Identification and Community Profiling of *Vaccinium membranaceum* Root-associated Fungi Over an Elevation Gradient in BC's Eastern Rocky Mountains

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Abstract

Root-associated fungi, including ericoid mycorrhizas, are important to the health of alpine and northern forest ecosystems. These symbiotic root-fungus associations form with shrubs in the family Ericaceae. This is the first report of an attempt to profile the fungal community structure associated with roots of *Vaccinium membranaceum* (huckleberry) over a biogeoclimatic (BEC) zone elevation gradient in BC's eastern rocky mountains. Four biogeoclimatic zones were targeted on McBride peak: the alpine, ESSF, ICH and SBS. Fungal associates were grown in culture and fungal DNA analyzed directly from the roots. Both techniques generated community profiles that demonstrated differences in root-associated fungal community structure between each of the four zones. The higher elevation alpine and ESSF hosted fungi known to form ericoid mycorrhizas. Lower elevation ICH and SBS tended to host more common fungi. The diversity patterns generated have potential implications in climate change.

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List of Abbreviations

AM: arbuscular mycorrhiza
BC: British Columbia
CCA: Canonical Correspondence Analysis
CWD: coarse woody debris
DNA: deoxyribonucleic acid
ECM: ectomycorrhiza
ESSF: Engelmann spruce sub-alpine fir
ERM: ericoid mycorrhiza
FWD: forest woody debris
ICH: Interior cedar hemlock
ISSR-PCR: inter simple sequence repeat polymerase chain reaction
LH-PCR: length heterogeneity polymerase chain reaction
LMA: Leaf mass to area ratio
NMS: Nonmetric multidimensional scaling
NTFP: non-timber forest product
PerMANOVA: permutational multivariate analysis of variance
RFLP: restriction fragment length polymorphism
SBS: sub-boreal spruce
UPGMA: unweighted pair group method with arithmetic mean
VPT: variable percentage threshold

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Chapter 1

"One of the main reasons the world faces a global environmental crisis is the belief that we human beings are somehow separate from the natural world in which we live, and that we can therefore alter its physical, chemical, and biological systems without these alterations having any effect on humanity"

Kofi Annan

"In myriad ways humanity is linked to the millions of other species on this planet. What concerns them equally concerns us. The more we ignore our common health and welfare, the greater are the many threats to our own species. The better we understand and the more rationally we manage our relationship to the rest of life, the greater the guarantee of our own safety and quality of life"

E.O. Wilson

Introduction

This work is motivated by and entrenched within the idea that understanding ecosystems benefits humanity. Regardless of how humanity chooses to see ecosystem services, we are at the mercy of the ecology that surrounds us. The ecosystem can thrive without us, yet we cannot survive without it.

With the awareness of potential impacts of global climate change (IPCC 2007), it is becoming more crucial than ever to increase fundamental understanding of ecosystem processes so that we may predict and possibly mitigate the inevitable changes. The soil in boreal and northern temperate climates is storing a sizable portion of the earth's carbon (Smith and Read 2008). Since these forests cover 70% of the northern hemisphere, their ecosystems are globally important (Read 2002). Soil dynamics, including in large part mycorrhizal symbioses, are an important part of this globally crucial system.

Researchers in ecology and soil science have generally accepted the role of symbiosis in root systems (Sapp 2004), and studies exploring the structure and function of mycorrhizas have flourished. It has become evident that associations of fungus and plant roots as partners have the potential, in some ecosystems, to drive above ground diversity (Read *et al.* 2004). In nitrogen limited boreal and heath environments, mineralization of nitrogen is reduced by the presence of plants capable of intercepting nitrogen from freshly senescing plant matter; an ability conferred to plants through ericoid and ectomycorrhizal associations. Thus fungal symbioses drive the competitive exclusion of plants that do not have mycorrhizas, or are otherwise able to access nitrogen via fixation, in these ecosystems (Read *et al.* 2004). Mycorrhizas as drivers of ecosystems thus warrant intensive study.

Ericoid mycorrhizas (ERM) are fungal symbioses formed with roots of most members of the order Ericales, including Ericaceae and Epacridaceae (Peterson *et al.* 2004). These plants form an important component of the understory of northern temperate and boreal forest and dominate in alpine, arctic and heath environments (Smith and Read 2008). Of these, black huckleberry (*Vaccinium membranaceum* Dougl. Ex Torr.) is a non-timber forest product (NTFP) that has eluded cultivation. Huckleberry fungal associations may be at the root of this.

Mountain slopes provide a natural gradient of abiotic factors that may be exploited for the study of mycorrhizal symbiosis. A slope face may have similar plants throughout that are growing under very different above and below ground conditions. Because of the variability in precipitation and temperature, mountain slopes often consist of belts of biogeoclimatic zones. These mountain slopes are excellent proxies for ecological structuring over large latitudinal spreads. An elevation gradient may have an alpine tundra landscape at its peak and be dense with trees and understory vegetation at its base. At McBride peak (McBride, BC), *V. membranaceum* is found from the base of the slope to the top of the mountain allowing for the exploration of the impact of the different biogeoclimatic zones and associated abiotic factors on the *V. membranaceum* root-associated fungal community. Differences of adaptation to the plants' particular environment are expressed not morphologically but within the context of their fungal symbionts. Furthermore, changes in community structure may represent potential future shifts in response to climate change. As temperatures increase biogeoclimatic zones are generally expected to shift up mountain slopes and northward, and as such plant communities and their soil microorganisms can be expected to change with them. The

results of this study will contribute to understanding the potential range of changes within soil ecosystems in response to climate change.

The objectives of this study were:

- To assess changes in fungal diversity of *Vaccinium membranaceum* root-associated fungi over an elevation gradient as defined by targeting four biogeoclimatic zones (alpine, ESSF, ICH, SBS) along a mountain slope

and

- To correlate changes in abiotic factors over that elevation gradient to changes in fungal diversity.

Literature Review

Mycorrhizas are a globally important symbiosis formed by soil fungi and plant roots. A fungus colonizes plant root cells and an interface between the two is created; the resultant bidirectional nutritional exchanges potentially confer benefits to both partners. The fungus is better suited to extracting minerals from the soil, its mycelia are orders of magnitude smaller than plant roots and can penetrate areas otherwise inaccessible to plants (Peterson *et al.* 2004), and have specialized enzymes that release nutrients. In return, the plant provides a space for the fungus within its root cells and interstitial spaces, protecting it from a diverse and challenging soil environment that is home to many predators with competing interests. The fungus is heterotrophic whereas the plant is able to photosynthesize and can trade its carbon to the fungus.

The mycorrhizal relationship exists in a continuum of interactions that are important because they impact large scale ecology in natural habitats (Brundrett 2004; Read *et al.* 2004). Mycorrhizal associations can be balanced where both partners benefit

or exploitive where only one partner benefits. When a fungus has no apparent effect on the plant, the relationship is termed endophytic (Brundrett 2004). The most extreme example of exploitive relationships is the achlorophyllous plant that acquires carbon from mycorrhiza via a common mycelial network that connects it to another photosynthesizing plant (Cullings *et al.* 1996). Furthermore, the relationship ranges from obligatory as in the case of AM (arbuscular mycorrhizas), to loose, as is probably the case with dark septate endophytes, where the fungus can thrive within roots but also in the soil or among other plant parts. Common mycelial networks add a significant layer of complexity and connectedness to ecological landscapes. Links can occur between multiple plants and by multiple fungi (Simard and Durall 2004). Furthermore, mycorrhizas are impacted by and have impacts upon herbivore behaviour (Gehring and Whitman 2002) as well as other soil heterotrophs (Gange and Brown 2002). Mycorrhizal associations are important because of their potential range of impacts on plants and ecosystems.

Seven categories of mycorrhiza are recognized, with AM (arbuscular mycorrhiza) being the most widespread, and ECM (ectomycorrhiza) occurring on some gymnosperms, dicotyledons and one monocotyledon genus (Brundrett 2002). Other categories, including arbutoid, monotropoid, orchid and ericoid are restricted to specific plant families (Brundrett 2002). Ericoid fungi are found on the roots of plants in the order *Ericales*, including Ericaceae in the Northern Hemisphere and Epacridaceae in the Southern Hemisphere (Smith and Read 2008). Ericoid mycorrhizas are found in northern temperate and boreal forests and predominate in nutrient poor habitats including heaths, the alpine and sub-alpine regions in mountainous regions as well as the arctic (Read 2002; Smith and Read 2008).

Structure of the ericoid symbiosis

According to molecular clock estimates, the ERM condition arose 140 mya in the Cretaceous period which is consistent with the currently accepted notion that the Ericales originated in that time period (Cullings 1996). Like all mycorrhizal associations, ERM are distinguished by intimate and specific intracellular contact between the fungus and host (Smith and Read 2008).

Hair roots are a characteristic feature of ericaceous plants. These minute lateral roots have no secondary growth and are composed of a narrow vascular cylinder, a layer or two of cortical cells and a single layer of epidermal cells (Peterson *et al.* 2004). Hair roots are typically 100μm to <50μm in diameter (Smith and Read 2008). Epidermal cells are penetrated by fungal hyphae and become enlarged with the increased numbers of organelles such as mitochondria, plastids and endomembrane components, as well as an enlarged nucleus (Peterson *et al.* 2004). Epidermal cells are a feature of young hair roots and disappear as the root ages, they may be sloughed off and act as propagules of the fungus in the soil (Ashford *et al.* 1996). Cortical cells thicken and become the outer surface of the older roots (Smith and Read 2008). Ericoid hyphae penetrate root epidermal cells individually, possibly functioning as separate units (Perotto *et al.* 2002). Conventional wisdom regarding this individual penetration has recently been challenged by observations of lateral hyphal connections between adjacent epidermal cells using confocal scanning laser microscopy (CSLM). It has thus been suggested that one individual hyphal penetration may result in colonization of more than one epidermal cell (Massicotte *et al.* 2005).

Colonization results in an increase in cytoplasmic volume of the root cell. Although the fungus penetrates the cell wall, it does not penetrate the cell plasma

membrane. Once hyphae enter, the fibrillar sheath disappears. Hyphae coil and branch inside the cell forming hyphal complexes (Peterson *et al.* 2004). Two types of hyphal complexes have been observed: loose and compact, the latter densely packed and taking up most of the epidermal cell volume (Massicotte *et al.* 2005). The cell membranes of both plant and fungus remain continuous and their cytoplasms do not mix. An apoplastic compartment between the membranes of both partners is formed (Dexheimer and Pargney 1991). The coiling and branching of these membranes greatly increases the surface area available for nutrient exchange (Peterson and Massicotte 2004). The extent of the colonization will vary, although it is often high in field collected samples. It is hypothesized that there is a high turn-over rate of hyphal complex formation in ERM (Peterson *et al.* 2004).

Initiation of the colonization process is probably fungus and host specific. Plant roots secrete a mucilaginous compound and ericoid mycorrhizal fungal cell walls produce an exocellular fibrillar sheath both probably involved in the recognition process. The specifics of this remain ambiguous (Straker, 1996). The fibrillar sheath disappears from the internal fungal structures after recognition takes place. When fungi encounter a non-host plant, the interaction produces a different deleterious and characteristically necrotrophic response in the plant. *Rhizoscyphus ericae* invasion of *Trifolium pratense* (clover) shows no disappearance of the fibrillar sheath (Straker, 1996). Furthermore, there is no characteristic swelling of the cytoplasm, rather a degeneration of cytoplasm and organelles as well as rupture of the plasmalemma occurs (Bonfante-Fasolo *et al.* 1984). Compatible colonization is thus different from other non-compatible invasions and indicates some level of specificity between the partners.

Ericoid fungi

There are several fungi that are known to form ericoid mycorrhizas. *Rhizoscyphus ericae* (Zhang and Zhuang 2004) of the order Helotiales, Ascomycota (Perotto *et al.* 2002), a teleomorph of the anamorph *Scytalidium vaccinii* Dalpé, Litten & Sigler (Egger and Sigler 1993) was originally described as *Pezizella ericae* then transferred to *Hymenoscyphus ericae. Oidiodendron* spp. with teleomorphs in the order Onygenales (Perotto *et al.* 2002) have also been identified as ericoid mycorrhizas. These taxa are the most commonly identified ERM fungi (Allen *et al.* 2003; Chambers *et al.* 2000; Hambleton and Currah 1997; Monreal *et al.* 1999; Perotto *et al.* 2002; Read 1996; Sharples *et al.* 2000).

There are probably many other fungi that form ERM, but identification of fungi forming these associations is limited by the lack of distinguishing observable morphologies as is typical in ECM associations. Fungi can be isolated from roots and grown in culture. While identification and manipulation of the specimen is easy with this approach, it excludes fungi that do not grow on laboratory media. Analyzing DNA directly extracted from roots circumvents the culturing issue, but creates its own set of challenges. There is, for instance, no reference sample with associated morphology. Berch *et al.* (2002) found that *Gaultheria shallon* (Pursh.) was predominately colonized by an unculturable basidiomycete. Therefore, the finding that *Rhizoscyphus ericae* and *Oidiodendron* spp. are often isolated from ericaceous plant roots does not preclude the existence of other common ERM forming fungi that are unculturable.

Rhizoscyphus ericae can be an attractive partner for an ericaceous plant due to its metabolic diversity. *Rhizoscypus ericae* has been shown to scavenge both inorganic nitrogen (as ammonia and nitrate) and phosphorus (Read 1996). *Rhizoscyphus ericae*

has been shown to produce many extracellular enzymes that degrade structural components of plant litter (Read and Perez-Moreno 2003) providing a source of otherwise inaccessible organic nitrogen and phosphorus. When necessary, this fungus can obtain its own carbon from pectins and lignins (Peterson *et al.* 2004). *Rhizoscyphus ericae* is able to tolerate water stress (Chen *et al.* 2003) and furthermore, can tolerate heavy metals such as arsenic (Sharples *et al.* 2000), cadmium (Perotto *et al.* 2002), zinc (Martino *et al.* 2000) and copper (Gibson and Mitchell 2005, Monni *et al.* 2000). Benefits of *Rhizoscyphus ericae* colonization could include better access to nutrients as well as heavy metal resistance.

Some *Oidiodendron* spp. are recognized to form ERM with multiple potential hosts (Lacourt *et al.* 2001). Although less thoroughly characterized than *R. ericae*, *O. maius* has been shown to play a role in nitrogen acquisition when forming ERM with *Gaultheria shallon* (Xiao and Berch 1999).

Dark Septate Fungi

Dark septate fungi are frequently found on ericaceous plant roots. Isolated often, they rarely sporulate and produce dark to mouse grey mycelium in culture. They are called 'endophytes', a term which means they do not appear to help or harm the plants they inhabit (Peterson *et al.* 2004). Structurally, dark septate fungi appear different from ericoid fungi. They present both distinct melanized hyphae with septa as well as thinner hyaline hyphae. They form extensive networks around and within roots and are known to colonize both epidermal and cortical cells and vascular tissues as well (Peterson *et al.* 2004). Dark septate fungi form microsclerotia which are easy to identify and diagnostic of their presence in field collected samples (Peterson *et al.* 2004).

Dark septate fungi are ubiquitous. Besides their associations with ericaceous plants, they are also found on the roots of 600 trees and plant species (Mandyam and Jumpponen 2005). They do not seem to be limited to associations with plant roots. Menkis *et al.* (2004) report isolating *Phialocephala* spp. from healthy and decaying roots, coarse woody debris (CWD) and fine woody debris (FWD), healthy stems and freshly cut woody surfaces of *Picea abies* (L.) Karst., *Pinus sylvestris* L. and *Betula pendula* L. in Sweden and Lithuania. Menkis *et al.* (2004) theorize that dark septate fungi may alter strategies to coincide with the life stages of trees: from mycorrhizal-like colonization of healthy roots and stems to decomposition in declining and dead wood. It appears that dark septate fungi are able to thrive in disparate ecological niches.

Further evidence is provided by Addy *et al.* (2000) who isolated dark septate fungi across a sand to wetland gradient. They found that dark septate fungi show no preference for either extreme and thrive in both very wet and very dry conditions (Addy *et al.* 2000). The *Phialocephala* spp. collected at each of their sites showed little sequence variability suggesting that there is little specificity among strains for particular environmental conditions. Thus they do not seem to be limited by habitat or geographic range. Piercey *et al.* (2004) isolated *Phialocephala fortinii* with near identical sequences from geographically distant locations: from southern Alberta at 49° N to Nunavut at 78° N. Experiments examining their roles as mycorrhizal fungi produce contradictory results (Mandyam and Jumpponen 2005). Depending on the experimental conditions and the identity of the plant being tested, they can show positive, neutral or negative host responses (Jumpponen 2001). Jumpponen (2001) argues that, because of this and because they are capable of forming structures and interfaces that would allow for symbiosis, they should be considered mycorrhizal. Even though their function remains

elusive, their sheer abundance must have ecological impact and thus cannot be ignored (Mandyam and Jumpponen 2005).

Field Study Rationale

Many of the functions, or lack thereof in the case of dark septate endophytes, attributed to root-associated fungi, are based on laboratory experiments. Given complex systems like plant-fungus symbioses, the tendency has been to plan reductionist experiments simplifying the system so that effects can be clearly demonstrated. Such designs demonstrate potential effects occurring in natural habitats. They do not, however, show how those potentials are expressed (Read 2002). For instance, it has been shown that *R. ericae* grown in culture changes its nutritional preferences and metabolizing abilities after storage (Grelet *et al.* 2005). The fungus, in this case, has adapted to its new environment: the medium in the petri dish. What it may have been able to do under field selection pressures is potentially lost. Increasing the complexity of the system under study corresponds to an increase in relevance at the expense of precision. Thus field studies are the most relevant and least precise of all experimental designs (Read 2002). The challenge, then, is to measure appropriate variables that will help explain differences observed in the field. Naturally occurring gradients of vegetation, forest productivity, microclimate, soil nitrogen availability, and soil pH have been exploited to show differences in mycorrhizal assemblages in changing field environments (Bougoure et al. 2007; Mulder and de Zwart 2003; Nilsson et al. 2005). Gradients occur naturally on mountain slopes and have been used to assess ectomycorrhizal communities (Kernaghan and Harper 2001). The slopes of British Columbia's interior rocky mountains are not only appropriate for this purpose, they have some unique ecological features that can be incorporated into studies.

Northern Wet-belt Forests

In BC, plant landscapes are recognized as belonging to 14 biogeoclimatic zones (Meidinger & Pojar 1991). This ecosystem classification provides a framework of reference for ecological studies on temperature, moisture and aspect, and by inference on fungal communities associating with mycorrhizal plants. Each zone is, broadly speaking, a plant community that is an expression of the temperature and precipitation regimes present at that location. In this study, zones are considered proxies for different habitats of *V. membranaceum* root-associated fungal communities. While the host remains constant, climate variables and plant communities differ based on BEC zone classifications. The study site is located in the northern wet-belt forest. Northern wet-belt forests include the very wet subzone of the Sub-boreal Spruce (SBS) Zone and the wet and very wet subzones of the Interior Cedar-Hemlock (ICH) and Engelmann Spruce-Subalpine Fir (ESSF) Zones. Throughout this study, the subzones sampled are referred to by their general BEC zone designations. The very wet subzone of the SBS is characterized by high annual precipitation and high growing season precipitation. It also has the lowest mean annual temperature of all the SBS units (DeLong 2003). This subzone is dominated by hybrid white spruce (*Picea glauca* (Moench) Voss × *Picea engelmannii* Parry ex Engelm.) and subalpine fir (*Abies lasiocarpa* Hook. Nutt.) (DeLong 2003). The SBS is contiguous with the wet and very wet subzones of the ICH at higher elevations. The very wet subzone of the ICH is characterized by less annual precipitation than the very wet SBS. This subzone is dominated by western red cedar (*Thuja plicata* Donn ex D. Don) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg). At higher elevations, the very wet ESSF zone is characterized by sub-alpine fir and Engelmann spruce (*Picea engelmannii* Parry Ex. Engelm.) (Coupé *et al.* 1991). Due to their steep

topography, wet-belt forests provide a unique opportunity to study impacts of elevation as a proxy of temperature and precipitation as a selection pressure on mycorrhizal systems and provide a platform for extrapolation under scenarios of climate change.

The plant host: *Vaccinium membranaceum*

Vaccinium membranaceum grows between elevations of 600 and 3500m above sea level. It is known as the western black huckleberry (Small and Catling 2005). It is a deciduous shrub measuring 0.3 to 1.5m tall that produces creamy pink to yellowish flowers 5-6mm in size from April to June. They are found in foothills to montane regions from southern Yukon and NWT to Wyoming in the south (Kershaw *et al.* 1998). *V. membranaceum* is rhizomatous, forming extensive clumps (Small and Catling 2005). It can be differentiated from other *Vaccinium* spp., which include wild and cultivated blueberries, by their finely toothed ovate leaves and characteristically black to deep purple fruit. It is a non-timber forest product (NTFP) as it is not currently cultivated, only collected in the wild. Traditionally used by First Nations peoples, the berries are eaten raw, dry or cooked and the roots and leaves have been used for medicinal purposes to relieve gout and alleviate diabetes mellitus (Stevens and Darris 2000).

Approaches to the study of root-associated fungi

Traditionally, the study of fungal diversity has relied on collecting sporocarps or examining differences in root morphology as well as isolating fungi from roots. Fungal fruiting patterns have not been shown to represent underground diversity. Root morphology remains useful but is restricted to ECM and cannot be used to distinguish ERM. Not all fungi are culturable, and those that do not grow on media are missed in approaches that use this approach exclusively. Berch *et al.* (2002) found that 65% of their *Gaultheria shallon* ericoid roots were colonized by a non-culturable basidiomycete:

Sebacina vermifera. Allen *et al.* (2003) found that 74% of their colonized roots yielded no culturable fungi. In contrast, an Australian study of *Epacris pulchella (Epacridaceae)* roots found only 8% of their roots yielded no culturable fungi (Bougoure and Cairney 2005). It is recommended that a combination of isolation and directly extracted DNA be used to capture the most diversity (Allen *et al.* 2003; Berch *et al.* 2002; Bougoure and Cairney, 2005).

Molecular Approaches

The rRNA operon has features that make it a good tool for differentiating fungi. Coding regions are conserved and consequently are good targets for fungal specific primers. Non-coding regions tend to vary between genus and species, allowing for differentiation and identification of the fungi. There are two intergenic spacers between the 18S rRNA gene and 28S rRNA gene that flank the 5.8S rRNA gene, called ITS1 (intergenic spacer 1) and ITS2 (intergenic spacer 2) (Figure 1). Amplifying this region is the starting point for both sequencing and identification as well as community profiling techniques. Although ideal for

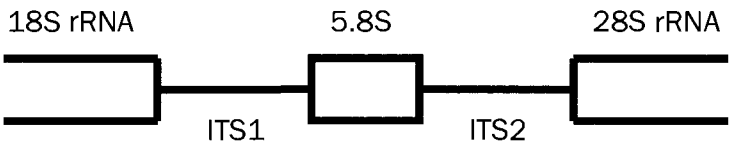


Figure 1. Schematic of rDNA ITS region

identification of some fungi, its not effective for all. The Sebacinales are known to have very diverse ITS regions and this region cannot be relied on solely for their identification. In the case of these fungi, rDNA large subunit (LSU) data is needed to supplement the

ITS identifications (Selosse *et al.* 2007). The ITS region cannot be used for quantification because its copy numbers vary between fungi, but the specifics of those differences that would aid in quantification are not known (Anderson and Cairney, 2004). Thus the abundance of a particular ITS genotype may not directly reflect the abundance of the fungus it resided in. Furthermore, identification to the species level is not always possible due to lack of information in GenBank. The fungal ITS region is less variable than the RNA spacer region in bacteria, and which is widely used in bacterial community profiling (Anderson and Cairney, 2004).

Length-Heterogeneity PCR (LH-PCR)

Length-Heterogeneity Polymerase Chain Reaction (LH-PCR) is a microbial community profiling technique. Developed to evaluate bacterial diversity, it is also known as Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett 1999). Adapted for fungal diversity assessments (Ranjard *et al.* 2001), this technique is used to profile communities for comparison between sites. Intergenic spacers flanking the 5.8S RNA are targeted with a PCR reaction containing fluorescent labeled primers. Amplified fragments are analyzed using an automated sequencer with laser detection. The profile of peaks generated from a community is a fingerprint that represents the diversity of fungi within that community (Ranjard *et al.* 2001). It has been used to effectively compare fungal communities (Lejon *et al.* 2005; Ranjard *et al.* 2001). Profiling methods are known to underestimate the diversity of a community because they can fail to detect rare species (Bent et. al. 2007).

Statistical analysis

Multivariate data were collected for this study. Several statistical approaches were employed and are detailed below.

Non-metric Multi-dimensional Scaling (NMS)

Most ordination methods make multivariate assumptions that are not appropriate for ecological data. Non-metric multidimensional scaling (NMS) is a preferred method for analyzing plant ecological data because it avoids certain pitfalls. For instance, NMS does not assume that there are linear relationships between variables (McCune and Grace 2002). NMS seeks to represent the data in the lowest number of dimensions while retaining the relative order of distances between objects. The resultant multidimensional plot shows similar objects closer together than more dissimilar objects. Unlike eigenvector techniques, NMS does not seek to maximize the variability represented by each axis; in NMS, the axes are arbitrary (Legendre and Legendre 1998). Another advantage of using NMS for ecological data is that it helps to relieve the zero-truncation problem. Once a species is absent from a habitat, we cannot tell how 'disfavourable' that environment is to that species; there is no 'negative' abundance. Zero-truncation makes it difficult to use species abundance as a measure of favourability of habitat. NMS uses ranking to linearize relationships between distances in species space, and this helps to relieve the zero-truncation problem (McCune and Grace 2002).

Any distance measures can be chosen in NMS. A good ecological distance measure is Sørensen's Index. This measure is a value ranging from zero to one. Zero represents identical items; the larger the value, the greater the difference between the two objects. The Sørensen's measure also assumes symmetry in that the distance from A to B is the same as B to A. Distances are based on non-Euclidean mathematics: fuzzy sets theory where the proportion coefficients are used as distance measures of the intersection of two fuzzy sets of quantitative data. However, NMS is adaptable to any distance measure that is appropriate for ecological data sets (McCune and Grace 2002).

The procedure starts with a distance matrix using the previously selected distance measure and the data are assigned positions in ordination space (either randomly or based on scores from a previous ordination). This initial configuration (X) is then normalized and a new matrix (D) is calculated. A matrix of dissimilarity coefficients is created. The dissimilarity coefficient matrix is then rank ordered. Elements of D which do not satisfy the monotonicity constraint are then replaced successively. The amount of movement required to achieve monotonicity is measured and used to determine "stress". Stress is the difference between the original dissimilarity matrix and the distances between objects reordered in ordination space. The lower the stress, the better the fit. The goal of the iteration procedure is to minimize stress. Objects are consecutively moved in direction of steepest descent (i.e., greatest reduction in stress) in ordination space until a minimum reduction in stress is achieved with each iteration. The process is repeated (iterated) until stability is achieved or until a certain predetermined number of iterations are performed (McCune and Grace 2002).

A Monte Carlo test is used to evaluate whether the NMS procedure is extracting a pattern that could happen by chance. The original data set is randomized and the NMS procedure repeated. Stress between the two analyses is compared. The original data should produce a stress that is lower than the randomized data. A p-value is derived by comparing stresses and expresses the probability of getting such a result with the original data set by chance (i.e., Type 1 error) (McCune and Grace 2002).

The best solution in NMS has low stress and is stable. Ecological data often presents a stress of 10-20; a value of over 20 suggests the graph produced is not very reliable. Plots of stress vs iteration number should be examined to determine stability. A

final instability of 10^{-3} should be considered maximal for graphical interpretation purposes (McCune and Grace 2002).

Objects close together on an NMS plot are more similar to each other than objects further apart. Each axis will represent a portion of the variance. Correlations to other variables can be overlaid from another matrix (McCune and Grace 2002).

perMANOVA

Non-metric multidimensional scaling represents relationships graphically; to test for statistical differences between groups the perMANOVA procedure can be used. This non-metric version of an ANOVA test avoids several key assumptions of multivariate ANOVA's. There is no requirement of multivariate normality or homogeneity of variance which are often difficult to achieve in ecological data. A distance measure can be selected and should mirror that used in NMS. Statistical significance is determined by a Monte Carlo test (McCune and Grace 2002).

Indicator Species Analysis

With ecological data, differences detected using perMANOVA can be characterized with Indicator Species Analysis. In this simple approach, a species is a perfect indicator if it is always present in one group (faithful) and never present in any other group (exclusive). Each species is evaluated with respect to this criterion and given a score based on how good of an indicator species it is. Confidence in the result is determined by a Monte Carlo test and reported as a p-value. If perMANOVA answers the question "are the groups different?", then indicator species analysis answers the question "how are they different?" (McCune and Grace 2002).

Canonical Correspondence Analysis

Canonical Correspondence Analysis (CCA) is an ordination technique that uses environmental variables to constrain the species matrix data. It is an excellent technique to demonstrate how sampled species are related to the values of environmental variables from the same sites. It is an eigenvector technique that extracts principal axes using a reciprocal averaging algorithm with the addition of a constraint at each calculation step. The constraining step uses standardized environmental variables from a second matrix and applies an ordinary least squares multiple regression. These are then used as predicted values in the subsequent calculation of species scores (Quinn and Keough 2002). As such, this technique excludes from its analysis community structure data that is unrelated to the environmental variables (McCune and Grace 2002).

This is an *a priori* approach. Canonical correspondence analysis assumes the environmental variables tested are independent variables and that the species matrix represents dependant data (Økland 1996). Variation that cannot be explained by the environmental variables is ignored (McCune and Grace 2002). Implicit within this is the assumption that there are no other independent variables that could affect the species data. The purpose of ecological studies is often to find the variables that impact community composition. By ignoring variation that does not fit with the environmental variables that happened to have been measured (often out of convenience), any indicators of additional effects are removed (Økland 1996). As such, CCA offers no additional information about community structure. Its function is solely to explain species variation in terms of the measured environmental variables.

Canonical Correspondence Analysis is vulnerable to dramatic shifts in results with the inclusion of irrelevant or noisy environmental data (McCune 1997). Once the number of environmental variables approaches the number of sites, the constraints on the axis become so weak as to become no different than random variables (McCune and Grace 2002). Environmental variables are also assumed to have linear relationships, and as such, the data must be transformed to satisfy this requirement (Quinn and Keough 2002). Environmental variables used in constraining should be few and have clear linear trends.

Canonical Correspondence Analysis is sensitive to abundances, weighing rare species heavily. It also falls prey to the problem of distortion of data if species at the end of each gradient are very dissimilar to each other. It will group these samples as more similar by a common lack of identical species, the resulting graph may curve on itself like a horseshoe. This technique is often used in examining species diversity differences along gradients. Because of the distortion problem, it is not good for large gradients where species at the ends of the gradients are completely different from each other (Quinn and Keough 2002).

Recognizing the limitations of this technique, it is possible to use it synergistically with other ordination techniques. Configurations that agree between constrained and non-constrained ordinations indicate strong real trends (Økland 1996). Still, these are only graphical representations. Follow up analysis, such as multivariate ANOVA, are needed to characterize group structure (Quinn and Keough 2002).

Chapter Two: Methods

Study site

McBride peak (53° 20 N, 120° 07 W) is located just outside the town of McBride in the Interior range of the Canadian Rockies in east-central British Columbia. The southwest facing slope of McBride peak encompasses four biogeoclimatic zones: the alpine, ESSF, ICH and SBS. The subzones of each BEC zone are described in the introduction. Transects were created to target the midpoint of each zone, thus spacing between transects is not equidistant along the slope. The start and end points of each transect were recorded with GPS unit and those points used in ClimateBC to calculate mean annual temperature (MAT), mean annual precipitation (MAP), mean summer precipitation (MSP) and the number of frost free days (NFFD) taken as the average Climate BC output for the two GPS points (Wang *et al.* 2007).

The alpine zone used in this study (Figure 2A) was probably the lower range of the true alpine-tundra biogeoclimatic zone, as there were several tree islands of Whitebark pine (*Pinus albicaulis* Engelm.) and subalpine fir (*Abies lasiocarpa*), although stunted, in the surrounding area. The AT is by definition treeless (Pojar and Stewart, 1991). The area sampled was dominated by Mountain Heather (*Cassiope mertensiana* Bong D. Dong., and *Phyllodoce* sp.). The canopy was open. The elevation sampled was 1923 ± 10m. The MAT was 0.5°C, the MAP was 1262mm, the MSP was 587mm and the NFFD was 129.

The ESSF was dominated by stunted subalpine fir (*Abies lasiocarpa*), Whitebark pine (*Pinus albicaulis*) and Engelmann spruce (*Picea engelmannii*)(Figure 2B). The canopy was mostly open, although the trees were larger and thus provided more shade than in the alpine landscape. The understory was dominated by *Rhododendron albiflorum* Hook. and *Menziesia ferruginea* Smith. The organic layer of the soil was

shallow (3-5 cm deep). There were more rocks at this elevation that made excavation of plants more difficult. The elevation sampled was 1801 ± 7.5m, about mid-way through the zone. The MAT was 0.5°C, the MAP was 1229mm, the MSP was 586mm and the NFFD was 129.

The ICH was the mid elevation biogeoclimatic zone in this study (Figure 2C). Climax forests in the ICH would be dominated by western hemlock (*Tsuga heterophylla*) and western red cedar (*Thuja plicata*) (Ketcheson *et al.* 1991). Due to recent fire disturbance history, as inferred by charcoal in the soil pit and younger seral stands, these were present but sparse especially at higher elevations of the zone. The overstory was dominated by trembing aspen (*Populus tremuloides* Michx.) and paper birch (*Betula papyrifera* Marsh.). The understory was dense with large *Rhododendron albiflorum, Menziesia ferruginea* and willows (*Salix* spp.) *The V. membranaceum* plants were difficult to excavate due to the density and size of other roots present in this soil. *Vaccinium membranaceum* plants were mostly shaded at this elevation. The elevation sampled was 1224 ± 10.4m. This is the upper elevation limit for the ICH. In the field, mid points of each BEC zone were targeted. Thus this appeared to be the best place to sample *in situ*. The MAT was 0.4°C, the MAP was 1491mm, the MSP was 558mm and the NFFD was 130.

The lowest elevation sampled, the SBS, had a more open understory than the ICH stand but and a more closed overstory (Figure 2D). The SBS was dominated by Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and white spruce. Root systems within the soil at this elevation were prolific rendering excavation of *V. membranaceum* plants challenging. It was, however, easier than the ICH to excavate because the understory

Figure 2. Photographs of the sampled landscape. A. Alpine Tundra. B. ESSF. C. ICH. D. SBS

Table 1. List of plants and lichens present at each sampling transect.

AT	ESSF	ICH	SBS
Abies lasiocarpa Hook. Nutt.	*Abies lasiocarpa* Hook. Nutt.	*Abies lasiocarpa* Hook. Nutt.	*Acer glabrum* Torr.
Cassiope mertensiana (Bong.)D.Don	*Arctostaphylos alpina var. rubra* (Rehd.&Wilson) Fern.	*Anaphalis margaritacea* (L.) Benth.&Hook. f. ex. CB Clarke	*Actaea rubra* (Ait.) Willd.
Cladina sp.	*Arnica cordifolia* Hook.	*Betula papyrifera* Marsh.	*Alnus viridis* ssp.*sinuata* (Regel) A. & D. Löve
Lycopodium sp.	*Cladina sp.*	*Chimaphila umbellata* (L.) Bart.	*Amelanchier alnifolia* Nutt.
Phyllodoce sp.	*Cladonia borealis* S.Stenroos	*Clintonia uniflora* (Menzies ex J.A. & J.H. Schult.) Kunth	*Betula papyrifera* Marsh.
Pinus albicaulis Engelm.	*Epilobium augustifolium* L.	*Cornus canadensis* L.	*Chimaphila umbellata* (L.) *Bart.*
Rhododendron albiflorum Hook.	*Menziesia ferruginea* Smith.	*Epilobium augustifolium* L.	*Clintonia uniflora* (Menzies ex J.A. & J.H. Schult.) Kunth
Vaccinium caespitosum Michx.	*Pinus albicaulis* Engelm.	*Goodyera* sp.	*Cornus canadensis* L.
Valeriana sitchensis Bong.	*Picea engelmannii* Parry ex. Engel.	*Linnaea borealis* L.	*Epilobium augustifolium* L.
Veratrum viride W.Ait.	*Phyllodoce sp.*	*Lycopodium* sp.	*Galium sp.*
	Rhododendron albiflorum Hook.	*Menziesia ferruginea* Smith.	*Lonicera utahensis* S. Wats.
		Pinus contorta Dougl. Ex. Loud.	*Mahonia* sp.
		Populus tremuloides Michx.	*Menziesia ferruginea* Smith.
		Salix sp.	*Monotropa uniflora* L.
		Sorbus sp.	*Picea glauca* (Moench) Voss × *Picea engelmannii* Parry ex Engelm.
		Spiraea betulifolia Pall.	*Populus balsamifera* ssp. *trichocarpa* (T. & G.) Bradyshaw
		Streptopus sp.	*Prosartes hookeri* Torr.
		Thuja plicata Donn. Ex. D. Don	*Pseudotsuga menziesii* (Mirbel) Franco
		Tsuga heterophylla (Raf.) Sarg.	*Pyrola asarifolia* Michx.
			Rosa acicularis Lindl.
			Shepherdia canadensis (L.) Nutt.
			Smilacina racemosa (L.) Desf.
			Spiraea sp.
			Vaccinium myrtilloides Michx.

vegetation was less dense. Plants excavated at this elevation were shaded. A complete list of plants found at each site (excluding *V. membranaceum*) can be found in Table 1. The elevation sampled was 875 ± 11.2m. The MAT was 0.1°C, the MAP was 1353mm, the MSP was 600mm and the NFFD was 125.

Harvesting plants

McBride peak is accessible by a road (4-wheel drive only) that terminates just below the AT zone. In July of 2006, four 100m transects were laid out perpendicular to the slope of the mountain, each transect targeting a biogeoclimatic zone. Ten black huckleberry plants, selected as the closest plant to each ten meter interval along the transect, were harvested with their surrounding soil, for a total of 40 plants. The entire root system was targeted. Excavations of plants yielded soil blocks approximately 0.5 x 0.5m x 20cm (Figure 3A). A 12cm TDR water probe was used to assess soil moisture at time of harvest. Measurements were taken at each of the four corners of an excavation site, averaged, and reported per plant (Figure 3B). For leaf mass to area (LMA) analysis, ten randomly selected leaves were taken from each plant and stored in plastic bags at -20°C. Plants were double-bagged and kept in coolers for transport to the lab, where they were stored at 4°C.

Harvesting hair roots

Each *V. membranaceum* plug containing soil and its root system was soaked in tap water for 24-48 hours to loosen the soil. Over the course of 60 days, all the plants were processed in the same manner: roots were massaged free of soil, other roots and rocks, and all attached lateral hair roots were harvested, cut into 1-5cm pieces and assigned randomly to one of three processing approaches: 1) surface sterilized

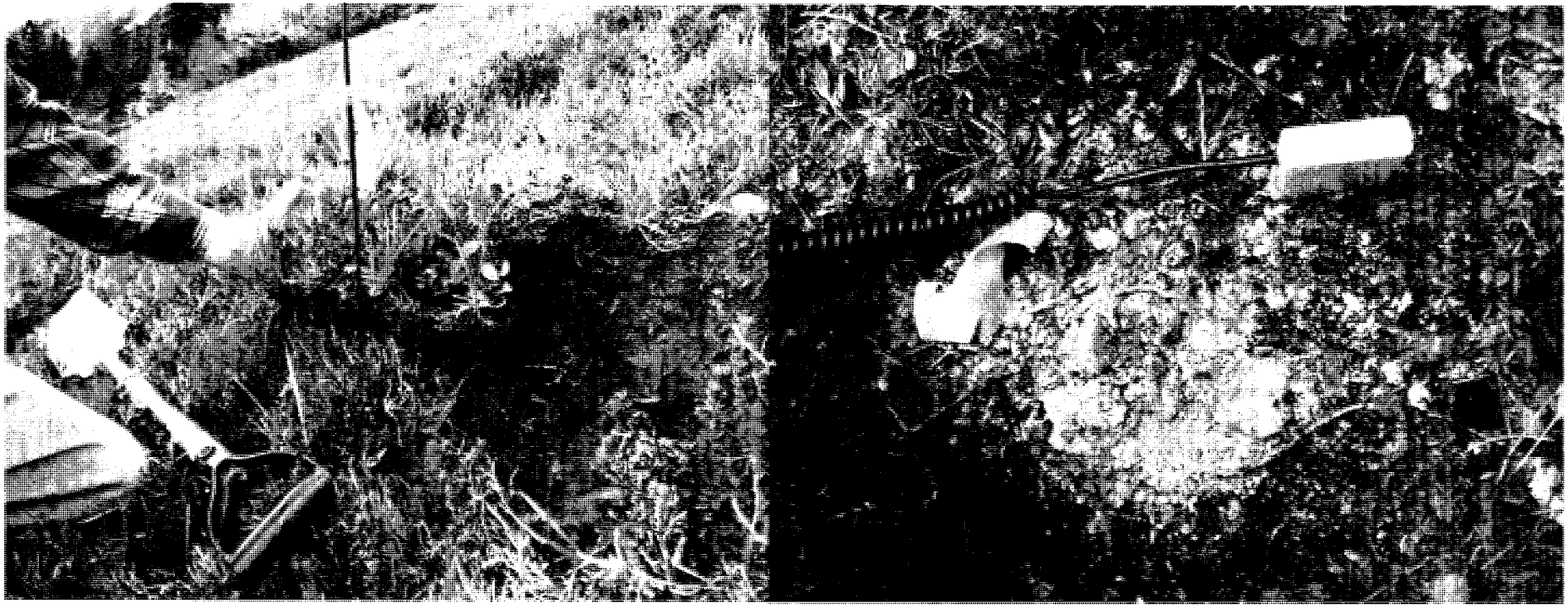


Figure 3. Photographs of the sampling process. A. Excavating *V. membranaceum* plants. B. Taking soil TDR measurements after plant excavation.

immediately for culturing, 2) frozen at -20°C for DNA extraction, and 3) preserved in 50% ethanol for microscopy.

Fungal isolation

Twelve randomly selected root pieces from each plant were surface sterilized in 10% H_2O_2 for 45 seconds, washed 3 times in sterile dH_2O and plated onto full strength Potato Dextrose Agar (PDA), as per the protocol of Berch *et al.* (2002), at six 3cm root pieces per plate. Plates were examined once a week and growing fungi were sub-cultured onto individual plates. Sub-cultured fungi were monitored for contamination, cultured again if necessary and discarded if contamination could not be eliminated. Cultures were grown for 6 months at room temperature (20 °C) then stored at 4°C.

Isolates were named to identify the plant from which they originated. This convention is used throughout the text. Names begin with a letter (i.e. A, E, I, S) which refers to a biogeoclimatic zone (i.e. alpine, ESSF, ICH, SBS). The letter is followed by a number which references the number of the plant the fungus was originally isolated from. Plant numbers range from 1-10 along each transect. The second number following a dash denotes the chronological order in which the fungus was isolated and is largely irrelevant, other than to denote it as different from another isolate from the same plant.

A total of 461 isolates were cultured from the surface sterilized roots, with cultures representing every plant harvested. These were categorized based on morphological features. Ninety representative cultures were first sequenced as described below, and this revealed that many of these cultures were *Phialocephala fortinii* (the most common dark septate endophyte [Peterson *et al.* 2004]) and were easily recognized based on morphology in culture. Thus a second round of

sequencing excluded obvious *P. fortinii* cultures and focused on all other morphologies, aiming to sequence each type of culture obtained from each harvested *V. membranaceum* plant. After the second round of sequencing, both *P. fortinii* and *Cryptosporiopsis* sp. could be readily identified by culture morphology and were counted as isolated from their respective plants even if those specific cultures were not identified with sequencing. The numbers and identities of cultures isolated for each plant are summarized in the results.

Sequencing fungal cultures

The DNA from 298 cultures (out of 461) was extracted using Nucleospin multi-96 plant kit (MJS BioLynx) according to the manufacturer's protocols. Six positive controls (*Cladosporium* sp., *Endoconidioma*, *Geomyces pannorum*, *Knufia* sp., *Sclerotinia sclerotiorum*, *Tilletia barclayana*) were used per 96-well plate.

Extracted DNA was amplified using the forward primer ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and reverse primer LR6 (5'CGCCAGTTCTGCTTACC3'), targeting the ITS1 and ITS2 region of rDNA. In a 10µL reaction, consisting of 0.1mM of dNTP's, 0.8µM of each primer and 1x Titanium Taq™ buffer (including 3.2mM $MgCl_2$) and 0.1ng Titanium Taq™ Polymerase, 1µL of template was added. The thermocycler conditions were 95°C initial denaturation, then 95°C for 1 minute, followed by annealing at 58°C for 30s and a 72°C extension for 2 minutes for 40 cycles. A final of extension of 72°C for 8 minutes was included before being held at 10°C. Bands were visualized using the E-gel® 96 high-throughput agarose electrophoresis system (Invitrogen).

Sequencing was performed using the BDT System (Brinkman Instruments, CA). Forward primers used were ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS5,

reverse was ITS4 (5'TCCTCCGCTTATTGATATGC3'). A final volume of 10μL contained 0.875x sequencing buffer v3.1, 0.125x BDT sequencing mixture v3.1 and 0.16μM primer as well as less than 20ng of PCR template. Sequences were edited and forward and reverse sequences aligned using Sequencher 4.2.2 (Gene codes corp). Edited sequences were compared to GenBank using BLAST searches (www.ncbi.nlm.nih.gov) to find the closest named fungus.

LH-PCR

Roots (1/3 of entire root system of each huckleberry plant) stored at -20°C (stored no more than 2 months) were pulverized by immersion in liquid nitrogen and ground with a mortar and pestle. The mortars and pestles were washed with soap and water, immersed in 3M HCl for 24 hours and washed again with soap and water prior to use in order to ensure no cross contamination between samples. DNA was extracted from the crushed roots using the UltraClean Soil DNA extraction (MoBio Laboratories Inc) kit following the alternative protocol for maximum yields. Spectrophotometric analysis of extractions showed very low amounts of DNA in some cases with concentrations ranging from 1-50ng/μL.

Each plant was subjected to extraction twice and was amplified with one set of primers targeting the ITS1 region of fungal ribosomal DNA for LH-PCR. The primers used were ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and green dye (D3) labeled NLB4 (5'-5D3-GGATTCTCACCCTCTATGAC-3') (Invitrogen Inc). Each 30μL reaction contained 3μL of genomic DNA (no single dilution worked for every sample, thus some samples amplified successfully with no dilution, some with 1/10, 1/20, or 1/50), 1x PCR Buffer, 2mM dNTP's, 25mM $MgCl_2$, 10μM of each primer and 3U of Platinum Taq DNA Polymerase (Invitrogen). Thermocycler conditions were as follows: 4 minutes

denaturation at 94°C followed by 35 cycles of denaturing, annealing and extension at 93°C for 35 seconds, 52°C for one minute and 72°C for 1.5 minutes respectively. The final extension was 5 minutes at 72°C. Reaction products were visualized with ethidium bromide staining in 1% agarose gel electrophoresis and subjected to fragment analysis with the Beckman Coulter CEQ™ 8000 Fragment Analysis System (Beckman-Coulter Inc.).

Fragment Analysis

Fragments were binned and analyzed in the AFLP (amplified fragment length polymorphism) program of the Beckman Coulter CEQ™ 8000 Fragment Analysis System (Beckman Coulter Inc.). Analysis parameters were set for the 600bp size standard quartic model. A 3bp bin size was selected in accordance with Beckman-Coulter Inc equipment detection limits (Beckman Coulter 2004) for the 600bp ladder. All directly extracted DNA samples were analyzed together to eliminate the possibility of discrepancy between program settings on the fragment analysis system. Profiles were then examined manually and flawed profiles, some including those with problems such as ladder failures or the detection of incorrect dyes, were deleted.

The Variable Percentage Threshold (VPT) method (Osborne *et al.* 2006) was used to score peaks in LH-PCR profiles generated by the AFLP program. Osborne et al (2006) have demonstrated that the VPT method outperforms other approaches when tested on replicate PCR data. Variability of the fluorescence signal between profiles, due to the uncertainty of amount of LH-PCR product applied in each run, is taken into account when selecting which peak to retain and which to discard. The total area of a profile is divided by different values (divisors), and those peaks below the divisor value eliminated. Different divisors are tested and the correlation examined until the

relationship between the divisor and the number of peaks retained is weakest. The point at which correlations between the total area and number of peaks retained is weakest is theoretically the point at which signal is distinguished from noise. The optimal divisor for each profile is thus found and peaks with areas less than this value are removed from the profile before further analysis. The VPT method was applied to fragment lengths ranging from 300-681bp in size.

Eighty profiles were kept for further analysis: 40 duplicate root samples. These are not true replicates. Each duplicate sample was extracted from a different set of hair roots from one plant. One species of fungus could have been present in one sample and not the second; thus a peak in only one of the two samples does not imply incongruence between samples, rather it indicates the presence of that species within only one of the two sub-sampled root systems. The duplicate profiles are thus additive and were performed to boost the quality of information obtained: overall, fragments common to both duplicate profiles accounted for only 20% of the fragments detected. The result was a matrix of presence/absence data for x distinct fragments on each of the 10 plants harvested at the 4 elevations.

Linking cultures with LH-PCR

The LH-PCR technique was paired with sequencing to determine if individual fragments correspond to a particular culture. A selection of cultures were subjected to LH-PCR in duplicate, and sequenced as per above. Representatives of each identified taxon were selected for LH-PCR analysis.

Statistical analysis of Matrices

The sequencing and direct DNA analysis approaches both yielded data in matrix format that could be visualized using non-metric multidimensional scaling (NMS). Three matrices were analyzed. Presence/absence data from the culturing approach was summarized in one matrix, as was presence/absence data from the direct DNA analysis. The number of cultures isolated per plant was used as an abundance measure for a third matrix. Although the LH-PCR approach does generate data on amount of DNA detected, it is not an appropriate abundance measure. The copy number of ITS1 differs between species of fungi, thus the amount detected cannot be used as a measure of abundance of a fungus. Furthermore, the difficulty of preferential amplification in PCR adds another level of uncertainty that precludes using this method as an abundance measure.

All 3 matrices were calculated on the basis of the Sørensen distance measure. Ordinations were performed using PC-ORD v5.0. (McCune and Grace 2002) run on autopilot in the 'slow and thorough' mode, using random starting coordinates. Monte Carlo tests were run for each matrix, with 250 randomized runs to determine the chance ($p=0.04$ in all cases) that a similar stress could have been obtained by chance. The dimensionality of the solution is selected once the addition of more dimensions provides only small reductions in stress. No solutions above a final stress of 20 or above an instability value 0.03 were considered. Solutions for ecological community data with stress values between 10-20 are common; the lower the value, the more reliable the solution (McCune and Grace 2002). Differences between richness of fragments and cultures and abundance of cultures from the four elevations were determined using a non-parametric multivariate ANOVA

(permANOVA). Results were deemed significant when $p<0.05$ and somewhat significant when $p<0.1$.

Indicator species analysis

Indicator species analysis was applied to the fungal community data to assess the value of a species (or fragment) to indicate the conditions present at a particular elevation. This intuitive measure, ranging from 0 to 100, applies as value of 100 to a perfect indicator. A perfect indicator species or fragment would always be present at a particular elevation (faithful) and would never occur at another elevation (exclusive) (McCune and Grace 2002). Relative abundance of a species or fragment within an elevation compared to other elevations is determined and multiplied by the relative frequency (proportion of species or fragments in each elevation that contain that species or fragment). Since the two values are multiplied, both values must be high to result in a high indicator species value. A Monte Carlo randomization was used to determine statistical significance (McCune and Grace 2002).

Canonical Correspondence Analysis

Canonical Correspondence Analysis (CCA) was used to assess the potential for the environmental variables to explain the variability in fungal community structure. Since this ordination technique is sensitive to irrelevant environmental data, only those variables found to differ along the elevation gradient were used in the analysis. These included water content, pH of both organic and mineral soils, age of plants, C:N ratio of mineral soil and LMA. All values tested were z-scores with outliers replaced by means for the elevation (group).

Diversity measures of LH-PCR fragments and cultures

Both LH-PCR fragment and culture data were summarized in terms of alpha, beta and gamma diversity. Alpha diversity is the average richness at each elevation. Gamma diversity is the sum of all unique species or fragments at that elevation. Beta diversity as per Whittaker (1960) (β_W) is reported as the ratio of gamma to alpha diversity. This approach to measuring beta diversity is included here because it is the most common metric reported in the literature (Koleff *et al.* 2003) and so could facilitate comparisons to other studies. In addition $\beta_{sorensens}$ and β_{sim} (Koleff *et al.* 2003) are included. $Beta_{sorensens}$ is a broad-sense measure that emphasizes species in common and differences in richness between the fungal communities. $Beta_{sim}$ is a narrow-sense measure that emphasizes gains and losses of species between sites and is thus more sensitive to composition changes than $\beta_{sorensens}$. With both measures, high values represent low beta diversity (high similarity) and conversely, low values represent high beta diversity (low similarity between root associated communities).

Plant characteristics

Age determination

Vaccinium membranaceum is a multi stemmed shrub. All large stems attached to the same root system were sectioned by hand using razor blades and stained for 1 minute in 10% potassium iodine solution and mounted in lactoglycerol on slides and examined with an Olympus CH30 light microscope. Rings, defined as the change in vessel dimension from early wood to late wood, were counted to determine the age of each plant. The oldest shoot was taken to be the age of the plant.

Leaf Mass per Area (LMA)

The leaves collected at the time of harvest were thawed in a refrigerator (4°C), patted dry and average area determined using a LI-3100 Area meter (LI-COR Lincoln Nebraska). Leaves were then dried to a constant weight at 105°C, their final weight determined on a Sartorius MC410S analytical balance. Average mass was divided by the average area of the ten leaves from each plant to yield a LMA value for each plant. Relatively higher ratios represent smaller and thicker leaves that would be present in high light saturation circumstances, whereas lower ratios represent larger and thinner leaves typical of shaded closed-canopy environments.

Soil characteristics

Soil analysis

Prior to soaking in tap water, a random sample of soil from at least 4 different locations on the soil plug was taken and air dried. Mineral and organic fractions from soil blocks associated with each plant were collected separately and sieved; the organic fraction being kept free of leaf litter. Soils were ground in a coffee grinder and used for pH and carbon and nitrogen determination. The pH measurements were performed in duplicate using an Orion 710A pH meter, diluting the organic soil 2:1 in dH_2O and 1:1 for the mineral soil. Total organic carbon and total nitrogen content of both the organic and mineral soil fractions were determined using a Fison NC 1500 Elemental Analyzer.

Fungal Characteristics

Fungal Colonization Assessment

Approximately half of the roots stored in 50% ethanol were used to determine the extent of fungal colonization. Roots were first rinsed 3 times in sterile dH_2O and then

cleared in 5% KOH at 60°C for 12 hours. To remove the KOH, roots were again rinsed 3 times in sterile distilled water and then stained with 0.03% Chlorazol Black E (Sigma C-1144) in 1:1 lacto-glycerol at 60°C for 3 hours. Roots were stored at 4 °C in 1:1 lacto-glycerol. Percent colonization was determined by using a modification of the Brundrett (1996) method for examining arbuscular colonization of root sections in a dissecting microscope. Although low magnification may be adequate to assess arbuscular colonization, it is insufficient for ericoid colonization. The protocol by Brundrett (1996) involves floating roots in a petri dish containing grid. The points at which the roots intersect the grid are assessed as colonized or not, for a total of 100 intersections expressed as percent colonized. The following is a modification of the Brundrett (1996) method designed to be used with a compound microscope to accommodate the greater magnification needed to determine colonization of ericoid hair roots.

Stained roots sections were mounted on glass slides in lacto-glycerol as the mounting medium and assessed under 400x magnification with the Olympus CH30 light microscope. To mimic spreading root sections on grid, roots sections were arranged parallel to the length of the slide and the eyepiece micrometer used as a line of intersection for the roots. Scrolling through the roots was accomplished by moving the stage a set distance, following gradations on the stage, for each observation. Cells intersecting the micrometer line were determined to be colonized by classic, or non-typical ericoid morphology or dark septate (Peterson *et al.* 2004; Massicotte *et al.* 2005). Percent colonization values include all types of colonization noted. It was also noted whether there were hyphae on the surface of the cells, however, these observations were not included in the determination of the percent

colonization values. One hundred random intersections of the fine roots were used to determine the final value for each plant. Only young lateral roots were used, roots over 6 cells thick were excluded from the assessment.

Statistical analysis of plant, soil and fungus characteristics

All of the plant, soil and univariate fungal variables were tested for differences between elevations using one-way ANOVA's. A post hoc Tukey's HSD analysis was used because it is a conservative measure and more likely to reveal true differences between groups (Zar 1999). Variables were sorted into plant characteristics (age and leaf mass to area), soil characteristics (water content, pH-organic and pH-mineral soil, and C:N ratio-organic and C:N ratio-mineral soil) and fungus characteristics (extent of colonization, richness of fragments and richness of cultures). A $p<0.05$ was considered significant, whereas $p<0.1$ was considered potentially significant within the context of field-collected, ecological data .

Chapter 3: Results

LH-PCR

The total number of fragments (ranging from 310 to 681bp) generated from all 40 root samples was 57 (Figure 4). The alpine samples generated 20 fragments, 3 of which were exclusively found in the alpine samples. The ESSF root samples generated 27 fragments, 5 exclusive to that biogeoclimatic zone. The ICH samples yielded the most fragments (36) and the most exclusive fragments (10). The SBS root samples yielded 29 fragments, 4 of which were unique. Plant root samples from individuals yielded between 1-20 fragments each. Although the mean number of fragments isolated per plant from each elevation increases in value with decreases in elevation (Alpine=4.4 fragments; ESSF=6.7; ICH=7 and SBS=8.3) (Table 2), there is no significant difference between them based on a one-way ANOVA. The variability of numbers of fragments found per plants was high enough to obscure a difference.

Cultures

A total of 379 cultures (out of 461 fungi isolated) were successfully identified by sequencing the ITS1-5.8S-ITS2 region of ribosomal genes. The remaining 82 cultures represent: those that were not successfully sequenced (18); were sequenced but did not produce a confident identification in GenBank (5); were cultures that died (29); were contaminated (6); those that were morphologically similar to *P. fortinii* and excluded from sequencing (24). Morphologically unique cultures were targeted for sequencing. Appendix 1 contains photographs of the sequenced fungi. Between 1-6 distinct cultures were isolated from each plant. Overall, twenty discernable taxa were identified, 8 of which were isolated on only one occasion. Eleven taxa were isolated from the alpine, 4 of which were exclusive to the alpine. The ESFF generated 10 taxa,

only one of which was exclusive to that elevation. The ICH generated 8 taxa, 2 of which were exclusive to the ICH. The lowest elevation SBS generated 9 taxa of which 3 were exclusive.

The most commonly isolated fungus was *Phialocephala fortinii*, representing 52% of the cultures isolated and found at all four elevations. *Meliniomyces* spp. and *Cryptosporiopsis* spp. were also found at all four elevations. *Meliniomyces* spp. was found most frequently in the ESSF representing 20% of the cultures isolated at that elevation. *Cryptosporiopsis* spp. was found most frequently in the SBS, representing 47% of all the taxa isolated from the roots of plants at this lowest elevation. *Rhizoscyphus ericae* was found most frequently at high elevations, representing 21% of the isolates from the alpine. It was not found in the SBS. Table 3 summarizes the total number of each culture found at each elevation. Figure 5 shows the relative frequency out of 10 of those fungi isolated from each elevation. *Phialocephala fortinii* had a relative frequency ranging from 1 (isolated from every plant) to 0.7 (isolated from 7 out of 10 plants). In contrast to the mean of LH-PCR fragments found, the mean number of cultures found per plant per elevation decreases with decreasing elevation (Alpine=3.5 cultures; ESSF=3.2; ICH=2.7 and SBS=2.4) (Table 2), but again, there is no significant difference between the means based on a one-way ANOVA.

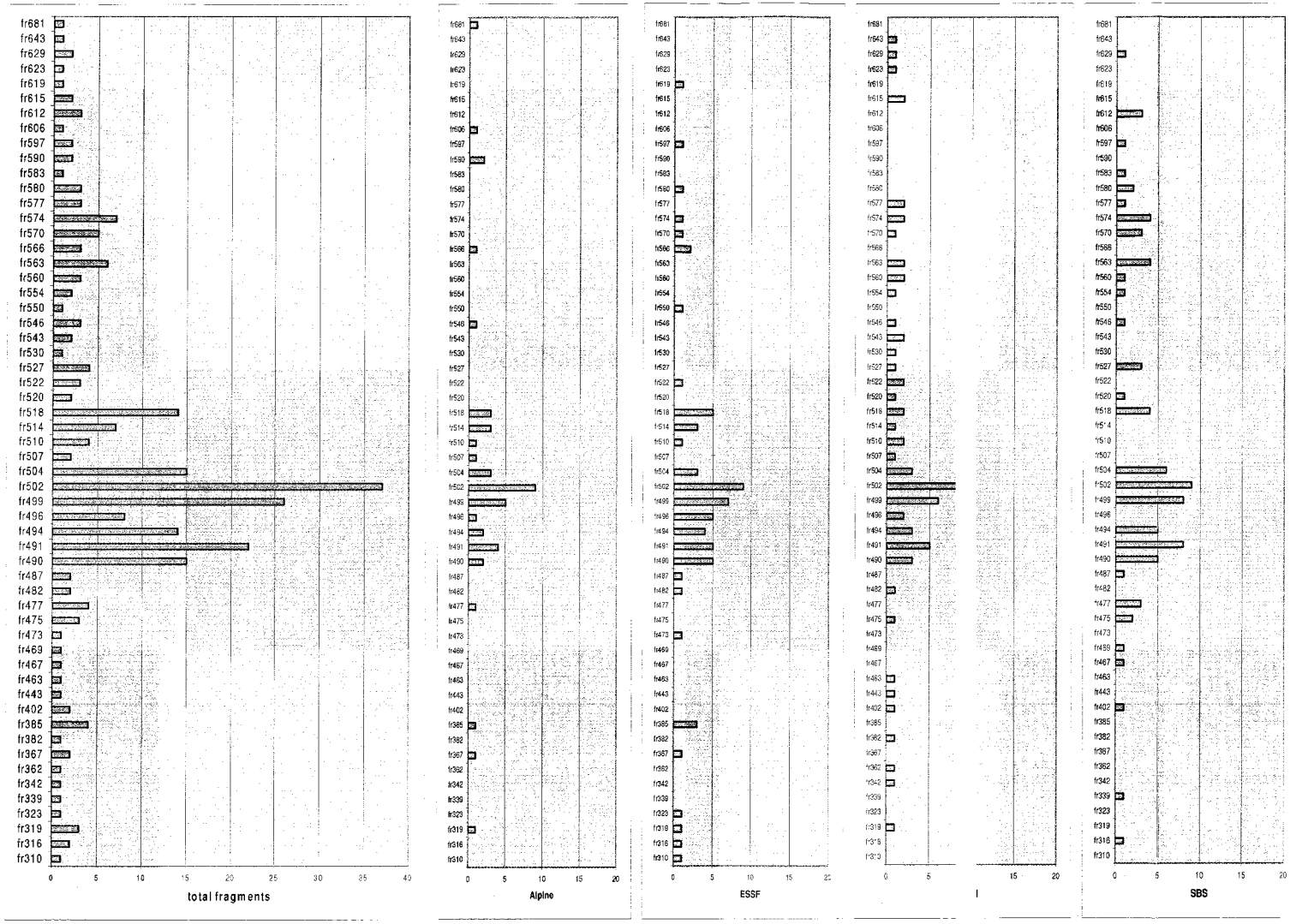


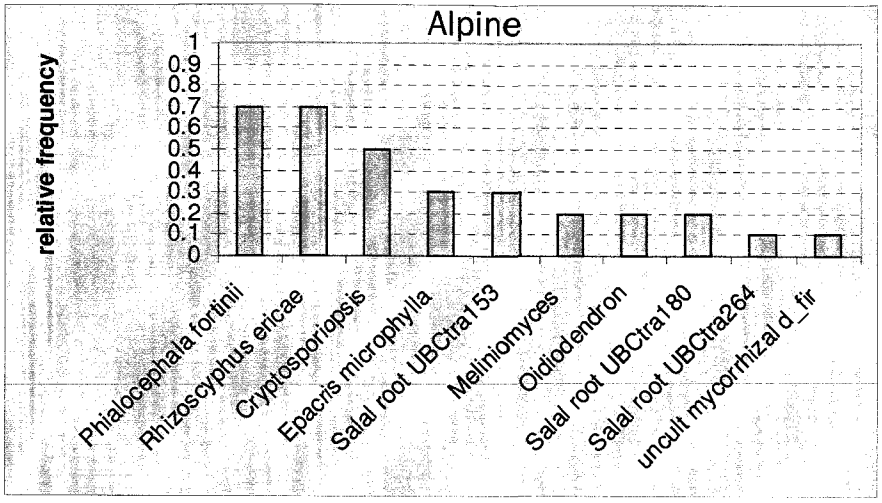
Figure 4. Total number of fragments generated from all root samples (left panel) using LH-PCR, followed (left to right) by total fragments fror ll Alpine, ESSF, ICH and SBS plants.

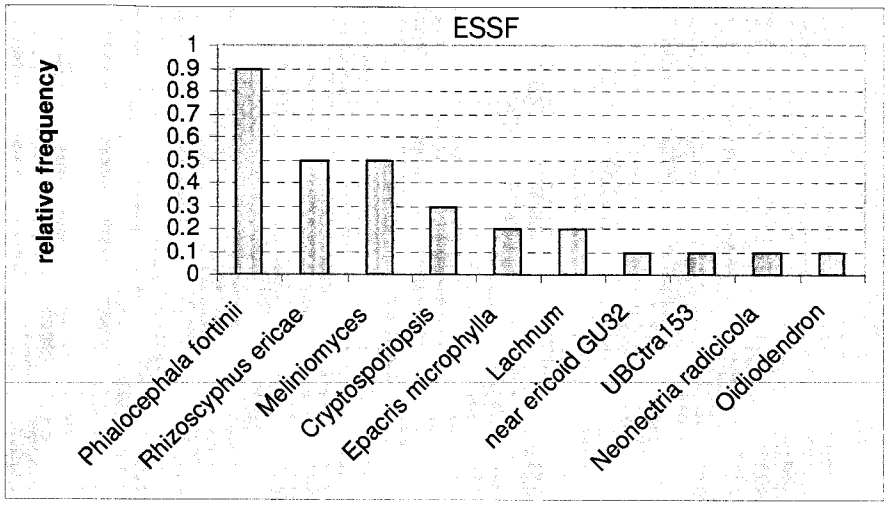
Table 2. Average species and fragment richness per plant (α) and its standard deviation (SD) using both culturing and direct fungal DNA analysis (LH-PCR), beta (β= γ/ α), and gamma diversity (γ=total species richness per elevation).

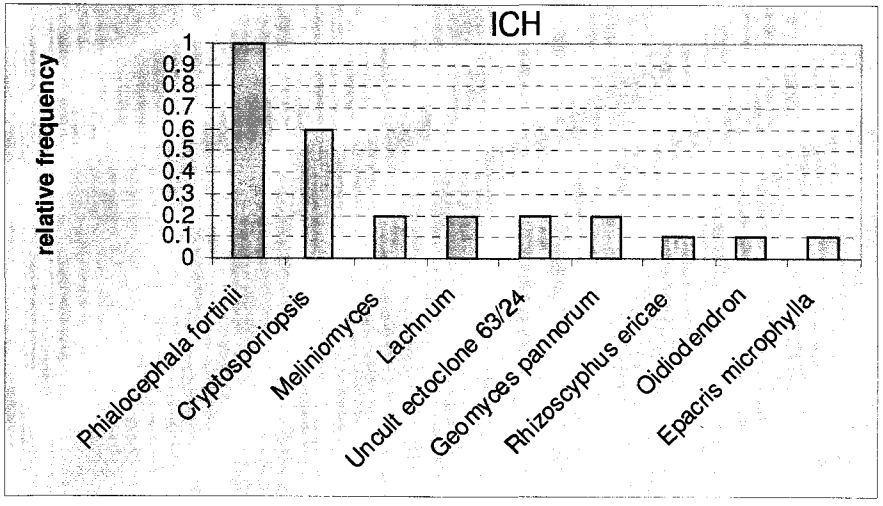
Cultures				LH-PCR			
	α (SD)	β	γ		α (SD)	β	γ
Alpine	3.5 (1.35)	3.1	11	Alpine	4.4 (3.75)	4.5	20
ESSF	3.2 (1.4)	3.1	10	ESSF	6.7 (4.88)	4.0	27
ICH	2.7 (0.82)	3.3	9	ICH	7.0 (5.75)	5.3	36
SBS	2.4 (0.97)	3.75	9	SBS	8.3 (1.89)	3.5	29

Table 3. Summary of numbers of the 20 unique cultures isolated and sequenced from *V. membranaceum* plants at all four elevations.

Reference name	Total	alpine	ESSF	ICH	SBS
Phialocephala fortinii	196	29	65	75	27
Rhizoscyphus ericae	23	15	7	1	0
Cryptosporiopsis spp.	75	11	8	19	37
Meliniomyces spp.	30	2	25	2	1
Oidiodendron spp.	14	2	9	3	0
Neonectria radicicola	10	0	2	0	8
Lachnum sp.	7	0	4	2	1
Epacris microphylla root-fungi	5	3	1	0	1
Salal root UBCtra153	5	3	2	0	0
Geomyces pannorum	2	0	0	2	0
Salal root UBCtra180	2	2	0	0	0
Salal root UBCtra264	2	2	0	0	0
uncult mycorrhizal d_fir	1	1	0	0	0
ectoclone 879/18	1	1	0	0	0
ericoid endophyte GU32	1	0	1	0	0
Uncult ectoclone 63/24	1	0	0	1	0
Gyoerffyella sp.	1	0	0	0	1
Rhizoscyphus aggregate	1	0	0	0	1
Leptodontidium orchidicola	1	0	0	0	1
Totals	379	71	124	105	78







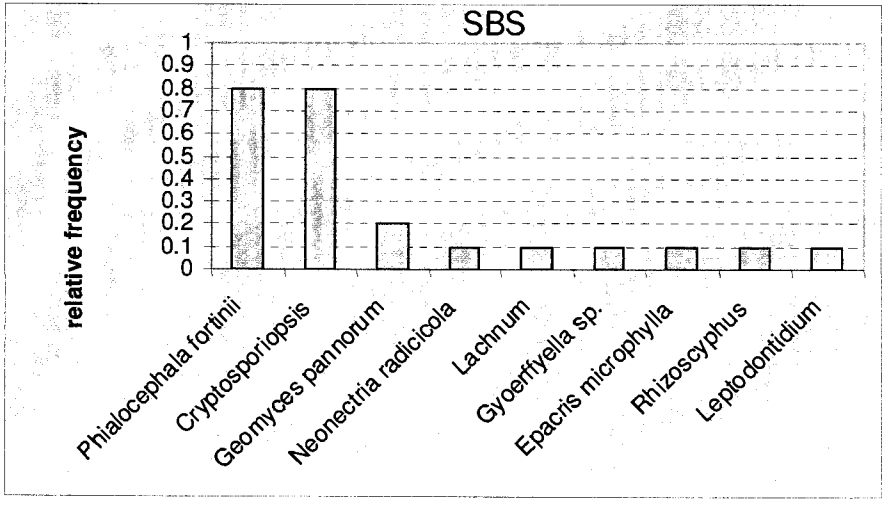


Figure 5. Proportion of plants at each elevation (alpine, ESSF, ICH, and SBS) harbouring cultured fungi.

Diversity measures of LH-PCR fragments and cultures

The diversity indices of $\beta_{sorensens}$ and β_{sim} (Table 4) are used to compare each elevation based on both analysis techniques. For both approaches, LH-PCR and cultures, both diversity indices show that the alpine and SBS have the highest beta diversity between them (they are most different from each other). The diversity indices also agree that the alpine and ESSF have the lowest beta diversity (they are most similar). Differences between the ESSF and ICH are technique dependant: cultured fungi are more different between these elevations than LH-PCR fragments. The final comparison between ICH to SBS shows that these two elevations are more different based on LH-PCR fragments than based on cultured fungi. Comparing the alpine and ICH, the culture data shows a discrepancy between the two beta diversity measures. $Beta_{sim}$, which focuses on gains and losses of species between elevations indicates that they are more similar, whereas $\beta_{sorensens}$, which places greater emphasis on species in common, indicates that these elevations are more different from each other.

Table 4. Beta diversity using Broad-sense ($\beta_{sorensens}$) and narrow-sense (β_{sim}) measures to compare presence/absence fungal community structure data using both culturing and direct DNA approaches between elevations.

	Cultures		LH-PCR	
	Beta-Sorensens	Beta-sim	Beta-Sorensen's	Beta-sim
Alpine vs ESSF	0.609	0.737	0.667	0.700
ESSF vs ICH	0.459	0.538	0.632	0.667
ICH vs SBS	0.594	0.655	0.444	0.444
Alpine vs ICH	0.444	0.632	0.500	0.556
Alpine vs SBS	0.375	0.474	0.400	0.444
ESSF vs SBS	0.464	0.481	0.632	0.667

Thus it appears that in this case, there are fewer species in common and greater gains and losses of species between elevations. Finally, comparing the ESSF and

SBS shows that these elevations are similar based on LH-PCR fragments and different based on culturable fungi. Overall, β_{sim} reports higher similarities between elevations in all but one case.

Linking identified cultures with LH-PCR

Several different taxa generated the same length fragment when analyzed with the LH-PCR technique (Table 5). Lengths of fragments were further analyzed by comparing lengths as determined by LH-PCR and those determined by counting the number of base pairs as determined by sequencing (Table 6). The lengths of fragments do not match between the two techniques. The discrepancy ranges from 1 to 9 base pairs. Identification through sequencing demonstrated that multiple species can produce fragments of the same length in LH-PCR. Some fragments seem more likely than others to represent a particular species; fragment 491 was produced by 3 isolates of *P. fortinii* (Table 5), but also by an *Oidiodendron* species. *Phialocephala fortinii* also produced a 484, 489, and 494bp fragment. These isolates do not differ based on a UPGMA tree (Figure 6). *Phialocephala fortinii* was the most frequently isolated culture (Table 2), and 500bp the most common fragment (Figure 4), but surprisingly, there were no *P. fortinii* cultures that produced fragments of this size.

Cryptosporiopsis sp. produced 497 and 500bp sized fragments, but no others. Likewise, *Epacris microphylla* produced only 500 and 502bp sized fragments. No species seemed to produce all of the fragments sizes. Only *Lachnum pygmaeum* was faithful in producing only one sized fragment.

The 500bp fragment was produced by 6 different isolates (only 5 are listed because one of the isolates could not be identified with certainty using GenBank).

Table 5. Cultures identified using sequencing and their corresponding LH-PCR fragments. Culture identifications beginning with A denote Alpine samples, E denote ESSF, I for ICH and S for SBS.

LH-PCR Fragment	Name by sequencing ID	Culture ID
494	*Phialocephala fortinii*	A4-3
	Uncultured clone (DQ233886.1)	A8-3
	Lachnum pygmaeum	I1-4
	Salal root associate UBCtra305 *(AF149076.1)*	I10-2
	Lachnum pygmaeum	S7-4
480	*Geomyces pannorum*	S5-13
484	*Phialocephala fortinii*	A1-5
489	*Oidiodendron sp.*	A2-4
	Meliniomyces	E5-31
	Phialocephala fortinii	I7-1
491	*Phialocephala fortinii*	E3-3
	Oidiodendron maius	I1-9
	Phialocephala fortinii	I6-3
	Phialocephala fortinii	S6-14
	Phialocephala fortinii	S9-3
497	*Cryptosporiopsis*	A1-15
	Rhizoscyphus ericae	A8-1a
	Uncultured mycorrhizal fungus (EF195481.1)	A10-9
	Rhizoscyphus ericae	E4-5
	Salal root associate UBCtra153 (AF149078.1)	E7-14
	Ericoid endophyte (AF252837)	E9-11
500	*Epacris microphylla*	A2-1
	Cryptosporiopsis	A5-7
	Cryptosporiopsis	A7-1
	Salal root associate UBCtra153 (AF149078.1)	E3-1
	Meliniomyces	E5-26
	Rhizoscyphus ericae	E6-4b
	Cryptosporiopsis	I3-1
	Meliniomyces	I8-9
	Cryptosporiopsis	S3-5
	Neonectria radicicola	S5-12
502	*Meliniomyces*	A3-6
	Epacris microphylla	S1-2

Table 6. Comparisons of lengths of ITS2 segments of identified cultures as determined using LH-PCR and sequencing.

Culture	Length by LH-PCR	Length by sequencing
Phialocephala fortinii	494	489
Uncultured clone (DQ233886.1)	494	490
Phialocephala fortinii	484	490
Oidiodendron sp.	489	491
Phialocephala fortinii	491	491
Rhizoscyphus ericae	491	494
Uncultured mycorrhizal fungus (EF195481.1)	491	494
Rhizoscyphus ericae	491	494
Salal root associate UBCtra153 (AF149078.1)	491	497
Ericoid endophyte (AF252837)	491	493
Epacris microphylla	500	498
Cryptosporiopsis	500	489
Cryptosporiopsis	500	499
Salal root associate UBCtra153 (AF149078.1)	500	498
Rhizoscyphus ericae	500	494
Meliniomyces	502	498

It appears as though this fragment may be the most common ITS2 fragment length. It is likely that it represents more unculturable fungi.

Phylogenetic analysis of cultures

Phylogenetic trees using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) were generated for ITS sequences from the most commonly isolated fungi (Figures 6-9). In general, very little sequence variation was found between different isolates of the same taxa. The four most commonly isolated fungi were compared using phylogenetic trees. *Phialocephala fortinii* ITS sequences do not differ across the elevation gradient (Figure 6). *Cryptosporiopsis* sp. ITS sequences show some aggregation based on elevation (Figure 7). GenBank sequences for *C. radicicola* and *C. ericae* were included in this tree to characterize the affinities among sequences in

the tree. Most of the Cryptosporiopsis sp. isolated are related to *C. ericae*, and these are representatives from the ESSF, ICH and SBS. Several SBS isolates group together with *C. radicicola*. Alpine isolates tend to group out from the rest and appear to be related to neither *C. ericae* or the less predominant *C. radicicola*. One alpine isolate, A8-1, stands out as least related to the whole group. *Rhizoscyphus ericae* did not differ between or within the alpine and ESSF (Figure 8). *Meliniomyces* sp. was found mostly in the ESSF. Isolates of *Meliniomyces* from other elevations do not seem to differ greatly from those found in the ESSF (Figure 9).

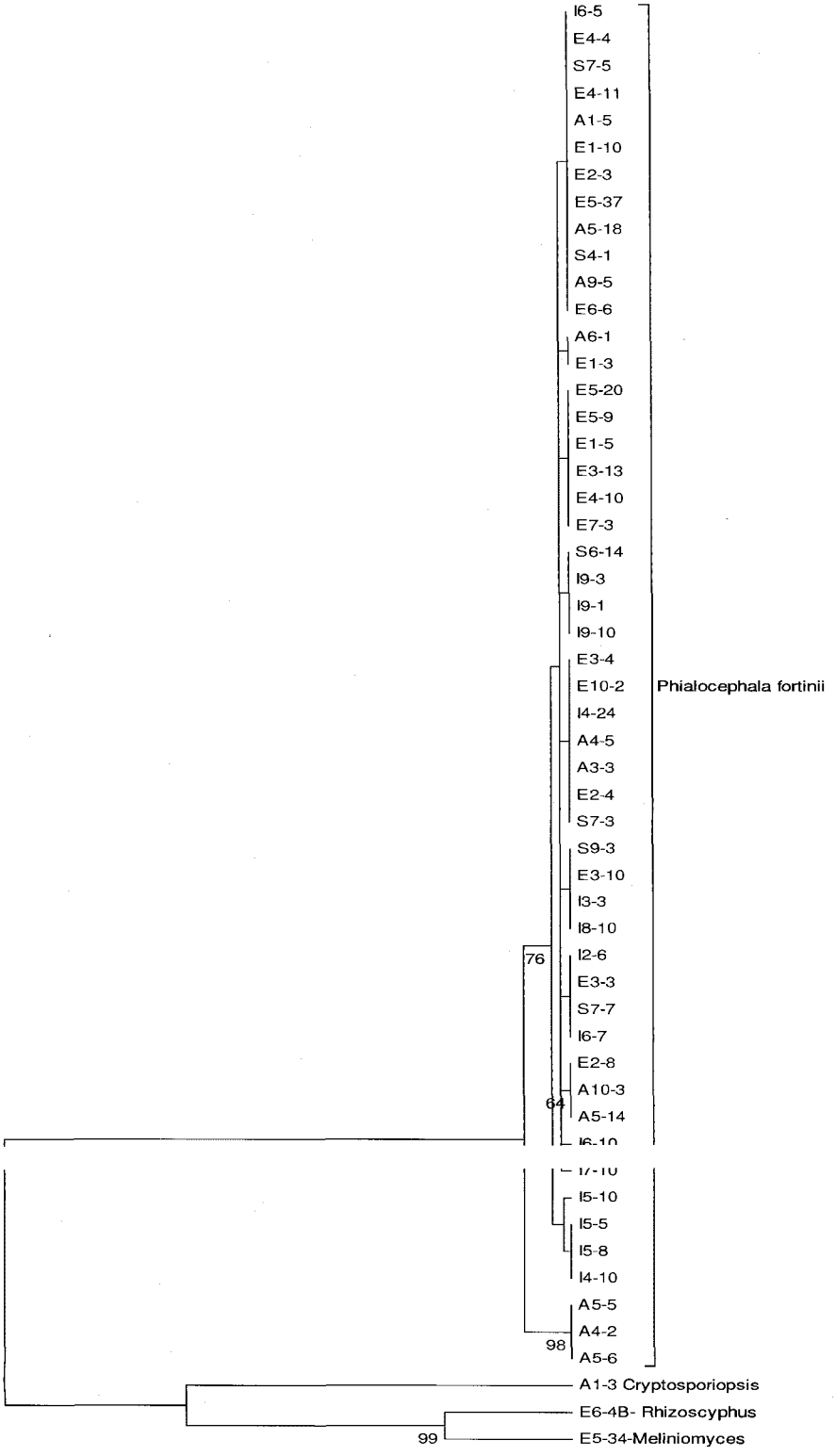


Figure 6. UPGMA phylogenetic tree comparing *Phialocephala fortinii* isolates from all elevations rooted to other significant isolates. Bootstrap performed with 1000 replicates and values over 50 reported.

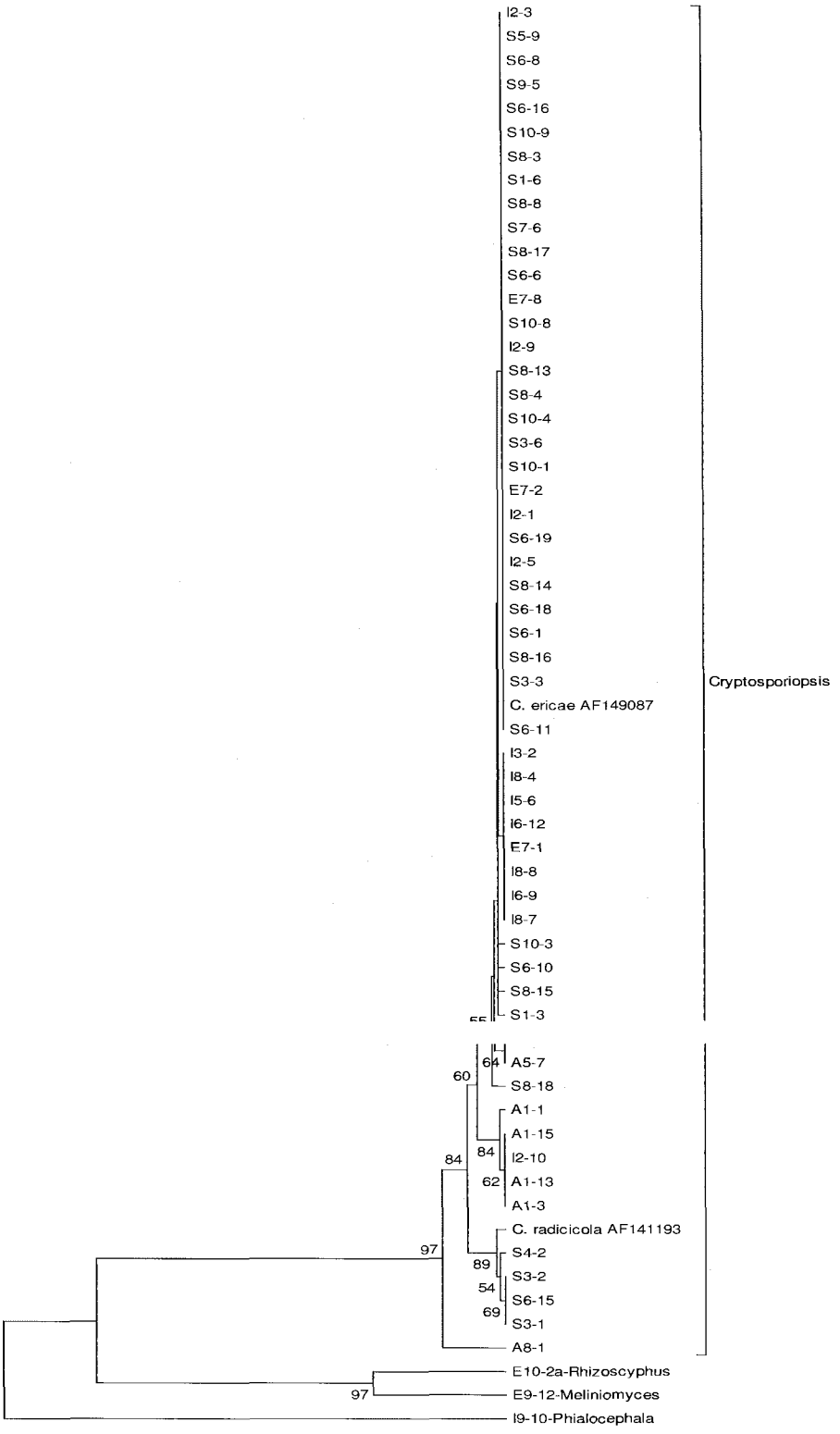


Figure 7. *Cryptosporiopsis* UPGMA phylogenetic tree with bootstrap values

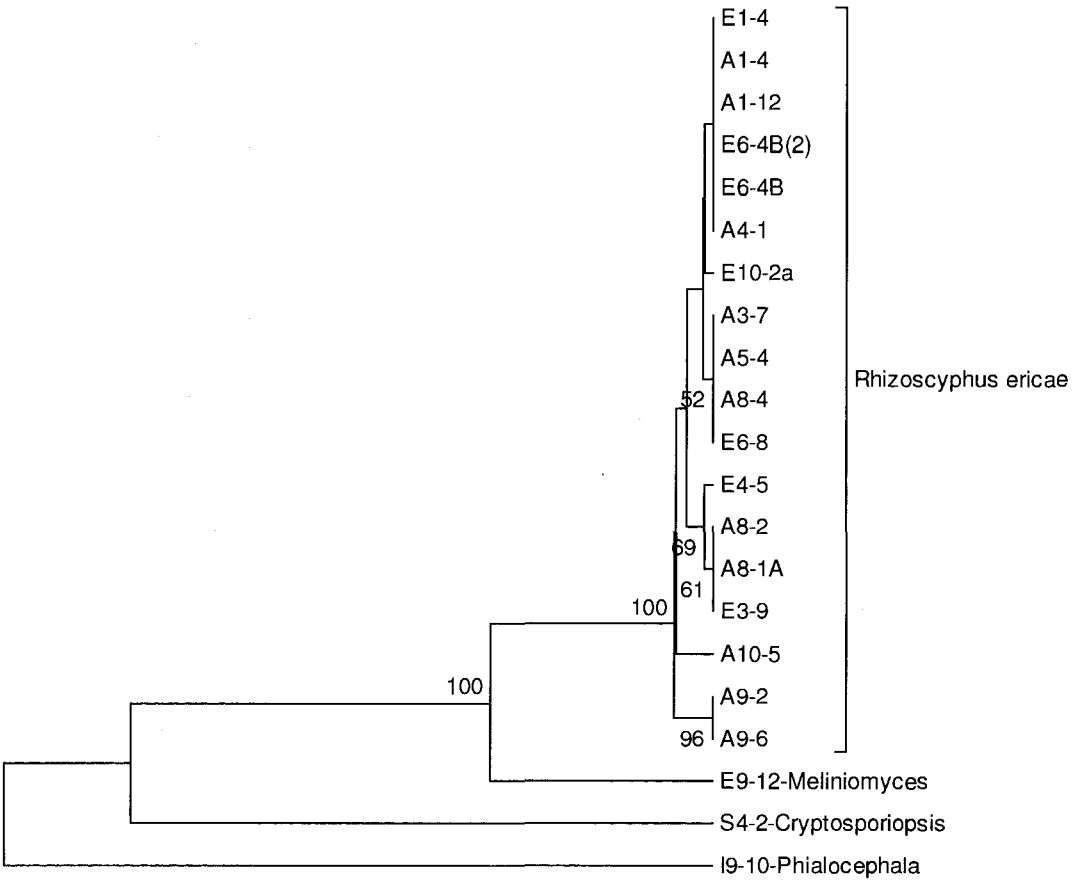


Figure 8. UPGMA phylogenetic tree of *Rhizoscyphus ericae* ITS sequences from alpine (A1, A denotes alpine, 1 denotes first plant harvested in transect) and ESSF (E denotes ESSF) *V. membranaceum* plants.

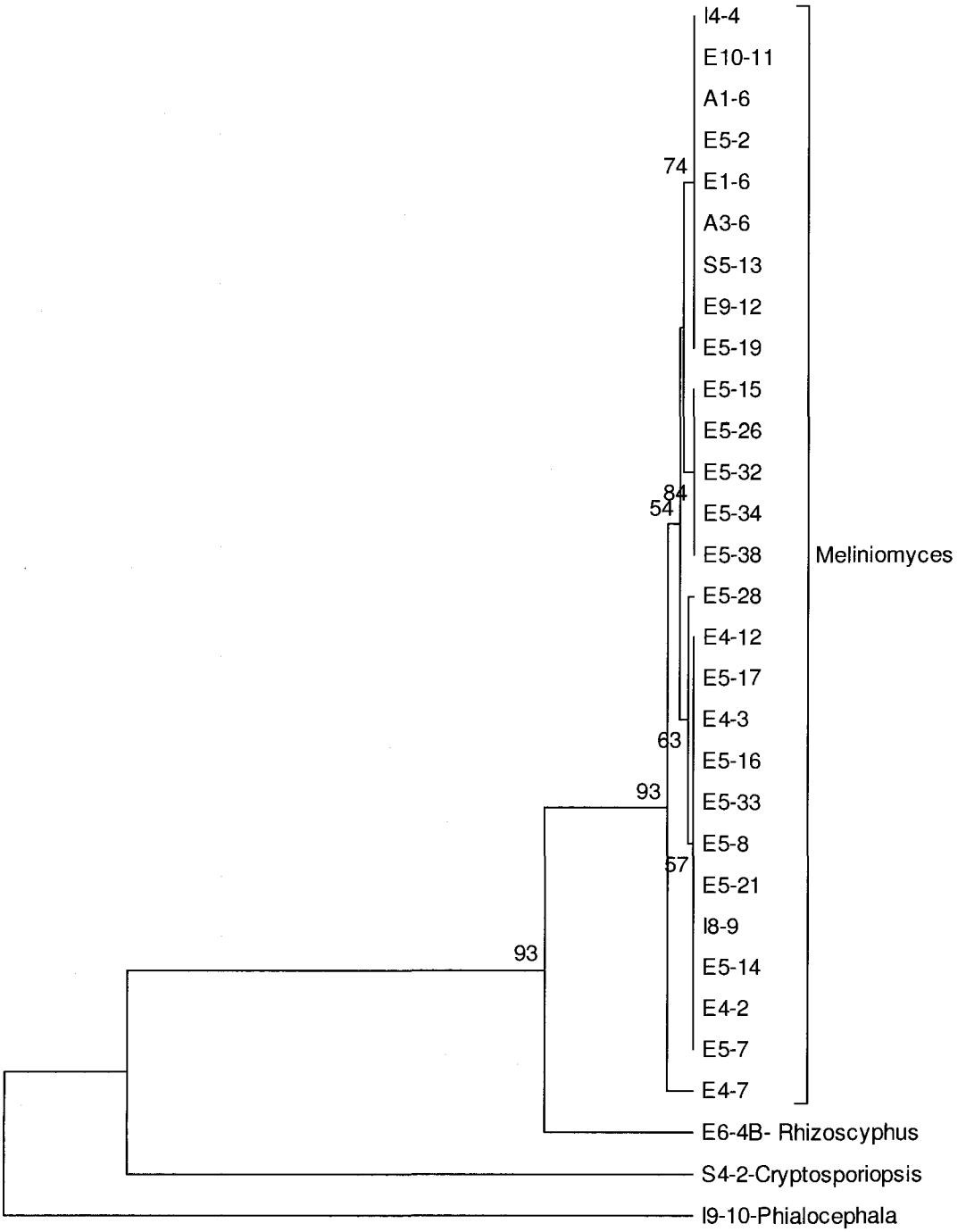


Figure 9. UPGMA phylogenetic tree of *Meliniomyces* ITS sequences from alpine (A1, A denotes alpine, 1 denotes first plant harvested in transect) and ESSF (E denotes ESSF) and ICH *V. membranaceum* plants.

LH-PCR Multivariate approaches

Differences in the community structure of fungi associated with *V. membranaceum* roots as detected by LH-PCR were analyzed using NMS. Presence/absence of fragments generated from each plant was used to produce the plot in Figure 11. A final stress of 15.6 for a 3-dimensional solution with an instability of 0.002872 was found. Each point on the plot in Figure 11 represents the suite of fragments found on the roots of one plant. The Axis 1 & 2 projection shows the SBS plants tend to group together. The arrows in this projection also indicate the direction and relative strength of the correlation between this grouping of plants and two environmental variables: pH of organic soil (Pearson and Kendall correlation, r^2=0.120) and leaf mass to area ratio (r^2=0.123). Thus, the grouping of SBS plants tends to be associated with increased pH values of the organic fractions of soil and a decrease in the leaf mass to area ratio (Appendices 3 & 4). These correlations are not very strong. As illustrated by the Axis 1 & 3 and Axis 2 & 3 projections of Figure 11, there is considerable overlap between the points, such that the SBS grouping is not very distinct. Thus the structure of the community associated with SBS plants resembles the communities of some plants from other elevations.

Whereas Figure 11 is a graphical NMS representation of fragment profiles associated with the roots of plants at particular elevations, differences in the profiles between elevations were also tested using multivariate ANOVAs (perMANOVA). According to the perMANOVA analysis, the SBS differs significantly from the alpine and ESSF (both, $p<0.05$) and somewhat from the ICH ($p=0.15$). Figure 10 includes a schematic of these LH-PCR differences as well as differences detected based on

culture data (below). Figure 10 represents the overlaps in community structure over the mountain side and demonstrates graphically that these differences exist as a gradient from the top to the bottom of the slope.

Furthermore, indicator species shows two significant indicators: fragment 496 for ESSF ($p<0.05$) and fragment 612 ($p<0.1$) for the SBS.

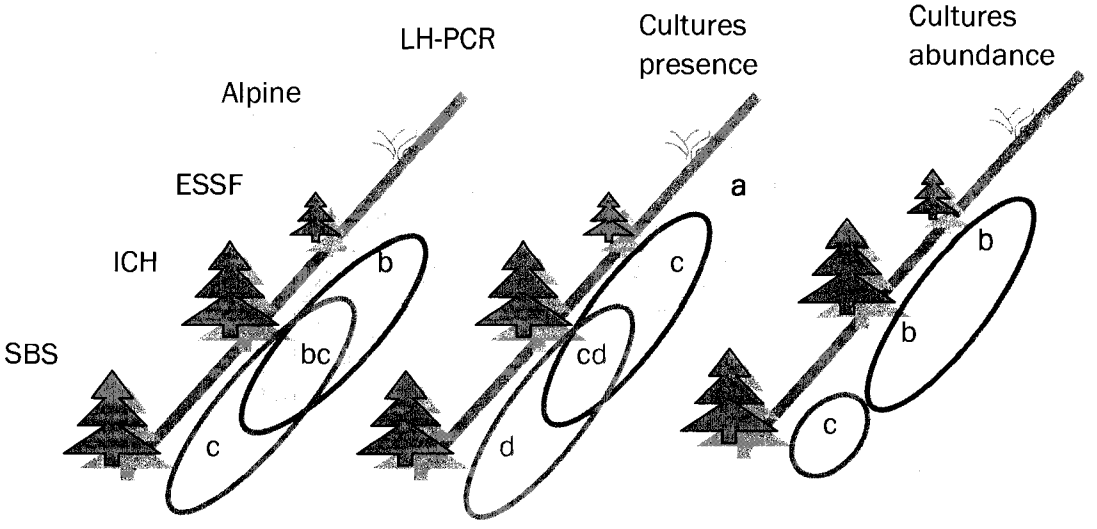


Figure 10. Schematic of multivariate differences between elevations based on three approaches: LH-PCR of fungal DNA extracted directly from roots; presence/absence data based on culturing from roots; and abundance based on numbers of cultures isolated from each plant over the four elevations tested. Results shown are those where $p<0.05$. Diagram is not to scale.

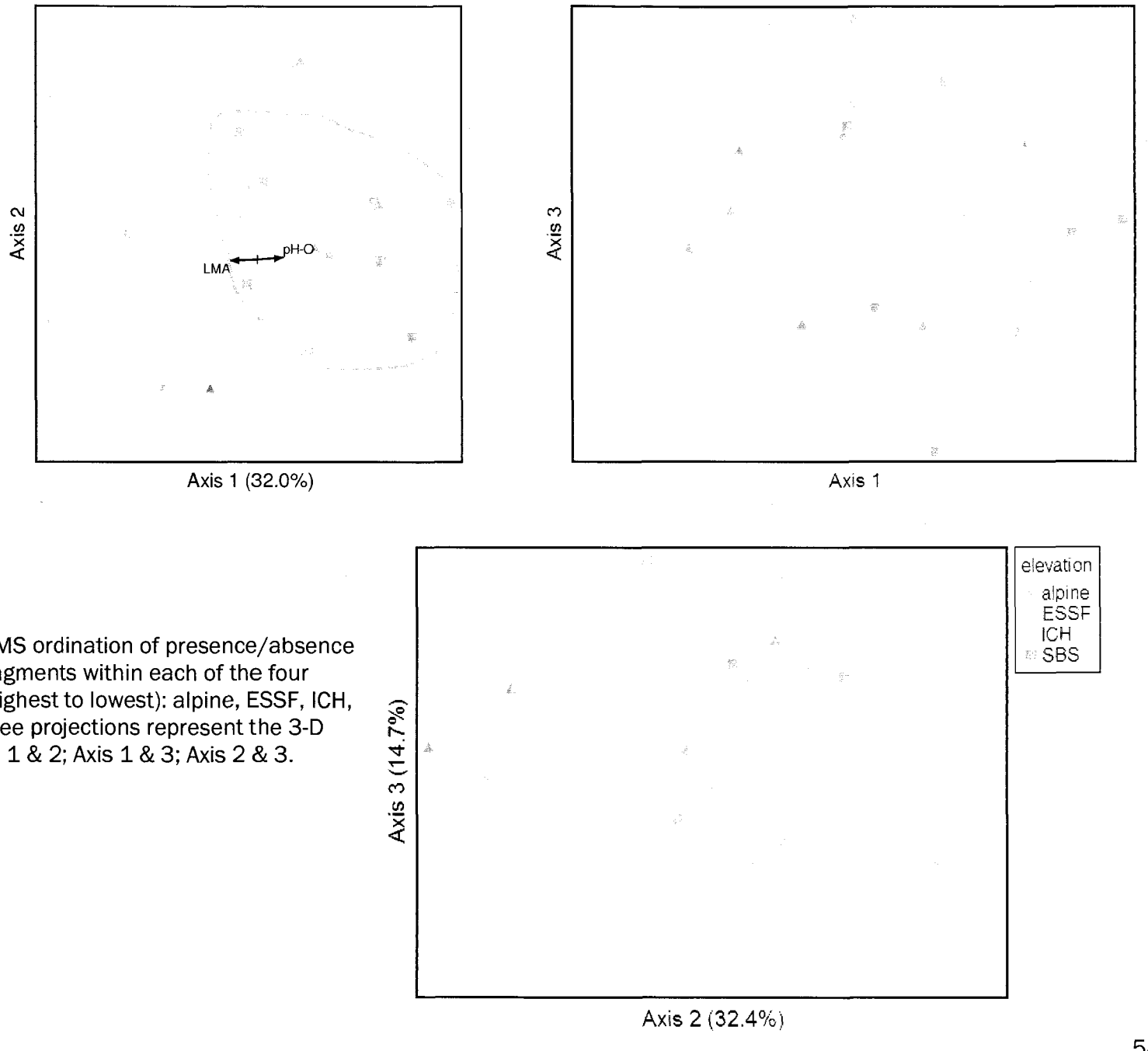


Figure 11. NMS ordination of presence/absence of LH-PCR fragments within each of the four elevations (highest to lowest): alpine, ESSF, ICH, and SBS. Three projections represent the 3-D solution: Axis 1 & 2; Axis 1 & 3; Axis 2 & 3.

Multivariate approaches to Cultures

Two data sets were used in NMS of culture data: presence/absence and abundance. The NMS ordination of presence/absence data yielded a 2-D solution (Figure 12). This analysis had a final stress of 19.53 and an instability of 0.00002. This graph also shows that the SBS plant root communities tend to group together. Only six points representing SBS plant root community structure appear in Figure 12. *Cryptosporiopsis* sp. and *P. fortinii* were isolated from roots of five out of ten plants in the SBS: thus in presence/absence data, these five plants appear identical and thus overlap in the ordination graph. Pearson and Kendall correlations show that differences represented by Axis 1 in this graph are correlated with increases in pH of both soil fractions (organic and mineral) and carbon to nitrogen ratios of the mineral soil. Also, the grouping is correlated with a decrease in leaf mass to area ratios.

Comparing the elevations using perMANOVA shows that adjacent elevations are not different from each other. The alpine and the ESSF do not differ, nor does the ICH and ESSF, nor the ICH and SBS. The differences found are between the alpine and the ICH and SBS ($p<0.01$). The ESSF is also significantly different from the SBS ($p<0.01$). These differences are illustrated in Figure 10.

Abundances were calculated based on the relative number of identical cultures isolated from each plant. Ordination of identified cultured fungi from each plant using the same parameters as above yielded an unstable NMS results with repetitive trails. Comparing elevations with perMANOVA (Figure 10) demonstrated significant differences between the alpine and ESSF ($p<0.05$), the alpine and ICH ($p<0.05$) and between the ESSF and ICH ($p<0.01$). The alpine differed somewhat

from the SBS (p=0.062). Differences between the ESSF and ICH were not significant (p=0.128) and between the ICH and SBS (p=0.183).

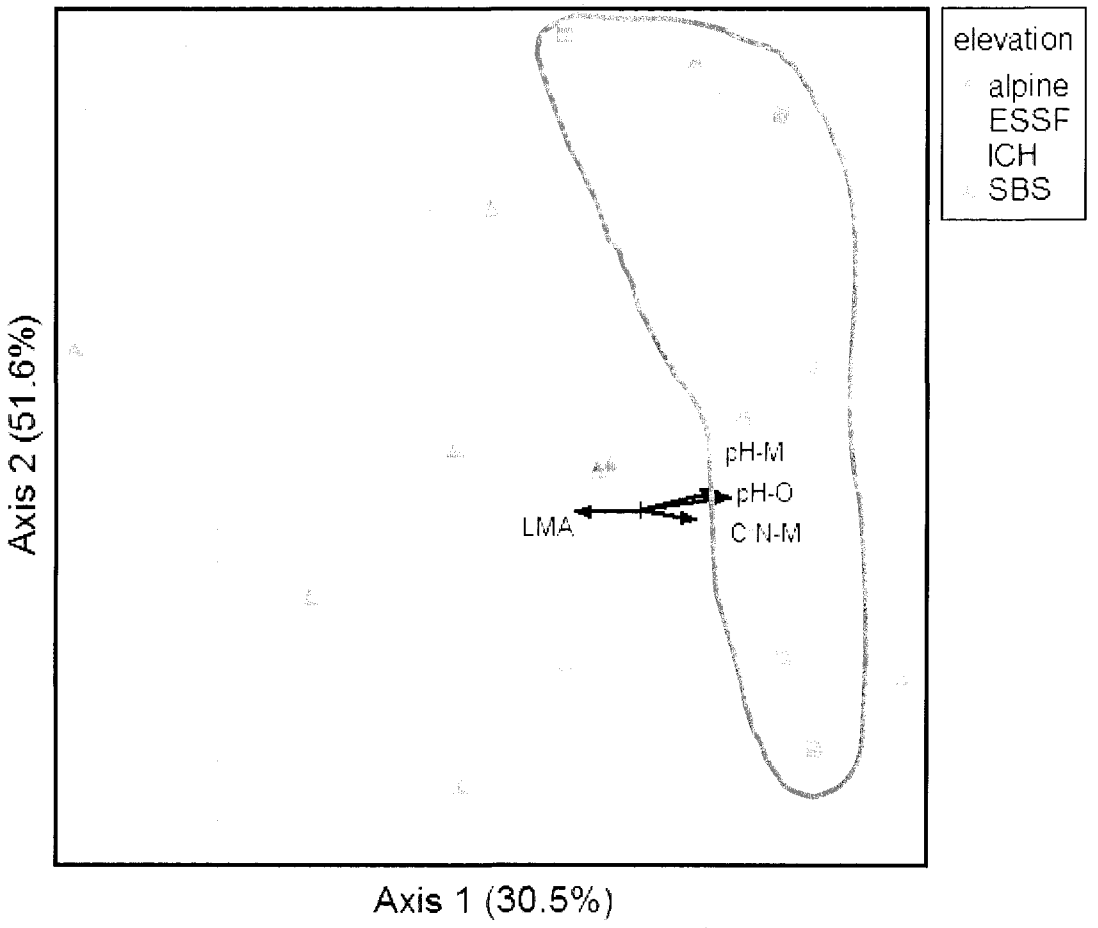


Figure 12. NMS ordination of presence/absence of cultures isolated at each of the four elevations (highest to lowest): alpine, ESSF, ICH and SBS. Associated primarily with Axis 1, Pearson and Kendall correlations for environmental variables are pH of organic soil (r^2=0.221), pH of mineral soil (r^2=0.185), C:N ratio of mineral soil (r^2=0.135) and LMA (r^2=0.157).

Using the presence/absence data, the only significant indicator species was found for the alpine: *Rhizoscyphus ericae* (p=0.051). When abundances are considered, *Cryptosporiopsis* is an indicator for the SBS (p=0.040) and *Meliniomyces* is an indicator for the ESSF (p=0.028). *Phialocephala fortinii* is somewhat of an

indicator of the ICH ($p=0.174$), but it is present at every elevation and thus a poor indicator species.

Canonical Correspondence Analysis

The LH-PCR and both interpretations of the culturing approaches demonstrate the same result with CCA (Figures 13-15). In all 3 cases, CCA indicates the fungal community structure is different between high and low elevations. The LH-PCR data (Figure 13) clearly separates the ICH and SBS (low elevations) from the alpine and ESSF (high elevations). The low elevation root associated fungal community structure variability is best explained by increasing soil pH (of both mineral and organic soils). The high elevation fungal community structure is associated with increased age of *V. membranaceum* plants, increased sun leaves (by leaf mass to area ratios), and wetter soil.

The matrix of presence/absence of cultures isolated from roots of plants at each of the elevations as analyzed by CCA shows the same trend as the LH-PCR results (Figure 14). Fungal community structure partitions between high and low elevations. The correlations to environmental variables are also the same with the addition of carbon to nitrogen ratios increasing with low elevations.

Figure 15 shows the CCA analysis of the abundance of cultures data. Here, like in the previous two analyses, the CCA graphs show that root-associated fungal community structure partitions based on elevation. The high elevation alpine and ESSF overlap and are distinct from the lower elevations ICH and SBS, which also overlap. The environmental variables that correlate to the changes are also the same as the presence/absence of cultures data in Figure 14. High elevation communities correlate with higher soil moisture, higher proportion of sun leaves and older plants.

Lower elevation communities correlate with high soil pH values and higher mineral soil carbon to nitrogen ratios.

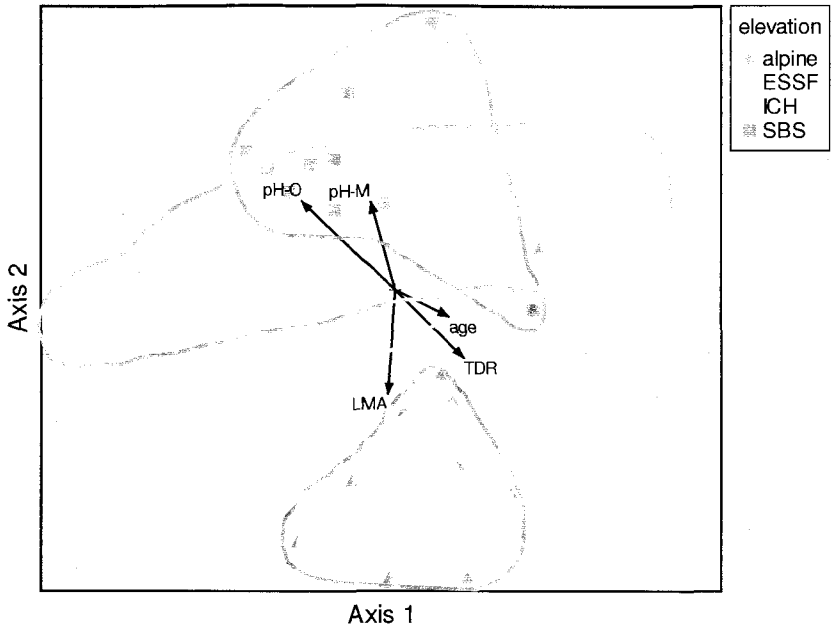
Mantel tests

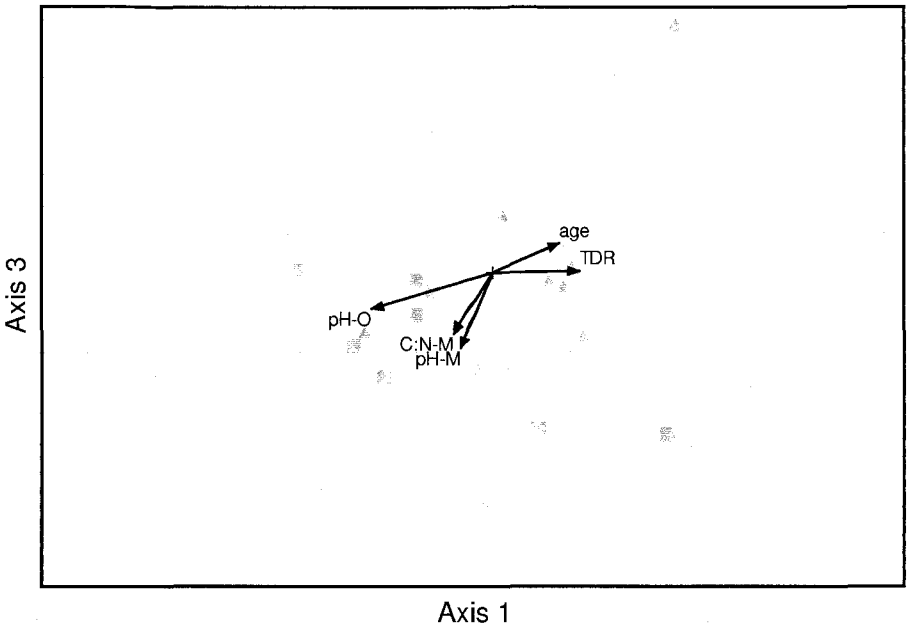
The mantel test was used to determine if plant and soil characteristics correlate to observed differences between fungal communities (McCune and Grace 2002). Two matrices, one containing fungal community data (any one of the three) and one containing environmental variables are compared to see if a positive association exists between the two. In the case of both types of culture data, a weak positive association was detected by the Mantel test ($r=0.218$, $p=0.002$, relative abundance of cultures; $r=0.199$, $p=0.001$ for presence/absence culture data). The LH-PCR of fungal DNA data demonstrated no such association.

Plant, soil and fungus characteristics

Plant and soil characteristics as well as colonization by ERM were compared between the four elevations. The plant variables measured were age and leaf mass to area ratios (LMA). The soil characteristics determined were percent water content at time of excavation, and the pH and C:N ratios for both mineral and organic soils. Extent of colonization by ericoid and dark septate fungi was also determined. Of these factors, six were found to differ significantly ($p<0.05$) between elevations using one-way ANOVA's (Figure 16).

Plant characteristics differed between elevations. Higher elevation *V. membranaceum* plants (alpine and ESSF) were significantly older than their lower elevation (ICH and SBS) counterparts. Leaf mass to area ratios were highest in the alpine, followed by the ESSF and then both low elevations which did not differ





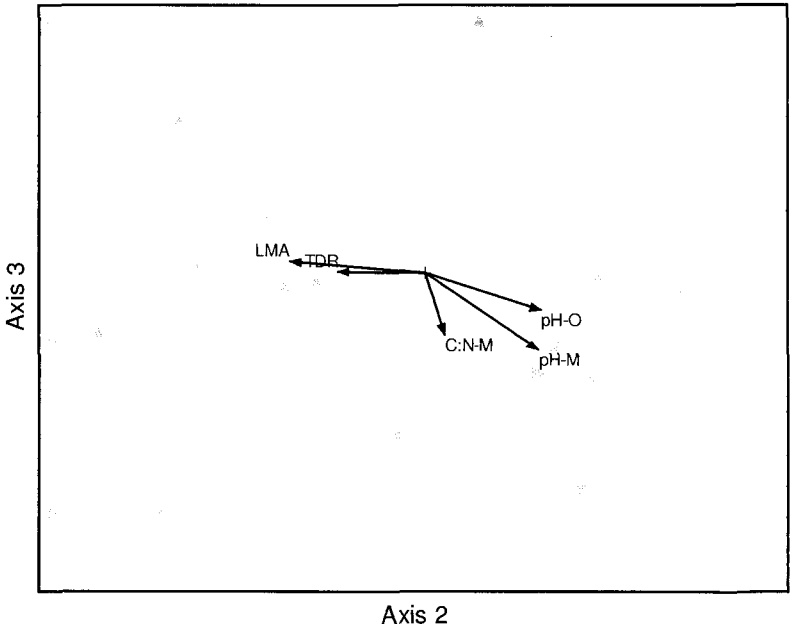
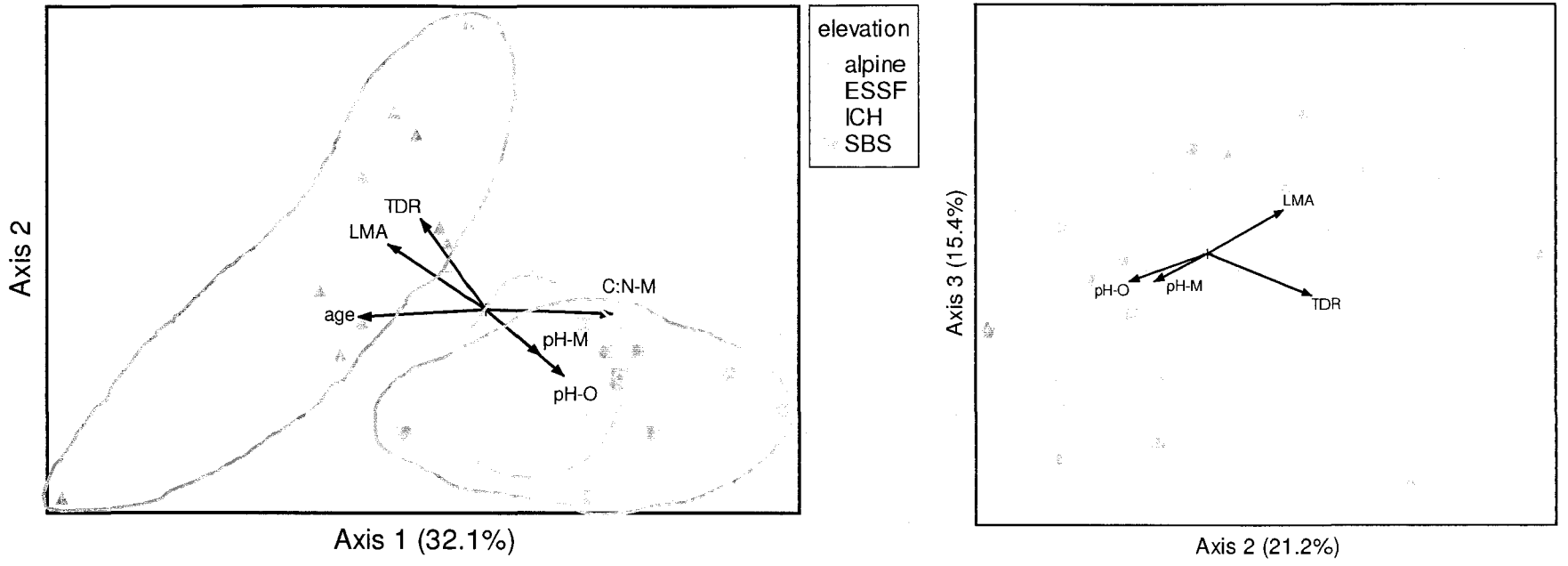


Figure 13. Canonical Correspondance Analysis ordination of presence/absence of LH-PCR fragments within each of the four elevations (highest to lowest): alpine, ESSF, ICH, and SBS. Confidences based on 1000 Monte Carlo runs for eigenvalues $p<0.1$, Species-environmental correlations $p<0.1$.



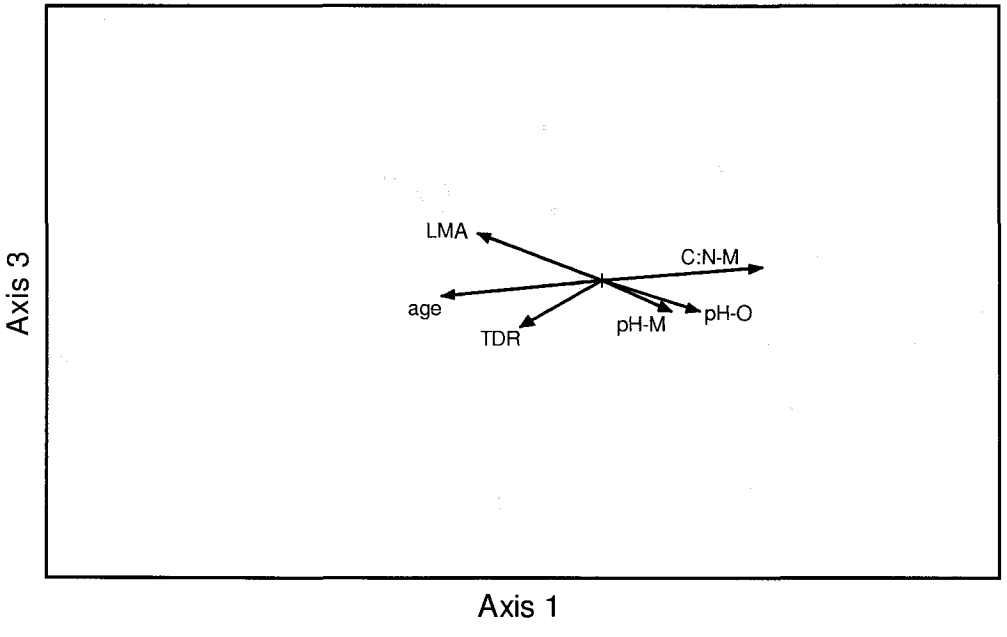


Figure 14. Canonical Correspondance Analysis ordination of presence/absence of cultures isolated at each of the four elevations (highest to lowest): alpine, ESSF, ICH, and SBS. Confidences based on 1000 Monte Carlo runs for eigenvalues $p<0.1$, Species-environmental correlations $p<0.1$.

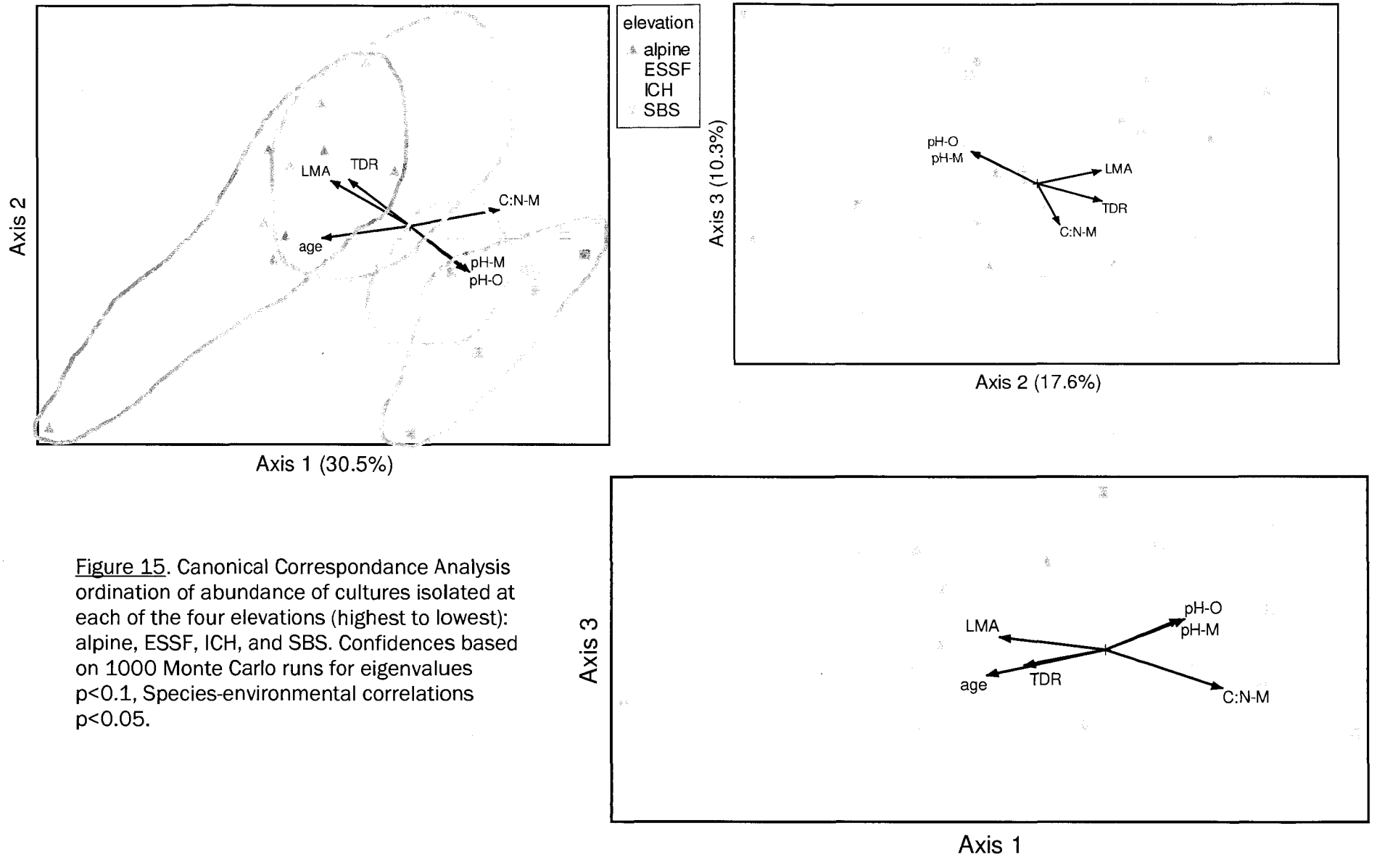


Figure 15. Canonical Correspondance Analysis ordination of abundance of cultures isolated at each of the four elevations (highest to lowest): alpine, ESSF, ICH, and SBS. Confidences based on 1000 Monte Carlo runs for eigenvalues $p<0.1$, Species-environmental correlations $p<0.05$.

significantly from each other. The plants at the low elevations were thus equally shaded whereas the ESSF was partially shaded and the alpine plants experienced full sun; these measurements conform to field observations.

Soil characteristics also differed between elevations. At the time of excavation, the higher elevation *V. membranaceum* plants (alpine and ESSF) had significantly wetter soils than their lower elevation (ICH and SBS) counterparts. Alpine and ESSF organic horizons were more acidic than ICH organic horizons which were more acid than the lowest elevation SBS. The mineral soils, however, did not differ between the alpine, ESSF and ICH, but were more acidic than the SBS mineral soils. Finally, the C:N ratios were lower in the alpine than in the SBS, with overlap in the mid elevations. The C:N ratio values of the organic horizons ranged from 16.5 to 74.8 across elevations. No difference in ratios was detected, which may have been due to the high variability within groups.

The extent of colonization of roots by ericoid and dark septate fungi did not differ convincingly between the elevations (Appendix 3). The alpine (mean=46.3 ± 15.2%) and ICH (mean=45.1 ± 13.8%) are somewhat more colonized than the ESSF (mean=34.2 ± 13.1%)and SBS (mean=34.6 ± 14.9%) ($p<0.1$ in each case). These differences only become apparent after the removal of two potential outliers in a data set that is highly variable. Thus these apparent differences may not be real. The extent of colonization values represent a sum of ericoid and dark septate microsclerotia recorded during observation. Both of these morphologies were considered separately and neither differ between the elevations. Variability remains

high for these values as well (with the exception of the dark septate morphology observed on ESSF and SBS plant root samples, which have a low variability).

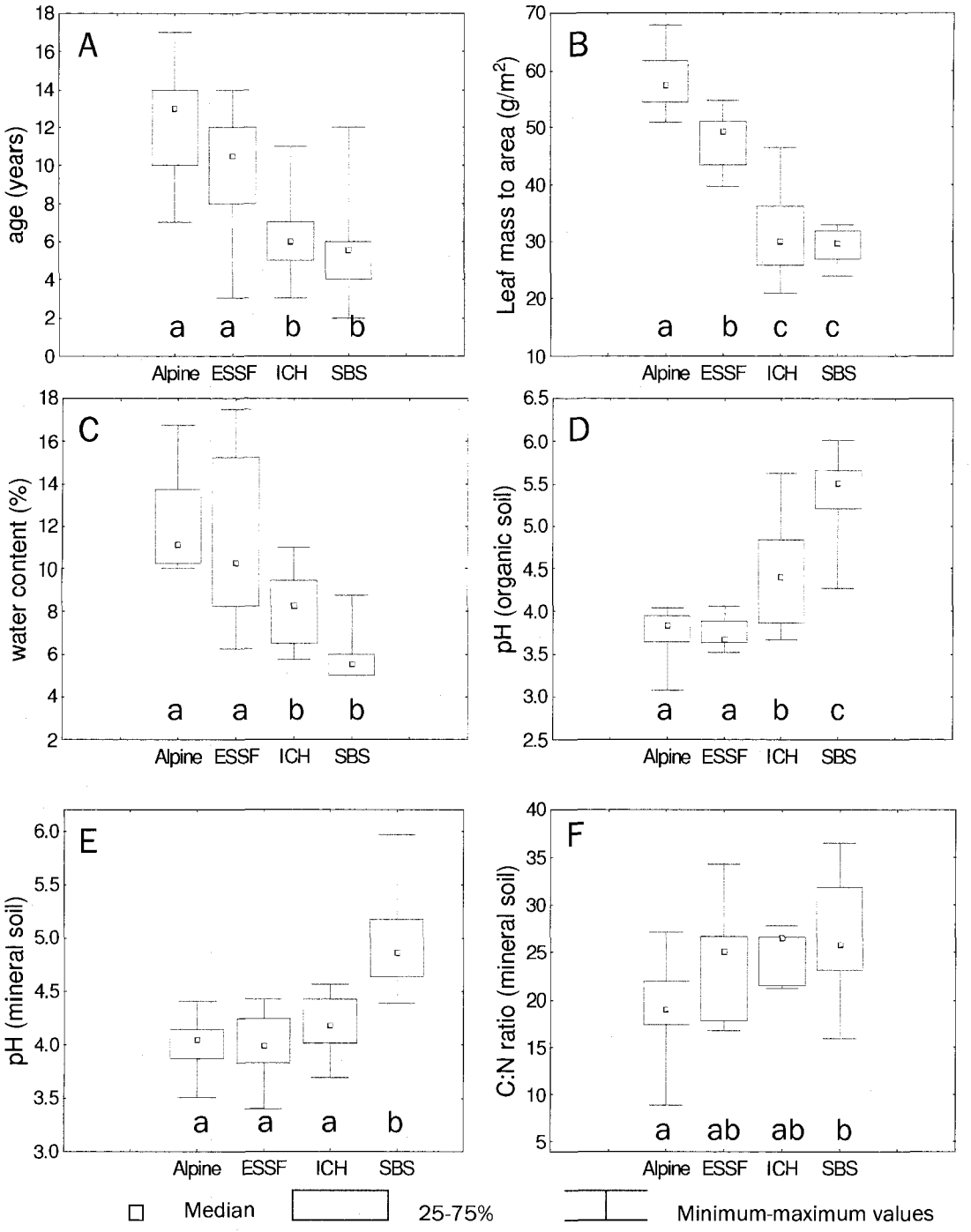


Figure 16. Box and whisker plots of *V. membranaceum* plant and soil characteristics illustrating medians and data spread. **A**) Age of plants in-the alpine (12.2 ± 3.30 years), ESSF (9.5 ± 3.5 years), SBS (6.2 ± 2.1 years), and ICH (5.8 ± 2.8 years); **B**) LMA values for plants in the alpine (58.2 ± 5.4g/m^2), ESSF (47.9 ± 4.8g/m^2), ICH (31.2 ± 7.3g/m^2) and SBS (29.2 ± 2.3g/m^2); **C**) soil water content at time of harvest in the alpine (12.1 ± 2.36%), ESSF (11.5 ± 4.06%), ICH (8.2 ± 1.83%) and SBS (5.8 ± 1.18%) **D**) pH of the organic layer of soil in the alpine (3.74 ± 0.29), ESSF (3.73 ± 0.20), ICH (4.46 ± 0.65) and SBS (5.40 ± 0.50) **E**) pH of the mineral layer of soil in the alpine (4.00 ± 0.27), ESSF (3.97 ± 0.33), ICH (4.17 ± 0.29) and SBS (4.96 ± 0.49) **F**) C:N ratios of the mineral soil layer in the alpine (19.06 ± 5.19), ESSF (24.41 ± 5.78), ICH (25.08 ± 2.65) and SBS (26.78 ± 6.56).

Chapter 4: Discussion

The purpose of this study was to characterize changes in fungal community structure associated with *V. membranaceum* hair roots across a BEC zone elevation gradient and to correlate those changes with environmental variables across that gradient. There were differences between the high elevation (alpine and ESSF) and low elevation (ICH and SBS) fungal communities, with considerable overlap. The SBS fungal community tended to group together. Correlations were weak and limited to two variables measured. The higher elevation (alpine and ESSF) fungal communities were correlated with indicators of an open canopy (LMA), whereas the lower elevation (ICH and SBS) fungal communities was correlated with an increase in pH of the organic soil at lower elevations.

Most of the interpretation of the results is focused on the NMS analysis. The objectives of this study were met using statistical approaches that do not weigh equally. NMS is best suited for this exploratory data set (Quinn and Keough 2002) and is primarily used in interpretation. The second approach, CCA, is important in that it corroborates the patterns generated by NMS. CCA does not weigh equally to NMS. The two techniques for assessing the fungal community, Length Heterogeneity-PCR and culturing, complement each other. These methods are assumed to contribute information equally to the conclusions.

LH-PCR

The NMS ordinations of the LH-PCR data demonstrate a grouping of the SBS plants. This grouping is corroborated by the CCA analysis. The perMANOVA analysis also indicates that the SBS is significantly different from both of the two highest elevation sites: the alpine and ESSF. Both the NMS and perMANOVA show considerable

overlap in root-associated fungal community structure between the elevations. In light of the pattern of fragments generated, it is not surprising that the NMS analysis found that there are overlaps in the community structure. Many fragments are common to all four elevations. The diagram representing significant differences based on perMANOVA suggests that there is a gradient from the top to the bottom of slope in terms of similarity of fungal community structure between these sites. Adjacent elevations tend to be alike (not significantly different from each other) but do differ from elevations further apart.

Each elevation has several fragments that are exclusive to it. The ICH, with 10, has the greatest number of exclusive fragments. The ESSF has 5, the SBS has 4 and the alpine has 3. Only two fragments, however, are significant indicator 'species' (fragment 612 is an indicator of the SBS and fragment 496 is an indicator of the ESSF). Indicator 'species' (fragments, in this case), must be found on most plants in an elevation and must not be found at other sites. Although there are many exclusive fragments (22 total), they appear to represent rare fungi. Furthermore, the cultured fungi generated LH-PCR fragments ranging from 484-502bp in length. The fragments that were exclusive to each elevation did not fall into this range. It thus appears that the LH-PCR technique was able to detect more rare taxa of fungi than the culturing method.

Lejon *et al.* (2005) used LH-PCR to examine fungal and bacterial community structure in forests of Norway spruce (*Picea abies*), Douglas fir (*Pseudotsuga menziesii*), and oak (*Quercus sessiflora*) and mixed stands in Morvan, France. They found 100 fragments per fungal profile when examining soil samples with LH-PCR. Our study found a maximum of 20 per profile. The technique used by Lejon *et al*

(2005) was slightly different (using both ITS1 and ITS2) and their substrate was soil, which could account for the difference. Soil is known to contain a great diversity of fungi including decomposers, parasites, mycorrhizal fungi and in different states such as spores and mycelia (Stamets 2005). It is likely that soil would yield more fragments in LH-PCR than thin hair roots where extraneous soil material had been meticulously removed.

Fragments generated by LH-PCR do not necessarily represent one taxon. Nor do they correspond directly to length as determined by sequencing. This may be due to changes in fragment length due to the inclusion of the fluorophore in fragment analysis (Ritchie *et al.* 2000). LH-PCR can, however, discriminate between different communities of fungi (Ranjard *et al.* 2001).

Cultures

Four fungi were most commonly isolated from surface sterilized roots. *Rhizoscyphus ericae* is a known ERM (Pearson and Read 1973) and was a significant indicator of the alpine; its occurrence decreased with elevation and none were isolated from the lowest elevation. *Meliniomyces* sp. was most frequently isolated from plant roots in the ESSF. It was isolated at least once in every other elevation. *Cryptosporiopsis* spp. were found in abundance in the SBS. *Phialocephala fortinii* was most frequently isolated and was found at every elevation. The largest proportion of *P. fortinii* was found in the ICH (71%), followed by the ESSF (52%), the alpine (41%) and the SBS (35%). *Oidiodendron* sp. and *Neonectria radicicola* were also common, but found in fewer numbers than the above fungi.

Ordination with NMS of the data generated from culturing approach demonstrates similar patterns to those found with the LH-PCR approach. Figure 12

shows the grouping of SBS plants based on the presence and absence of cultures isolated from their roots. This pattern is corroborated by the CCA ordination for presence/absence data and also by the less robust abundance measure, namely plate enumeration. Four out of 10 SBS plants yielded only *Cryptosporiopsis* sp. and *P. fortinii* cultures. Furthermore, perMANOVA demonstrates that the differences in community structure follow a gradient along the slope of the mountain where high elevation communities differ from low elevations. This is also true for the culture abundances data set.

Phialocephala fortinii was isolated at every elevation and in abundance from the middle elevations. It has been shown to be a root-associate of numerous trees (Mandyam and Jumpponen 2005) and isolated from other plant parts (Menkis *et al.* 2004). A fungus with such a range of substrates may be easier to isolate because it is able to grow under various conditions, including saprotrophically. The reason there may be an abundance of cultures from these elevations may be related to its propensity to grow on potato dextrose agar, as opposed to ericoid fungi which may be unable to grow without a host presence. Interestingly, more *P. fortinii* isolates were found in the ESSF and ICH (65 and 75, respectively) than in the alpine and SBS (29 and 27). Since the isolating techniques were identical, it seems that there may be more *P. fortinii* mid-slope than at the extreme high or low elevations, on the same host. This result is especially interesting in light of the fact that the alpine and ESSF zones were located close together, that they would differ so considerably in yielding *P. fortinii* cultures. It seems to indicate that these two zones differ somewhat in terms of *V. membranaceum* root-associated fungal communities. Regardless, *P. fortinii* was found on most *V. membranaceum* plants in this study. Its ubiquity may be

attributed to its many adaptations and abilities (Addy *et al.* 2000, Piercey *et al.* 2004). Its preference for the mid-range of the gradient may indicate less fitness at the extremes.

In the SBS, *Cryptosporiopsis* spp. increase in abundance relative to the other higher elevations. This finding contrasts previous studies that have indicated that *Cryptosposiopsis* sp. is rarely isolated (Sigler and Gibas 2005; Verkley *et al.* 2003) and often failed to be detected (Allen *et al.* 2003; Berch *et al.* 2002) in ericaceous plants in North America. It has been demonstrated as an endophyte of aspen (*Populus tremuloides* Michx.) (Wang *et al.* 2007). *Cryptosporiopsis* spp. have various unique qualities such as transforming food industry by-products (Huszcza et al. 2008; Kostrzewa-Susłowa *et al.* 2007). They have also been isolated from healthy aerial plant parts (Wang *et al.* 2007). The results of our study indicate that *Cryptosporiopsis* spp. can be found in abundance in a North American ericaceous plant, and that this abundance seems to be habitat specific (i.e., the SBS) when it comes to associations with *V. membranaceum*.

The only other fungus isolated from all four elevations, besides *P. fortinii* and *Cryptosporiopsis* spp., was *Meliniomyces* spp. *Meliniomyces* spp. is the name given to the '*Hymenoscyphus ericae* aggregate' of morphologically distinct fungi often isolated from ericaceous roots and related to *Rhizoscyphus* (formerly *Hymenoscyphus*) *ericae*. *Meliniomyces variabilis* Hambleton & Sigler has recently been demonstrated to form typical ericoid mycorrhizas with roots of *Vaccinium* microcuttings (Vohnik *et al.* 2007). *Meliniomyces* spp. were found to be associated with many ericoid and non-ericoid hosts and thus may not be host-specific (Hambleton and Sigler 2005). *Meliniomyces* spp. may have some mycorrhizal

affinities, sometimes showing an ability to form ECM, sometimes ERM (Hambleton and Sigler 2005).

Oidiodendron spp. were isolated from two plants in the alpine, and one each from the ESSF and SBS. *Oidiodendron maius* forms ERM, but other species do not (Hambleton and Currah 1997, Lacourt *et al.* 2001). *Oidiodendron maius* was isolated from the ESSF and SBS. A determination of species for the alpine samples was not possible from the sequences obtained (due to quality of sequences). This genus is widespread and has been isolated worldwide (Rice and Currah 2005), so it is surprising that so few representatives were found in this study.

Not all fungi can be isolated in culture (Berch *et al.* 2002). Those that can be isolated are not necessarily mycorrhizal fungi (Smith and Read 2008). In this study, surface sterilization with dilute hydrogen peroxide was used to eliminate adhering fungi and surface hyphae, the assumption being that only those fungi present within plant root cells would be isolated on media plates. In practice, this has been shown to also effect fungi within roots and may reduce the numbers of isolates obtained (Smith and Read 2008). It could be that the profile of cultured fungi that were found using this approach is more a reflection of the fungi that were resistant to the hydrogen peroxide treatment, than what was in the root originally. Thus, the fact that 52% of the isolated fungi were *P. fortinii* could be a function of its resistance to the surface sterilization technique.

Abundance values are influenced by the sample size of the roots used to generate them. It is very difficult to determine the extent of hair root presence in a root system. Roots could have become detached during excavation or during washing. Estimates of root mass were probably flawed and thus not incorporated in

the analysis of abundance data. Root size estimates would have been needed to determine sample sizes appropriate to each population to apply correction factors post sampling (Quinn and Keough 2002). Abundance values are therefore not given much weight in the interpretation of the results in this study.

Soil, Plant and Fungal Variables

The second objective of this thesis was to correlate changes in root-associated fungal community structure to measured environmental variables. High elevation (alpine and ESSF) *V. membranaceum* plants were older and experienced more sun than the lower elevation (ICH and SBS) plants. The age of the plants may be impacted by the fire history of the site, as charcoal was found when digging in the ICH. High elevation soils were wetter and more acidic and possessed relatively less carbon than lower elevations. The trends appear linear along the BEC zone elevation gradient.

Of all the variables measured, only leaf mass to area ratios and pH of the organic soil correlated to changes in community structure. And these correlations were not very strong. Although environmental variable trends were strong, the lack of good correlation to fungal community structure may be due to the structure of community data itself which show overlap between the elevations. The lack of apparent structure in the data may be responsible for the poor correlations (McCune and Grace 2002).

Natural Gradients

This is the first report of ERM community structure over an elevation gradient. Similar studies that exploit natural gradients to find patterns of mycorrhizal community structure have been done (Bougoure *et al.* 2007; Kernaghan and Harper 2001; Mulder and de Zwart 2003; Nilsson *et al.* 2005). Bougoure *et al.* (2007)

examined both *Calluna vulgaris* (L.) Hull and *Vaccinium myrtillus* L. root-associated fungal community structure along a heath to forest gradient in Scotland using denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism and cloning and sequencing. They found the fungal community composition did not differ for *V. myrtillus* between their sites, whereas it did for *C. vulgaris*. Both were shown to have a distinct fungal profile and high fungal diversity associated with their roots. It appears as though root associated fungal communities are different between hosts and may vary with environmental conditions, but again, that is host dependent. Nilsson *et al.* (2005) explored the dominance of mycorrhizal types across a short nutritional gradient. By use of in-growth mesh bags and phospholipid fatty acid analysis, they found that in nutrient poor habitats, ERM and ECM dominate and there is more fungal biomass than in nutrient rich habitats. Arbuscular mycorrhizal fungi dominated in the more nutrient rich portion of their study gradient. Their results suggest that the dominant mycorrhizal categories change from ERM to ECM to AM as nutrient status increases along a gradient. Kernaghan and Harper (2001) found that ECM species richness and diversity decreased with increases in elevation along their gradient in the Alberta Rockies. They collected and used DNA sequencing to identify sporocarps. They found that host specific ECM tended to be found in ECM habitats, namely the subalpine forest. The alpine did harbour ECM fungi, but they tended to be generalists. Similarly, in this study, known ERM fungus (i.e., *R. ericae*) were found predominately in habitats with an abundance of ericaceous plants. At lower elevations, other hosts were present. There, fungi that exhibit other capabilities (i.e., *P. fortinii*) and are not specifically ERM were more likely to be found.

This study has exploited a natural gradient to gain understanding of ERM *in situ*. Much is known about fungi from controlled laboratory studies which are valuable but can never replace real world field studies (Smith and Read 2008). From laboratory-based studies, it is known that *R. ericae* is able to tolerate water stress (Chen *et al.* 2003). It can be inferred from this study that *R. ericae* confers this ability to its host. From the CCA analysis in this study, it appears, however, that the *R. ericae*-rich alpine zone correlates with wetter soil. At the time of this study, *R. ericae* did not seem to be associated with conferring water stress tolerance to this particular host. This first study of ERM associated with *V. membranaceum* across a natural elevation gradient joins other studies that attempt to understand the dynamics of mycorrhizal systems in natural settings.

Diversity measures of LH-PCR fragments and cultures

The LH-PCR method found more richness than the culturing method as demonstrated by higher alpha diversity values. Of note is the higher standard deviation of alpha diversity values in the LH-PCR approach. This prevents meaningful comparisons between elevations with the β_W measure. The number of fragments found per plant with LH-PCR ranged from 1-20, whereas the number of cultures obtained per plant ranged from 1-6. Plants with high fragment numbers did not correspond to those that had high numbers of cultured fungi. Gamma diversity was about 3 fold higher for LH-PCR fragments than cultures. With both alpha and gamma diversity measures, the differences could be attributed to direct DNA extraction and LH-PCR capturing more fungi. Berch *et al.* (2002) found most of their *Gaultheria shallon* roots yielded few culturable fungi but were colonized and yielded fungal DNA. Length-heterogeneity PCR fragments could also represent spores associated with the roots or saprotrophs

that were captured in the DNA extraction step. The fragment analysis captures more diversity but the ericoid or root-associated status of the fungi detected is not known, especially since these fragments were not clones and therefore not identified in this study.

Beta diversity is a measure of similarity between elevations. The alpine and ESSF are very similar by all measures and techniques. The results for the next transition, from ESSF to ICH are technique dependant. Culturable fungi are more different between these elevations than LH-PCR fragments. The final transition between ICH to SBS shows that these two elevations are more different based on LH-PCR fragments than based on culturable fungi. Considering the extremes of the gradient, the alpine compared to the SBS, both beta diversity measures and both techniques indicate that these elevations are most different from each other. Comparing the alpine and ICH, the culture data shows a discrepancy between the two beta diversity measures. $Beta_{sim}$, which focuses on gains and losses of species between elevations indicates that they are more similar, whereas $\beta_{sorensens}$, which places greater emphasis on species in common, indicates that these elevations are more different from each other. Thus it appears that in this case, there are fewer species in common and greater gains and losses of species between elevations. Finally, comparing the ESSF and SBS shows that these elevations are similar based on LH-PCR fragments and different based on culturable fungi. Overall, β_{sim} reports higher similarities between elevations in all but one case. Using more than one measure of similarity can reveal more about the data than one measure alone.

Beta diversity measures are similarity indices. The NMS analysis performed in this study was based on $\beta_{sorensens}$, as recommended by McCune and Grace (2002).

The resultant structure in the NMS plots was minimal. The LH-PCR raw data could be interpreted as changing in terms of species gained and lost from one elevation to the next. Since $\beta_{sorensens}$ does not emphasize these differences, it may not be the best distance measure for this data set. Richness did not differ between elevations (both techniques) based on one-way ANOVA's. The high variability between the numbers of fungi detected to be associated with *V. membranaceum* plant roots likely inhibits elucidating differences. The program used to run the NMS analysis, PC-ORD, does not include an option to use a narrow-sense beta diversity measure to generate the distance matrix in the first step of the analysis. Exploring the data with β_{sim} as the distance measure could yield better NMS plots.

Linking sequenced cultures with LH-PCR

Length-heterogeneity PCR appears to underestimate actual fungal diversity. The 500bp fragment appears to be produced by 6 different isolates; six different taxa are considered as one in this analysis. Nonetheless, by the diversity measures, LH-PCR is still producing considerably higher alpha diversity than that observed with isolating fungi from surface sterilized roots. Each LH-PCR produced fragment may not, in fact, represent one taxon.

Furthermore, the lengths of the fragments do not always match between LH-PCR and sequencing. Differences of a couple of base pairs may be expected (Ritchie *et al.* 2000). Some values differ considerably beyond the error inherent in the procedure (example, the *Cryptosporiopsis* A5-7 LH-PCR fragment length is 500bp, but is 489bp by sequencing). This demonstrates that the LH-PCR technique should not be used to identify individual taxa from a profile. Profiles taken as a whole in representing root-associated fungal community can be compared between elevations

because these errors presumably are carried throughout the procedure. Despite the caveats, this technique has been demonstrated as effective in distinguishing fungal community structure (Ritchie *et al.* 2000, Ranjard *et al.* 2001) and in our study revealed interesting insights.

Phylogenetic analysis of cultures

The existence of distinct subpopulations of *P. fortinii* between elevations was not found. This may be because *P. fortinii* has low ITS variability (Addy *et al.* 2000; Grünig *et al.* 2002; Menkis *et al.* 2004). Examining a latitudinal transect of Canada, Piercey *et al.* (2004) did not find distinct subpopulations for *P. fortinii* associated with *Salix* spp. between latitudes. They did, however, find subpopulations within their sites. Considering how close spatially our sites were, it is surprising that there was not more than one population type associated with *V. membranaceum* within and between elevations. Of course, the effect observed by Piercey *et al.* (2004) could be host specific, as they examined *Salix* spp. Furthermore, sequencing may be insufficient to effectively separate cryptic species (Grünig *et al.* 2004). Grünig *et al.* (2004) found evidence of the existence of multiple cryptic *P. fortinii* species using RFLP (restriction fragment length polymorphism) and ISSR-PCR (inter simple sequence repeat polymerase chain reaction). Their study, like the latitudinal transect study of Piercey *et al.* (2004) found identical subpopulations in study sites separated by 5km.

Likewise, we did not find subpopulations of *Cryptosporiopsis* spp that were distinct to specific sites. Sequencing ITS regions may be insufficient for some species within this genus. Wang *et al.* (2007) suggest that sequencing mitochondrial rDNA or β-globulin gene may be necessary to elucidate the relationship of the closely related

C. radicicola and *C. ericae*. These two species are very similar in terms of both ITS sequences and morphology.

Rhizoscyphus ericae and *Meliniomyces* spp. also demonstrated no fractionation of populations based on comparison of ITS sequences with UPGMA. In the case of these two fungi, there were fewer genotypes overall and fewer isolates that spanned more than one elevation. The phylogenetic graphs demonstrate that both these fungi are probably monospecific.

Canonical Correspondence Analysis (CCA)

Canonical correspondence analysis is a good addition to this study. It helps to confirm NMS results. The best indicator of finding trends in community structure is when constrained (i.e., CCA) and non-constrained ordinations (i.e., NMS) corroborate each other (Økland 1996). On its own, CCA is insufficient for this analysis because it assumes the species data is dependant on the environmental variables tested (McCune and Grace 2002). Furthermore, the CCA of both LH-PCR and cultures show near identical trends: distinct clusters that separate the high and low elevation fungal community structures. As with the NMS analysis, CCA demonstrates that the two technical approaches for measuring the fungal community agree with each other.

Patterns of diversity

This study considers a local community, an elevation within a BEC zone, and a regional community as defined as the slope of McBride peak. According to Kneitel and Chase (2004), patterns of diversity over local and regional scales can be used to understand community structure and dynamics. The fact that *P. fortinii* is found in abundance in all these elevations supports previous observations that it is able to adapt to a variety of root habitats (Grünig *et al.* 2004; Piercey *et al.* 2004). Because

P. fortinii and *R. ericae,* and *P. fortinii* and *Meliniomyces* spp. coexist, there must exist various niches within a root system. Trade-offs are individual characters manifest within an environmental context (Knietel and Chase 2004). A trade-off may be an ability to utilize one resource and not another. Thus coexistence may be dependant on the two organisms accessing different resources in a shared space. Abiotic factors may limit the ability of an organism to access a particular resource. Resistance to drought or low temperatures may give one organism better access over a less tolerant one. Life strategies with respect to predation are another trade-off. Finally, temporal variation may allow species to coexist. In this case, organisms may have abilities to withstand difficult conditions, through generation of dormant spores for example (Kneitel and Chase 2004). Diversity patterns may point to mechanisms of coexistence. Of all possible trade-offs, this study did attempt to characterize abiotic factors. Based on CCA, *R. ericae* and *Meliniomyces* may have traits that make them better competitors under the conditions of high light, higher soil moisture and older *V. membranaceum* plants.

This study did not attempt to look at resource competition or predation or temporal variation. And while these factors could easily be at play, they would, given the ephemeral nature of the subject organisms, be very challenging to determine. While niche theory is a valuable theoretical framework for understanding community dynamics, if trade-offs are ignored, similar patterns of diversity can be predicted based on variable rates of dispersal alone (Hubbell 2001).

Dispersal mechanisms could be used to explain the patterns described in this study. Both *Cryptosporiopsis* (Wang *et al.* 2007) and *P. fortinii* (Menkis *et al.* 2004) have been found in aerial parts of plants. It could be that they are able to release

spores from above ground. Fungi that are restricted to soil and root environments may have more limited dispersal ranges. If they are obligate root residents, they may not have the capacity to colonize at a distance. Thus these fungi may be more widely distributed if they are indeed able to aerially disperse.

Climate Change

This study demonstrates that fungal root-associated communities can differ across a BEC zone gradient. Although these effects may be host-specific (Bougoure *et al.* 2007), fungi such as *R. ericae* appear to be restricted to the higher alpine elevation site. A significant warming trend, measured in decades, is expected to elevate climate variables (temperature and precipitation) associated with BEC zones northward and up mountain slopes (Hamann and Wang 2006). Whether and how the associated plant communities will respond is the subject of current inquiry (eg. Iverson *et al.* 2004; Saxe *et al.* 2000). Presumably, the alpine will be reduced in size while other BEC zones migrate upwards. Different outcomes are possible with changes in precipitation. Should BEC zones migrate upwards, this could effectively reduce the pool of (apparently) less abundant and alpine restricted fungi such as *R. ericae*. If dispersal is indeed the main mechanism generating observed patterns of diversity (Hubbell 2001), then species such as *R. ericae,* could become restricted to ever decreasing mountain-top islands and potentially become threatened. Unlike *P. fortinii* and *Cryptosporiopsis*, which appear to be capable of aerial dispersal, *R. ericae* may be restricted to propagation within the soil and thus unable to hop between mountain top islands.

Furthermore, in boreal and heath environments, carbon is easily stored because it is a nitrogen-limiting environment. Mycorrhizal fungi sequester organic

nitrogen as soon as it becomes available and remaining litter is low in nitrogen and thus poor substrate for decomposers. Organic carbon is thus stored. Such environments have low mean annual temperatures and evapotranspiration rates. Should temperature rise due to climate change or nitrogen deposition increase due to pollution, the dynamics could shift to favour mineralization of nitrogen thus making the carbon pools more accessible to saprotrophs. This could then release the stored carbon into the environment exacerbating the CO_2 problem (Read *et. al* 2004). Shifting this particular mycorrhizal-driven ecosystem could have global implications. Increases in carbon emissions under different scenarios have been modeled to demonstrate that CO_2 feedbacks within the atmosphere, because they are buffered by land and sea uptake of carbon, are not linear (Boer and Arora 2009). If the mycorrhizas in this ecosystem are selected against due to shifting ecological zones, their functional role in sequestering nitrogen and thus acting as a large carbon storage bin could be reduced, perhaps eliminated. Tipping the scales in favour of nitrogen mineralization in these ecosystems could be the trigger to accelerate CO_2 emissions globally.

Conclusion

Vaccinium membranaceum root-associated fungal community structure differs over an elevation gradient of 1000m on McBride peak, BC. The highest elevation is most different from the lowest elevation with a gradual gradient of change over the slope. Elevations closest together tend to be most alike. Results from both techniques used in this study, LH-PCR and culturing, demonstrated this pattern. *Rhizoscyphus ericae* and *Meliniomyces* were most commonly found at high elevation and *Cryptosporiopsis* sp. were found mostly in the lower elevations. The same strain of *P. fortinii* was found

in abundance throughout the slope. Strong correlations to environmental variables were not found and the cause of the observed community structure patterns are thus deserving of further investigations.

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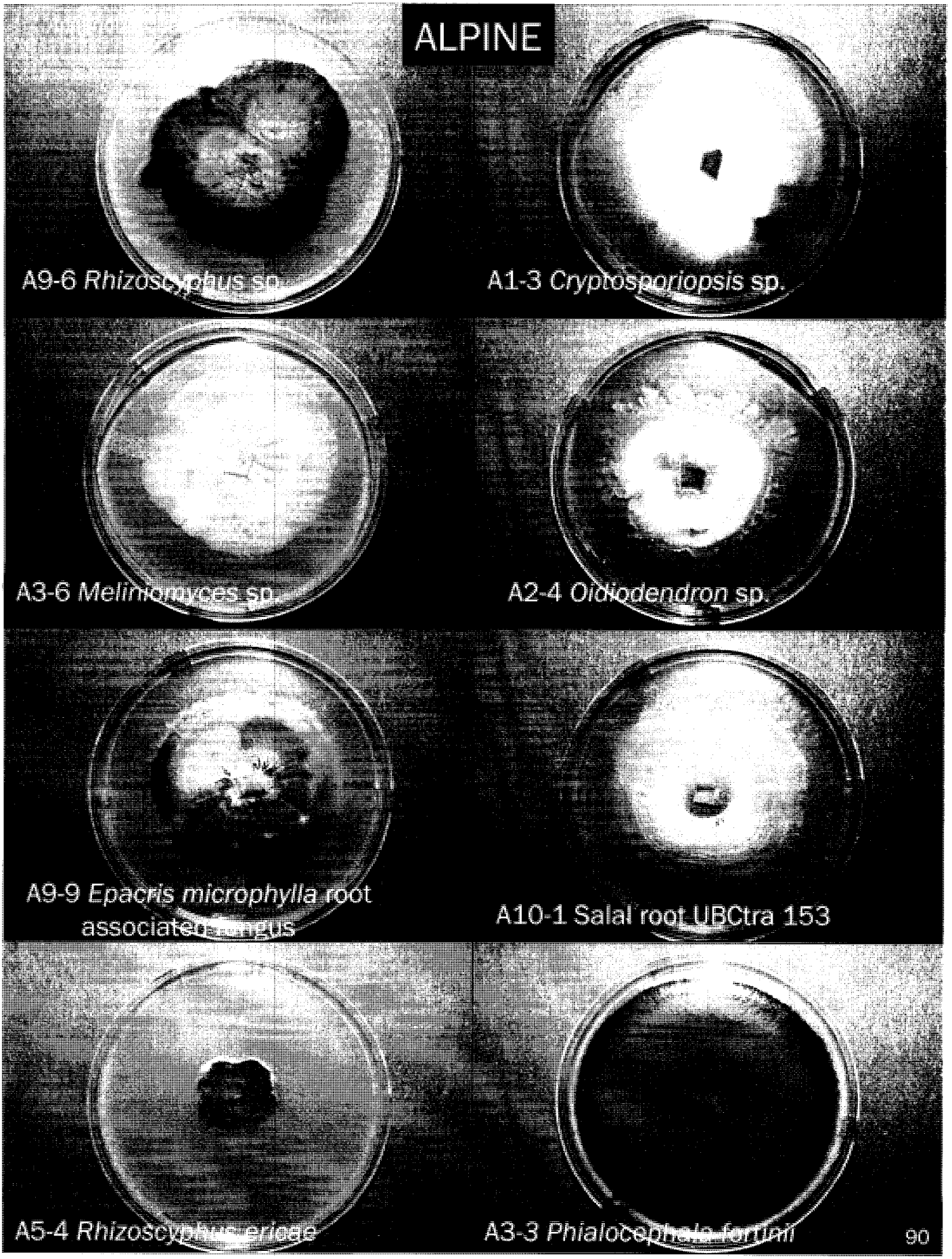
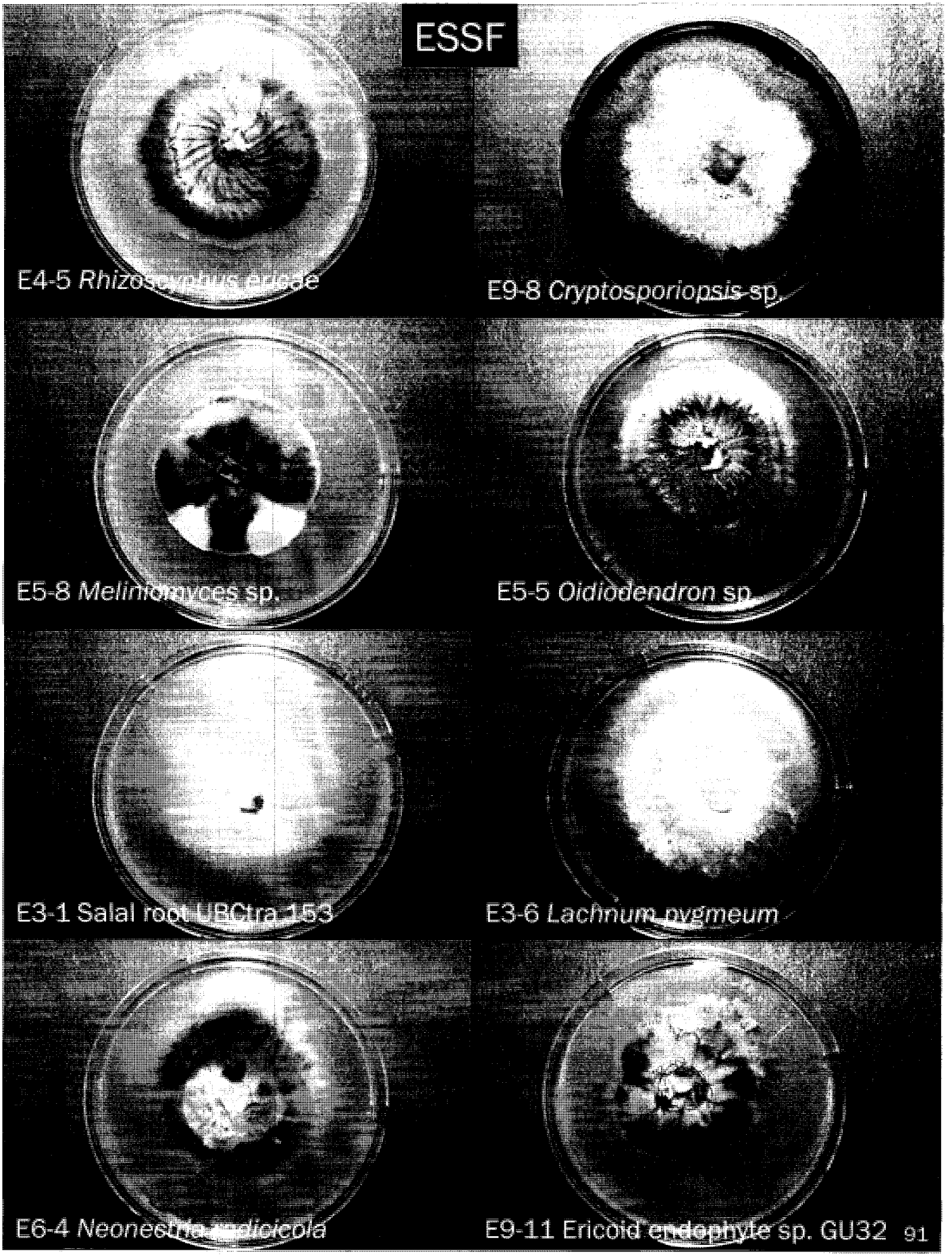
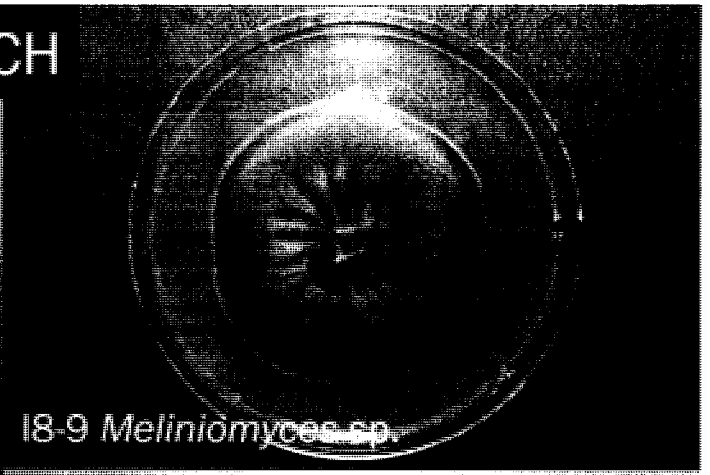
ALPINE


A9-6 *Rhizoscyphus* sp.
A1-3 *Cryptosporiopsis* sp.
A3-6 *Meliniomyces* sp.
A2-4 *Oidiodendron* sp.
A9-9 *Epacris microphylla* root associated fungus
A10-1 Salal root UBCtra 153
A5-4 *Rhizoscyphus ericae*
A3-3 *Phialocephala fortinii*


ESSF
E4-5 Rhizoscyphus ericae
E9-8 Cryptosporiopsis sp.
E5-8 Meliniomyces sp.
E5-5 Oidiodendron sp.
E3-1 Salal root UBCtra-153
E3-6 Lachnum pygmeum
E6-4 Neonectria radicicola
E9-11 Ericoid endophyte sp. GU32

ICH

I6-9 *Cryptosporiopsis* sp.



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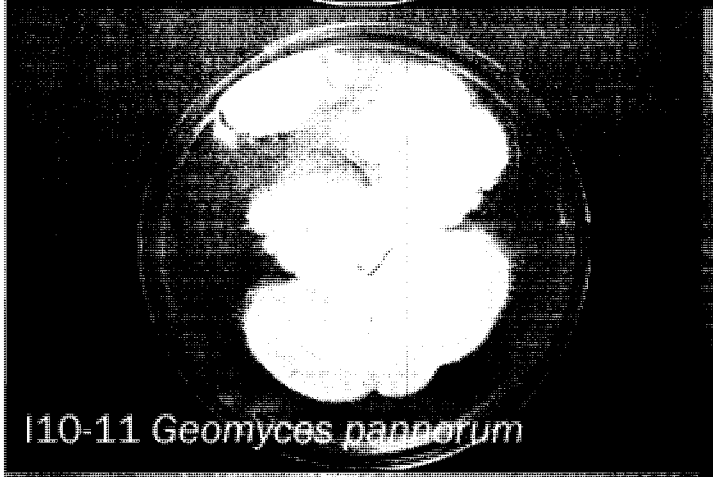


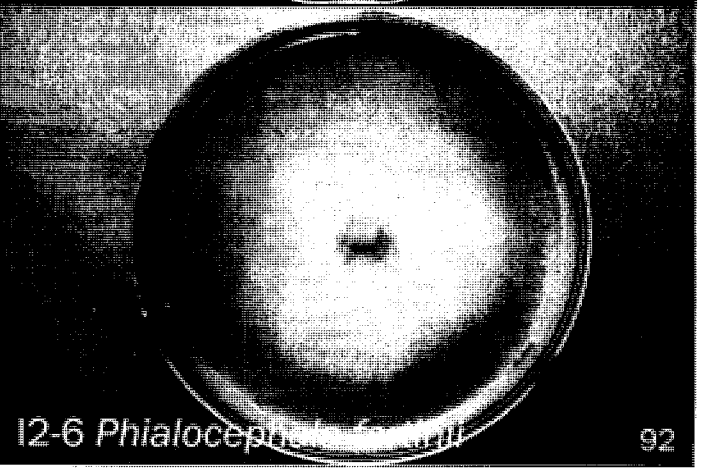
I10-11 *Geomyces pannorum*

I5-5 *Phialocephala fortinii*

I1-4 *Lachnum pygmeum*

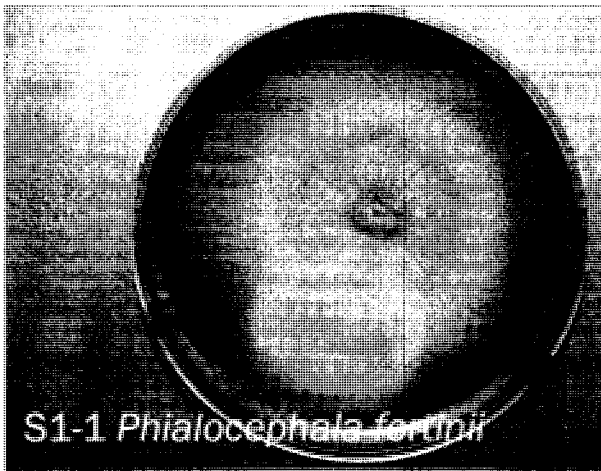
I10-2 Salal root UBCtra 305

I4-8 uncultured ectomycorrhizal clone63/24



I2-6 *Phialocephala fortinii*

SBS



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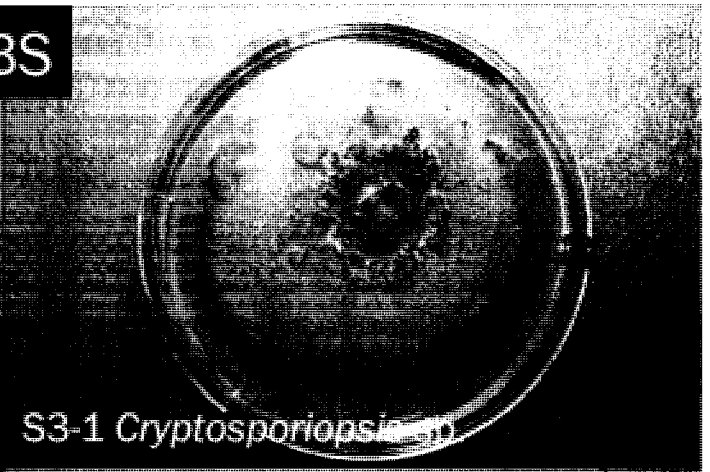

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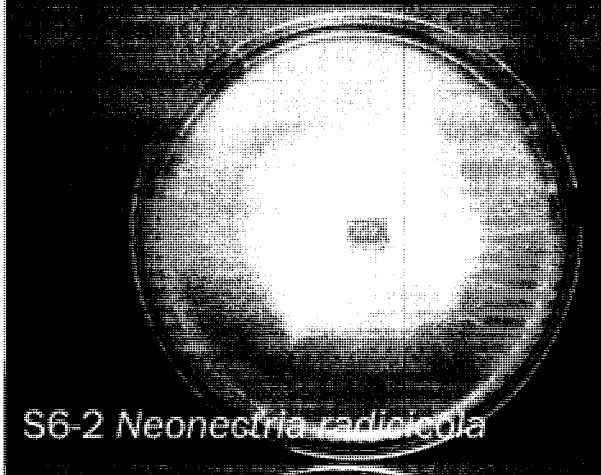
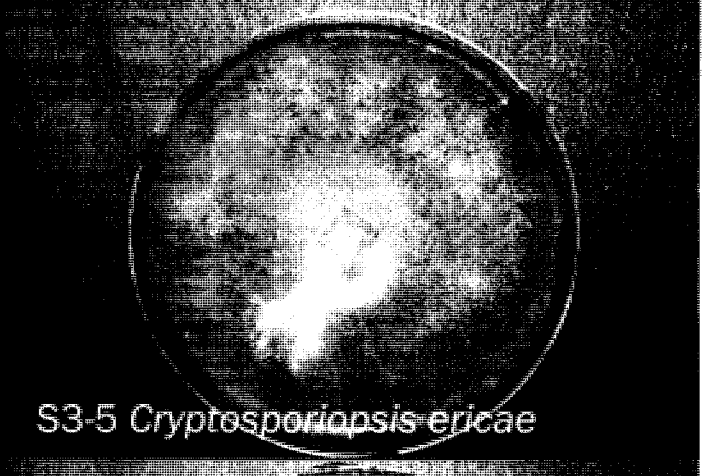


S6-2 *Neonectria radicicola*

S8-9 *Rhizoscy[illegible]gate*

S7-4 *Lachnum pygmeum*



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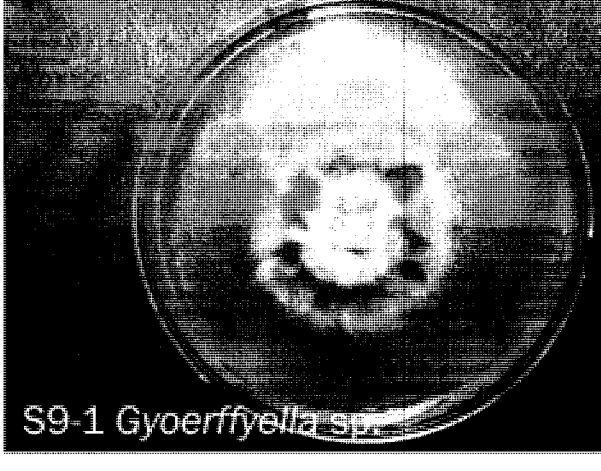

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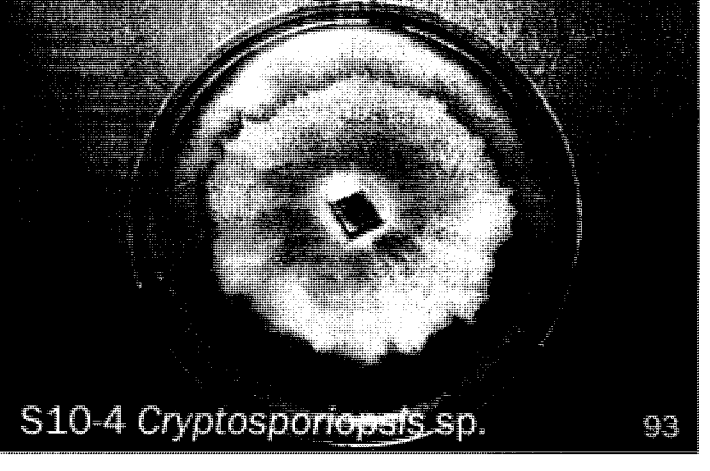

S10-4 *Cryptosporiopsis* sp.

Appendix 2: Summary of cultures found per plant.

Alpine Summary	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Phialocephala fortinii	3		3	4	14	3			1	1
Rhizoscyphus ericae	3		1	1	1			4	4	1
Cryptosporiopsis spp.	5				2		1	1	2	
Meliniomyces sp.	1		1							
Oidiodendron spp.		1	1							
Epacris microphylla		2							1	1
Salal root UBCtra180		1					1			
Salal root UBCtra153						1			1	1
Salal root UBCtra264										2
uncult mycorrhizal d_fir										1

ESSF Summary	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Phialocephala fortinii	9	9	8	5	5	10	6		6	5
Rhizoscyphus ericae	2		1	1		2				1
Cryptosporiopsis spp.							4		4	1
Meliniomyces spp.	1			4	17				1	2
Oidiodendron sp.					9					
Epacris microphylla			1				1			
Lachnum sp.	1		3							
near ericoid GU32									1	
UBCtra153							1			
Neonectria radicicola					1					

ICH Summary	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10
Phialocephala fortinii	8	1	1	20	9	10	7	4	7	8
Rhizoscyphus ericae				1						
Cryptosporiopsis spp.		6	3		1	3	2	4		
Meliniomyces sp.				1				1		
Oidiodendron mauis	3									
Epacris microphylla										1
Lachnum sp.	1	1								
Uncult ectoclone 63/24			1	1						
Geomyces pannorum						1				1

SBS Summary	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Phialocephala fortinii	3	3	4	1		1	5	5	5	
Cryptosporiopsis sp.	3		5	1	1	12		6	2	7
Lachnum pygmaeum							1			
Geomyces pannorum					1					
Neonectria radicicola					6	2				
Gyoerffyella sp.									1	
Epacris microphylla	1									
Rhizoscyphus aggregate								1		
Leptodontidium orchidicola					1					

Appendix 3: Measured plant characteristics

Raw data summarizing leaf mass per area, ages and percent colonization for all plants in this study.

Plant ID	LMA	age	% colonization
A1	59.52	15	55
A2	61.61	10	66
A3	56.34	17	36
A4	54.36	13	29
A5	57.51	14	23
A6	64.5	10	42
A7	67.96	13	38
A8	50.95	11	67
A9	51.77	7	58
A10	57.12	24	49
E1	51.07	3	20
E2	46.44	8	41
E3	47.49	5	40
E4	42.88	12	26
E5	51.18	8	34
E6	39.65	14	27
E7	50.91	11	18
E8	43.36	13	--
E9	54.73	11	38
E10	51.6	10	64
I1	32.52	5	56
I2	46.39	6	62
I3	25.68	11	28
I4	28.47	7	42
I5	28.13	7	28
I6	20.86	6	49
I7	25.71	3	37
I8	31.57	5	31
I9	36.12	7	54
I10	37.08	5	64
S1	29.61	4	46
S2	32.92	12	24
S3	25.47	2	33
S4	29.49	6	30
S5	29.79	5	14
S6	29.41	6	19
S7	23.92	4	38
S8	32.52	4	33
S9	26.88	9	66
S10	31.88	6	43

Appendix 4: Measured soil characteristics

Raw data summarizing water content of soil at time of harvest, pH of organic (pH-O) and mineral (pH-M) soil layers, and carbon to nitrogen ratios for organic (C:N-O) and mineral (C:N-M) soil layers determined for most plants in this study. The shaded grey cells indicate places where a value was unobtainable (frequently due to the absence of one of the two soil layers in that sample); the mean of all measurements at that elevation was used in its place in the multivariate analysis only (NMS and permANOVA). Continued next page.

plant ID	% H_2O	pH-O	pH-M	C:N-O	C:N-M
A1	10.75	3.64	--	16.5	--
A2	13.75	3.08	3.82	31.31	19.09
A3	10	4.04	4.1	28.87	22.09
A4	10	3.93	--	25.57	--
A5	10.25	3.81	4.42	26.07	27.12
A6	15.25	3.68	4.2	30.73	21.61
A7	12.25	3.97	4.08	26.35	17.53
A8	16.75	3.85	3.92	30.85	17.26
A9	11.25	3.47	3.51	25.04	18.85
A10	11	3.95	4.01	18.19	8.93
E1	7.25	3.52	3.95	33.57	16.85
E2	9.5	4.06	4.3	37.3	30.63
E3	6.25	--	4.11	--	24.33
E4	10.25	3.89	3.83	28.64	24.06
E5	15.25	3.66	3.51	70.91	34.31
E6	13	3.67	4.25	32.82	26.69
E7	10.25	3.99	4.03	24.4	17.83
E8	17.5	3.63	4.43	74.77	26.55
E9	17.25	3.52	3.93	38.23	17.22
E10	8.25	3.7	3.4	34.44	25.65
I1	7	4.84	4.56	28.17	26.47
I2	5.75	5.62	4.43	26.09	25.52
I3	9.5	4.16	--	26.94	--
I4	11	3.67	--	33.22	--
I5	10.5	4.74	4.04	28.89	21.19
I6	6.25	3.87	4.18	27.14	26.51
I7	9.25	3.71	3.69	31.18	26.56
I8	8.5	4.14	4.02	30.51	21.48
I9	8	4.66	4.32	34.38	27.83
I10	6.5	5.15	--	27.37	--

Continued from previous page.

plant ID	% H_2O	pH-O	pH-M	C:N-O	C:N-M
S1	5	5.58	5.23	21.68	24.14
S2	5.75	6.01	5.97	23.92	16
S3	6.75	5.21	4.83	24.68	23.24
S4	5.75	5.96	5.11	28.73	33.18
S5	5.25	5.14	–	24.1	–
S6	5	5.2	4.6	30.14	30.58
S7	6	5.43	4.4	24.68	36.61
S8	5	4.27	–	45.17	–
S9	5	5.66	4.89	26.54	27.37
S10	8.75	5.59	4.68	26.19	23.13

Appendix 5: Aerial Photograph of McBride Peak