

Project Description

Introduction

The genus *Calostoma* comprises a morphologically unusual group of gasteroid fungi (puffballs). The genus name, "beautiful mouth", aptly describes this genus, which forms a brightly colored raised opening at its apex, from which it releases its spores (FIG 1a-b). The genus has been placed in multiple fungal groups including its own family, the Calostomataceae. Recent molecular studies have placed this genus in the suborder Sclerodermatineae of the Boletales, a large order of fungi that includes many ectomycorrhizal species, as well as a few saprotrophic species (Hughey et al 2001, Binder and Bresinsky 2002). Most Boletales have a characteristic cap and stalk morphology (boletoid), while others, including *Calostoma*, are puffballs that form spores internally (gasteroid) (FIG 1). *Calostoma* has been interpreted by some authors (Miller and Miller 1988, Bougher and Syme 1998) as being saprotrophic (litter/wood decomposing). However, based on its relationship to the Boletales, we suspect the genus to be ectomycorrhizal (ECM).

While the molecular ecology and phylogeny of boletoid and some gasteroid Boletales have been well investigated (Dahlberg and Stenlid 1990; Kretzer et al 1996; Binder et al 1997; Kretzer and Bruns 1997, 1999; Grubisha et al 2001), recent studies in the Sclerodermatineae have been few. No studies have explored the ecology or taxonomic structure of *Calostoma* in depth. Twenty-nine species have been described in *Calostoma*, which are distributed from the Americas to Asia and Australasia, but so far only two species (*C. cinnabarinum* and *C. ravenellii*) have been included in any phylogenetic study (Hughey et al 2001, Binder and Bresinsky 2002).

The proposed research has three major goals: **1.** Describe the ecological role of *Calostoma* using molecular methods and analysis of stable isotopes. As part of this work, the plant hosts of temperate and tropical species will be identified and patterns of host switching will be resolved. **2.** Test the monophyly of *Calostoma* and resolve its higher-level phylogenetic position within the Sclerodermatineae. **3.** Infer species-level relationships within the genus using phylogenetic analyses of a comprehensive sampling of *Calostoma* taxa, including material collected in Southeast Asia (Thailand and Malaysia). Thus, the proposed research represents an integrated study of the phylogeny, taxonomy and ecology of an enigmatic group of basidiomycetes.



FIGURE 1. a) *Calostoma cinnabarinum* from eastern US (Mike Wood). b) *C. sarasinii* from Malaysia. (D. Desjardin). c) *Scleroderma cepa* (Mike Wood, MykoWeb.com) d) *Gyroporus purpurinus* (Kuo, M. (2003, August). MushroomExpert.Com: http://www.bluewillowpages.com/mushroomexpert/gyroporus_purpurinus.html). Use NSF FastLane to view this image in color.

Background Information

• Morphology, Taxonomy, and Systematics

Morphology and Taxonomic History - *Calostoma* fruiting bodies form a globose spore bearing head that is composed of three layers. The outer layer forms a thick gelatinous cuticle, or a cuticle that forms scales. Upon maturity, the outer layer peels away or dries up to

expose the tough and persistent second layer. At the apex of this layer is a brightly colored, star shaped ostiole with raised ridges that split to expose the spores inside. The final layer consists of a spore sac. *Calostoma* fruiting structures are sessile or are elevated on a stalk composed of gelatinized, interwoven strands. Because of its unusual morphology, *Calostoma* has been allied with numerous groups of gasteromycetes, including Phalloideae (stinkhorns), *Geastrum* (earthstars), and *Tulostoma* (the stalked puffballs); it has also been placed in its own family Calostomataceae (Masse 1888, Burnap 1897, Fischer 1990, Cooker and Couch 1928, Miller and Miller 1988, Li Fan et al 1994). Twenty-nine species have been described in *Calostoma*.

Molecular Phylogenetics - Hughey et al (2000) performed a phylogenetic analysis of ribosomal genes (rDNA), which showed that *Calostoma cinnabarinum* and *C. ravenellii* are placed within the Boletales. Later, Binder and Bresinsky (2002) included these species in their study of gasteroid Boletales. Binder and Bresinsky's analysis placed the genus within a well-supported lineage of gasteroid fungi, which they recognized as the suborder Sclerodermatineae. Besides *Calostoma*, several other genera of gasteroid fungi are placed within the Sclerodermatineae, including the thick-skinned puffball *Scleroderma* (FIG 1c), the earthstar-like *Astraeus* and the chambered puffball *Pisolithus*. Surprisingly, the boletoid genus *Gyroporus* (FIG 1d) was placed as the sister group of *Calostoma* (Binder and Bresinsky 2002). However, this relationship received only 56% bootstrap support, with each group represented by only two species and both relegated to long branches. In summary, *Calostoma* has been shown to be a member of the Sclerodermatineae (Boletales), but its precise placement is poorly resolved, only two out of 29 species have been analyzed, and only rDNA sequences have been used to study the relationships of the genus. Thus, there are still many open questions regarding the higher-level and infrageneric relationships of *Calostoma*.

- Ecology and Biogeography

Hypothesized Ecological Status - Because of its prior taxonomic classifications, *Calostoma* has been described as saprotrophic. Habitat records from the literature and herbarium collections, however, tend to be limited. Where complete descriptions are available there is a suggestion of an ECM lifestyle for *Calostoma* species. *Calostoma cinnabarinum*, which is the most common species of *Calostoma* in North America, fruits on soil in mixed woods associated with *Quercus*, *Fagus* and *Carya* (Fagales). *Calostoma* species have also been collected in highland forests of Costa Rica under *Quercus* (R. Halling pers com). Three species of Australian and New Zealand *Calostoma* are associated with *Nothofagus* (Fagales, Nothofagaceae) and *Eucalyptus* (Myrtaceae) as indicated on collections. Habitat descriptions for almost all Asian species lack sufficient detail on plant associations with information limited to the substrate. One exception is a single report of *C. junghuhinii* from Nepal (Miller and Cotter 1988) in a mixed dicot forest with *Quercus semicarpifolia*. Other tropical members of the Boletales have been associated with plant families Dipterocarpaceae, Myrtaceae and Fagaceae (Trappe 1962, Molina et al 1992, H. Besl pers com). *Calostoma* from Malaysia has been associated with *Castanopsis* species (H. Besl pers com). Most ecological information that can be gathered about *Calostoma* suggests a predominant association with the family Fagaceae, however host switching to the family Myrtaceae is documented from Australian collections.

Distribution and Collections - *Calostoma* has a disjunct distribution. It occurs in Central Asia, Southeast Asia, and Australasia, as well as eastern North America, Central America, and northern South America. There are no reports of *Calostoma* spp. from Africa or Europe. This Australasian-Asian-American distribution resembles that of certain other fungal groups, such as *Lentinula* (Hibbett et al 2001) and *Cyttaria* (Korf 1983).

There appears to be a high level of endemism within the genus. Three species of *Calostoma* (*C. cinnabarinum*, *C. lutescens*, *C. ravenelii*) occur from North America down through Central America and into northern South America. American species of *Calostoma* are collected frequently and are well represented in several herbaria. There are three species that occur in Australia and New Zealand. Collections of these can be found in the Herbarium at the Royal Botanical Garden, Melbourne. Collections of a purportedly new Australian species have been made recently (Tom May, pers com). Within the last 30 years, numerous species of *Calostoma* from China have been reported or described (Liu Po et al 1975, 1984, Liu Bo 1985, Li Fan 1994). Most of these are available from Chinese herbaria. Southeast Asia is a region of high diversity for *Calostoma*. Up to seven species have been described from the region (Bodjin 1938, Lim 1969), five of these being endemic. Most of the herbarium material of *Calostoma* from Southeast Asia was collected in the early to mid 1900's. These collections are probably too old to serve as sources of DNA, so it will be necessary to obtain new collections for molecular studies.

Molecular and isotopic analysis of fungal nutritional modes - Molecular approaches are now routinely used to study the ecology of ECM fungi (Gardes and Bruns 1993, Bruns et al 1998, Horton and Bruns 2001), with analyses of the internal transcribed spacers (ITS) of rDNA being especially popular. Analyses of stable isotopes can also be used to determine whether a fungus is saprotrophic or mycorrhizal (Hobbie et al 1999, 2001, 2002). Nitrogen and carbon isotopes in the environment can serve as potential tracers of metabolism and nutrient cycling in fungi. Environmental samples from a variety of fungal fruiting bodies show that saprotrophic fungi are higher in $\delta^{13}\text{C}$ than ECM fungi (Hobbie et al 1999). Gleixner et al (1993) explained the reason for this by tracing the deposition of carbon isotopes to the cellulose in cell walls. Nitrogen isotopes are typically higher in ECM fungi than saprotrophic fungi. As a result, measurements of the relative abundance of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ can provide support for a particular mode of nutrition. Hobbie et al (2001) created a combined index ($\Delta_{\text{CN}} = \delta^{13}\text{C}_{\text{AVE}} - \delta^{15}\text{N}_{\text{AVE}}$), which is defined as the difference between average fungal $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in the study. A combined index regression line can help distinguish between clusters representing saprotrophic and mycorrhizal fungi when individual fungal isotope values are plotted on a chart (See Preliminary Results, FIG 2b). Analysis of radiocarbon (^{14}C) is also useful, but the assays are cost prohibitive (Hobbie et al 2002). In order to effectively compare the difference in isotopic levels between fungi, specimens of ECM fungi, saprotrophic fungi, and plant tissues from the same locality must be analyzed (Hobbie et al 1999, 2001, 2002).

Proposed Research

The proposed research addresses the ecology and evolution of the genus *Calostoma*, and is divided into three linked studies that are organized according to the three major goals described in the introduction:

- **Goal 1: Describe the ecological role of *Calostoma* using molecular methods and analyses of stable isotopes, and identify the plant hosts of temperate and tropical species.** - This study will describe the ecological role of *Calostoma* using molecular methods and analysis of stable isotopes. This research involves field-work that will be conducted in North America (Massachusetts) and Southeast Asia (Thailand and Malaysia; see description of Goal 3 for details). We have experience using all of the methods proposed here to describe the ecology of *C. cinnabarinum* (see Preliminary Results).

Sampling—general methods - *Calostoma* fruiting bodies will be collected and soil cores will be extracted from beneath the fruiting bodies. ECM root tips will be separated from soil core roots using a dissecting microscope. Root tips from a single collection will be

pooled based on morphology, stored in TE buffer, and refrigerated until molecular analysis can be performed. DNA will be extracted from fruiting bodies with the remaining tissues dried and stored as vouchers. Macroscopic descriptions and images of fresh ectomycorrhizal root tips of *Calostoma* spp. will be made using a dissecting scope. Microscopic description of the mantle hyphae and images of the stained ectomycorrhizal root tips will also be taken using a compound light microscope and follow protocols described by Agerer et al (1998).

1a. Isotopic Analysis - For isotopic analysis specimens of both ectomycorrhizal and saprotrophic fungi, as well as foliage from local ECM-associated plants, will be collected in the same localities as *Calostoma* fruiting bodies. Sporocarps and plant material will be identified at least to genus, dried, and kept as vouchers. A portion of the samples will be ground into a fine powder using liquid nitrogen at Clark University. Stable carbon and nitrogen isotope abundances will be measured at the University of New Hampshire in the isotope ratio mass spectrometer facility run by Erik Hobbie (pers com). Stable isotope abundances will be reported as $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ ‰ (Hobbie et al 1999). Data for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ will be compared across samples. A chart plotting individual fungal $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values and a regression line derived by the combined index (Hobbie et al 2001) will be used to graphically distinguish where *Calostoma* falls among clusters of identified mycorrhizal and saprotrophic fungi.

1b. Molecular Analysis - Two to three ECM root tips will be used for extraction following protocols described by Ken Cullings (1992) with some modification. The polymerase chain reaction (PCR) will be performed using the protocols of Vilgalys and Hester (1990). PCR and cycle sequencing reactions will use ITS primers ITS1F and ITS4B (Gardes and Bruns 1993). *Calostoma*-specific primers will be created to aid screening of ectomycorrhizal root tips for the presence of *Calostoma* spp. Plant-specific primers ITS 1-plant and ITS 4-plant (Muir & Schlötterer 1999) will be used to identify plants associated with *Calostoma* spp. PCR products will be purified with GeneClean glassmilk (Q-BIOgene www.qbiogene.com). Sequence editing will be performed using Sequencher v 3.1.1 software (GeneCodes Corp, Ann Arbor, Michigan).

ITS sequences obtained from ECM root tips and *Calostoma* fruiting bodies will be aligned with closely matching sequences obtained through a BLAST search of the GenBank database. Initial alignments will be performed in Clustal X 1.81 (Thompson et al. 1997) with additional alignment by hand in MacClade v 4.03 (Maddison and Maddison 2001). Phylogenetic trees will be estimated using PAUP* v 4.0b (Swofford 2002; see Goal 2 for discussion of analytical approaches) and will be used to match sequences. PCR products from root tips obtained using *Calostoma*-specific primers will be visualized using gel electrophoresis and will be screened for the presence of *Calostoma* DNA. Sequences obtained using ITS plant-specific primers will be used as queries in BLAST searches of GenBank, and phylogenetic analyses of the retrieved sequences will be used to identify species or genera of plants associated with *Calostoma* spp.

• **Goal 2. Test the monophyly of *Calostoma* and resolve its higher-level phylogenetic position within the Sclerodermatineae.** - This study will take a multi-locus approach, using six of the seven genes that are being sampled in Assembling the Fungal Tree of Life (AFTOL) project, to estimate the phylogeny of *Calostoma* within the context of the Sclerodermatineae.

2a. Sampling of taxa and loci - 37 species representing 8 genera from the suborder Sclerodermatineae have been chosen, with 3 other species of Boletales included from the AFTOL project (TABLE 1). These 37 representatives of the Sclerodermatineae are chosen based on their relationship to *Calostoma* (Binder and Bresinsky 2002), their taxonomic representation as type species and the availability of viable material or DNA. This sampling scheme will help identify different patterns of evolution within the Sclerodermatineae,

identify the sister group to *Calostoma*, and resolve the position of outliers should the genus prove to be non-monophyletic. Eight species of *Calostoma* have been chosen to represent geographic and morphological variation within the genus. Two species occur in the Americas, *Calostoma cinnabarinum* and *C. ravenelii*. Another two species occur in Australia and New Zealand, *C. fuscum* and *C. rodwayi*. *Calostoma japonicum* can be found in Japan and mainland Asia. *Calostoma junghuhinii* ranges from Nepal to Southeast Asia. *Calostoma insignis* is found in Thailand and Indonesia and *C. sarasinii* is found in Malaysia. Of these taxa only *C. cinnabarinum* and *C. insignis* form a thick gelatinous cuticle. *Calostoma cinnabarinum*, *C. ravenelii*, *C. fuscum* and *C. japonicum* form ellipsoid spores while the rest form globose spores. Spore features on these taxa range from reticulate to echinulate. Preliminary nLSU analysis of an unknown fungus resembling *Diplocystis* places it within the Sclerodermatineae. This association will be confirmed by including specimens of identified *Diplocystis wrightii* in this analysis.

Laboratory protocols will follow those outlined in section 1b above. Regions for study, RNA polymerase II subunits (RPB1 and RPB2), elongation factor 1- α (EF1- α), ATPase subunit 6 (atp-6), ITS and nLSU have been shown to be informative at various phylogenetic levels for basidiomycete fungi (Matheny et al 2002; Liu et al 1999; O'Donnell et al 1998, 2001; Rehner 2001; Kretzer and Bruns 1999). Oligonucleotide primers for PCR and cycle sequencing reactions have been created for each region as part of the AFTOL project and are available online (<http://ocid.nacse.org/research/aftol/primers.php>). Cloning of PCR products from RPB2 will be necessary for sequencing based on observations of intron length polymorphisms in *Calostoma cinnabarinum* (B. Matheny pers com).

2b. Phylogenetic analysis - Combining data from different loci can improve or reduce phylogenetic accuracy depending on whether or not the loci have the same phylogenetic history, and evolve according to similar models (Bull et al. 1993, Cunningham 1997, Downton and Austin 2002). A preliminary analysis of the above regions will be performed to assess their combinability. Preliminary analysis of the Boletales using combined AFTOL loci show dramatic improvement for support of clades compared to nuclear rDNA alone (M. Binder pers com).

Creation of data sets for all analyses will be performed using Clustal X 1.81 (Thompson et al. 1997) with re-positioning of sequences by hand in MacClade v 4.03 (Maddison and Maddison 2001). Amino-acid

sequences will be used to aid in initial alignment of protein coding genes. Preliminary analyses will use the Shimodaira-Hasegawa (SH) test to compare constrained tree topologies from different loci for strongly supported conflict (SH 1999). Topologies for testing conflicts will be created for each locus using Maximum Parsimony (MP) and Bayesian analysis of individual loci (see below). Genes used in the complete

TABLE 1: Taxon sampling for phylogenetic study of *Calostoma* within the Sclerodermatineae

Sclerodermatineae
<i>Astraeus hygrometricus</i> * ^a , <i>pteridis</i>
<i>Boletellus merulioides</i> * ^a , <i>exiguus</i> , <i>rompelii</i>
<i>Phlebopus beniensis</i> , <i>brasiliensis</i> , <i>portentosus</i> , <i>sudanicus</i> , <i>sulfureus</i>
<i>Calostoma cinnabarinum</i> * ^a , <i>fuscum</i> *, <i>insignis</i> , <i>japonicum</i> , <i>junghuhunii</i> , <i>ravenelii</i> , <i>rodwayi</i> , <i>sarasinii</i> *
<i>Gyroporus castaneus</i> *, <i>cyanescens</i> , <i>purpurinus</i> , <i>rosealbus</i>
<i>Pisolithus albus</i> , <i>arrhizus</i> , <i>tinctorius</i> *
<i>Scleroderma areolatum</i> *, <i>bovista</i> , <i>cepa</i> , <i>citrinum</i> *, <i>dictyosporum</i> , <i>echinatum</i> , <i>geaster</i> , <i>meridionale</i> *, <i>sinnamariense</i> , <i>verrucosum</i>
<i>Veligaster columnaris</i>
<i>Diplocystis wrightii</i>
Other Boletales
<i>Aureoboletus thibetanus</i> * ^a , <i>Boletellus projectellus</i> * ^a , <i>Paxillus vernalis</i> * ^a

*taxa chosen for preliminary sequencing and analysis. ^aAFTOL species.

Underlined species indicate DNA or herbarium material obtained

analysis of all 38 taxa will be chosen based on the amount of support in the individual gene tree topology and presence of strong conflict detected with the SH test. Sequences from all combinable loci will be combined into a single data matrix for estimation of the species phylogeny.

Phylogenetic analyses will be performed using MP and maximum likelihood (ML) methods in PAUP* v 4.0b (Swofford 2002) and Bayesian analysis performed with MrBayes version 3.0 (Huelsenbeck and Ronquist 2001). Prior to these analyses, the combined data set will be divided into partitions that will be analyzed and assigned different models of evolution (ML, Bayesian analysis). MP analyses will treat gaps as missing data and will use 1000 heuristic searches with MAXTREES set to auto-increase, random taxon addition sequences, branch swapping set to TBR, and saving all most parsimonious trees. Parsimony bootstrap analysis will be performed using 1000 replicates with 10 random taxon sequence additions per replicate. Models of evolution will be determined using likelihood ratio tests implemented in ModelTest v 3.06 (Posada and Crandall 1998). ML analysis will use trees generated in MP as starting trees and TBR branch swapping. Bayesian analysis will operate under the same evolutionary model selected for ML. Bayesian analysis will employ up to 8 chains and run for at least one million cycles sampling every 100th tree. Trees sampled prior to convergence on a stable log likelihood value will be eliminated as part of the initial burn-in. Posterior probabilities will be generated using a 50% majority rule consensus of all trees produced in the Bayesian analysis. Statistical support for branches in the tree will be based on MP bootstrap and posterior probability values (Alfaro et al 2003, Cummings et al 2003, Huelsenbeck et al 2002). A SH test of constrained trees will be used to test the monophyly of *Calostoma* and possible sister group relationships.

• **Goal 3: Infer species-level relationships within the genus using phylogenetic analyses of a comprehensive sampling of *Calostoma* taxa** - Morphological data and phylogenetic species recognition (Taylor et al 2001) will be used to address species limits. Attempts to culture *Calostoma cinnabarinum* from either tissues or spores have been unsuccessful (M. Binder pers com), for this reason the biological species concept will not be employed.

3a. Sampling of taxa and loci - Individual and multiple samples from each of approximately 29 species of *Calostoma* will be obtained from herbarium samples or field collections. To authenticate herbarium samples, morphological characters from collections will be compared to descriptions in the literature. When possible, DNA will be extracted from type specimens and ITS sequences used to authenticate collections. Herbarium samples of species from New Zealand, China, Thailand and Malaysia have already been sampled (see Preliminary Results). Loans from the Royal Botanical Garden in Melbourne, Australia have been arranged (Tom May pers com).

AFTOL loci that will be efficient in analyzing relationships at low taxonomic levels will be chosen using the preliminary analysis methods described above. The ITS region will probably provide resolution at the infrageneric level, based on results in other genera of fungi eg. *Lentinula* (Hibbett 1995), *Suillus* (Kretzer et al 1996), *Gasterosuillus* (Kretzer and Bruns 1997), *Gymnopus* (Wilson et al 2004). Intron sequences in protein-coding genes will also be investigated as sources of characters. Phylogenetic analyses will be performed as described above. Congruence of multiple gene genealogies will be used to assess species limits (Taylor et al 2001).

3b. Fieldwork - Fieldwork in Southeast Asia is proposed to support both the taxonomic and ecological aspects of the proposed research. A minimum of seven species of *Calostoma* is reported from Thailand through Malaysia and Indonesia (Bodjin 1938, Lim 1969). Collecting is proposed in Thailand and Malaysia. A formal affiliation with Dr. Dennis Desjardin and Dr. Kevin D. Hyde at the Mushroom Research Center, Pa Pae, Chiang Mai

Province, Thailand has been established as a base of operations (see Supplementary Documentation). Malaysian collecting sites where high diversity of *Calostoma* species are reported are Pahang, Cameron Highlands, and Gunung Beremban, mountain rain forests composed of *Castinopsis* (H. Besl pers com). Dr. Dennis Desjardin has established Malaysian contacts and formal affiliations with these contacts are being established at the time of this proposal. Primary collecting will be carried out where *Calostoma* species are known to occur based on habitat descriptions, collection information, and field observations (H. Besl pers com). Other species of Boletales, including members of the Sclerodermatineae, are also likely to be found and will be collected. Location (GPS), habitat description, date, and macromorphological data including photographs will be recorded for each specimen collected. Collections will be identified, dried, and stored at the Mushroom Research Center, Thailand with tissue samples necessary for phylogenetic and isotopic analysis brought back to the US for processing. Ecological studies of *Calostoma*, including molecular and isotopic analyses, will follow methods described above.

Timetable

June 2005:	Complete ecological studies of <i>C. cinnabarinum</i> , Fieldwork in eastern US, Visit herbaria, DNA Sequencing.
2005-2006:	DNA sequencing, Field studies in Thailand and Malaysia (May 10 - June 10 2006), isotope analysis of tropical <i>Calostoma</i> .
2006-2007:	DNA sequencing, Data analysis, Preparation of dissertation defense (Spring 2007).

Significance

Intellectual Merit - This study will provide ecological and phylogenetic information for a morphologically unusual and largely understudied group within the Boletales. Ecological patterns of host switching between tropical and temperate ECM species will be explored. Information on species limits will facilitate future taxonomic and ecological studies within the group.

Broader Impact - Results of ecology and phylogenetic studies will be contributed to the Tree of Life web project promoting fungal systematics and enhanced understanding of an understudied group. Results from these studies will be presented at national (MSA 2005-2007) and international (IMC 2006) conferences.

Preliminary Results

- Ecology of *Calostoma cinnabarinum* - Fruiting bodies of *C. cinnabarinum* have been sampled from four locations in Massachusetts. Fruiting bodies were collected along with soil cores from beneath the fruiting bodies. Soil samples were stored for later use. Danielle Stehlik, an undergraduate at Clark University, received training in methods of fungal ecology and assisted with collection of fruiting bodies and soil cores.

One location, where *C. cinnabarinum* and multiple species of ECM and saprotrophic fungi were fruiting in abundance, was chosen for the isotopic study. Thirteen fungal fruiting bodies were collected. Six mycorrhizal specimens were identified in the genera *Lactarius*, *Gyroporus*, *Scleroderma* (2), *Cortinarius* and *Boletinus*. Four saprotrophic specimens represent the genera *Armillaria* (2), *Lycoperdon*, and *Trametes*. Three collections of *C. cinnabarinum* were made. In addition, samples from five plant specimens representing genera *Quercus*(3), *Pinus*, and *Carya* were made. Isotopic values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were generated for all fungi. Analysis of the data show the biggest difference between ECM and saprotrophic fungi in $\delta^{15}\text{N}$ with *C. cinnabarinum* falling well within expected levels for ECM fungi (FIG 1a). Values for $\delta^{13}\text{C}$ did not show a significant difference between ECM and

saprotrophic fungi. A combined index of $\delta^{15}\text{N} = \delta^{13}\text{C} + \Delta_{\text{CN}}$, where $\Delta_{\text{CN}} = 29.2\%$, creates a regression line (FIG 2b) and shows clear distinction between the two clusters of fungi with *C. cinnabarinum* clearly falling into the ECM cluster.

For molecular analysis ECM root tips were separated from the soil cores with water and forceps. Tips were separated into individual eppendorf tubes based on morphology. ECM root tip sequences from ITS1-F and ITS4-B primers were generated, compared to GenBank sequences via a Blast search, and incorporated into a data matrix with ITS sequences of *C. cinnabarinum*. A neighbor-joining tree was generated to visually compare the level of similarity between sequences (FIG 3). Results show that several root tip ITS sequences are highly similar to the sequences of *C. cinnabarinum*. *Calostoma* specific primers were developed and have shown in a preliminary analysis to amplify only *Calostoma* DNA and not the DNA of closely related genera. PCR reactions using specific primers on root tips identified as *Calostoma* show clear bands on an electrophoresis gel. Similar analyses using root tips from different ECM species show multiple banding patterns. Additional studies will be performed to establish specificity of the primers and test alternate hypotheses for the ecology of *C. cinnabarinum*.

- Molecular phylogenetic analyses - New ITS sequences have been obtained for 4 species of *Calostoma* and 2 from different genera within the Sclerodermatineae (*Astraeus*, *Gyroporus*). New nrLSU sequences have been obtained for 4 species of *Calostoma*. Drs. Manfred Binder and Brandon Matheny have generated six of the seven AFTOL loci from *Calostoma cinnabarinum*.

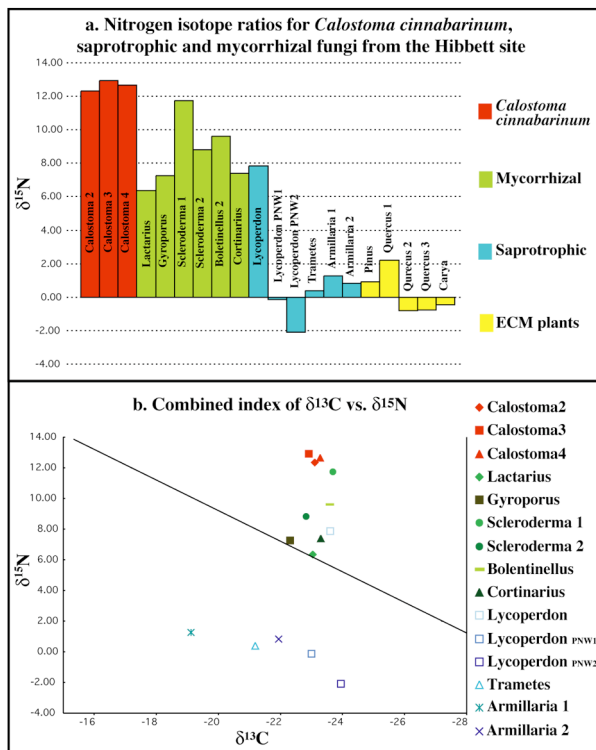


FIGURE 2. Stable isotope analysis to determine nutritional mode of *Calostoma cinnabarinum*. a) Comparison of nitrogen stable isotope values among fungal fruiting bodies. b) Combined index (regression) is drawn to separate ECM (solid, green) from saprotrophic (open, blue) fungi. Use NSF FastLane to view this image in color.

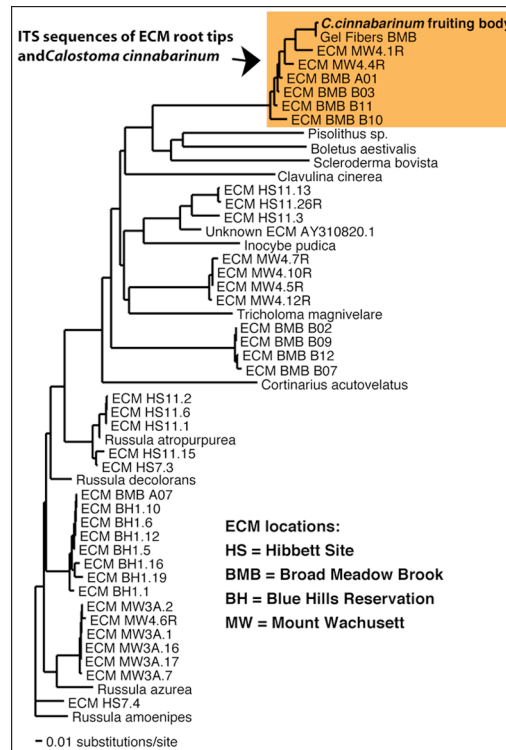


FIGURE 3. Neighbor Joining tree with "p"-corrected distances of ITS regions 1 and 2 for ECM root tips collected under *Calostoma cinnabarinum* fruiting bodies. Use NSF FastLane to view this image in color