoo et al., 2006; Smith and Read, 2008). Ectomycorrhizae function as a mutualism whereby plants provide photosynthate to fungi, while fungi may enhance drought tolerance, disease resistance, and nutrient uptake to plants (Trappe and Strand, 1969; Zak, 1971; Duddridge et al., 1980; Smith and Read, 2008). Despite the polyphyletic nature of both plant and fungal symbionts in the ECM symbiosis, the overall morphology of ECM roots is similar in most host–fungus combinations (Hibbett et al., 2000; Agerer, 2006; Tedersoo et al., 2006; Wang and Qiu, 2006). The basic morphology of an ECM root consists of three common features (Smith and Read, 2008): (1) a mantle of fungal hyphae that ensheathes the plant root tip, (2) a labyrinthine matrix of fungal hyphae, the Hartig net, that grows between epidermal and cortical cells in the root, and (3) a network of extraradical fungal hyphae that grows into the surrounding soil and leaf litter.

One unusual deviation from the basic ECM morphology is the tuberculate ectomycorrhiza (TECM). TECM are dense aggregates of ECM root tips enclosed in a "peridium-like sheath" or "rind" of hyphae (Melin, 1923; Trappe, 1965). The fungal covering forms a barrier that prevents the ECM roots from directly contacting the surrounding leaf litter and soil. Although TECM have sometimes been referred to as coralloid or compound ectomycorrhizae (e.g., Masui, 1926; LePage et al., 1997), we use the term tuberculate ectomycorrhiza (TECM) as defined by Trappe (1965).

The morphology and ecology of TECM have been studied extensively in Pinaceae (*Pseudotsuga* and *Pinus* spp.), where tubercules are formed exclusively by mycobiont species of *Rhizopogon* and *Suillus* (Suillineae, Boletales) (Melin, 1923; Trappe, 1965; Zak, 1971, 1973; Keller, 1992; Li et al., 1992; Massicotte et al., 1992; Olsson et al., 2000; Paul et al., 2007). Studies have focused on the Pinaceae–Suillineae TECM because they are widespread and abundant in the northern hemisphere (Hirose et al., 2004; Kretzer et al., 2004; Paul et al., 2007) and because most *Suillus* and *Rhizopogon* species can be cultured on artificial media and easily studied in the laboratory and greenhouse (Melin, 1923; Zak, 1971; Keller, 1992). The exact function of TECM is not yet known, but presumably the morphology of the TECM offers a functional advantage over the normal ECM root morphology. In particular, TECM may enhance the nutritional status of host plants by harboring nitrogen-fixing bacteria (Paul et al., 2007), provide physical or chemical protection from plant pathogens or root-feeding insects (Zak, 1965, 1971), or act as a site for fungal nutrient storage (Göbl, 1967).

Although the Pinaceae–Suillineae TECM interactions have received extensive attention, TECM have also been reported from a variety of angiosperms: *Eucalyptus* (Myrtaceae) from Australia (Dell et al., 1990), *Photinia* (Rosaceae) from the USA (Grand, 1971), *Quercus* (Fagaceae) from Japan (Masui, 1926), and both *Castanopsis* (Fagaceae) and *Engelhardtia* (Juglan-

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daceae) from Taiwan (Haug et al., 1991). Although the morphology of angiosperm tubercules has been studied in detail, the fungal symbionts have not been identified. Most studies have suggested that these TECM fungi are basidiomycetes because of their morphological similarities to Pinaceae–Suillineae TECM. Dell et al. (1990) also noted that rhizomorphs on *Eucalyptus* tubercules are similar to those of the brown rot fungus *Serpula lacrymans* (Boletales). Studies of *Eucalyptus* and *Castanopsis* tubercules reaffirmed that the symbionts are basidiomycetes because TECM hyphae have dolipore septa (Dell et al., 1990; Haug et al., 1991).

Of the angiosperm TECM studied to date, only Masui (1926) identified a putative fungal symbiont; boletoid sporocarps associated with *Quercus* tubercules were tentatively identified as *Boletus luteus* L. (current name *Suillus luteus*). Unfortunately, Masui's identification was based on a "rather old" specimen and his description included morphological features consistent with a *Suillus* species (a "viscid pileus" and "granular black dots" on the stipe). Although the identity of Masui's sporocarps remains unknown, it is safe to assume that this *Quercus* TECM fungus was not a species of *Rhizopogon* or *Suillus* (Suillineae). These genera belong to the suborder Suillineae, a monophyletic clade that is morphologically, chemically and phylogenetically unique within the Boletales (Besl and Bresinsky, 1997; Binder and Hibbett, 2006). Furthermore, species of *Rhizopogon* and *Suillus* form specific ECM associations with Pinaceae and no *Rhizopogon* or *Suillus* species have been confirmed as ECM symbionts of *Castanopsis*, *Engelhardtia*, *Eucalyptus*, *Photinia*, or *Quercus* (Molina and Trappe, 1994; Wu et al., 2000). Neither *Rhizopogon* nor *Suillus* form ECM with *Eucalyptus,* even when tested in pure-culture synthesis trials (Malajczuk et al., 1982, 1984).

In summer and autumn of 2007 and 2008, we collected TECM beneath *Quercus* species in a tropical forest near Xálapa, Mexico and in mixed hardwood forests in Massachusetts, USA. These tubercules were morphologically similar to published descriptions of other angiosperm TECM and also to the TECM formed by *Rhizopogon* or *Suillus* on Pinaceae tree hosts. We also encountered large numbers of sclerotia (aggregations of fungal tissue that function as survival structures) in the soil surrounding tubercules. Sclerotia have not been previously reported with TECM of angiosperms or gymnosperms. The discovery of abundant TECM and sclerotia with *Quercus* posed several key questions that are addressed in this study: (1) what is the identity and geographical distribution of the *Quercus* TECM fungal symbiont, (2) how is the *Quercus* TECM symbiont phylogenetically related to the fungi that form TECM with Pinaceae (*Rhizopogon* and *Suillus*), and (3) did the TECM morphology evolve once or several times?

Fungal ITS rDNA sequences of TECM and sclerotia matched those derived from *Boletus rubropunctus* Peck sporocarps collected in Massachusetts, USA. Plant ITS and *trnL* sequences from tubercules confirm that the TECM host trees are *Quercus* species. We use fungal DNA sequences from five gene regions (mt-LSU, *atp6*, 18S, ITS, and 28S rDNA) to unambiguously identify the fungal symbiont as a member of the suborder Boletineae (Boletales). No member of this lineage has previously been reported to form TECM, suggesting that this unique morphology has evolved separately in two distinct suborders of Boletales (Suillineae and Boletineae). The occurrence of TECM with distantly related angiosperm and gymnosperm ECM host plants indicates that TECM formation is probably controlled by the fungal symbiont.

MATERIALS AND METHODS

Field sampling of tubercules, sclerotia, and sporocarps—Tuberculate ectomycorrhizae and sclerotia from the USA were collected at six sites in three conservation areas of Middlesex County, Massachusetts: (1) Whipple Hill (Lexington) on 26 October 2007 and 7 August 2008 (MES114–MES116, MES248–MES250), (2) Arlington Great Meadows (Lexington) on 19 and 26 August 2008 (MES257, MES261, MES262), and (3) Estabrook Woods (Concord) on 5 October 2008 (MES285). Elevation at these sites ranges between 20 and 50 m above sea level. Tubercules and sclerotia were found beneath *Quercus* spp. (primarily *Q. alba* L., but also *Q. rubra* L. and *Q. velutina* Lam.) in mixed hardwood forest with diverse ECM plants including *Betula* (Betulaceae), *Pinus* (Pinaceae), and *Fagus* (Fagaceae). Sporocarps of Boletineae spp. were collected at Whipple Hill and Arlington Great Meadows on several dates in July and August 2008.

Tubercules from Veracruz State, Mexico were collected near Xálapa, on the road to San Andres Tlapelhuayuán near Rancho Viejo (elevation 1500 m above sea level) on 16 September 2007 (MES117). This site is a tropical, mesic forest dominated by *Quercus* species (*Q. xalapensis* Humb. and Bonpl., *Q. sartorii* Liebm., *Q. leiophylla* A. DC) with a subdominant ECM host, *Carpinus* sp. (Betulaceae). Sclerotia and sporocarps were not collected in Mexico.

Sporocarps and sclerotia were collected, described, and photographed on the day they were found. Small pieces of fresh tissue were placed in DNA extraction buffer. We attempted to grow the TECM fungal symbionts in pure culture by plating surface-sterilized sclerotia and TECM pieces on several types of agar media. Specimens of tubercules, sclerotia, and sporocarps were dried and deposited at Havard University's Farlow Herbarium (FH).

Morphological examination—Fresh TECM specimens were cleaned with a brush, sectioned with a sterile scalpel, and examined with a hand lens or dissecting microscope and light microscope. Tubercules were studied fresh or after rehydration overnight in water. TECM were sectioned by hand or with a freezing microtome then viewed in water, 3% KOH, and Melzer's reagent. Representative sections were later stained by heating in Congo red or cotton blue (Sime et al., 2002).

Molecular protocols—Fragments of TECM, sclerotia, and sporocarps were ground with micropestles, and DNA was extracted using a modified CTAB method (Gardes and Bruns, 1993). PCR was performed with high resolution *Taq* polymerase (Invitrogen, Carlsbad, California, USA) to amplify DNA from multiple regions according to published protocols: (1) ITS rDNA with primers ITS1f and ITS4b (Gardes and Bruns, 1993), (2) 28S rDNA with primers LROR and LR5F (Hopple and Vilgalys, 1994; Tedersoo et al., 2008), (3) 18S rDNA with primers NS1, NS8, nu-SSU-1536, and nu-SSU-0817 (White et al., 1990; Borneman and Hartin, 2000), (4) *atp6* with primers atp6–1 and atp6–2 (Kretzer and Bruns, 1999), and (5) the mitochondrial large subunit (mt-LSU) with primers ML5 and ML6 (Bruns et al., 1998). Plant ITS rDNA sequences were generated with primers ITS.LEU, ITS1, and ITS4 (White et al., 1990; Baum et al., 1998) and for chloroplast DNA with primers trnTc and trnTf (Taberlet et al., 1991). PCR products were visualized on 1.5% agarose gels with SYBR Green I (Molecular Probes, Eugene, Oregon, USA). Successful amplicons were cleaned with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Sequencing was performed with the described primers using the Big Dye Sequencing Kit v.3.1 (Applied Biosystems, Foster City, California, USA) on an ABI13730xl sequencer (Applied Biosystems). Sequences were edited with the program Sequencher v.4.1 (Gene Codes, Ann Arbor, Michigan, USA) and aligned with the program Clustal_X (Chenna et al., 2003) in the program Mesquite v.1.1 (Maddison and Maddison, 2006).

Phylogenetic analyses of multigene data set—To explore the placement of the TECM symbiont, we used baseline data from published phylogenetic studies of Boletales (Bruns et al., 1998; Kretzer and Bruns, 1999; Binder and Bresinsky, 2002; Binder and Hibbett, 2006). We conducted phylogenetic analyses on a multigene data set from five DNA regions (mt-LSU, *atp6*, 18S, 5.8S, and 28S rDNA) including a backbone of 37 Boletales for which sequences from all five DNA regions were available (Binder and Hibbett, 2006). We used BLAST searches and neighbor-joining trees to evaluate whether phylogenetic placement was similar with all DNA regions. Because TECM consistently grouped within Boletineae or Boletineae-Paxillineae, we combined data from all five DNA regions into a single alignment and performed maximum parsimony (MP) and maximum likelihood (ML) analyses. For ML analysis, we performed 10 replicate runs using the program Garli v. 0.951 (Zwickl, 2006). An appropriate

model of nucleotide evolution was selected using the Akaike information criterion (AIC) with the program Mr. Modeltest (Nylander, 2004). The general time reversible GTR+I+G model was selected with rates generated by Mr. Modeltest. We used this model because it was appropriate for the majority of the data set (18S, 5.8S, and 28S rDNA). Maximum likelihood bootstrapping was performed using 500 replicate searches, the GTR+1+G model, and rates estimated by Garli v. 0.951. We also conducted a heuristic MP search using the program PAUP* 4.0b10 (Swofford, 2001) with 100 random taxon addition replicates, equal weighting of all characters, gaps treated as missing data, tree-bisection-reconnection (TBR) branch swapping and MulTrees on. This search was followed by MP bootstrap analysis using 1000 replicate heuristic searches with random taxon addition.

RESULTS

Overall morphology of TECM—Tubercules are locally abundant at sites in the USA and Mexico, with hundreds of specimens often detected at each site. TECM are found in clusters at the interface between the leaf litter and soil or in the top few centimeters of mineral soil. *Quercus* TECM are morphologically similar to previously described angiosperm TECM (Table 1). Tubercules are generally spherical to subglobose and ca. 2–10 mm in diameter (Fig. 1). Fresh specimens are firm to rubbery in texture and resemble basidiomycete "false truffles" except that they are attached to roots. The exterior surface of tubercules are smooth to pustulate, white to cream in color, and become yellowish to light brown in age or when dried. Three key TECM characters are obvious in freshly sectioned tubercules: the peridial hyphal sheath, tightly packed ECM roots, and protruding rhizomorphs. The TECM are often associated with white to yellowish-green sclerotia, that are sometimes attached to the TECM via rhizomorphs. Exterior surfaces of tubercules, including rhizomorphs, are sometimes covered with adhering soil particles. TECM are sometimes found with tubercules from the previous growing season; these old tubercules are dry, brown, and lack a fungal sheath. Because both morphological and molecular data indicate that fungal symbionts at all sites are closely related, the morphological descriptions are combined.

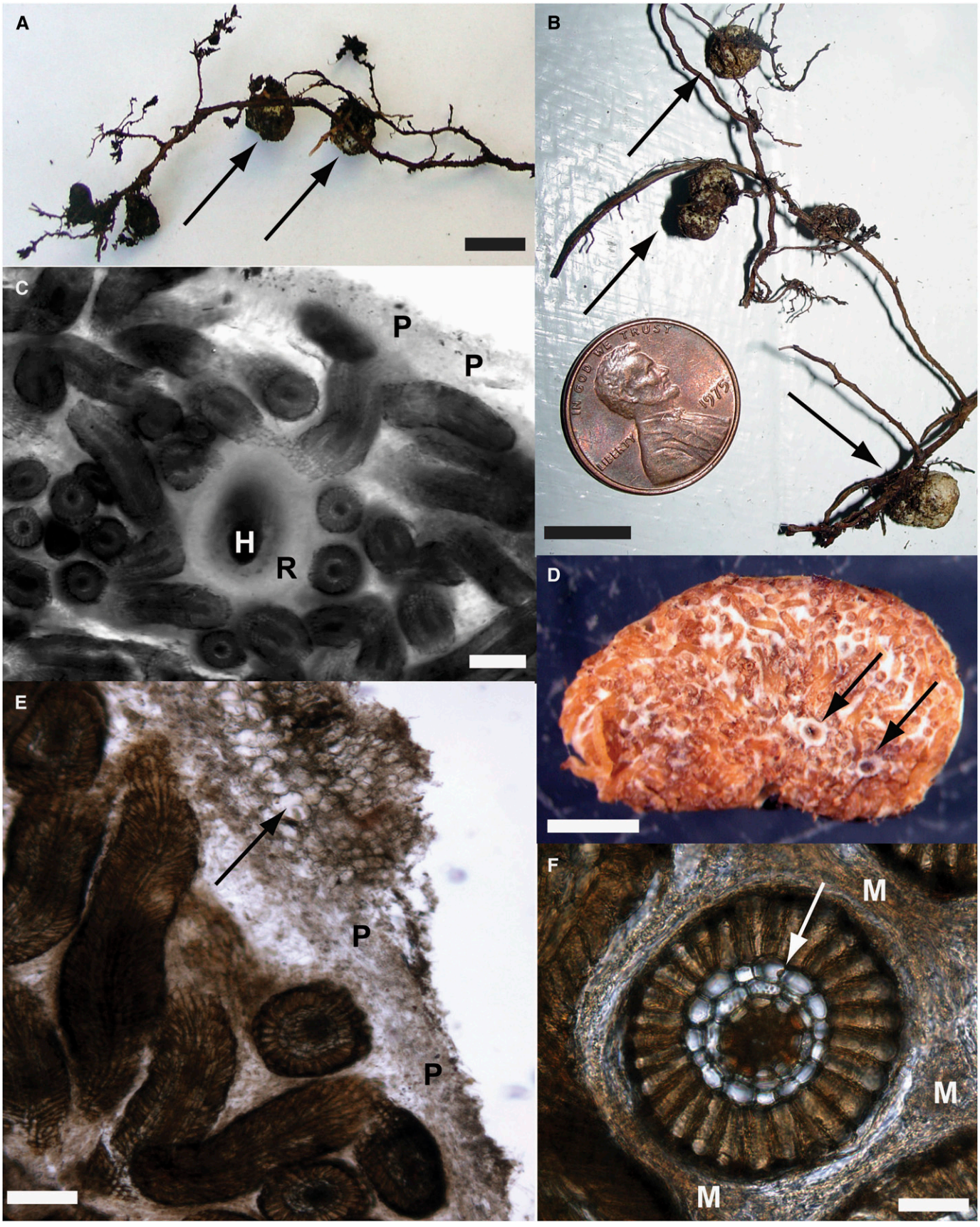
Peridial sheath—Each tubercule is fully enclosed in a peridium or sheath of hyphae that is usually 100–125 μm thick (range 80–230 μm). The peridium is a well-organized layer consisting of tightly packed, thin-walled hyphae that are 2.5–4 μm in diameter. In section, peridia showed no obvious reaction to KOH or Melzer's reagent, and no cystidia or clamp connections were detected. The peridium sometimes peels away from the root-filled interior of the TECM during sectioning. Peridial hyphae mostly run parallel to the surface of the sheath, but in some specimens small, felty wefts of hyphae protrude from the peridial surface. Between the outer layer of the peridium and the mycorrhizal roots, there is a layer of prosenchyma that is less well organized than the outer peridium. In some cases, this prosenchyma is directly attached to ECM fungal mantles, but other times it gives rise to loosely packed hyphae and air spaces between roots. Sometimes aggregations of an unidentified crystalline substance are present within the prosenchyma matrix.

Ectomycorrhizal roots—Tubercules are filled with healthy, entangled ECM roots that are mostly 100–160 μm in diameter, with occasional roots up to 200 μm in diameter (Fig. 1). ECM roots are packed tightly together but the distance between adjacent roots ranges from 2–100 μm. Sometimes the spaces are filled with ECM mantles, but at other times the spaces between roots are filled with loosely arranged hyphae. ECM mantles

TABLE 1. Comparison of tuberculate ectomycorrhizae morphology on various host plants. Each report of tuberculate ectomycorrhizae on angiosperms is listed separately but Pinaceae–Suillineae tuberculate ectomycorrhizae are listed as a composite for comparison purposes.

Host	Plant family	Location	Color	Diameter (mm)	Sclerotia	Rhizomorphs	Heavily infected roots?	Reference	ECM symbiont
A) Angiosperm									
Photinia glabra[a]	Rosaceae	North Carolina, USA	creamy white	7–45	no	no[a]	no[a]	Grand (1971)	unknown
Eucalyptus pilularis	Myrtaceae	Queensland, Australia	pale yellow	5–20	no	yes	yes[b]	Dell et al. (1990)	Boletales
Castanopsis borneensis	Fagaceae	Taiwan	white	4–10	no	yes	yes	Haug et al. (1991)	Basidiomycota
Engelhardtia roxburghiana	Juglandaceae	Taiwan	white	4–10	no	yes	yes	Haug et al. (1991)	Basidiomycota
Quercus pausidentata	Fagaceae	Tajimi, Japan	white	4–15	no	yes	yes	Masui (1926)	Boletales (see text)
Quercus spp.	Fagaceae	USA, Mexico	white	2–10	yes	yes	yes	this publication	*Boletus rubropunctus*
B) Gymnosperm									
Pinus, *Pseudotsuga*	Pinaceae only	Native to northern hemisphere	white, buff, peach, yellow, gray or silvery	2–40	no	yes	no	Melin (1923), Trappe (1965), Zak (1971, 1973), Mleczko and Ronikier (2007)	*Suillus* and *Rhizopogon* species

[a] *Photinia* TEM were "mature or overmature" and "in various stages of desiccation" when they were collected by Grand (1971).
[b] Called "partly decomposed roots" by Dell et al. (1990)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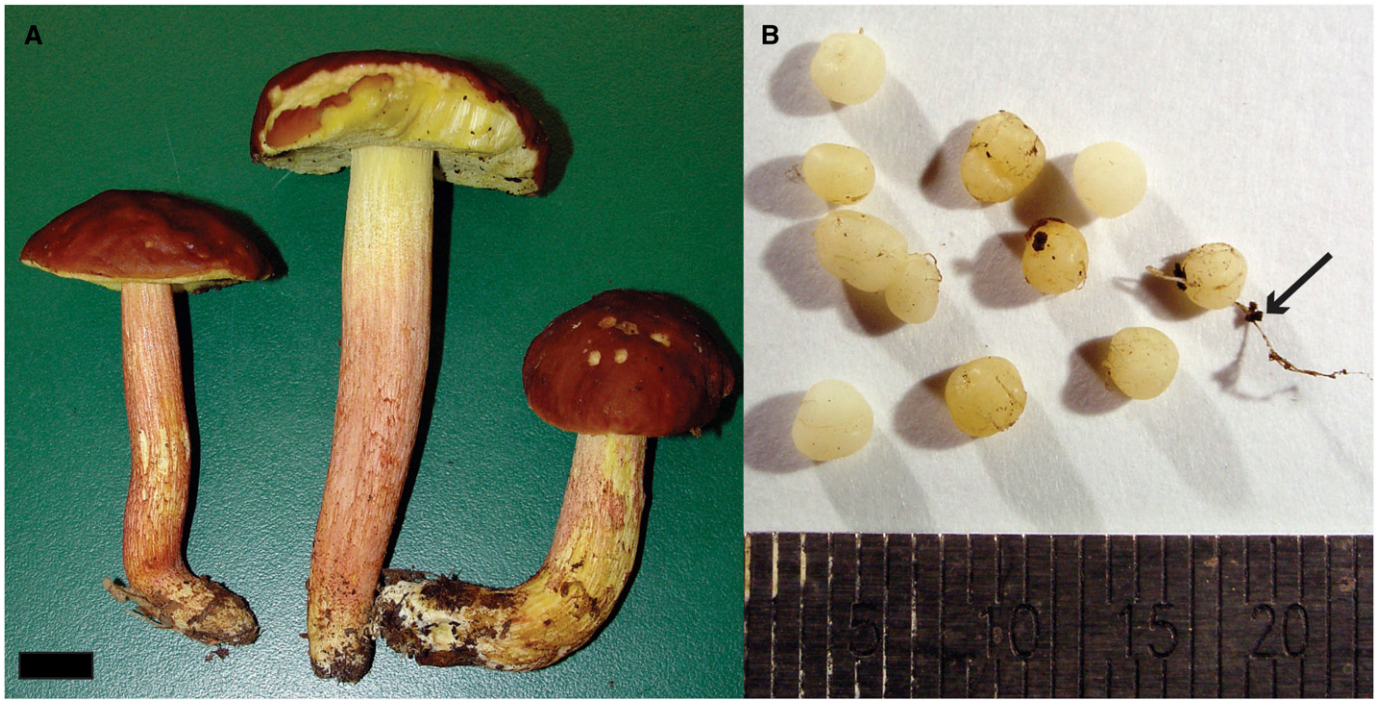

Fig. 2. *Boletus rubropunctus* Peck. (A) Fresh sporocarps from collection MES242. Bar = 1 cm. (B) Fresh sclerotia from collection MES261. Arrow: attachment to rhizomorph (scale in mm).

consist of a layer of prosenchyma averaging 15–25 µm thick (range 10–80 µm). Sometimes several ECM roots share a single, fused mantle. Typical Hartig nets with intercellular hyphae (0.5–1 µm diameter) stain strongly in cotton blue and can be seen in outer cell layers of most roots. Hartig nets are sometimes attached to vesicles (ca. 3–5 µm diameter) between epidermal cells.

Rhizomorphs, peridial pustules, and sclerotia—Each tubercule has one or a few rhizomorphs attached to its surface (Fig. 2). The rhizomorphs are concolorous with the peridium, usually branching dichotomously, and occasionally are encrusted with a crystalline substance. Rhizomorphs are ca. 100–200 µm in diameter, but with occasional sections swollen up to 320 µm. Rhizomorphs have notable internal structure and can be classified as "F-type" or "Boletoid" rhizomorphs (Agerer, 2006). Rhizomorphs have two distinct hyphal types; thin-walled, small-diameter hyphae and thick-walled, large-diameter vessel hyphae (Smith and Read, 2008). The thin-walled hyphae (0.6–1.5 µm diameter) form the outer layer of rhizomorphs. These hyphae appear gelatinized and are often twisted and tightly bound together. Vessel hyphae have thick cell walls (ca. 1.8–2.5 µm) and are 10–30 µm in diameter, with occasional cells up to 40 µm in diameter. The transverse septa of vessel hyphae are irregularly spaced and appear degraded.

Another notable feature of tubercules is the pustules on the peridial surface (Fig. 1). Peridial pustules are raised above the peridium and are often yellowish. The pustules range greatly in size (50–400 µm) and occur irregularly on the peridium. In cross-section, they appear as circular aggregations of enlarged, thick-walled cells surrounded by peridial hyphae. The peridial hyphae near pustules are atypical; they tend to be more highly branched and twisted, are wider (up to 6.5 µm), and have bulbous hyphal tips (ca. 8–10 µm) (e.g., "knee-like" swellings; Mleczko and Ronikier, 2007).

Tubercules are found in close association with sclerotia that are subglobose to lobate and 0.5–2 mm in diameter (Fig. 2). Sclerotia are sometimes attached directly to tubercules via rhizomorphs. Sclerotia are white when young but turn creamy to light yellowish or yellowish-green with age. This color change that accompanies sclerotia maturation apparently occurs because the exterior hyphae become more gelatinized over time. When viewed in Melzer's reagent, this layer of gelatinous hyphae does not absorb stain, whereas the interior becomes slightly reddish or dextrinoid. Sclerotia consist of hyaline, tightly packed, thin-walled hyphae that are irregularly septate and 3–5 µm in diameter. After we discovered *Boletus rubropunctus* sclerotia, we noticed that they were always associated with TECM collections. When we returned to several Massachusetts TECM collecting sites after *Quercus* had lost their leaves (November

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Fig. 1. Morphology of *Quercus–Boletus rubropunctus* tubercules. (A, B) Macroscopic appearance of fresh tubercule specimens still attached to *Quercus* roots. Bar = 10 mm. (C) Cross section of *Quercus* tuberculate ectomycorrhiza. Prominent "heavily infected root" (H) is surrounded by dense ring of aggregated hyphae (R). P = peridium (P). Bar = 100 µm. (D) Cross section of fresh tubercule. Areas between roots are packed with fungal hyphae. Arrows: "heavily infected roots". Bar = 1 mm. (E) Cross section of tuberculate ectomycorrhiza peridium (P). Arrows: peridial pustule with inflated cells. Bar = 100 µm. (F) Cross section of typical mycorhizal root within a tubercule showing healthy mantle (M). The thickened cell walls are due to the presence of a hartig net with swollen hyphae that resemble vesicles (arrow). Bar = 25 µm.

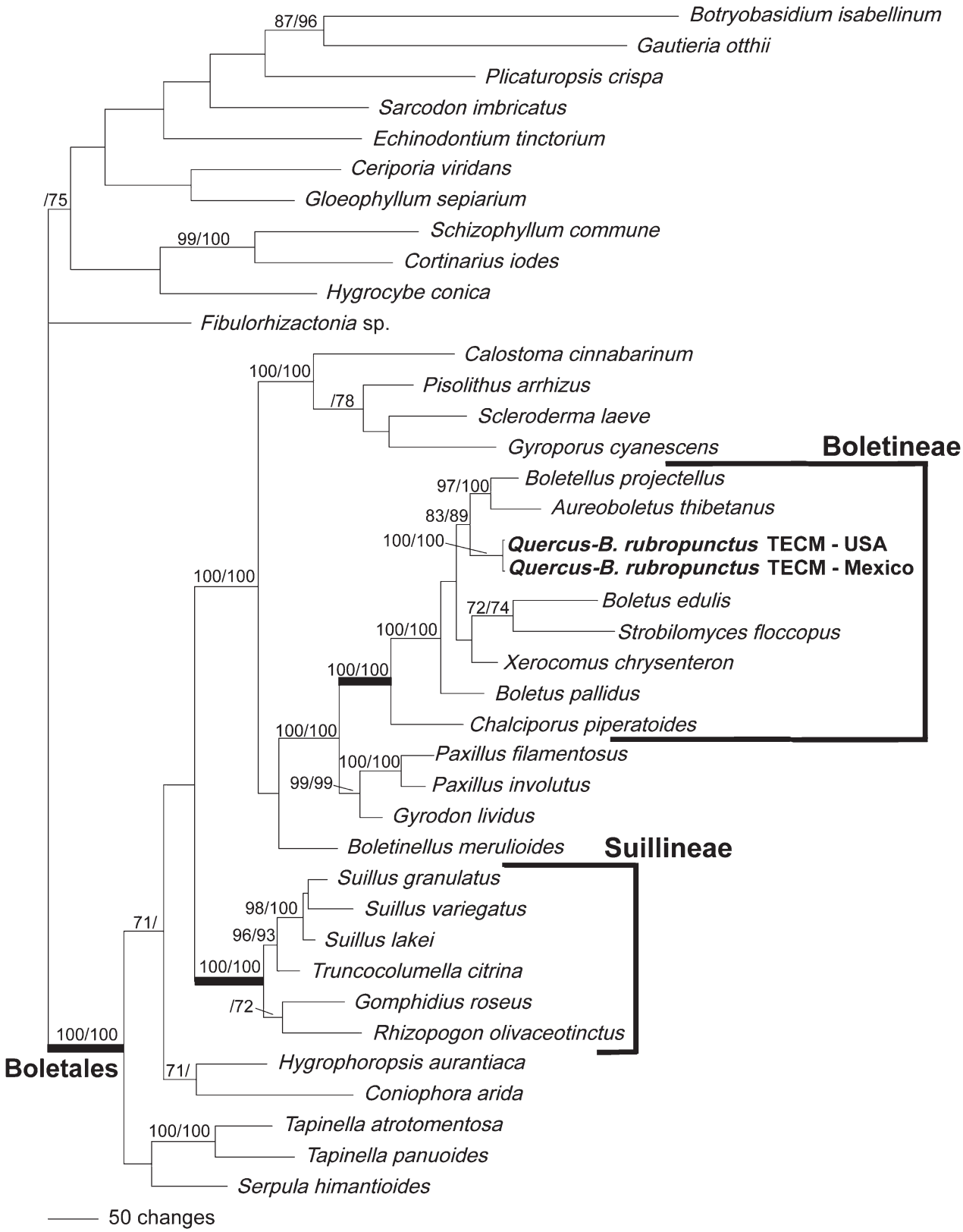

Fig. 3. One of four most parsimonious trees showing the phylogenetic position of the *Quercus* tuberculate ectomycorrhizal symbiont, *Boletus rubropunctus*, based on 3484 bp from five DNA regions (mt-LSU, *atp6*, 18S, 5.8S, and 28S rDNA). Maximum parisomony/maximum likelihood bootstrap support is indicated above the nodes with strong support of three relevant clades (Boletales, Boletineae, Sullineae) highlighted by thickened nodes and brackets.

2008), we were unable to detect sporocarps or TECM but still encountered abundant sclerotia in the soil. Sclerotia of a second, unidentified species of *Boletus* were also detected at Arlington Great Meadows in Massachusetts (Appendix 1). These sclerotia are larger, irregular in shape, and bright orange but microscopically similar to *Boletus rubropunctus* sclerotia (Appendix S1, see Supplemental Data with the online version of this article).

Heavily infected roots—The vast majority of roots within tubercules are healthy, but one or a few roots have an unusual morphology matching the "heavily infected roots" described by Masui (1926) and Haug et al. (1991) (Fig. 1). As seen in cross-section, heavily infected roots are encased in a ring of dense pseudoparenchymal tissue 20–30 µm thick that intergrades with more loosely packed hyphae at the edges. The dense halo of pseudoparenchyma is arranged in a radiating pattern, but individual cells are pressed so tightly as to make recognition of individual hyphae impossible in thick sections.

ITS sequence data from plant and fungal symbionts—We identified plant DNA sequences by performing BLAST searches against GenBank. Plant sequences from Massachusetts TECM confirm that *Boletus rubropunctus* can associate with *Quercus* from both the *Lobatae* (red oak) and *Quercus* (white oak) subgenera (Manos et al., 1999). A *trnL* intron sequence from specimen MES114 was 99% similar (680/684 bp) to *Quercus pubescens* Willd., a European white oak. A partial ITS sequence from specimen MES285 was 98% similar (252/257 bp) to two American red oaks, *Quercus laevis* Walter and *Q. falcata* Michx.. An ITS sequence from Mexican specimen MES117 was 100% similar (486/486 bp) to *Quercus rugosa* Née., a tropical to subtropical American white oak.

We generated fungal ITS sequences from five TECM collections (Massachusetts, USA: MES114–MES116, MES250; Xálapa, Mexico: MES117) and one sclerotium (Massachusetts, USA: MES261). The ITS sequences from tubercules and the sclerotium were more than 99% similar to those of *Boletus rubropunctus* sporocarps collected at two Massachusetts sites (MES242, Whipple Hill; MES256, Arlington Great Meadows). Although ITS sequences from *Boletus rubropunctus* were 84–88% similar to species of *Boletus*, *Xerocomus*, and *Octavianina,* we abandoned attempts at phylogenetic inference based on ITS because of frequent, unalignable indels and potential ITS paralogs (Halling et al., 2008).

Fungal ITS sequences from all USA collections were identical except for a C/T transition in ITS1 (sclerotium MES261) and a dual C/T peak in ITS2 (sporocarp MES256). The ITS of Mexican TECM was identical to USA collections except for four nucleotides: two C/T transitions in ITS1, one C/T transition in ITS2, and one C/G transversion in ITS2.

Sporocarp data—We collected sporocarps of ca. 20 species of Boletineae at TECM sites and sequenced ITS for 12 species of *Boletus*, *Tylopilus*, *Xanthoconium*, and *Xerocomus* (Appendix 1). Two collections of *B. rubropunctus* (Fig. 2) matched the descriptions of Peck (1897), Singer (1947) (as *Leccinum rubropunctum*), and Bessette et al. (2000). Our collections were also morphologically similar to *B. rubropunctus* specimens at New York Botanical Garden (NY) identified by Roy Halling (accessions 13529, 13531, 792788, 817393). *Boletus rubropunctus* has a chestnut red to reddish-brown cap with yellow context. The cap is viscid when moist and often wrinkled when young. The pore surface is yellow to brownish-yellow with circular pores. The stipe is solid, yellow, and often tapered or curved at the base. The most notable feature is the red punctate dots that cover the stipe (Fig. 2).

Phylogenetic inference based on the multigene data set—The multigene data set consisted of five DNA regions from 41 taxa, including 28 Boletales, 11 outgroups, and two TECM specimens (MES116, MES117). After exclusion of ambiguously aligned characters, the nucleotide matrix was 3484 bp in length and included 889 parsimony informative characters. Parsimony analysis generated four equally most parsimonious trees (length = 4839 steps, CI = 0.391, RI = 0.524) that differed only in minor rearrangements (Fig. 3). Both MP and ML bootstrapping supported most of the key nodes in the phylogeny and both methods indicated that *B. rubropunctus* is nested within the strongly supported, monophyletic Boletineae. Species of the other TECM-forming genera (*Rhizopogon* and *Suillus*) were placed in a monophyletic Suillineae clade. The order Boletales was strongly supported as a monophyletic group. All TECM sequences for both plants and fungi have been submitted to GenBank (Appendix 2).

DISCUSSION

Diverse angiosperms from around the globe form TECM, but no previous reports have identified any of the angiosperm TECM fungal symbionts. This report of TECM formation by *Boletus rubropunctus* on the roots of *Quercus* is the first documentation of TECM formation by a member of the suborder Boletineae. Our multigene phylogeny indicates that *B. rubropunctus* is nested within the Boletineae and is only distantly related to *Rhizopogon* and *Suillus,* the two genera previously known to form TECM (Fig. 3). The fact that TECM are morphologically similar, whether formed by species in the suborder Boletineae on angiosperms or by those in the suborder Suillineae on gymnosperms, suggests either that TECM-formation is an ancient feature that was present in an ectomycorrhizal ancestor of the Boletineae and Suillineae (≥50 million years ago based on the fossil record and molecular dating estimates; LePage et al., 1997; Bruns et al., 1998) or that TECM-formation evolved separately in two clades of Boletales. Although we cannot be certain which evolutionary scenario is correct, a large number of independent losses of TECM formation would be required to explain our phylogeny of the Boletales (Fig. 3). TECM formation in such widely divergent taxa is probably due to convergent evolution, suggesting that the TECM morphology is an adaptive feature that has originated multiple times due to some selective advantage.

Based on the strong morphological similarities between the *Quercus–Boletus rubropunctus* TECM described here and the angiosperm tubercules from previous reports (Table 1), we assume that most or all angiosperm TECM are formed by species in the suborder Boletineae. All reported angiosperm TECM are morphologically similar; they are small and light-colored with an obvious fungal sheath stuffed with entangled, mostly healthy ECM roots. Furthermore, ECM species of Boletineae are widespread in both the northern and southern hemispheres and are confirmed symbionts of the three common angiosperm TECM host genera (e.g., *Castanopsis*, *Eucalyptus, Quercus*) (Agueda et al., 2008; Halling et al., 2008). Except for *Photinia* TECM, all angiosperm tubercules are clearly associated with thick, highly differentiated, boletoid rhizomorphs. TECM are consid-

ered "long distance exploration types" (sensu Agerer, 2001) because they form rhizomorphs and thus probably extend great distances from the host roots via rhizomorph growth (Agerer, 2006). These rhizomorphic systems aid in plant uptake of phosphorous (Kammerbauer et al., 1989), reduce the effects of drought (Duddridge et al., 1980), and facilitate the colonization of new seedlings (Brownlee et al., 1983). Many species of Boletineae have "long-distance exploration" mycorrhizas (e.g., rhizomorphs, clustered ECM roots), including species of *Boletus* (Agueda et al., 2008), *Leccinum* (Montecchio et al., 2006), and *Chamonixia* (Raidl, 1999).

Although the *Quercus–Boletus rubropunctus* TECM and other angiosperm TECM share key features with Pinaceae-Suillineae TECM (e.g., thick fungal sheaths, boletoid rhizomorphs, crystaline encrustations on their hyphae) (Table 1; Massicotte et al., 1992; Mleczko and Ronikier, 2007), angiosperm TECM also have unique morphological features. The most notable aspect of *Quercus–Boletus rubropunctus* tubercules are the visually striking "heavily infected roots." These are degraded roots surrounded by a dense ring of fungal hyphae that penetrate into lysed cortex cells near the root tips (Fig. 1). Nearly identical structures have been reported in tubercules of *Quercus*, *Castanopsis*, *Engelhardtia*, and *Eucalyptus* but never from Pinaceae–Suillineae TECM (Table 1). Both Masui (1926) and Haug et al. (1991) detailed the microscopic morphology of "heavily infected roots." Although their exact function remains unclear, Masui (1926) suggested that the "heavily infected roots" might indicate that the fungus is parasitizing the plant.

Sclerotia function as asexual propagules, survival structures to tolerate cold temperatures or drought, and possibly as sites of nutrient storage (Cotter and Miller, 1985; Kohn and Grenville, 1987; LoBuglio, 1999). Although sclerotia have not been previously documented with other TECM, *B. rubropunctus* sclerotia were abundant near *Quercus* tubercules. We also collected morphologically distinct sclerotia of a second *Boletus* species at one site (Appendix S2, see Supplemental Data with the online version of this article). There is only one description of sclerotia formation by a species of *Boletus;* Giltrap (1979) reported that *B. porosporus* formed sclerotia in pot cultures with *Betula pendula*. Unfortunately, these data have never been published. Sclerotia have, however, been detected with many other genera of Boletales, including both saprotrophic (e.g., *Boletinellus*: Cotter and Miller, 1985; *Hygrophoropsis*: Hutchison, 1991) and ECM genera (e.g., *Paxillus*: Grenville et al., 1985; *Pisolithus*: Grenville et al., 1985; *Melanogaster*: Wiedmer et al., 2004; *Austropaxillus*: Palfner, 2001; *Leccinum*: Montecchio et al., 2006). Sclerotia have previously been used to obtain axenic cultures of other Boletales (Grenville et al., 1985), but our attempts to culture sclerotia were unsuccessful. The phylogenetic breadth of sclerotia formation within the Boletales and the abundant sclerotial production by some species (e.g., *Pisolithus tinctorius*, *B. rubropunctus*) suggests that they may play a greater role in the life histories of ectomycorrhizal Boletales than has previously been appreciated. Furthermore, sclerotia probably play an important role in the clonal growth and persistence of fungal individuals. Undetected sclerotia formation might explain the large, persistent fungal genets formed by some species in the suborder Boletineae (e.g., >100 m^2 for *Xerocomus chrysenteron*) (Fiore-Donno and Martin, 2001).

Except for the work of Masui (1926) previous studies of angiosperm TECM are based on only one or a few collections (Table 1), implying that TECM occurrence might be rare or localized. In contrast, we frequently encountered *Quercus–Boletus rubropunctus* TECM at six collection sites (separated by >100 m) in mixed forests of Massachusetts, indicating that these structures are common. The abundance of the *Quercus–Boletus rubropunctus* tubercules in a tropical forest more than 3600 km from the New England sites demonstrates that this interaction is also geographically widespread. Despite the different habitats and the long distance between the collection sites, *Quercus–Boletus rubropunctus* TECM from Mexico and the USA were morphologically similar and had nearly identical DNA sequences at five loci (mt-LSU, *atp6*, 18S, ITS, and 28S rDNA). These observations from roots corroborate sporocarp data. *Boletus rubropunctus* is reported across the eastern USA, from New England through the Appalachian range and along the Gulf Coast (Singer, 1947; Bessette et al., 2000; collections at FH, NY, TENN, and MICH). Our data confirms that the range of *B. rubropunctus* and its TECM also extends into Mexican *Quercus* forests, following a biogeographical distribution common to many ECM fungi (Mueller and Strack, 1992; Halling et al., 2008; Morris et al., 2008, 2009).

The discovery of TECM formation in two major clades of Boletales raises the question: what is the selective advantage of the tuberculate morphology? Zak (1965, 1971) hypothesized that the hyphae that cover the tubercules might serve a defensive purpose by suppressing pathogens or limiting insect damage. Zak (1971) tested whether *Rhizopogon* TECM isolates antagonized root pathogens. Three oomycetes and two basidiomycetes were inhibited, but five other pathogens were unaffected, suggesting that protective effects might be limited to a subset of natural enemies. Zak (1965) noted that root-feeding aphids infested several *Pseudotsuga* ECM morphotypes but did not feed on tubercules. He suggested that the TECM sheath might form a physical barrier or that the fungal symbiont might produce repellent compounds. Although TECM-forming fungi could inhibit rhizosphere pathogens or herbivores, there is contrasting evidence that tubercule formation might actually make the fungal symbiont more vulnerable to attack by fungal parasites in the genera *Gomphidius* and *Chroogomphus* (Gomphidiaceae) (Agerer, 1990; Olsson et al., 2000). Another hypothesized advantage of tubercules is as a site for nutrient storage. Although this idea has seldom been tested, Göbl (1967) found more P and K in *Pinus* TECM than in individual ECM root tips, and Dell et al. (1990) found evidence of protein and lipid reserves in *Eucalyptus* TECM hyphae.

The most intriguing suggested function of TECM is that they might be analogous to nodules of Leguminosae such as *Acacia*, *Alnus*, and other nodule-forming plants by providing a suitable anaerobic environment for nitrogen-fixation by bacteria (Frey-Klett et al., 2007; Paul et al., 2007). Nitrogen-fixing bacteria have been isolated from inside *Suillus* tubercules (Paul et al., 2007) and from the exterior surfaces of both *Rhizopogon* and *Suillus* TECM (Li and Hung, 1987; Li et al., 1992; Li and Strzelczyk, 2000; Timonen and Hurek, 2006). Furthermore, there is some evidence of N fixation associated with Suillineae TECM in field and greenhouse studies (Richards and Voigt, 1964; Paul et al., 2007), and culture-independent studies have found some evidence of N-fixing bacterial genes associated with tubercules (Izumi et al., 2006; Timonen and Hurek, 2006). However, most N-fixing bacteria are localized in the TECM sheath (Massicotte et al., 1992; Li et al., 1992; Timonen and Hurek, 2006; A. Kretzer, SUNY-College of Environmental Science and Forestry, personal communication), and some studies provide only weak evidence of N-fixation (Li et al., 1992) so the significance of tubercules for N-fixation remains

unclear. Given that N-fixing bacteria have been isolated from diverse ECM fungi (Frey-Klett et al., 2007) and that specificity between bacteria and ECM fungi appears low (Burke et al., 2008), it is plausible that TECM are sites of bacterial N fixation simply because of their large surface area relative to other mycorrhizal types. Whatever the adaptive advantage of tubercules, the widespread and abundant *Quercus–Boletus rubropunctus* TECM described in this report offers the possibility of future comparative studies to elucidate these effects.

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APPENDIX 1. Voucher information and BLAST results for sporocarps and sclerotia of Boletineae species found at tuberculate ectomycorrhizae collecting sites in Massachusetts, USA.

Voucher	Species name	Collection location	GenBank no.	Highest BLAST hit	Similarity, bp (%)
A) Sporocarp					
MES-228	*Boletus bicolor*	Whipple Hill	FJ480439	*Xerocomus pruinatus*	303/364 (83)
MES-229	*Boletus frostii*	Whipple Hill	Not sequenced	—	–
MES-230	*Tylopilus* sp.	Whipple Hill	FJ480445	*Boletus aestivalis*	666/712 (93)
MES-231	*Gyroporus castaneus*	Whipple Hill	Not sequenced	—	—
MES-232	*Boletus* cf. *innixus*	Whipple Hill	FJ480443	*Xerocomus* sp. UE-2006a	491/532 (92)
MES-233	*Tylopilus felleus*	Whipple Hill	Not sequenced	—	—
MES-234, MES-247	*Boletus* sp.	Whipple Hill	FJ480436	*Phylloporus rhodoxanthus*	662/666 (99)
MES-235	*Xerocomus* sp.	Whipple Hill	FJ480441	*Boletus erythropus*	529/620 (85)
MES-236	*Boletus* sp.	Whipple Hill	Not sequenced	—	—
MES-240	*Tylopilus plumboviolaceous*	Whipple Hill	Not sequenced	—	—
MES-241	*Retiboletus ornatipes*	Whipple Hill	Not sequenced	—	—
MES-242	***Boletus rubropunctus***	Whipple Hill	FJ480427	*Xerocomus subtomentosus*	433/489 (88)
MES-243	*Boletus* sp.	Whipple Hill	Not sequenced	—	—
MES-244	*Boletus* cf. *subvelutiples*	Whipple Hill	FJ480442	*Boletus erythropus*	525/558 (94)
MES-245	*Xerocomus* cf. *chrysenteron*	Whipple Hill	FJ480437	*Xerocomus chrysenteron*	153/167 (91)
MES-246	*Boletus* cf. *pallidus*	Whipple Hill	FJ480440	*Boletus pallidus*	577/583 (98)
MES-258	*Boletus* sp.	Whipple Hill	Not sequenced	—	—
MES-259	*Tylopilus* cf. *sordidus*	Whipple Hill	Not sequenced	—	—
MES-254	*Xanthoconium* cf. *affine*	Arlington Great Meadows	FJ480435	*Phylloporus rhodoxanthus*	711/716 (99)
MES-255	*Leccinum* cf. *rugosiceps*	Arlington Great Meadows	Not sequenced	—	—
MES-256	***Boletus rubropunctus***	Arlington Great Meadows	FJ480428	*Xerocomus subtomentosus*	433/489 (88)
B) Sclerotia					
MES-260	*Boletus* sp.	Arlington Great Meadows	FJ480444	*Boletus mirabilis*	265/314 (84)
MES-261	***Boletus rubropunctus***	Arlington Great Meadows	FJ480429	*Xerocomus subtomentosus*	433/489 (88)

APPENDIX 2. (A) Plant DNA sequences from *Quercus–Boletus rubropunctus* tuberculate ectomycorrhizae and (B) fungal DNA sequences from *Quercus–Boletus rubropunctus* tuberculate ectomycorrhizae and *B. rubropunctus* sporocarps and sclerotia.

Origin	Voucher	DNA region	GenBank accession	Source	Location
A) Plant host					
Quercus sp.	MES114	*trnL* intron	FJ480448	Tubercule	USA
Quercus sp.	MES285	ITS	FJ480449	Tubercule	USA
Quercus sp.	MES117	ITS	FJ480446	Tubercule	Mexico
B) Fungal symbiont					
B. rubropunctus	MES116	mtLSU	FJ480422	Tubercule	USA
B. rubropunctus	MES116	18S	FJ480426	Tubercule	USA
B. rubropunctus	MES116	*atp6*	FJ480424	Tubercule	USA
B. rubropunctus	MES116	ITS, 28S	FJ480434	Tubercule	USA
B. rubropunctus	MES117	mtLSU	FJ480421	Tubercule	Mexico
B. rubropunctus	MES117	18S	FJ480425	Tubercule	Mexico
B. rubropunctus	MES117	*atp6*	FJ480423	Tubercule	Mexico
B. rubropunctus	MES117	ITS, 28S	FJ480433	Tubercule	Mexico
B. rubropunctus	MES114	ITS	FJ480432	Tubercule	USA
B. rubropunctus	MES115	ITS	FJ480431	Tubercule	USA
B. rubropunctus	MES250	ITS	FJ480430	Tubercule	USA
B. rubropunctus	MES261	ITS	FJ480429	Sclerotium	USA
B. rubropunctus	MES242	ITS	FJ480427	Sporocarp	USA
B. rubropunctus	MES256	ITS	FJ480428	Sporocarp	USA