

Ectomycorrhizal fungi

Developing Selective Medium for Ectomycorrhizal Fungi Isolation

Field Collection, Attempted Isolation And Reagent Testing

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Honors Thesis



A milky cap showing aberrant growth

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Abstract:

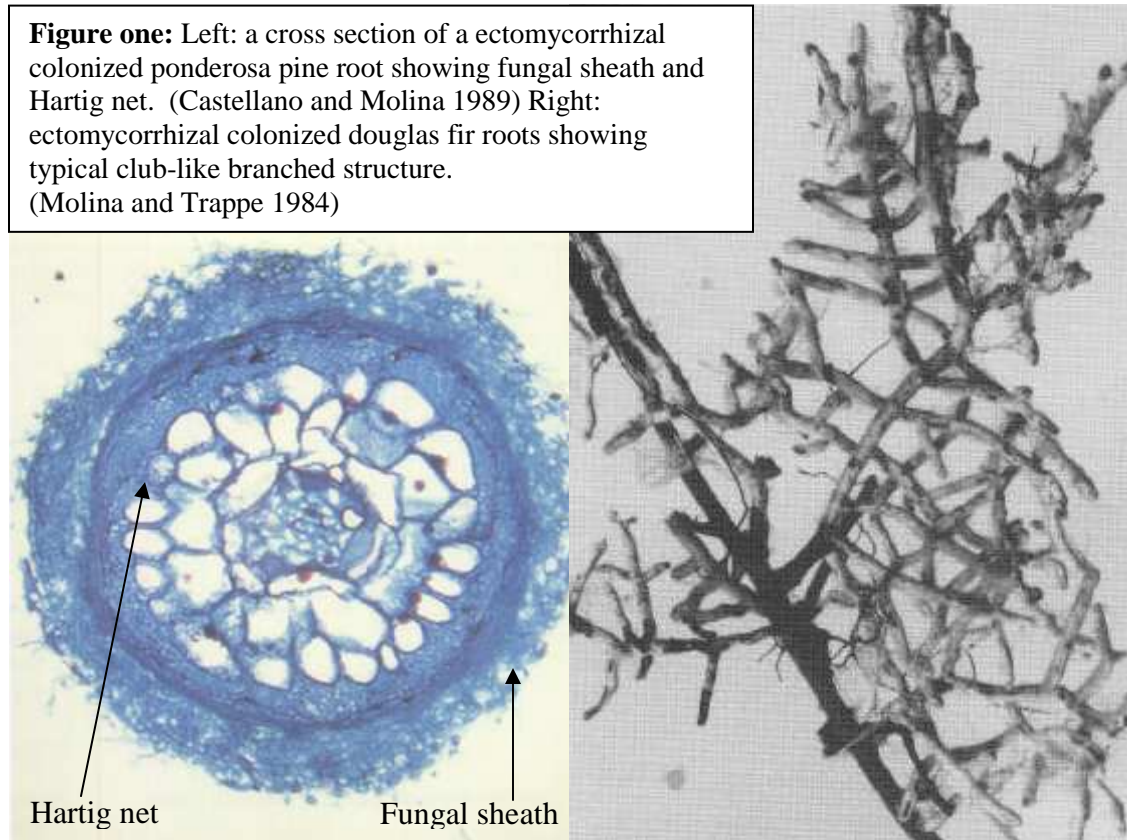
Ectomycorrhizal fungi are a group of organisms that enter into mutualistic associations with many plants, greatly benefiting the ability of these plants to compete for necessary resources. Not only do these fungi assist plant growth, but many species of ectomycorrhizal fungi produce edible fruiting bodies of economic importance. Efforts to study these organisms have been hampered by the difficulties involved in isolating pure cultures since their tissue contains many contaminants. This study incorporated work with ectomycorrhizal fungi including field collection, isolation attempts and the testing of the fungicide benomyl for use in selective isolation medium. Field collection was highly successful with many specimens being procured. However, isolation attempts yielded only a variety of contaminants. These were used in the later fungicide testing along with a purchased culture of *Armalariella mellea*, a species closely related to several important ectomycorrhizal species. Inhibition testing revealed that the fungicide benomyl holds promise for use in selective medium because the mold contaminants were inhibited at concentrations much lower than those which inhibited *Armalariella mellea*.

Background:

The ectomycorrhizal association is a mutualism (a relationship in which all participants benefit) between a fungus and a plant that is very important to many forest ecosystems. The word "mycorrhizae" loosely translated means "fungus roots" and is an apt description of this association. The fungal component of this mutualism can belong to either the phylum *Ascomycota* or *Basidiomycota* and can be referred to as the "mycobiont." Formation of the ectomycorrhizal association begins as the mycobiont

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infects a growing plant root. As the root matures, a fungal sheath envelops and encases the root. The plant does not produce root hairs, which are small hair-like structures required for the plant to take up water and nutrients in the absence of an ectomycorrhizal mutualism. Instead of root hairs, the fungal hyphae, one-cell-thick fungal threads that constitute the vegetative structure of the organism, enter the cortex of the root and form a network around the cortical cells called a Hartig net (see figure 1). At no point do they penetrate the cell wall. Ectomycorrhizal formation is accompanied by increased branching of the root as it grows. These morphological characteristics distinguish ectomycorrhizal associations from other mycorrhizal fungus-plant mutualisms (Amaranthus et. al. 1993, Castellano and Molina 1989, Molina and Trappe 1984).



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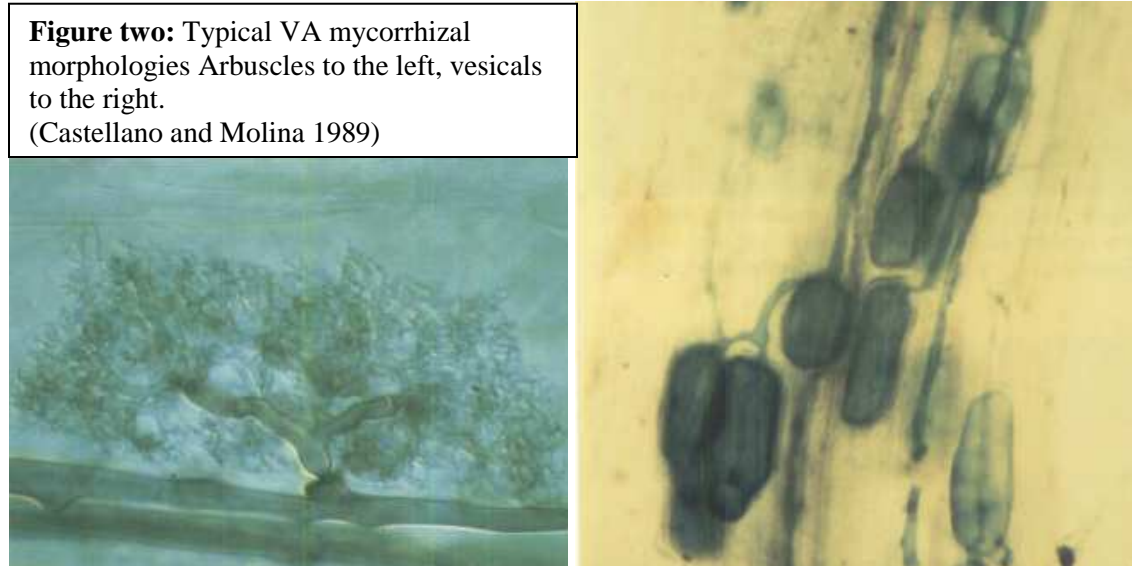
This study only examines ectomycorrhizal fungi, but there are forms of fungus-plant mutualism that produce other diagnostic morphologies. These morphologies include ectendomycorrhizal morphology, vesicular appendages and arbuscular appendages. These last two morphologies are often found together leading to the designation “vesicular-arbuscular (VA) mycorrhizae.” The ectendomycorrhizal association is a subset of ectomycorrhizal symbiosis in which the fungal sheath is thinner than normal. These fungal hyphae do penetrate the cell wall of some, but not all, cortical cells. This is most common in the phylum *Ascomycota* (Castellano and Molina 1989, Molina and Trappe 1984).

Vesicular-arbuscular mycorrhizae are completely dissimilar from the ectomycorrhizae. These mycobionts belong exclusively to the phylum *Zygomycota*. As ectomycorrhizal fungi colonize a root its morphology is fundamentally changed, but no such alterations occur during VA mycorrhizal formation. During root colonization by VA mycorrhizae each individual hyphal thread penetrates the root at a single point only and no sheath or Hartig net formation occurs. The hyphae penetrate the cell wall of cortical cells and produce two types of structures: vesicles and arbuscles (see figure 2). A vesicle is a small bulbous sack used to store lipids and reproduce asexually. An arbuscle is a cluster of thin, hair-like radiating processes specialized for nutrient exchange (Castellano and Molina 1989, Molina and Trappe 1984).

Whereas ectomycorrhizal fungi primarily function to help the plant acquire water and some phosphorus, the VA mycorrhizae primarily assist with nutrient uptake. The VA fungi all produce small, hypogeous (beneath the ground surface) fruiting bodies. The

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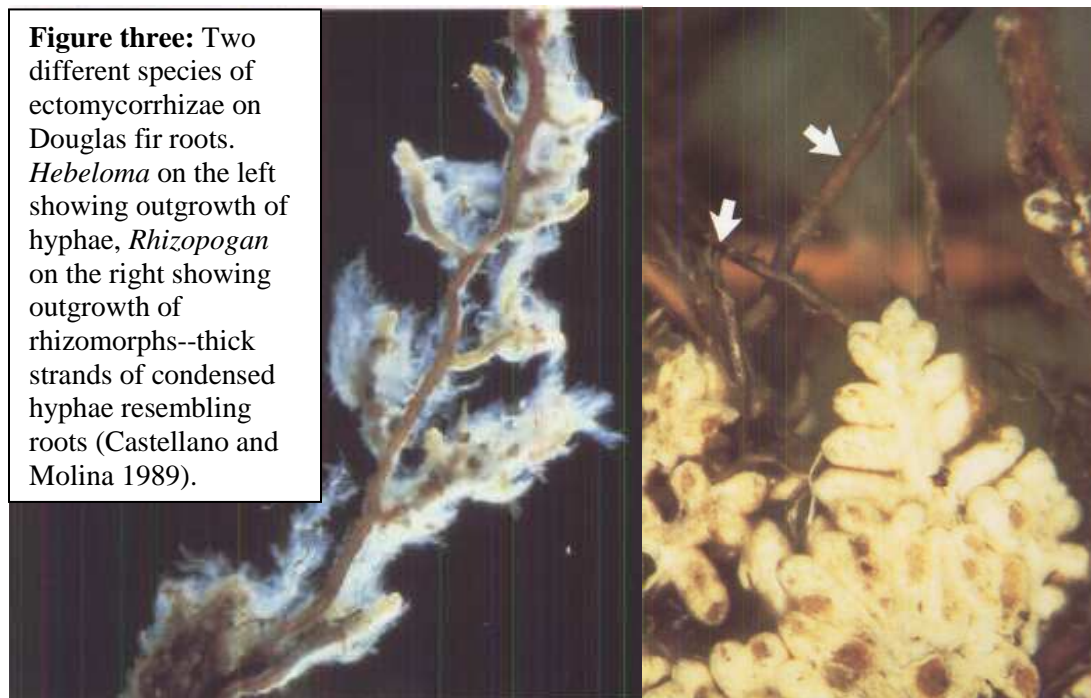
ectomycorrhizal fungi are of primary importance to temperate forest trees; the VA fungi are of primary importance to cedars, a handful of temperate hardwoods, tropical forests and herbaceous species (Castellano and Molina 1989, Molina and Trappe 1984).



While most plants can survive in the absence of a mycorrhizal mutualism, such associations are very important to the vigor and competitive ability. The hyphae of the mycobiont extend out from the Hartig net (see figure 3) and extract resources, mostly water and some minerals such as phosphorus, from a much larger volume of soil than could the root hairs of an un-infected root. This increased volume of soil colonized can be on the order of 10^2 - 10^3 . In addition to the water and phosphorus uptake, the plant symbiont benefits the physical barrier to pathogen infection provided by the fungal sheath. Another function attributed to some mycorrhizal fungi is the production of antimicrobial agents. These beneficial fungi can also contribute to detoxification of contaminated soil and increased resistance to soil acidity as well as drought and heat. The exact set of benefits varies from one species to another. The mycobiont benefits because the plant provides it with glucose as a carbon source. As the fungus and plant interact,

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the mycobiont becomes enriched in ^{15}N and depleted in ^{13}C (in terms of isotope relative abundance) compared to the plant it associates with. This occurs because some of the enzymes involved in the transfer of nutrients and sugars are partially isotope selective. This phenomenon has been used to study fungi that have never been successfully isolated into pure culture (Assche et. al. 1999, Brendemuch and Ruchle 1981, Castellano and Molina 1989, Hobbie et. al. 2001, Molina and Trappe 1984).



The ectomycorrhizal mutualism involves interactions with soil microbes as well as with plants. It has been observed that certain bacteria associate with ectomycorrhizae and directly encourage the establishment of mycorrhizal associations. Bacteria known to assist mycorrhizal development are given the general descriptor "mycorrhizal helper bacterium" (Chen et. al. 2003, Churin et. al. 1999, Duponnois and Plenchette 2003). These and other bacteria often associate with the sporocarp (fruiting body) tissue as well as the hyphae (Alstrom et. al. 1993). In a study of the bacterial flora of the

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ectomycorrhizal fungus *Suillus grevillei* sporocarps, the presence of twenty-seven distinct culturable bacteria species were revealed, the most prevalent genera being *Pseudomonas*, *Bacillus* and *Streptomyces*. Further dual-culture experimentation involving the bacteria isolates and *S. grevillei* showed that the *Pseudomonas* isolates tended to be "helper bacterium." The *Bacillus* strains tend to have little or no effect on the fungus, although some studies have shown them to promote plant growth independently of any interactions with mycorrhizae (Chanway et. al. 1996), while *Streptomyces* tended to inhibit the fungus (Luppi-Mosca et. al. 1996). Similar observations were made in a study of bacteria incorporated in *Tuber borchii* (a species of truffle) ectomycorrhizae (Agnolucci et. al. 2002). Alstrom et. al. suggests that bacterial presence within sporocarps may be incidental and although this was not addressed in the Luppi-Mosca et. al. (1996) study it would be a logical explanation for the presence of inhibitory microbes. Though beyond the scope of this paper, it is worth mentioning that important bacteria-mycobiont interactions also influence other forms of mycorrhizal symbiosis (Berta et. al. 2004, Bothe et. al. 2002, Chen et. al. 2003, Parks and Schmitt 1997).

The importance of ectomycorrhizae has been demonstrated in numerous case studies. One of these is the case of introducing *Pinus radiata*, Monterey pine— a North American indigenous species, to new areas. There are three populations of *P. radiata* in four counties along the Californian coast and two additional subspecies found on Guadalupe and Cedros Islands off the Mexican coast (Cope 1993). This species has been used in attempts to establish plantations in New Zealand. All attempts failed except those in which the trees were provided with suitable mycobionts from their native range (Molina and Trappe 1984).

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Another example of the importance of ectomycorrhizae to forest trees is the inhibition of tree regeneration in rhododendron thickets. Because of this inhibition, large stands of rhododendron can cause problems for forest management. Two recent studies by the same research group have produced evidence that inhibitory effects are directed at the ectomycorrhizae mycobionts of forest trees. Rhododendron form ericoid mycorrhizal associations (a subset of VA mycorrhizae) but do not form ectomycorrhizae. The production of chemicals (called allelopathy) or environmental conditions which selectively inhibit ectomycorrhizal formation removes the competitive advantage of overstory species (Clinton et. al. 1996 and Clinton et. al. 1999).

A third case for the importance of mycorrhizal fungi is the assistance they render in tree nurseries. Even in a nursery setting, where the seedlings are provided with a constant supply of water and nutrients under optimum conditions, the seedling must quickly colonize its local soil in order to take advantage of these provisions. Seedlings that are grown in the presence of mycorrhizal fungi accomplish this task faster than those with no mycobionts available. Because of this, the use of mycorrhizal fungi has been shown to lead to faster, more consistent growth and higher-grade seedlings. The inverse of this has also been observed where seedlings planted in fumigated beds have shown nutrient deficiencies even though soil chemistry was at optimum levels (Brendemuch and Ruchle 1981, Castellano and Molina 1989, Molina and Trappe 1984).

Observations regarding the out-planting success of nursery-grown mycorrhizae-colonized seedlings within their native range have been mixed. Some reports are entirely positive while others show the mycorrhizae-colonized seedlings have no clear advantage. These mixed results remain somewhat enigmatic but probably reflect a lack of knowledge

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regarding the details of environmental requirements for specific mycobionts

(Brendemuch and Ruchle 1981, Castellano and Molina 1989, Molina and Trappe 1984).



Figure four: Bicolor bolete: an edible ectomycorrhizal species growing near a yellow birch root in Worlds End State Park.

In addition to the importance of ectomycorrhizal fungi to forest ecology many of these organisms constitute natural resources of high economic value. The valuable members of this group are the edible species, the harvest and sale of which contributes large

sums of money annually to the national economy and is an important source of income to many rural citizens. For example, Kutara and Lettman (2005) estimated that wild mushroom wholesaling from Washington and Oregon states grosses between two hundred million and four hundred million dollars each year, although the authors describe this as an underestimation. While not all wild mushrooms sold are mycorrhizal species, these numbers illustrate the importance of the industry as a whole. Some ectomycorrhizal species of economic importance include *Basidiomycetes* such as the king bolete (related to the bicolor bolete in figure 4), the chanterelle and the matsutakes. Economically important edible, ectomycorrhizal *Ascomycetes* include such well-known and valuable fungi as the morels and the truffles. The heavy harvesting of mycorrhizal fungi in some areas has led to concerns about conservation. Some harvesters practice techniques such as pulling mushrooms vertically out of the ground and forest floor raking

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that disrupt the hyphae and can cause production and species diversity to decline (Amaranthus 1993, Danell et. al. 2003, Kutara and Lettman 2005, Parks and Schmitt 1997).

Since mycorrhizae are required if plants are to thrive, fungi dispersal can influence plant dispersal. In several studies done on pioneer species which colonize abandoned waste places and fields, those that successfully colonized had mycorrhizae (many of these studies dealt with herbaceous species and VA mycorrhizae). Often small animals and insects that have consumed ripe sporocarps act as vectors for the dispersion of these fungi. This is especially important for the dispersal of hypogeous species such as the truffles which only fruit below the ground. Epigous species, fungi which produce spores above the ground, also use wind dispersal to spread their spores (Castellano and Molina 1989, Clagidge and Colgen 2002, Molina and Trappe 1984, Ponder 1992, Rothwell and Vogel 1982).

The majority of ectomycorrhizal fungi are obligate symbionts. One known exception to this is *Morchella* genus. Recent evidence suggests that *Morchella* can exist as both saprobes and as symbionts (Buscot and Kottke 1990, Dahlstrom et. al. 2000). Because these fungi are typically obligate symbionts, isolation into pure culture is extremely difficult. Isolation is possible both from sporocarp tissue and from colonized roots but growth is very slow *in vitro* (Daniel and Fries 1990, Eric 1994, Degawa et. al. 2001, Harvey 1991, Marx and Zak 1964). Danell et. al. (2003) cited *in vitro* growth rates at 0.5 mm per day for the chanterell. The weakness of this growth is so pronounced that after several years of subculturing in medium the fungi begin to lose their ability to colonize plant roots. If strains are to be maintained, a cycle of inoculation onto a growing

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plant followed by re-isolation between periods of subculturing is necessary (Grove et. al. 1993).

As already stated, since the fungal tissue incorporates bacteria, environmental samples are necessarily contaminated with a myriad of organisms. This requires any isolation attempt to include washing and surface sterilization steps to remove these contaminants. If these procedures fail the desired organism will be killed or a contaminant will survive and rapidly overwhelm the slower growing fungus specimen (Daniel and Fries 1990, Eric 1994, Degawa et. al. 2001, Harvey 1991, Marx and Zak 1964). Inhibitory contamination is not limited to bacteria. Undesired fungal contaminants have been shown to interact *in vitro* with mycorrhizal fungi in inhibitory, competitive and directly parasitic ways (Girlanda and Varese 1995). These problems are summarized by Eric (1994) "The research on the physiology of the edible ectomycorrhizal *basidiomycete Cantharellus cibarius* has not been intensive due to the difficulties in obtaining pure mycelium. The fruit bodies... are heavily infested with *Pseudomonas fluorescens* and other bacteria and molds." A similar quotation from Hobbie et. al. (2001) is as follows: "Many putatively mycorrhizal fungi have proven impossible to culture and therefore their mycorrhizal status cannot be directly proven..."

It would stand to reason that if a medium could be designed to selectively inhibit the growth of some common contaminants it would be of great aid isolating ectomycorrhizal fungi. One compound which holds promise for such use is the fungicide benomyl. Benomyl is a benzimidazole derivative that binds microtubules. This binding leads to problems with transport within the cell, spindle fiber poisoning and cell cycle arrest. Specific benzimidazole derivatives selectively bind different forms of

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microtubules. Derivatives of this compound have been designed to target cancer, helminthes and fungi. (IPCS INCHEM) This fungicide has been used in selective medium at concentrations of 10 ppm for the isolation of wood-rotting *Basidiomycetes* (Carey and Hull 1989) and at concentrations of 20ppm for the selective isolation of mucoralean fungi from soil (Botha et. al. 2000). Although neither of the above are target organisms of this study, these works demonstrate the potential benomyl has for selecting against soil contaminants *in vitro*. A third study demonstrated that the addition of benomyl to soil increases the ability of the ectomycorrhizal *Bacidiomycete Pisolithus tinctorius* to form mycorrhizae *in vivo* (Pawuk and Barnett 1981). The purpose of this study was to evaluate the possibility of using benomyl as a selective agent to aid in the isolation of ectomycorrhizal fungi.

Materials and Methods:

Field collection:

Specimens were collected from the field beginning early summer 2005 when sporocarp production was first observed. Many of these collecting trips were conducted with the assistance of a recent Lycoming College alumni Rain Bell who also aided in field identification. All collecting was conducted in Lycoming and Sullivan counties (Pennsylvania) and continued until sporocarp production ceased in the fall of 2005. Nineteen possible mycorrhizal species were collected. Each specimen was inserted into a screw-cap vial of sterile deionized water in the field and held in refrigeration until isolation was attempted.

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Isolation attempt one:

The first isolation attempt was loosely based on procedures that appear in the literature (Daniel and Fries 1990, Degawa et. al. 2001, Marx and Zac 1964) but was adapted to accommodate materials and reagents available at the time. From each specimen, a piece of tissue was torn or cut and immersed in 95% ethanol for one to two minutes for surface sterilization. The tissue was then further dissected and placed on growing medium. The growing medium used was Modified Melin-Norkrans (MMN) medium (Harvey 1991). The composition of this medium was glucose 10 g, $(\text{NH}_4)_2\text{HPO}_4$ 0.25 g, KH_2PO_4 0.5 g, MgSO_4 0.0732 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0662 g, NaCl 0.025 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.02 g, Thiamine HCl 1×10^{-4} g and agar 10 g in one liter of deionized water. The quantities of MgSO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were altered from the quantities given in the citation to account for differing waters of hydration. Additionally, 10 ml of 0.5% chloroamphenicol and the same of chlorotetracycline were added to the isolation medium for a total concentration of 0.05 g/l for each antibiotic. (Skaar and Stenwig 1996). All incubations of fungi were done at room temperature, approximately 20°C.

DNA identification of isolates:

The polymerase chain reaction (PCR) amplification of the 16s rRNA gene (for bacteria) or the ITS rRNA regions (for fungi) proceeded as follows. A heavy inoculum was removed from each culture and introduced into 100 µl of sterile deionized water and subjected to two freeze-thaw cycles in a cold block frozen at -80°C and a hot block at 42°C. One µl of frozen-thawed cells was then added to a reaction mixture of 12.5 µl Ex Taq pre mix (a solution of enzyme and nucleotides), 4 µl each of two primers and 3.5 µl of deionized water. The reaction mixture was then covered with 30 µl of mineral oil in

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preparation for amplification. The PCR was conducted in a thermocycler with the following conditions: one cycle of three minutes at 94°C, one minute at 50°C and one minute at 72°C. This was followed by thirty-five cycles of 94°C for one minute, 50°C for one minute and 72°C for one minute. The last cycle was 94°C for one minute, 50°C for one minute and 72°C for ten minutes. For bacteria the primers

A: 5'-CGGCCAGACTCCTACGGGAGGCAGCAG-3' and

B: 5'-GCGTGGACTACCAGGGTATCTAATCC-3' were used. These primers amplify a variable region of the bacterial 16s rRNA (Newman 2000). For fungi the primers

ITS1-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

were used (Jasalavich et. al. 2000). These amplify the internal transcribed spacer (ITS) variable regions between the fungal large and small subunits of the rRNA. Primers were manufactured by Sigma-Genosys. Ten bacteria and nine fungal samples were so amplified, though for one fungal sample no PCR product was produced (Botton et.al. 1996).

After amplification, the reaction mixture was separated via agarose gel electrophoresis. The DNA was visualized using ethidium bromide and ultra violet light. The single bands were excised from the gel and the DNA purified using QIAquick spin columns (produced by Qiagen) according to the manufacturer's instructions.

The purified PCR products of the eighteen samples successfully amplified in the previous steps was sent to Gene Gateway LLC for direct sequencing using primer A for bacteria and ITS1 for fungi. The results obtained from five bacteria genes sequenced with primer A were not acceptable; four of these were re-sequenced using primer B. The sequencing results were returned via e-mail and analyzed using the NCBI Nucleotide-

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nucleotide BLAST search tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). Each sequence (less any meaningless values at the end of a sequence) was used as a search term and probable identity was assigned using the highest scoring matches.

Reagent testing for selective medium:

The fungicide benomyl (95% Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate; Aldrich) was added to MMN medium at five concentrations: 0 g/l, 0.002 g/l, 0.01 g/l, 0.05 g/l and 0.1 g/l. The fungicide was delivered to the medium in the minimum amount of 95% denatured ethanol required to dissolve the sample. This was done before the medium was autoclaved. At all concentrations except the highest (0.1 g/l benomyl) the fungicide produced a clear solution in the ethanol, immediately made the medium cloudy upon addition but entered and remained in solution after addition and brief agitation. Some of the benomyl initially re-crystallized at the 0.1 g/l concentration, but fully dissolved in the autoclave and remained in solution thereafter.

Four species of mold and one *bacidiomycete* were tested for inhibition on the media above described. Three replicates were performed for each combination of species and benomyl concentration for a total of seventy-five plates. Four mold species including *Penicillium thomii*, *Aspergillus nidulans*, *Epicoccum nigrum* and a possibly novel organism referred to as “mold isolate number 09” (M09) were inoculated at a single point in the middle of each plate with an inoculating loop measuring approximately 4 mm. Inoculation proceeded from plates of low concentration of benomyl to plates of higher concentration and the loop was washed in 95% denatured ethanol before beginning a new set of plates. The loop was flamed between each inoculation in keeping with good

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aseptic technique. *Armillariella mellea* was acquired in pure culture from Carolina Biological Supply and subcultured/maintained on MMN and TSA medium. This species was inoculated at a single point in the center of each plate using a small piece of agar cut from the edge of a colony actively growing on MMN medium. As the size of these agar chips varied, an initial measurement was taken at the time of inoculation and recorded as “day 0.” All incubations were at room temperature (20°C).

Data was gathered at days 0 (for *A. mellea* only), 2, 4, 7, 9 and 11 after inoculation. Data collection consisted of measuring the diameter of each actively growing colony with a graduated caliper; measurements were estimated to the nearest millimeter. Measurements were taken by viewing the colony through the bottom of the plate. A lighted magnifying glass was used in cases where the hyphae at the colony edges were difficult to see. This was done consistently for the plates containing *A. mellea* and *E. nigrum*, but not the others. For each colony, two measurements were taken perpendicular to each other and the mean recorded.

Results:

Field collection:

The field collection phase of this research was highly successful with many important ectomycorrhizal species being found. In total nineteen species were collected from Lycoming and Sullivan counties (Pennsylvania); most of these were collected with accompanying field notes, the others were “collections of opportunity” and field notes were not able to be taken. Many of the collections originated in Worlds End State Park as the author found this region to be quite rich in fungal biodiversity. Identification was attempted in the field, but given that many species bear great resemblance to one another

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accurate identification was not always possible. Several specimens have only been assigned probable genera. Field identification was aided by the book National Audubon Society Field Guide to Mushrooms (1981) by Gary H. Lincoff. All of the scientific names assigned to the specimens below are given in this source. See the appendix for table detailing the identity, location and environment of these specimens.

Isolation attempt:

Several attempts to isolate pure culture ectomycorrhizal fungi from the field collections resulted in growing pure cultures of mold and bacteria species but none of the target organisms. Nine mold specimens (numbered M01-M09—see table 1) were saved and identified for later use in fungicide testing. Likewise, eleven bacteria specimens were saved and identified. However, during identification (discussed in greater detail later) many of these specimens became contaminated and had to be re-purified. A list of all identifications based on DNA sequencing results is provided in table two. No further work was done with the bacteria isolates.

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Table one: descriptions of all mold isolates and the sporocarp specimens from which they originated.

ID#	Source	Description
M01	Tissue from an unknown milky cap	Black growth, inhibited the growth of M02 which occurred on the same plate.
M02	Same specimen as M01	White growth, was inhibited by M01.
M03	King bolete tissue	White, very tall aerial mycelium was produced. This specimen turned the agar a yellowish color.
M04	Chantrell tissue	White and pale green growth. Abundant spores and small, yellowish structures are produced after an extended period of growth.
M05	Bicolor bolete tissue	Black growth
M06	Tissue from an unknown russula	White growth
M07	Bicolor bolete tissue	Specimen was observed producing concentric rings of growth.
M08	Black morel tissue	Green growth, this specimen was observed producing concentric rings of growth
M09	Chantrell tissue	Very dark green growth

Identification:



Eighteen pure culture isolates were identified using PCR amplification of the ITS regions one and two in the case of fungi; a fragment of the 16S rRNA small subunit was amplified in the case of bacteria samples. The first identification attempt was executed using the bacteria isolates. The PCR amplification proceeded properly, but when the product was analyzed via agarose gel electrophoresis many of the bands were doubled, leading to the conclusion that

the isolates were not pure cultures. These cultures were repeatedly streaked on TSA plates until pure cultures were obtained. The majority of bacteria isolates were identified successfully using primer A for sequencing. In five samples, the initial sequencing did not produce a usable product and for four of these the DNA was sequenced a second time using primer B. These four were identified as *Ochrobacter tritici*, *Pseudomonas spp.* (the nineteen top scoring BLAST results were uncultured or unidentified, possibly novel), and two isolates of *Pseudomonas syringae*. The other samples were identified based on the first sequencing results. All results are listed in table two.

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Table two: probable identifications resulting from gene analysis of isolates.

ID#	Probable Identification	ID#	Probable Identification
M01	<i>Cladosporium elatum</i>	M02	<i>Aspergillus nidulans</i>
M03	<i>Epicoccum nigrum</i>	M04	<i>Penicillium thomii</i>
M05	<i>Cladosporium cladosporioides</i>	M06	No PCR produce acquired
M07	<i>Penicillium lividum</i>	M08	<i>Penicillium corylophilum</i>
M09	Likely novel organism. Possible relationship to the genus <i>Cladosporium</i>		

Probable identifications for bacteria isolates

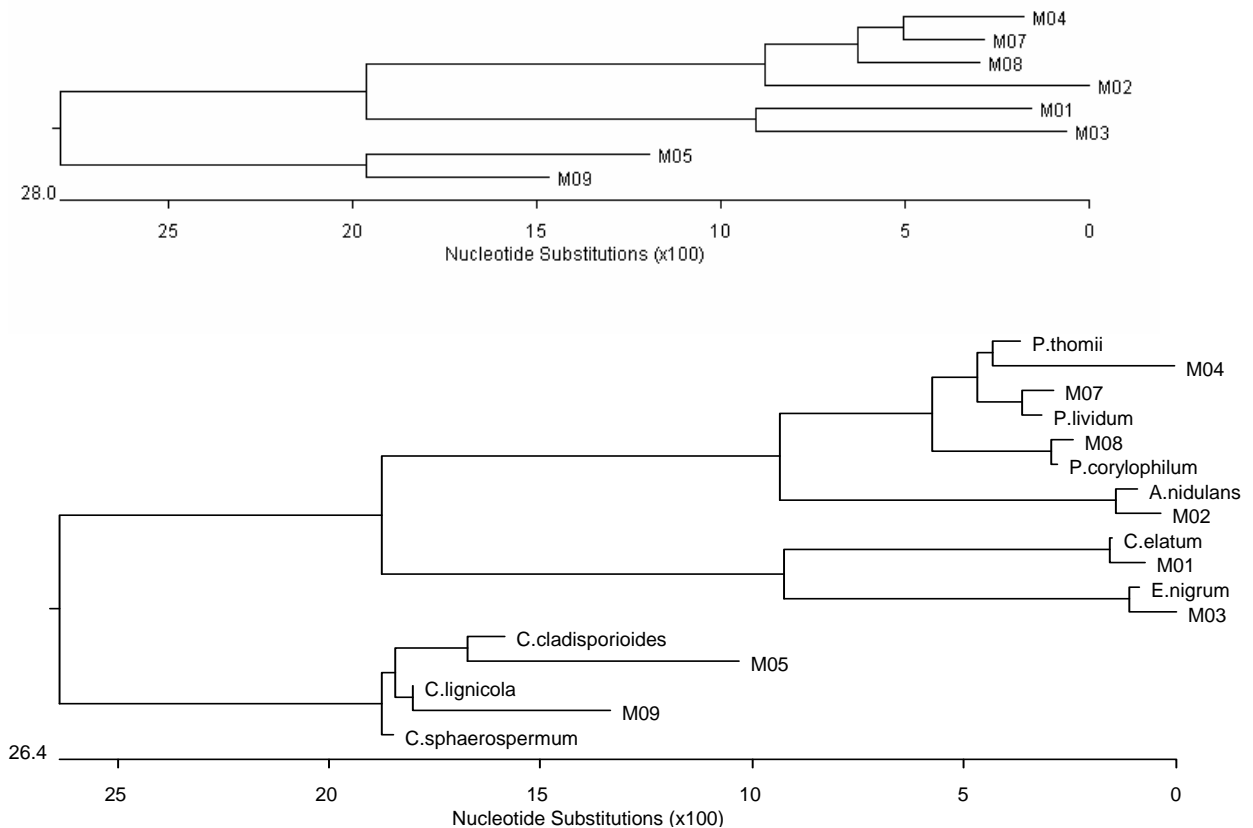
Escherichia spp. (probable *E. coli*, two isolates), *Ewingella americana* (two isolates), *Staphylococcus epidermis*, *Orchrobacter tritici*, *Pseudomonas syringae* (two isolates), *Pseudomonas spp.* (possibly novel)

The fungal samples were identified using amplification of the ITS regions. The primer ITS1 binds near the 3' end of the 18s rRNA (or 17s rRNA in some cases). The primer ITS4 binds near the 5' end of the 28s rRNA gene. These probes capture the end of the 18s rRNA subunit → ITS region 1 → 5.6s rRNA subunit → ITS region 2 → lastly the beginning of the 28s rRNA. The ITS regions are variable which allows for identification (Botton et.al. 1996, Jasalavich et. al. 2000). A PCR product was achieved for all fungal specimens with the exception of M06 (see figure 5).

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The fungal DNA sequences were aligned using the clustalW method on DNASTar Lasergene MegAlign software (see figure 6 for phylogenetic trees, alignments are shown in appendix 2). The isolates from the genera *Aspergillus* and *Penicillium* were closely bracketed. One member of the genus *Cladosporium* was bracketed with *Epicoccum nigrum*, the other with isolate M09. This suggests that M09 is closely related to the genus *Cladosporium*, but it is questionable whether or not it belongs to this taxon.

Figure six: Above- phylogenetic tree of mold isolates generated using the sequencing results acquired in this study. Bellow- a tree comparing the mold isolates to sequences of known organisms extracted from the NCBI database.



Fungicide testing:

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The fungicide benomyl inhibited the mold isolates tested at concentrations much lower than those which inhibited the basidiomycete *Armillariella mellea*. The mean diameter growth of *A. mellea* at benomyl concentrations 0, 0.002 and 0.01 g/l were 0.82, 0.82 and 0.85 mm/day respectively with measurable growth occurring at day two. There is no statistically significant difference between these values (ANOVA score $P=0.8697$). At benomyl concentration 0.05 g/l growth was visible at the surface of the original agar pieces beginning day four, but growth was extremely slow, averaging 0.15 mm/day over the eleven-day period. Small, sclerotia-like (small, dense growths) structures were observed growing on the sides of the agar pieces. No growth of any kind was observed at the fungicide concentration of 0.1 g/l

The molds *Penicillium thomii* and *Aspergillus nidulans* produced visible growth on the 0 g/l benomyl plates by day two. The mean diameter growth rates were 21.52 and 1.09 mm/day respectively. In this study growth was quantified by measuring the diameter of a single colony, which was difficult in the case of *P. thomii* because sporulation was abundant and the spores spread easily. The *Penicillium* spores did not adhere well to the inoculating loop and multiple colonies were produced in a wide line between the edge of the plate and the point where deliberate inoculation occurred because spores fell from the loop. Initially there was a central mass to measure (though this still represented multiple colonies), but by day seven the entire plate had been filled with growth. With the exception of *A. nidulans* on the 0.002 g/l benomyl plates, benomyl plates containing these two molds produced light tan structures on day two where the agar was touched with the inoculating loop (see figure nine). There was never any hyphal extension observed on most of these plates. There was no statistically relevant difference

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between the benomyl-containing plates for either *P. thomii* (ANOVA score $P=0.3669$) or *A. nidulans* (ANOVA score $P=0.2416$). A slide was made from a *P. thomii* 0.002 g/l plate. The tan masses had formed a pellet which was lifted from the plate with an inoculating loop, placed onto a drop of water and chopped into smaller pieces with the loop. The slide was then stained with lactophenol cotton blue, which stains the fungal cell wall. The spores on this plate had germinated and attempted to form hyphae, but these hyphae were highly abnormal. This abnormal growth included short, swollen hyphae with many isolated bulbous swellings along their length. When measuring the diameter of the tan masses, both positive and negative changes in diameter were noted—presumably only due to the margin of error in measurement. The 0.002 g/l benomyl *A. nidulans* plates mirrored the plates with abnormal hyphae just described except that during the last week of observation a small amount of hyphal growth was observed. This growth was quite slow and only on the last day of observation did the hyphae approach edge of the initial tan masses. The mold isolate M09 grew at a mean rate of 1.18 mm/day in the absence of benomyl and produced no visible structure or growth of any sort in the presence of it.

The mold *Epicoccum nigrum* was slow to germinate—one 0 g/l benomyl plate and two 0.002 g/l benomyl plates germinated on day four, the remainder of the plates at these concentrations germinated by day seven. The mean diameter growth for benomyl 0 plates was 3.15 mm/day, the mean growth rate for 0.002 g/l benomyl plates was 3.03 mm/day. These rates are not statically significant (ANOVA score $P=0.7399$). No structures or growth of any sort were observed on the other plates.

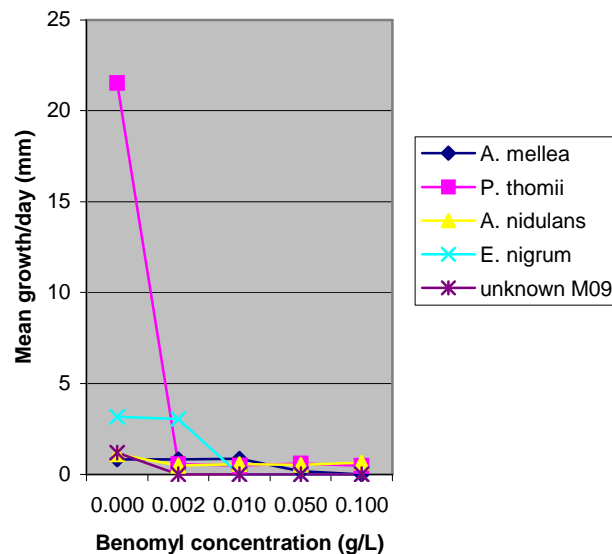
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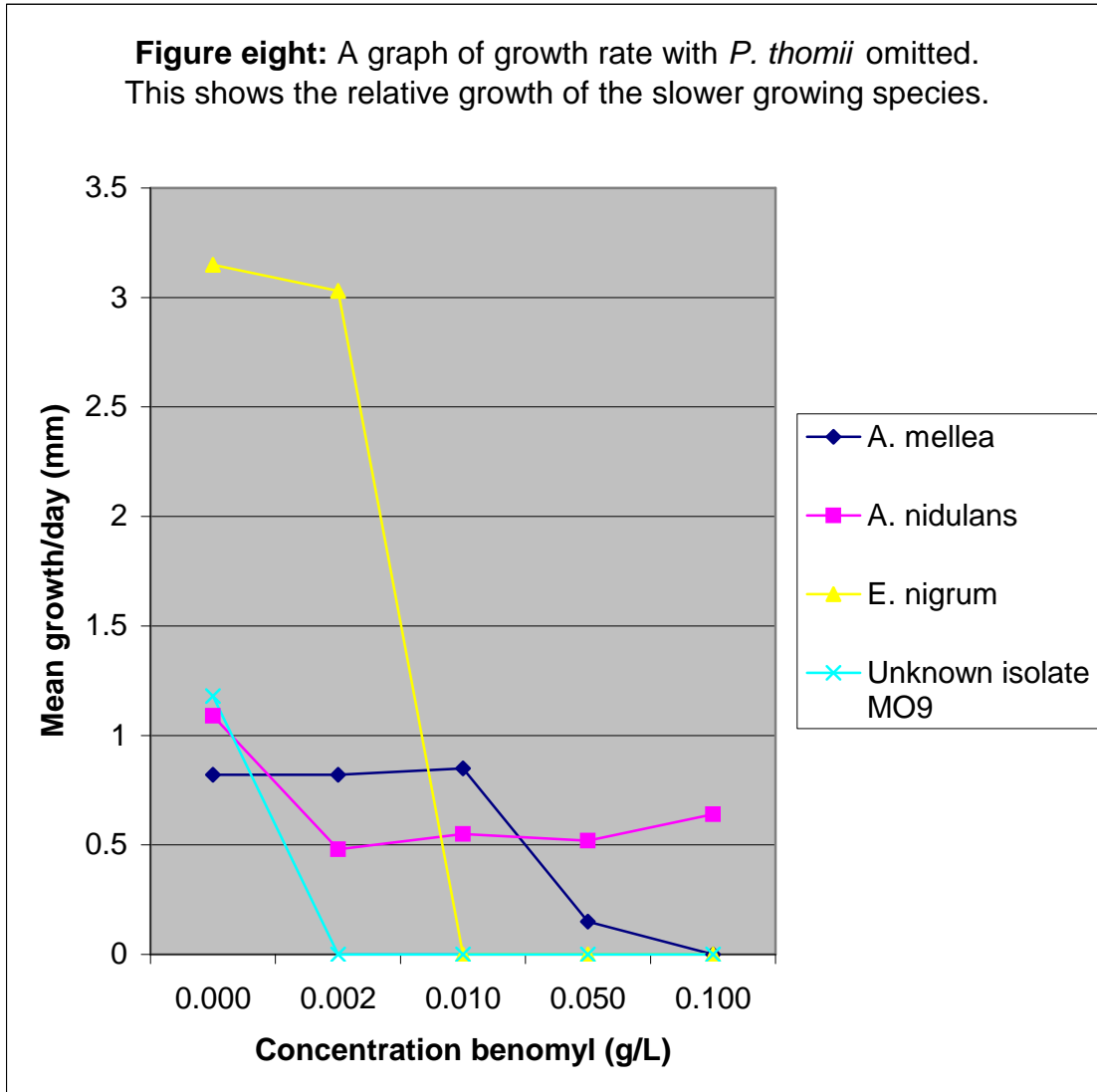
To summarize, the results of the benomyl inhibition study showed that the growth rates of the undesirable mold contaminants (see table 3) were drastically or completely reduced at relatively low concentrations of benomyl. At the same concentrations that inhibited all mold species used, the target fungus *A. mellea* showed no relevant change in its growth rate. This species was not strongly inhibited until the concentration of benomyl was quite high. Only the highest concentration of benomyl completely halted the growth of *A. mellea*. These results are shown in table three, figures seven and eight.

Table three: mean growth rates for entire two-week period.

Benomyl concentration	<i>A. mellea</i>	<i>P. thomii</i>	<i>A. nidulans</i>	<i>E. nigrum</i>	Unknown M09
0 g/l	0.82	21.52	1.09	3.15	1.18
0.002 g/l	0.82	0.55	0.48	3.03	0.00
0.01 g/l	0.85	0.48	0.55	0.00	0.00
0.05 g/l	0.15	0.58	0.52	0.00	0.00
0.1 g/l	0.00	0.45	0.64	0.00	0.00

Figure seven: A graph of growth rate for all species tested.





Discussion:

The isolation procedure produced no usable cultures of ectomycorrhizae and was abandoned. This necessitated the purchase of the *A. mellea* specimen for use in the benomyl experiment. Experimentation with fungal isolation is ongoing at the time of this writing utilizing a laboratory protocol published by Degawa et. al. (2001) which involves three vortexings in three 1ml portions of 0.01% tween 80 solution followed by a thirty minute wash in 100 ml of 0.005% tween 80 solution in a shaken flask. This was

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followed by a one-minute surface sterilization with 1% hypochlorite solution and three subsequent rinsing in deionized water. All solutions were autoclaved prior to use. This cleaning and surface sterilization procedure is done on small fragments of sporocarp tissue, which were further dissected in a sterile petrie dish after the cleaning had been completed. The final tissue fragments are placed on nutrient medium, several to a plate. This attempt will not yield any concrete results in time for inclusion in this paper.

The specimen *A. mellea* was selected because of availability. After it became obvious that a pure culture would need to be purchased, a search for such specimens was done. *Armillariella mellea*, from Carolina Biological Supply was the only culture found at a reasonable price. The species *A. mellea* is itself a forest pathogen, infecting root systems. This species is common in both temperate and tropical areas of the world. Even though this species is not itself mycorrhizal, it is closely related to several important mycorrhizal species. This specimen was sold under the genus name “*Armillariella*” which is a synonym for the genus name “*Armillaria*” (*Tricholomataceae*). The genus “*Tricholoma*” has been divided out of the genus “*Armillaria*.” The nomenclature of these organisms is rather confused. Many of the members of this “group” of organisms are important mycorrhizal species and some are highly valued edibles. Two examples of these are *T. bakamatsutake* and *T. matsutake* (the highly valued matsutake mushroom) isolated in the study by Degawa et. al. (2001) as well as parasitic species such as *A. mellea*. When our culture was received and subcultured, it showed a long delay time between tissue plating and growth, followed by relatively slow growth which are characteristics expected of mycorrhizal species. Given all of these reasons, it was

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decided that *A. mellea* would be a reasonable organism for reagent testing. (Burdshall and Volk, Degawa et. al. 2001, Lincoff 1981)

The results of the fungicide testing were encouraging. Most of the mold species tested were strongly inhibited at the 0.002 g/l concentration of benomyl. Only one mold grew on this medium but it was completely inhibited on the 0.01 g/l concentration. The *A. mellea* showed equivalent growth on 0, 0.002 and 0.01 g/l concentrations. Growth was reduced but not eliminated on the 0.05 g/l concentration. This shows that *A. mellea* is more resistant to the fungicide used than the mold contaminants. If repetitions of this experiment demonstrate equal benomyl resistance among a large sample of ectomycorrhizal fungi, than this reagent would be a viable selective agent for use in the isolation of these organisms.

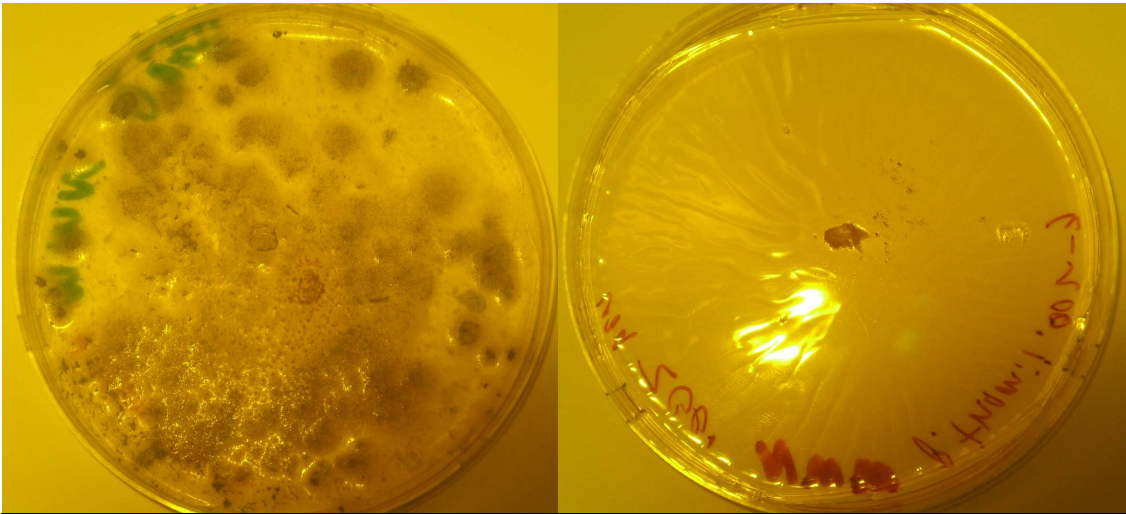


Figure nine: Left: *P. thomii* on 0 g/l benomyl plate. Right: *P. thomii* on 0.002 g/l benomyl plate

More research is needed to advance the goal of developing a final isolation medium for ectomycorrhizal fungi. The first requirement is to repeat this study using a larger sample of ectomycorrhizal genera. In regard to the mold contaminants, the research presented here suggests that the optimal concentration of fungicide lies between

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0.002 and 0.10 g/l. As it is logical that an isolation medium should utilize the most mild conditions practical, it would be beneficial to do further research with the species *E. nigrum* using MMN and a gradient of benomyl concentrations between 0.002 g/l and 0.01 g/l to determine the minimum concentration required to completely inhibit the growth of this species. A question of importance not dealt with in this study is the problem of bacterial contamination. The bacterial strains isolated from the field specimens were able to survive a combination of antibiotic additives designed for mold isolation from silage. (Skaar and Stenwig 1996) Two possible explanations include antibiotic resistance with the bacteria or improper addition of the antibiotics to the medium. It would be useful to know which of these is correct. Lastly, since the morels and truffles are ectomycorrhizal fungi belonging to the phylum *Ascomycota*, it would be of interest to determine the response of these organisms to benomyl exposure. This could potentially benefit the study of these economically important species.

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Appendix one:

Table giving details of field collected specimens.

Field identification	General location	Notes
Black morel	Northern Lycoming county	Rich mesic soil along a ridge top. The fungi were found within a grove of large pole/small sawtimber sweet cherry. May 12, 2005
Unknown russula (<i>Russula spp.</i>)	Worlds End State Park. Sullivan County.	This specimen had a red cap with white gills and was found along the banks of the Loyalsock creek.
Yellow patches amanita (<i>Amanita flavoconia</i>)	Worlds End State Park. Sullivan County.	This specimen was found in a mixed stand of small sawtimber, which included maple, sweet birch and black birch with a beech brush understory. This location was upland but near the Loyalsock creek.
Unknown white mushroom	Elkland township, Sullivan County	The fruiting body was white having a bell-shaped cap with gills and no obvious veil. The base of the stype was swollen but no true “death’s cup” was observed. There was a bitter odor to the specimen. The over story consisted of hemlock and sugar maple.
Two-color bolete (<i>Boletus bicolor</i>)	Worlds End State Park. Sullivan County.	This specimen was found growing directly alongside a yellow birch root. The over story was primarily yellow birch but also included eastern hemlock, black cherry and ironwood/muscle wood with a beech brush understory near the confluence of two first order streams. July 17, 2005
Possible bitter bolete (<i>Tylopilus felleus</i>)	Worlds End State Park. Sullivan County.	Found near the bicolor bolete, but the overstory contained sugar maple and the understory hobble bush vibernum. July 17, 2005
Scaly vase chanterelle (<i>Gomphus floccosus</i>)	Worlds End State Park. Sullivan County.	Found near the bicolor bolete in a similar environment. July 17, 2005

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Field identification	General location	Notes
Russula, variable or tacky green. (<i>Russula variata</i> or <i>R. aeruginea</i>)	Worlds End State Park. Sullivan County.	A russula with a damp, light green surface on the cap. Found near the bicolor bolete in a similar environment. July 17, 2005
Cinnabar red chanterelle (<i>Cantharellus cinnabarinus</i>)	Worlds End State Park. Sullivan County.	Found along the base of a large sawtimber white ash. Sugar maple and eastern hemlock were also present. This location was on the edge of a shallow drywash near and leading to the Loyalsock creek. July 17, 2005
Unknown bolete	Rickets Glen State Park. Sullivan County.	Found in a hemlock stand alongside Lake Jean. July 18, 2005
Small chanterelle (<i>Cantharellus minor</i>)	Worlds End State Park. Sullivan County.	This specimen was found in the same location as the yellow patches amanita. July 25, 2005
Unknown milkey cap (<i>Lactarius spp.</i>)	Worlds End State Park. Sullivan County.	This specimen was found in the same location as the yellow patches amanita. July 25, 2005
Chantrell waxy cap (<i>Hygrophorus camarophyllus</i>)	Worlds End State Park. Sullivan County.	Found near the bicolor bolete in a similar environment. July 25, 2005
Chantrelle (<i>Cantharellus cibarius</i>)	Worlds End State Park. Sullivan County.	This was found near the bicolor bolete but the location was along the steeply sloping banks of one of the first order streams. The nearest tree was a sugar maple. Black birch was also present in the overstory. July 25, 2005
Chrome footed bolete (<i>Tylopilus chromapes</i>)	Worlds End State Park. Sullivan County.	Found near the bicolor bolete in a similar environment. July 25, 2005

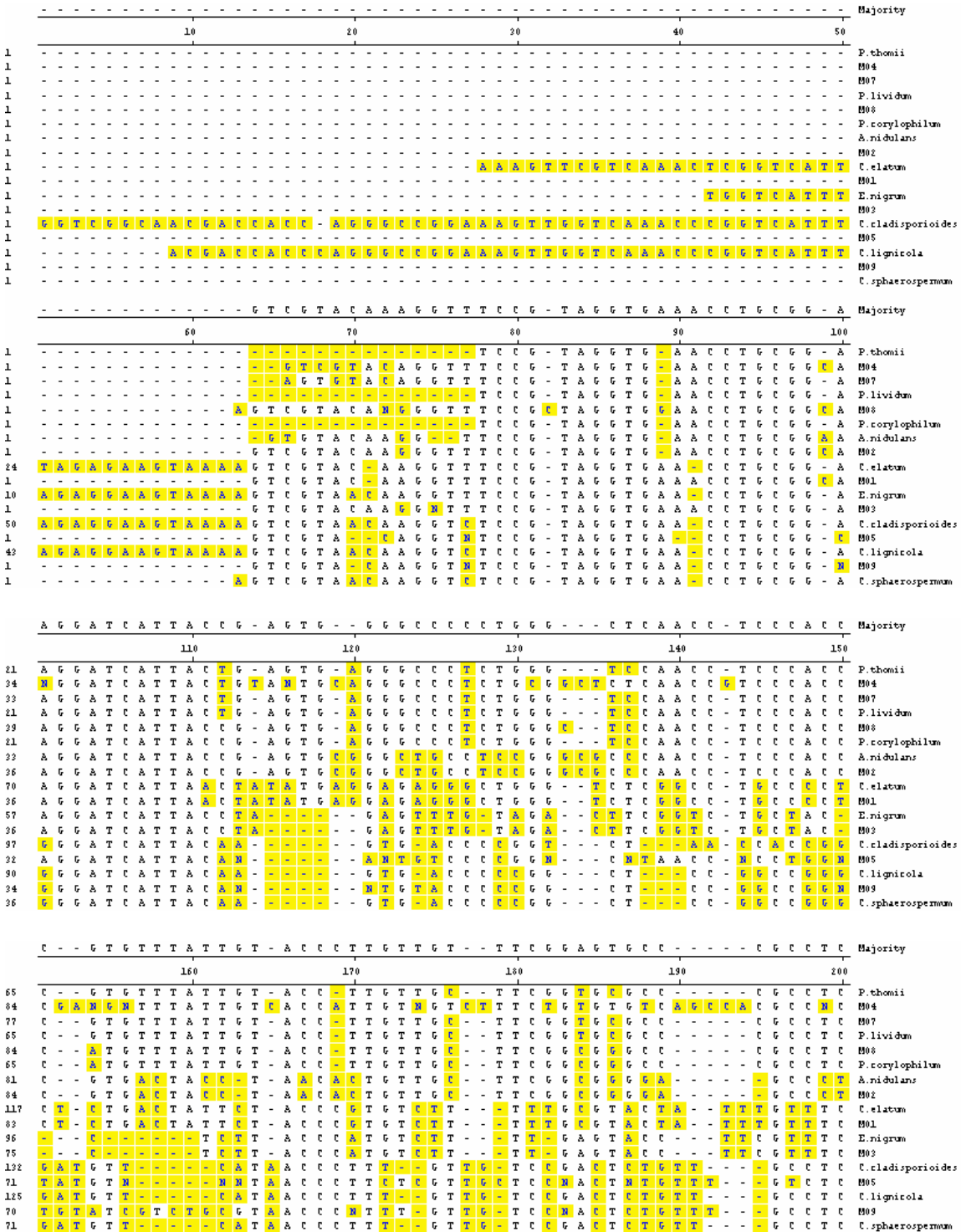
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Field identification	General location	Notes
Desepive milkey (<i>Lactarius deceptivus</i>)	Worlds End State Park. Sullivan County.	Found along the bank of a small second order stream near its confluence with the Loyalsock creek under a mixed hardwood canopy.
Pigskin poison puffball (<i>Scleroderma citrinum</i>)	Worlds End State Park. Sullivan County.	Found several rods from the bank of the Loyalsock creek.
King bolete (<i>Boletus edulis</i>)	N/A	Specimen donated by Rain Bell.

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Appendix two:

Multiple sequence alignment showing unknown molds sequencing results and known sequences extracted from the NCBI database. Produced using Lasergene MegAlign software and the clustalW method.



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410		420		430		440		450																																											
281	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	P. thomii	
320	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	C	T	G	G	T	A	T	M04
293	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	C	T	G	G	T	A	T	M07
281	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	P. lividum	
301	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M08	
282	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	P. corylophilum	
276	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	A. nidulans	
280	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M02	
318	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	U. elatum	
285	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M01	
287	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	E. nigrum	
266	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M03	
340	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	U. cladisporioides	
291	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	A	M	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M05	
338	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	U. lignicola	
300	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	M09	
284	A	A	T	T	C	A	-	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	T	G	A	A	C	G	C	A	C	A	T	T	G	C	C	C	C	C	C	T	G	G	T	A	T	U. sphaerospermum	

T C C G G G G G G C A T G C C T T G T T C G A G C G T C A T T T C T G C C C - T C A A G C C C C G G C T Majority																																																	
460		470		480		490		500																																									
330	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	P. thomii
269	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	M04
342	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	M07	
330	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	P. lividum	
350	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	M08	
321	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	P. corylophilum	
285	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	A. nidulans	
229	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	M02	
267	T	C	C	A	T	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	-	T	C	A	A	G	C	C	C	T	T	T	G	C	U. elatum	
324	T	C	C	A	T	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	-	T	C	A	A	G	C	C	C	T	T	T	G	C	M01	
326	T	C	C	A	T	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	-	T	C	A	A	G	C	C	C	T	T	T	G	C	E. nigrum	
215	T	C	C	A	T	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	C	-	T	C	A	A	G	C	C	C	T	T	T	G	C	M03	
389	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	T	C	A	E	C	-	T	C	A	A	G	C	C	T	C	T	G	C	U. cladisporioides
340	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	T	C	A	E	C	-	T	C	A	A	G	C	C	T	C	T	G	C	M05
387	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	T	C	A	E	C	-	T	C	A	A	G	C	C	T	C	T	G	C	U. lignicola
350	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	T	C	A	E	C	-	T	C	A	A	G	C	C	T	C	T	G	C	M09
333	T	C	C	G	G	G	G	G	C	A	T	G	C	C	T	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	T	C	A	E	C	-	T	C	A	A	G	C	C	T	C	T	G	C	U. sphaerospermum

T G - G T G T T G G G C G - C T - G T C C C G C C - - - C G - G G G G A C G G G C C T G A A A G C Majority																																																	
510		520		530		540		550																																									
279	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	P. thomii		
416	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	M04	
391	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	M07	
279	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	P. lividum	
399	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	M08	
280	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	C	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	P. corylophilum	
275	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	-	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	A. nidulans	
279	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	-	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	M02	
416	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	-	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	U. elatum	
382	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	-	-	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	M01
285	T	G	-	G	T	G	T	T	G	G	G	C	G	-	C	T	-	G	T	C	C	C	C	-	-	-	-	-	-	-	G	G	G	G	A	C	G	G	G	C	C	T	G	A	A	A	G	C	E. nigrum
364	T	G	-	G	T	G	T	T	G	G	G																																						

Ectomycorrhizal fungi

Multiple sequence alignment of Ectomycorrhizal fungi. The alignment is organized into blocks with positions 610-650, 660-700, 710-750, 760-790, and 800-830. Majority consensus sequences are shown on the right, with specific nucleotide positions indicated above the lines.

Block 1 (610-650):

Majority	610	620	630	640	650
670	C T C T - G T A G G C C C G G C C G G - C G C C A G C C G A C A A C C - - - T C A T C C T T T T				
699	C T C T - G T A G G C C C G G C C G G - C G C C A G C C G A C A A C C A - - A T C A T C C T T T T				
697	C T C T - G T A G G C C C G G C C G G - C G C C A G C C G A C A A C C C - - A T C A T C C T T T T				
675	C T C T - G T A G G C C C G G C C G G - C G C C A G C C G A C A A C C C - - A T C A T C C T T T T				
695	C T C T T G T A G G C C C G G C C G G - C G C T T G C C G A C A A C C - - - A T C A A T C T T T T				
676	C T C T T G T A G G C C C G G C C G G - C G C T T G C C G A C A A C C - - - A T C A A T C T T T T				
669	C T C G A T T A G G G C C C G G C C G G G C G C C A G C C G A C C G T C T C C A A C C A T T T T T				
672	C T C G A T T A G G G C C C G G C C G G G C G C C A G C C G T C T C C A A C C A T T T T T				
610	C T T C - - T G G T C T T G A A A T G G T - T G G C C G T C C A T T A A G C - - - C T A T A C A T T T				
677	C T T C - - T G G T C T T G A A A T G G T - T G G C C G T C C A T T A A G C - - - C T A T A C A T T T				
678	C T T T - - G C A C T C A T A A C G A - - C G A C G T C C A A - - A G T - - - A C A T T T T T A C -				
657	C T T T - - G C A C T C A T A A C G A - - C G A C G T C C A A - - A G T - - - A C A T T T T T A C -				
621	C T A A A G G G T G T T - C G G G A G G C T A C G T C - - G A A A A A C - - - A A C C C C A T T T C				
672	C T A A A G G G T G T T - C G G G A G G C T A C G T C - - G A A A A A C - - - A A C C C C A T T T C				
619	C T A A A G G G T G T G C T A C C G G G A G G C C A C G T C - - G A A A A A C - - - A A A C C C A T T T C				
692	C T A A A G G G T G C C A C A G G G A G G C C A C G T C - - M A A A A A C - - - M A A C C C A T T T C				
665	C T A A A G G G T G C C A C C G G G A G G C C A C G T C - - G A A A A A C - - - A A A C C C A T T T C				

Block 2 (660-700):

Majority	660	670	680	690	700
616	T C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
655	T C A G G T T G A C C C T C G - G A T C A A M G T A G G G A T A C C C G C T G A A C T T A A G C A T - T				
692	- C A G G T T G A C C C T C G A G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
620	- C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
641	T C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
622	T C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
618	- C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
622	- C A G G T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
652	- G C T C T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
619	- G C T C T T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
619	- A C T C T T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
498	- A C T C T T T G A C C C T C G - G A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A - A T				
665	T A A G G T T G A C C C T C G G - A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
617	T A A G G T T G A C C C T C G G G - A T C A G G C A G G A T A C C C M C T G A M M T T A A G C A T A T				
664	T A A G G T T G A C C C T C G G - A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				
627	T A A G G T T G A C C C T C A G - A T C A M G T A M G G A T A C C C G C T G A A C T T A A T C A T A M				
610	T A A G G T T G A C C C T C G G - A T C A G G T A G G G A T A C C C G C T G A A C T T A A G C A T A T				

Block 3 (710-750):

Majority	710	720	730	740	750
665	C A A T A A G C C G G G G A A X				
693	C A T A A A G C C G G A G G A A A G A A A C C C A A T A G G G A T T G C C T C				
691	C A T A A A A N G C C G G A M G G A A A T T				
681	C A M A A A A M C C G G M A G M A A A M G				
668	C A T A A G C C G G A G G A A A A G A A A C C C A A T A G G G A T T A C C T C				
690	T A A A A A				
671	C A A T A A G C C G G A G G A A A G A A A C C C A A C A G G G A T T G C C T C				
666	C A A T A A G C C G G A G G A				
670	M A A A A A M G G G G A A G M A A A				
600	C A A T A A C				
667	A T A A A M M C M G G A A E G A A				
667	C A A T A A G C C G G A G G A				
645	A A A A A M C G M G G M M A G M A A A				
614	C A A T A A C				
667	C M T M M M G M C G G M G G A A A G C T C G C C M T T M C A M M A A C A A A M M A A M T T T T G A				
613	C A A T A A C				
676	C A T M M M M M C C G G A G G A G M C T T T M G				
659	C A A T A A G C C G G A G G A A				

Block 4 (760-790):

Majority	760	770	780	790
602	X X			
623				
601				
605				
608				
596				
608				
579				
588				
606				
584				
580				
564				
620				
617	C C N C M A A A M A A N G T M G G A M T M M C C C G C M G A A C T T A A M C M T A T C A			
619				
598				
573				