AN ABSTRACT OF THE THESIS OF

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Post-fire Pezizales often fruit seasonally approximately six weeks after a fire in successional groups over the course of two years. Two methods, pure culture synthesis and PCR-based identification, were used to determine if some of these species were mycorrhizal.

Eleven fungal isolates, *Anthracobia melaloma, Gyromitra infula, Helvella compressa, Morchella sp., Pseudorhizina californica, Rhizina undulata, Sarcosphaera coronaria, Sphaerosporella brunnea, Trichophaea hemisphaeriodes, Wilcoxina mikolae* var. *tetraspora,* and an undescribed genus and species, were tested with two sugar levels (no dextrose and 5 g l^{-1}) for their ability to form mycorrhizae with two tree species (Douglas-fir and ponderosa pine) in an aseptic system for the pure culture synthesis experiment. Four fungi were included from the USDA Record of Decision. Containerized seedlings of Douglas-fir and ponderosa pine were grown for approximately four months. Only *Wilcoxina*

mikolae var. *tetraspora* colonized the root tips. However, when colonizing ponderosa pine, ectendomycorrhizae were formed and on Douglas-fir, ectomycorrhizae were formed.

For the PCR-based identification study, post-fire Pezizales were examined for mycorrhizal formation with ponderosa pine *in vivo* on the Malheur National Forest. Fruiting bodies of post-fire Pezizales were collected from a prescribed burn site. Root tips were extracted from soil cores taken underneath fruiting bodies of post-fire Pezizales. Restriction fragment length polymorphisms (RFLPs) were compared between fruiting bodies and fungal symbionts on root tips. No matches were found; therefore, identities of these fungal symbionts were determined by DNA sequence analyses using the 18s, ITS 1, and 5.8s regions of the nuclear ribosomal DNA. The Pezizalean mycobionts were *Geopora cooperi*, *Geopora sp.*, and *Wilcoxina rehmii.*

Outcomes from both studies were inconclusive as to the mycorrhizal status of the post-fire Pezizales examined. Fungi that are probably not mycorrhizal include *Helvella compressa, Scutellinia sp., Tricharina sp.*, the undescribed taxon, and species of *Anthracobia, Morchella,* and *Peziza,* found from burned areas. This study used PCR-based methods to determine the ascomycetous community *in vivo*. A database to help determine the ascomycete mycobionts was created.

Mycorrhizal Status of Post-Fire Pezizales

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kei E. Fujimura, Author

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Chapter 1

Introduction - Comprehensive Literature Review

The purpose of this study was to determine if post-fire Pezizales are mycorrhizal by two proven and important methods: pure culture synthesis (PCS) and polymerase chain reaction (PCR)-based identification. Although many post-fire fungi are widespread and occur in several forest types, their ecological functions remain largely unknown. In the following discussion, topics covering ponderosa pine forests, in which this project was conducted, definition and ecology of post-fire ascomycetes, definition of mycorrhizae, difficulties and methods of studying ectomycorrhizal communities, and descriptions of methods will be reviewed.

1.1 Ponderosa Pine Forests

Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) forests are often characterized as forests in which the trees are widely spaced and park-like. Ponderosa pines are drought resistant and often associated with warm, dry summers and cold winters (Agee 1998). These forests are adapted to low intensity, high frequency fires that maintain their park-like appearance (Agee 1998). Prior to

European settlement, Native Americans managed these forests using this fire regime, thereby probably improving ecosystem robustness (Everett et al. 1994). This fire regime 1) improved fire tolerance of the landscape by favoring fire tolerant species and elevating the crown base of the trees above the level reached by flames of low intensity fire, 2) created wider spacing between trees, 3) improved forage conditions by increasing shrubs, grasses, and forbs in the understory, 4) improved tolerance to forest pathogens and insects, and 5) increased landscape diversity (Everett et al. 1994). It created even-aged pine forests at the stand level, but uneven-aged at the landscape level (Agee 1994).

Ponderosa pines in turn have adapted to survive low-intensity fires that smolder at the base and rarely reach the crown. These trees have thick bark, protecting the trunk, and deep tap roots, providing water in these droughty ecosystems (Agee 1998).

The USDA Forest Service imposed the Fire Exclusion Policy on all federal land in the early 1900s, following European traditions of forestry where fires were treated as a threat (Agee 1994). Fires were thought to threaten lives, property, and timber reserves and considered an agent that killed saplings under large trees (Agee 1994). However, fire regimes have a major influence in the rate and direction of plant succession (Ahlgren and Ahlgren 1960); changes in an established fire regime can cause major alterations for ecosystems. Ponderosa pine ecosystems were altered partly because their fire regimes changed from frequent, low-intensity fire to infrequent, moderate to high-intensity fire. In moist conditions fires would not

carry through stands of thick understories as they did before fire suppression. This alteration was due to heightened relative humidity near the ground that increased the moisture content in dead needles. Under dry conditions, fires burn more intensely from larger fuel loads (Agee 1994). In addition, because of this new fire regime, smoke potential intensified due to higher fuel loads, and the recovery period after a fire lengthened (Everett et al. 1994). With increased periods between fires, aboveground nutrients can accumulate in organic matter and be lost with a hot fire (Everett et al. 1994). Fire intensity is correlated with soil changes. As fire intensity increases, so do changes in the soil (Vázquez et al. 1993, Ahlgren and Ahlgren 1965). Contrary to conditions before fire suppression, severe fires would more likely cause some soils to erode and run off (Ahlgren and Ahlgren 1960). Nutrients in soil tend to be lost due to volatilization with increasing temperatures, nitrogen, phosphorus, and sulfur being more susceptible than other nutrients (Agee 1993).

Once fire frequency was reduced in ponderosa pine forests, regeneration under the larger trees changed the mosaic of groups of even-aged trees on a landscape level to an evenly spread of thickets of saplings (Agee 1994). Ponderosa pine growth was stunted when compared to trees before fire suppression (West 1969). After fire suppression, many trees never exceeded diameters greater than several centimeters after 60-80 years of growth (Agee 1994). This dense understory created additional stress on trees during the dry season. When the larger trees were removed, stands remained too dense and stagnated, increasing their

susceptibility to western pine beetle (*Dendroctonus brevicomis* LeConte), mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Agee 1994), western spruce budworm (*Choristoneura occidentalis* Freeman), and Douglas-fir tussock moth (*Orgyra pseudotsugata* McDunnough) (Everett et al. 1994). In addition, this congestion predisposes trees to dwarf mistletoe, which spreads rapidly (Everett et al. 1994). Everett et al. (1994) reported dwarf mistletoe causes more loss of tree growth and increased mortality than any other disease or insect. This increased number of dead trees adds to fuel loads that were not previously part of ponderosa pine ecosystems (Everett et al. 1994, Agee 1994).

Increased fuel load changes fire behavior in ponderosa pine forests. When trees are well spaced, low intensity fires spread horizontally, carried by the pine needle litter and grass. After fire suppression, horizontal fires become more intense and vertical, with burning of branches and ladder fuels, as well as needles and grass. Fires reach higher and crowns can be torched (Agee 1994). As fire intensity increases, the fire tolerance of these forests decreases (Agee 1994).

Reports on general effects of fire on fungi demonstrate great variability. Fungal fruiting was reduced after a wildfire compared to before the fire, wildfires burning more intensely than prescribed fires (Vázquez et al. 1993). Ahlgren and Ahlgren (1965) saw no decrease in fungal fruiting in prescribed fires when compared to conditions before the fires, but concluded that fungal fruiting depended on fire severity (Ahlgren and Ahlgren 1960). However, fungi vary greatly in fruiting patterns, depending on taxa examined. For example, reports of

increased fruiting (Ahlgren and Ahlgren 1960, Seaver and Clark 1909) are for post-fire fungi. Conversely, reports of decreased fruiting are often fungal pathogens (Ahlgren and Ahlgren 1960, Agee 1994). Wright and Tarrant (1958) reported that the percentage of seedlings with mycorrhizae was lower on burned than unburned sites. They also observed that for one-year old seedlings found in more intense burns, mycorrhizae did not occur in the upper soil layers.

According to Everett et al. (1994), by the 1960s, the public wanted change in the fire suppression policy. They attributed this change to changes in the public's view of natural ecosystems. The public wanted clean water and air, conservation of wildlife and plants and their habitats, and wise use of renewable resources, and showed more awareness of disease and insect outbreaks and severe fires. They saw these factors as threats to loss of fish, birds and mammals by overuse of the land and loss of habitat. The Forest Service recommended that frequent, low to moderate-intensity fires be returned to landscapes that historically had this fire regime. In 1978, prescribed burning became Forest Service policy for these ecosystems (Agee 1994).

1.2 Ecology and Definition of Post-fire Ascomycetes

The Ascomycota are characterized by producing spores in sac-like cells (asci) containing ascospores. In contrast the Basidomycota produce club-shaped cells (basidia) on which spores are borne. The Ascomycota include many classes, including the Euascomycetes (Luttrell 1951). The subclass Discomycetes are characterized by production of apothecial ascocarps, and forcible spore discharge

or forms thought to be derived from them (Weber et al. 1997) in which spores may or may not be forcibly discarded. Orders of the Discomycetes are further divided as having either operculate asci, in which spores exit from a subcircular opening or slit at or just below the apex, or inoperculate asci. The asci of members of the Pezizales have an apical operculum, or may have evolved from ancestral types with operculate asci. However, most hypogeous and a few epigeous members of this order have lost their ability to forcibly discharge their spores (Trappe 1979), many have evanescent asci, and depend on mycophogy for spore dispersal (Maser et al. 1978). Post-fire Pezizales include members of several families (El-Abyad and Webster 1968, Petersen 1970). Post-fire fungi are defined as "species that under external conditions [are] found exclusively on burnt ground" (Petersen 1970). Other names synonymous to "post-fire" are "fireplace fungi" (Petersen 1970) and "phoenicoid fungi" (Carpenter and Trappe 1985). Post-fire fungi seem to fruit seasonally in successional groups after a fire for up to two years (Petersen 1970).

Questions remain as to why many of the post-fire Pezizales are found only after a fire, and why they fruit in a successional pattern. El-Abyad and Webster (1968) proposed competition as the main biological factor and that these fungi compete better because of their tolerance of basic soils. Seaver (1909) and Moore and Korf (1963) hypothesized that these fungi cannot compete successfully with other fungi and bacteria in non-burned soil. Daubenmire (1949) based the competition on low nitrogen requirements of post-fire fungi, which freed these fungi from competition of fungi with higher nitrogen requirements.

Petersen (1970) suggested other reasons why post-fire fungi fruit only after a fire 1) properties of ash, 2) change of competition conditions, 3) germination of dormant spores due to heating, and 4) formation of toxins. He concluded that sporocarp occurrence was based on three factors: 1) release of nutrients from roots of killed trees and herb, 2) reduction of biotic competition through heat, and 3) formation of an ash layer. Zak and Wicklow (1980) supported Petersen and found that without ash, biotic factors associated with unburned soils, significantly lowered the diversity of the post-fire Pezizalean community. These biotic factors seemed to prevent certain species from dominating post-fire soils. They further suggested that ash inhibited microorganisms, which produce antibiotics that inhibit post-fire Pezizalean growth and compete for nutrients. They felt that germination of dormant spores was the main contributing factor. "To cope with the ephemeralness of habitat, production of dormant spores evolved to secure germination when the next fire occurs" (Zak and Wicklow 1980). These dormant spores would germinate at opportune times when prospective competing microbes are reduced.

Recent studies of post-fire Pezizales focussed on functions of these fungi. Danielson (1984) was the first to report that a post-fire species of the Pezizales, *Sphaerosporella brunnea* (Alb. & Schwein. : Fr.) Svr e k & Kubi k, forms mycorrhizae with various hosts. He questioned past presumptions about the role of post-fire Pezizales as only saprotrophs. Warcup (1990) reported three species of post-fire Pezizales, *Lachnea vinosobrunnea* (Berk. & Br.) Sacc., *Muciturbo*

reticulatus Talbot, and *Pulvinula tetraspora* (Hansford) Rifai formed mycorrhizae with seedlings of *Melaleuca uncinata* R. Br. ex Aiton *f.* and *Eucalyptus obliqua* L. Hér. in an open pot study.

Egger (1986) and Egger and Paden (1986a, 1986b) used pure culture synthesis (PCS) to determine if certain post-fire Pezizales were pathogenic, saprotrophic, or biotrophic on *Pinus banksiana* Lamb. To test for pathogenicity, they visually assessed the seedlings for disease by the following criteria: root or stem rot, chlorosis, and stunted shoot growth. To determine if the fungi were saprotrophic, the fungi were assayed for enzymes that could degrade various substrates. To test for biotrophism, root tips were morphotyped by looking for the "classical" mycorrhizal morphology of Hartig net and mantle. They concluded that most post-fire Pezizales tested appeared to be opportunistic decomposers or facultative biotrophs.

Of the species that Egger and Paden (1986a) found to be biotrophic, only *Sphaerosporella brunnea* was clearly mycorrhizal, confirming the findings of Danielson (1984). Egger and Paden also found that *Geopyxis carbonaria* (Alb. & Schw.) Sacc., *Trichophaea hemisphaerioides* (Mont.) Graddon, and *Anthracobia maurilibra* (Cooke) Boud. may be mycorrhizal or weakly pathogenic, perhaps depending on environmental conditions. They reported two species to be pathogenic, *Rhizina undulata* Fr. and *Pyropyxis rubra* (Peck) Egger. With these conclusions, Egger and Paden (1986a) echoed Molina and Palmer's (1982) caution that findings from PCS studies may be exaggerated when compared to what would

happen *in vivo* but saw this technique as an important initial step to determine if a fungus is capable of biotrophic interactions.

Recently Vrålstad et al. (1998) reported *Geopyxis carbonaria*, a fungus common and abundant after severe fires, to form mycorrhizae on *Picea abies* Karst. They suggested that *G. carbonaria* needed not only a suitable host but also a severe fire for fruiting to occur. They postulated that severe fires changed the environment (Peterson 1970, Zak and Wicklow 1980, El-Abyad and Weber 1968), disrupted mycorrhizal associations (Wright and Heinselman 1973, Harvey et al. 1994), reduced competition in the upper rhizosphere (Wright and Heinselman 1973, Harvey et al. 1994), and altered physiochemical conditions (Wicklow and Hirschfield 1979). These changes allowed copious fruiting of *G. carbonaria*. Another possible strategy for post-fire fungi to survive burns is that some mycorrhizae are deep enough to avoid heat penetration (Vrålstad et al 1998, Mikola et al. 1964).

1.3 Definition of Mycorrhizae

The definition of mycorrhizae addresses two criteria: function and structure. The functional definition refers to the ecological role of mycorrhizae; the structural definition serves as a means to categorize mycorrhizae. Trappe (1994) provides a comprehensive functional definition when he defines mycorrhizae as "dual organs of absorption formed when symbiotic fungi inhabit healthy absorbing organs (roots, rhizomes or thalli) of most terrestrial plants and many aquatics and epiphytes." This definition includes the significance of the absorptive nature of mycorrhizae,

emphasizes a mutualistic relationship, and refers to the "near omnipresence" of mycorrhizae (Trappe 1994). The structural definition distinguishes between penetration of the cortical cells of the plant host by the fungus (endomycorrhizae, including arbuscular mycorrhizae [AM]), and no penetration of the cortical cells (ectomycorrhizae). AM characteristics include an arbuscule, a haustorium-like structure thought to be the site of nutrient exchange between fungus and plant. AM may or may not have a vesicle, a sack-like structure thought to store extra nutrients. These fungi are found only the Glomales in the subphylum Zygomycota.

Ectomycorrhizae have a Hartig net of hyphae that enclose the outer cortical cells and a mantle of hyphae that cover the rootlets. Ectomycorrhizal fungi often change the root tip morphology. For example, ectomycorrhizae often appear as swollen, branched, and discolored root tips. Ectomycorrhizal fungi belong mostly to the Basidiomycota, with some in the Ascomycota and a few in the Zygomycota. Further structural definitions have arisen to accommodate other unique structures (e.g., ectendomycorrhizae, arbutoid mycorrhizae, or ericoid mycorrhizae. See Smith and Read (1997) for further descriptions).

1.4 Analysis of the Ectomycorrhizal Community

Most ectomycorrhizal community studies have been based on sporocarp production. Although Luoma et al. (1997) found that mycorrhizal frequency of *Hysterangium sp.*, *Lactarius rubrilacteus* Hesler & A.H. Sm, *Leucogaster rubescens* Zeller & Dodge, and *Truncocolumella citrina* Zeller increased with

sporocarp biomass on the stand level, the correlation between sporocarp production and the fungal species' corresponding mycorrhizae is not well supported.

Gardes and Bruns (1996a) reported that the most abundant fruiters aboveground do not necessarily equate with the fungi colonizing most of the root tips. Although *Amanita franchetti* (Boudier) Fayod and *Suillus pungens* Thiers & Smith appeared to be the most abundant from aboveground surveys, the belowground community was quite different. *A. franchetti* was found in about 60% of the soil cores but was only found on about 10% of the root tips. *S. pungens,* the highest producer of sporocarps, was found in only 20% of the soil cores and less than 5% on root tips. The most abundant mycorrhizal fungus on root tips was *Russula amoenolens* Romagnesi, which produced few fruiting bodies. The second most abundant mycorrhizal fungus on root tips was what Gardes and Bruns labeled 'Boletoid-1'. The DNA sequence of this fungus could not be matched to any known generic sequences entered in GenBank, but it seemed to belong in the Boletaceae. Gardes and Bruns (1996a) concluded that mycorrhizal communities cannot be determined by surveys of sporocarps. This study also indicated how little is known about the belowground ecosystem and what is known aboveground cannot be extrapolated to what is unknown belowground.

Taylor and Alexander (1989) cautioned about assessing mycorrhizal communities from sporocarps. They found in a 43-year old Sitka spruce stand (*Picea sitchensis* (Bong.) Carr.) less than 5% of mycorrhizal morphotypes belonged to species that fruited most abundantly. By use of molecular techniques, the

mycorrhizal fungi can be identified at least to family, whereas in previous studies based on morphotypes could only identify a few distinct mycorrhizal fungal species. Using molecular methods, Gardes and Bruns (1996a) estimated from morphotyping studies that at least half the mycorrhizal types were formed with unknown fungi.

1.5 Identification of the Mycobiont

Identification of the mycobiont is essential in understanding structure, function, and communities of mycorrhizae. The inability to identify most mycorrhizal fungi from mycorrhiza morphotypes has hindered understanding of the basic ecology of mycorrhizal fungi (Gardes and Bruns 1993). Agerer (1986) credited Dominik (1959) for creating the first key to identify mycorrhizae. However, his key very vague, and his intent was to lump mycorrhizae into morphological groups (Trappe 1967). Trappe (1967) created a key to classify mycorrhizae by hyphal characteristics that would lead to a specific mycobiont for more distinctive mycorrhizae. The less distinctive fungi would fall into general groups. Trappe (1967) also noted that to gain a positive identification, field collections along with a pure culture synthesis (PCS) of the fungi would be needed. Tracing the mycelium to the fruiting body, to determine if that fruiting body is the mycobiont, is difficult and the results uncertain, especially if it is the only method used (Trappe 1957, 1967).

Zak (1973) gave four methods for identifying the mycobiont. In PCS, a seedling is inoculated with a pure culture from a fungus to determine if

mycorrhizae form. In a second method the fungus is isolated from mycorrhizae, grown in culture, and compared to known cultures from sporocarps. This method has limitations in that collections of known fungal cultures are inadequate, and not all mycorrhizal fungi can be cultured (Zak 1973). Zak's third method was to trace the rhizomorphs and hyphae to the sporocarp, a difficult procedure with considerable uncertainty (Trappe 1957). The fourth method is to link the sporocarp to the underlying mycorrhizae. In this method, sporocarps and the mycorrhizae beneath them are both collected. The tissues of the sporocarp and mycorrhizae are then compared. This method is limited to when and what sporocarps are present (Zak 1973).

Agerer succeeded in tracing hyphae or rhizomorphs from sporocarp to mycorrhizae for identification of mycorrhizal fungi from root tips (Agerer 1987-1993). He carefully takes soil cores from the base of sporocarps, ensuring that roots and rootlets are connected with the fruit body or base of the stipe, and then cuts off the stipe (Agerer 1987-1993). He soaks the soil core in water, and uses dissecting needles, forceps, and paintbrushes to carefully remove the soil to show the hyphal connection between the mycorrhizae and the fruit body or base of the stipe. Agerer then records and photographs characteristics of the mycorrhizae and gives the mycorrhiza a Latin name. Results of his work are published in the "Color Atlas of Ectomycorrhizae" (Agerer 1987-1993). Agerer's philosophy incorporates the idea that anatomical studies of mycorrhizae can provide information on their

physiological capabilities, and that a comprehensive characterization is needed to understand this correlation (Agerer 1994).

A new method for identifying the mycobiont is by molecular techniques. For mycorrhizal research, PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) is commonly used. According to Dannell (1994), before PCR, no reliable methods existed to identify mycorrhizal fungi from root tips. Molecular techniques reduce problems that occurred in morphotyping and isolating mycorrhizal fungi (Egger 1995). Morphotyping is problematic due to intraspecific variation within taxa, and isolating mycorrhizal fungi can be a challenge because they often do not grow in culture. In addition, mycorrhizae may differ in morphology when associated with different hosts. For instance, Taylor and Bruns (1997) found root tips of the non-photosynthetic orchid *Cephalanthera austinae* (A. Gray) Heller colonized by a member of Thelephoraceae. The root tip had penetration of the cortical cells and no mantle. However, root tips of trees near *C. austinae* were colonized by the same fungus colonizing its tips but had a mantle, Hartig net, and lacked intracellular penetration. The identity of the mycobiont was confirmed by matching RFLP patterns and sequence analyses from both the nuclear ribosomal DNA (nrDNA) and mitochondrial ribosomal DNA (mt rDNA).

1.6 Pure Culture Synthesis

Pure culture synthesis (PCS) may be used to determine if a fungus has the potential to be mycorrhizal and as a means to identify the mycorrhizal fungus from a root tip. This method entails obtaining a pure culture by isolating the fungus

from spores, sporocarp, mycelium, or mantle and growing it on a sterile medium. Seedlings are then inoculated with the fungus in an aseptic system. If the fungus colonizes the root, then it is probably mycorrhizal. If the fungus does not colonize the root, then the question remains unresolved. Unfortunately, AM fungi and many ectomycorrhizal fungi do not grow in culture. Pure culture synthesis methods were developed by a pioneer in mycorrhizal research, Elias Melin (Molina and Palmer 1982). His basic techniques are still applied in many PCS studies.

Duddridge (1986) argued against the PCS method. Her premise was that the excess sugar used in PCS studies induced mycorrhiza formation that may not occur in natural settings. Duddridge (1986) argued that excess sugar may explain why ectomycorrhizal fungi in PCS studies tend to have a wide host range. In a previous study by Duddridge and Read (1984), SEM/TEM techniques were used to demonstrate that transfer cells may be produced in the presence of exogenous sugar. Transfer cells transport carbohydrates from the media to the fungus, and then to the plant. They argued that this transfer of sugar from media (or substrate) to plant would not occur in nature. Duddridge (1986) advised against the use PCS techniques when studying specificity or compatibility of mycorrhizal fungi.

Jumpponen and Trappe (1998) examined the excess sugar argument. They added different levels of sugar to the inoculum in both closed and open pot systems. They hypothesized that increased growth in an aseptic culture system was due to fungal respiration. As the glucose concentration increased, then the host plant biomass substantially increased. However, this increase in glucose

concentration did not significantly increase *Pinus contorta* biomass when no inoculum was added. They asserted that increase of fungal respiration and not the addition of glucose *per se*, increased CO_2 levels, consequently increasing plant biomass. More support for this argument came from the results of their open pot experiment. The same strain of inoculum did not significantly increase plant biomass as it did in the closed system. They concluded that the combination of carbohydrate transfer by the fungus and elevated CO_2 concentrations due to fungal respiration, resulted in increase of plant biomass in closed systems. Their study supported Duddridge's conclusion with statistical evidence for exercising caution in PCS studies (A. Jumpponen, pers. comm.).

1.7 PCR-Based Identification

PCR-based methods are good for large, comparative studies because only a small amount of DNA is needed, and many samples can be processed in a relatively short time (Bruns et al. 1991). DNA can be extracted from cultures, sporocarps, and mycorrhizae (Egger 1995). Nuclear ribosomal DNA (nrDNA) is often used for fungal studies because molecular markers are universally conserved among organisms, variability is high between species and minimal within a species (Egger 1995), and the ribosomal repeat is a multi-copy gene (Gardes and Bruns 1993). The nrDNA is comprised of: 1) the small subunit (18s region), 2) the internal transcribed spacer (ITS1) region, a non-coding region of the gene, 3) the 5.8s region, 4) the ITS 2 region, a non-coding region, 5) the large subunit (28s region),

6) the intergenic spacer (IGS), a non-coding region; this sequence is then repeated, starting with the small subunit.

The small subunit of nrDNA (18s region) is effective for studying distantly related organisms (White et al. 1990), and intergeneric studies (O'Donnell et al. 1997). The ITS region is helpful for identifying species within a genus (White et al. 1990). It is readily amplified with universal primers that are fungal specific, allowing amplification of fungal DNA from mixed extracts, such as plant and fungal DNA extracted from ectomycorrhizal root tips (Gardes and Bruns 1996b). The 5.8s gene is helpful as an initial screen to determine if sequences are contaminated, to place asexual fungi in appropriate subphyla, and identify mycorrhizal fungi (Cullings and Vogler 1998). The IGS region is suitable for population studies (Jorgenson and Cluster 1998). The mitochondrial ribosomal DNA (mt rDNA) is another gene often used in fungal research. A sequence database using about 350 base pairs of mt rDNA has been developed for determining taxa at the ordinal and family levels for basidiomycetes (Bruns et al. 1998).

Once the ITS region is amplified, endonucleases are used to digest the PCR product into fragments (Egger 1995). For example, the restriction enzyme *Hae* III will cleave the DNA when the sequence 'GGCC' appears and will cut between the G and C. Often for ectomycorrhiza studies, at least three restriction enzymes are used to reveal different species. Digestion of DNA by restriction enyzmes followed by fragment separation by electrophoresis produces restriction fragment

length polymorphisms (RFLPs). Restriction fragment length polymorphisms appear as banding patterns created by the different sizes (and somewhat on the charge of the molecule) of DNA fragments that are a function of the occurrence of restriction sites in the ITS sequence. Restriction fragment length polymorphisms of unknown root tips should be compared to RFLPs of suspected matching sporocarps, so knowing the fungi and their different habitats is crucial if the mycorrhizal fungi are to be identified (Gardes and Bruns 1996b). This method may not work for closely related species, and it may identify the same species as different because of intraspecific variation. If the latter problem occurs, then another coding region is recommended (Gardes and Bruns 1996b). In general, RFLPs can be efficient aids in identifying fungal symbionts by comparing patterns of unknowns to patterns of identified fungi.

If no RFLP matches identified the fungi, then DNA sequences can be used. Computer programs align the sequences to known sequences stored in databases (Egger 1995). For unknown ascomycetes, sequences from regions from the nrDNA are often used. For instance, the database GenBank has sequences for members of the Pezizales for the 18s region, partial 28s region, and ITS region; sequences from the small subunit are the most frequent. Sequences of the small subunit have been used for intrageneric and intergeneric studies (O'Donnell et al. 1997, Landvik and Eriksson 1994, Norman and Egger 1996) and subordinal analyses (Landvik et al. 1997, Harrington et al. 1999). When the 5' end of the 28s region is used for analysis, the whole or partial 18s region and ITS region are also included. This

combination is helpful for testing hypotheses that include a broad range of organisms (Bruns et al. 1991, Cullings and Vogler 1998) and answers inter- and intraordinal questions (O'Donnell et al. 1997, Norman and Egger 1996, Holst-Jensen et al. 1997, Egger 1996).

Sequence data are analyzed to assess phylogenetic relationships. A particularly useful tool for such analysis is PAUP (Phylogenetic Analysis Using Parsimony, Swofford 1999). Parsimony, which chooses the simplest hypothesis that requires the fewest steps and assumptions to find relationships, is one of the most common methods used for phylogenetic studies (Swofford et al. 1996). Maximum parsimony methods evaluate the probability that the evolutionary history will give rise to observed data. It is robust to many violations and assumptions in the model and has lower variances than other phylogenetic methods. Maximum parsimony is supported by bootstrap values, which are based on the concept that the variance can be estimated by repeatedly resampling randomly chosen data from the original data set. Bootstrapping eliminates variances for sampling distributions that are either unknown or difficult to derive analytically. Bootstrap values, usually reported as a percent, are the number of pseudosamples (usually 100 pseudosamples) in which a certain grouping will occur.

1.8 Conclusion

The function of many post-fire fungi remains unknown. This project will address the functional question for some of these species, or shed light as to the function of some non post-fire ascomycetes. This study is one of the first to

identify ascomycetous mycorrhizae using PCR-based identification methods. Ponderosa pine forests in which ponderosa pine is the sole member of the Pinaceae, provide a convenient study area because their roots are easy to differentiate from root tips of other potential hosts found at the site. Drawbacks of this study include the lack of ascomycete-specific primers and understanding the needs of ascomycetes to successfully grow in culture and axenically colonize root tips.

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Chapter 2

Lack of Mycorrhizal Colonization by Post-fire Pezizales in a Pure Culture Synthesis Experiment

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2.1 INTRODUCTION

Sporocarps of post-fire fungi appear seasonally in successional groups of species in forests, and then seemingly disappear after two years (Petersen 1970). Many of these fungi belong to families in the Pezizales (El-Abyad and Webster, 1968, Petersen 1970). Although they are presumed to be saprotrophic, their ecosystem function is not well known.

Danielson (1984) showed that the post-fire species *Sphaerosporella brunnea* (Alb. & Schwein. : Fr.) Svr ek & Kubi k formed mycorrhizae with containerized seedlings of jack pine (*Pinus banksiana* Lamb.). Furthermore, he suggested that other epigeous species in the Pezizales may be mycorrhizal (Danielson 1984). Warcup (1990) demonstrated that 3 species (*Lachnea vinosobrunnea* (Berk. & Br.) Sacc., *Muciturbo reticulatus* Talbot, and *Pulvinula tetraspora* (Hansford) Rifai) of post-fire Pezizales formed mycorrhizae with containerized seedlings of *Melaleuca uncinata* R. Br. ex Aiton *f.* and *Eucalyptus obliqua* L. Hér. Dahlstrom *et al.* (in press) demonstrated the ability of some species of *Morchella*, a well-known post-fire genus, to form mycorrhizae in aseptic conditions.

This study was conducted to expand upon previous findings of mycorrhiza formation by species in the Pezizales. Four species were included from the Record of Decision (USDA 1994) to determine the ecosystem function of and address conservation concerns for rare fungi within the range of the Northern spotted owl (*Strix occidentalis caurina* deVesey). A second objective of the study was to test

the selected fungi for host specificity with two ecologically and economically important tree species in the Pacific northwestern region of North America, ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) and Douglas-fir (*Pseudotsuga meziesii* (Mirb.) Franco).

Formation of mycorrhizae by some ascomycetes can be difficult to detect because of scant mantle and Hartig net formation (Ingleby *et al.* 1990, Dahlstrom *et al.* in press). Consequently, mycorrhiza formation by many ascomycetes has been largely ignored or possibly overlooked. Pure culture synthesis (PCS), an aseptic technique, was selected as a method for easily detecting mycorrhiza formation with the selected tree hosts. Pure culture synthesis has been instrumental in studying the biology of fungi, including post-fire fungi, for several decades (Molina 1979, Molina and Palmer 1982, Danielson 1984, Egger 1986, Egger and Paden 1986, Dahlstrom *et al.* in press). It has been criticized because sugar provided in the media may permit colonization that would not occur in nature (Duddridge 1986). Therefore, a third objective of our study was to examine the effects of dextrose levels on mycorrhizal development.

2.2 Materials and Methods

2.21 Fungal Inocula

Isolates of eleven species of Pezizales (Table 2.1) were tested for their ability to form mycorrhizae with Douglas-fir and ponderosa pine. All were

post-fire fungi except *Helvella compressa, Morchella sp, Sarcosphaera coronaria.* and. *Pseudorhizina californica.*

TABLE 2.1: SPECIES OF FUNGAL INOCULUM

Species	Collection Number[1]	Isolate Source	Medium[2]
Anthracobia melaloma (Alb. & Schwein. : Fr.) Arnauld	KEF 1	Fresh spores	MYA
**Gyromitra infula* (Schaeff. : Fr.) Quel.	NSW 8132	Fresh spores	MYA
**Helvella compressa* (Snyder) N.S. Weber	KEF 9	Tissue explant	MYA
Morchella sp.	NSW 7702	Dried spores	PDA
**Pseudorhizina californica* (Phillips) Harmaja	NSW 6661	Dried spores	PDA
Rhizina undulata Fr. : Fr.	NSW 8125	Dried spores	PDA
**Sarcosphaera coronaria* (Jacq.) J. Schrot.	NSW 7728	Dried spores	PDA
Sphaerosporella brunnea (Alb. & Schwein. : Fr.) Svr ek & Kubi k	NSW 6793	Dried spores	PDA
Trichophaea hemisphaeriodes (Mouton) Graddon	NSW 7166	Dried spores	PDA
Wilcoxina mikolae var. *tetraspora* (Yang & Wilcox) Yang & Korf	CSY-57	Unknown	MMN
Undescribed taxon	NSW 8130	Fresh spores	MYA

[1]Collections from CSY: C.S. Yang, KEF: K.E. Fujimura, NSW: N.S. Weber.
[2]MYA = malt yeast agar; PDA = potato dextrose agar; MMN = Modified Melin-Norkrans

Isolates from fresh specimens were from spores or sporocarp tissue (Table 2.1). Spore isolates were obtained by suspending 0.5 – 1 cm^3 sections from the apothecium, hymenium side down, over sterile Petri dishes containing malt yeast agar (MYA) (Stamets 1993), potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) or modified Melin-Norkrans (MMN) agar (Marx 1969) for 3-5 minutes. Warming the sporocarp helped stimulate spore release. To ensure even distribution of spores, the apothecium was suspended for about fifteen seconds over different areas of agar. Tissue isolates were obtained by aseptically removing a small piece (1-2 mm^2) of sterile tissue from the sporocarp and placing it on nutrient agar in a sterile Petri dish. Preserved spores from N.S. Weber (NSW) collections were previously stored on glass cover slips 1cm in diameter, as described by Weber (1995).

Isolates were incubated between 4 and 20 months. Two vegetatively colonized 9 mm diameter discs of each isolate were cut from agar plates and transferred into sterile flasks containing 100 ml of liquid media corresponding to the media on which they were isolated. Fungi were grown in liquid media for 4-8 weeks and swirled 3-4 times per week.

2.22 Seedling Preparation

Seeds of ponderosa pine and Douglas-fir were surface sterilized in 30% hydrogen peroxide in a covered sterile container for 50-55 minutes, rinsed with sterile deionized water (dH_2O), aseptically sown in Petri dishes containing 25 ml of PDA, left at room temperature (19-21° C) for one week, and checked for

contamination. Seeds were then cold stratified (4-5° C) for 6-8 weeks to ensure even germination, then left at room temperature for one week to germinate.

2.23 Pure Culture Synthesis

The PCS protocol followed that of Molina (1979) and Dahlstrom *et al.* (in press) with minor adjustments. Seedlings were grown in 30 x 300 mm glass test tubes topped with 50 ml beakers. Each tube was filled with a mixture of 110 cc vermiculite, 10 cc peat moss, and 70 ml of MMN with either no dextrose or 5 g l^{-1} of dextrose, then autoclaved one hour, and left to cool to room temperature. Individual axenic seeds with protruding radicles were placed in tubes. Seedlings were allowed to grow for 4-6 weeks before fungal inoculation.

Fungi in liquid media were blended in a sterilized Waring™ blender for about eight seconds. 100 ml of blended mixture were filtered through a 7 cm Buchner funnel containing Whatman's qualitative filter paper. Fungal mycelium on the filter was rinsed once with 100 ml of sterile dH_20 to further remove remaining nutrient medium. Fungal mycelium on the filter paper was placed in a beaker containing either MMN with no dextrose or MMN with 5 g l^{-1} of dextrose. Once the fungal mycelium was shaken off the filter paper, the filter paper was removed. As the medium with fungi was constantly stirred, 10 ml aliquots were pipetted into six test tubes per tree species. Test tubes were randomly placed in racks with the bottom one third of the tube submerged in a water bath, set at 16-20° C. Seedlings grew under fluorescent-incandescent light of an average of 157.6

μmol m^{-2} s^{-1} with a 16-hour photoperiod (Dahlstrom *et al.* in press). Racks were repositioned in the water bath every two to three weeks.

2.24 Experimental Design

The experiment consisted of a 2 x 2 factorial design with two sugar levels in the inoculation media (MMN with ½ strength dextrose [5 g l^{-1}], and MMN with no dextrose) and two tree species (Douglas-fir and ponderosa pine). Six replications for each treatment combination of sugar level and tree species were used for the eleven test fungi, and for a noninoculated control for each treatment combination. Two of the eleven test fungi, *Wilcoxina mikolae* var. *tetraspora* and *Sphaerosporella brunnea*, have been well-established as mycorrhizal fungi (Mikola 1965, Laiho 1965, Yang and Korf 1985, Egger and Fortin 1990, Egger 1996, Danielson 1984, Egger and Paden 1986) and served as positive controls. One month separated trials of three to four fungi to provide time for assessing colonization of seedlings approximately the same age.

2.25 Mycorrhizal Assessment

Four to five months after inoculation, seedlings were removed intact from tubes and substrate. Substrate was gently washed from the root with dH_2O. Number of colonized and total number of root tips were counted. Roots were examined by stereomicroscopy to determine if mycorrhizae had formed. Root tips in 0.01 g trypan blue diluted in 10 ml of lactoglycerol were heated with a match for a few seconds to quicken staining reaction, then rinsed with dH_2O. Free hand

sections were mounted in lactoglycerol and examined with a compound microscope for mantle, Hartig net, and inter- and intracellular penetration by hyphae.

To confirm the identity of the fungal symbionts, we used polymerase chain reaction (PCR)-based identification methods to compare restriction fragment length polymorphisms (RFLP) patterns of fungal material from the root tips with the original sporocarp, following the protocol of Gardes and Bruns (1993). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was amplified using primer pairs ITS1f and ITS4 (Gardes and Bruns, 1993). Endonucleases, *Dpn* II and *Hinf* I, were used for restriction digests.

2.26 Statistical Analysis

For isolates that successfully colonized root tips, t-tests assuming unequal variance were conducted to determine significant differences between sugar treatments, with $\alpha = 0.05$. Differences due to sugar treatments were assessed by the percentage of total root tips colonized. To determine if presence of the fungus affected number of root tips, number of tips was used in the analysis.

2.3 RESULTS

Only *Wilcoxina mikolae* var. *tetraspora*, one of our positive controls, formed mycorrhizae with the two host species. On ponderosa pine, *W. mikolae* formed ectendomycorrhizae with thin mantles, Hartig net, and hyphal coils in the cortical cells. On Douglas-fir, ecto- but not ectendomycorrhizae were detected, and root tips were more brown than orange-brown. Colonization did not occur with the

other fungi. Although roots looked swollen with *Sphaerosporella brunnea*, no colonization was detected and little mycelium grew in the tubes. Other fungi that did not grow well when added to PCS tubes were *Helvella compressa, Rhizina undulata* when added to Douglas-fir, and *Pseudorhizina californica* when added to Douglas-fir; all other fungi grew after inoculation. When fungi were present on the roots, hyphae were abundant along the primary root, with no sign of penetration. Root tips and primary roots of seedlings inoculated with *Helvella compressa* and an undescribed genus and species were lignified. Also, conidia were found with the undescribed taxon.

Some of the PCS tubes containing *Morchella sp., Trichophaea hemisphaeriodes, Rhizina undulata, Sarcosphaera coronaria,* and *Wilcoxina mikolae* were contaminated. The contaminant found with *Morchella sp.* was an unidentified actinomycete. For PCS tubes with *R. undulata* and *T. hemisphaeriodes*, the contaminant was cottony white and the root tips were black. Also a dark blue stain on the base of the root tips was found with some of the seedlings with *R. undulata.* The contaminant found with one seedling for each sugar treatment for *W. mikolae* and seedlings with *S. coronaria* was indicated by a slimy, white substance on the roots. No contamination was detected in tubes or on roots with the undescribed taxon, although some of the roots looked necrotic, and Douglas-fir with dextrose in the media appeared stunted in some of the tubes.

Wilcoxina mikolae colonized 14% of the Douglas-fir root tips in MMN with no dextrose compared to no colonization with ½ the strength dextrose (p-value =

0.0007) (Table 2.2). No difference in percent of colonization root tips of ponderosa pine was detected between dextrose treatments (p-value = 0.59) (Table 2.2).

TABLE 2.2: T-TEST ANALYSIS FOR PERCENT COLONIZATION BY *WILCOXINA MIKOLAE* BETWEEN DEXTROSE TREATMENTS ON SEEDLING HOSTS WITH UNEQUAL VARIANCE

Treatment	Number of replicates	Percent colonized	Standard Error	T-stat	p-value
Wilcoxina mikolae on Douglas-fir: MMN with no dextrose	6	14%	0.003	6.36	0.0007
Wilcoxina mikolae on Douglas-fir: MMN with ½ strength dextrose	6	0%	0		
Wilcoxina mikolae on ponderosa pine: MMN with no dextrose	5	26%	0.078	0.56	0.59
Wilcoxina mikolae on ponderosa pine: MMN with ½ strength dextrose	5	21%	0.048		

The mean number of Douglas-fir root tips was greater in the *Wilcoxina*-inoculated MMN with dextrose treatment compared to the non-inoculated control with dextrose (p-value = 0.0012) (Table 2.3). The difference between the mean number of colonized root tips of other plant treatments and uncolonized root tips of the controls were significantly different as expected (Table 2.4). No difference in mean number of root tips was detected between dextrose treatments for non-inoculated seedlings of ponderosa pine (p

-value = 0.23) or Douglas-fir (p-value = 0.12) (Table 2.4). The mean number of root tips with ½ strength dextrose appears greater than of seedlings with no dextrose for inoculated seedlings of ponderosa pine, but this result is weakly supported (p = 0.088) (Table 2.4).

DNA from cultures and hyphae found in PCS tubes of *Pseudorhizina californica, Helvella compressa,* and *Sphaerosporella brunnea* did not successfully amplify. For *S. brunnea*, the sparse hyphae on roots and in PCS tubes indicated the concentration of fungal DNA was too low for proper PCR priming. Although DNA concentrations did not appear to limit *H. compressa* and *P. californica*, the inability to amplify may be due to the lack of appropriate primers. Although fungal specific primers exist, they do not always effectively amplify ascomycetes. For the undescribed taxon, the mycelia were either fluffy and white or matted thick and yellow and appeared denser than the white hyphae. According to the RFLP patterns, both types were identical to the original fruiting body.

2.4 Discussion

Out of eleven species of Pezizlaes examined, only one, *Wilcoxina mikolae* formed mycorrhizae with the tested tree hosts. *Wilcoxina mikolae* is a member of a group of mycorrhizal fungi known as E-strain fungi, which form mycorrhizae having intracellular penetration, weakly developed mantles, and wide hyphae for the Hartig net (Laiho 1965). E-strain fungi were originally thought to be

TABLE 2.3: T-TEST ANALYSIS FOR MEAN NUMBER OF ROOT TIPS BETWEEN INOCULATED AND NON-INOCULATED SEEDLINGS, WITH UNEQUAL VARIANCE.

Treatment	Number of replicates	Mean number of root tips	Standard Error	T-stat	p-value
Wilcoxina mikolae on Douglas-fir: MMN with no dextrose	6	61	227.2	2.08	0.04
No fungal inoculum on Douglas-fir: MMN with no dextrose	5	29.2	977.2		
Wilcoxina mikolae on Douglas-fir: MMN with dextrose	6	124.67	49.5	5.63	0.0012
No fungal inoculum on Douglas-fir: MMN with dextrose	5	10	5.52		
Wilcoxina mikolae on ponderosa pine: MMN with no dextrose	6	198.5	6503.5	3.56	0.005
No fungal inoculum on ponderosa pine: MMN with no dextrose	6	67.3	1640.7		
Wilcoxina mikolae on ponderosa pine: MMN with ½ strength dextrose	6	263.83	5526.2	6.47	0.0003
No inoculum on ponderosa pine: MMN with ½ strength dextrose	6	51.67	921.5		

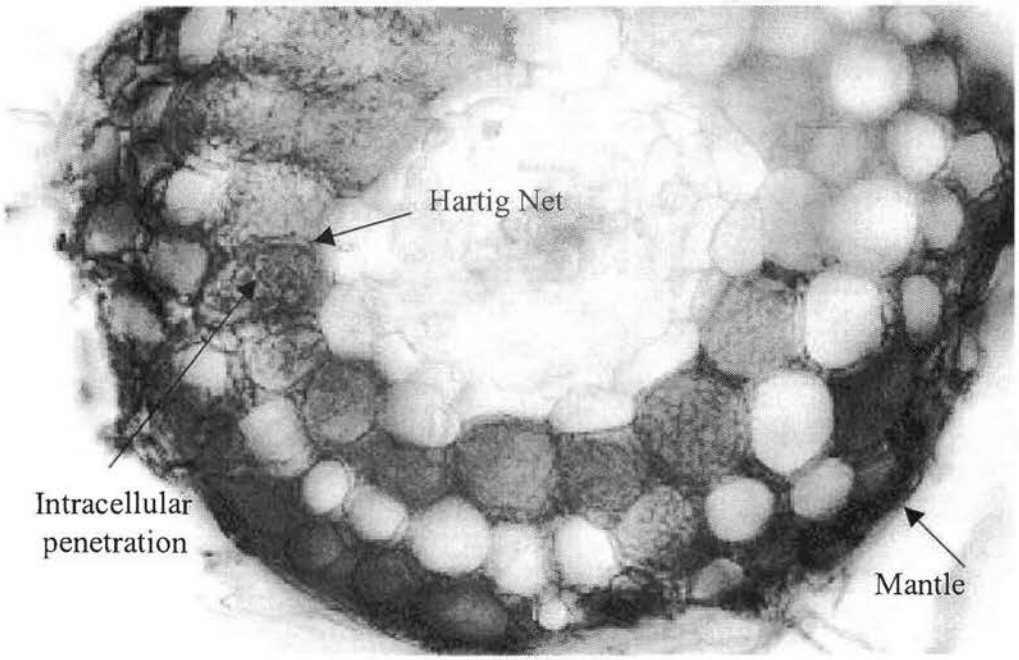
TABLE 2.4: T-TEST ANALYSIS FOR MEAN NUMBER OF ROOT TIPS BETWEEN DEXTROSE TREATMENTS, WITH UNEQUAL VARIANCE.

Treatment	Number of replicates	Mean number of root tips	Standard Error	T-stat	p-value
Wilcoxina mikolae on Douglas-fir: MMN with no dextrose	6	61	227.2		
				-3.01	0.012
Wilcoxina mikolae on Douglas-fir: MMN with ½ strength dextrose	6	124.7	2449.8		
Wilcoxina mikolae on ponderosa pine: MMN with no dextrose	6	198.5	6503.5		
				-1.46	0.088
Wilcoxina mikolae on ponderosa pine: MMN with ½ strength dextrose	6	263.8	5526.2		
No fungal inoculum on Douglas-fir: MMN with no dextrose	5	29.2	977.2		
				1.35	0.12
No fungal inoculum on Douglas-fir: MMN with ½ strength dextrose	5	10	30.5		
No fungal inoculum on ponderosa pine: MMN with no dextrose	6	67.3	1640.7		
				0.76	0.23
No inoculum on ponderosa pine: MMN with ½ strength dextrose	6	51.7	921.5		

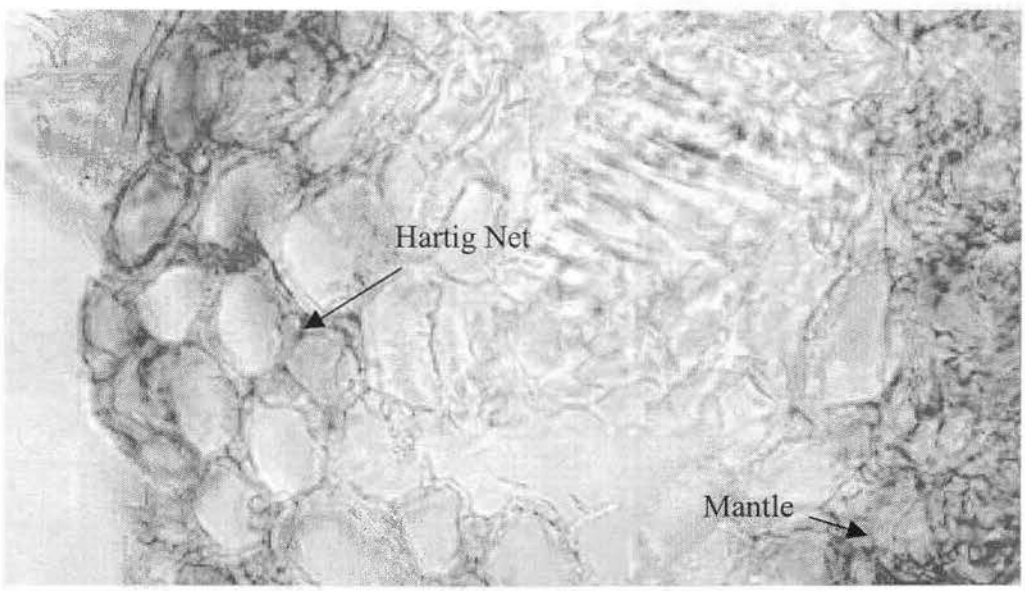
represented by one species (Mikola 1965, Wilcox 1971). Yang and Wilcox (1984) successfully identified one teleomorph of E-strain fungi, naming it *Tricharina mikolae*. Yang and Korf (1985) realized that *T. mikolae* was morphologically and trophically distinct from other species of *Tricharina* and described the new genus, *Wilcoxina*. After further investigation, E-strain fungi were thought to be either *Wilcoxina mikolae* var. *mikolae* or *W. rehmii* (Egger and Fortin 1990, Egger et al. 1991). Differences between the two species include production of chlamydospores and ecology; *W. mikolae* is chlamydosporic mainly from disturbed areas, and *W. rehmii* is non-chlamydosporic in undisturbed soils or soils amended with peat (Egger and Fortin 1990, Egger *et al.* 1991). Interestingly, *W. mikolae* var. *tetraspora* was postulated to be a hybrid of *W. mikolae* var. *mikolae* and *W. rehmii* (Egger and Fortin 1990, Egger *et al.* 1991).

In our experiment, *Wilcoxina mikolae* var. *tetraspora* formed ectendomycorrhizae with ponderosa pine and ectomycorrhizae with Douglas-fir, with scant mantles for both formations (See Figure 2.1). With an unspecified species of E-strain, Laiho (1965) also found ectendomycorrhiza formation with ponderosa pine and ectomycorrhiza with Douglas-fir. Laiho (1965) detected no mantle formation. The physiological differences in mantle formation seen in our study compared to Laiho (1965) may have been because Laiho used a different E-strain fungus than *W. mikolae* var. *tetraspora*. Fungus and plant combinations apparently determine the formation of ecto- or ectendomycorrhizae (Wilcox *et al.* 1983, Molina *et al.* 1992).

FIGURE 2.1 CROSS-SECTION OF *WILCOXINA MIKOLAE* ON DOUGLAS-FIR AND ON PONDEROSA PINE



2.1a Ponderosa pine colonized by *Wilcoxina mikolae* with no dextrose



2.1b Douglas-fir colonized by *W. mikolae* with no dextrose

Figure 2.1, Continued

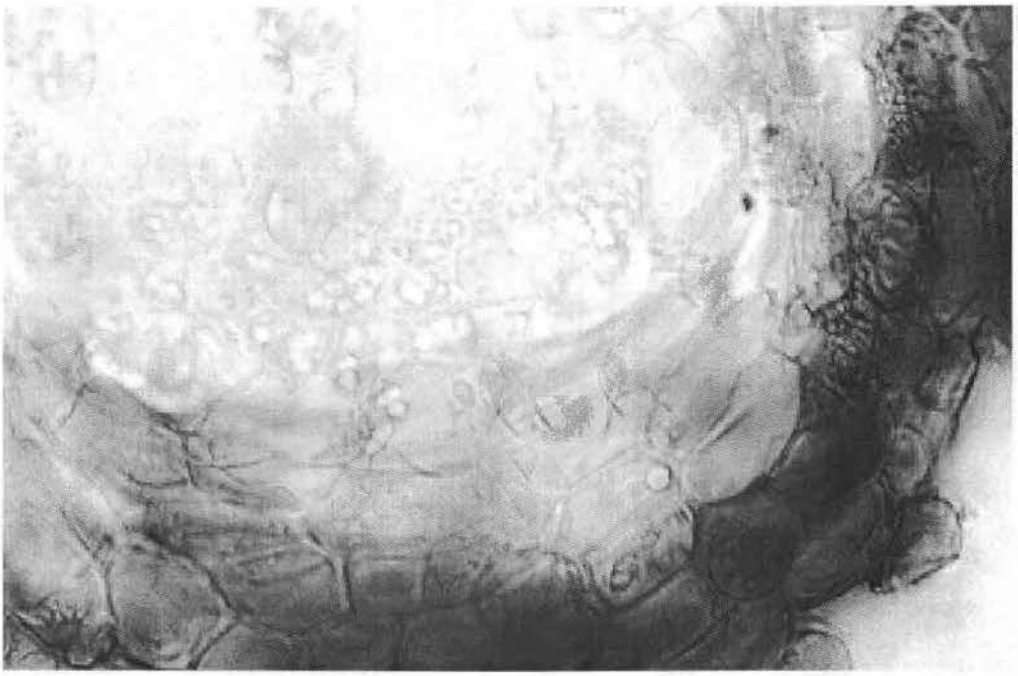


Figure 2.1c Douglas-fir colonized by *Wilcoxina mikolae* with half the strength dextrose

We found that the addition of dextrose to the media made no difference to the colonization of ponderosa pine but appeared to inhibit colonization of Douglas-fir by *W. mikolae*. These results contrast with those of Duddridge (1986) who suggested that the availability of an external source of carbon to the fungus results in increased colonization by the fungus. Furthermore, Duddridge (1986) observed antagonistic intracellular penetration of cortical cells by *Suillus grevillei* (Klotzsch) Sing. and postulated that this antagonism was aggravated by exogenous sugar. Although colonization by *W. mikolae* includes intracellular penetration, it is reported as an ectendomycorrhizal fungus with no apparent antagonism towards the

host (Mikola 1965, Laiho 1965, Wilcox *et al.* 1983, Egger 1996, Egger and Fortin 1990).

Our finding that the percent of colonized root tips of ponderosa pine did not differ when dextrose or no dextrose was added to the medium may be because 'early-stage fungi' have low or no requirements for sugar (Gibson and Deacon 1990). Early-stage fungi defined by Gibson and Deacon (1990) colonize root tips of seedlings in nurseries or glasshouse conditions and occupy younger root tips of expanding root systems of older trees. Our experiment was conducted on seedlings, so our mycobiont would fit Gibson and Deacon's definition of an early-stage fungus. These studies are further supported by Jumpponen and Trappe (1998) who reported that the combination of carbohydrate transfer by the fungus and elevated CO_2 concentrations due to fungal respiration resulted in increase of plant biomass in closed systems. They found in their PCS experiment with *Phialocephela fortinii*, an ascomycete also categorized as an early-stage fungus, and *Pinus contorta* that the increase in glucose concentration positively correlated with host plant biomass, but did not significantly increase when no inoculum was added. They argued that the increase in plant biomass resulted of an increase of CO_2 due to fungal respiration and not the addition of glucose *per se*, which was supported by the results of their open pot experiment where the same strains of inocula did not significantly increase plant biomass. These studies differ from Duddridge (1986) who argued that in PCS experiments excess sugar may benefit

the fungus so that colonization of root tips may occur *in vitro*, widening host ranges when compared *in vivo*.

Wilcoxina mikolae formed mycorrhizae with Douglas-fir when no dextrose was added to the medium but did not when dextrose was added to the medium. Hutchinson and Piché (1995) found when full concentrations of glucose were added, excess sugar inhibited colonization for certain plant and fungal combinations. They suggested that elevated levels of sugar increased mycelial growth, which released fungal metabolites that were toxic to the seedlings. They argued that this toxicity explained inhibition of colonization. In our case, a stronger explanation is that the medium supplied sufficient carbon so the fungus did not require carbon from its tree host. If fungal metabolites were toxic, then the plant would have shown signs of protection such as lignification (Molina and Trappe 1982), production of phenols (Hutchinson and Piché 1995), death of host cells (Duddridge and Read 1984), or even death of seedlings.

Species that probably do not form mycorrhizae with Douglas-fir and ponderosa pine are *Helvella compressa* and the undescribed taxon. Seedlings with *H. compressa* or the undescribed taxon developed lignified root tips, and their dark brown color likely indicates formation of phenols (Molina and Trappe 1994, Hutchinson and Piché 1995). Phenols produced by the host may indicate incompatibility between plant and fungus because the phenols limit intra- and intercellular penetration (Molina and Trappe 1994).

We found no colonization of root tips by *S. brunnea*, our second positive control. Danielson (1984) observed *S. brunnea* forms mycorrhizae with several hosts including ponderosa pine. Lack of colonization in our study may be due to insufficient amounts of inoculum indicated by the lack of hyphae; a second inoculation may have resulted in colonization. Danielson (1984) also tested *Anthracobia melaloma, Sphaerospora minuta, Trichophaea contradicta,* and *T. abundans*. None of these fungi colonized roots, although hyphae of *A. melaloma, T. abundans,* and *T. contradicta* were abundant in the containers; this is consistent with our study for *A. melaloma* and *T. abundans.*

Another reason for the lack of colonization by *S. brunnea* may be due to the choice of medium. Fungal and plant ectomycorrhizal associations are strongly influenced by the nutrient status of the synthesis medium (Duddridge 1986). Danielson (1984) used a medium that was nutrient poor compared to MMN in his PCS experiment proving that *S. brunnea* forms mycorrhizae. Egger and Paden (1986) used the same medium as Danielson for their experiments and came to the same conclusions as Danielson.

An unidentified actinomycete was found in tubes of seedlings inoculated with *Morchella*. Though actinomycetes inhibit growth of parasitic and mycorrhizal fungi, they can also enhance the growth of certain mycorrhizal fungi (Becker *et al.* 1999). In our study the actinomycete clearly did not enhance the growth of *Morchella.*

We selected MMN as the medium for our study because past PCS experiments with MMN have been successful (Marx 1969, Molina 1979, Molina and Palmer 1982). It has been commonly used when testing basidiomycetes for mycorrhizal colonization and has been effective in investigating whether an ascomycete, *Morchella*, forms mycorrhizae with various hosts (Dalhstrom *et al*, in press). Prior to inoculation, fungi were grown on MYA or PDA, depending on which medium the fungus grew most vigorously. Only *Wilcoxina mikolae* was grown on MMN. However, all fungi were transferred to MMN before seedlings were inoculated. Perhaps this change prevented the fungi from colonizing roots.

Warcup (1990) reported that some post-fire Pezizales form mycorrhizae with eucalyptus seedlings. However, he did not show that the fungus colonizing the root was the original fungus of interest. Now that the ability to confirm root tip colonization with original inocula is relatively simple with PCR-based identification, a follow-up experiment from Warcup's study would be useful for understanding the biotrophic status of post-fire Pezizales.

PCS can provide valuable information about mycorrhizae. However, as Molina and Palmer (1982) and Egger and Paden (1986) asserted, failure in PCS emphasizes how little we know about fungi and their needs. The mycorrhizal potential for many epigeous ascomycetes is poorly understood. Because many of the studies of ectomycorrhizae have focused on basidiomycetes, the number of epigeous ascomycetous mycobionts may be underestimated (Danielson 1984, Vrålstad *et al.* 1998). Differences seen in our study compared to other PCS studies

that included the same species of fungi emphasize how different media may affect fungal and plant interactions.

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Chapter 3

Ascomycetous Mycorrhizae on Ponderosa Pine (*Pinus ponderosa)* after a Prescribed Burn in Eastern Oregon.

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3.1 INTRODUCTION

After a wildfire or prescribed burn, a series of macromycetes characterestic of phoenicoid habitats fruit. Post-fire Pezizales start fruiting about 6 weeks after a fire and continue to fruit in successional groups for about 2 years before seemingly disappearing from the landscape (Petersen 1970). Their ecological role was presumed to be saprotrophic until Danielson (1984) observed not only saprotrophic capacities, but also mycorrhiza formation by *Sphaerosporella brunnea* (Alb. & Schwein. : Fr.) Svr ek & Kubi kwith jack pine (*Pinus banksiana* Lamb.). Deacon and Fleming (1992) hypothesized that *S. brunnea* colonizes seedlings while saprotrophically receiving organic carbon and that other post-fire fungi may have similar strategies. Carpenter *et al.* (1987) postulated that post-fire fungi may survive heat disturbances as mycorrhizae, then grow from mycorrhizae into the substrate.

Egger (1986) and Egger and Paden (1986) addressed the ecological role of post-fire Pezizales and concluded that most of the species they examined were saprotrophic, with some species pathogenic and some mycorrhizal. Vrålstad *et al.* (1998) reported that *Geopyxis carbonaria* (Alb. & Schw.) Sacc. formed mycorrhizae with Norway spruce. Warcup (1990) found three species of post-fire Pezizales (*Lachnea vinosobrunnea* (Berk. & Br.) Sacc., *Muciturbo reticulatus* Talbot, and *Pulvinula tetraspora* (Hansford) Rifai) formed mycorrhizae with *Melaleuca uncinata* R. Br. ex Aiton *f.* and *Eucalyptus obliqua* L. Hér. With the exception of Vrålstad *et al.* and Carpenter *et al.*, these studies have been *in vitro*.

Vrålstad *et al.* focussed on one species and examined root tips from windblown trees; Carpenter *et al.* focussed on successional fruiting of fungi after the eruption of Mt. St. Helens. In our study, we examined the ecological role of post-fire Pezizales *in vivo*.

Mycorrhizae, the symbiotic relationship between fungi and plants, are often defined structurally by their morphology. Mycorrhizae are often separated into two major categories, the endomycorrhizae and ectomycorrhizae, depending on hyphal penetration or no penetration of plant root cortical cells, respectively. Other traits of endomycorrhizae may include arbuscules, vesicles, and lack of a mantle. Ectomycorrhizae have a Hartig net, hyphae that surround the cortical cells, and a mantle, hyphae that cover the root tip. The morphological classification can be refined by recognition of a group known as ectendomycorrhizae. These have a Hartig net, mantle, and intracellular penetration by the hyphae. This morphological group includes E-strain mycorrhizae, which were first described by Mikola (1965) and Laiho (1965). Laiho (1965) characterized E-strain mycorrhizae for *Pinus* as those with intracellular penetration, weakly developed mantle, and wide hyphae in the Hartig net. For our purposes, we will follow this definition when discussing E-strain mycorrhizae. The term ectendomycorrhizae will refer to the general definition of morphologies that include a Hartig net, mantle, and intracellular penetration.

Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) forests are known for their spacious, park-like appearance. These forests consist of mosaics of clumps of

even-aged trees. Ponderosa pines are drought-resistant often associated with cold winters and warm, dry, droughty summers (Agee 1998). Their thick bark and deep taproots protect them from low intensity, high frequency fires, which smolder at the base and rarely reach the crown (Agee 1998). The USDA Forest Service practices prescribed burnings to mimic the natural fire regime of eastern Oregon (Everett *et al.* 1994, Agee 1994). These forests are ideal sites to examine ectomycorrhizae of ponderosa pine *in vivo.*

The project was part of a larger, integrative study of the effects of prescribed fires on insects and ectomycorrhizal communities. This project focussed on a small section of the ectomycorrhizal community, a group of ascomycetes. Our objective was to determine if post-fire Pezizales form mycorrhizae on ponderosa pines. A secondary goal was to develop tools for identifying mycorrhizal ascomycetes.

3.2 Materials and Methods

3.21 Site Description

The study site is located on a ponderosa pine forest on the Burns Ranger District of the Malheur National Forest, Harney Co., OR. The oldest ponderosa pines on the site are 100-200 years old. The site is dry in the summer, receiving on average 0.48 inches of rain between June and October in both 1997 and 1998 (Oregon Climate Service <http://www.ocs.orst.edu>). Maximum temperatures averaged 27° C in the summer (June – September), and 4° C in the winter

(November – February) (Oregon Climate Service). Other common plants include mountain mahogany (*Cercocarpus ledifolius* Nutt.) and elk sedge (*Carex geyeri* Boott.).

The site is divided into four stands, designated as Kidd Flat, Trout, Driveway 14, and Driveway 17. The distance between Kidd Flat and Trout is about 3.2 km. The distance between the two Driveway stands is less than 1 km. Kidd Flat and Trout are 14.5 km away from the two Driveway stands. The plots on Kidd Flat face east northeast; on Trout they are on a ridge top; on Driveway 14 they face west; and on Driveway 17 southeast.

3.22 Study Design

Each stand, 8-12 ha, was prescribe-burned in fall, 1997. One 240 m permanent transect line was established with 6 permanent plots, approximately 40 m apart per stand. The ponderosa pine closest to each 40 m mark that would most likely survive the prescribed fire was the point of reference for each plot and was marked with an aluminum tag. Areas within the dripline of the marked tree were designated as plots that were circular and variable in size, depending on the dripline (tree canopy).

Root tips were collected by taking soil cores. Location of cores was determined by presence of post-fire Pezizalean fruiting bodies that were closest to marked trees. Fungi are not distributed evenly over the landscape, and the location of mycorrhizal root tips is even more difficult to determine (Gardes and Bruns 1996). Soil cores 5 cm in diameter and 15 cm in length were collected under

Pezizalean fruiting bodies to increase chances of finding colonization by these post-fire Pezizales. If no fruiting bodies were present on the plot, cores were taken directly south of the marked tree, within its dripline. If the area around the marked tree was not burned, then the area around the closest ponderosa pine with burned ground was sampled. One to two soil cores were taken from each plot, 8 cores per stand, 32 cores for the entire site. The 7^{th} and 8^{th} cores were collected from plots that had more than one post-fire Pezizalean species or fungal mat. Soil cores were placed in plastic bags, stored in an ice chest for no more than three days, and transferred to a 4° C refrigerator until root tips were examined and lyophilized, which was within two weeks after collection. Soil cores were collected in June and July 1998.

Fruiting bodies of post-fire Pezizales were collected from all plots in June 1998. None were found in July 1997 due to dry conditions. For DNA extraction, pieces of fruiting bodies with a surface area of 1 mm x 1 mm, or single specimens of small fruiting bodies were stored in 95% ETOH for less than one month. Fresh characteristics of remaining fruiting bodies were recorded. Specimens were then dried on a food dehydrator and deposited in the Oregon State University Mycological Herbarium.

3.23 Laboratory Analysis

Soil cores were soaked in water for at least two hours, gently rinsed, and poured through a 0.5 mm sieve (No. 35 USA standard testing sieve, W.S. Tyler,

Inc.) which was used to catch root tips. The root tips saved were stored in water in a 200 ml beaker at 4-5° C.

Root tips were categorized roughly following the system of Agerer (1987-1995). Categorization was based on color, unifurcation vs. bifurcation of root tip, thickness of mantle, and presence or absence of rhizomorphs. Root tips were then lyophilized for 24 to 36 hours and were frozen until needed for DNA extraction.

DNA extraction, polymerase chain reaction (PCR), and restriction fragment length polymorphisms (RFLP) fragmentation followed the protocol of Gardes and Bruns (1993) with minor adjustments. For extractions, one or two root tips, 1 mm x 1 mm of the surface area of sporocarp tissue, or a single sporocarp were placed in 300 µl of CTAB with 2 µl of β-mercaptoethanol. Samples were quickly frozen on modular blocks that were previously frozen by dry ice or liquid nitrogen and thawed for three cycles, then ground with a micropestle. Tissues were again quickly frozen, then incubated at 65° C for 30-60 minutes. Three hundred microliters of chloroform were added, samples were centrifuged for 15 minutes, and the supernatant was transferred to new tubes.

For mycobionts that did not successfully amplify, an extra step of purifying the DNA was taken, following Lee's (1987) protocol, with minor adjustments. New samples had equal amounts of phenol to chloroform (300 µl: 300 µl) added to root tips after breaking of tissue and incubation. Samples were centrifuged for 15 minutes, then supernatant was transferred to new tubes. Three hundred microliters

of chloroform were then added to the tubes, centrifuged for 5 minutes, and the supernatant transferred to new tubes.

Five hundred microliters of cold isopropanol were mixed with the supernatant to precipitate the DNA. Samples were stored in -20° C freezer for at least 3 hours, then centrifuged for 10 minutes. Isopropanol was poured from tubes, and then 500 µl of cold ethanol were mixed in to clean the samples. Samples were centrifuged for 5 minutes, and the ethanol was then poured from tubes. Once tubes were completely dry, DNA was resuspended in TE buffer for at least 20 minutes, then stored in a freezer until further use. Extractions were done at least three times for each morphotyped group, or until amplification was successful.

Primer pairs for the ITS region of the nuclear ribosomal DNA (nrDNA) were used for PCR-RFLP analysis. Fungal specific primer ITS 1f (Gardes and Bruns 1993) and universal primer ITS 4 (White *et al.*1990, Gardes *et al.* 1991) were the main primers used. Primer pair ITS 1f and NL6A (Egger 1995), NL6A specific for ascomycetes, were also tested. However, the results from primer pair ITS 1f and ITS 4 produced cleaner and brighter bands. Samples successfully amplified with primer pair ITS 1f and ITS 4 were also amplified with primer pair ITS 1f and ITS 4b (Gardes and Bruns 1993). ITS 1f and ITS 4b primers are specific for basidiomycetes (Gardes and Bruns 1993) and were used to screen out basidiomycetous mycobionts. Primer pair ML5 and ML6 (Gardes and Bruns 1993) were also used to screen out potential basidiomycetes. For samples that did not amplify with ITS 1f and ITS 4, other primers were tested. DNA from fruiting

bodies successfully amplified with primer pair ITS 1 and ITS 2 (White *et al.* 1990). However these primers did not successfully amplify fungal DNA off root tips. Other primer pairs that did not amplify fungal DNA from root tips were ITS 2/ ITS 5 (White *et al.* 1990) and ITS 4/ITS 5.

PCR amplifications were done by the following program: 94°C (30 sec); [93°C (35 sec); 55°C (53 sec); 72°C (30 + 5 sec/cycle) x 34 cycles]. Successful amplification was verified with gel electrophoresis with 3% or 2% agarose gels. Gels were run at 110 V for about 40 minutes, stained in ethidium bromide (ETBR) and rinsed in deionized H_2O (dH_2O) for 10 minutes each. Gels were then placed in the MultiImage™ Light Cabinet, Alpha Innotech®. Photos were taken by AlphaImager™ AlphaEase™ vers. 3.24 (Alpha Innotech Corp. 1996) and printed by Sony® Digital Graphic Printer UP-D890.

Once the ITS region was amplified, PCR products were cut with restriction enzymes. RFLPs were created with endonucleases *Alu* I, *Hinf* I, and *Dpn* II. Band sizes were measured with a 100 bp DNA Ladder for a standard and use of AlphaImager™ AlphaEase™ software. AlphaImage™ and photos taken by Sony® Digital Graphic Printer UP-D890 recorded all RFLPs. When RFLP types did not match sporocarps, sequencing was done for each RFLP type. Species level identification was determined by identical RFLP matches for all three endonucleases.

For sequencing, DNA was amplified with the PCR by use of primers for the gene region of interest. Success of amplification of DNA was verified by gel

electrophoresis of 5 µl of PCR products. Qiagen® QIAquick PCR Purification Kit™ protocol was used to clean PCR products. To verify success of purification, 2 µl of purified PCR product were gel electrophoresed, and the concentration of the sample was adjusted if necessary. Samples were sequenced on a 377 ABI automated sequencer with appropriate primers at the Center for Gene Research and Biotechnology at Oregon State University. The resulting sequences were checked for accuracy and then aligned using PAUP 3.1 (Swofford 1993) and PAUP* (Swofford 1999).

The 5.8s region of the rDNA was sequenced as the final means to sort out the basidiomycetes. Sequences were placed in an existing database to differentiate taxa between plant, animal, and fungal DNA, with some resolution between ascomycetes and basidiomycetes (Cullings and Volger 1998). ITS 1f and ITS 4 primers were used to amplify and sequence the ITS region. Neighbor-joining analysis was conducted as suggested by Cullings and Vogler (1998).

The first 1726 bp of the 18s region (small subunit) of the nrDNA was sequenced for six RFLP types. This region was used to place mycobionts to family, and if possible, genus. Primers NS1, NS2, NS3, NS4, NS5, NS8, NS21 and NS24 (White *et al.* 1990) were used for amplification and sequencing. NS3, NS4, NS5, and NS8 successfully amplified most fungal DNA from root tips. In a couple of cases, they amplified plant DNA; for which fungal specific primer pairs NS21 and NS24 were used for amplification and sequencing. All sequences were

compared to sequences in GenBank <http:// www.ncbi.nlm.nih.gov> to verify that sequences were from fungal DNA and not plant DNA (blast search).

Sequences of mycobionts were added to the database of sequences included in Spatafora *et al.* (1998) with some minor changes. Our database continued for 576 bp beyond the 1150 bp used by Spatafora *et al.* (1998), for a total of 1726 bp. In addition, sequences of ascomycetous fungi, which were either downloaded from GenBank (see Table 3.1) or produced for this study (see Table 3.2), were added to the database.

TABLE 3.1 LIST OF SPECIES USED IN 18S SEQUENCE ANALYSIS AND ACCESSION NUMBERS FROM GENBANK.

Taxon	GenBank Accession Number
Aleuria aurantia	U53371
Alternaria alternata[a]	U05914
Aspergillus fumigatus[a]	U55626
Athelia bombacina[a]	M55638
Balsamia vulgaris	AF054905
Barssia oregonensis	U42657
Blastomyces dermatitidis[a]	M63096
Botryosphaeria rhodina[a]	U42476
Botryosphaeria ribis[a]	U42477
Candida albicans[a]	UM60302.1
Capronia pilosella[a]	U42473

Table 3.1, Continued

Cheilymenia sp.[b]	
Coccidioides immitis[a]	M55627
Cryptococcus neoformans[a]	D12804
Cudonia confusa[a]	Z30240.1
Dothidea hippophaeos[a]	U42475
Dothidea insculpta[a]	U42474
Genea intermedia[b]	
Geopora cooperi[b]	
Geopyxis carbonaria	U62011
Gyromitra esculenta[a]	U42648
Hypocrea lutea[a]	D14407
Inermisia aggregata	Z30241
Lamprospora maireana	AF061719
Lasiobolus sp.[b]	
Leotia lubrica[a]	L37536
Leucoscypha oroarctica	AF061724
Microascus cirrosus[a]	M89994
Morchella elata	U42641
Neottiella rutilans	AF061720
Neurospora crassa[a]	X04971
Octospora wrightii	AF061722

Table 3.1, Continued

Orbilia delicatula	U72603
Otidea leporina	U53381
Peziza badia[a]	L37539
Phialocephela fortinii	L76626
Plectania rhytidia	AF061723
Pleospora rudis[a]	U00975
Pseudorhizina californica	U42650
Pyronema domesticum	U53385
Rhizina undulata	U42664
Saccharomyces cerevisiae[a]	Z75578
Sarcosoma globosum	U53386
Sarcosphaera coronaria	AF133157
Sclerotinia sclerotiorum[a]	L37541.1
Scutellinia scutellata	U53387
Spathularia flavida[a]	Z30239
Sphaerosporella brunnea	U53388
Spongipellis unicolor[a]	M59760
Sporomia lignicola[a]	U42478
Talaromyces flavus[a]	M83262
Taphrina deformans[a]	X69852
Tarzetta catinus	U53389

Table 3.1, Continued

Trichophaea hybrida	U53390
Tuber gibbosum[a]	U42663
Underwoodia columnaris	U42658
Wilcoxina mikolae	U62014
Wynnella sylvicola	U42655
Xylaria carpophila[a]	Z49785

[a]Taxa used in Spatafora *et al.* 1998
[b] Sequences determined for this study.

TABLE 3.2 LIST OF SPECIES USED FOR SEQUENCE ANALYSIS AND THEIR SUBSTRATE AND LOCALITY.

Taxon	Substrate & Locality	NSW[1] Accession Number
Neottiella sp.	On damp soil, top of spur trail. Mt. Hood National Forest, Hood River Co., OR	6676
Geopora cooperi	Solitary, emergent on gravely soil along dirt road. *Tsuga, Larix, Thuja,* and *Pinus* forest. Priest River Experimental Forest, Bonner Co., ID	7527
Cheilymenia sp.	Scattered to gregarious on damp rotting plant material under *Alnus* with assorted forbes in seepage area. Downstream from Brundage Reservoir along Brundage Creek, Payette National Forest, Adams Co, ID	7552
Genea intermedia	Scattered to gregarious, hypogeous in loose soil near *Pseudotsuga menziesii*. Paul M. Dunn Research Forest, Benton Co., OR	8187
Lasiobolus sp.	Gregarious on aged deer droppings in *Pinus contorta* forest burned in 1996. Umatilla National Forest, Umatilla, Co., OR	8223

[1]NSW = N.S. Weber

Maximum parsimony was performed by PAUP 3.1.1 (Swofford 1993) and PAUP* (Swofford 1999) according to Spatafora *et al.* (1998). Only parsimony-informative characters were used, with all characters having equal weight. Gaps were treated as missing characters, and multistate taxa were interpreted as uncertainties. Ten heuristic replicate searches were done, by branch swapping with tree-bisection-reconnection and an initial random seed number. Weighted parsimony analysis favoring transitions over transversion was also conducted (1:1.4 [TS: TV]). Weighting by a step matrix followed the procedure of Spatafora *et al.* (1998).

For intergenic clarification, sequences from the partial 18s and ITS 1 region were used. The ITS region is more variable than the 18s region and is helpful for inter- and intrageneric studies (Vrålstad *et al.* 1998, Norman and Egger 1996, Gardes and Bruns 1993, Cullings and Volger 1998). The 3' end was amplified and sequenced with NS5 and NS8; the ITS 1 region was amplified with ITS 1f and ITS 4 and sequenced with ITS 1f and ITS 2.

Maximum parsimony was used for the partial 18s and ITS 1 sequence analysis. Seventy-one of 284 characters were parsimony informative. Sequences for *Wilcoxina mikolae* var. *mikolae* (Yang and Wilcox) Yang and Korf, *W. rehmii* (Yang and Wilcox) Yang and Korf, *Tricharina gilva* (Boud. in Cooke) Eckblad, and *Sphaerosporella brunnea* were downloaded from GenBank (see Table 3.3). *Sphaerosporella brunnea*, known to form mycorrhizae (Danielson 1984, Egger and

Paden 1986), was used as the outgroup. One hundred replicates were used to obtain bootstrap values.

TABLE 3.3 LIST OF SPECIES AND ACCESSION NUMBERS FROM GENBANK OF TAXA INCLUDED IN PARTIAL 18S/ITS1 SEQUENCE ANALYSIS AND RFLP ANALYSIS.

Species	Accession Number
Wilcoxina mikolae	U38635
Wilcoxina rehmii	U38567
Wilcoxina alaskana	U38583.1
Tricharina gilva	U38629
Sphaerosporella brunnea	U38587
Trichophaea hybrida[a]	NSW[b] 7162
Geopora sp. A[a]	NSW 7225
Geopora sp. B[a]	NSW 7341
Geopora sp. C[a]	NSW 7342
Geopora sp. D[a]	NSW 8296
Geopora cooperi[a]	NSW 7527
Geopora clausa 1[a]	JMT[c] 5438
Geopora clausa 2[a]	JMT 7420

[a]Sequence determined from this study
[b]NSW = N.S. Weber accession number
[c]JMT = J.M. Trappe accession number

For intrageneric clarification, RFLPs were produced from dried sporocarps of selected fungi we thought might be related to the unknown mycobionts. Sporocarp materials for 5 *Geopora spp.* were obtained from the collections of N.S. Weber and J.M. Trappe (see Table 3.3). Extractions from *Wilcoxina rehmii* were made from cultures provided by K.F. LoBuglio.

3.3 Results

Fruiting bodies of five genera of post-fire Pezizales were found. *Tricharina* occurred in all four stands; *Anthracobia* was found at Trout and Driveway 17, *Peziza* at Kidd Flat and Trout, *Morchella* only at Kidd Flat, and *Scutellinia* only at Driveway 14. One species each of *Anthracobia, Scutellinia*, and *Peziza* was found, two of *Tricharina*, and three of *Morchella*. Number of species was determined by RFLP analysis. Number of cores taken beneath sporocarps of each genus is listed in Table 3.4.

Out of 89 morphotyped root tips, 24 were determined likely to be basidiomycetes by use of primer pair ITS 1f and ITS 4b. Twenty-seven of the root tips did not amplify with any primer pairs tested. Thirty-five of the tips amplified with ITS 1F and ITS 4 and did not amplify with ITS 1F and ITS 4b or ML5 and ML6. Of the 35 remaining tips, 15 unique RFLP types were observed. Distribution of these 15 RFLP types is shown in Table 3.5 and band sizes in Table 3.6.

TABLE 3.4 NUMBER OF COLLECTIONS OF SPOROCARPS AT EACH STAND, AND NUMBER OF CORES TAKEN BENEATH SPOROCARPS OF EACH FUNGAL GENUS. NUMBER OF CORES TAKEN BENEATH GENUS ARE IN PARENTHESES.

	Genus				
Stand	*Anthracobia*	*Morchella*	*Peziza*	*Scutellinia*	*Tricharina*
Driveway 14	0	0	0	(1)	(5)
Driveway 17	6(4)	0	0	0	10(1)
Kidd Flat	0	(1)	(2)	0	8(4)
Trout	3(2)	(2)	1(0)	0	(3)
Total Number of Collections	9	3	3	1	26
Number of Cores/Genus	6	3	2	1	13

TABLE 3.5 LOCALITY OF RFLP TYPES (O = RFLP TYPES FOR MYCORRHIZAL ASCOMYCETES, X = RFLP TYPES FOR MYCORRHIZAL BASIDIOMYCETES)

	RFLP TYPE														
Stand	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Driveway 14	X						X	O					X	O	X
Driveway 17		X	X				X								
Kidd Flat					X	O	X	O	X	O	O	O			
Trout				X											

TABLE 3.6 RFLP BAND SIZES FOR ASCOMYCETOUS MYCOBIONTS

	Restriction Enzyme									
RFLP TYPE or Species	*Alu* I		*Dpn* II			*Hinf* I				
Type 6	695		235	325		370				
Type 8	200	4	209	314		130	212	279		
Type 10	216	323	208	303		133	222	288		
Type 11	625		240	330		100	180	373		
Type 12	676		213	332		138	220	290		
Type 14	194	420	178	243	275	141	233	297		
Wilcoxina rehmii	209	445	222	330		132	228	299		
Geopora cooperi	620		227	319		98	187	384		
Geopora clausa 1	625		245	340		70	92	119	171	213
Geopora clausa 2	630		257	340		70	97	144	171	195
Geopora sp. A	642		241	334		114	209	417		
Geopora sp. B	634		151	170	236	110	205	398		
Geopora sp. C	551		236	326		112	198	398		

No RFLPs from DNA extracted from fruit bodies matched those from the mycorrhizae. Based on sequence analysis of the 5.8s region, only 6 of 15 RFLP types were ascomycetes, representing 16% of the total biomass of all root tips

collected. The remaining RFLP types were most likely basidiomycetes. Descriptions of the mycorrhizal morphology of six ascomycetes are listed in Table 3.7.

NS1 and NS2, which amplified the 5' end of the small subunit, did not sequence cleanly; thus, the first 600 characters of the 5' end of the small subunit were not used in the final sequence analysis. Of the remaining 1126 characters, 640 were excluded because of hypervariablity, and 257 were parsimony-informative. The four most parsimonious trees of 921 steps with a consistency index (CI) = 0.439 and retention index (RI) = 0.686 were deduced. The bootstrap consensus tree is shown in Fig. 3.1. The uncorrected 'p' distance for the two unknown RFLP types supports their affinity to *Wilcoxina mikolae* and *Trichophaea hybrida* (see Table 3.8). RFLP Types 6 and 11 showed close affinity to *Geopora cooperi*, supported by a very high bootstrap value of 95 (see Fig. 3.1) and the uncorrected 'p' distances are listed in Table 3.8.

To determine if the mycobionts of RFLP types 8 and 10 were *Wilcoxina* or *Trichophaea*, the partial 18s and ITS 1 regions were amplified. Species of *Wilcoxina* and *Tricharina* were downloaded from GenBank (Table 3.3). Types 8 and 10 were closely affiliated with *Wilcoxina rehmii* with less than five changes (see Fig.3. 2). A bootstrap value of 100 supports the two clades of the two mycorrhizal fungi and *Wilcoxina rehmii* (see Fig. 3.2) from one most parsimonious tree of 129 steps with a CI of 0.7377 and RI of 0.6923. All 66 parsimony informative characters were used. For further support, RFLP patterns were

TABLE 3.7 MORPHOLOGICAL DESCRIPTION OF ASCOMYCETOUS MYCOBIONTS

RFLP TYPE	Color	Mantle	Intracellular Penetration & Hartig Net	General Shape	Hyphal measurements & Description
Type 6	Yellowish to Orangish brown.	Thin mantle, net prosenchyma to net syrenchyma	Yes	Bifurcate.	Hyaline, outlined in black. Diameter = 4.2 – 13.3 µm
Type 8	Ranged from bright orange to reddish brown.	Thin to no mantle, net syrenchyma, thickness = 21 - 84 µm	Yes	Both unifurcate and bifurcate.	Hyaline. Diameter = 4.9 – 7.7 µm
Type 10	Reddish brown.	Thin, thickness = 21 - 84 µm	Yes	Bifurcate.	Hyaline. Diameter = 2.1 - 8.4 µm
Type 11	Reddish brown.	Thin, net prosenchyma	Yes	Multiple branched, knobby.	Hyaline.
Type 12	Root tips were very similar to type 11, reddish-brown.	Thin, net syrenchyma, thickness = 14 - 70 µm	Yes	Multiple branched, knobby.	Brown. Diameter = 3.5 - 6.3 µm
Type 14	Orangish-brown.	Thin to none, net prosenchyma, thickness = 28 - 700 µm	Yes	Bifurcate. Hyphae bulbous	Hyaline. Diameter = 3.5 - 9.8 µm

FIGURE 3.1 BOOTSTRAP CONSENSUS FROM A MAXIMUM PARSIMONIOUS ANALYSIS TREE, BASED ON THE 18S REGION OF NRDNA

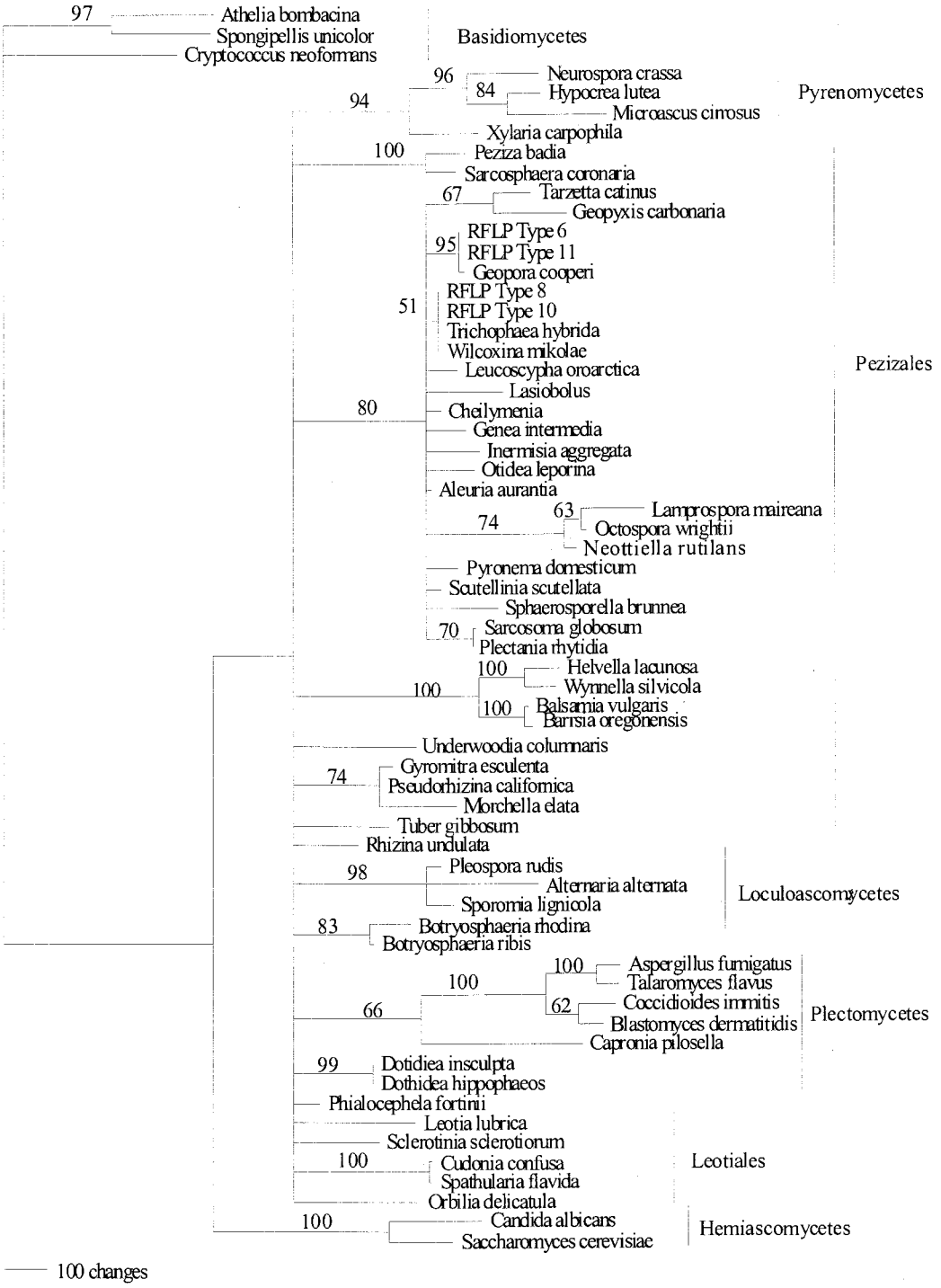
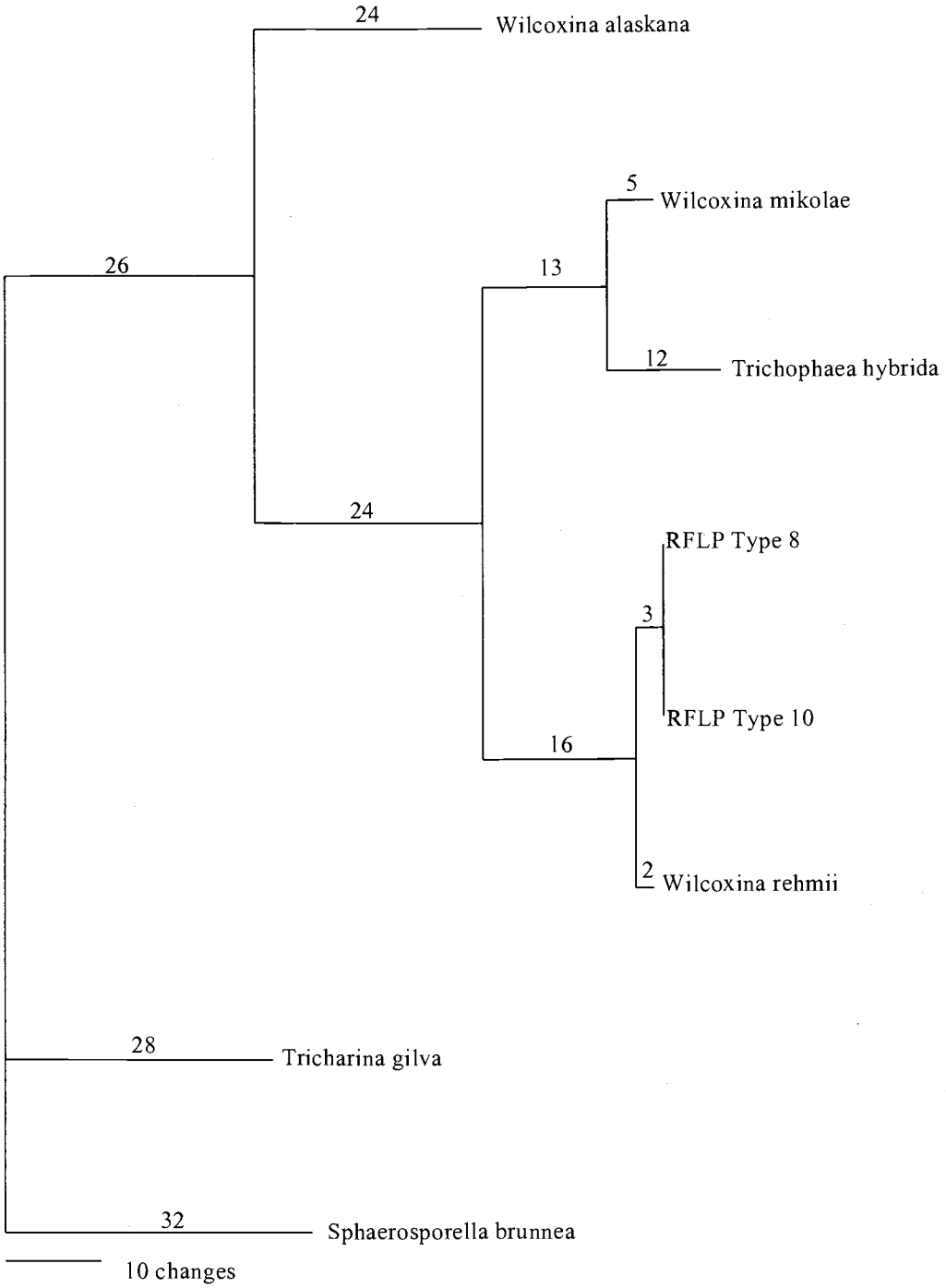


TABLE 3.8 UNCORRECTED 'P' DISTANCES FROM THE 18S REGION OF THE NRDNA FOR *WILCOXINA MIKOLAE* VAR. *TETRASPORA*, *TRICHOPHAEA HYBRIDA*, *GEOPORA COOPERI* AND RFLP TYPES 6, 8, 10 AND 11.

	Geopora cooperi	*Trichophaea hybrida*	*Wilcoxina mikolae* var. *tetraspora*
RFLP Type 6	0.00121	--------	--------
RFLP Type 8	--------	0.00000	0.00095
RFLP Type 10	--------	0.00103	0.00286
RFLP Type 11	0.00101	--------	--------

FIGURE 3.2 MAXIMUM PARSIMONIOUS ANALYSIS, BASED ON THE PARTIAL 18S REGION AND ITS 1 REGION.



matched between a culture collection of *W. rehmii* and Type 8. When the restriction enzyme *Alu* I was used, there was a slight variation between *W. rehmii* and Type 10.

RFLP patterns for Type 11 and *Geopora cooperi* matched for all three enzymes and also for a fourth restriction enzyme, *Hae* III. RFLP patterns did not match between Type 6 and any of the *Geopora spp.* analyzed (see Table 3.6). Identity of *Geopora clausa* (Gilkey) Burds. is uncertain because the RFLPs did not match between the two different collections. Type 6 is tentatively identified as *Geopora sp.* Analysis of the partial 18s region and ITS 1 region was not necessary for these root tips, because only one genus was possible.

Sequences for RFLP Type 14 were unattainable using primers NS 3, 4, 5, 8, 21, and 24. Although NS 21 and NS 24 successfully amplified fungal DNA for RFLP Type 12, there were still too few informative characters for analysis to be successful. Sequences for RFLP Type 12 were not used and deleted from the database.

The remaining six RFLP types were found only on two of the four stands, Kidd Flat and Driveway 14 (see Table 3.5). *Wilcoxina rehmii* was the most prevalent ascomycetous type and occurred in both stands, in June and July on the Driveway 14 stand but only in July on the Kidd Flat stand. *Geopora sp.* was also found in both stands and on the same plot as *W. rehmii* for the Driveway stand. *Wilcoxina sp., Geopora cooperi*, and RFLP Type 12 occurred only on the Kidd Flat stand, and Type 14 was found only on the Driveway stand.

3.4 Discussion

We found no root tips colonized by species of post-fire fungi. These early fruiting post-fire fungi are likely saprobic, especially those ten species whose sporocarps were found. We extracted multiple cores from below most fungal genera found (see Table 3.4) to increase chances of finding roots colonized by these genera. However, we collected rootlets only a few months after the fire, so it is still possible that other post-fire Pezizales fruiting later than our time frame may form mycorrhizae.

Post-fire Pezizales served as the impetus for this study. We could not clarify the mystery of the ecological role of these fungi. It has been suggested that post-fire mycorrhizal fungi may colonize roots deep in the soil and not in the upper 10 cm as do many ectomycorrhizal fungi (Vrålstad *et al.* 1998, Mikola *et al.* 1964). Post-fire mycorrhizal fungi may colonize roots deeper than 15 cm, the length of our soil cores. Also, the possibility exists that the fruiting body may be produced some distance from the colonized roots (Gardes and Bruns 1996).

Although post-fire Pezizales did not appear to be forming mycorrhizae in our cores, we did observe effects of prescribed burning on mycorrhizae. RFLP pattern comparisons from pre-treatment data collected from the same marked tree (J.E. Smith, unpublished data) indicate that mycobionts from the stands after the prescribed fires were not present on the pre-treatment stands. Our identified mycobionts are not categorized as post-fire Pezizales. *Geopora cooperi* in particular is widely distributed with the Pinaceae and not reported as a post-fire or

post-disturbance fungus (Burdsall 1968, States and Gaud 1997, Maia 1996). Gehring *et al.* (1998) reported *G. cooperi* on root tips of pinyon pines in nutrient poor, volcanic cinder soils but not in nutrient rich, sandy loam soil.

Interestingly, all six ascomycetous RFLP types were Pezizales and E-strain mycorrhizae. Previous studies suggested that one fungal genus formed E-strain mycorrhizae (Mikola 1965, Laiho 1965, Egger and Fortin 1990). Subsequent studies have shown that E-strain fungi are not limited to one genus (*Wilcoxina*), but also include *Humaria* and *Geopora* (Egger, pers. comm.) Our study supports this latter report and Danielson's (1982) suspicions that *Geopora* could be a teleomorph of E-strain fungi.

Wilcoxina rehmii is our most common ascomycetous mycobiont. Although the bootstrap value from the 18s tree was low (see Fig. 3.1), the uncorrected 'p' distance showed that our RFLP types were closely affiliated to *Trichophaea hybrida* and *Wilcoxina mikolae* (see Table 3.8) and initially appeared to be closer to *Trichophaea hybrida*. Further analysis with the more variable gene region, ITS1, revealed an apparent identity of our mycobiont with *W. rehmii*. When associated with *Picea glauca* and *Pinus banksiana*, *W. rehmii* is reported to be associated with unburned soils with high organic matter or soils amended with peat; although it can be found on disturbed soils (Egger *et al.* 1991, Egger 1996). The soils on our study site were not amended with peat and were burned.

Our findings concur with earlier observations by Yang and Korf (1985) who suggested that *W. rehmii* the most common species of *Wilcoxina,* is found on

unspecified soils and coniferous litter and is mycorrhizal with the Pinaceae. Since our root tips were from burnt soils, it was expected that our mycobionts would have been *Wilcoxina mikolae*, which has been reported from burned soils (Yang and Korf 1985, Egger *et al.* 1991, Egger 1996). However, not many collections of *Wilcoxina*, only one paratype for North America (Yang and Korf 1985) have been reported so its distribution and ecology are not fully understood.

Subsequent RFLP patterns and outcome from the 18s/ITS 1 region analysis verify that Type 8 is *W. rehmii*. However, our RFLP patterns for Type 10 differ with the restriction enzyme *Alu* I. Since the variation is small, about 100 bp discrepancy, the difference is probably due to intraspecific variation in the ITS region, which occurs infrequently (Gardes and Bruns 1991, Kårén *et al.* 1998), but should be monitored when comparing mycobionts simultaneously over sites (Kårén *et al.* 1998). Either *W. rehmii* forms mycorrhizae on burned soils but does not fruit there or Type 10 is an undescribed species of *Wilcoxina*, closely related to *W. rehmii*.

E-strain mycorrhizae occur in nurseries and disturbed soils (Mikola 1965, Laiho 1965, Danielson 1991, Danielson and Pruden 1989). The fungi that form this root morphology are thought to be early colonizers of a site because, unlike ectomycorrhizae, ectendomycorrhizae have spaces of exposed roots because their mantle is often patchy, so competing mycorrhizal fungi may take over. The converse is unlikely to occur because ectomycorrhizal fungi cover the whole tip (Allen and Allen 1992). Wilcox (1971) also postulated that the transformation

from ectendomycorrhizae to ectomycorrhizae was due to ectomycorrhizal fungi outcompeting the ectendomycorrhizal fungi. He also suggested that the same species could be forming both ectendomycorrhizae and ectomycorrhizae. This latter explanation is unlikely since it would require the hyphae to change from the coarse hyphae he had observed with ectendomycorrhizae to the thinner hyphae he observed with ectomycorrhizae. Danielson and Pruden (1989) observed E-strain as behaving as a multi-stage fungus on urban spruce, noting that it may have occurred due to reduced competition. How often ectendomycorrhizae, including E-strain mycorrhizae, occur in natural, undisturbed forests is unknown because they are often overlooked and not recorded. Descriptions of *Geopora cooperi* or *Wilcoxina rehmii* mycorrhizae in undisturbed habitats would help verify the distribution of E-strain mycorrhizae.

From analyzing sequences from the 18s region, we initially concluded that RFLP types 11 and 6 were likely to be *Trichophaea hybrida.* However, further analysis of the partial 18s region and ITS 1 region revealed this not to be so. Apparently, more than one gene region should be analyzed to avoid this type of error. Another misleading assumption is if mycorrhizae do not amplify with basidiomycetous specific primers, ITS 1f and ITS 4b, then remaining mycobionts are most likely to be ascomycetes (Gehring *et al.* 1998). However, even though 41% (35/86) of our mycobionts did not amplify with ITS 1F and ITS 4b, only 43% of those mycobionts were ascomycetes, compared to 52% as previously reported

(Gehring *et al.* 1998). Fifty-seven percent (20/35) of the morphotyped root tips that did not amplify with basidiomycete specific primers were basidiomycetes.

This study focussed on the mycorrhizal ascomycetes by use of molecular tools. The section of the 18s region used for our phylogenetic analysis sufficed to identify most of our mycobionts. However, more precise tools are obviously needed. Our primers did not work for two of our RFLP types. When we used fungal specific primers, sequence data were insufficient for accurate analysis. Furthermore, ascomycete-specific primers as efficient as basidiomycete-specific primers are needed. These tools are important if we are to gain a comprehensive understanding of the ectomycorrhizal fungal community.

3.5 Acknowledgments

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Summary

The purpose of this thesis was to examine if post-fire Pezizales form mycorrhizae with two common trees found in the Pacific Northwest, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco.) and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.). This question was approached by two methods whose outcomes were similar: the mycorrhizal status of most of the species examined is inconclusive.

Taxa that are probably not mycorrhizal fungi include *Helvella compressa* (Snyder) N.S. Weber, *Anthracobia, Morchella spp.* found from burned areas, *Peziza sp.* found from burned areas, *Scutellinia, Tricharina*, and an undescribed genus and species. This study supports previous assumptions that *Tricharina* is not mycorrhizal, but saprotrophic (Yang and Korf 1985, Egger 1996).

The pure culture synthesis study reemphasized the plasticity of mycorrhizal morphology, which appears to depend on the host plant. In this study, *Wilcoxina mikolae* var. *tetraspora* (Yang & Wilcox) Yang & Korf formed E-strain mycorrhizae with ponderosa pine but ectomycorrhizae with Douglas-fir. E-strain mycorrhizae are defined as having Hartig nets, thin to no mantles, intracellular penetrations, and coarse hyphae (Laiho 1965). Ectomycorrhizae are defined to have Hartig nets, mantles, and no intracellular penetration. Mycorrhizal colonization with Douglas-fir was affected by the amount of dextrose in that colonization by *W. mikolae* was inhibited when excess dextrose was added to the

medium. However, dextrose did not affect colonization by *W. mikolae* with ponderosa pine.

Occurrence of fungal sporocarps was not correlated with occurrence of those fungi on roots underlying the sporocarps. Possible reasons for this include 1) spatial, e.g. the mycorrhizae may be lateral to the sporocarp rather than directly beneath, or the symbiont of interest may be deeper in the soil, and 2) temporal, e.g. mycorrhizae may have formed by these fungi either before or after we collected. This study reemphasized the difficulty of assessing the ectomycorrhizal community and the inadequacies of sampling methods that accurately describe this community. We did find that all the ascomycetous mycorrhizae from this study were of the E-strain type. We also found that formation of E-strain mycorrhizae is not restricted to *Wilcoxina* but includes other genera such as *Geopora*.

Future research directions include determining the number of ectomycorrhizal root tips that need to be assessed to adequately understand the ectomycorrhizal community. Another area is the development of ascomycetous-specific primers that work effectively when plant DNA is present. While examining the *Wilcoxina spp.*, we became aware that more phylogenetic work is necessary to understand relationships between *Wilcoxina*, *Trichophaea* and other related taxa. To understand more about post-fire Pezizales, data should be collected not only a couple of months of the first season after the fire but should continue in intervals up to two years, because post-fire Pezizales fruit in succesional groups up to two years (Petersen 1970).

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