The ectomycorrhizal status of *Calostoma cinnabarinum* determined using isotopic, molecular, and morphological methods

Andrew W. Wilson, Erik A. Hobbie, and David S. Hibbett

Abstract: *Calostoma cinnabarinum* Corda belongs to the suborder Sclerodermatineae (Boletales), which includes many well-known ectomycorrhizal basidiomycetes, but the genus *Calostoma* has been described as saprotrophic. This study combines isotopic, molecular, and morphological techniques to determine the mode of nutrition of *C. cinnabarinum*. $\delta^{13}C$ and $\delta^{15}N$ measurements were compared among co-occurring *C. cinnabarinum*, ectomycorrhizal fungi, saprotrophic fungi, and ectomycorrhizal plants. Isotopic profiles of *C. cinnabarinum* resembled those of ectomycorrhizal fungi but not those of saprotrophic fungi. For molecular analyses, ectomycorrhizal root tips were extracted from soil cores collected beneath *C. cinnabarinum* fruit bodies. Nuclear ribosomal internal transcribed spacer (nrITS) sequences were obtained from ectomycorrhizal root tips using fungal-specific primers and screened against *C. cinnabarinum* nrITS sequences. Ectomycorrhizal root tips had nrITS sequences that matched *C. cinnabarinum* fruiting bodies. Root tips colonized by *C. cinnabarinum* were also described morphologically. Several morphological characters were shared between fruiting bodies and ectomycorrhizal root tips of *C. cinnabarinum*. Results of isotopic, molecular, and morphological analyses indicate that *C. cinnabarinum* is ectomycorrhizal. Molecular analysis and observations of plant associations suggest that *C. cinnabarinum* forms ectomycorrhizae with *Quercus*.

Key words: Calostoma, nrITS, ectomycorrhizae, saprotrophic, isotope, molecular ecology, fungal ecology.

Résumé : Le *Calostoma cinnabarinum* Corda appartient au sous-ordre des Sclerodermatinae (Bolétales), qui inclut plusieurs espèces de basidiomycètes bien reconnues, mais on a décrit le genre *Calostoma* comme saprophyte. Les auteurs ont déterminé le mode de nutrition du *C. cinnabarinum* à l'aide de techniques isotopiques, moléculaires et morphologiques. Ils ont comparé les mesures des $\delta^{13}C$ et $\delta^{15}N$ entre le *C. cinnabarinum*, des champignons ectomycorhiziens, des champignons saprophytes et des plantes ectomycorhiziennes, poussant sur un même site. Les patrons isotopiques du *C. cinnabarinum* ressemblent à ceux des champignons ectomycorhiziens, mais non à ceux de champignons saprophytes. Afin de conduire des analyses moléculaires, des apex ectomycorhiziens ont été extraits de carottes de sol récoltées sous des fructifications de *C. cinnabarinum*. En utilisant des amorces fongiques spécifiques on a obtenu des séquences nucléaire ribosomal espaceur transcrit interne (nrITS), à partir des apex ectomycorhiziens, et on les a comparées avec les séquences nrITS du *C. cinnabarinum*. Ces séquences nrITS des apex correspondent à celles de fructifications du *C. cinnabarinum*. On présente également une description des apex racinaires colonisés par le *C. cinnabarinum*. Les résultats des analyses isotopiques, moléculaires et morphologiques indiquent que le *C. cinnabarinum* est ectomycorhizien. Les analyses moléculaires et l'observation des associations végétales suggèrent que le *C. cinnabarinum* forme des ectomycorhizes avec des *Quercus*.

Mots-clés : Calostoma, nrITS, ectomycorhizes, saprophytes, isotope, écologie moléculaire, écologie fongique.

[Traduit par la Rédaction]

Introduction

The genus *Calostoma* consists of morphologically unusual species of basidiomycetes that fruit on the ground in temperate and tropical forests composed of Fagaceae, Nothofagaceae, Myrtaceae, and Dipterocarpaceae. *Calostoma* species form gastroid fruiting bodies often with brightly colored peristomes (ridged ostioles) and gelatinized tissues. There are 26 species of *Calostoma* in the CABI database (www.indexfungorum.org/). Three of these species occur in Northern and Central America, and the rest are found in Asian and Australian regions. Recent molecular studies have placed this genus in the Sclerodermatineae, a suborder of the Boletales (Hughey et al. 2000; Binder and Bresinsky 2002). The Boletales are mostly ectomycorrhizal with some saprotrophic members, while all core Sclerodermatineae genera — *Scleroderma* (Godbout and Fortin 1983; Buée et al. 2004), *Pisolithus* (Moyersoen and Beever 2004), *Astreus* (Danielson 1984), *Gyroporus* (Agerer 2002) — are considered ectomycorrhizal. Hughey et al. (2000) described *Calostoma cinnabarinum* Corda as a litter decomposer after Miller and Miller (1988), but this assumption is most likely based on previous taxonomic comparisons with the saprotrophic *Tulostoma* (Burnap 1897) and with *Geastrum* (Massee 1888), which have been previously described as saprotrophic (Kreisel 1969; Miller and Miller 1988; Sunhede 1989), but

Received 15 February 2007. Published on the NRC Research Press Web site at canjbot.nrc.ca on 14 June 2007.

A.W. Wilson[1] and D.S. Hibbett. Department of Biology, Clark University, Worcester, MA 01610, USA.
E.A. Hobbie. Complex Systems Research Center, Morse Hall, University of New Hampshire, Durham, NH 03824, USA.

[1]Corresponding author (e-mail: anwilson@clarku.edu).

 doi:10.1139/B07-026

more recently described as ectomycorrhizal (Agerer and Beenken 1998). However, Hughey et al. (2000) acknowledged that the classification of *Calostoma* as saprotrophic was probably based on previous taxonomic placement and suggested that the ecological role of this fungus should be further investigated.

Saprotrophic and ectomycorrhizal fungi often acquire carbon and nitrogen from different sources. This difference in nutrient acquisition is observed in studies of stable carbon (^{13}C) isotope abundances in fruiting bodies of known saprotrophic and mycorrhizal fungi, with saprotrophic fungi being several parts per mille (‰) enriched in ^{13}C relative to mycorrhizal fungi (Högberg et al. 1999; Hobbie et al. 1999, 2001; Kohzu et al. 1999; Henn and Chapela 2001; Taylor et al. 2003; Trudell et al. 2004; Hobbie 2005; Hart et al. 2006). In addition, because the creation of transfer compounds such as amino acids by mycorrhizal fungi results in the transfer of ^{15}N-depleted nitrogen to host plants and the retention of ^{15}N-enriched nitrogen in mycorrhizal fungi, ectomycorrhizal fungi that supply nitrogen to their host plants can be enriched in ^{15}N relative to co-occurring saprotrophic fungi (Hobbie et al. 1999, 2001, 2005; Kohzu et al. 1999; Henn and Chapela 2001; Taylor et al. 2003; Trudell et al. 2004; Hobbie 2005). The source of nitrogen in the environment may also affect ^{15}N enrichment in ectomycorrhizal fungi (Gebaur and Taylor 1999). Although values of ^{13}C and ^{15}N content vary between sites and across species of fungi, Hobbie et al. (2001) and Taylor et al. (2003) used measurements of isotopic values from known ectomycorrhizal and saprotrophic fungi to infer nutrient acquisition in fungi of uncertain ecological role. These studies suggest that ^{13}C and ^{15}N measurements could be used to infer whether *Calostoma* resembles ectomycorrhizal fungi or saprotrophic fungi in its nutrient acquisition strategy.

Molecular methods have been used in many studies to determine the identity of mycorrhizal fungi colonizing the root tips of plants (Gardes and Bruns 1993, 1996; Cullings et al. 1996; Dahlburg et al. 1997; Chambers et al. 1998; McKendrick et al. 2000; Bidartondo et al. 2002). The nuclear ribosomal internal transcribed spacer (nrITS) regions can be sequenced from ectomycorrhizal fungi found on root tips with the use of fungal-specific primers (Gardes and Bruns 1993) and can be used to identify ectomycorrhizal mantles to the level of species (Horton and Bruns 2001). By using these techniques, the identity of ectomycorrhizal root tips can then be compared with the identified fruiting bodies. Morphological features of mantles vary among species and can also provide characters for identification (Agerer 1987–1996; Horton and Bruns 2001). *Calostoma cinnabarinum* has yet to be successfully cultured despite repeated attempts with vitamin-amended media (M. Binder, personal communication, 2003).

Calostoma cinnabarinum produces fruiting bodies on soil in forests dominated by ectomycorrhizal trees. Moreover, phylogenetic analyses place *C. cinnabarinum* within the Sclerodermatineae, which is dominated by ectomycorrhizal taxa (Hughey et al. 2000; Binder and Bresinsky 2002). Therefore, it is probably ectomycorrhizal, but direct evidence is lacking. This study used isotopic, molecular, and morphological techniques to resolve the nutritional mode of *C. cinnabarinum*.

Materials and methods

Sampling

Calostoma cinnabarinum fruiting bodies and soil cores were collected from four locations in Massachusetts, USA (Table 1). Samples of fruiting bodies and plant foliage used in the isotopic analysis were collected from the Upton site. The samples consisted of three fruiting bodies of *Calostoma*; six fruiting bodies of the ectomycorrhizal genera *Boletinellus*, *Cortinarius*, *Gyroporus*, *Lactarius*, and *Scleroderma*; and four fruiting bodies of the saprotrophic genera *Armillaria*, *Lycoperdon*, and *Trametes*. In addition, a total of five foliage samples were collected from three ectomycorrhizal plant genera (*Quercus*, *Carya*, *Pinus*) for isotopic analysis. The fruiting bodies were dried and stored as vouchers. Soil cores of approximately 500–1000 mL were extracted from beneath fruiting bodies to about 15 cm below the surface. Ectomycorrhizal root tips were separated from roots using a dissecting microscope. Root tips extracted from soil cores were pooled based on morphology, stored in Eppendorf tubes in 1× Tris–EDTA buffer, and refrigerated. Samples for molecular and isotopic analyses were collected from August through October of 2003.

Isotopic analysis and sample preparation

Isotopic analyses in this study follow the methods described in Hobbie et al. 1999 and 2001 and Taylor et al. 2003. For the analyses, samples of ectomycorrhizal fungi, saprotrophic fungi, and foliage from ectomycorrhizal plants were identified to genus. Samples were dried at 50 °C. Approximately 250 mg of each sample was ground to a fine powder using a mortar and pestle with liquid nitrogen. At least half of the sampled fruiting body or plant material was kept as vouchers. Up to two replicates per sample were analyzed for $\delta^{13}C$ and $\delta^{15}N$ at the University of Virginia on a Carlo Erba elemental analyzer coupled to a Micromass Optima mass spectrometer (Fisons/VG/Micromass, Manchester, UK). The internal standards for isotopic and concentration measurements were acetanilide and apple leaves (NIST 1515). Stable isotope abundances are reported as

$$\delta^{15}N \text{ or } \delta^{13}C\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000$$

where R is $^{15}N/^{14}N$ or $^{13}C/^{12}C$ of either the sample or the reference standards of atmospheric N_2 for nitrogen and Pee-Dee belemnite for carbon. The standard deviation of isotopic measurements of the internal standards used was 0.2‰.

Statistical analyses were performed using STATISTICA v. 7.1 (StatSoft Inc., Tulsa, Oklahoma) to analyze the similarity of isotope values among *Calostoma*, ectomycorrhizal fungi, and saprotrophic fungi. A one-way ANOVA of the raw data was followed by a Tukey HSD test for unequal sample sizes.

DNA extraction, PCR, and DNA sequencing

For each of the four sites listed in Table 1, DNA was collected from ectomycorrhizal root tips using extraction protocols described in Cullings (1992) with some modification. DNA was extracted from dried *C. cinnabarinum* fruiting bodies using the E.Z.N.A. fungal DNA miniprep kit (Omega Bio-tek, Inc., Doraville, Georgia).

Table 1. Location and identity of fruiting body and ectomycorrhizal sequences, including group associations with GenBank and UNITE sequences used.

	Group			
Site or source	Boletales (Fig. 2*a*)	*Inocybe* (Fig. 2*b*)	*Russula* (Fig. 2*c*)	Unknown
Upton *	*Calostoma cinnabarinum*, AY854064[†]	HS11.3, DQ493548 HS11.13, DQ493549 HS11.20, DQ493550 HS11.26, DQ493551	HS7.3, DQ493552 HS7.4, DQ493553 HS7.14, DQ493554 HS11.1, DQ493555 HS11.2, DQ493556 HS11.6, DQ493557 HS11.15, DQ493558	
Broad Meadow Brook*	BMB A01, DQ493559 BMB B03, DQ493560 BMB B10, DQ493561 BMB B11 DQ493562 Rhizomorphs, DQ493563[‡]		BMB A07, DQ493564	BMB B02, DQ493565 BMB B07, DQ493566 BMB B09, DQ493567 BMB B12, DQ493568
Blue Hills Reservation*		BH3.27, DQ493569 BH3.28, DQ493570	BH1.1, DQ493571 BH1.5, DQ493572 BH1.6, DQ493573 BH1.10, DQ493574 BH1.12, DQ493575 BH1.16, DQ493576 BH1.17, DQ493577 BH1.19, DQ493578	
Mt. Wachusett*	MW4.10, DQ493579 MW4.12, DQ493580	MW4.5, DQ493587 MW4.7, DQ493588 MW4.10, DQ493589 MW4.12, DQ493590	MW3A.1, DQ493581 MW3A.2, DQ493582 MW3A.7, DQ493583 MW3A.16, DQ493584 MW3A.17, DQ493585 MW4.6, DQ493586	
GenBank sequences	*Boletus aestivalis*, AY130295.1 *Pisolithus* sp., AB099922.1 *Scleroderma bovista*, AB099901.1	*Inocybe nitidiuscula*, AJ534934.1 *Inocybe pudica*, AY228341.1 *Inocybe* sp., AY751558.1 Unknown EcM, AY310820.1[§]	*Russula ammoenipes*, AY061656.1 *Russula atropurpurea*, AY061654 *Russula azurea*, AY061660.1 *Russula decolorans*, AY194601.1	
UNITE sequences	*Boletus calopus*, UDB000659 *Pisolithus arhizus*, UDB001206 *Scleroderma areolatum*, UDB00121	*Inocybe lanuginosa*, UDB000615 *Inocybe napipes*, UDB000017 *Inocybe soluta*, UDB000630 *Inocybe stellatospora*, UDB000610	*Russla vinosa*, UDB000350 *Russula amethystina*, UDB000303 *Russula atropurpurea*, UDB000313 *Russula brunneoviolacea*, UDB000013 *Russula caerulea*, UDB000335 *Russula integra*, UDB000355 *Russula ochroleuca*, UDB000295 *Russula raoultii*, UDB000328 *Russula solaris*, UDB000302	

*Locations are as follows: Upton, Mass.; Broad Meadow Brook, Worcester, Mass.; Blue Hills Reservation, Milton, Mass.; Mt. Wachusett, Princeton, Mass.

[†]Sequences from fruiting bodies.

[‡]Sequences from rhizomorphs.

[§]An unknown ectomycorrhizal species.

Table 2. $\delta^{13}C$ and $\delta^{15}N$ mean values (± standard deviations) fungal groups and plant foliage and collected from the Upton site.

	$\delta^{13}C$	$\delta^{15}N$
Sample means (‰)		
Calostoma cinnabarinum (n = 3)	–23.1 (±0.2)	–12.6 (±0.3)
Mycorrhizal (n = 6)	–23.1 (±0.5)	–8.5 (±2.0)
Saprotrophic (n = 4)	–21.0 (±1.3)	–0.4 (±0.9)
Plant foliage (n = 4)	–27.4 (±1.4)	–0.2 (±1.3)
Tukey post hoc probabilities		
Calostoma vs. mycorrhizal fungi	0.999*	0.007
Calostoma vs. saprotrophic fungi	0.019	<0.001
Mycorrhizal vs. saprotrophic	0.006	<0.001

Note: $\delta^{13}C$ and $\delta^{15}N$ values of fungal groups were compared using a Tukey HSD test for unequal sample sizes.

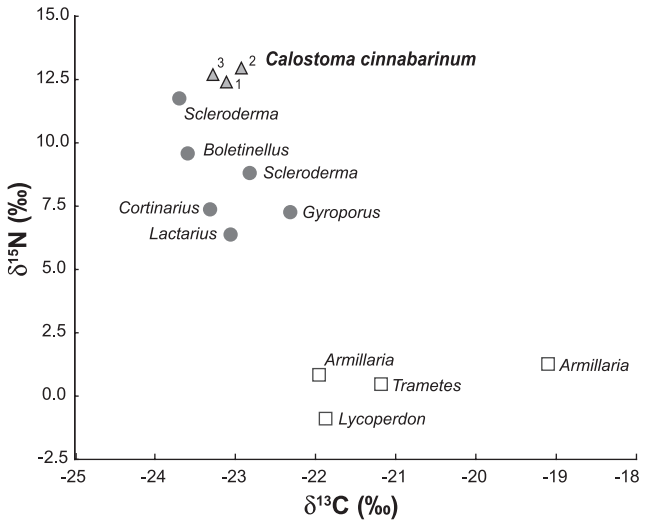
*Group comparisons are not significantly different ($P > 0.05$) from each other.

The polymerase chain reaction (PCR) and cycle sequencing were performed with primers ITS1F and ITS4B (Gardes and Bruns 1993) on a MJ Bioworks PTC 200 DNA engine thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California). Plant-specific primers ITS1-plant and ITS2-plant (Muir and Schlötterer 1999) were used for host plant identification. Cycle sequencing protocols were as follows: denature step of 94 °C for 2 min; 35 cycles of 30 s at 92 °C, 1 min at 55 °C, and 30 s at 72 °C; final extension of 72 °C for 5 min. Sequencing products were purified with GeneClean glassmilk (Q-BIOgene, www.qbiogene.com) and run on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, California). Sequence editing was performed using Sequencher v. 3.1.1 (Gene Codes Corp., Ann Arbor, Michigan).

Molecular analyses

Fungal nuclear ribosomal internal transcribed spacers (nrITS) 1 and 2 and 5.8S sequences were generated from ectomycorrhizal root tips collected from each sampling site (Table 1) and were used as queries in BLAST searches of the GenBank (www.ncbi.nlm.nih.gov/) and UNITE (unite.ut.ee/; Kõljalg et al. 2005) databases. Identified sequences with the highest E score from the BLAST query were obtained from GenBank. The two closest-related sequences from UNITE queries were also obtained for analysis. These sequences were aligned with ectomycorrhizal sequences using Clustal_X 1.81 (Thompson et al. 1997) with default settings, followed by manual alignment using MacClade v. 4.03 (Maddison and Maddison 2001). Two sets of distance analyses of DNA sequences were performed. First, a set of 70 sequences, including all the newly generated sequences and the sequences obtained from GenBank and UNITE, were analyzed together. ITS evolves rapidly, making it difficult to assess homology in alignments of distantly related taxa. The purpose of this first analysis was not to provide a rigorous phylogenetic estimate, but simply to group identified nrITS sequences and unidentified ectomycorrhizal nrITS sequences into clusters of similar sequences. Subsequently, subsets of similar sequences from the 70 sequences were realigned and subjected to independent analyses. The distance analyses used a neighbor-joining (NJ) method that employed a Kimura two-parameter model of evolution. These analyses were performed using PAUP* v. 4.0b (Swofford 2002). An NJ bootstrap analysis was performed using 1000 bootstrap replicates and the same distance analysis parameters described above.

Fig. 1. Nitrogen and carbon stable isotope values for fungi from the Upton site. Ectomycorrhizal fungi are represented by shaded circles; saprotrophic fungi are represented by open squares; samples for *Calostoma cinnabarinum* are represented by shaded triangles.



Morphological analysis

Ectomycorrhizal root tips molecularly identified as *C. cinnabarinum* were described using protocols and terminology from Ingleby et al. (1990) and Agerer (1991). Macromorphological features were observed and photographed with tissue immersed in water, using a dissecting microscope. Micromorphological features were observed using a compound microscope. For micromorphological features, root tips were processed in a combination of 10% and 50% KOH and then stained with cotton blue. Permanent slides were made with a 50% glycerol solution. Both macromorphological and micromorphological features were described with the aid of a checklist developed by the British Columbia Ectomycorrhizae Research Network (www.pfc.forestry.ca/biodiversity/bcern/index_e.html).

Results

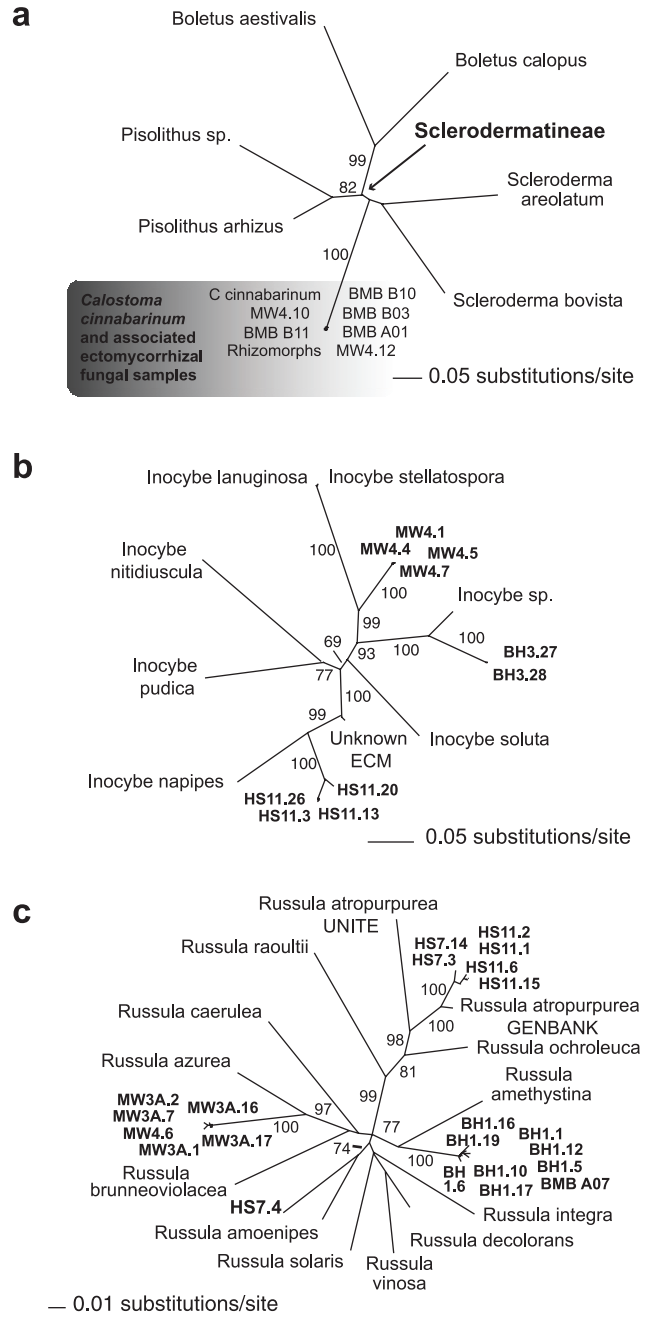
Sampling

Forty-eight *C. cinnabarinum* fruiting bodies were collected from four sites in Massachusetts. Nine fruiting bodies from a single location were collected from the Upton site. Another eight fruiting bodies were collected from two locations at Broad Meadow Brook, nine fruiting bodies were collected from two different locations at Blue Hills Reservation, and 22 were collected from four different locations at Mt. Wachusett.

Isotopic analysis

Thirteen fruiting bodies representing *Calostoma*, ectomycorrhizal fungi and saprotrophic fungi, along with five foliage samples, were collected from the Upton site. Mean isotope values and standard deviations of fungal groups and plant foliage are listed in Table 2. Isotopic values for

Fig. 2. Unrooted NJ trees of ectomycorrhizal root tip nrITS sequences and the nearest sequence matches from BLAST and UNITE databases. Three basidiomycete groups are identified: (*a*) Boletales, (*b*) *Inocybe*, and (*c*) *Russula*. Numbers adjacent to branches represent statistical support determined by NJ Kimura 2 parameter bootstrap analysis using 1000 replicates.



C. cinnabarinum are compared with ectomycorrhizal fungi and saprotrophic fungi in Fig. 1.

Saprotrophic fungi differed from both the known ectomycorrhizal fungi and *C. cinnabarinum* in $\delta^{13}C$ values (Fig. 1, Table 2). On average, *C. cinnabarinum* was 2.1‰ depleted in ^{13}C relative to saprotrophic fungi and did not differ from ectomycorrhizal fungi ($P = 0.999$; Table 2). Plants were depleted in ^{13}C by 4.2‰ relative to ectomycorrhizal fungi and by 6.2‰ relative to saprotrophic fungi.

The $\delta^{15}N$ values differed substantially among all three fungal groups (Fig. 1). *Calostoma cinnabarinum* values were 4.1‰ higher than ectomycorrhizal and 12.2‰ higher than saprotrophic fungi. Saprotrophic fungi and plant foliage differed in $\delta^{15}N$ values by 0.2‰.

Molecular analysis

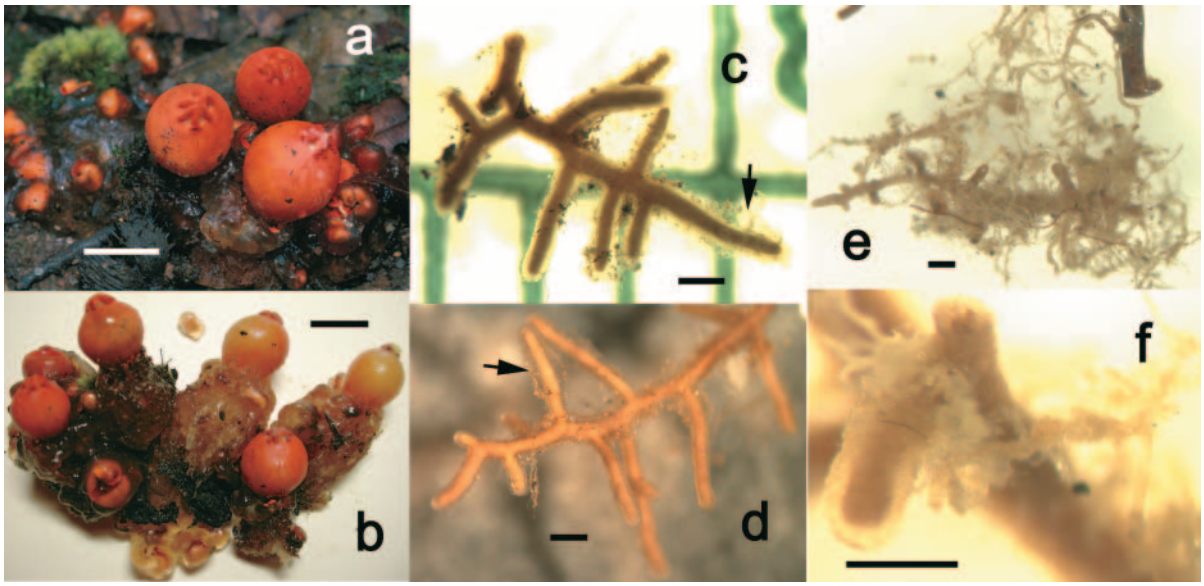
Sequences of nrITS were obtained from 43 ectomycorrhizal root tips collected from all four sites described in Table 1. Sequences of *C. cinnabarinum* fruiting bodies from the Upton site were obtained for primary identification of ectomycorrhizal sequences. The GenBank search yielded 10 identified sequences and an unknown ectomycorrhizal species (Table 1). GenBank sequences for *Inocybe* sp., *Russula atropurpurea*, *Russula azurea*, and the unknown ectomycorrhizal species received *E* values of 0.0 in BLAST searches. The seven remaining GenBank sequences received *E* values from as low as 1×10^{-128} (*Russula amoenipes*) to a high of 1×10^{-73} (*Boletus aestivalis*). The UNITE search yielded 16 sequences (Table 1).

An initial NJ tree was created from a complete alignment of all 43 ectomycorrhizal sequences, *C. cinnabarinum* nrITS, and all 27 species obtained from online databases (data not shown). The nrITS data matrix was 787 characters in length, of which 512 were removed because of ambiguities in aligning variable nrITS regions. The results of this initial NJ tree indicate that three fungal groups and one unidentified fungal sequence (sequenced from four ectomycorrhizal root tips) (Table 1) are represented in the soil cores underneath *C. cinnabarinum* in this study. The three identified fungal groups represent the genera *Inocybe* and *Russula* and the order Boletales. Sequences of the three identified fungal groups were realigned, and individual unrooted NJ trees were created (Fig. 2). Sequence alignments for these three groups were submitted to TreeBase (www.treebase.org/treebase/, study accession number S1718).

The Boletales group (Fig. 2*a*) is represented by *C. cinnabarinum*, six ectomycorrhizal sequences, a rhizomorph sequence, and six species from GenBank and UNITE in a data matrix of 749 characters. The six ectomycorrhizal and rhizomorph sequences are >99% similar to the sequence from the *C. cinnabarinum* fruit body. These ectomycorrhizal sequences were extracted from soil cores from Broad Meadow Brook and Mt. Wachusett. Sequences from *C. cinnabarinum* fruit bodies from these locations were >99% similar to the sequence from the fruit body from the Upton site. Gelatinous rhizomorphs were collected from the soil cores. They were observed most frequently from Broad Meadow Brook soil cores but rarely from Mt. Wachusett soil cores. The rhizomorphs were not observed in soil cores from the remaining two sites. In separate observations, the rhizomorphs were seen attached to *C. cinnabarinum* fruit bodies and to ectomycorrhizae. The nrITS sequence of the rhizomorphs matched the sequence of *C. cinnabarinum.*

The unknown ectomycorrhizal sequence found in this study retrieved sequences identified as uncultured Russulaceae isolate (DQ061931.1, *E* value = 3×10^{-176}) and *Rhizoctonia* sp. (DQ093652.1, *E* value = 1×10^{-166}) in searches of GenBank. This group was placed on a long branch in the initial NJ analysis of nrITS sequences (data not shown) and

Fig. 3. (*a* and *b*) *Calostoma cinnabarinum* fruiting bodies from Mt. Wachusett (scale = 1 cm). (*c* and *d*) *Calostoma cinnabarinum* ectomycorrhizal root tips. Black arrows indicate extent of gelatinous cuticle (scale = 0.5 mm). (*e* and *f*) Gelatinous rhizomorphs from *C. cinnabarinum* (scale = 0.5 mm). Photo credits: *a*, P.B. Matheny; *b*, M. Binder; *c–f*, A.W. Wilson.



did not fall among any groups identified below. The *Inocybe* group (Fig. 2*b*, Table 1) is represented by six ectomycorrhizal sequences from this study and by seven species from GenBank and UNITE in a 867 character data set. The *Russula* group represents the largest and most diverse populations within the soil cores collected underneath *C. cinnabarinum* fruit bodies (Fig. 2*c*, Table 1). This group is represented by 21 ectomycorrhizal sequences and by 13 species from GenBank and UNITE in a 678 character data set. None of the ectomycorrhizal sequences from either the *Inocybe* or the *Russula* group could be identified to species from the data available in the GenBank and UNITE databases.

Plant-specific primers used on *C. cinnabarinum* ectomycorrhizae obtained ITS1, 5.8s, and ITS2 sequences from all *C. cinnabarinum*-identified ectomycorrhizal root tips. All five sequences (DQ860279–DQ860283) were >98% similar in identity. The sequence was subjected to a BLAST search of GenBank, which retrieved the top three hits of *Quercus incana* (AY456170), *Q. buckleyi* (AF174631), and *Q. rubra* (AF098478).

Morphological analysis

Ectomycorrhizae of *C. cinnabarinum* are characterized by a thick gelatinous cuticle interwoven with thin, branched hyphae suspended in the gelatinous matrix and mantles with yellow orange to red orange colorations. Rhizomorphs are gelatinized, are the same consistency as the basidiome stipe, and have undifferentiated, clamped hyphae.

Macromorphological features

Mycorrhizal systems irregularly monopodial-pinnate, 5–20 mm in length, up to 10 branches per 10 mm, rarely occurring in enriched mineral soil and mineral soil (Figs. 3*c*, 3*d*). Ectomycorrhizal mantles in fresh root tips have a thick glutinous, gelatinous cuticle to which soil particles adhere. Most soil particles are easily washed off, remaining soil particles typically embedded in gelatinous matrix. Main axes 0.5–1 mm in diameter;branches straight to slightly bent, cylindrical, 1–8 mm in length by 0.5–0.7 mm in diameter, young tips orange yellow to light red orange, older tips red orange to brown orange. Mantle surface easily observed through translucent gelatinous cuticle, surface texture smooth to velvety with a matte luster, mantle gradually more transparent with age, host generally visible through mantle. Rhizomorphs absent on some root tips but frequent on others, generally rare, 15–100 µm in diameter, light grey orange in color, attachment restricted to a single point on mantle, surface smooth, branching frequent, round or flat in cross section, rubbery-gelatinous with texture similar to that of the fruiting body stipe; when frequent, rhizomorphs form dense interwoven clusters around root tips. No chemical reactions observed with KOH or Meltzer's reagent.

Micromorphological features

Mantle 48–100 µm thick, gelatinous cuticle 30–100 µm thick, type A-C (Agerer 1991). Outer mantle layers felt-prosenchyma, with gelatinous matrix, no special pattern discernable, hyphae cylindrical, 1.5–4 µm in diameter, smooth, hyaline, septate with clamps, hyphal junctions with 30°–60° angles, and embedded in gelatinous matrix. Middle and inner layers a net-prosenchyma to a net-synenchyma, hyphae cylindrical, 3–4 µm in diameter, smooth, hyaline, septate with clamps. Rhizomorphs forming loose to smooth-undifferentiated strands, hyphae 4–7 µm in diameter, smooth, hyaline, septate with clamps. No chemical reactions observed with KOH or Meltzer's reagent.

Material examined

BMB B10 and BMB B11 were collected from soil under collection AWW140, 9 September 2003, Broad Meadow Brook Wildlife Sanctuary, Worcester, Massachusetts; MW4.10 and MW 4.12 were collected from soil under col-

lection AWW152, 17 October 2003, Mt. Wachusett, Princeton, Massachusetts.

Discussion

Results of both molecular and isotopic analyses indicate that *C. cinnabarinum* is ectomycorrhizal. Characterization of *C. cinnabarinum* ectomycorrhizal root tips and rhizomorphs reveal that they share several morphological characters with the fruiting bodies, such as a clear gelatinous cuticle and rubbery gelatinous tissues.

Isotopic analysis

Because potential differences in site nutrient composition and in nutrient acquisition and use among taxa could complicate interpretation of isotopic data, Taylor et al. (2003) advised caution when using isotope values to infer the trophic status of a fungus. To avoid these pitfalls, a conservative approach that uses both ^{15}N and ^{13}C analyses of multiple taxa from all ecological groups occurring in the same location is suggested (Taylor et al. 2003). This study used such a scheme with material collected from a single location with multiple representatives from ectomycorrhizal and saprotrophic taxa.

Calostoma cinnabarinum was significantly higher in $\delta^{15}N$ than both ectomycorrhizal fungi and saprotrophic fungi (Fig. 1, Table 2). High $\delta^{15}N$ values in specific ectomycorrhizal taxa are correlated with proteolytic capabilities (Lilleskov et al. 2002) or with a high degree of host specificity (Hobbie et al. 2005), whereas high $\delta^{15}N$ values are unusual in saprotrophic fungi. Interestingly, in both the latter study and others (Taylor et al. 2003; Hart et al. 2006), ectomycorrhizal fungi of the Boletaceae had higher $\delta^{15}N$ values than other co-occurring ectomycorrhizal fungi. The $\delta^{15}N$ values suggest that *C. cinnabarinum* acquires and processes nitrogen in a fashion similar to that of ectomycorrhizal fungi. Although this study was not designed to address the specific causes of isotopic values for *C. cinnabarinum*, it is also possible that *C. cinnabarinum* has proteolytic capabilities. Other studies suggest that differences in ^{15}N abundance among fruiting bodies may result from the availability of soil nitrogen in different locations (Trudell et al. 2004) and different fractionation processes among different ectomycorrhizal fungi (Hobbie and Colpaert 2003; Hobbie et al. 2005). Whether fractionation in *C. cinnabarinum* occurs from source differences or from an increase in internal fractionation is unknown.

ANOVA results of $\delta^{13}C$ suggest that *C. cinnabarinum* is ectomycorrhizal (Table 2). Average *C. cinnabarinum* $\delta^{13}C$ values and ectomycorrhizal $\delta^{13}C$ values did not differ from each other, but were lower than saprotrophic fungi. The average $\delta^{13}C$ value for an ecological group is a more reliable reflection of nutrient acquisition than $\delta^{15}N$, since $\delta^{13}C$ values among taxa of a specific ecological group are less variable than $\delta^{15}N$ values (Hobbie et al. 2001; Hart et al. 2006). Combining $\delta^{15}N$ and $\delta^{13}C$ measurements in Fig. 1 shows that *C. cinnabarinum* samples cluster closely with samples from ectomycorrhizal fungi. The juxtaposition and analysis of $\delta^{15}N$ and $\delta^{13}C$ measurements between fungal groups represented in Fig. 1 and Table 2 suggest that *C. cinnabarinum* is ectomycorrhizal.

Molecular analysis

Seven of 43 ectomycorrhizal root tip sequences obtained matched ITS sequences of *C. cinnabarinum* (Fig. 2*a*), and these seven were collected from two of the four study locations (Table 1). This is direct evidence identifying *C. cinnabarinum* as an ectomycorrhizal fungus.

The remaining 36 root tip sequences collected from beneath *C. cinnabarinum* fruiting bodies represent a diverse ectomycorrhizal mycota. *Russula* has the largest representation, with approximately four species colonizing ectomycorrhizal root tips found in the soil cores (Fig. 2*c*). Many studies characterizing ectomycorrhizal communities have reported similar results (Gardes and Bruns 1996; Dahlburg et al. 1997; Kernaghan et al. 1997; Horton and Bruns 1998; Horton et al. 1999; Stendell et al. 1999). *Inocybe*, with three species (Fig. 2*b*), makes up the second-largest ectomycorrhizal group, followed by an unidentified ectomycorrhizal species (Table 1). The observation that *C. cinnabarinum* is poorly represented in the soil cores relative to other ectomycorrhizal taxa is not unprecedented. In several studies (Gardes and Bruns 1996; Dahlburg et al. 1997) of ectomycorrhizal communities, aboveground abundance of fruiting bodies corresponded poorly with belowground community structure. Gardes and Bruns (1996) studied the ectomycorrhizal community within a forest of *Pinus muricata*. Their results showed that *Suillus pungens* is similar to *C. cinnabarinum* in having a high aboveground representation but low belowground representation in the ectomycorrhizal community.

A symbiotic association of *Quercus* spp. with *C. cinnabarinum* is reasonable based on the flora in the proximity of fruiting bodies. Three of the four locations in this study were deciduous forests dominated by *Fagus* and *Quercus* species. At one (Upton site), *Fagus* was absent, leaving ectomycorrhizal hosts to be *Quercus*, *Pinus*, or *Carya*. Since no *Calostoma* ectomycorrhizae were collected from this site, we cannot identify the host. However, we assume that the most likely host would be the *Quercus* species, considering the host sequence matches from the other sites. Additional justification for this assumption comes from observations of *Quercus* spp. with *C. cinnabarinum* in Costa Rica (R. Halling, personal communication, 2004) and with *Calostoma miniata* in China (Z.-L. Yang, personal communication, 2006). Though this study suggests that *Quercus* is a primary host, the possibility that *C. cinnabarinum* is symbiotic with other ectomycorrhizal plant hosts cannot be ruled out.

Anecdotal evidence indicates that the genus *Calostoma* still associates with the order Fagales in regions where *Quercus* hosts are absent. *Calostoma rodwayii* has been described as ectomycorrhizal on *Nothofagus* in southern Australia (T. Lebel, personal communication, 2006). *Calostoma* species have also been collected in New Zealand *Nothofagus* forests. *Calostoma sarasinii* and *Calostoma berkeleyi* were recently collected by D. Desjardin and A. W. Wilson in Malaysian forests dominated by *Castanopsis* and *Dipterocarpus* species. Ectomycorrhizal symbioses between *Calostoma* species and host species other than Fagales remains a possibility.

Acknowledgements

We thank Brandon Matheny, Brian Perry, Paula Hartzell,

and Jason Slot for locating the *Calostoma cinnabarinum* populations used in this study; Brandon Matheny and Manfred Binder for the photographs of *C. cinnabarinum* fruiting bodies from Mt. Wachusett; Danielle Stehlik for assistance in the field and laboratory; Camilo Khatchikian and Rebecca Louzan for assistance with statistical analyses; John Baker for helpful comments on the manuscript; and Steve Macko for assistance with isotopic analyses. This study was funded by a National Science Foundation Doctoral Dissertation Improvement Grant to DSH and AWW (DEB-0508716), as well as grants DEB-0228657, DEB-0444531, DBI-0320875, and DEB-0235727.

References

Agerer, R. 1987–1996. Colour atlas of Ectomycorrhizae. Einhorn-Verlag Eduard Dietenberger, Schwäbisch, Gmünd.

Agerer, R. 1991. Characterization of ectomycorrhiza. Methods Microbiol. **23**: 25–73.

Agerer, R. 2002. *Gyroporus cyanescens*. *In* Colour atlas of Ectomycorrhizae. *Edited by* R. Agerer. Einhorn Verlag, Schwäbisch Gmünd. pp. 1–156.

Agerer, R., and Beenken, L. 1998. *Geastrum fimbriatum* Fr. + *Fagus sylvatica* L. Descriptions of Ectomycorrhizae, **3**: 13–18.

Bidartondo, M.I., Redecker, D., Hijri, I., Wiemken, A., Bruns, T.D., Dominguez, L., Sérsic, A., Leake, J.R., and Read, D.J. 2002. Epiparasitic plants specialized on arbuscular mycorrhizal fungi. Nature (London), **419**: 389–392. doi:10.1038/nature01054. PMID:12353033.

Binder, M., and Bresinsky, A. 2002. Derivation of a polymorphic lineage of Gasteromycetes from boletoid ancestors. Mycologia, **94**: 85–98.

Buée, M., Vairelles, D., and Garbaye, J. 2004. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus sylvatica*) forest subject to two thinning regimes. Mycorrhiza, **15**: 235–245. PMID:15221576.

Burnap, C.E. 1897. Contributions from the cryptogamic laboratory of Harvard University XXXVIII: Notes on the genus *Calostoma.* Bot. Gaz. **23**: 180–192. doi:10.1086/327486.

Chambers, S.M., Sharples, J.M., and Cairney, J.W.G. 1998. Towards a molecular identification of the *Pisonia* mycobiont. Mycorrhiza, **7**: 319–321. doi:10.1007/s005720050199.

Cullings, K.W. 1992. Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. Mol. Ecol. **1**: 233–240.

Cullings, K.W., Szaro, T.M., and Bruns, T.D. 1996. Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites. Nature (London), **379**: 63–66. doi:10.1038/379063a0.

Dahlburg, A., Jonsson, L., and Nylund, J.E. 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. Can. J. Bot. **75**: 1323–1335.

Danielson, R.M. 1984. Ecotmycorrhizal associations in jack pine stands in northeastern Alberta. Can. J. Bot. **62**: 932–939.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 113–118. PMID:8180733.

Gardes, M., and Bruns, T.D. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can. J. Bot. **74**: 1572–1583.

Gebaur, G., and Taylor, A.F.S. 1999. ^{15}N natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. New Phytol. **142**: 93–101.

Godbout, C., and Fortin, J.A. 1983. Morphological features of synthesized ectomycorrhizae of *Alnus crispa* and *A. rugosa.* New Phytol. **94**: 249–262. doi:10.1111/j.1469-8137.1983.tb04498.x.

Hart, S.C., Gehring, C.A., Selmants, P.C., and Deckert, R.J. 2006. Carbon and nitrogen elemental and isotopic patterns in macrofungal sporocarps and trees in semiarid forests of the southwestern USA. Funct. Ecol. **20**: 42–51. doi:10.1111/j.1365-2435.2005.01058.x.

Henn, M.R., and Chapela, I.H. 2001. Ecophysiology of ^{13}C and ^{15}N isotopic fractionation in forest fungi and the roots of the saprorphic-mycorrhizal divide. Oecologia, **128**: 480–487. doi:10.1007/s004420100680.

Hobbie, E.A. 2005. Assessing functions of soil microbes with isotopic measurements. *In* Microorganisms in soils: roles in genesis and functions. *Edited by* F. Buscot and A. Varma. Springer, New York. pp. 383–398.

Hobbie, E.A., and Colpaert, J.V. 2003. Nitrogen availability and colonization of mycorrhizal fungi correlate with nitrogen isotope patterns in plants. New Phytol. **157**: 115–126. doi:10.1046/j.1469-8137.2003.00657.x.

Hobbie, E.A., Macko, S.A., and Shugart, H.H. 1999. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia, **118**: 353–360. doi:10.1007/s004420050736.

Hobbie, E.A., Weber, N.S., and Trappe, J.M. 2001. Mycorrhizal vs saprotrophic status of fungi: the isotopic evidence. New Phytol. **150**: 601–610. doi:10.1046/j.1469-8137.2001.00134.x.

Hobbie, E.A., Jumpponen, A., and Trappe, J. 2005. Foliar and fungal ^{15}N:^{14}N ratios reflect development of mycorrhizae and nitrogen supply during primary succession testing analytical models. Oecologia, **146**: 258–268. doi:10.1007/s00442-005-0208-z. PMID:16096847.

Högberg, P., Plamboeck, A.H., Taylor, A.F.S., and Fransson, P.M.A. 1999. Natural ^{13}C abundance reveals trophic status of fungi and host origin of carbon in mycorrhizal fungi in mixed forests. Proc. Natl. Acad. Sci. U.S.A. **96**: 8534–8539. doi:10.1073/pnas.96.15.8534. PMID:10411910.

Horton, T.R., and Bruns, T.D. 1998. Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). New Phytol. **139**: 331–339. doi:10.1046/j.1469-8137.1998.00185.x.

Horton, T.R., and Bruns, T.D. 2001. The molecular revolution on ectomycorrhizal ecology: peeking into the black-box. Mol. Ecol. **10**: 1855–1871. doi:10.1046/j.0962-1083.2001.01333.x. PMID:11555231.

Horton, T.R., Bruns, T.D., and Parker, V.T. 1999. Ectomycorrhizal fungi associated with *Arctostaphylos* contribute to *Pseudotsuga menziesii* establishment. Can. J. Bot. **77**: 93–102. doi:10.1139/cjb-77-1-93.

Hughey, B.D., Adams, G.C., Bruns, T.D., and Hibbett, D.S. 2000. Phylogeny of *Calostoma*, the gelatinous-stalked puffball, based on nuclear and mitochondrial ribosomal DNA sequences. Mycologia, **92**: 94–104.

Ingleby, K., Mason, P.A., Last, F.T., and Fleming, L.V. 1990. Identification of Ectomycorrhizas. HMSO Publication Centre, London. Institute of Terrestrial Ecology (ITE) Res. Publ. No. 5.

Kernaghan, G., Currah, R.S., and Bayer, R.J. 1997. Russulaceous ectomycorrhizae of *Abies lasiocarpa* and *Picea engelmannii.* Can. J. Bot. **75**: 1843–1850.

Kohzu, A., Yoshioka, T., Ando, T., Takahashi, M., Kobqa, K., and Wada, E. 1999. Natural ^{13}C and ^{15}N abundance of field-collected fungi and their ecological implications. New Phytol. **144**: 323–330. doi:10.1046/j.1469-8137.1999.00508.x.

Kõljalg, U., Larsson, K.-H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Hoiland, K., Kjoller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Vralstad, T., and Ursing, B.M. 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. **166**: 1063–1068. doi:10.1111/j.1469-8137.2005.01376.x. PMID:15869663.

Kreisel, H. 1969. Grundzüge eines natürlichen systems der Pilze. Gustav Fischer Verlag/Cramer, Jena, Germany.

Lilleskov, E.A., Hobbie, E.A., and Fahey, T.J. 2002. Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. New Phytol. **154**: 219–231. doi:10.1046/j.1469-8137.2002.00367.x.

Maddison, D.R., and Maddison, W.P. 2001. MacClade 4, version 4.03. Sinaur, Sunderland, Mass.

Massee, G. 1888. A monograph of the genus *Calostoma*, Desv. (*Mitremyces*, Nees). Ann. Bot. (Lond.), **2**: 25–45.

McKendrick, S.L., Leake, J.R., Taylor, D.L., and Read, D.J. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. New Phytol. **145**: 539–548. doi:10.1046/j.1469-8137.2000.00592.x.

Miller, O.K., and Miller, H. 1988. Gasteromycetes: morphological and developmental features with keys to the orders, families, and genera. Mad River Press, Eureka, Calif.

Moyersoen, B., and Beever, R.E. 2004. Abundance and characteristics of *Pisolithus* ectomycorrhizas in New Zealand geothermal areas. Mycologia, **96**: 1225–1232.

Muir, G., and Schlötterer, C. 1999. Limitations to the phylogenetic use of ITS sequences in closely related species and populations — a case study in *Quercus petraea* (Matt.) Liebl. [online]. Available from webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm [accessed 18 July 2006].

Stendell, E.R., Horton, T.R., and Bruns, T.D. 1999. Early effects of prescribed fire on the structure of the ectomyorrhizal fungus community in the Sierra Nevada ponderosa pine forest. Mycol. Res. **103**: 1353–1359. doi:10.1017/S0953756299008618.

Sunhede, S. 1989. Geastraceae (Basidiomycotina). Morphology, ecology and systematics with special emphasis on the North European species. Syn. Fung. **1**. Fungiflora, Oslo.

Swofford, D.L. 2002. PAUP*. Phylogenetic analysis using parisimony (*and other methods), version 4 beta 10. Sinauer, Sunderland, Mass.

Taylor, A.F.S., Fransson, P.M., Högberg, P., Högberg, M.N., and Plamboeck, A.H. 2003. Species level patterns in ^{13}C and ^{15}N abundance of ectomycorrhizal and saprotrophic fungi sporocarps. New Phytol. **159**: 757–774. doi:10.1046/j.1469-8137.2003.00838.x.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**: 4876–4882.

Trudell, S.A., Rygiewicz, P.T., and Edmonds, R.L. 2004. Patterns of nitrogen and carbon stable isotope ratios in macrofungi, plants and soils in two old-growth conifer forests. New Phytol. **164**: 317–335. doi:10.1111/j.1469-8137.2004.01162.x.