Molecular Identification of Ericoid Mycorrhizal Fungi

by

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Abstract

Molecular techniques facilitated the identification of ericoid mycorrhizal fungi associated with an ericaceous plant, salal (*Gaultheria shallon* Purch), dominant in some reforestation sites in the Canadian West Coast. The polymerase chain reaction (PCR) was used to amplify the DNA coding for the internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal repeat of 28 fungal isolates. Restriction fragment length polymorphism (RFLP) patterns of these isolates obtained using a set of four restriction enzymes were compared and a synoptic key that differentiates these isolates into 14 groups was created. A fungal specific primer (ITS1-F) was used to amplify DNA of salal mycorrhizae obtained under axenic conditions with specific combinations of salal plants and fungal inoculum. Comparison among RFLP patterns of the fungal isolates with salal mycorrhizae showed that the fungal specific primer (ITS1-F) allowed the preferential amplification of the fungal component of the mycorrhizal association. RFLPs of fungal isolates and salal mycorrhizae were identical.

Mycorrhizal root fragments from a salal plant were collected at a reforestation site on Vancouver Island. From one set of root fragments, DNA was extracted and amplified by PCR. Subsequent RFLP patterns obtained indicated the presence of a selection of various fungi. A second set of 5 m m mycorrhizal roots fragments were plated on Petri dishes and 20 fungal isolates were obtained. The in vitro capacity to form mycorrhizae with salal was tested for all isolates and only five were confirmed mycorrhizal with salal. Known RFLP patterns were detected from two new isolates: one matched the RFLP

pattern produced by *Oidiodendron* species and the other matched a nonsporulating fungus (Unknown 2) described from previous work. The RFLP patterns of the other 18 newly isolated sterile fungi, including three isolates that formed in vitro mycorrhizae with salal, were different from those of all known ericoid mycorrhizal fungi. The fifteen new isolates that did not form ericoid mycorrhizae in vitro corresponded with seven RFLP patterns and their roles in association with salal roots is unknown.

To create a method that would allow the direct assesment of the presence of known ericoid mycorrhizal fungi in salal roots collected from the field, the internal transcribed spacer ITS2 region and 3' end of the 5.8S rRNA gene (400 base pairs) of 24 fungal isolates were amplified and sequenced. Sequence data analysis segregated the mycorrhizal isolates into two main groups, one including species of *Oidiodendron,* and the second including *Hymenoscyphus* and allied taxa. Accordingly, three different specific primers were designed, the first to amplify *Oidiodendron* species, and the second and third, to identify clusters of isolates from the *Hymenoscyphus* group. Tests performed using the new primers with fungal DNA mixtures of known mycorrhizal isolates and non-mycorrhizal fungal isolates detected only the fungal DNA.

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Dedication

Lovingly dedicated to my mother Hilda and my daughters Gilda and Elisa.

Chapter 1

Literature review

Introduction

Mutualistic symbiotic associations between fungi and plant roots are known as mycorrhizae. Fungi (mycobiont) associated with roots (phytobiont) are adapted to explore the soil around the root system and to acquire water and mineral nutrients. Phytobionts photosynthesize and produce energy- rich carbon compounds (Kendrick, 1992). The fungus-root interaction develops after a series of events leading to the formation of a new functioning structure establishing a nutrient exchange interface (Peterson and Farquhar, 1994).

Growth of mycorrhizal plants is almost always enhanced compared to that of non-colonized plants (Harley, 1969). Mycorrhizal associations are common in nature and therefore of great importance in various biomes (Harley and Smith, 1983), as they may regulate plant competition (Fitter, 1977), plant communities and succession (Allen, 1984; Francis and Read, 1995). Ecological factors such as climate, edaphic conditions, and anthropogenic activities can influence the success of particular types of mycorrhizae. Complex interactions between mycorrhizal species within and across ecosystems have yet to be understood (Read, 1991).

Identification of the fungal symbionts of some groups of mycorrhizae can be very difficult because the morphology of fungus-root associations formed by different fungi is not distinct between the species. Use of traditional culturing techniques to isolate mycorrhizal fungi may be selective for some species over others present at sampling time. Furthermore, some cultures of fungal isolates remain indefinitely as sterile mycelium. Therefore, the study of species

composition and dynamics of mycorrhizal communities using traditional culturing techniques is very difficult. Two additional important factors add to the complexity of studies of mycorrhizal communities. The first is that ecosystems such as forest or alpine type are composed of mixed plant species, each of which may be associated with totally different mycorrhizal fungi, and individual plants may be associated with various mycorrhizal fungi. The second is that plant roots are surrounded by soil organic and inorganic particles that harbor additional microbial species, many with roles yet to be defined. At present, molecular techniques can be used to identify fungal symbionts in an efficient and precise manner *in situ* and to clarify phylogenetic relationships (Giovannetti and Gianinazzi-Pearson, 1994). Furthermore, molecular techniques are being used to gain better understanding of the complex mechanisms controlling development and integration of both symbionts to produce a functional structure (mycorrhiza), and operation at the cellular and molecular level. Use of molecular characters of mycorrhizal fungi will allow ecologists to monitor and characterize changes in the fungal communities after anthropogenic interference.

In this literature review mycorrhizae are described, and their symbiotic components and interaction with ecosystems are discussed. Within this context, the application of molecular techniques to study mycorrhizal fungi are reviewed. In particular, the ericoid mycorrhizal fungi associated with salal (*Gaultheria shallon* Pursh) plants present at a reforestation site on Vancouver Island, British Columbia, are discussed.

Types of Mycorrhizae

The different types of mycorrhizae have been classified according to growth of fungal vegetative filaments (hyphae) in relation to the host root tissue

(Kendrick, 1985). Adhesion occurs upon contact of the fungal hyphae with root cells. The basic mechanisms controlling the developmental stages of any type of mycorrhizae are largely unknown. Depending on the fungus and plant taxa, the fungus hyphae undergoes changes and produces specialized structures on the root surface in order to penetrate between or into epidermal and cortical cells of plant roots (Tagu *et al.*, 1993; Martin *et al.*, 1995; Lambais and Mehdy, 1995; Perotto *et al* .,1995).

The ectomycorrhiza is characterized by fungal hypha growth around the root, forming a fungal mantle or sheath. The hyphae of the ectotrophic type only penetrates and grows between cortical cells to produce the hyphal network called Hartig net. Around 2000 species of host plants form ectomycorrhizae, including some of the most economically important forest trees that belong to the families of the Pinaceae (pine, spruce, fir etc.), Fagaceae (beech, oak, southern beech), Betulaceae, Myrtaceae (eucalyptus), Dipterocarpaceae and Leguminoseae (Isaac, 1992). The identified fungi associated with these plants are mainly basidiomycetes (around 5000 species) involving fungi of the orders of *Agaricales, Aphyllophorales, Gautieriales, Hymenogastrales, Lycoperdales, Melanogastrales, Phalleles* and *Sclerodermatales* . Also, two orders of the Ascomycetes form ectomycorrhizal associations; the *Pezizales* and the *Elaphomycetales* (Kendrick, 1985).

In general, endotrophic mycorrhizae are the most common. In this association, hyphae of the fungal symbiont enter the cortical cells of the host root and microscopic intracellular interfaces are produced. Usually, only a loose network of hyphae can be observed on the root surface (Jackson and Mason, 1984). Arbuscular, ericoid, monotropoid and orchid mycorrhizae represent the three main types of typical endotrophic mycorrhizae.

The most common kind of endotrophic mycorrhizal association is the

arbuscular mycorrhiza (AM). Phylogenetic analysis using DNA sequences of genes that code for small ribosomal subunit of rRNA estimate that ancestral endomycorrhizal fungi originated in the Paleozoic era, between 353-462 Myr ago (Simon *et al*., 1993). These fungi may have been important in the colonization of land by plants (Pirozynski and Dalpé, 1989). About 1000 genera of plants have been reported to associate with AM fungi, corresponding to around 80% of vascular plants species (Harley, 1989). These include most of the agriculturally important crops, herbs, and tropical trees (Kendrick, 1992). About 130 fungal species of AM fungi have been described, based on spore morphology and asexual spore-bearing structures formed at the tip of undifferentiated hyphae. These fungi belong largely to the class Zygomycetes, order Glomales and include six genera; *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* (Morton and Benny, 1990).

Orchidaceous mycorrhizae are formed by thousands of species of the Orchidaceae and the fungi are members of the basidiomycetes, often of the genus *Rhizoctonia.* This association is very important for successful seedling establishment. In orchidaceous mycorrhizae, the fungus supplies carbon to a subterranean, achlorophyllous plant structure (Peterson and Farquhar, 1994).

Some types of mycorrhizae display characteristics of both endotrophic and ectotrophic mycorrhizae. These are known as ectendomycorrhizae. Often a small mantle is present, but a Hartig net does not necessarily exist. Intracellular penetration of cortical cells occurs and hyphal coils are formed (Harley and Smith, 1983; Isaac, 1992). Interestingly, fungi of the same species can form either ectomycorrhiza or ectendomycorrhizae depending on the host plant. For example, a fungus of the genus *Wilcoxina* (E-strain), forms ectendomycorrhizae with *Pinus* and *Larix* species, but ectomycorrhizae with *Picea, Betula, Abies, Tsuga, Pseudotsuga* and *Populus* (Laiho, 1965; Scales and Peterson, 1991;

Peterson and Farquhar, 1994). Arbutoid and monotropoid mycorrhizae, named after the group of host plants, have ectendomycorrhizal characteristics.

The term "arbutoid mycorrhizae" has been used to described associations of plants of the order Ericales, including plants of the genera *Arbutus, Arctostaphylos* and *Pyrola* (woody shrubs and trees), forming mycorrhizal associations with fungi of the basidiomycetes (Zak, 1976; Molina and Trappe, 1982) and Ascomycetes (Fusconi and Bonfante-Fasolo, 1984).

Monotropoid mycorrhizae are formed between plants of the family Monotropaceae , which include achlorophyllous herbaceous plants, and fungi of the basidiomycetes (Isaac, 1992).

Ericoid mycorrhiza

The ericoid mycorrhiza is accepted as one of the common types of endotrophic association and is present in the majority of the Ericales (Harley, 1969). Plants involved are of the genera *Calluna, Rhododendron, Erica, Vaccinium, Gaultheria* and also some genera in the families Epachridaceae and Empetraceae (Isaac, 1992). Ericaceous plants predominate on mor humus rich soils of high latitude and altitude, forming dwarf-shrub heaths. They also grow as understorey species of the boreal forest, and in mediterranean climates (Read, 1992). These plants have fine roots (hair roots) composed of one to three layers of cortical cells around a small stele (Isaac, 1992). The fungal hyphae are present around the root surface where they form a loose weft of mycelium (Stoyke and Currah, 1991). Cortical cells are penetrated, and microscopic intracellular interfaces are formed. Host cell plasma membrane and an interfacial matrix surround the hyphae (Bonfante-Fasolo and Gianinazi-Pearson, 1982). Apparently, colonization of each epidermal cell takes place independently from adjacent epidermal cells (Bonfante-Fasolo and Gianinazi-Pearson, 1982).

Colonized cells are filled with dense coils (Brook, 1952). The morphology of ericoid mycorrhizae formed by different fungi looks similar in all the species of ericaceous hosts.

Ericaceous plants have the capacity to grow and sometimes become dominant in acid (pH 2 to pH 4), strongly leached, nutrient poor soils with a low rate of organic matter decomposition (Read, 1984). In these soils, mineral forms of nitrogen (N) and phosphorus (P) are limiting or absent, even though total organic forms of N and P are high (Read, 1991). Ericoid mycorrhizal fungi have the ability to provide access to nutrients (N and P) that are part of soil organic molecules (organic phosphates, amino acids, peptides and proteins) to roots of ericaceous plants (Bajwa *et al.*,1985; Leake and Read, 1989; Read, 1984; Read and Bajwa, 1985). For example, the well-known ericoid mycorrhizal fungus, *Hymenoscyphus ericae* (Read) Korf & Kernan, has some ability to degrade chitin (Leake and Read, 1990), lignin (Haselwandter *et al.*, 1990), and tannins (Leake, 1987). In addition, *H. ericae* produces an iron-specific hydroxamate siderophore that protects the plant from toxic effects when iron is present in excess concentration (Schuler and Haselwandter, 1988; Shaw *et al.*, 1990).

Fungi forming ericoid mycorrhizae are found in the hyphomycetes and in two orders of the Ascomycetes; the Onygenales and the Leotiales. The taxonomy of several sterile ericoid mycorrhizal fungi is still unknown (Haselwandter 1987; Pearson and Read, 1973; Perotto *et al.*, 1990, 1994, 1995; Xiao, 1994).

In the Leotiales, the taxonomy of the first ericoid mycorrhizal isolate was determined in pot culture of *Calluna vulgaris* (L.) Hull, when the formation of sexual structures, apothecia, confirmed the perfect state of the species *Hymenoscyphus ericae* (Read) Korf & Kernan (Read, 1974). Later, French ericoid mycorrhizal isolates obtained from *Rhododendron* and *Calluna* roots formed apothecia similar to the ones produced by two English isolates of *H. ericae* (Vegh

et al., 1979). Although several isolates from North America have been putatively classified as *H. ericae* , the sexual stage has never been observed in the field in North America (Litten *et al.*, 1985).

Although not isolated from ericaceous roots, a group of fungi of the families *Myxotrichaceae* and *Gymnoascaceae* (Onygenales) formed ericoid mycorrhizae in vitro with *Vaccinium angustifolium* (Dalpé, 1989). The Myxotrichaceae includes the species *Myxotrichum setosum* (Eidam) Orr, Kuehn & Plunkett, and *Pseudogymnoascus roseus* Raillo, with *Oidiodendron* and *Geomyces* anamorphs, respectively (Currah, 1986). The Gymnoascaceae includes the species *Gymnascella dankaliensis* (Castellani) Currah, which has an *Oidiodendron* anamorph (Currah, 1986).

The first ericoid mycorrhizal fungus identified in the hyphomycetes was the species *Oidiodendron griseum* Robak isolated from roots of *Vaccinium angustifolium* and *V. corymbosum* L., (Couture *et al.* , 1983). Later, other fungal species of the genus *Oidiodendron* formed ericoid mycorrhizae under axenic conditions, including *O. rhodogenum* Robak (Dalpé, 1986), *O. maius* Barron (Douglas et al., 1989), *O. chlamydosporicum* Morral, *O. citrinum* Barron, *O. flavum* Szilvinyi, *O. pericoinioides* Morral, and *O. scytaloydes* (Dalpé, 1991). Other identified hyphomycetes include the species *Scytalidium vaccinii* (Dalpé, 1989), *Stephanosporium cerealis* (Thum.) Swart (Dalpé, 1991), and one unusual mycorrhizal species, *Acremonium strictum* W. Gams (Xiao, 1994). Some basidiomycetes may form ericoid mycorrhizae with roots of *Calluna* (Bonfante-Fasolo and Gianinazzi-Pearson, 1979; Bonfante-Fasolo, 1980). A species of *Clavaria* was detected in thin sections of roots of *Rhododendron* by an immunocytochemical detection technique (Mueller *et al,*. 1986). However, inoculation of *Rhododendron* plants with the fungus *Clavaria argillacea* had previously failed (Read, 1983).

Some of the ericoid mycorrhizal fungi grow slowly in culture compared to other rhizosphere fungi. Many of these remain sterile indefinitely under laboratory conditions (Read, 1983). Since positive identification and classification of fungal species requires the presence of reproductive structures, their absence is a main obstacle to the study of the biodiversity of sterile ericoid mycorrhizal isolates or to the study of fungal population dynamics in ecosystems in which ericaceous plants are dominant.

Isolation of the fungal symbiont from surface-sterilized ericoid mycorrhizal roots was first performed by Bain (1937) and it has since been used routinely. Pearson and Read (1973) successfully isolated a large number of dark slow-growing mycelia from suspensions of cortical cells of fine roots of ericaceous plants which were sampled at a series of sites, surface-sterilized and macerated. In the later study, for practical reasons, only some of the isolates obtained per site were selected to test their capacity to synthesize ericoid mycorrhiza (Pearson and Read, 1973). Presently, even though the techniques of Bain (1937) and Pearson and Read (1973) allow successful isolation of sterile mycelium from ericaceous roots, researchers are confronted with similar practical problems as in the past, due to the lack of reproductive structures in the isolates. Due to practical laboratory considerations, researchers testing the capacity of new isolates to form ericoid mycorrhizae in vitro , usually have to select a fraction of the isolates per study site. Selection of sterile isolates is based on cultural and colony characteristics (Pearson and Read, 1973; Stoyke and Currah, 1991; Xiao, 1994). These approaches can lead to selection of isolates that do not fully represent the biodiversity of ericoid mycorrhizae fungi from a given site.

In order to overcome the difficulties of isolation and identification, an immunocytochemical procedure was used to detect in situ fungi of the genera

Clavaria and *Pezizella* present in the roots of *Rhododendron.* Using an immunogold labeling technique, three distinct fungal types were detected in thin sections of these roots (Mueller *et al.*, 1986). The presence of more than one ericoid mycorrhizal fungus in a single root segment has also been reported in a study of fungal isolates grown out of surface-sterilized roots (Hambleton and Currah, 1994), as well as in a study involving molecular techniques (Perotto *et al.*, 1994).

Use of molecular biology techniques to study mycorrhizae

At present, a range of cellular and molecular technologies are being used to study mycorrhizal fungal/plant interactions. These include symbiosis-regulated genes, as well as several aspects of individual symbionts (plant or fungus) such as taxonomy, evolution, phylogeny, genetics, physiology, and molecular ecology (Gianinazzi-Pearson *et al.*, 1995; Simon *et al.*, 1993; Lambais and Medhy, 1995; Perotto et al., 1995; Martin *et al.*, 1995; Gardes and Bruns, 1993; Bruns *et al.*, 1991). Molecular biological techniques allow detection of biosynthesis of enzymes formed *de novo* in fungal mycelium as well as in plant cells during establishment and development of the fungal/plant mycorrhizal interaction (Isaac, 1992).

Temporal or spatial expression of symbiosis-regulated fungal and plant gene-products is being characterized for ectomycorrhizae, arbuscular mycorrhizae, and ericoid mycorrhizae symbioses. For example, genes for the production of fungal cell wall polypeptides are produced during the early stages of interaction between *Eucalyptus* spp. and *Pisolithus tinctorius* (Martin *et al.*, 1995).

Studies on arbuscular mycorrhiza (AM) interactions have been carried out (recognition, compatibility or resistance) using mutant pea plants unable to form

root nodules (nod$^-$) and also resistant to colonization by mycorrhizal fungi (myc$^-$) (Duc *et al.*, 1989).

Plant defense genes which have been identified operating in arbuscular mycorrhizae include those that code for chitinases and *B*-1,3 glucanases. Those types of enzymes degrade fungal cell walls. Also, those genes that code for enzymes such phenylalanine ammonia lyase, chalcone synthase and chalcone isomerase have been identified and they are involved in the biosynthesis of flavonoid phytoalexins, and biosynthesis of proteins like hydroxyproline-rich glycoproteins, which are involved reinforcing the plant cell wall (Spanu *et al.*, 1989; Dumas-Gaudot *et al.*, 1992; Harrison and Dixon, 1993, 1994; Lambais and Medhy, 1993; Volpin *et al.*, 1994; Franken and Gnadinger, 1994; Bowles, 1990). Lambais and Mehdy (1993) showed transient induction of endochitinase and *B*-1,3 endoglucanase activity followed by suppression under both high and low P. Possibly the ability to form a mycorrhiza may be controlled by a differential expression of these defense genes depending on the P availability to the plant. Further understanding of the effect of P availability on gene expression of fungal and plant symbionts of AM mycorrhizae could provide a basis for management decisions in sustainable agricultural systems. The studies of gene regulation in arbuscular mycorrhizae could guide investigations into regulation of other mycorrhizal types, including ericoid mycorrhizae.

Molecular approaches to the study of taxonomy, phylogeny and ecology of mycorrhizae

Efforts to obtain isolates and identify different mycorrhizal fungal taxa from plant roots are only partially successful, and labor intensive. Also, field-sampled mycorrhizal roots are surrounded by inorganic soil particles (sand, silt and clay), organic material (humus, plant and animal debris), water, and

different kinds of rhizosphere microorganisms. Furthermore, mycorrhizal roots may host a diverse community of mycorrhizal fungi. In addition to traditional isolation techniques, the task of studying mycorrhizal communities can be undertaken by using molecular techniques which may provide greater specificity and sensitivity in analysis of mycorrhizal fungi on soil samples and complex mixtures of plant and fungal species. One of these techniques involves in vitro amplification of minute amounts of specific fragments of DNA. Such DNA amplification is accomplished by using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The PCR reaction involves sequential cycles of reactions that accomplish the following three essential steps: denaturation of the targeted DNA template, annealing of two synthetic oligonucleotide primers to the denatured DNA strands, and primer extension by a thermostable DNA polymerase. The primer sequences define the specificity of the reaction, since they anneal to sequences flanking the target DNA. Newly synthesized DNA products of each serial reaction serve as templates for the next set of reactions. The amplification is carried out exponentially, which explains why twenty cycles of PCR yields about a million-fold increase in copies of the target DNA fragment (Erlich, 1989; Mullis, 1990; Henson and French, 1993).

Since its invention, use of the PCR reaction has expanded to all fields of biological sciences because it offers many advantages to molecular biologists. These advantages include the fact that minute amounts of DNA or mixed DNAs can be used and amplified products can be kept frozen indefinitely. Various other molecular protocols have been improved by PCR. For instance, tedious conventional molecular cloning protocols and template preparation for sequencing can be circumvented by using PCR (White *et al.*, 1989).

Molecular characterization of biological specimens can be accomplished by using PCR products in analyses such as restriction fragment length

polymorphism (RFLP) of the ribosomal DNA, random amplification of polymorphic DNA (RAPD), DNA-sequencing, and the use of taxon-specific primers and probes.

RFLP analysis involves the digestion of PCR products with restriction endonucleases that recognize and cleave specific sequences of base pairs, and the separation of the fragments by gel electrophoresis, producing a banding pattern that allows comparison between isolates. RFLP analysis has been used in combination with morphological characteristics to identify sterile septate fungi isolated from roots of a subalpine shrub heath in the Alberta Rocky Mountains (Stoyke *et al.*, 1992). By using a known ectomycorrhizal fungus (*Cenococcum geophilum* Fr.), an ericoid mycorrhizal fungus (*H. ericae* (Read) Korf & Kernan), and three known root endophyte species (*Phialocephala fortinii* Wang and Wilcox, *Phialocephala dimorphospora* Kendrick , and *Phialophora finlandia* Wang and Wilcox, Stoyke *et al.* (1992) demonstrated that two-thirds of the isolates were closely related or conspecific with *P. fortinii* and five of the isolates represented species different from known ones.

Likewise, colony morphology and molecular techniques, including the PCR reaction, RFLP patterns and sequencing analysis of the ribosomal DNA, were used to investigate the anamorph-teleomorph connection between the known ericoid mycorrhizal fungi *Scytalidium vaccinii* (anamorph) and *H. ericae* (teleomorph) (Egger and Sigler, 1993). Morphological as well as molecular techniques were used to investigate the fungal biodiversity present in roots of five individual plants of *Calluna vulgaris* obtained from an ericaceous heath in northern Italy (Perotto *et al.*, 1994). Molecular data obtained using the RAPD technique showed that different ericoid mycorrhizal fungal species as well as individuals of the same species can coexist in the same root system (Perotto *et al.*, 1994; Perotto *et al.*, 1995).

Ectomycorrhizal fungi, in particular *Laccaria* spp., have been studied using RFLPs of the nuclear ribosomal RNA genes (Armstrong *et al.*, 1989; Gardes *et al.*, 1990; Henrion *et al.*, 1992). RFLP patterns from the ribosomal RNA genes were isolate-specific for nine isolates of *L. bicolor* and *L. laccata* collected from different geographic locations of North America. They did not show uniform dominant band patterns which allowed separation of the isolates at the genus or species level (Armstrong *et al.*, 1989). In spite of the RFLP pattern variability shown by the *Laccaria* spp., Armstrong *et al.* (1989) suggested that the RFLP fingerprinting technique could be useful to compare patterns of new isolates with previously obtained isolate-specific RFLP patterns. RFLP analysis done on European and North American isolates of *Laccaria* spp., including 29 isolates of *L. bicolor*, eight of *L. laccata*, three of *L. proxima*, and two of *L. amethystina*, showed that the RFLP patterns distinguished the four *Laccaria* spp., and four biological species within *L. laccata* (Gardes *et al.*, 1990). In a more detailed study, the internal transcribed spacer regions (ITS) of the ribosomal repeat, and the mitrochondrial genomes were amplified and evaluated for length, RFLP analysis (*Pst* I, *Hind* III, *Mbo* I, and *Rsa* I) and sequence analysis. Those analyses identify strains, species and genera of ectomycorrhizal fungi, including 22 isolates of *Laccaria* s spp., and seven other ectomycorrhizal fungi, including the genus *Amanita, Boletus, Hebeloma, Paragyrodon, Suillus* and *Thelephora* (Gardes *et al.*, 1991). Findings of this study showed that RFLP analysis of the ITS region did not distinguish between four species of *Laccaria*, but RFLPs produced by the restriction enzyme *Mbo* I could discriminate between all the different fungal genera. Gardes *et al.* (1991) performed PCR and RFLP analyses on ectomycorrhizae from nursery-grown seedlings of Jack pine (*Pinus banksiana*) and black spruce (*Picea mariana*) previously inoculated with *L. bicolor* 0101. The RFLP patterns of *Mbo* I and *Rsa* I indicated that a fungal contaminant was

present in some of the Jack pine mycorrhizae, and the RFLP pattern corresponded to the one produced by the isolate *Thelephora terrestris* 0095. Furthermore, a mixed *Mbo* I RFLP pattern of one mycorrhiza of Jack pine suggested the coexistence of two fungi in a single root system, since the restriction fragments corresponded to a combination of both *T. terrestris* 0095 and *L. bicolor* 0101 (Gardes *et al.*, 1991).

Morphological characteristics, as well as restriction fragment patterns (RFLPs), were used to clarify whether taxa of the E-strain mycorrhizal fungi isolated from roots have teleomorphs in the genus *Wilcoxina* (Egger and Fortin, 1990). Results of the previous study indicated that the morphology of the taxa studied could be correlated with the RFLP analysis, and that the majority of E-strain isolates belong in two species of the genus *Wilcoxina*, *W. mikolae* and *W. rehmii* (Egger and Fortin, 1990).

RAPD analysis requires the use of single arbitrarily chosen oligonucleotide primers in a PCR reaction that results in the amplification of random segments of genomic DNA. The segments generated by the RAPD products can be used as molecular markers. RAPD markers can be used to map traits, fingerprint individuals, and for analysis of molecular taxonomy and population genetics (Welsh and McClelland, 1990; Williams *et al.*, 1990; Rafalski *et al.*, 1991). The RAPD technique has been used for genetic analysis of homokaryons representing each of four mating-types obtained from a single basidiome of *Laccaria bicolor* (Doudrick *et al.*, 1995).

Direct DNA sequencing can be carried out on PCR products using a DNA sequencing machine (Gibbs *et al.*, 1989). The sequencing reaction requires the use of a PCR-product as a template, one primer, a nucleotide mixture and thermostable polymerase. Sequencing data can be used for phylogenetic studies and to create a data-base that can be used to design more specific molecular tools

such as genus- or species-specific probes or primers. Partial DNA sequences of the ITS region of the ribosomal genes using direct DNA sequences were used to study intraspecific variation between three isolates of *Laccaria bicolor* , and interspecific variation with the species of *L. laccata, L. proxima* and *Thelephora terrestris* . Furthermore, based on the intraspecific variation between *L. bicolor* isolates, a probe was created that was specifically able to distinguish one isolate from another, based on a single base difference (Gardes *et al.*, 1991).

Molecular studies have also been done on the fungi forming ectendomycorrhizae known as the E-strain (Laiho and Mikola, 1964). Sequence analysis of the nuclear-encoded ribosomal RNA genes studied the phylogeny of taxa of the E-strain mycorrhizal fungi of the genus *Wilcoxina* and their relationship to the genus *Tricharina,* both of the order Pezizales (Egger, 1996). In this study, phylogenetic analysis clearly segregated the genus *Wilcoxina* from *Tricharina,* suggesting they should be kept as separate genera. *W. rehmii* and *W. mikolae* could be distinguished by RFLP analysis (Egger and Fortin, 1990; Egger *et al.*, 1991) as well as by ascocarp morphology (Yang and Korf, 1985b), and culture morphology (Yang and Korf, 1985a; Egger and Fortin, 1990). Furthermore, a new species of *Wilcoxina* was revealed by the phylogenetic study, described in the study as *Wilcoxina* sp. nov.

To study ectomycorrhizae and rust fungi, two taxon specific-primers, one specific for fungi (ITS1-F) and the other for basidiomycetes (ITS4-B), were designed for the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit (Gardes and Bruns, 1993). Their results showed that when paired with the universal primer ITS1 or fungal-specific primer ITS1-F, the basidiomycete-specific primer (ITS4-B) efficiently amplified DNA from all basidiomycetes tested, but the DNA's of the ascomycetes were not amplified. Although with certain plants the basidiomycete-specific primer (ITS4-B)

produced a small amount of PCR product, in mixtures of fungal and plant DNAs, the fungal DNA was amplified preferentially, apparently excluding the amplification of the plant DNA. The results of amplification with the fungal-specific primers (ITS1-F and ITS4-B) of mycorrhizae collected immediately below a basidiocarp of *Amanita francheti* showed the same RFLP pattern as the basidiocarp collected above them. Another mycorrhiza sample amplified with the same primers, yielded a RFLP pattern different from that obtained from the basidiocarp collected on the surface, and the fungus could not be identified (Gardes and Bruns, 1993).

Fungal-specific primers can be used to study mycorrhizal fungi in different ecosystems to avoid misidentification of the fungal partner and to avoid the need to obtain a culture of the mycobiont (Gardes and Bruns, 1993). The fungal specific primer ITS1-F (Gardes and Bruns, 1993) was selected as a molecular tool for use in the present research to identify ericoid mycorrhizal fungi (Gardes and Bruns, 1993).

Ericoid mycorrhizal fungi in reforestation sites of the West Coast of Canada

In the coastal forest ecosystems of Canada, salal (*Gaultheria shallon* Pursh, Ericaceae) is an abundant understory plant. Salal is a dominant species in the coastal forest ecosystems of British Columbia, covering an estimated forest area of 100,000 hectares, including the maritime subzone of the Coastal Western Hemlock (CWH) biogeoclimatic zone (Weetman *et al.*, 1990). To avoid slash accumulation and heavy cover by salal plants, and to obtain better conifer regeneration, cutover areas of old-growth cedar-hemlock forest, known as CH phase, were slashburned and planted with Sitka spruce (*Picea sitchensis* (Bong.) Carr.). However, after 6 to 8 years, the spruce plantations showed poor growth and signs of chlorosis and stagnation, indicating severe nutrient deficiency. In

contrast, after clear cutting and slashburning, salal plants grew quickly without showing signs of nutrient deficiency, and became the dominant species of the sites, reaching a maximum cover of about 60% within four years (Messier and Kimmins, 1991). Two main factors seem to have affected tree growth at the CH sites; the low availability of inorganic N, and the dominant presence of salal (Messier 1991; Weetman *et al.* 1989). The salal root system is composed of rhizomes that produce numerous fine roots. The rhizomes and roots account for a large below-ground biomass located mainly at the organic layer of the CH sites; this organic layer varied in thickness between 20 and 40 cm (Messier 1991; Messier and Kimmins 1991). The dominance of salal at the CH sites presents a serious problem for the forest industry (Weetman *et al.*, 1989). In order to understand the problem and find a solution to the reforestation problems at the CH sites, a long-term research effort was initiated at the maritime subzone on the Coastal Western Hemlock (CWH) bioclimatic zone, the Salal Cedar Hemlock Integrated Research Program (SCHIRP).

Interestingly, forest trees responded to nitrogen fertilization at the CH site while salal did not seem to show deficiency in nitrogen (Weetmann et al., 1989). The main source of nitrogen at the CH sites is in the organic layer (Messier, 1991). In Europe, work on the dominance of *Calluna vulgaris* (L.) Hull), an ericaceous plant, has shown that the associated ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan, provides the ericaceous host with nutrients (Read, 1987). Studying the role of ericoid mycorrhizal fungi in the dominance of salal thus became part of the Salal Cedar Hemlock Integrated Research Program (SCHIRP) (Xiao, 1994).

Xiao (1994) isolated fungi from salal roots sampled at the SCHIRP site and tested the capacity of the isolates to form ericoid mycorrhizae with salal plants under axenic conditions. Using colony morphology and cultural characteristics,

the sterile isolates that formed ericoid mycorrhizae with salal were grouped and named **Unknown 1** and **Unknown 2.** The identity of the sterile isolates could not be confirmed, even though cultures of the isolates in the **Unknown 2** had a colony morphology resembling that of two known *Hymenocyphus ericae* isolates (100 and 101). Two other groups of ericoid mycorrhizal fungi were identified to the species level since they formed reproductive structures. One of the sporulating species, *Oidiodendron griseum* Robak, produced conidia on long stalked conidiophores (Barron, 1962). *O. griseum* had been described before as an ericoid mycorrhizal fungus of *Vaccinium* but this was the first report of it forming ericoid mycorrhizae with *Gaultheria* (Couture *et al.* 1983; Xiao, 1994). The second sporulating species produced simple phialide pegs and was identified as *Acremonium strictum* W. Gams (Xiao, 1994). Although ubiquitous in nature, this was the first time that *A. strictum* isolates were reported to be mycorrhizal with an ericaceous plant (Xiao, 1994). Colonization morphology of *A. strictum* was defined as pseudomycorrhizal because it was different from the typical ericoid mycorrhizae, with the fungal hyphae loosely arranged inside the cortical cells of salal, instead of forming typical hyphal complexes (Xiao, 1994).

The previous study also showed that the ericoid mycorrhizal fungi isolated from the SCHIRP site could utilize different forms of organic nitrogen such as the amino acid glutamine, the peptide glutathione and/or protein. In vitro studies also found that growth of three ectomycorrhizal species associated with Western hemlock was inhibited by the four taxa of ericoid mycorrhizal fungi of salal isolated at the SCHIRP site. Taken together these results suggest that the association of salal with ericoid mycorrhizal fungi in the clearcut CH reforestation sites in British Columbia may favor the growth of salal (Xiao, 1994).

Since forest sites may be composed of overlapping types of mycorrhizal fungal communities, including ericoid, arbuscular, ecto-, and entendo-

mycorrhizal fungi, molecular techniques used alongside traditional culturing techniques would help to study fungal population dynamics and community structure. The assessment of the impact of anthropogenic intervention on mycorrhizal populations in processes such as logging, could be done at a faster rate using molecular techniques. Periodic assessment of performance and geographic distribution of mycorrhizal inocula, including sterile isolates, will be possible. Also, fungal taxonomic diversity within a single root system as well as between root systems can be possible by using techniques such RFLP, RAPD, sequencing of selected genes, or molecular probes.

Objectives

Xiao's research (1994) provided important information about the identity and role of ericoid mycorrhizal fungi present at reforestation sites in British Columbia. However, questions concerning taxonomy, biodiversity, phylogeny, and ecology of the ericoid mycorrhizal fungi of salal still need to be addressed.

The focus of this thesis's research was to develop molecular techniques and to use them concurrently with traditional techniques to expeditiously evaluate the presence of fungal isolates associated with roots of ericaceous plants in reforestation sites. Such molecular techniques could be used to evaluate ericoid mycorrhizea present in forest and alpine ecosystems. The objectives of this thesis are as follows:

(a) To use molecular techniques in addition to traditional methods to determine the genetic identity of known ericoid mycorrhizal fungi and some root-associated fungi.

(b) To create a synoptic key for identification of ericoid mycorrhizal isolates, based on an RFLP pattern database.

(c) Using the PCR reaction, to establish if fungus-specific primers would

amplify DNA of the mycobiont from synthesized mycorrhizae and from mycorrhizae obtained in the field.

(d) To test the usefulness of the RFLP database for identification of newly acquired isolates from the field.

(e) Using sequence data, to design taxon-specific ericoid mycorrhizal primers.

(f) Using sequence analysis, to develop an understanding of the phylogenetic relationships between the taxa of the ericoid mycorrhizal fungi studied.

Chapter 2

Restriction Fragment Length Polymorphism of Ericoid Mycorrhizal Fungi

Abstract

To facilitate the identification of ericoid mycorrhizal fungi associated with salal (*Gaultheria shallon* Pursh) and to generate a database of ericoid mycorrhizal RFLP patterns, the polymerase chain reaction (PCR) was used to amplify the DNA that codes for the entire internal transcribed spacer region (ITS1 and ITS2) and the 5.8S rRNA gene of the nuclear ribosomal repeat of 34 fungi. Restriction fragment length polymorphism (RFLP) patterns of fungal PCR products obtained using a set of four restriction enzymes were compared and a synoptic key that divides these fungi into 16 groups was created. A fungal specific primer (ITS1-F) allowed preferential amplification of fungal DNA from axenically synthesized mycorrhizae obtained with specific combinations of salal plants and fungal inoculum. RFLPs of fungal isolates from pure culture and from axenic mycorrizae were identical.

From a single field-collected salal rhizome, DNA was extracted from a series of approximately 5 mm long root fragments of mycorrhizal roots and amplified by PCR, using a fungal specific primer (ITS1-F). Restriction digests of fungal DNAs yielded complex fragment patterns, suggesting that more than one fungus was associated with each 5 mm root fragment. Also, to establish whether

RFLP screening would be useful in identification of field isolates, 20 fungi were cultured from 3 mm mycorrhizal roots fragments from the same rhizome. Five of the 20 isolates formed mycorrhizae in resynthesis experiments, two of which had similar colony morphology and RFLP patterns to known mycorrhizal fungi. Two ericoid mycorrhizal fungi lacked diagnostic morphological characters and exhibited new RFLP patterns. These experiments suggest that RFLP patterns may be useful in revealing diversity among cultured ericoid mycorrhizal fungi that lack diagnostic sporulation features.

Introduction

One type of plant root endotrophic association, the ericoid mycorrhiza, is present in the plants of the order Ericales (Harley, 1969). The exact number and diversity of ericoid mycorrhizal fungi remains uncertain and only a small number of taxonomically diverse ascomycetes have formed ericoid mycorrhizae under in vitro conditions (Straker, 1996). Several studies indicate that both dark and light sterile mycelial isolates form ericoid mycorrhiza but no reproductive structures (Freisleben, 1933, 1934; Bain, 1937; Couture *et al.*, 1983; Pearson and Read, 1973; Xiao, 1994). Lacking reproductive structures, sterile fungi cannot be classified even when they are grown in pure culture. Furthermore, the morphology of the symbiotic structure (ericoid mycorrhiza) is similar in all host plants. Molecular biology techniques, such as PCR used to amplify specific DNA regions, random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLPs), and DNA sequencing, used in

combination with morphological and cultural properties, can contribute to understanding the taxonomy, phylogeny and ecology of ericoid mycorrhizal fungi.

Known ericoid mycorrhizal fungi include those in the class Ascomycetes, order Leotiales and order Onygenales. The ericoid species *Hymenoscyphus ericae* (Read) Korf & Kernan (Read, 1974) has a *Scytalidium* anamorph. *Myxotricum setosum* (Eidam) Orr, Kuehn & Plunkett (Dalpé, 1989) and *Gymnascella dankaliensis* (Castellani) Currah (Dalpé, 1989) both have *Oidiodendron* anamorphs, and *Pseudogymnoascus roseus* Raillo (Dalpé, 1989; Xiao, 1994) has a *Geomyces* anamorph. In the Hyphomycetes, species of the genera *Oidiodendron, Stephanosporium, Scytalidium* and *Acremonium* have been proven to form ericoid mycorrhiza in synthesis experiments, including: *O. citrinum* Barron (Dalpé, 1986), *O. chlamydosporicum* Morrall (Dalpé, 1991), *O. maius* Barron (Xiao, 1994), *O. flavum* Szilvinyi amend. Barron (Dalpé, 1991; Xiao, 1994), *O. griseum* Robak (Couture *et al.*, 1983; Dalpé, 1986; Xiao, 1994), *O. rhodogenum* Robak (Dalpé, 1986), *O. scytaloides* Gams & Soderstrom (Dalpé, 1991), *O. periconioides* Morrall (Dalpé, 1991), *Scytalidium vaccinii* Dalpé, Litten and Sigler (Dalpé, 1989; Xiao, 1994), *Stephanosporium cerealis* (*Thum.*) Barron (Dalpé, 1986), and *Acremonium strictum* (Xiao, 1994). Using transmission electron microscopy, dolipore septa characteristic of basidiomycetes were observed in roots of *Calluna vulgaris* L. (Bonfante-Fasolo, 1980) and *Rhododendron* (Peterson *et al.*, 1980). Furthermore, using an immunocytochemical detection technique, *Clavaria* species was detected in thin sections of *Rhododendron* roots (Mueller *et al.*,

1986). However, to the present, experiments to synthesize ericoid mycorrhiza from basidiomycetes have failed.

Molecular techniques have been used to study mycorrhiza, including both ecto- and endomycorrhizal type, helping to clarify fungal taxonomy, phylogeny and population ecology of the different fungal groups (Gardes *et al.*, 1990; Gardes *et al.*, 1991; Stoyke *et al.*, 1992; Perotto *et al.*, 1994; Egger and Sigler, 1993). Evidence obtained from cultural and from molecular data including RFLP patterns and partial ribosomal RNA gene sequences was used to suggest that the ericoid mycorrhizal fungi, *Scytalidium vaccinii* and *H. ericae*, are anamorph and teleomorph stages of a single taxon (Egger and Sigler, 1993). In the Alberta Rocky Mountain region, sterile septate fungal isolates from roots of subalpine shrub heath were characterized and grouped by colony morphology. In an attempt to identify these isolates, RFLP analysis of 18 selected cultural groups were compared to the ectomycorrhizal fungus *Cenococcum geophilum* Fr., the ericoid mycorrhizal fungus *H. ericae*, and the root endophytes *Phialocephala fortinii* Wang and Wilcox, *Phialocephala dimorphospora* Kendrick, and *Phialophora finlandia* Wang and Wilcox (Stoyke *et al.*, 1992). RFLP analysis showed that two-thirds of the isolates were conspecific with or closely related to *P. fortinii*. In a natural heathland in northern Italy, ericoid mycorrhizal isolates obtained from roots of *Calluna vulgaris* were studied using conventional and PCR-RAPD techniques (Perotto *et al.*, 1994). The study concluded that several ericoid fungal species were present in a single plant root system, and that high genetic polymorphism exists among the same species of ericoid mycorrhizal fungi. Also,

fungal isolates with the same DNA fingerprint were detected in roots growing distant from each other (Perotto *et al.*, 1994).

Salal (*Gaultheria shallon* Pursh), an ericaceous shrub, is the most abundant understory plant in the coastal forest ecosystem of the USA Pacific Northwest. In Western Canada, on certain reforestation sites located in the Coastal Western Hemlock (CWH) biogeoclimatic zone, salal has become a dominant species. On these sites, poor growth of conifers (Sitka spruce, Western hemlock, Western red cedar and Amabilis fir) includes symptoms such as chlorotic foliage and growth check after 6 to 8 years. In order to find a solution to this problem, the Salal Cedar Hemlock Integrated Research Program (SCHIRP) was initiated in a maritime subzone on the Coastal Western Hemlock (CWH) biogeoclimatic zone, located between Port Hardy and Port McNeill, in northern Vancouver Island, British Columbia. Those old-growth sites, prior to clearcutting and slash burning, had two major overstory species, western red cedar (*Thuja plicata* Donn), and western hemlock (*Tsuga heterophylla* (Raf.) Sarge), and are known as CH forest type (Prescott and Weetman, 1994). Forest floors of the CH forest type are low in N availability and the salal understory produces tannins that further interfere with N mineralization (Keenan, 1993; Minore 1983; Prescott and Weetman, 1994). At the SCHIRP site, most roots of plants are found in the organic layer (Prescott and Weetman, 1994). One of the factors affecting the dominance of salal plants in CH reforestation sites in British Columbia may be salal's association with ericoid mycorrhizal fungi (Prescott and Weetman, 1994). As part of the SCHIRP project, Xiao (1994) isolated and identified fungi that

formed ericoid mycorrhiza with salal. Axenic studies showed that all the ericoid mycorrhizal fungi isolated at the SCHIRP site have the capacity to use simple forms of organic nitrogen, such as the amino acid glutamine, and *Oidiodendron griseum* and an unidentified fungus (Unknown 2) utilized the peptide glutathione. *Acremonium strictum* utilized a protein as a source of nitrogen, bovine serum albumin (Xiao, 1994). Furthermore, dry weights of axenically-grown mycorrhizal salal plants showed that the amino acid glutamine was a good source of N for colonized salal plants, and that the peptide was a good source of N for salal plants inoculated with *Oidiodendron griseum.* Salal plants inoculated with *Acremonium strictum* grew well in all sources of N, including glutamine, glutathione, BSA and $(NH_4)_2$ HPO_4. Also, results of *in vitro* studies of the interactions among four ericoid mycorrhizal fungi of salal and three species of ectomycorrhizal fungi associated to Western hemlock showed that all three ectomycorrhizal fungi were inhibited by the ericoid mycorrhizal fungi of salal (Xiao, 1994).

Fungal colony morphology and when possible, asexual fruiting bodies, were used to characterize the ericoid mycorrhizal fungi of the SCHIRP site (Xiao, 1994). The most commonly found species sporulated and was identified as *Oidiodendron griseum* Robak. The second most common sporulating species was *Acremonium strictum* W. Gams. Colonization morphology produced by the isolates of *A. strictum* was different than the typical ericoid mycorrhizae and it was defined as a pseudomycorrhizal association. *A. strictum* in salal produced fungal hyphae loosely arranged inside the cortical cells of salal instead of

producing the typical hyphal complexes (Xiao, 1994). Two other groups of isolates with distinct cultural and morphological colony characteristics did not sporulate under laboratory conditions and they were named Unknown 1 and Unknown 2. According to Xiao (1994), colony morphology of taxon Unknown 2 resembled that of two well known ericoid mycorrhizal isolates of the species *Hymenoscyphus ericae* (100 and 101) obtained from Dr. D. J. Read. In the absence of sexual or asexual reproductive structures, however, it was only possible to speculate further about the identity of the two sterile taxa. Taxonomic diversity and distribution of ericoid mycorrhizal fungi in this ecosystem is still not clear. The present research is aimed toward developing molecular techniques to characterize known and new ericoid mycorrhizal isolates as well as toward developing molecular techniques to study the distribution of ericoid mycorrhizal fungi *in situ*, directly from colonized roots taken from the field.

Objectives

The main goals of this research were:

(a) To use molecular characters, in this case RFLP patterns, in addition to already established morphological and cultural characters, to evaluate the genetic identity of currently known ericoid mycorrhizal fungal isolates and some root associated fungi.

(b) To develop, based on a RFLP patterns database, a synoptic key for identification of ericoid mycorrhizal isolates.

(c) To determine whether fungus-specific primers would amplify the

fungal partner only from DNAs of axenically synthesized mycorrhizae.

(d) To determine whether a fungus specific primer would amplify a single fungus from mycorrhizae obtained from the field.

(e) To assess whether the RFLP database will be useful in identification of known ericoid mycorrhizal fungi among a newly acquired set of isolates from colonized field roots.

Materials and Methods

Fungal isolates

The 34 fungal isolates used in this study were obtained from the University of Alberta Mycological Herbarium (UAMH), from the Canadian National Collection of Fungus Cultures (DAOM), from Dr. D. J. Read (UK) and from G. Xiao (UBC) and M. Monreal (UBC), Departments of Botany and Soil Science of the University of British Columbia (Table 2.1).

Table 2.1 Fungal isolates used in this study, source of isolates, mycorrhizal status, and isolation data.

Fungal isolate	Source	Mycorrhizal status	Isolation data
Acremonium strictum	UBC S232	+	Roots of *Gaultheria shallon*, Vancouver Island,B.C., Canada.
	UBC S214	+	" "
	UBC S228	+	" "
Hymenoscyphus ericae	UAMH 6561	+	Roots of *Vaccinium angustifolium*, Maine, USA.
	UAMH 6045	+	Roots of *Vaccinium angustifolium*, Windsor, Ontario, Canada.
	UAMH 6598	+	Roots of *Vaccinium angustifolium*, Alma, Quebec, Canada.

Table 1 Continued.

Hymenoscyphus ericae	UAMH 6663	+	Roots of *Vaccinium corymbosum*, Alma, Quebec, Canada.
	READ,D. J. 101	+	Roots of *Calluna vulgaris*, Bolsterstone, U K.
	UAMH 6735	+	" "
Oidiodendron citrinum	UAMH 1525	+	Soil of cedar bog , Aberfoyle, Ontario, Canada.
Oidiodendron flavum	UAMH 1524	+	" "
Oidiodendron griseum	UAMH 4080	+	Wood chips and bark in logging truck, Westlock, Alberta, Canada.
	UBC S4	+	Roots of *Gaultheria shallon*, Vancouver Island, B.C. , Canada.
	UBC S18	+	" "
	UBC S80	+	" "
	UBC M10	+	" "
Oidiodendron maius	UAMH 1540	+	Peat soil cedar bog, Guelph, Ontario, Canada.

Table 1 Continued.

Oidiodendron periconioides	DAOM 197506	+	Soil, Candle Lake, Saskatchewan, Canada.
Oidiodendron scytaloides	UAMH 6521	+	Forest soil, Sweden
Phialocephala fortinii	UAMH 6815	-	Roots of *Cassiope mertensiana*, Jasper National Park, Alberta , Canada
Phialocephala dimorphospora	UAMH 7527	-	Slime in pulp mill, New Brunswick, Canada.
Phialophora finlandia	UAMH 7454	+	Roots of *Pinus sylvestris* seedling, Finland.
Pseudogymnoascus roseus	UAMH 1658	+	*Vaccinium angustifolium* seedlings.
Scytalidium vaccinii	UAMH 5828	+	Roots of *Vaccinium angustifolium*, Maine, USA.
Unknown 1	UBC S9	+	Roots of *Gaultheria shallon*, Vancouver Island, B.C., Canada.
	UBC S222	+	" "
	UBC S245	+	" "

Table 1 Continued.

Unknown 2	UBC S246	+	Roots of *Gaultheria shallon,*
	UBC S203	+	Vancouver Island, B.C., Canada.
	UBC S226	+	" "
	UBC M5	+	" "
Unknown 3	UBC M8	+	Roots of *Gaultheria shallon,* Vancouver Island, B.C., Canada.
Unknown 4	UBC M20	+	Roots of *Gaultheria shallon,* Vancouver Island, B.C., Canada.

DAOM: Canadian National Collection of Fungus Cultures. Agriculture and Agri-Food Canada, Central Experimental Centre, Ottawa, Ontario, Canada.
UAMH: University of Alberta Mold Herbarium and Culture Collection, Edmonton, Alberta, Canada.
UBC: Isolates of G. Xiao (1994) and M. Monreal (1994), Departments of Botany and of Soil Science, respectively, University of British Columbia, B.C., Canada.

DNA extraction, PCR and RFLP patterns of isolates

Prior to DNA extraction, fungal isolates were grown for three weeks at 25 C in the dark on modified Melin Norkrans agar (MMN) (Marx, 1969). DNA extractions were carried out on scraped mycelium from the edge of the fungal colony (Lee and Taylor, 1990). The DNA region including the internal transcribed spacer 1 (ITS 1), the 5.8S rRNA gene, and the internal transcribed spacer 2 (ITS 2) of the nuclear ribosomal repeat was amplified using PCR (Mullis and Faloona, 1987).

PCR conditions for DNA amplification from pure fungal culture were a denaturation step at 95 C for 2 min., followed by 30 cycles of denaturation at 95 C for 1 min., annealing at 48 C for 1 min., and an initial extension at 72 C for 45 sec. Subsequent extension times were increased by 4 sec per cycle. The final extension step was 10 min. To test for contamination, negative controls were used in every PCR reaction. PCR products were agarose purified. Purified product was reamplified with the same primers to increase amount of product.

RFLP analyses were performed on 8 µl of PCR product, following instructions given by the restriction enzymes manufacturers. A total of 8 enzymes were used: ***Msp I*** (New England Biolabs), ***Rsa* I** (New England Biolabs), ***Cfo* I** (Promega), ***Alu* I** (Promega), ***Pst* I** (New England Biolabs), ***Bam I*** (Boehringer-Mannheim), ***Hind* III** (Boehringer-Mannheim), and ***Eco*R I** (New England Biochemical). The restriction fragments were electrophoresed in 0.5 % (wt/vol) Synergel (Diversified BioTech)/ 1 % (wt/vol) agarose gel, run at 80 V for 4 hours. The gels were stained for 30 min in a solution of ethidium bromide,

destained in distilled water for 30 min, and photographed under a UV transilluminator. Restriction fragment sizes were estimated using the method described by Southern (1979).

Synthetic salal mycorrhizae

To obtain axenically grown ericoid mycorrhizal roots, salal seeds were surface sterilized in 30% hydrogen peroxide and rinsed in sterilized distilled water. Drained seeds were placed on the surface of water agar (10 g l^{-1}) in Petri dishes that were sealed with a strip of parafilm. The growth chamber was kept at a maximum of 25 C under 18 h light at 310 μmol m^{-2} sec^{-1} and 6 h dark (Xiao and Berch, 1992). After leaf emergence, one seedling of salal was placed at the center of a Petri dish with half of the media disc cut out. The growth medium consisted of 0.1 g l^{-1} bovine serum albumin (BSA) and 8 g l^{-1} of Difco Bacto agar, basic salts of MMN, with the exclusion of mineral-N, thiamine, malt extract, and glucose. Fungal plugs of 5 m m^3 were placed on the cut edge of the medium at about 1 cm from the salal plant. Several Petri dishes were inoculated with each fungal isolate. The fungi used were *Scytalidium vaccinii* (UAMH 5828), *Oidiodendron griseum* (UBC S4), Unknown 1 (UBC S222), Unknown 2 (UBC S246) and the root endophytes, *Phialocephala fortinii* (UAMH 6815) and *Phialophora finlandia* (UAMH 7454). The Petri dishes were kept in the growth chamber for three to four months. Roots were examined under the light microscope to see if they were mycorrhizal.

PCR and RFLP of synthesized mycorrhizae

At harvest, mycorrhizal roots were washed in distilled water and DNA was extracted. Because of the need for preferential amplification of fungal DNA from mycorrhizae of salal roots, a fungal specific primer, ITS1-F, flanking the 5' end of the ITS1 spacer (Gardes and Bruns, 1993) and universal primer, ITS4, flanking the 3' end of the ITS2 region (White *et al.*, 1990) were used. Flanking the same DNA region, the universal primers ITS 5 and ITS 4 (White *et al.*, 1990) were used to amplify salal DNA and these PCR products were used as controls. PCR conditions were an initial denaturation step at 95 C for 2 min., followed by 30 cycles of denaturation at 95 C for 1 min., annealing at 52 C for 1 min. and extension at 72 C for 45 sec, increasing the extension time of each cycle by 4 sec per cycle and ending with a final extension step of 10 min. PCR products were agarose purified and reamplified to increase the amount of product. PCR products were digested with a set of four endonucleases (*Rsa* I, *Cfo* I, *Alu* I, and *Msp* I). DNA fragments generated by the digestion were electrophoresed as described for fungal isolates. To test for DNA contamination of PCR reaction mixtures, negative and positive controls were used in every PCR reaction. The negative controls contained only distilled water. Undigested salal PCR product, undigested fungal PCR product, the digests of pure fungal isolates, and the negative control, were electrophoresed alongside digestion fragments generated by digested DNAs of mycorrhizae synthesized by a known fungal taxon.

Field samples of salal mycorrhizae

In October 1994, salal roots were collected at the SCHIRP study site, along a trail in Block 7, a reforestation plot of an old-growth Cedar Hemlock (CH) cutover, dominated by growth of salal (Prescott and Weetman, 1994; Xiao, 1994). In the laboratory, salal fine roots were selected from one underground shoot (rhizome) of approximately 8 cm. They were washed in distilled water and examined under the light microscope to determine the presence of ericoid mycorrhizae. Two approaches, direct PCR amplification and culturing, were used to study the presence of fungi in these ericoid mycorrhizal roots. For the direct amplification, DNA was extracted from one set of twentyone 5 mm root fragments. A second set of thirty 3 mm root fragments was used for fungal culturing. DNA extraction (Lee and Taylor, 1990) was followed by PCR and RFLP analysis.

Molecular study of field ericoid mycorrhizal roots via direct DNA extraction

DNA was extracted from a set of twenty root fragments, each approximately 5 mm long. Each root fragment was placed in 1.5 centrifuge tubes, covered by 30 µl lysis buffer (50 mM Tris-HCl (pH 8.0), 50 mm EDTA, 3% SDS, 1% B-mercaptoethanol) and the root tissue was crushed with a micropestle. Lysis buffer was added to each centrifuge tube to complete a volume of 750 µl and incubated at 65C for 1 h (Lee and Taylor, 1990). Dry pellets of DNA were suspended in 20 µl TE (low EDTA) and placed in a water bath at 60 C for 5 min.

Before PCR amplification, 10 µl of the DNA stock solutions were further diluted in 45 µl of sterile distilled water, resulting in a 5.5 fold dilution. The diluted DNAs were PCR amplified using fungal-specific primer ITS1-F and primer ITS4. The resulting band was purified by cutting each agarose band and melting the agarose in 100 µl of sterile distilled water and reamplified (PCR) to increment the yield of PCR product. RFLP analysis followed after digestion with the enzyme ***Msp* I.**

RFLP of fungi in pure culture, isolated from ericoid mycorrhizae

Sixty root fragments of about 3 mm lengths were excised and placed on Petri plates with either potato dextrose agar (PDA) or MMN. Three root fragments were placed in each plate. A total of twenty plates was used, ten with PDA and ten with MMN. Fungal isolates growing from these root pieces were transferred to new plates after a week. They were grown for another two weeks and DNA was extracted. This was followed by PCR and RFLP analysis. Each isolate of the new fungal cultures was inoculated onto several Petri dishes with axenically grown salal seedlings to determine if the isolate formed ericoid mycorrhizae.

Results

Length variation of PCR amplified DNAs from fungi in pure culture

The length of the PCR products obtained by using the fungal specific primer ITS 1- F and the universal primer ITS 4 varied between 980 bp and 590 bp (Fig. 2.1). Three of the isolates representing the species *Acremonium strictum* (UBC S232, S214, and S228) and *Phialocephala dimorphospora* (UAMH 7527) had fragment lengths that varied in size between 630 and 620 bp. Three of the putative North American isolates of *Hymenoscyphus ericae* (UAMH 6561, 6045, and 6598), as well as *Scytalidium vaccinii* (UAMH 5828) and *Phialophora finlandia* (UAMH 7454) were 980 bp long. New sterile isolates from the SCHIRP site , Unknown 3 (M8 UBC), and Unknown 4 (M19 UBC and M20 UBC) were 980 bp (Fig. 2.3) and 1100 bp (Fig. 2.4) long respectively. The remaining 22 isolates, including three isolates of *H. ericae* (UAHM 6663, UAHM 6735, and Read, D. J. 101) had similar lengths of 590 bp.

Restriction fragment length polymorphism of fungal isolates from pure culture

Restriction patterns from endonucleases with six base pair recognition sites were not variable enough to be useful in identification of fungal isolates. No restriction fragments were produced by ***Pst*** I (CTGCA/G) in any of the isolates. The restriction enzyme ***Bam*** **HI** (G/GATCC) produced two fragments of 510 and 130 bp in only one isolate, *Phialocephala dimorphospora* (UAMH 7527). The enzyme ***Hind*** **III** (A/AGCTT) produced two fragments of 840 bp 160 bp in

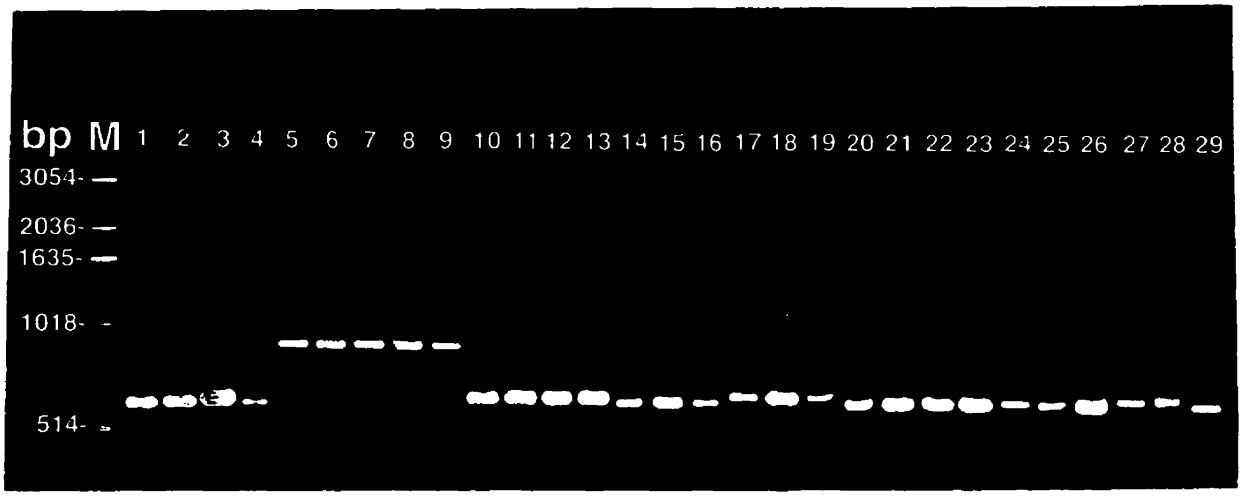

Figure 2.1 PCR products of the ITS region of 29 isolates of ericoid mycorrhizal fungi and root associated fungi. M = fragment size marker; lane 1 = *H. ericae* (Read, D.J. 101); lane 2 = *H. ericae* (UAMH 6735); lane 3 = *P. dimorphospora* (UAMH 7527); lane 4 = *H. ericae* (UAMH 6663); lane 5 = *H. ericae* (UAMH 6598); lane 6 = *S. vaccinii* (UAMH 5828); lane 7 = *H. ericae* (UAMH 6561); lane 8 = *H. ericae* (UAMH 6045); lane 9 = *P. finlandia* (UAMH 7454); lane 10 = *P. fortinii* (UAMH 6815); lanes 11, 12, and 13= **Unknown 2** (UBC S246, UBC S203, UBC S226); lanes 14, 15, and 16 = **Unknown 1** (UBC S9, UBC S222, UBC S245); lanes 17, 18, and 19 = *A. strictum* (UBC S232, UBC S214, UBC S228); lanes 20, 21, and 22 = *O. griseum* (UBC S4, UBC S18, UBC S80); lane 23 = *O. griseum* (UAMH 4080); lane 24 = *O. periconioides* (DAOM 197506); lane 25 = *O. flavum* (UAMH 1524); lane 26 = *O. maius* (UAMH 1540); lane 27 = *O. citrinum* (UAMH 1525); lane 28 = *O. scytaloides* (UAMH 6521); lane 29 = *P. roseus* (UAMH 1658).

two of the *Hymenoscyphus ericae* isolates (UAMH 6598 and 6561) and in *Scytalidium vaccinii* (UAMH 5828). The enzyme ***EcoR* I** (G/AATTC) produced two fragments in all the isolates studied (Table 2.2).

However, four endonucleases that cleave the DNA when they recognize a sequence of 4 base pairs worked effectively; ***Rsa* I** (GT/AC), ***Cfo* I** (GCG/C), ***Alu* I** (AG/CT), and ***Msp* I** (C/CGG). Each enzyme produced a number of fragments of different sizes which were used as character states in a synoptic key. Based on brightness of the bands some of the DNA digests were composed of two fragments of approximately the same size, and these are described as "doublet" in the synoptic key and in Table 2.2. The 34 fungal isolates fell into sixteen RFLP groups and numbers 1 to 16 were assigned to each one of them (Table 2.2).

The restriction enzyme ***Msp* I** revealed the most variation, generating different patterns for 14 out of the 16 RFLP groups of isolates (Fig. 2.2). The RFLP pattern generated by Group 15 (**Unknown 3**, isolate UBC M8) and Group 16 (**Unknown 4**, isolates UBC M19 and UBC M20) are shown on Fig. 2.3 and Fig. 2.4, respectively. Based on RFLP patterns of fungal isolates from the SCHIRP site, six morphological groups of the ericoid mycorrhizal fungi including *Acremonium strictum, Oidiodendron griseum,* **Unknown 1** (UBC S9, UBC S222, UBC S245) (Fig. 2.4), **Unknown 2** (UBC S246, UBC S203, UBC S226, and UBC M5), **Unknown 3** (UBC M8), and **Unknown 4** (UBC M19, and UBC M20), can be distinguished. Sterile isolates **Unknown 1, Unknown 2, Unknown 3,** and **Unknown 4** remain unidentified as their RFLP patterns did not match any identified mycorrhizal isolates used in this study (Table 2.2).

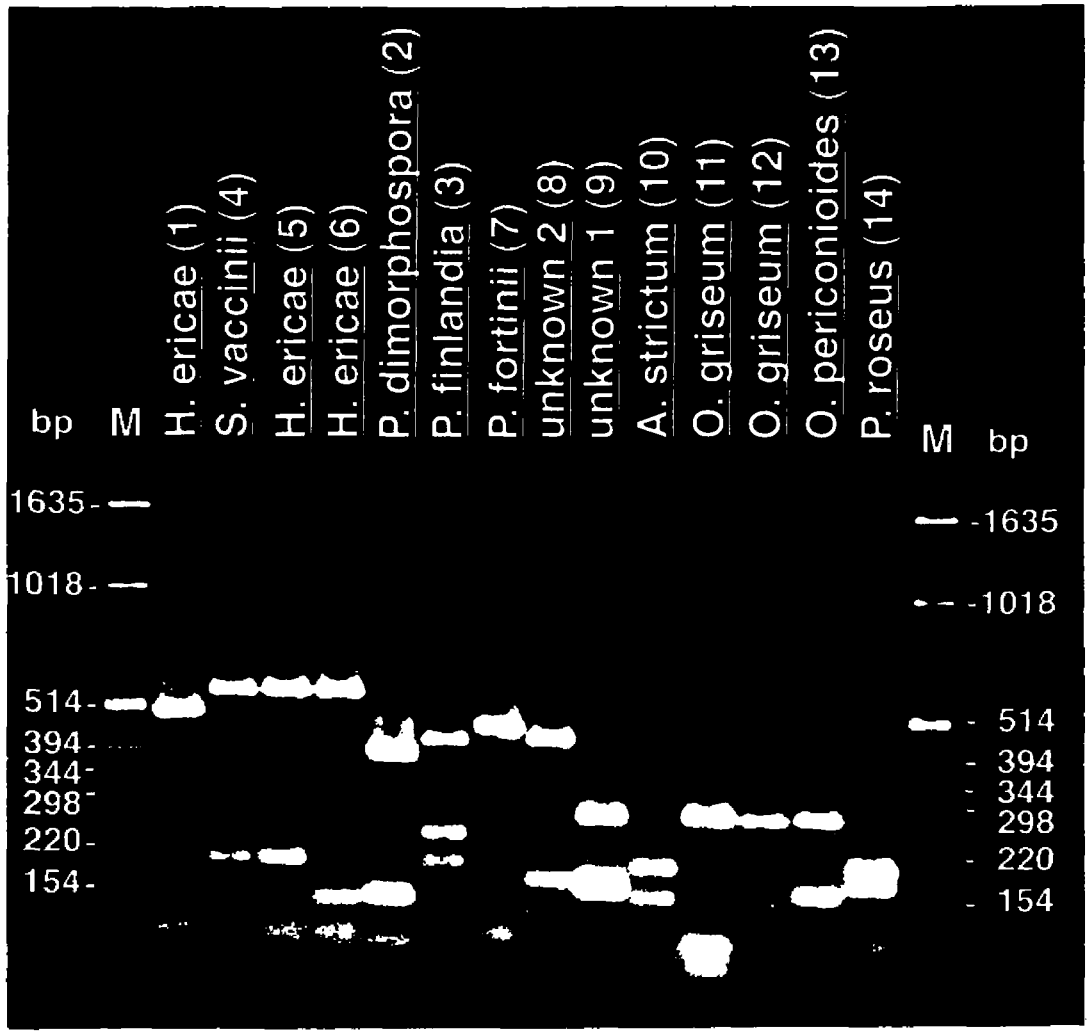

Figure 2.2 RFLP patterns of PCR-DNAs of fungal isolates digested with the restriction enzyme ***Msp* I. M** = molecular fragment size marker; lane 1 = Group 1 (*H. ericae* , UAMH 6735); lane 2 = Group 4 (*S. vaccinii* , UAMH 5828) ; lane 3 = Group 5 (*H. ericae* , UAMH 6561); lane 4 = Group 6 (*H. ericae*, UAMH 6045) lane 5 = Group 2 (*P. dimorphospora*, UAMH 7527); lane 6 = Group 3 (*P. finlandica*, UAMH 7454); lane 7 = Group 7 (*P. fortinii*, UAMH 6815); lane 8 = Group 8 (**Unknown 2**, UBC S246); lane 9 = Group 9 (**Unknown 1**, UBC S9); lane 10 = Group 10 (*A. strictum*, UBC S232); lane 11 = Group 11 (*O. griseum*, UBC S4); lane 12 = Group 12 (*O. griseum*, UAMH 4080); lane 13 = Group 13 (*O. periconioides*, DAOM 19706); lane 14 = Group 14 (*P. roseus*, UAMH 1658)

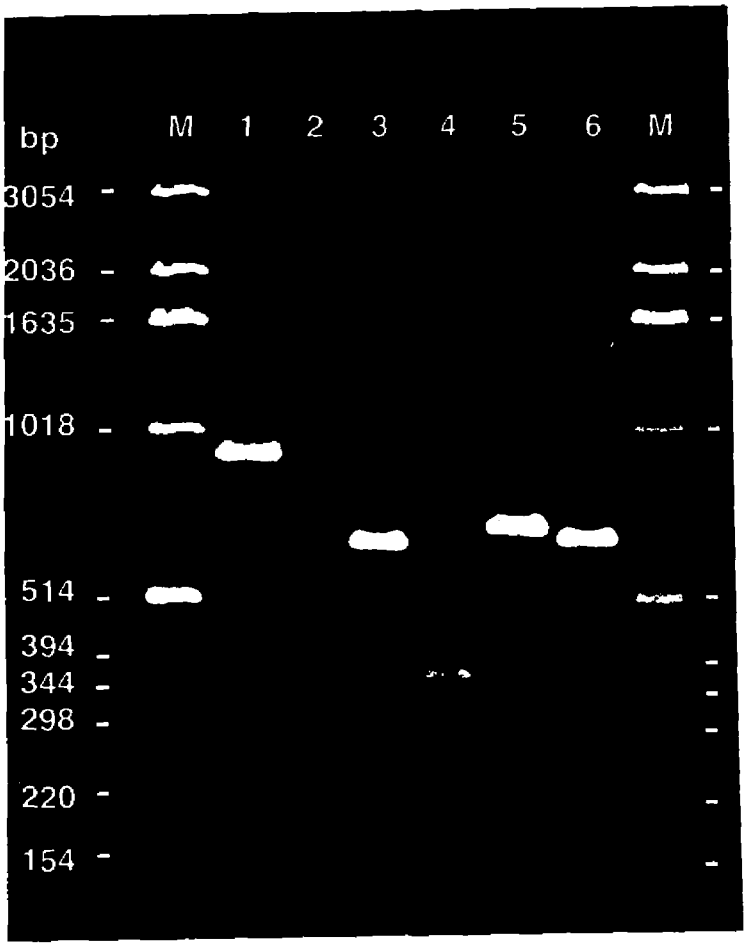

Figure 2.3 RFLP patterns of PCR products of fungal isolate **Unknown 3** (UBC M8, Group 15), using the restriction enzymes ***Alu* I, *Rsa* I, *Cfo* I** and ***Msp* I**. **M** = molecular fragment size marker; lane 1 = undigested fungal DNA; lane 2 = negative control (no DNA template); lane 3 = digest with ***Alu* I**; lane 4 = digest with ***Rsa* I**; lane 5 = digest with ***Cfo* I**; lane 6 = digest with ***Msp* I**.

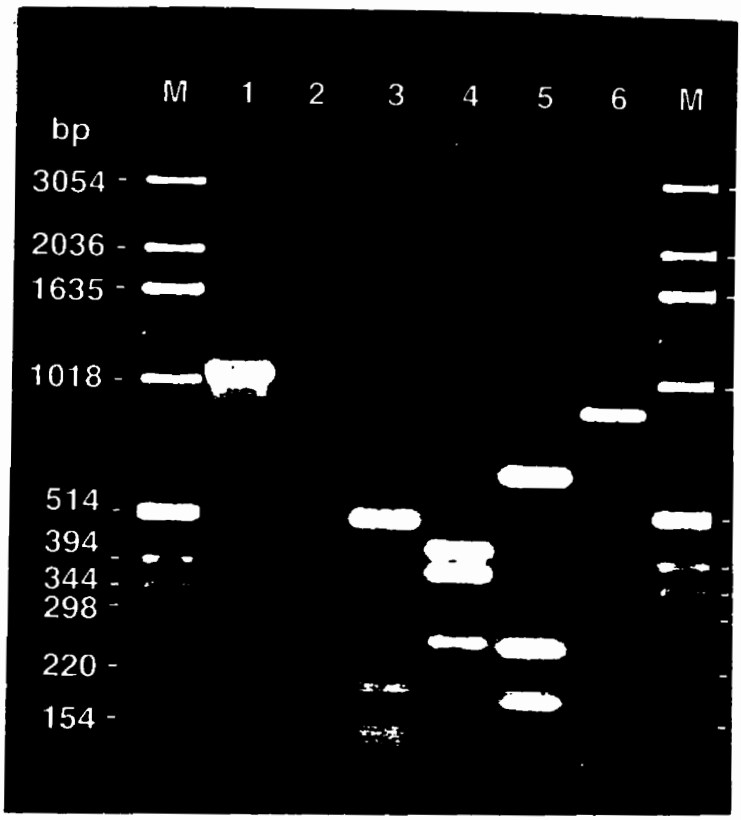

Figure 2.4 RFLP patterns of PCR products of fungal isolate **Unknown 4** (UBC M19 and M20, Group 16), using the restriction enzymes ***Alu* I, *Rsa* I, *Cfo* I** and ***Msp* I. M** = molecular fragment size marker; lane 1= undigested fungal DNA; lane 2 = negative control (no DNA template); lane 3 = digest with ***Alu* I**; lane 4 = digest with ***Rsa* I**; lane 5 = digest with ***Cfo* I**; lane 6 = digest with ***Msp* I**

The synoptic key including the restriction enzymes, the number and size of fragments produced, and the corresponding assignment to RFLP groups number are as follows:

***Msp* I:**

2 fragments of 920 and 115 (doublet) bp.....16

or 670 and 140 (doublet) bp.....15

or 485 and 125 bp.....1

or 460 and 130 bp.....7

or 450 and 180 bp.....8

or 260 and 170 (doublet) bp.....9, 13

3 fragments of 560, 205 and 125 (doublet) bp.....4, 5

or 560, 150 and 115 (doublet) bp.....6

or 385, 160 and 110 bp.....2

or 270, 145 and 135 bp.....11

4 fragments of 415, 220, 195 and 110 bp.....3

or 260, 150, 120 and 110 bp.....12

or 210, 180, 120 and 110 bp.....14

or 195, 160, 140 and 130 bp.....10

***Cfo* I:**

2 fragments of 220 and 180 (doublet) bp.....12

or 250 and 190 (doublet) bp.....1, 8, 14

or 260 and 160 (doublet) bp.....10

or 320 and 250 bp.....9

or 700 and 225 bp.....15

3 fragments of 345, 150, and 110 bp.....2, 7

or 490, 250 and 190 bp.....4

or 610, 240 and 180 bp.....16

or 360, 250 and 190 bp.....3

or 315, 250 and 205 bp.....5

or 740, 250 and 190 bp.....6

or 190 (doublet), 130 and 110 bp.....11, 13

4 fragments of 360, 250, 180 and 120 bp.....3

***Alu* I:**

1 fragment of 620 bp.....2

or 540 (doublet) bp.....3

or 590 bp..... 8, 10

2 fragments of 400 and 195 bp.....14

or 390 and 210 bp.....1, 9, 12, 13

or 480 and 150 bp.....7

or 450 and 150 bp.....11

or 670 and (175 doublet) bp.....15

or 740 and (150 doublet) bp.....5

or 740 and 118 (doublet) bp.....6

3 fragments of 580, 210 and 145 (doublet) bp.....4

4 fragments of 500, 190, 150 (doublet) and 125 bp.....16

***Rsa* I:**

1 fragment of 590 bp.....10

2 fragments of 360 and 240 bp.....1, 8

or 360 and (320 doublet) bp.....3

or 340 and 230 bp.....9, 14

or 450 and 210 bp.....2

or 450 and 150 bp.....11

3 fragments of 250, 360 and 425 bp.....16

or 240, 230, 150 bp.....7, 12, 13

or 210, 245 and 355 bp.....15

or 360, 290, and 240 bp.....4, 5, 6

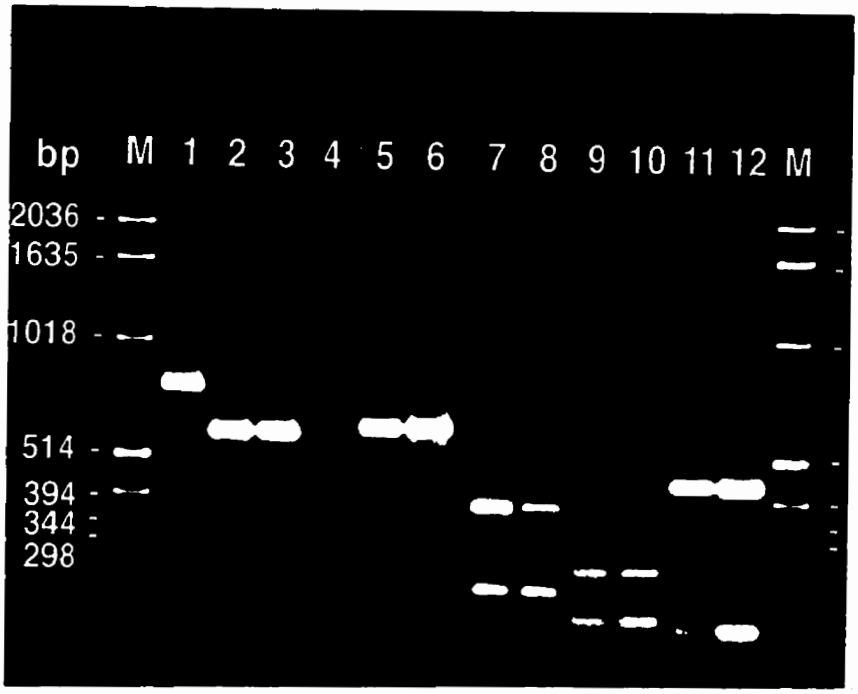

Figure 2.5 Identical RFLP patterns from synthesized ericoid mycorrhizae and from fungi in pure culture for isolate UBC S9 (**Unknown 1**). M = molecular fragment size marker; **1** Salal PCR product; **2** undigested fungal DNA; **3** undigested mycorrhizal DNA. Odd lanes 5-12 = fungal DNA; even lanes 5-12 = mycorrhizal DNA. Salal was amplified with universal primers ITS4 and ITS5. Fungal and mycorrhizal DNAs were amplified with fungus specific primer ITS1-F and ITS4. Restriction enzymes used for the digests were: 5, 6 = *Alu* I; 7, 8 = *Rsa* I; 9,10 = *Cfo* I; 11,12 = *Msp*I.

Table 2. 2 RFLP group number assigned to fungal species with the same RFLP pattern, fungal species and source of isolate, fragment length of PCR product and fragment length of digest produced by restriction enzymes ***Msp* I, *Cfo* I, *Alu* I, *Rsa* I** and ***Eco* R I.**

RFLP group number	Fungus species and isolate source	Total length of PCR products (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*Eco*R I
1	*Hymenoscyphus ericae*	590	485	250	390	360	345
	UAMH 6663		125	190 d	210	240	285
	Hymenoscyphus ericae	590					
	Read, D.J. 101, U.K.						
	Hymenoscyphus ericae	590					
	UAMH 6735						
2	*Phialocephala dimorphospora*	620	385	345	620	450	345
	UAMH 7527		160	150		210	285
			110	110			

Table 2 Continued.

RFLP group number	Fungus species and isolate source	Total length of PCR producs (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*Eco*R I
3	*Phialophora finlandia* UAMH 7454	980	415 220 195 110	360 250 190 d	540 d	360 320 d	660 285
4	*Hymenoscyphus ericae* UAMH 6598	980	560 205 125 d	490 250 190	580 210 145 d	360 290 240	660 285
	Scytalidium vaccinii UAMH 5828	980					
5	*Hymenoscyphus ericae* UAMH 6561	980	560 205 125 d	315 250 205 d	740 150 d	360 290 240	660 285

Table 2 Continued.

RFLP group number	Fungus species and isolate source	Total length of PCR producs (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*Eco*R I
6	*Hymenoscyphus ericae* UAMH 6045	980	560 150 115 d	740 250 190	740 118 d	360 290 240	660 285
7	*Phialocephala fortinii* UAMH 6815	590	460 130	345 150 110	480 150	240 230 150	345 285
8	**Unknown 2** UBC S246, UBC S203, UBC S226, UBCM5	590	450 180	250 190 d	590	360 240	345 285
9	**Unknown 1** UBC S9, UBC 222, UBC S245	590	260 170 d	320 250	390 210	340 230	325 285

Table 2 Continued.

RFLP group number	Fungus species and isolate source	Total length of PCR producs (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*Eco*R I
10	*Acremonium strictum* UBC S232, UBC S214, UBC S228	630	195 160 140 130	260 160 d	590	590	345 332
11	*Oidiodendron citrinum* UAMH 1525	590	270 145 135	190 d 130 110	450 150	450 150	345 285
	Oidiodendron griseum UBC S4, UBC S18, UBC S80	590					
	Oidiodendron maius UAMH 1540	590					

Table 2 Continued.

RFLP group number	Fungus species and isolate source	Total length of PCR produc (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*EcoR* I
11	*Oidiodendron sp.* UBC M10	590					
12	*Oidiodendron flavum* UAMH 1524	590	260 150 120 110	220 180 d	390 210	390 210	345 285
	Oidiodendron griseum UAMH 4080	590					
13	*Oidiodendron periconioides* DAOM 197506	590	260 170 d	190 d 130 110	390 210	240 230 150	ND
	Oidiodendron scytaloides UAMH 6521	590					

Table 2 Continued.

RFLP group number	Fungus species and isolate source	Total length of PCR producs (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
			Msp I	*Cfo* I	*Alu* I	*Rsa* I	*Eco*R I
14	*Pseudogymnoascus roseus* UAMH 1658	590	210 180 120 110	250 190 d	400 195	360 220	ND
15	**Unknown 3** UBC M8	980	670 140 d	700 225	670 175 d	355 245 210	ND
16	**Unknown 4** UBC M19, UBC M20	1100	920 115	610 240 180	500 190 150 d 125	425 360 250	ND

DAOM: Canadian National Collection of Fungus Cultures. Agriculture and Agri-Food Canada, Ottawa, Ont., Canada.
UAMH: University of Alberta Mold Herbarium and Culture Collection, Edmonton, Alberta, Canada.
UBC: Isolates of G. Xiao (1994) and M. Monreal (1994), Department of Soil Science, University of British Columbia, B.C., Canada.
ND: Not determined and **d:** possible doublet.

Synthesized salal mycorrhizae

All tested known ericoid mycorrhizal species formed synthetic mycorrhizae. Also, the known ectendomycorrhizal species *Phialophora finlandia* (UAMH 7454) formed the typical ericoid mycorrhizal morphology in salal roots. However, the non-mycorrhizal isolate of *Phialocephala fortinii* (UAMH 6815) formed intracellular sclerotia instead of the typical ericoid mycorrhizal coils (Fig 2.7 b).

Amplification and RFLP in DNAs from mycorrhizae

The fungal DNAs of synthesized ericaceous mycorrhizal roots were preferentially amplified over the salal DNA when the fungal specific primer ITS1-F (Gardes and Bruns, 1993) and the universal primer ITS4 were used. However, PCR products of salal DNAs were obtained when the universal primers, ITS5 and ITS4 (White *et al.*, 1990) were used. These primers produced a fragment of 780 bp for salal DNA, which was 200 bp longer than the fungal DNAs without inserts and about 200 shorter than the isolates with inserts (Fig. 2.6, lane marked **S**).

All the RFLP patterns generated from the PCR products of mycorrhizal DNAs were identical to the RFLP's generated by the pure isolates (Fig. 2.6). RFLP patterns produced by *Phialocephala fortinii* (UAMH 6815) were identical to the ones produced by digest of the PCR products of DNA extracts of the intracellular sclerotia (Fig 2.7b).

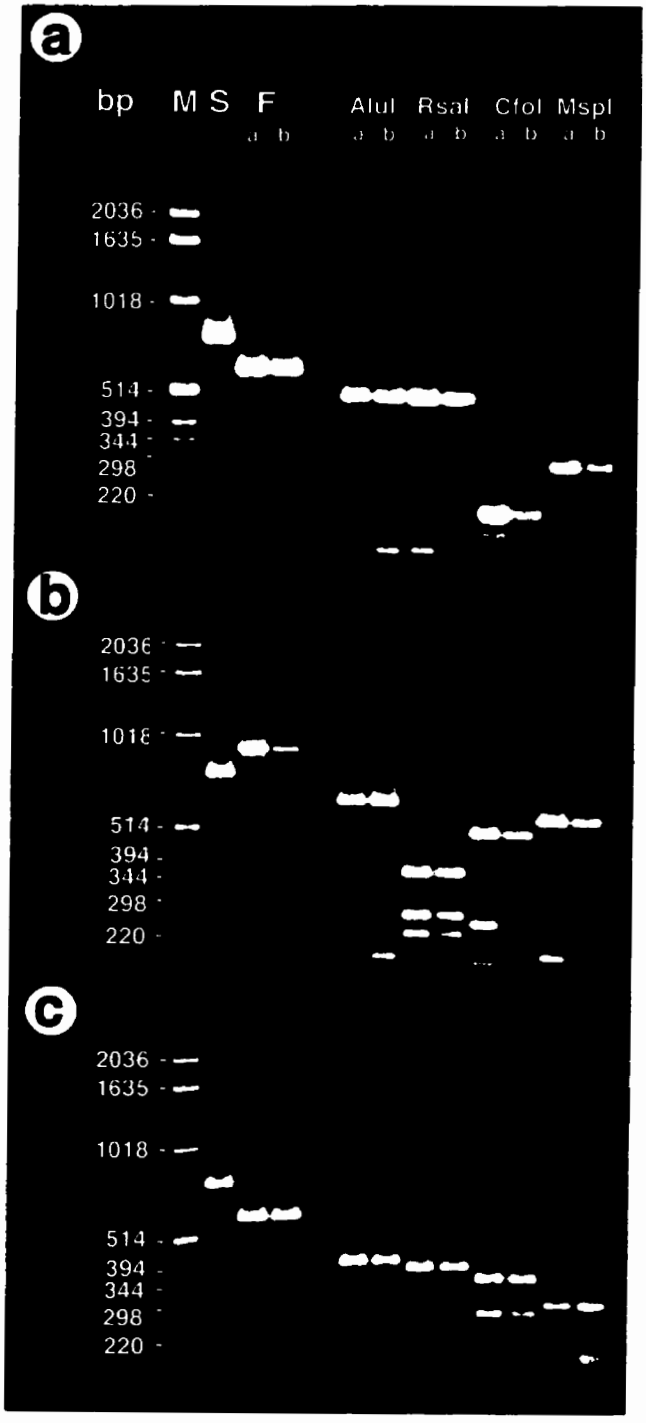

Figure 2.6 Identical RFLP patterns from synthesized ericoid mycorrhizae and from fungi in pure culture. Salal DNA was excluded from PCR amplification of mycorrhizae with a fungal specific primer. **S** = Salal PCR product amplified with universal primers; **F a** = undigested fungal DNA amplified with fungal-specific primer; **F b** = undigested mycorrhizal DNA amplified with a fungal-specific primer; under each enzyme name, **a** = digested fungal DNA; **b** = digested mycorrhizal DNA. **3a** = Group 11(*O. griseum*, UBC S4); **3b** = Group 4 (*S. vaccinii*, UAMH 5828); **3c** = Group 9 (**unknown 1**, UBC S9); Restriction enzymes were***Alu* I, *Rsa* I, *Cfo* I** and ***Msp* I**; **M** = molecular fragment size marker

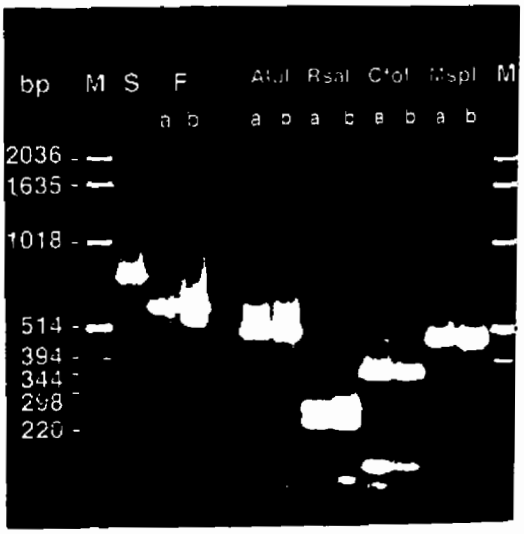

Figure 2.7a Identical RFLP patterns of the endophytic fungus *Phialocephala fortinii* (UAMH 6815) from pure culture and from dual cultures with salal. **S** = Salal PCR product amplified with universal primers ; **F a** = undigested PCR product of fungal DNA amplified with a fungal-specific primer. **F b** = PCR product from DNAs of salal roots with formation of intracellular sclerotia using a fungal-specific primer; **M** = molecular fragment size marker. Restriction enzymes used were ***Alu* I, *Rsa* I, *Cfo* I** and ***Msp* I**.

Figure 2.7b Cortical cells of salal roots showing intracellular sclerotia (500x).

RFLP of mycorrhizal salal sampled in the field via direct DNA extraction

PCR amplification of the ITS regions from field-collected mycorrhizae using ITS4 and the fungal specific primer ITS1-F produced multiple, faint bands varying between 580 and 1000 bp. The initial PCR product was agarose purified and single bands from each sample were selected for re-amplification. Re-amplification produced a single, bright band for some DNAs. However, restriction digests of the re-amplified products yielded complex, dim patterns (Fig. 2.8). Since the combined sizes of the restriction fragments exceeded the initial size of the PCR product, at least two different DNAs must have been present in each amplification.

In an attempt to amplify a single fungal ITS region out of the mixed DNAs, the initial PCR product was serially diluted 10, 100, 1000, 10,000 and a 1,000,000 fold. Each of the dilutions served as template for PCR re-amplification. The re-amplified PCR products were 580 bp. long, indicating that a fungus DNA, not the salal DNA had been amplified (Fig. 2.9). However, even after a 1,000,000 fold dilution, the RFLP pattern showed too many bands to be consistent with the digestion of a single fungal DNA (Fig. 2.9).

RFLP of fungi in pure culture isolated from field ericoid mycorrhizal roots

In addition to direct amplification of DNAs of ericoid mycorrhizal root fragments subsampled from a single rhizome, a second set of molecular data was obtained from 20 pure fungal isolates obtained from sixty 3 mm root fragments. The new set of isolates generated 11 different RFLP patterns using the restriction

enzymes *Alu* I, *Rsa* I, *Cfo* I, and *Msp* I (Fig. 2.10). Synthesis experiments confirmed the capacity to form mycorrhizae of isolates UBC M5, UBC M8, UBC M10, UBC M19 and UBC M20. Again, the enzyme *Msp* I proved to be a good diagnostic enzyme distinguishing two known ericoid mycorrhizal RFLP patterns, Group 11 (Table 2.2) (*Oidiodendron* species, isolate UBC M10) and Group 8 (Table 2.2) (**Unknown 2**, isolate UBC M5). Three new sterile isolates generated two new RFLP patterns and they were included in the RFLP synoptic key as Groups 15 (isolate UBC M8) and 16 (isolate UBC M20). Isolate UBC M19 had similar RFLP pattern and colony characteristics to isolate UBC M20. The isolate UBC M19 formed ericoid mycorrhiza with salal under axenic conditions, however it was tested once, compared to two consecutive tests performed with isolate UBC M20 (Table 2.2). The rest of the isolates, a total of 15, produced seven new RFLP patterns, but failed to form ericoid mycorrhizae.

Discussion

PCR products of the isolates

Fragment length of PCR products of most of the isolates in this study were similar within species, with only small variation between genera, 40 bp at the most (Fig. 2.1). However, a set of North American isolates including pre-existing isolates of *H. ericae, S. vaccinii, P. finlandia,* and the new isolates UBC M8, UBC M19 and UBC M20 produced PCR products between 390 and 510 bp longer than the rest of the isolates (Fig. 2.1). In a study on ectomycorrhizal fungi, Gardes *et al.* (1991) used the primers ITS1 and ITS4 to amplify the entire ITS region. They

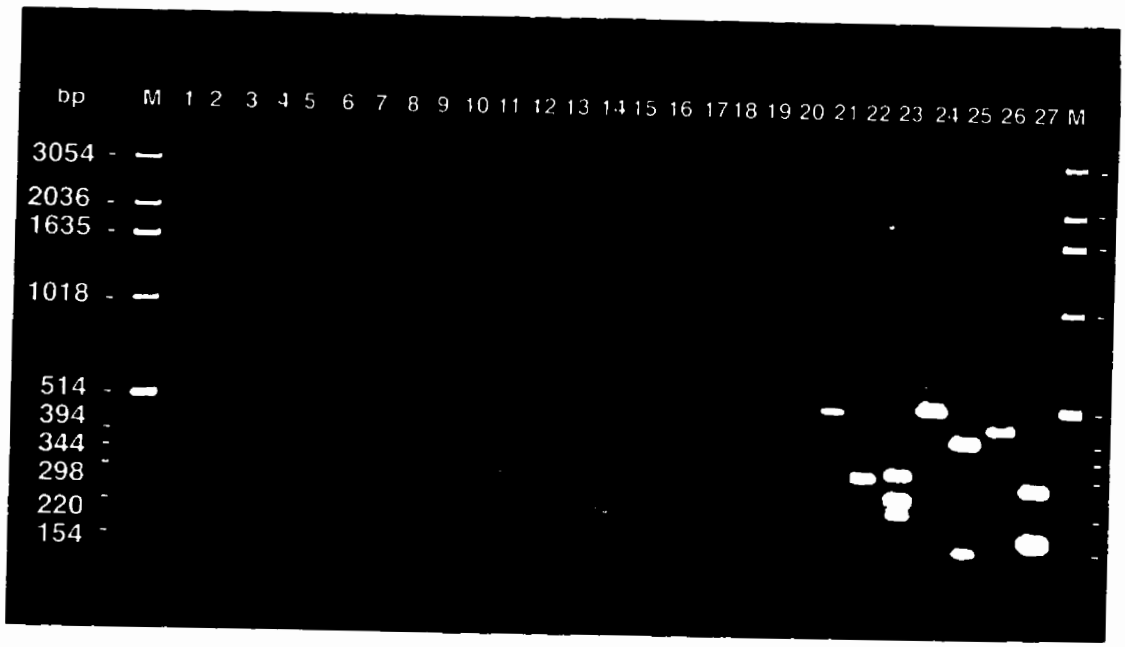

Figure 2.8 Comparison of dim, complex RFLP patterns from field collected ericoid mycorrhizae (Lanes 1 to 21) with bright, clear RFLP patterns from pure salal DNA (lane 23) and from pure fungal DNA (lanes 22, *O. griseum* (UBC S4); lane 24, *H. ericae* (UAMH 6735); lane 25, *P. dimorphospora* (UAMH 7527); lane 26, **Unknown 2** (UBC S246); lane 27, **Unknown 1** (UBC S9)). The universal primers ITS4 and ITS5 were used to amplify salal DNA. For the other samples, fungal-specific primer ITS1-F was used in the PCR amplifications, and PCR products were digested with *Msp I*.

Figure 2.9 Restriction digests of PCR product from field collected ericoid mycorrhizal DNAs show complex banding patterns (7-10, 13 -14) even after several serial dilutions of template DNA. DNA extracted from field-collected mycorrhizae was PCR amplified once and the product was diluted, re-amplified, and digested. Lanes 3-6 show the bright undigested PCR bands from a single, re-amplified mycorrhizal DNA. Templates for the reactions in lanes 3-6 were 10, 1000, 10,000 and 1,000,000 fold dilutions of the initial PCR product, respectively and digests of these mycorrhizal DNAs are in lanes 7-10, respectively. Templates for reactions in lanes 11 and 12, were respectively, 1000 and 10,000 fold dilutions of a second reamplified mycorrhizal sample, with their digests in lanes 13 and 14. Lanes 15 and 16 show digests of pure fungal DNAs that were the closest known matches to patterns in 13 and 14. Note that the field mycorrhizae produced more RFLP bands than the single fungal DNAs. Lanes M = molecular fragment size marker; 1 = undigested salal; 2 = negative control; 15-17, restriction digests; 15 = *O. griseum*, UBC S4; 16 = *P. roseus* UAMH 1658; 17 = digested salal DNA. *Msp* I was used in all digests.

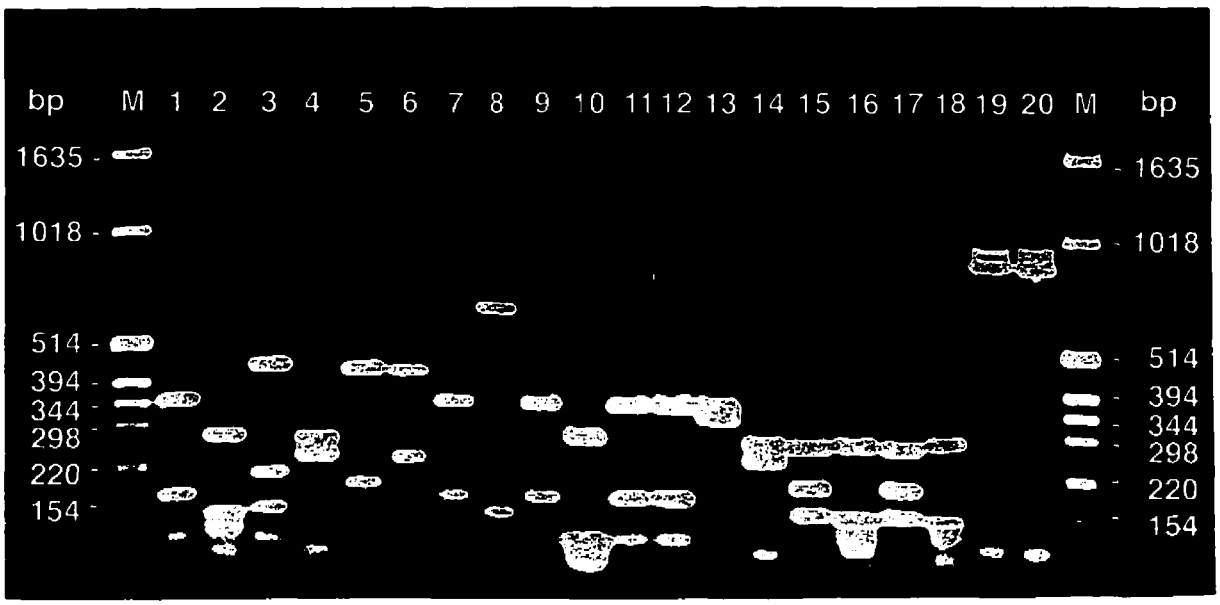

Figure 2.10 Eleven different RFLP patterns from 20 fungal isolates from mycorrhizae of a single salal rhizome (lanes 1-20). Of the twenty, five formed mycorrhizae with salal in vitro . Mycorrhizal isolates include four sterile, unidentifiable isolates: lane 5, **Unknown 2** (UBC M5); lane 8, **Unknown 3** (UBC M8); lanes 19, 20, **Unknown 4** (UBC M19 and UBC M20). One of the mycorrhizal isolates sporulated: lane 10, *Oidiodendron* sp. (UBC M10). **M** = molecular fragment size marker.

found that PCR products of ITS region varied in length between 600-800 bp for fungi of different genera. However, length of PCR products of the ITS region was approximately the same among 20 isolates belonging to four species of the genus *Laccaria*.

RFLP synoptic key of known ericoid mycorrhizal fungi

Known ericoid mycorrhizal fungal isolates from the SCHIRP site can be identified by RFLP patterns generated with four restriction enzymes. RFLP patterns of Groups 8 (**Unknown 2**), 9 (**Unknown 1**), 10 (*Acremonium strictum*), 11 (*Oidiodendron griseum*), 15 (**Unknown 3**), and 16 (**Unknown 4**) coincided with four morphological groups of fungal isolates obtained by Xiao (1994) and two additional new groups obtained by Monreal. Interestingly, new isolates UBC M8 and UBC M20 represent previously unreported ericoid mycorrhizal fungi for the extensively-surveyed SCHIRP site (Xiao, 1994). Because the RFLP patterns of sterile isolates from the SCHIRP site (**Unknown 1, Unknown 2, Unknown 3,** and **Unknown 4**) differed from the patterns of all known mycorrhizal species, they remain unidentified species.

Six species of the genus *Oidiodendron* generated only three groups of RFLP patterns (Table 2.2), indicating either that this molecular method is not specific enough to differentiate between the species of this genus or that there are taxonomic problems at the species level. Isolates of *Oidiodendron griseum* from the SCHIRP site (UBC S4, UBC S18, and UBC S80) had the same pattern as two other species, *O. citrinum* (UAMH 1525) and *O. maius* (UAMH 1540) (RFLP

Group 11), while *O. scytaloides* (UAMH 6521) and *O. periconioides* (DAOM 197506) shared the same RFLP pattern (RFLP Group 13). However, an *O. griseum* isolate (UAMH 4080) from Alberta, Canada had the same RFLP pattern (RFLP Group 12) as an isolate of *O. flavum* (UAMH 1524), from Ontario, Canada and a different pattern than other *O. griseum* isolates (RFLP Group 11). The isolate UAMH 4080 was probably misidentified (Sigler, personal communication). Detailed taxonomic study of species and isolates in *Oidiodendron*, including observations of colony morphology and asexual structures, could help to clarify these molecular findings.

In contrast to the uniformity of RFLP patterns among *Oidiodendron* species, isolates of the species *Hymenoscyphus ericae* segregated into four RFLP patterns (RFLP Groups 1, 4, 5, and 6) (Table 2.2). Either *H. ericae* is polymorphic or isolates are misidentified. Supporting the hypothesis that *H. ericae* amplified ITS regions are polymorphic, Egger and Sigler (1993) found insertions in the ribosomal RNA genes of most of the North American isolates with the exception of only one isolate (UAMH 6663) that segregates into RFLP pattern Group 1 along with two European isolates (UAMH 6735 and Read, D.J. 101). Less probably, the PCR product size difference may indicate taxonomic heterogeneity among *Hymenoscyphus ericae* isolates. Egger *et al.* (1995) demonstrated that one isolate, UAMH 6562, had a large putative group I intron in the 5' end of the 18S gene. This intron would be included in the DNA region I amplified with primers ITS1-F and ITS4. Introns can be lost or gained through horizontal transfer and the presence or absence of the intron could explain the length variation among

strains of *H. ericae* . Intron sequences evolve very quickly, and changes in intron sequence could explain the diversity of RFLP patterns found in H. ericae isolates. The North American isolates of *H. ericae* have been identified based only on colony type, the formation of arthroconidia (schizolitic dehiscence) forming zigzag chains, and their capacity to form mycorrhizae with various Ericales (Egger and Sigler, 1993; Litten *et al.*, 1995; Perotto *et al.*, 1990). In contrast, the European isolate of *H. ericae* (Holotypus IMI 182065) produced sexual fruiting structures, small apothecia (1 mm diameter) when seedlings of *Calluna vulgaris* L. Hull. were grown and inoculated with fungus in soil sterilized by gamma irradiation (Read, 1974). Also in France, ericoid mycorrhizal fungi obtained from *Rhododendron* and *Erica* produced sterile mycelia and apothecia under controlled laboratory conditions, similar to the ones produced by the English isolates *H. ericae* (Vegh *et al.*, 1979). However, apothecia have never been observed in North American isolates of *H. ericae*, either in the field or under culture and so isolate identification remains tentative.

The RFLP patterns of endophytic species *Phialocephala fortinii* (UAMH 6815) and *Phialocephala dimorphospora* (UAMH 7527), Groups 2 and 7 respectively, were different than all the ones produced by the ericoid mycorrhizal fungi of this study. Since these endophytic species generate distinct RFLP patterns, they can be separated from ericoid mycorrhizal fungi even though they also inhabit ericaceous roots. *Phialocephala fortinii* forms intracellular sclerotia in the cortical cells of salal roots rather than hyphal coils (Fig 2.7b). It also forms sclerotia on roots of *Menziesia ferruginea* (Ericaceae) (Stoyke and Currah, 1991)

and in *Lupinus latifolius* (Leguminosae) (O'Dell *et al.*, 1993). As reported in previous studies *P. fortinii* it is clearly not a typical ericoid mycorrhizal fungus, though it could easily be detected in roots. *P. fortinii* is associated with roots of subalpine dwarf shrub heath, including plants of the Ericaceae (Stoyke *et al.*, 1992).

RFLP identification of in vitro salal mycorrhizae

DNA extracted from in vitro mycorrhizae and amplified with a fungal specific primer combination produced clear, bright RFLP patterns (Fig. 2.5, 2.6). Patterns from the mycorrhizae were identical to those produced by DNA digests of fungi grown in pure culture (Fig. 2.5, 2.6). These tests were necessary since mycorrhizae, composed of plant and fungal cells, have mixed DNAs. Furthermore, the fungal specific primer (ITS1-F) had been reported to exclude DNA amplification for some, but not of all, plant species (Gardes and Bruns, 1993). This was the first time the primer ITS 1-F was tested with salal plants and salal DNA was clearly not amplified (Fig. 2.5, 2.6).

Limits to direct amplification and RFLP identification of fungi from field-collected mycorrhizae.

Complex RFLPs resulted from digests of DNA amplified from field collected mycorrhizae, suggesting that more than one fungus was present in each 5 mm root segment (Fig. 2.8). That the complex patterns indicated the presence of organisms other than fungi cannot be ruled out. However, several pieces of

evidence indicate that DNAs from fungi predominated. Roots were initially washed, eliminating most of the organisms clinging to their outside surfaces. Before extracting DNA, each root was examined microscopically. Since the hair root fragments were only about 2 mm in diameter and since the cortical cells are clear (for example, see Fig. 2.7b), many of the cells were observed directly. No contaminating organisms were seen. Bacteria were certainly present but they lack the ITS spacer regions that that would have been amplified with our primers. Plant DNA was excluded from amplification with a fungus-specific primer, ITS1-F. Confirming that salal DNA was not amplified, the size of the PCR bands from the mycorrhizae, about 600 bp (Fig. 2.9), were significantly smaller than the bands produced by amplified salal (Fig. 2.9). The 600 bp PCR band size is common among ascomycetous fungi (Fig. 2.1).

The diversity of fungi cultured from roots collected at the same site corroborates the results from direct amplification. Twenty different fungi, five of which formed ericoid mycorrhizae in vitro were isolated from sixty segments, aproximately 3 mm each, of the fine roots of an 8 cm salal rhizome. Finding high diversity is also consistent with reports of very diverse populations of mycorrhizal and non-mycorrhizal endophytes in individual root systems of *Calluna vulgaris* that were collected on natural heathlands in northern Italy (Perotto *et al.*, 1994).

The diversity of ericoid mycorrhizal fungi on a small scale complicates fungal identification following direct PCR amplification. This contrasts with the situation in ectomycorrhizae, where a single fungus can usually be amplified

from a single root (Gardes and Bruns, 1993). Possible approaches to untangling the diversity include PCR amplification followed by cloning to separate the different DNAs; culturing of fungi to obtain pure fungal cultures; or as discussed in CH. 3, design of specific primers that will selectively amplify a specific, known ericoid mycorrhizal fungus from a root.

Testing the synoptic key

Listed first in the synoptic key, the restriction enzyme ***Msp* I** generated the greatest number and most varied lengths of fragments among the isolates studied, including 14 RFLP groups out of a total of 16. Therefore, ***Msp* I** is recommended to be used first as a diagnostic enzyme to study new ericoid mycorrhizal isolates, followed in order by ***Cfo* I**, ***Rsa* I** and ***Alu* I**. Afterwards, use the RFLP synoptic key to check if fragment sizes correspond to patterns of known isolates. If the isolate fits a known RFLP pattern, the next step would be to confirm identity with morphological characteristics and synthesis of mycorrhiza on salal or other Ericaceae. In a further step, amplified DNA fragments of new isolates, including ITS1, the 5.8S rRNA region, and ITS2 could be sequenced and results compared with available sequence data (GenBank).

The RFLP synoptic key proved to be useful to screen out the known ericoid mycorrhizal fungi UBC M5 (Group 8) and UBC M10 (Group 11) from the 20 new isolates from the SCHIRP site. The listing of known RFLP patterns should allow faster screening of large number of isolates with efficient use of manpower and laboratory resources. Cultured new isolates matching known

mycorrhizal RFLP patterns can be speedily selected, and their ability to form mycorrhizae confirmed through inoculation of host plants. The synoptic key is not complete. Out of 20 fungal cultures, three new ericoid mycorrhizal fungi were detected with new RFLP patterns, indicating that even well-studied sites are likely to have additional unknown species.

A total of fifteen isolates which grouped under 7 new RFLP patterns failed to form mycorrhizae with salal plants. Because no one knows how many fungi might associate with salal roots, the mycorrhizal potential of new isolates with unknown RFLP patterns should be tested. However, laboratory conditions used to confirm synthesis of mycorrhiza with new isolates may not be ideal for all taxa, and even fungi that do not form mycorrhizae in vitro may be mycorrhizal in nature. Further work developing new conditions for ericoid mycorrhizal synthesis would help to sort out the role of fungi associated with salal roots.

The direct use of PCR and RFLP techniques for field ericoid mycorrhiza proved to have a limited application. Although suggesting the presence of a large diversity of fungi in salal roots, their taxonomic classification could not be confirmed. In order to detect known taxonomic groups of ericoid mycorrhizae from roots sampled in the field, the second part of this research concentrated on more specific molecular technique, the design of specific primers, based on DNA sequences of the ITS2 region of the ribosomal repeat genes.

Chapter 3

Taxon Specific Primers to Identify Ericoid Mycorrhizal Fungi

Abstract

In order to create a method that would detect the presence of known ericoid mycorrhizal fungi within a mixed fungal population, the internal transcribed spacer 2 (ITS2) of 27 fungal isolates was amplified and sequenced. Analysis included sequence data of nine fungi from GenBank. Sequence data analysis segregated the mycorrhizal isolates into two main groups; the ***Oidiodendron* group** and the ***Hymenoscyphus* group**. To distinguish three commonly encountered mycorrhizal isolates from each other and from other non-mycorrhizal fungi, three different specific primers were designed. The new primers anneal to the 3′ end of the ITS2 region, approximately 45 bp upstream of the 5′ end of the 28S rRNA gene. For PCR reactions the new primers were paired with the fungal specific primer ITS1-F to anneal to the 3′ end of the 18S rRNA gene. Tests performed using the new primers with fungal DNA mixtures of known mycorrhizal isolates and non-mycorrhizal fungal isolates detected only the targeted fungal DNA.

Introduction

The taxonomic identification of fungal symbionts of ericoid mycorrhizae cannot be done *in planta* since the morphology of fungus-root associations formed by different fungal species are alike under microscopic examination, showing the typical presence of hyphal coils in the cortical cells of the plant's fine roots. The use of traditional culturing techniques to identify isolates obtained from ericoid mycorrhizae limits taxonomic classification to the species forming sexual or asexual reproductive structures. Apothecial formation confirmed the perfect state of the ericoid mycorrhizal species *Hymenoscyphus ericae* (Read) Korf and Kernan, and formation of conidiophores and conidia confirm identity for species of *Oidiodendron* (Couture *et al.*, 1983). However, the taxonomy of several sterile mycelia of ericoid mycorrhizal fungi isolated over the years remains unresolved (Haselwandter 1987, Perotto *et al.*, 1994, 1995; Stoyke and Currah, 1991; Xiao, 1994).

Using cultural and morphological colony characteristics Xiao (1994) found two new sets of sterile ericoid mycorrhizal isolates and named them **Unknown 1** and **Unknown 2**. Xiao (1994) indicated that colony morphology of **Unknown 1** isolates did not resemble any of the known ericoid mycorrhizal species, although **Unknown 2** isolates resembled the species *Hymenoscyphus ericae*. However, Xiao (1994) could not confirm this hypothesis since the isolates remained sterile. In Chapter 2, Monreal described an additional two fungi, **Unknown 3** and **4**, distinguished from each other and from previous unknowns by RFLP patterns.

Several studies of morphology of intracellular hyphae of ericoid

mycorrhizae showing simple septa with the presence of Woronin bodies suggest that the endophytes are Ascomycetes (Pearson and Read, 1973; Bonfante-Fasolo and Gianinazzi-Pearson, 1979).

Known ericoid mycorrhizal fungi are taxonomically diverse and include the species *Hymenoscyphus ericae* (Read) Korf & Kernan (Read, 1974) in the Leotiales, *Myxotricum setosum* (Eidam) Orr, Kuehn & Plunkett, and *Gymnascella dankaliensis* (Castellani) Currah in the Onygenales (Dalpé, 1989), and hyphomycetes belonging to *Stephanosporium, Scytalidium, Acremonium,* and *Oidiodendron.* Several *Oidiodendron* species form mycorrhizae in pure culture, including *O. griseum* Robak, *O. citrinum* Barron, *O. chlamydosporicum* Morrall, *O. maius* Barron, *O. flavum* Szilvinyi amend. Barron, *O. rhodogenum* Robak, and *O. scytaloides* Gams & Soderstrom (Couture *et al.*, 1983; Dalpé, 1986; Dalpé, 1991; Xiao, 1994).

Some studies suggest that a basidiomycete of the genus *Clavaria* may also form ericoid mycorrhizae (Englander and Hull, 1980; Peterson *et al.*, 1980; Mueller *et al.*, 1986). Also, observations of colonized cells indicated the presence of basidiomycetes in *Calluna* and *Rhododendron* (Bonfante-Fasolo, 1980; Peterson *et al.*, 1980; Mueller *et al.*, 1986). Up to the present, however synthesis experiments with fungi of the genus *Clavaria* have failed (Read, 1983; Mueller *et al.*, 1986); for example, *Rhododendron* plants inoculated with *Clavaria argillacea* failed to form mycorrhizae (Read, 1983).

If the ITS regions of the ribosomal genes of ericoid mycorrhizae can be preferentially amplified from field-collected roots using fungal-specific primers

and PCR, then the mycorrhizal fungi could be identified by RFLP or sequence analysis. Specific primers and PCR-based detection have been used successfully for fungi. The internal transcribed spacer regions ITS1 and ITS2 of the ribosomal genes of DNAs from ectomycorrhizae and rust-infected tissues were preferentially PCR amplified using the fungus specific primer ITS1-F and the basidiomycete-specific primer ITS4-B (Gardes and Bruns, 1993). Gardes and Bruns (1993) study showed that the fungal components in ectomycorrhizal roots and rust-infected tissue were amplified efficiently using basidiomycete-specific primers and that the primers did not amplify ascomycete DNAs. When tested with 15 plant DNAs, the primers produced some PCR product in some of the plant species. Nevertheless, when fungus and plant DNA were present, the fungal DNA was amplified preferentially over plant DNA.

In Chapter 2 of this thesis the fungus specific primer ITS1-F and the universal primer ITS4 were used to exclude plant DNA while amplifying DNA from in vitro synthesized ericoid mycorrhizae. The RFLP patterns of PCR products differentiated among the fungal isolates. However, when the fungal-specific set of primers was used to amplify directly from field-collected ericoid mycorrhizae, more than one type of band was amplified. The mixed DNAs in the amplification suggested that more than one fungus may be present in close proximity in the mycorrhizae of salal roots in nature. Another obstacle in obtaining a single PCR product and clear RFLPs from DNAs of ericoid mycorrhizal roots as compared to ectomycorrhizae, might be the small amount of total fungal biomass available from colonized salal roots as compared to the

large amount of fungal biomass in ectomycorrhizae. In ectomycorrhizae the fungal biomass is collected from the mantle while the ericaceous mycorrhizal fungi present in salal roots are limited to the outer layer of cortical cells (Xiao, 1994). Another factor in favor of RFLP analysis of ectomycorrhizae was that both primers used to perform PCR on DNA were fungal specific primers; one was ITS1-F and the second was the basidiomycete-specific primer ITS4-B (Gardes and Bruns, 1993). Although RFLP analysis is a good molecular technique to differentiate among ericoid mycorrhizal isolates in pure culture, it does not distinguish field-collected ericoid mycorrhizal fungi from other root-associated fungi and therefore molecular techniques tailored to specific detection of known ericoid mycorrhizal fungi are needed.

Strategy for design of primers for specific amplification of ericoid mycorrhizal fungi.

In order to create a set of primers for ericoid mycorrhizal fungi, sequence data for the ITS2 region of a representative selection of known ericoid mycorrhizal fungi was needed. Having an alignment for the representative fungi would permit selection of sequence regions unique to each target fungus. Specific primers about 19 bp and complementary to the unique regions could then be designed. Ideally, these selective primers should anneal near the 3′ end of the ITS2 region, so that the combination of the specific primer and a fungal-specific primer (ITS1-F, Gardes and Bruns 1993) located at the 3′ end of the 18S gene would amplify the complete ITS1 region and most of the ITS2 region. That way,

either RFLP analysis or sequencing of the PCR product would permit verification that the target fungus had been amplified.

One objective of this research was to design a set of taxon-specific ericoid mycorrhizal primers, to be used concurrently with traditional culturing techniques, that would allow expeditious surveys of ericoid mycorrhizal fungi. A second objective was to use sequence data obtained in this study to explore phylogenetic relationships between the taxa studied.

Materials and Methods

Fungal isolates and DNA extraction

In this study, 27 fungal isolates were grown for three weeks at 25 C in the dark on modified Melin Norkrans agar (MMN)(Marx, 1969). After three weeks, DNA was extracted from each fungal isolate (Table 3.1) from mycelium scraped from the edge of the fungal colony (Lee and Taylor, 1990).

PCR amplification of DNA extracted from cultured fungal isolates

Using PCR (Mullis and Faloona, 1987), the region that contains the ITS1, 5.8S rRNA, and the ITS2 was amplified. A fungal specific primer, ITS1-F, was used for the region flanking the ITS1 spacer (Gardes and Bruns, 1993). A universal primer, ITS4 was used to flank the ITS2 region (White *et al.*, 1990).

PCR conditions were a denaturation step at 95 C for 2 min., followed by 30 cycles of denaturation at 95 C for 1 min., annealing at 48 C for 1 min., and an initial extension at 72C for 45 sec, with the extension time increased by 4 sec per

Table 3.1 Fungal isolates numbers used in the phylogram (Fig. 3.4), taxon name, taxon source, mycorrhizal status, and if applicable, the GenBank number.

Taxon number		Source of isolate	Mycorrhizal status	GenBank number
1	*Acremonium strictum*	UBC S232	+	
17	*Cladosporium fulvum*			L25430
36	*Fusarium sambucinum*			X65478
26	*Hymenoscyphus ericae*	READ,D. J. 101	+	
29	*Hymenoscyphus ericae*	UAMH 6735	+	
5	*Myxotrichum setosum*	UAMH 3835	+	
35	*Neurospora crassa*			M13906
3	*Oidiodendron chlamydosporicum*	UAMH 6520	+	
7	*Oidiodendron citrinum*	UAMH 1525	+	
12	*Oidiodendron flavum*	UAMH 1524	+	
14	*Oidiodendron griseum*	UAMH 4080	+	
9	*Oidiodendron griseum*	UBC S4	+	
8	*Oidiodendron griseum*	UBC S18	+	

Table 3.1 Continued

6	*Oidiodendron griseum*	UBC S80	+	
11	*Oidiodendron like sp.*	UBC M10	+	
10	*Oidiodendron maius*	UAMH 1540	+	
13	*Oidiodendron periconioides*	DAOM 197506	+	
15	*Oidiodendron rhodogenum*	UAMH 1405	+	
4	*Oidiodendron scytaloides*	UAMH 6521	+	
19	*Penicillium dendritricum*			L14502
25	*Phialocephala dimorphospora*	UAMH 7527		
23	*Phialocephala fortinii*	UAMH 6815		
24	*Phialocephala fortinii*	UAMH 6816		
31	*Phialophora finlandia*	UAMH 7454	+	
2	*Phialophora-like sp.*			X62991
16	*Pseudogymnoascus roseus*	UAMH 1658	+	
21	*Sclerotinia sclerotiorum*			M96382
22	*Sclerotinia trifoliorum*			U01218
30	*Scytalidium vaccinii*	UAMH 5828	+	

Table 3.1 Continued

18	*Talaromyces wortmannii*			L14507
20	*Thermomyces lanuginosus*			J02745
32	Unknown 1	UBC S9	+	
33	Unknown 2	UBC S246	+	
28	Unknown 4	UBC M20	+	
27	Unknown 3	UBC M8	+	
34	Unknown 5	UBC M5	+	

• GeneBank identification number of the isolate.

DAOM Canadian National Collection of Fungus Cultures. Agriculture and Agri-Food Canada, Central Experimental Centre, Ottawa, Ontario, Canada.

UAMH University of Alberta Mold Herbarium and Culture Collection, Edmonton, Alberta, Canada.

UBC Isolates obtained by G. Xiao (Ph.D. Thesis) preceded by letter S, Department of Soil Science, University of British Columbia, B.C., Canada.

UBC Isolates obtained by M. Monreal (Ph.D. Thesis) preceded by letter M, Department of Soil Science, University of British Columbia, B.C., Canada.

cycle. The final extension step was 10 min. To test for contamination, negative controls were used in every PCR reaction. PCR products were agarose gel purified. Purified product was reamplified with the same primers to increase amount of product.

DNA Sequencing

Sequences of the ITS2 region, including a portion of the 5.8 S gene, were determined in both directions by directly sequencing double-stranded PCR products with primers ITS3 and ITS4 (White *et al.*, 1990). Cycle sequencing reactions were carried out following Applied Biosystems (Mississauga, Ontario) instructions, using their PRISM™ TaqDyeDeoxy TM Terminator cycle sequencing kit. Following sequencing, excess dideoxy terminators were removed using Centri-Sep™ columns (Princeton Separations Inc., Adelphia NJ). Product was analyzed on an Applied Biosystems 373A DNA sequencer.

Analysis of DNA sequences

Assembly of sequences from opposite strands was carried out using the program CAP2 (Xiaoqiu, 1991). In addition, ITS2 sequences of 9 fungal taxa from GenBank were included in the alignment (Table 3.1). DNA sequences were aligned with the program Seqapp version 1.9a169 (D.G. Gilbert, c. 1993) and manually edited.

Gaps introduced to improve the alignment were coded as missing data and excluded from the phylogenetic analysis (Fig. 3.1). Characters that were

Figure 3.1 Sequence data of fungal taxa used in phylogenetic analysis. The underlined sequence regions are complementary to new primer sequences. Taxon names are abbreviated with the first three letters of the genus and the last four letters of the specific epithet, or if the fungus is unidentified, isolate numbers are given. Full names are given in Table 3.1. Lower case nucleotides were considered unalignable and were treated as missing and excluded from the analysis. Missing data are indicated by "N".

```
FUS.SAM.FSITSB      AAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCGCCAGTATTCT GGCGGGCATGCCTGTTCGAG
PHI.-LIKE.PSP58SRNA NNNNNNNNNNNNNNNNNNN NNNNNNNNNTTCAGTGAAT CATCGAATCTTTGAACGCAT ATTGCGCCCTCTGGTATTCC GGGGGGCATGCCTGTTCGAG
NEU.CRA.NEURGITR    GAAATGCGATAGGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCTCGCCAGTATTCT GGCGAGCATGCCTGTTCGAG
CLA.FUL.CLSRGB      GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCGTGGTATTCC GCGGGGCATGCCTGTTCGAG
TAl.WOR.TALRR58SJ   GAAATGCGATAAGTAATGTG AATTGCAGAATTCCGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCCTGGCATTCC GGGGGGCATGCCTGTCCGAG
PEN.DEN.PECRR58S    GAAATGCGATAAGTAATGTG AATTGCAGAATTCCGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCCTGGCATTCC GGGGGGCATGCCTGTCCGAG
SCL.SCL.SCEITSA     GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GGGGGGCATGCCTGTTCGAG
Scl.TRI.U01218      GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GGGGGGCATGCCTGTTCGAG
THE.LANU.TLAIRS     GAAATGCGATAAGTAATGTG AATTGCAGAATTCCGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCTCTGGTATTCC GGGGGGCATGCCTGTCCGAG
OID. CHLA           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. SCYT           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
MYX. SETO           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. PERI           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID.GRI.UAMH4080    GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. RHOD           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. CITR           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. GRI.UBCS80     GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. GRI.UBCS18     GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. MAIU           GAAATGCGATAAGTAATGCG GATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID.-LIKE.UBCM10    GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. GRI.UBCS4      GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
OID. FLAV           GAAATGCGATAAGTAATGCG AATTGCAGAATTCAGTGAGT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
PHI. FOR.UAMH6816   GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
HYM. ERI.READ101    GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
PSE. ROSE           GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCCTGGTATTCC GGGGGGCATGCCTGTCCGAG
HYM. ERI.UAMH6735   GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
UNKNOWN3.M8         GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
PHI. FOR.UAMH6815   GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
UNKNOWN4.M20        GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
PHI. DIMO           GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCTGTGGTATTCC GCAGGGCATGCCTGTTCGAG
PHI. FINL           GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATACCTGTTCGAG
UNKNOWN1.S9         GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCCGTTCGAG
UNKNOWN2.S246       GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
SCY. VACC           GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
UNKNOWN5.M5         GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCCTTGGTATTCC GAGGGGCATGCCTGTTCGAG
ACR.STR.S232        GAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCAC ATTGCGCCCGCCGGCACTCC GGCGGGCATGCCTGTCCGAG
```

```
                    <- 5.8S|   ITS2 ->
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PHI.-1IKE.PSP58SRNA CGTCATT AT??AACCACTCA AGCTCTCGCTTG?GTATTGG GG???T??TC??GCGG???? ???TCT??CGC??GGCCCCT ??????AAAAT?CAGTGGCG
NEU.CRA.NEURGITR    CGTCATT TC??AACCA?TCA AGCTCT?GCTTGCG??TTGG GG????A?TCC?GCGGCTGT CCG?????????????????CT c?????AAAAT?CAGTGGCG
CLA.FUL.CLSRGB      CGTCATT TC??A?CCACTCA AGCCTA?GCTTG?GTATTGG G?C????GTC??GCGG?T?T CCG?C??GCGCC?????T?T g?????AA?GT?CTCCGGC?
TAl.WOR.TALRR58SJ   CGTCATT TCTG??CC?CTCC AGCAC?GGC?TGGGTGTTGG G?C?GCTGTCCCC?C?????  CGGGAC?????ACG?CCCC? ??????AAAAG?CAGTGGCG
PEN.DEN.PECRR58S    CGTCATT TCTG??CC?CTCA AGCAC?GGCTTGTGTGTTGG G??tGTGGTCCCCTCCC??? ?TGG?CGGGGGACCTGCCC? g?????AAAGG?CAGCGG?C
SCL.SCL.SCEITSA     CGTCATT TC??AACC?CTCA AGCTCA?GCTTG?GTATTG? ??????AGTCC??A???TGT CAGTAATGG?CAGG?CTCT? ??????AAAAT?CAGTGGCG
SCL.TRI.U01218      CGTCATT TC??AACC?CTCA AGCTCA?GCTTG?GTATTG? ??????AGTCC??A???TGT CAGCAATGG?CAGG?CTC?T ??????AAAAT?CAGTGGCG
THE.LANU.TLAIRS     CGTCATT GC?GAACC?CTCA AGCAC?GGCTTGTGTGTTGG G?CCGCCGTCCCCTC???GT TTGGA?GGGG?ACGGGC?CT g?????AAAGG?CAGCGG?C
OID. CHLA           CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CCCC?G?CCCGC???T?? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?CAGTGGCG
OID. SCYT           CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CCCC?G?CCCGC???T?? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?CAGTGGCG
MYX. SETO           CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ??GC?G??GCC?GG?CCCC? ??????AAAGA?CAGTGGCG
OID. PERI           CGTCATT TC??AACC?CTCA AGCTCT?CCTTG?GTGTTGG G?CCC?AG?CCCG????T?? ?CG?CG??GCC?GG?CCCC? ??????AAAG?ACAGTGGCG
OID.GRI.UAMH4080    CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CCC?TG?CCCGC???T?? ??GC?G??GCC?GG?CCCC? ??????AAAG?ACAGTGGCG
OID. RHOD           CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CtCC?G?CCCCCG??T?? ?????G??GCC?GG?CCCC? ??????AAAGT?CAGTGGCG
OID. CITR           CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?TAGTGGCG
OID. GRI.UBCS80     CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?TAGTGGCG
OID. GRI.UBCS18     CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?TAGTGGCG
OID. MAIU           CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?CAGTGGCG
OID.-LIKE.UBCM10    CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?CAGTGGCG
OID. GRI.UBCS4      CGTCATT TC??AACC?CTCA AGCCTC?GCTTG?GTGTTGG G?CCC?TG?CCCGC?????? ?CGC?G??GCC?GG?CCC?T ??????AAAGA?TAGTGGCG
OID. FLAV           CGTCATT TC??AACC?CTCA AGCTCT?GCTTG?GTGTTGG G?CCC?TG?CCCGC???T?? ??GC?G??GCC?GG?CCC?T ??????AAAG?ACAGTGGCG
PHI. FOR.UAMH6816   CGTCATT T???AACCACTCA AGCCT?GGCTTG?GTATTGG GGC???ACGCGG???????? ???TCTCC?GC?GG?CCCTC ??????AAAAT?CAGTGGCG
HYM. ERI.READ101    CGTCATT AT?GA?CCACTCA AGCCTA?GCTTG?GTATTGG GG????T?T??CGCGG???? ???TCT??CGC?GG?CCCTT ??????AAAATT?AGTGGCG
PSE. ROSE           CGTCATT AC??AACC?CTCA AGCTCA?GCTTG?GTATTGG G?CCCC?G?CC?GAC????? ?C??CG??GC??GGGCCC?T ??????AAAGT?CAGTGGCG
HYM. ERI.UAMH6735   CGTCATT AT?GA?CCACTCA AGCCTA?GCTTG?GTATTGG GG????T?T??CGCGG???? ???TCT??CGC?GG?CCCTT ??????AAAAT?CAGTGGCG
UNKNOWN3.M8         CGTCATT AT??AACCACTCA AGTCTA?GCTTG?GTATTGG GG????T?T??CGCGG???? ???TCT??CGC?GG?CCCTT ??????AAAAT?CAGcGGCG
PHI. FOR.UAMH6815   CGTCATT T???AACCACTCA CGCCTA?GCGTG?GTATTGG GGC???ACGCGG???????? ???TCTCC?GC?GG?CCCTC ??????AAAATT?AGTGGCG
UNKNOWN4.M20        CGTCATT AT?GA?CCACTCA AGCCTA?GCTTG?GTATTGG GG????T?T??CGCGG???? ???TCc??CGC?GG?CCCCT ??????AAAAT?CAGTGGCG
PHI. DIMO           CGTCATT TA?GA?CCACTCA CGCCT?GGCGTG?GTATTGG GGC???ACGCGG???????? ???T?TCC?GC?GG?CCCTC ??????AAAAT?CAGTGGCG
PHI. FINL           CGTCATT AT??AACCACTCA AGCCTCGGCTTG?GTCTTGG GG????T?T??CGCGG???T ???T?TC??GC?GG?CCCTT ??????AAAAT?CAGTGGCG
UNKNOWN1.S9         CGTCATT AT??AACCCCTCA AGCCTA?GCTTG?GTGTTGG GGCCT???????GCTG???T ???T?aCCGGCa?G?CCCTT ??????AAAAT?CAGTGGCG
UNKNOWN2.S246       CGTCATT AC??AACC?CTCA AGCATT?GCTTG?GTATTGG G?C?TC?????CGCTGc??T cacCCa???GC?GGgCC?TT ??????AAAAT?CAGTGGCG
SCY. VACC           CGTCATT AT?GA?CCACTCA AGCCTA?GCTTG?GTATTGG GG????T?T??CGCGG???? ???TCT??CGC?GG?CCCTT ??????AAAAT?CAGTGGCG
UNKNOWN5.M5         CGTCATT AT??AACCACTCA AGCCT?GGCTTG?GTATTGG GG????TtTt?CGC??gTg? ???T?T??CGC?GG?CCCTT ??????AAAAT?CAGTGGCG
ACR.STR.S232        CGTCATT TC??AACC?CTCA GGACCCCCTTTC?GGGGGGG A??CCTGGTGCTGGGG?A?T CAGCGGCCTCCGGGCCCCTg tccccAAA?TTGAGTGGGG
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FUS.SAM.FSITSB      GTC???AC?GTCGAGC???T t?CC???A??tAGCGTAGTA AT?T???t???????acaca ????TC???G??T?T?ACT? ?GGTAAT?CGTCGC?GGCCa
PHI.-lIKE.PSP58SRNA GT?GCC??TGTCG?G?CTCT ???????A?CG??CGTAGTA ???ATACTCCTCGC?????? gt?CT????GGGT?CCGAC? AGGTCTACTTGCC???????
NEU.CRA.NEURGITR    G??GCT??CGTC?AG????T ???CaCAC?CGAGCGTGGTG ?????ACTC?T????acatc g??CTAT??GGTcGTGCGGc GGGTTC?TT?GCC?????gt
CLA.FUL.CLSRGB      ?T??????????GAGCaGTT ???CgTCTCTAAGCGTTGTG GCATATATT?TCGCtaaaga gttC?????GGG?CGGCTTT TGGCCG?TT???????????
TAl.WOR.TALRR58SJ   G?CG??CCGC????GTC??? GGG??TCCTCGAGCGTA?TG GGG???CTT?T??gtcac?t cg??CTC??GGGAGGGACTc ?GGT?CGGC?GC??TGG???
PEN.DEN.PECRR58S    G?AC?G?TCGCGTCC????? GG???TCCTCGAGCGTA?TG GGG???CTT?T??gtcac?t cg??CTC??GGGAAGGANN? ?GGTGCG?C?GC?GT???Ct
SCL.SCL.SCEITSA     G?C??ACCGCT?GGGTC?CT G?????AA?CG????TAGTA ATAT??CTC?TCGttacag? gttCTC???GG?T????GTg ??CTT??CT?GCC???????
SCL.TRI.U01218      G?CG??CCGCT?GGGTC?CT G?????AA?CG????TAGTA ATAT??CTC?TCGttacag? gttCTC???GG?T????GT? ?GCTT?C?T?GCC???????
THE.LANU.TLAIRS     GGCG?TCGCGTCC?GG???T ??CC?TC???GAGCGTA?TG GGG???CTC?T?Gtcac?gc g??CTC?AaGGA?GGGGTCc ?GG??CCGGGGCCATAGCCt
OID. CHLA           G?CAGCGC?CT?G?G???CT ???CC?AA??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCT??GGGG?C????T AGGC??GGTAGCC?T?GCC?
OID. SCYT           G?CAGCGC?CT?G?G???CT ???CC?AA??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCT??GGGG?C????T AGGC??GGTAGCC?T?GCC?
MYX. SETO           G?CGCCG?TCT?G?G?CTCT ??????AA??G??CGTAGTA ?CAA??CTC?TCG??????? ???CTCT??GGAG?CC???T ?GGC??GGT?GCCCT?GCC?
OID. PERI           G?CGCC???GTCGGG?CCCT ??????AA??G??CGTAGTA ?CAT??CTC?TCG??????? ???CTCTA?GGG??CCCCGT ?GGTGGCTT?GCC???????
OID.GRI.UAMH4080    GT?GCCGC?CT?G?G?CCCT G??????A??G??CGTAGTA ?CAT??CTC?TCG??????? ???CTCCA?GtGT?CCGGC? ?GGTGGCCT?GCC???????
OID. RHOD           G?CGCCG?TCT?G?G?CTCT ??????AA??G??CGTAGTA ?CAT??CTC?TCG??????? ???CTCTA?GGGT?CCTGC? ?GGTGGCTT?GCC???????
OID. CITR           G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGCA ?GGCAGCCT?GCC???????
OID. GRI.UBCS80     G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGC? ?GGCAGCCT?GCC???????
OID. GRI.UBCS18     G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGC? ?GGCAGCCT?GCC???????
OID. MAIU           G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGC? ?GGCAGCCT?GCC???????
OID.-LIKE.UBCM10    G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGCn nnnnnnnnnnnnnnnnnnnn
OID. GRI.UBCS4      G?CGCCGC?CT?G?G?CCCT ????C??A??G??CGTAGTA ?CAG??CTC?TCG??????? ???CTCCA?GGGT?CCGGC? ?GGCAGCCT?GCC???????
OID. FLAV           GT?GCCGC?CT?G?G?CCCT G??????A??G??CGTAGTA ?CAT??CTC?TCG??????? ???CTCCA?GtGG?CCCGGT ?GGTAGCCT?GCC???????
PHI. FOR.UAMH6816   G?CGCC??GGT??GGGCTCT ??????AA??G??CGTAGTA ?CAT??AcTCCCG??????? ???CTATA?GaGTTCCCCC? ?GGTGGCT?CGCC???????
HYM. ERI.READ101    GT?GCCA?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTATC?TCG??????? ???CTATT?GGGT?CCGGT? ?GGTTGCTT?GCC???????
PSE. ROSE           GT?GCCG?TC?CG?G???CT ???CC?????GAGCGTAGTA ??ATTCTTC?TCG??????? ???CTCT??GGAGGTCCGGT ?CGTGTGCTCGCC???????
HYM. ERI.UAMH6735   GT?GCCA?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTATC?TCG??????? ???CTATT?GGGT?CCGGT? ?GGTTGCTT?GCC???????
UNKNOWN3.M8         GT?GCCA?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTATC?TCG??????? ???CTATT?GGGT?CCGaTA ??GTTGCTT?GCC???????
PHI. FOR.UAMH6815   G?CGCC??GGT??GGgCTCT ??????AA??G??CGTAGTA ?CAT??AcTCCCG??????? ???CTATA?GaGTTCCCCC? ?GGTGGCT?CGCC???????
UNKNOWN4.M20        GT?GCCA?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTATC?TCG??????? ???CTATT?GGGT?CCGGT? ?GGTTGCTT?GCC???????
PHI. DIMO           G?CGCC??GGTA?GG?CCCT ??????AA??G??CGTAGTA A?AT??CTCCTCG?????7? ???CTATA?GGGT?CCTCTC ?GGTGGCT?CGCC???????
PHI. FINL           GT?GCCG?TCT??GG?CTCT ??????AA??G??CGTAGTA A?TT??CTC?TCG??????? ???CTATA?GGGT?CCaGGT ??GACcACCTGCC???????
UNKNOWN1.S9         GT?GCCA?TCT??GG?CTCT ??????AA??G??CGTAGTA A?TA??CTTCTCG??????? ???CTACA?GGGT?CCTGGT ?GGAT?ACTTGCC???????
UNKNOWN2.S246       GT?GCCG?TCgA?GG?CCCT G?????A???G??CGTAGTA AaTA???TCCTCG??????? ???CTATA?GGGa?CtCGGT ?GGACG?CTgGCC???????
SCY. VACC           GT?GCCA?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTATC?TCG??????? ???CTATT?GGGT?C?CGGT ?GGTTc?CTTGCC???????
UNKNOWN5.M5         GT?GCCG?TCT?G?G?CTCT ??????AA??G??CGTAGTA ??ATTTCTC?TCG??????? ???CTATA?GGGTTC?TGGT ?GGTT?ACTTGCC???????
ACR.STR.S232        GTCGCGCCGCAGCCTCCCCT GCGtaGTAGCACACCTCGCA cCGGAGAGCGGCTcggccac gccGTGAAaCcccCAATTTT TTAAGGTTGA?CCTCgGatc
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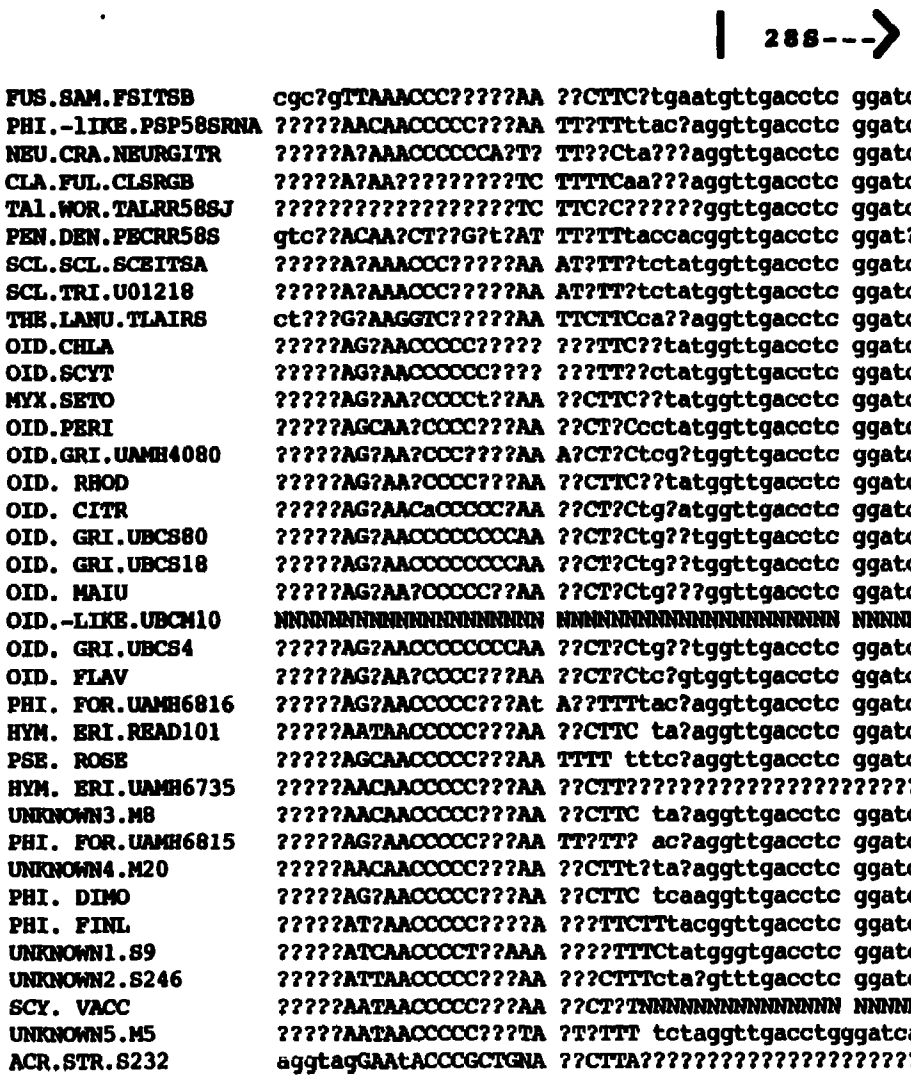


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FUS.SAM.FSITSB       cgc?gTTAAACCC?????AA ??CTTC?tgaatgttgacctc ggatca?
PHI.-1IKE.PSP58SRNA  ?????AACAACCCCC???AA TT?TTttac?aggttgacctc ggatcag
NEU.CRA.NEURGITR     ?????A?AAACCCCCCA?T? TT??Cta???aggttgacctc ggatca?
CLA.FUL.CLSRGB       ?????A?AA??????????TC TTTTCaa???aggttgacctc ggatcag
TAl.WOR.TALRR58SJ    ????????????????????TC TTC?C??????ggttgacctc ggatca?
PEN.DEN.PECRR58S     gtc??ACAA?CT??G?t?AT TT?TTtaccacggttgacctc ggat???
SCL.SCL.SCEITSA      ?????A?AAACCC?????AA AT?TT?tctatggttgacctc ggatcag
SCL.TRI.U01218       ?????A?AAACCC?????AA AT?TT?tctatggttgacctc ggatc??
THE.LANU.TLAIRS      ct???G?AAGGTC?????AA TTCTTCca??aggttgacctc ggatcag
OID.CHLA             ?????AG?AACCCCC????? ???TTC??tatggttgacctc ggatcag
OID.SCYT             ?????AG?AACCCCCC???? ???TT??ctatggttgacctc ggatcag
MYX.SETO             ?????AG?AA?CCCCt??AA ??CTTC??tatggttgacctc ggatcag
OID.PERI             ?????AGCAA?CCCC???AA ??CT?Ccctatggttgacctc ggatcag
OID.GRI.UAMH4080     ?????AG?AA?CCC????AA A?CT?Ctcg?tggttgacctc ggatcag
OID. RHOD            ?????AG?AA?CCCC???AA ??CTTC??tatggttgacctc ggatcag
OID. CITR            ?????AG?AACaCCCCC?AA ??CT?Ctg?atggttgacctc ggatcag
OID. GRI.UBCS80      ?????AG?AACCCCCCCCAA ??CT?Ctg??tggttgacctc ggatcag
OID. GRI.UBCS18      ?????AG?AACCCCCCCCAA ??CT?Ctg??tggttgacctc ggatcag
OID. MAIU            ?????AG?AA?CCCCC??AA ??CT?Ctg???ggttgacctc ggatcag
OID.-LIKE.UBCM10     NNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN NNNNNNN
OID. GRI.UBCS4       ?????AG?AACCCCCCCCAA ??CT?Ctg??tggttgacctc ggatcag
OID. FLAV            ?????AG?AA?CCCC???AA ??CT?Ctc?gtggttgacctc ggatcag
PHI. FOR.UAMH6816    ?????AG?AACCCCC???At A??TTTtac?aggttgacctc ggatcag
HYM. ERI.READ101     ?????AATAACCCCC???AA ??CTTC ta?aggttgacctc ggatcag
PSE. ROSE            ?????AGCAACCCCC???AA TTTT tttc?aggttgacctc ggatcag
HYM. ERI.UAMH6735    ?????AACAACCCCC???AA ??CTT??????????????????????
UNKNOWN3.M8          ?????AACAACCCCC???AA ??CTTC ta?aggttgacctc ggatcag
PHI. FOR.UAMH6815    ?????AG?AACCCCC???AA TT?TT? ac?aggttgacctc ggatcag
UNKNOWN4.M20         ?????AACAACCCCC???AA ??CTTt?ta?aggttgacctc ggatcag
PHI. DIMO            ?????AG?AACCCCC???AA ??CTTC tcaaggttgacctc ggatcag
PHI. FINL            ?????AT?AACCCCC????A ???TTCTTtacggttgacctc ggatcag
UNKNOWN1.S9          ?????ATCAACCCCT??AAA ????TTTCtatgggtgacctc ggatcgg
UNKNOWN2.S246        ?????ATTAACCCCC???AA ???CTTTcta?gtttgacctc ggatcag
SCY. VACC            ?????AATAACCCCC???AA ??CT?TNNNNNNNNNNNNNN NNNNNNN
UNKNOWN5.M5          ?????AATAACCCCC???TA ?T?TTT tctaggttgacctgggatcagg
ACR.STR.S232         aggtagGAAtACCCGCTGNA ??CTTA??????????????????????
```

written in lower case (a, t, g, or c) were considered unalignable and were also excluded from the analysis (Fig. 3.1). The final data set consisted of 350 aligned sites for 33 taxa.

Parsimony trees were inferred using the program PAUP 3.0s (Swofford, 1991). To speed up the analysis, sequences from three isolates, *O. scytaloides* (UAMH 6521) and *O. griseum* (isolates UBC S18 and UBC S4), were excluded from the parsimony search. Each of the excluded isolates was almost identical to an isolate sequence retained in the analysis (Fig. 3.1) so the absence of the three removed little phylogenetic information. Fifty heuristic searches were performed with random addition of the 30 remaining taxa. To estimate the support of the data for the branches, neighbor-joining was used to infer trees from 500 bootstrap replicates of the data set (PHYLIP 3.55c, Felsenstein 1993). The distance matrices for the bootstrapping were calculated using a Kimura correction with a transition/transversion ratio of two.

Primer design

To selectively amplify known ericoid mycorrhizal fungi from colonized salal roots, three taxon-specific primers were designed based on the sequence alignment and phylogenetic analysis. Each primer is composed of 19 nucleotides that were visually selected to be complementary to the 3' end of the ITS2 for the target isolate but not the other fungi (Fig. 3.1). The phylogenetic analysis of sequences grouped the ericoid mycorrhizal species of this study into two main groups, the ***Oidiodendron*** group and the ***Hymenoscyphus*** group (Fig. 3.4). To

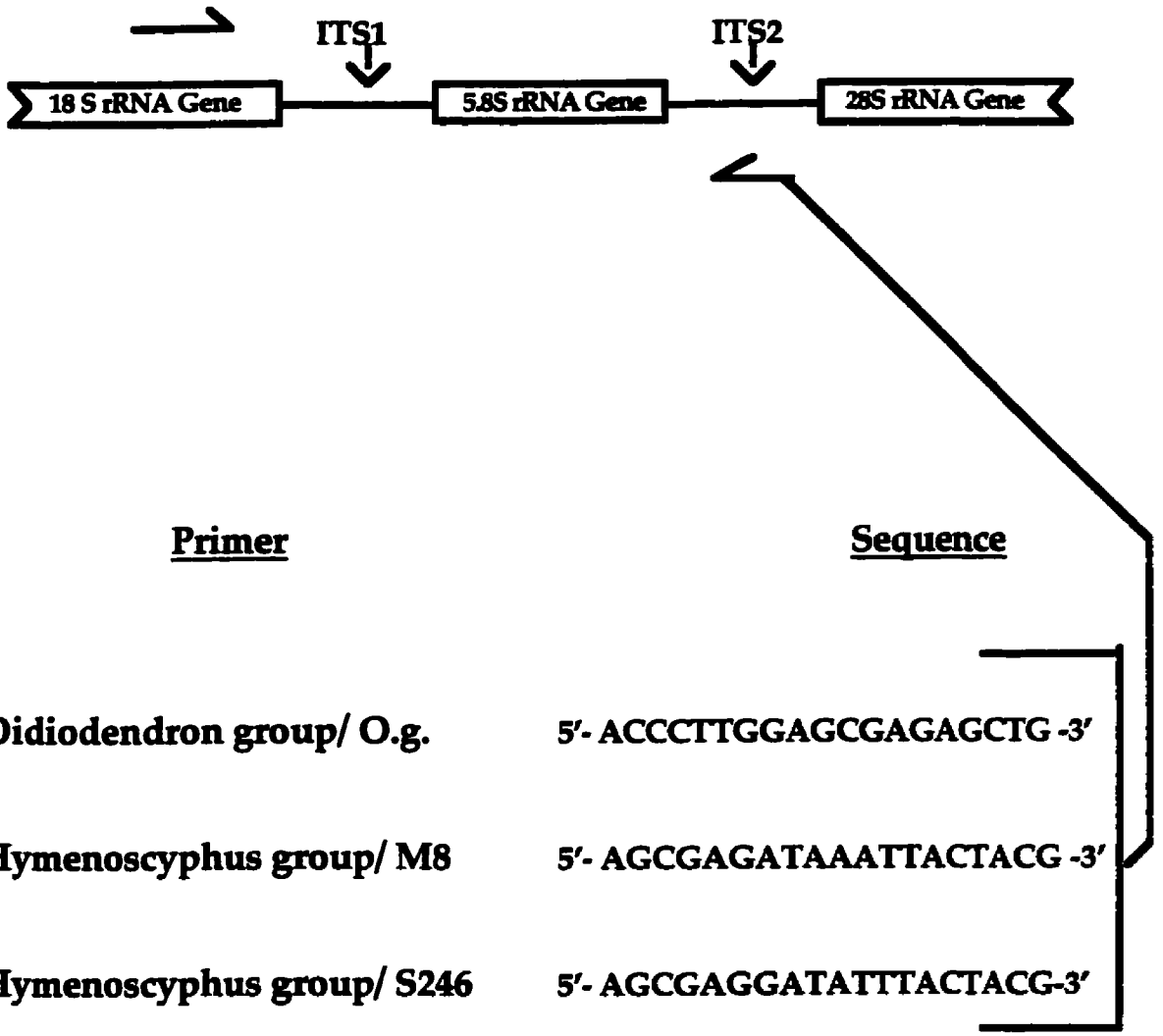

Figure 3.2 Set of three primers selected after sequence alignment and phylogenetic analysis.

amplify DNAs from fungi in the ***Oidiodendron*** group, primer **O.g.** was designed to match the sequences of the species *Oidiodendron griseum*. To amplify DNAs from the ***Hymenoscyphus*** group, primers **S246** and **M8** were designed as perfect matches for the sterile isolates Unknown 2 (UBC S246), and Unknown 3 (UBC M8) (Fig. 3.2), respectively. All three primers anneal to the 3' end of the ITS2 region, at 59 and 67 bp from the 28S gene for the primers **O.g.** and **S246** respectively (Fig. 3.2). Primers **M8** and **S246** anneal to homologous sites (Fig. 3.1). Each of these primers can be paired with the fungal specific primer ITS1-F (Gardes and Bruns, 1993) or the universal primers ITS5 or ITS1 in order to obtain a PCR product encompassing the ITS1 and most of the ITS2 regions of the ribosomal repeat.

Primers test for optimum PCR conditions and specificity with closely related taxa

To optimize stringency of primer binding, each new selective primer was tested for its ability to amplify target DNA at 52 C, 56 C, 59 C and 62 C. The ***Oidiodendron*** group specific primer **O.g.** was tested with *Oidiodendron* species; two *O. griseum* isolates, one *Oidiodendron* sp., and *Oidiodendron maius*. The primer **M8** was tested with unidentified isolates **Unknown 3, Unknown 4,** *S. vaccinii,* and *H. ericae* (Fig. 3.5). Primer S246 was tested using unidentified isolates **Unknown 1, 2** and **5,** and *H. ericae.*

Primer test with different fungal DNA concentrations

A set of serial dilutions of fungal DNA was used to establish the

minimum fungal DNA concentration at which the selective primers would produce a visible PCR product. Genomic fungal DNAs of the unidentified isolates UBC S246, UBC M8 and the species *Oidiodendron griseum* were used in concentrations ranging from 3 ng/μl to 8 x 10^{-6} ng/μl to test the PCR reaction with each primer set (Table 3.2).

Tests of primers with artificial mixtures of fungal DNAs

Since each of the three new primers represented a group of the ericoid mycorrhizal fungi used in this study, the primers were tested for their ability to amplify only the target DNA in artificial mixtures with DNA of ericoid mycorrhizal fungus of a non-target group. PCR tests for each set of primers were carried out with artificial mixtures of two fungal DNAs at a time, as well as with artificial mixtures of each fungal DNA with salal DNA (Table 3.3). The annealing temperature used was 56 C (Table 3.3).

Test of primer O.g. with fungi of diverse taxa

PCR reactions were performed to test the specificity of the three ericoid mycorrhizal specific primers using genomic DNAs of a wide range of non-mycorrhizal fungal taxa. The annealing temperature used was 56 C (Table 3.4). DNAs obtained from various fungal species (Table 3.4) were diluted 100 fold and amplified using the universal primers ITS4-ITS5. The PCR products were diluted 100-fold. The diluted product was then reamplified with two sets of primers: the universal primers (ITS4-ITS5) and a set including the fungal specific primer

Table 3.2 PCR products obtained with the primer **O.g.** when using *O. griseum* genomic DNA at different DNA concentrations.

DNA concentration in reaction mixture (ng/µl)	Total DNA per PCR reaction	PCR product
3.00	75.00 ng	+
1.50	37.50 ng	+
0.50	12.50 ng	+
0.25	6.25 ng	+
0.05	1.25 ng	+
0.025	0.625 ng	+
0.005	0.125 ng	-
0.001	0.025 ng	-
2×10^{-4}	5 pg	-
4×10^{-5}	1 pg	-
8×10^{-6}	0.2 pg	-

Table 3.3 Can new, specific primers distinguish target DNA from a DNA mixture? The left column gives the concentration of target and non-target DNA per PCR reaction. Specific primers used in each reaction are listed across the top. + indicates a single band of PCR product when the expected PCR band size for the two DNAs was the same; - indicates no amplification; -/+ indicates amplification of only the second DNA when the expected PCR band lengths of the two DNAs were different; +/+ indicates amplificaton of two DNAs with different PCR band sizes; +/- indicates amplification of only the first DNA; -/- indicates that neither DNA amplified.

DNA mixtures [ng/µl]	**PCR Products**		
S246/*O.griseum*	**Primer S246**	**Primer M8**	**Primer O.g.**
0 /6	-	-/-	+
1.5/4.5	+	-/-	+
3/3	+	-/-	+
4.5/1.5	+	-/-	+
6/0	+	-/-	-
S246/M8			
0/6	-/+	-/+	-/-
1.5/4.5	+/+	-/+	-/-
3/3	+/+	-/+	-/-
4.5/1.5	+/+	-/+	-/-
M8/*O. griseum*			
1.5/4.5	-/-	+/-	-/+
3/3	+/-	+/-	-/+
4.5/1.5	+/-	+/-	-/+
6/0	-/-	+/-	-/-
O.g / salal (3/3)	-/-	-/-	+/-
M8/salal (3/3)	+/-	+/-	-/-

Table 3.4 PCR products obtained from DNA of a variety of fungal isolates when using a set of universal primers and a set of a fungal specific primer ITS1-F with the group specific primer **O.g.**

Fungal isolate	Primers	
	Universal	O.g.
Aspergillus nidulans (Eidam) Winter	+	-
Botrytis cinerea Pers. :Fr. *f. lini* van Beyma	+	-
Caprionia pilosella (Karsten) E. Muller *et al.*	+	-
Cucurbidothis pitophila Petrack	+	-
Fusarium avenaceum (Corda: Fr.) Sacc	+	-
Neurospora terricola Gochenour and Backus	+	-
Oidiodendron griseum	+	+
Saccharomyces cerevisiae Hansen	+	-
Schizophyllum commune Vaill.:Fr.	+	-
Ustilago bullata Berk	+	-
Ustilago kolleri Wille	+	-

(ITS1-F) and the specific primer **O.g.**

Results

Sequence Analysis

The data matrix shown on Fig. 3.1 includes the sequence alignment for all the taxa used in this study. The nucleotide sequences include a portion of the 5.8S gene, the ITS2 region and the beginning of the 28S gene. All *Oidiodendron* isolates showed low levels of sequence divergence in the ITS2 region (approximately 180 aligned sites). The difference between *O. griseum* (UBC S18) and *O. maius*, and between *O. griseum* (UBC S18) and *O. citrinum* was only 0.5 percent. The percent basepair difference between *O. griseum* (UBC S18) and *O. flavum* was 9.4 percent. The percent basepair difference between *O. citrinum* and *O. maius* was 1 percent. Higher percent differences characterized other pairs of isolates, including the unknown taxa. The percent difference between *O. griseum* (UBC S18) and the taxon **Unknown 3** (UBC M8) and **Unknown 2** (UBC S246) was 15 percent and 12.8 percent, respectively.

For fungi of the ***Hymenoscyphus*** group, uncorrected pair-wise percent substitution among two of the unknown taxon, **Unknown 3** (UBC M8) and **Unknown 4** (M20) and *Hymenoscyphus ericae* (Read, D.J. 101 and UAMH 6735) varied between 0.55 and 2.77 percent (Table 3.5). However, the percent pair-wise substitution for *H. ericae* and the taxa **Unknown 1, 2** and **5** (UBC S9, UBC S246, and UBC M5) varied between 7.77 and 15.55 percent. All of the unknown isolates

showed pairwise percent substitution that varied between 6.11 percent and 11.66 percent when compared to *Phialophora finlandia* (Table 3.5).

Table 3.5 Uncorrected pair-wise percent substitution for fungi in the ***Hymenoscyphus*** group.

	PF7454	He101	Svacci	H6735	M8	M20	M5	S246
PF7454	-							
He101	8.33	-						
Svacci	6.66	2.77	-					
H6735	9.44	1.11	2.22	-				
M8	9.44	2.77	4.44	2.22	-			
M20	10.00	1.66	2.22	0.55	1.11	-		
M5	6.11	8.33	6.66	7.77	8.33	8.88	-	
S246	10.55	11.66	8.88	15.55	12.22	11.11	8.88	-
S9	11.66	11.11	7.77	12.22	10.00	10.00	10.55	13.88

Phylogenetic Analysis

Phylogenetic analysis of the ITS2 region of the nuclear ribosomal repeat using the program PAUP with 50 replicated heuristic searches (Swofford, 1991) generated 140 equally parsimonious trees of length 458 (Fig. 3.3). Ericoid mycorrhizal fungi clustered in two main groups, the ***Oidiodendron*** **group** and the ***Hymenoscyphus*** **group**. For the parsimony trees the consistency index was 0.623. Three endophytic *Phialocephala* species clustered apart from the ericoid mycorrhizal fungi (Fig. 3.3, 3.4). The ***Hymenoscyphus*** **group** segregated into two

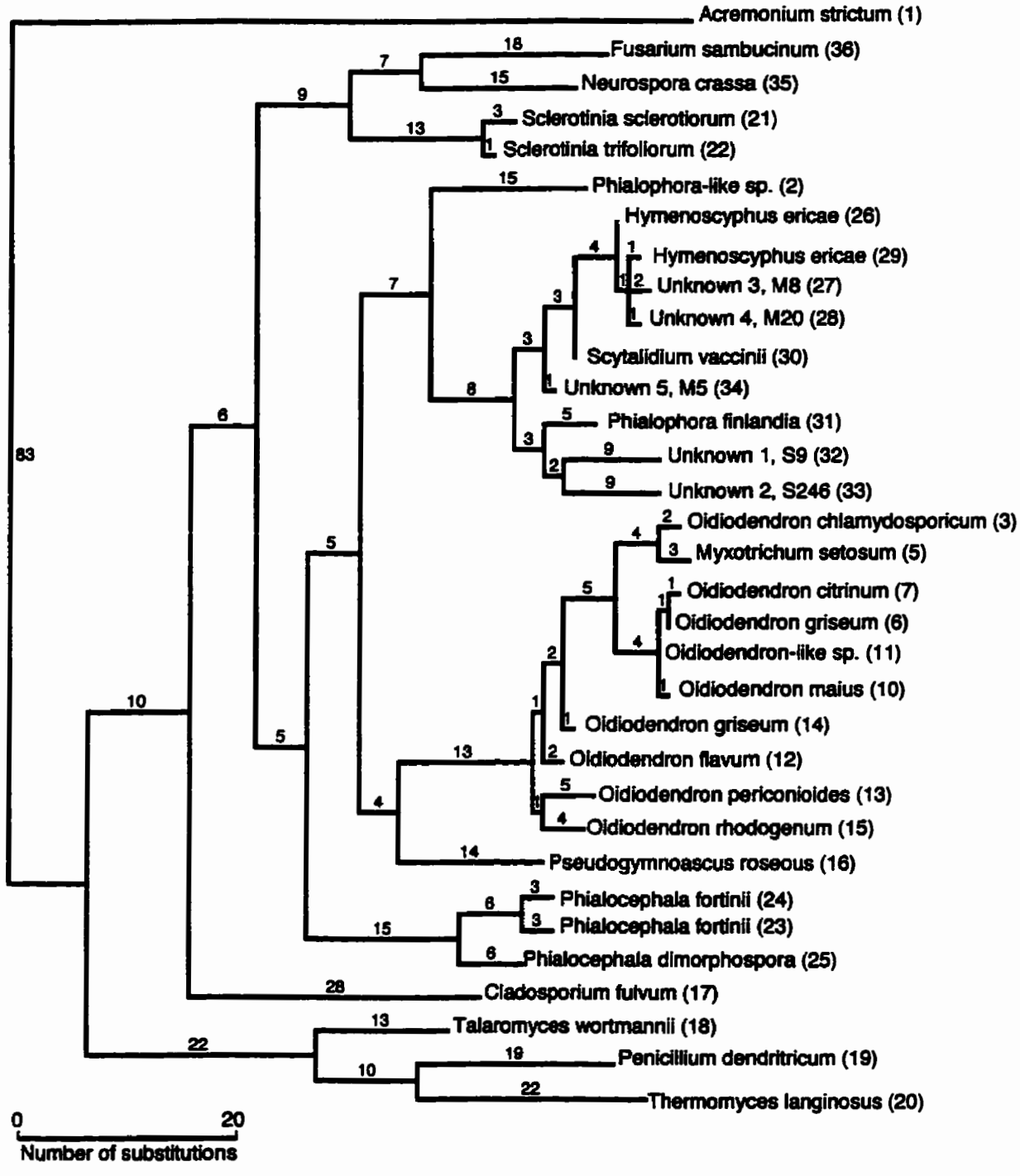

Figure 3.3 One of 140 equally parsimonious trees of length 458 found in 50 replicated heuristic searches with random addition of taxa using PAUP. In this phylogram, branch lengths are proportional to substitution numbers. The numbers are the number of changes that map to the branches.

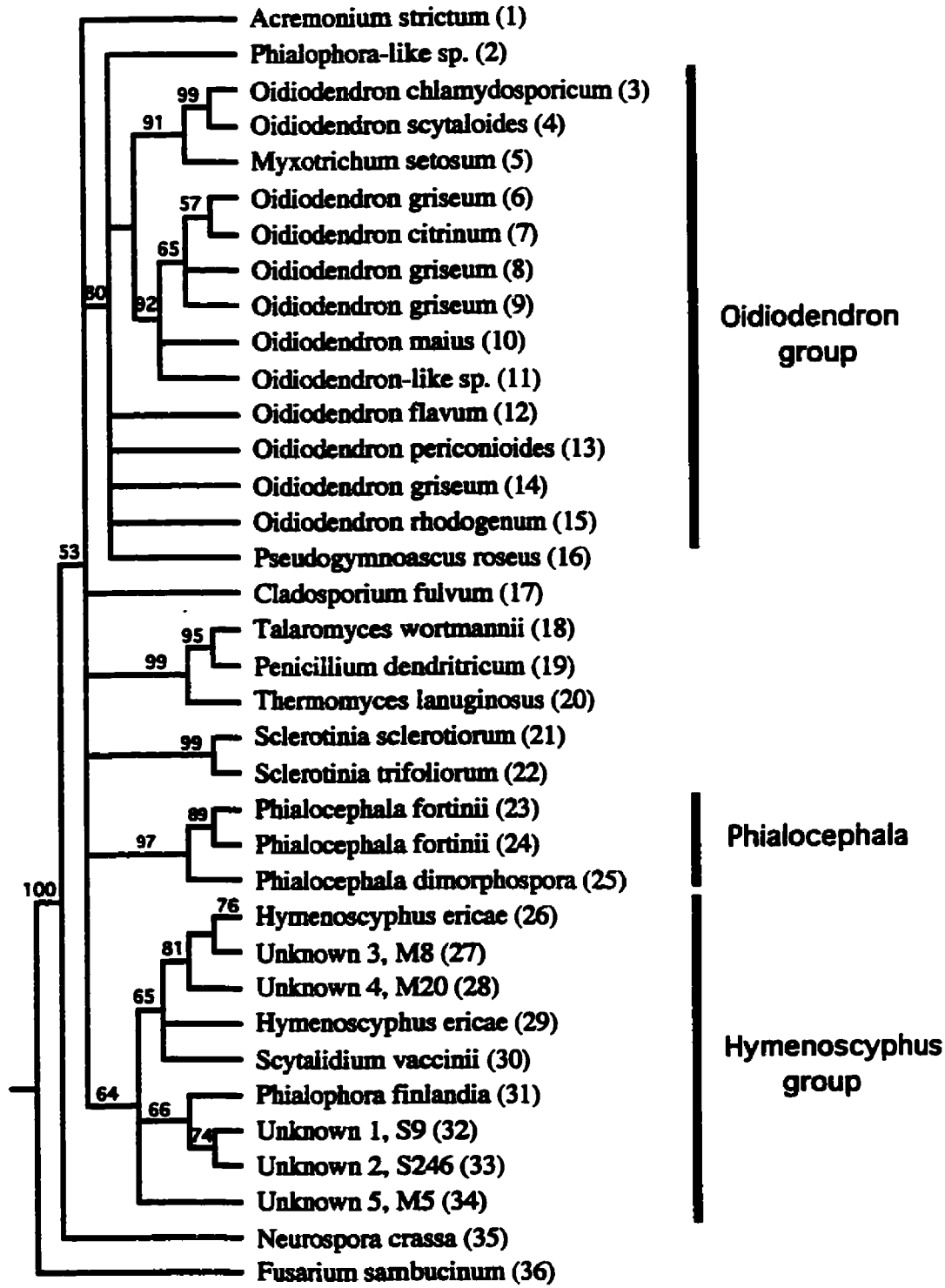

Figure 3.4 Consensus tree generated from neighbor-joining analysis of 500 bootstrap replicates. Percentage are bootstrap percentages. Only groups receiving 50% or more support are shown.

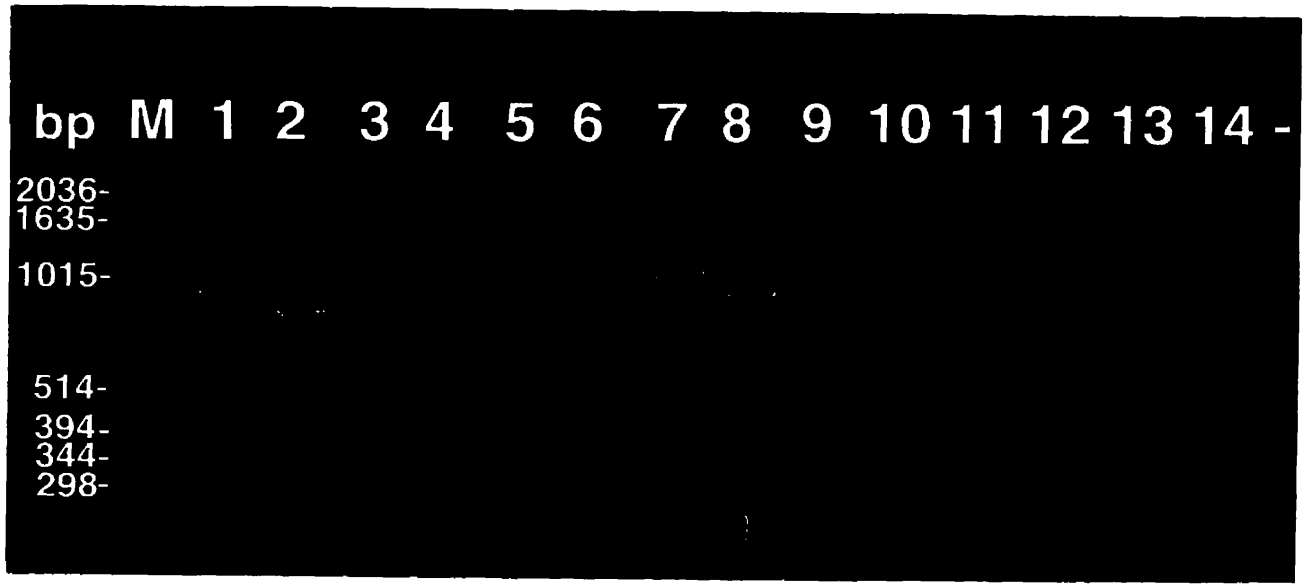

Figure 3.5 Comparison of PCR products from target fungal DNAs amplified using either the new selective genus-specific primer M8 or the fungus specific primer ITS1-F. For each pair of lanes, the first lane contains a longer fragment amplified with ITS1-F and ITS4. The second lane of each pair contains the shorter fragment amplified with genus-specific M8 and ITS4. The genomic templates and their dilutions were: Lanes 1-4; Unknown 4 (isolate M8). 1, 2, 10 fold dilutions; 3, 4, 100 fold dilution; 5-8, Unknown 3 (isolate M20), 5, 6, 10 fold dilution; 7, 8, 100 fold dilution; 11-12, *Scytalidium vaccinii*, 100 fold dilution; 13-14, *Hymenoscyphus ericae*. M = molecular size marker; -= negative control.

sub-groups in this phylogram; one branch included the species *Hymenoscyphus ericae*, *Scytalidium vaccinii* and sterile isolates (Fig. 3.3, 3.4). Isolates UBC M8 and UBC M20 clustered closely with the isolates of *H. ericae*. The other branch of the ***Hymenoscyphus* group** included the species *Phialophora finlandia* and sterile isolates UBC S246, and UBC S9 (Fig. 3.3, 3.4). The taxa of the ***Oidiodendron* group** clustered as follows; *Myxotrichum setosum* clustered with *Oidiodendron chlamydosporicum*. *Oidiodendron citrinum*, *O. griseum*, *O. maius* and the UBC M10 (*Oidiodendron*-like sp.) clustered together. An *O. griseum* isolate (UAMH 4080) that sometimes clustered closer to *O. flavum* (UAMH 1524) than to the other *O. griseum* isolates was probably misidentified (Sigler, personal communication). Although a member of the Onygenales, the isolate *Pseudogymnoascus roseus* was distant from the other species in the ***Oidiodendron* group.** The unusual ericoid mycorrhizal fungus *Acremonium strictum* was very different from all the other fungal taxa used in this analysis (Fig. 3.3).

The consensus tree generated by neighbor-joining bootstrap analysis with 500 replicates shows that the ***Hymenoscyphus*** and ***Oidiodendron*** groups receive 64% and 80% bootstrap support, respectively.

A cluster uniting the three isolates of the endophytic fungi of the genus *Phialocephala* received 97% support.

Within the ***Oidiodendron* group** the branch uniting *M. setosum*, *O. chlamydosporicum*, and *O. scytaloides* showed 91% support. The branch including the taxa *O. griseum* (UBC S4, UBC S18 and UBC S80), *O. citrinum* and

O. maius received 92% support. In the bootstrap consensus tree, the branching order of taxa including *O. flavum, O. periconioides, O. rhodogenum, O. griseum* (UAMH 4080) and *P. roseus*) was not resolved.

The ***Hymenoscyphus* group** included two sub-groups. One of the sub-groups of ***Hymenoscyphus*** included *H. ericae, S. vaccinii*, and the sterile isolates M8 and M20 with 65% support. Within this group, the cluster including isolates UBC M8, UBC M20 and *H. ericae* (Read, D.J. 101) received 81% support. The second ***Hymenoscyphus* sub-group** included the isolates *Phialophora finlandia* and the sterile isolates UBC S9 and UBC S246 with 66% support, with the two sterile isolates clustering closer together with 74% support. Although the sterile isolate UBC M5 clustered within the ***Hymenoscyphus* group**, it did not cluster with either sub-group.

Primer test for optimum PCR conditions and closely related taxa

Three selective primers were designed to amplify three different clusters of ericoid mycorrhizal isolates. ITS1-F has a calculated melting temperature of 60 C (Maniatis *et al.*, 1982). The three selective primers had calculated melting temperatures of 62 C, 52 C, and 54 C for **O.g.**, **M8**, and **S246**, respectively. When using annealing temperatures over 59 C, ITS1-F, M8, or S246 probably failed to anneal and a single-stranded PCR product resulted. Therefore, the selected annealing temperature was 56 C for each of the three selective primers paired with ITS1-F

Each of the three selective primers paired with the fungal specific primer

ITS1-F (Gardes and Bruns, 1993) amplified the ITS regions when tested with target DNA as follows: **primer O.g.** with ITS1-F amplified isolates UBC M10, *O. griseum* (UBC S18, UBC S80), and *O. maius* (UAMH 1540); **primer S246** with ITS1-F amplified isolates UBC S246, UBC S9 and UBC M5; and **primer M8** with ITS1-F amplified isolates UBC M8, UBC M20, *S. vaccinii* (UAMH 5828), and *H. ericae* (Read, D.J. 101) (Fig. 3.5).

Primer test for different fungal DNA concentrations indicated that the minimum concentration of genomic DNA was 0.025 ng/μl for each PCR reaction of a total volume 25 μl. That was equivalent to 0.625 ng of genomic DNA per PCR reaction (Fig. 3.8).

Tests of primer specificity with artificial mixtures of target and non-target ericoid mycorrhizal DNAs.

When their target DNA was present in the PCR reaction, all three selective primers, **O.g.** , **M8**, and **S246**, produced a PCR product. Target DNAs were amplified even when they constituted only 25% of an artificial DNA mixture (Fig. 3.6 and Fig. 3.7). Primers **O.g.** and **M8** showed higher specificity than primer **S246**, since primers **O.g.** and **M8** yielded a product only when their target DNA was present. However, when the primer **S246** was used in DNA mixtures of taxa M8 and S246, two bands were detected in electrophoresed agarose gel, one corresponding to the expected PCR product length of UBC M8 (approximately 980 bp) and the second corresponding to the expected length of UBC S246 (approximately 590 bp). These molecular data suggest that primer **S246** might

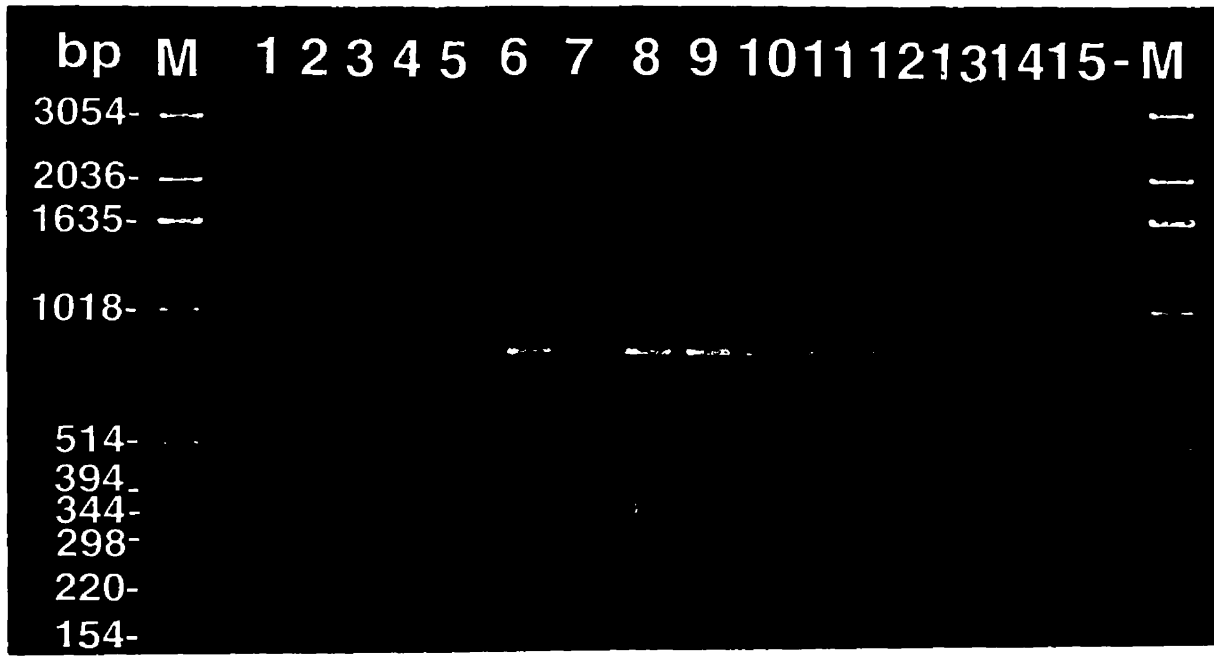

Figure 3.6 Use of genus-specific primer **M8** to amplify target DNA from a mixture of total genomic DNAs. PCR product formed only when target Unknown 3 (UBC M8) DNA was present (lanes 6-12, and 15). The combinations of the genomic templates in the reactions were: Lanes 1-5, with DNAs mixtures of Unknown 2 (UBC S246)/ *O. griseum* (UBC S18) with concentrations expressed in ng/μl; lanes 1, 6/0; 2, 4.5/1.5; 3, 3/3; 4, 1.5/4.5; and 5, 0/6; lanes 6-9, mixtures including target DNA with concentrations expressed in ng/μl target Unknown 3 (UBC M8)/ ng/μl Unknown 2 (UBC S246), lanes 6, 6/0; 7, 4.5/1.5; 8, 3/3; 9, 1.5/4.5. Lanes 10-12, mixtures in ng/μl target Unknown 3 (UBC M8)/ ng/μl *O. griseum* (UBC S18), lanes 10, 1.5/4.5; 11, 3/3; 12, 4.5/1.5. Lane 13, mixture included only salal DNA at a concentration of 6 ng/μl. Lane 14, mixture included salal DNA and *O. griseum* (UBC S18) with DNA concentrations of 3 ng/μl each. Lane 15, mixture included target DNA of Unknown 3 (UBC M8) and salal DNA at a concentration of 3 ng/μl each. Lane (-) = negative control. M = molecular size marker.

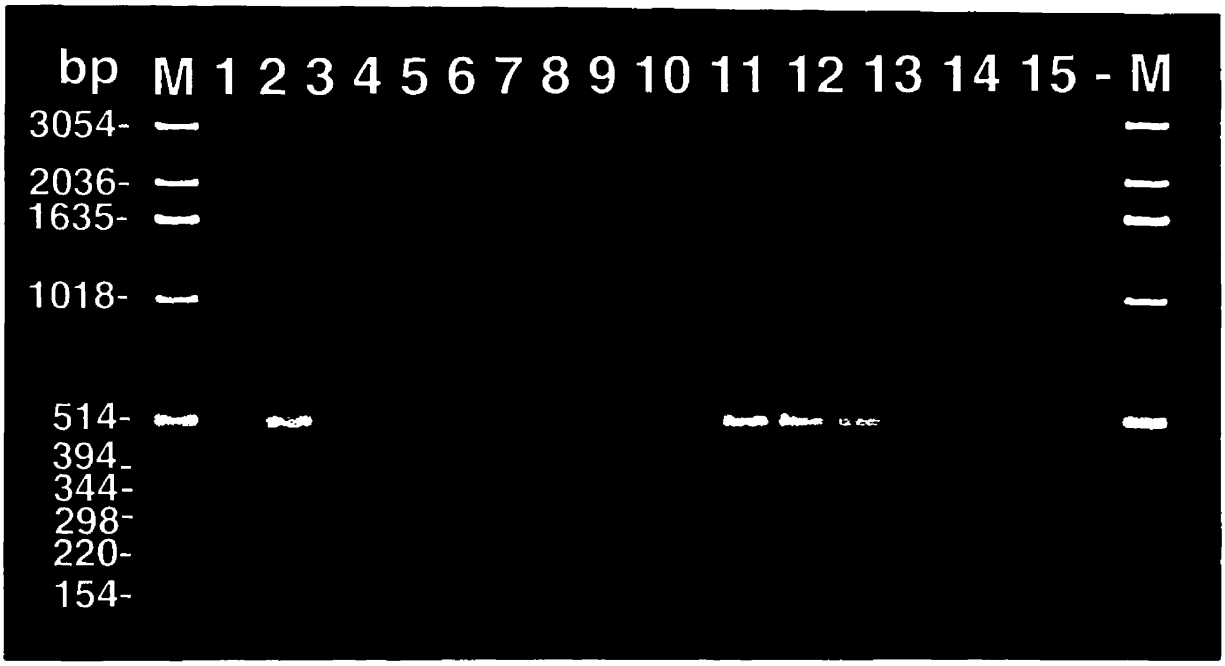

Figure 3.7 Use of genus-specific primer **O.g.** to amplify target DNA from a mixture of total genomic DNAs. PCR product formed only when target *Oidiodendron griseum* (UBC S18) DNA was present (lanes 1-4, 10-12, and 14). The combinations of the genomic templates in the reactions were: Lanes 1-5, mixtures in ng/μl of target *O. griseum* (UBC S18) and Unknown 2 (UBC S246); lanes 1, 6/0; 2, 4.5/1.5; 3, 3/3; 4, 1.5/4.5; and 5, 0/6. Lanes 10-12, mixtures in ng/μl of target *O. griseum* (UBC S18) and Unknown 3 (UBC M8); lanes 10, 4.5/1.5; 11, 3/3; 12, 1.5/4.5. Lanes 6-9, mixtures including non-target DNA with concentrations expressed in ng/μl target Unknown 3 (UBC M8) and Unknown 2 (UBC S246), lanes 6, 6/0; 7, 4.5/1.5; 8, 3/3; 9, 1.5/4.5. Lane 13, mixture included only salal DNA at a concentration of 6 ng/μl. Lane 14, mixture included salal DNA and target DNA *O. griseum* (UBC S18) with DNA concentrations of 3 ng/μl each. Lane 15, mixture included non-target DNA of Unknown 3 (UBC M8) and salal DNA at a concentration of 3 ng/μl each. Lane (-) = negative control. M = molecular size marker.

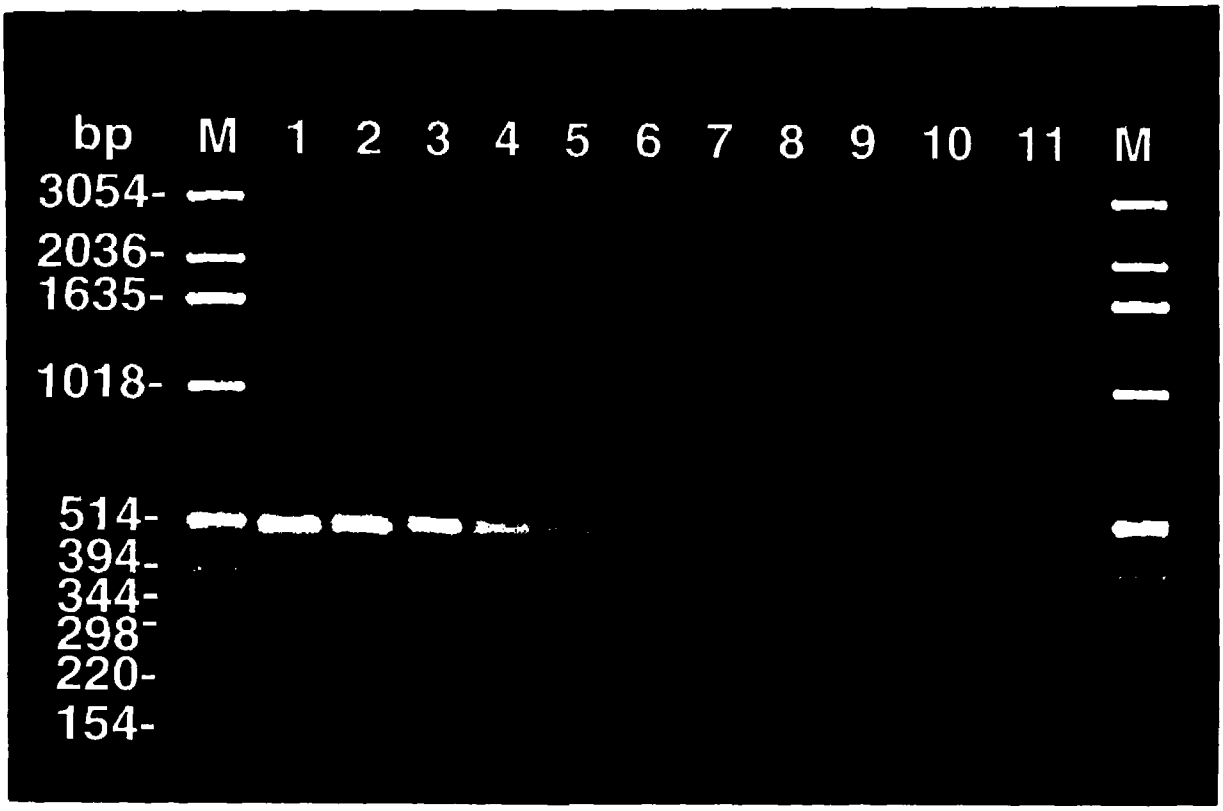

Figure 3.8 Establishing the minimum concentration of *Oidiodendron griseum* genomic template for successful amplification with the primer combination **O.g. /** and **ITS1-F.** DNA concentrations in the PCR reaction mixture, in ng/ μl, were: lane 1, 3.0; 2, 1.5; 3, 0.5; 4, 0.25; 5, 0.05; 6, 0.025; 7, 0.005; 8, 0.001; 9, 2×10^{-4}, 10, 4×10^{-5}, 11, 8×10^{-6}. The minimum concentration for amplifications was 0.025 ng/μl. M = Molecular size marker.

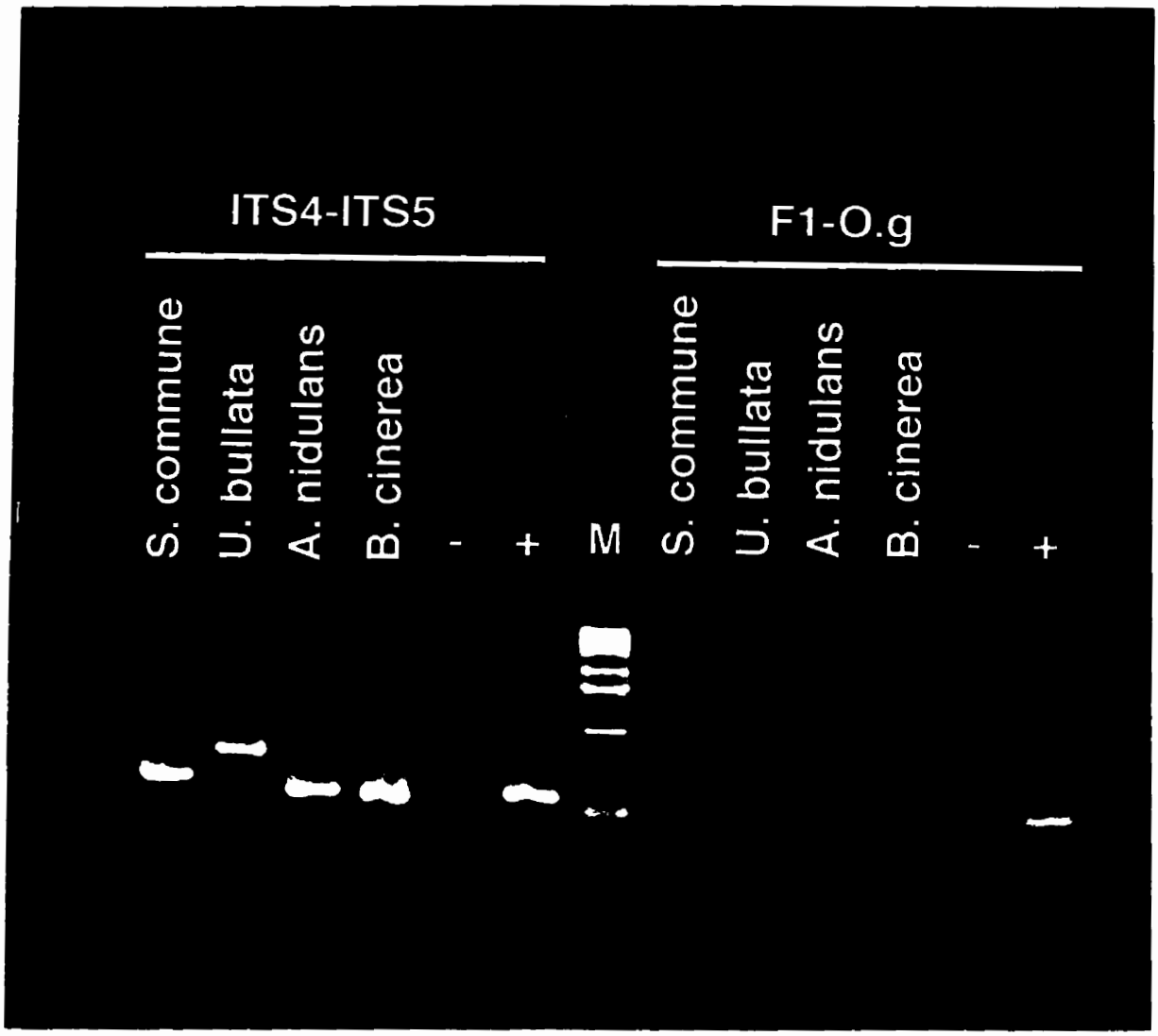

Figure 3.9 Universal primers **ITS4** and **ITS5** amplified DNAs from diverse fungi (lanes 1-4, 5); the genus specific primer combination **O.g./ITS1-F** amplified only target *O. griseum* DNA (lane 13). + = positive control, genomic DNA of *O. griseum*; - = negative control, no DNA; the test genomic DNAs were from: *Schizophyllum commune, Ustilago bullata, Aspergillus nidulans,* and *Botrytis cinerea.*

amplify a broad number of taxa within the ***Hymenoscyphus* group**. In contrast, primer **M8** did not amplify DNA of the taxon S246, indicating a higher degree of specificity within the ***Hymenoscyphus* group**. Primers **S246** and **M8** did not produce PCR product when tested with DNA of *O. griseum*. PCR products of artificial mixtures of each of the three taxa with salal DNA, as shown by length of bands in electrophoresed agarose gels, indicated that only the fungal component of the mixture was amplified each time. Therefore, salal DNA would not affect the performance of any of these new primers.

Tests of primer O.g. with fungi of diverse taxa.

Positive PCR products were obtained with DNAs of the fungal isolates of diverse taxa when amplified with the universal primers ITS1 and ITS4. When primers **ITS1-F** and **O.g.** were tested with a set of DNAs from diverse fungi, PCR products were obtained only when the fungus taxon present was *O. griseum* (Table 3.4, Fig. 3.9).

Discussion

Phylogenetic groups generated by data analysis

This molecular phylogenetic analysis of ericoid mycorrhizal fungal isolates supports the separation of the ericoid mycorrhizal isolates into two principal groups: the *Oidiodendron* group and the *Hymenoscyphus* group (Fig. 3.3, 3.4). In addition, the unusual ericoid mycorrhizal fungus *Acremonium strictum* W. Gam constitutes a third distantly related group. The three separate

clusters are consistent with morphological taxonomy, which would place *Oidiodendron* and teleomorphs *Myxotrichum* and *Pseudogymnoascus* in the Onygenales (Currah, 1985), *Hymenoscyphus* in the Leotiales (Korf, 1973) and *Acremonium strictum* probably in the Hypocreales (Kendrick and Carmichael, 1973).

The ITS2 region is highly variable. The high variability makes the region useful for recognizing closely-related species and for designing primers but it also leads to alignment ambiguity and convergent substitutions among sequences of distantly-related taxa. Where clusters in Fig. 3.3 conflict with other morphological and sequence-based phylogenies, high levels of sequence divergence are probably to blame. In the trees from the ITS2 regions, for example, *Hymenoscyphus* and *Sclerotinia* do not cluster together, even though they are both in the Leotiales (Korf, 1973). *Penicillium* and allies do not cluster with *Oidiodendron* and the Onygenales, even though they are all plectomycetes (Fennell, 1973). The polytomy at the base of the bootstrap tree (Fig. 3.4) reveals the lack of support for branching order of the deeper divergences.

Rooting the trees

Trees are shown with the root at the base of the pyrenomycetous taxa (Fig. 3.4) or midpoint rooted among the pyrenomycetes (Fig. 3.3). With the possible exception of *Cladosporium fulvum*, the isolates in the data set probably belong to the pyrenomycetes, the Leotiales or the plectomycetes. Based on the much longer and more conserved 18S rRNA gene sequences, the plectomycetes are clearly

monophyletic and Leotiales are probably also monophyletic (Gargas et *al.*, 1995) and therefore the root should not be within one of these higher groups. However, which groups should be basal remains unclear and placing the pyrenomycetes at the base was arbitrary.

Phylogenetics and the identity of the unknown ericoid mycorrhizal fungi

Sequences of five groups of sterile mycelial isolates (Unknowns 1-5) did not match any known fungi but the phylogenetic trees and sequence similarities suggest that they are probably Leotialean fungi related to *Hymenoscyphus*. Fungal isolates in the same species, and fungi in closely related species usually have almost identical ITS2 sequences. In our data set for example, the basepair differences between pairs of *Oidiodendron* species including *O. griseum, O. citrinum,* and *O. maius* were less than 1%. Small percent differences have also been reported for closely related taxa of *Armillaria* (Anderson and Stasovski, 1992). The differences among tree*Laccaria* species varied between 3% and 5%, and 1 to 2% within L. bicolor (Gardes *et al.*, 1991). The ITS regions of isolates *H. ericae* and its possible anamorph *S. vaccinii* differed by less than 3.5% (Egger and Sigler, 1993). Similarly, sterile fungi **Unknown 3** and **Unknown 4** differ from *H. ericae* by less than 3%, suggesting that they may be congeneric, or perhaps even conspecific, with *H. ericae.*

In contrast, the pairwise percent differences in our ITS2 data set among the sterile fungi **Unknown 1** and **Unknown 2** were high. *Hymenoscyphus ericae* was the closest teleomorph to **Unknown 1** and **Unknown 2** (Fig. 3.3, 3.4). However,

Unknown 1 and **Unknown 2** sequences differed from that of *H. ericae* by over 8%. In RFLP analysis (Ch.2), sterile mycelial isolate UBC M5 ITS region digests were identical to digests from **Unknown 2.** However, because sequence analysis showed that UBC M5 differed from **Unknown 1** and **2** by over 8% and from *H. ericae* by over 7%, the isolate was given its own group, **Unknown 5.** The phylogenetic trees show that the **Unknown 1, 2,** and **5** isolates probably belong to the ***Hymenoscyphus* group** (Fig. 3.3, 3.4). Possibly, the unknowns are all isolates of *H. ericae* and the species just has unusually high ITS2 substitution levels. If the correlation between ITS2 substitution levels and species limits are typical for fungi, however, Unknowns 1, 2, and 5 are not conspecific with each other, with *H. ericae* or any other identified ericoid mycorrhizal species. As the data base of sequences for identified Leotiales improves, it may become possible to identify the unknown isolates with confidence. At present, the unknowns serve to suggest that a cluster of Leotiales species distinct from *H. ericae* may be important in formation of ericoid mycorrhizae.

In the ***Hymenoscyphus* group,** the species *Hymenoscyphus ericae* is placed in the Leotiales (Read, 1974) and *Scytalidium vaccinii* Dalpé, Litten and Sigler was inferred as its possible anamorph based on molecular data (Egger and Sigler, 1993). In our analysis of ITS2 sequences, *S. vaccinii,* sterile isolates Unknowns 1-5 (UBC M8, UBC M20, UBC M5, UBC S9, UBC S246), and the ectendomycorrhizal species *Phialophora finlandia* all clustered closely with *Hymenoscyphus ericae.* Reported in Chapter 2, under axenic laboratory conditions, the isolate *P. finlandia* (UAMH 7527) produced typical ericoid mycorrhizal coils when

inoculated into axenically grown salal plants. Our results showing a close relationship between *P. finlandia* and *H. ericae* (Fig. 3.3, 3.4) are consistent with those of Stoyke *et al.* (1992), who reported that based on RFLP analysis of ITS regions, *H. ericae* (DAOM 185550) and *P. finlandia* (FAG-15) differed by fewer than 0.01 nucleotide substitutions per site. The authors found this result puzzling since colony morphology in the two species differs and *H. ericae* produces arthroconidia while *P. finlandia* produces phialoconidia.

Within the ***Hymenoscyphus* group**, sterile isolates Unknown 1 UBC S9 and Unknown 2 UBC S246 are particularly close to *P. finlandia* (Fig. 3.3; 3.4). Whether the Unknown 1 and 2 would resemble *P. finlandia* producing phialoconidia and ectendomycorrhizae with forest trees under appropriate conditions remains an open question. It might help, in order to provoke sporulation of the ericoid mycorrhizal sterile isolates, to keep them at zero degrees for six months in the refrigerator, in a similar manner that non-sporulating species were treated by Wang and Wilcox (1985). Wang and Wilcox (1985) placed 6-week-old cultures of sterile isolates that formed ectendomycorrhizae with pine on MMN agar plates and incubated them at low temperature (5 C) for 6 months to a year. Once sporulation was achieved, they described the species *Phialophora finlandia* and *Phialocephala fortinii.*

The genus *Acremonium* has over 100 species and they are ubiquitous in soils and plants (Domsch *et al.*, 1980). Because they are common in soil, the saprophytic *Acremonium* species would have to be screened out to permit molecular detection of the mycorrhizal species. Molecular phylogenetic studies

of the genus are in progress and the ITS sequence data base may soon be complete enough to design specific primers for the ericoid mycorrhizal *Acremonium* species (Glenn *et al.*, 1996).

The most parsimonious tree obtained with sequences analysis of the ITS2 region alone resolved the relationships among the ericoid mycorrhizal fungi of the ***Hymenoscyphus* group**, the ***Oidiodendron* group**, and the endophytic fungi of the genus *Phialocephala*. However, within each group, some branches of the tree did not have enough support to define the relationship among species of the group. To overcome that problem, the sequences of the ITS1 region will help to strengthen the analysis. Also, the analysis was not sensitive enough to show the relationship among the ***Hymenoscyphus* group**, the ***Oidiodendron* group** and the other groups of Ascomycetes. To define the relationship to other fungi from the Ascomycetes, sequences of the ITS1 and ITS2 region should be included in the phylogenetic analysis.

Ericoid mycorrhizal group specific primers

Newly designed specific primers selectively amplified the target DNA of fungal taxa of closely related groups, the ***Oidiodendron* group** and the ***Hymenoscyphus* group**. Primer **O.g.** specifically amplified only target DNA when tested in DNA mixtures of ericoid mycorrhizal fungi, i.e.; it did not amplify DNA of the ***Hymenoscyphus* group**, or DNA of a diverse selection of non-mycorrhizal fungi. Primer **M8**, amplified only target DNA, which included DNAs from *H. ericae* and Unknowns 3 and 4. Primer **M8** did not amplify DNAs

from Unknowns 1 and 2, the other taxa in the ***Hymenoscyphus* group**. Primer S246 was less specific and amplified DNAs of all the isolates tested from the *Hymenoscyphus* group. Primer **S246** can be used in sets of isolates involving a wider range of diversity of taxa represented in the ***Hymenoscyphus* group,** while primer **M8** may detect more readily taxa closely related to the species *Hymenoscyphus ericae.*

Use of these sets of primers might allow a faster screening of new isolates from forest and alpine ecosystems, in which plant species belonging to the Ericales are present. Furthermore, since most of the isolates of the ***Hymenoscyphus* group** remain sterile under laboratory conditions, primers **S246** and **M8** can be used as a molecular tools for detection alongside the traditional cultural and morphological techniques. At the same time, there is potential to further explore the use of these primers to detect related taxa present in root systems of other plant species.

However, with the exception of primer **M8,** the specificity of these primers is at the genus level, therefore the molecular methods to confirm the taxon identity from ericaceous roots taken from the field will have to be more stringent. Based on RFLP patterns from salal roots sampled at the SCHIRP site (Chapter 2), a large fungal diversity was detected, indicating the possibility that related taxa might be present in close proximity in the same root system (5 mm) and therefore more than one closely related fungal taxa DNA could be co-amplified.

Chapter 4

Synthesis

Territories occupied by evergreen dwarf-shrubs largely belonging to the Ericaceae are known as heathland. Ericaceous heaths occur in Europe, Africa (fynbos), Australia (kwongan), and North and South America (chaparral) (Read, 1991; Straker, 1996). A combination of geography (high altitudes and latitudes), climate (relative cool temperature regimes), soils (organic layer of mor humus type and/or podzolic inorganic soil types), and biotic (vegetation) and anthropogenic (deforestation) factors affect the formation and dominance of ericaceous heathlands (Gimingham, 1972; Read, 1991). Soils of such ericaceous systems have low pH, loss of base cations, accumulation of organic matter due to inhibition of mineralization, high content of organic acids, high C/N ratios and high solubility of metallic cations (Read, 1991). Since rates of ammonification and nitrification are low or completely inhibited in these systems, vegetation growth can be limited by the lack of inorganic forms of the major essential nutrients such nitrogen (N) and phosphorus (P) (Read, 1991). The association of mycorrhizal fungi with ericaceous plants is mainly a nutritional benefit since the ericaceous fungi access elements fixed in organic forms (Bajwa *et al.*, 1985; Read, 1984; Leake and Read, 1989).

In one coastal forest ecosystem of British Columbia, salal (*Gaultheria shallon* Pursh) becomes the dominant species on reforestation sites in which the previous major overstory species, western red cedar (*Thuja plicata* Donn) and of western hemlock (*Tsuga heterophylla* (Raf.) Sarge) have been clear cut and slashburnt. Reforestation efforts on such sites have been hampered by poor growth of conifers (Sitka spruce, western hemlock , western red cedar and Amabilis fir), as indicated by

symptoms such as chlorotic foliage and minimal height growth (Prescott and Weetman, 1994). To find a solution for this problem, the Salal Cedar Hemlock Integrated Research Program (SCHIRP) was initiated in an area of northern Vancouver Island. As part of the SCHIRP project, ericoid mycorrhizal fungi associated with salal roots were isolated (Xiao, 1994). Xiao (1994) isolated and identified the ericoid pseudomycorrhizal fungus *Acremonium strictum* W. Gams and three ericoid mycorrhizal fungi. One of these taxa was identified as *Oidiodendron griseum* Roback and the other two fungi remained sterile under laboratory conditions and were designated as **Unknown 1** and **Unknown 2.** *In vitro* studies of these isolates demonstrated that they can use organic forms of N, that the ericaceous mycorrhizal fungi were antagonistic to some ectomycorrhizal fungi of western hemlock, and it was proposed that they therefore may contribute to the dominance of salal in these systems (Xiao, 1994).

In comparison to other groups of mycorrhizal fungi, the ericoid mycorrhizal fungi are composed of a relatively small number of taxa, although taxonomically diverse (Starker, 1996). They are found in the Ascomycetes, including species in the order Helotiales and Onygenales (Pearson and Read, 1973, Dalpé, 1989). The best known species in the Helotiales is *Hymenoscyphus ericae* (Read) Korf & Kernan (Read, 1974). In North America several isolates have been putatively classified as *H. ericae* , although the apothecia have never been observed (Litten *et al.*, 1985). In addition, some taxa are Hyphomycetes, including species of *Oidiodendron, Scytalidium* and *Acremonium* (Dalpé, 1986, 1989, 1991; Xiao, 1994). Some sterile mycelia have also been reported to form ericoid mycorrhizae (Haselwandter, 1987; Pearson and Read, 1973; Perotto *et al.*, 1994, 1995; Xiao, 1994). Finally, there is some

indirect evidence of an ericaceous mycorrhizal association of a basidiomycete with *Rhododendron* plants (Mueller *et al.*, 1986).

So far primarily cultural and morphological techniques have been used to detect fungal taxa in mycorrhizae of ericaceous plants. However, ericoid mycorrhiza fungi grow slowly in culture compared to potential contaminants and many remain sterile under laboratory conditions which can limit the success of this approach (Read, 1983). Practical laboratory constraints make it difficult and time-consuming to process and to test the mycorrhizal potential of a large number of isolates, and usually only a sub-sample of all isolates is tested. When isolates remain sterile, they are usually selected only based on colony morphology. At present, the exact number of ericoid mycorrhizal fungal taxa is unknown (Straker, 1996) and we may be aware of a small percentage of them. In the present study, molecular techniques were used to study known ericoid mycorrhizal fungi with the aim to explore their possible application to the assessment of biodiversity, population dynamics and community structure of these fungi.

The polymerase chain reaction (PCR) that allows amplification of minute amounts of genomic DNA was used in this study (Mullis and Faloona, 1987). Restriction fragment length polymorphism (RFLP) and sequence analysis of the ITS1 and ITS2 region of the nuclear ribosomal RNA genes were used to identify ericoid mycorrhizal fungi and other endophytic fungi.

RFLP patterns segregate all known ericoid mycorrhizal fungi and various endophytic fungi into 14 Groups. The four morphological groups of fungi isolated by Xiao (1994) and two additional isolates obtained by Monreal at the SCHIRP site segregated into six different RFLP groups. This indicates that RFLP techniques could

be useful to search for known ericoid mycorrhizal fungi from reforestation sites dominated by salal.

Six *Oidiodendron* species generated only three RFLP patterns suggesting some degree of polymorphism within the genus. These patterns included *O. griseum, O. citrinum* and *O. maius* in RFLP Group 11, *O. flavum* and one isolate identified as *O. griseum* (UAMH 4080) in Group 12, and included *O. periconioides* and *O. scytaloides* in RFLP Group 13. The close relationship of *O. griseum, O. maius* and *O. citrinum* was later confirmed by sequence analysis. Also, a new isolate (UBC M10) obtained from the SCHIRP site produced RFLP pattern of Group 11 and sequence analysis that clustered it close to *Oidiodendron maius*. Sequence analysis confirmed that the isolate *O. griseum* (UAMH 4080) clustered closely to *O. flavum*, suggesting that the isolate had been misidentified.

At the SCHIRP site, twenty new isolates were all obtained from fragments of the same root system. RFLP results and axenic test with inoculated salal showed that five isolates formed ericoid mycorrhizae with salal and presented four different RFLP patterns, two previously known and two new ones. These results suggested that a relatively large number of taxa might be present in a fairly small amount of mycorrhizal roots. Additional direct PCR reaction and RFLP analysis of fungi from fragments of field roots confirmed that a large fungal biodiversity might be associated with salal roots. RFLP's of field root fragments produced many bands for each fragment analyzed indicating that two or more fungi were present in each selected mycorrhizal fragment.

Results indicated that the RFLP synoptic key created as part of this study will be useful to identify new isolates to the genus level. Morphological characterization

would have to be used to confirm the final identity of the fungus. RFLP analysis of synthetic mycorrhizae using more than one isolate might confirm the capacity of many fungi to associate with same ericaceous root system. The RFLP synoptic key could be useful in that case since the band lengths of the isolates used would be known.

Six isolates of *Hymenoscyphus ericae* segregated into four RFLP groups, suggesting large polymorphism within that species or misidentification of isolates. From four of the North American isolates of *H. ericae* only one had the same RFLP pattern as the European isolates (UAMH 6735 and READ, D J. 101) that apparently represent authenticated material. Large polymorphism among the *Hymenoscyphus ericae* isolates has been attributed in the past to the presence of insertions (Egger and Sigler, 1993). Egger *et al.* (1995) demonstrated that one isolate, UAMH 6562, had a large putative group I intron in the 5' end of the 18S gene. This intron would be included in the DNA region I amplified with primers ITS1-F and ITS4. Introns can be lost or gained through horizontal transfer and the presence or absence of the intron could explain the length variation among strains of *H. ericae.* Intron sequence could explain the diversity of RFLP patterns found in *H. ericae* isolates.

Analysis of sequence data showed that all known ericoid mycorrhizal fungi can be classified into two main groups: the ***Oidiodendron*** group and ***Hymenoscyphus*** group, with the exception of the pseudomycorrhizal fungus*Acremonium strictum* . Some species of the *Oidiodendron* group had pair wise percent differences of less than 1%. However, the ***Hymenoscyphus*** group branched into two sub-clusters. Molecular analysis suggest the species *Hymenoscyphus ericae , Scytalidium vaccinii* and two sterile isolates from the

SCHIRP site, Unknown 3 (UBC M8) and Unknown 4 (UBC M20), may be congeneric or conspecific. Interestingly, the second sub-cluster of the ***Hymenoscyphus*** group included the species *Phialophora finlandia* which clustered with several sterile isolates from the SCHIRP site including the taxa Unknown 1 (UBC S9), Unknown 2 (UBC S246), and Unknown 5 (UBC M5). Based on the typical ITS2 substitution levels for fungi, the fungi Unknown 1 (UBC S9), Unknown 2 (UBC S246), Unknown 5 (UBC M5), *H. ericae* and *P. finlandia* are not conspecific with each other. However, the molecular data indicating a close relationship among *Phialophora finlandia* and the sterile isolates of the SCHIRP site is important since it suggests that the techniques used by Wang and Wilcox (1985) to encourage sporulation of *P. finlandia* could be used to provoke sporulation on the sterile mycelia. This finding might have important ecological implications since *P. finlandia* is a known ectendomycorrhizal fungus. Some suggestion from this study that it might form ericoid mycorrhizae needs to be confirmed. At the same time the potential of the ericoid mycorrhizal sterile isolates to form ectendomycorrhizae with forest trees could be tested.

Sequence data was used to create ericoid mycorrhiza fungal specific primers. Primer tests indicated that the selected primers for the two sub-clusters of the ***Hymenoscyphus*** group, primer **S246** and **M8**, had different specificity within the targeted species of the ***Hymenoscyphus*** group. Primer **S246** amplified taxa of both sub-groups. Primer **M8** amplified DNAs of taxa closely related to the species *H. ericae* and it did not amplified taxon S246. Primer **O.g.** proved to be genus-specific and selective when tested with DNA of diverse fungal taxa.

The DNA sequences produced in this study of the ericoid mycorrhizal fungi could be used in the future to create fungal specific probes to be used under more stringent conditions. Also the sequences can be used as a data-base, alongside the RFLP synoptic key, to confirm the identity of new isolates of ericoid mycorrhizal fungi.

Communities of plants and mycorrhizal fungi form complex relationship and they are dependent on edaphic conditions where these root-fungal relationships develop. However, mycorrhizal relationships in complex edaphic conditions are difficult to study. Available molecular techniques can help disclose some of the unknowns of these interactions. Phylogeny, taxonomy, biodiversity, and ecology of each of the groups of mycorrhizal fungi can be addressed in a manner similar to that used in research presented here. New findings will provide an expanded database on which to rely to expeditiously evaluate the presence of fungal isolates associated with roots of ericaceous plants in reforestation sites and in undisturbed forest and alpine ecosystems.

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Appendix 1

Purification protocol for PCR products

This is a modified protocol to obtain high yields of pure PCR-DNA fragments (Qian, L. And M. Wilkinson. 1991), to be used with Taq Terminator Chemistry (Nucleic Acid_Protein Service Unit, University of British Columbia, Canada). The use of glass wool columns was introduced by Mr. Dean S. Mulyk (Ph.D. Student, Department of Botany, University of British Columbia, Canada).

Use 1.5 % Sea Plaque low melting agarose (FMC Bioproducts Rockland ME). When casting the gel, use combs big enough to hold 35 µl (x3 wells), 50 µl (x2 wells) or a 100µl well. The goal is to obtain between 1 to 5 µg of clean PCR products. Electrophorese at 80 volts for approximately 30 minutes. Stain the gel with EtBr (0.5 µg/ml) in a 100 ml bath for 10 minutes. Rinse in a distilled water bath for 10 minutes.

Prepare the glasswool filtration columns while electrophoresing the gel as follows: pierce the bottom of a 0.5 ml microcentrifuge tube with a 25 Gauge needle. With a pair of sterilized forceps, pack a small amount of autoclaved glass wool at the bottom of the tube. Place the 0.5 ml tube inside a labelled 1.5 ml microcentrifuge tube. Locate and cut the band(s) using a UV light transilluminator (use transparency to protect surface of transilluminator). When cutting the band, keep only fragments containing DNA and exclude as much of the excess agarose as possible. Place the slice(s) containing DNA into a 1.5 ml microcentrifuge tube. Depending on the amount of gel to be processed, use between 150 and 250 µl of TE buffer (10 mM TRIS at pH. 8.0 and 1 mM EDTA at pH 8.0).

To melt the gel, incubate the tube at 60 C for 10 minutes. Then tap the tube 6 times vigororously. Quick freeze the sample for 5 to 10 minutes (overnight if you need to) in a -70 C freezer. Thaw the sample and vigorously tap the tube to disrupt the gel structure. Centrifuge the sample at maximum speed (13,000 x rpm) for at least 1 minute. Look through the tube against the light to locate the agarose pellet. If some of the gel has not precipitated completely into a pellet, centrifuge again for a longer period of time (2 to 3 min.). The supernatant contains the DNA.

The supernatant is pipetted out from the microtube and put into the glass wool filtration columns. This is then spun at 3000 rpm for 45 seconds. To obtain close to 100% of the DNA use a "melted pipette tip (size P1000) to mash the gel pellet with 50 to 100 μl TE buffer. Place used glass wool column on a new label 1.5 microcentrifuge tube. Spoon the mashed agarose on top of glass column using the melted pipette tip. Repeat spinning process (3000 rpm for 45 sec). Collect all filtrates of a sample in one 1.5 ml microtube.

To precipitate the DNA add 3M NaAc (pH 5.2) at a rate of 10 % of the total filtrate volume and isopropanol (iced-cold) at a rate of 80 % of total filtrate volume. Mix the sample by inverting the capped tube several times (5x). Leave the sample sit at -20 C overnight (or at least 4 hours). Centrifuge the sample at maximum speed (13,000 rpm) for 15 to 40 minutes, preferable on refrigerated centrifuge (4 C). Pour off supernatant. Wash the DNA pellet with ice-cold 70% ethanol. Pour off supernatant. Vacuum dry the pellet (approximately 5 to 15 minutes). Care should be taken not to over dry the sample. Heat should not be applied when drying the pellet.

Resuspend the pellet in desire amount of milli-Q water (20 to 30 μl) depending on the concentration needed. The protocol of the 373 DNA Sequencer requires DNA suspended in water.

Calculate your DNA concentration by using a spectrophotometer equipped with a microcuvette which only requires 65 μl volume of sample (5 μl of suspended DNA in 60 μl distilled water). Another 5 μl of concentrated DNA can be run along side with a DNA marker with bands of known molecular weight, on 1 or 2 % agarose gel. Your sample is now ready to be sequenced. To the sequencing laboratory you need to send microtubes with the DNA templates and primers required for the sequencing reaction, specifying clearly their respective concentrations.

The Applied Biosystems, Inc. Amplitaq DyeDeoxy Terminator Cycle Sequencing provide a four base reaction premix of 9.5 μl. Total volume of the reaction is 20 μl, therefore there are 11.5 μl left to add the DNA template and primer. To obtain consistent results, keep primer (in excess of 3.2 pmoles) concentration and volume content for a given length of template in base pairs (Table A).

Table A

Length of DNA template (bp)	Amount of template (ng) to use with 3.2 pmoles primer mass.
100	10
200	20
300	29
400	39
500	49
600	59
700	69
800	78
900	88
1000	98
1100	108
1200	117
1300	127
1400	137
1500	147

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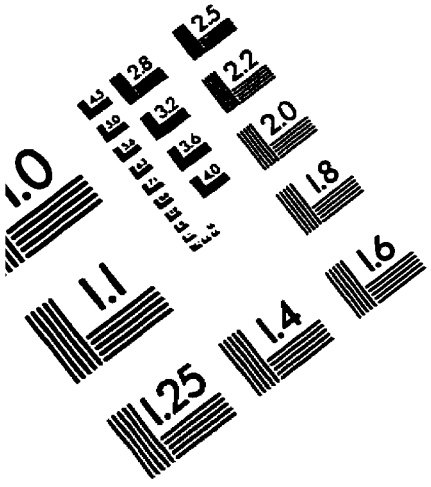
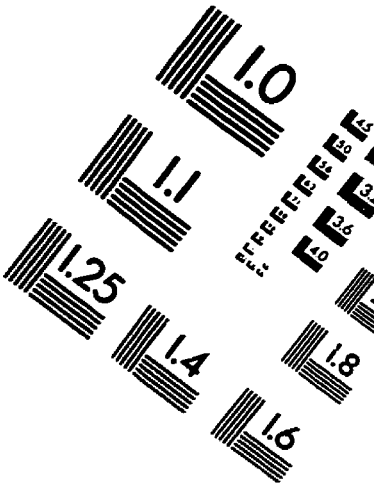
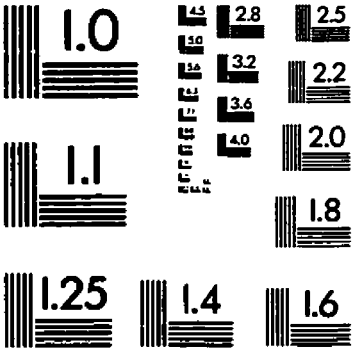
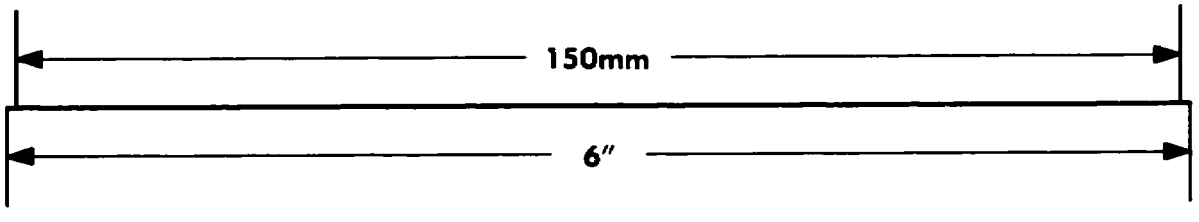
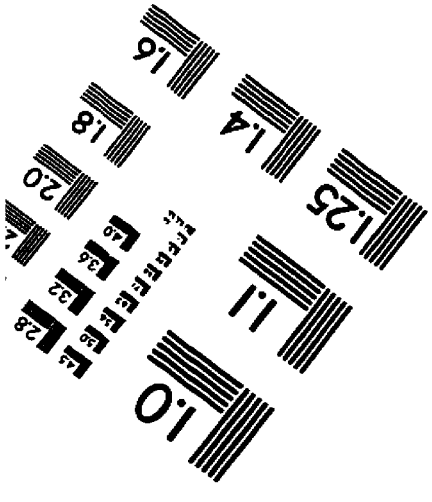
IMAGE EVALUATION
TEST TARGET (QA-3)

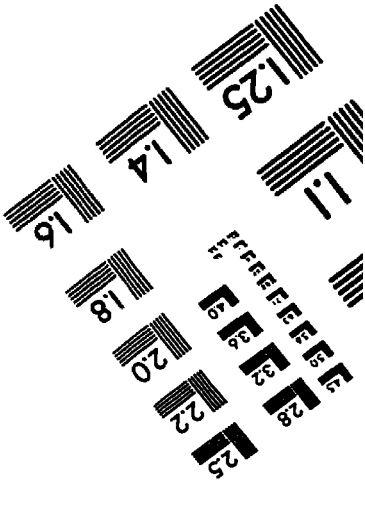












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