EFFECTS OF PRESCRIBED BURNING UPON MYCORRHIZAL FUNGAL DIVERSITY INHABITING THE ROOTS OF TWO AND A HALF-YEAR OLD BLACK SPRUCE (Picea mariana): MOLECULAR CHARACTERIZATION OF ECTOMYCORRHIZAL FUNGI VIA PCR/RFLP ANALYSIS

CENTRE FOR NEWFOUNDLAND STUDIES

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Effects of prescribed burning upon mycorrhizal fungal diversity inhabiting the roots of two and a half-year old black spruce (*Picea mariana*): Molecular characterization of ectomycorrhizal fungi via PCR/RFLP analysis.

by

Quentin F. Baldwin

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Memorial University of Newfoundland
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Abstract

Out-planted black spruce seedlings were harvested from unburned as well as low and high intensity prescribed burned sites to assess the effects of burning and fire intensity upon ectomycorrhizal (ECM) fungal diversity. The polymerase chain reaction (PCR), in conjunction with universal and fungal specific primers, was used to amplify a fragment spanning the two internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA belonging to ECM fungi. Sampling allowed for the harvesting of both mycorrhizal and non-mycorrhizal (NM) root tips. Despite the fact that some tips were classified as being NM, a PCR fragment was amplified from them. Staining of NM root tips showed there to be numerous types of endophytic hyphae surrounding and penetrating the root cortical cells. Restriction fragment length polymorphisms (RFLP) were used to classify specific fungal genotypes. Molecular data indicated that root tips harboring ECM fungi with distinct mantles (mantled tips), produced distinct RFLP genotypes compared to those root tips presumed to be non-mycorrhizal, or supporting mycorrhizas with thin/patchy mantles (exposed tips). Both NM tips, along with tips supporting thin/patchy-mantled fungi, displayed a wide variety of RFLP genotypes.

Both the Shannon-Wiener and the Simpson indices were used to assess diversity based upon the RFLP patterns. It was found that neither fire, nor its intensity, caused a significant change in the ECM fungal diversity and/or community structure. Mantled tips showed distinct RFLP clades, which corresponded to crude morphotype groups. Some of these clades showed several intraspecific polymorphisms representing a particular genotype. It appears that the Glide Lake study site has a very high degree of ECM, as well as endophytic, fungal diversity. The level of diversity within the endophytic genotypes was comparable to that of ECM genotypes. The study also indicates that traditional diversity indices (Shannon-Wiener and Simpson) are not well-suited for molecular data. Thus an index based upon phylogenetic distances, the *'Phi index'*, was constructed. The greater the degree of variability among the distances then the greater the diversity.

It would appear that foresters need not be overly concerned with prescribed burning, nor the intensity of these burns, affecting the ectomycorrhizal diversity of outplanted black spruce. However, this does not mean that other ECM fungi, which do not associate with black spruce, are not affected. Further studies are needed in order to investigate the effects of fire upon other hosts and their associated mycorrhizal fungi. As well, more rigorous testing is needed before the *Phi index* can be declared a better measure of diversity when using RFLP data.

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List of Abbreviations and Symbols Used

Adjusted Kruskal and Wallace statistic	H_a
Ammonium nitrogen	NH_4-N
Analysis of variance	ANOVA
Autoclaved, nanopure filtered	ANF
base pairs	bps
Calcium	Ca
Chi squared statistic	χ^2
deoxyadenosine triphosphate	dATP
deoxycytosine triphosphate	dCTP
deoxyguanine triphosphate	dGTP
Deoxyribonucleic acid	DNA
deoxythymine triphosphate	dTTP
double stranded Deoxyribonucleic acid	dsDNA
Ectomycorrhizal	ECM
Emanating hyphae	EH
Ericoid mycorrhizae	EM
Ethylenediaminetetraacetic acid	EDTA
external transcribed spacer	ETS
Hexadecyltrimethyl-ammonium bromide	CTAB
intergenic spacer	IGS
internal transcribed spacer	ITS
Large Subunit	LSU
Magnesium	Mg
Magnesium Chloride	$MgCl_2$
Microgram	µg
Micromolar	µM
Millimolar	mM
Nitrate nitrogen	NO_3-N
Phosphorus	P
Phylogeny Inference Package	PHYLIP
Polymerase Chain Reaction	PCR
Potassium	K
Potassium Hydroxide	KOH
Restriction Fragment Length Polymorphism	RFLP
Rhizomorph	R
Nuclear Ribosomal RNA gene	rDNA
Small Subunit	SSU
Sodium Chloride	NaCl
Sodium Hydroxide	NaOH
species	spp
Vesicular-arbuscular mycorrhizae	VAM

Chapter 1:

Introduction
and
Literature Review

1.1: General Introduction

Around the 19th century, foresters began to make use of prescribed burning as a means of clearing land that was scheduled for reforestation. This land may have harbored forests that were too old for harvest, or contained slash left behind from previous harvest (Neal *et al.*, 1965). At this time little was known about the effects of fire upon the soil, and the flora and fauna that rely upon the soil for existence. This prompted scientific investigation into the physical and chemical effects that prescribed burning has upon the biota and whether these effects were beneficial or detrimental.

1.2: Fire: Physical and chemical effects upon the soil

Natural fires, or wildfires, occur mainly during the drier summer months and are a most often a result of lightning strikes (Viro, 1974). The unpredictability of a wildfire makes it impossible to have control over the area in which they occur, the amount of destruction that ensues, or the intensity at which they burn. Fire does have beneficial effects on forests. It allows for the removal of old growth forests that have entered a state of decay or disease. This cleared land provides a new niche for flora and fauna that could not exist within the confined, light deprived forest floor typical of most old growth forests. As well the fire releases minerals and nutrients back into the soil which had previously been unavailable because they were tied up in cellular materials of living and decaying plants.

In the 19th century foresters began to implement fire use into their silvicultural procedures. Prescribed burning of clear-cuts allowed for the reduction of fire hazards,

opened new seedbeds, allowed easy access to the area for man and machinery, as well as created food sources for wildlife. However, even though prescribed burning has been used for the past century there has been little research to investigate the effects that it has upon the physical and chemical properties of soils. The research literature contains contradicting results, which is understandable since such things as fire intensity, temperature, fuel type and amount, and soil moisture content can all affect the outcomes of burning depending upon the ecosystem under investigation (Wells *et al.*, 1979).

Wildfires can be classified into three main categories; ground fire, surface fire and crown fire. Ground fires (or subsurface fires) burn duff, roots, wood and peat below the litter layer of the forest floor (Hartford and Frandsen, 1992). Surface fires are those in which the materials above the duff layer act as the fuel source (Merrill and Alexander, 1987). Crown fires occur on standing and supported forest materials above the ground (Merrill and Alexander, 1987). Prescribed burns are usually surface fires, and tend to be more restricted than wildfires with respect to certain parameters. For instance, wildfires usually have an erratic burning path that is controlled by the prevailing winds and the fuel type and load. It is not even uncommon to see unburned sections within the confines of a wildfire. Prescribed burns are more regulated, as foresters can model fire path and intensity with the input of information on fuel type, load and moisture level, as well as meteorological information. In essence, wildfires can be viewed as a patchwork of smaller fires, each with varying characteristics. Prescribed burns tend to be uniform in nature with no 'patch-like' pattern observed.

Fire changes physical properties not only of the above ground vegetation but indirectly these changes can cause below ground alterations, depending on the severity of the fire. A majority of Newfoundland's forests are conifer dominated or contain mixed deciduous/coniferous tree species. Within Canada's boreal forest, older forests tend to be white or black spruce dominated (Kelsall *et al.*, 1977). Due to the cooler climate, shorter growing season, and the resiliency of coniferous needles, the decay rate of organic matter in Canadian coniferous forest soils is slow (Viro, 1974). Thus most of these forests have a thick humus layer. This humus layer plays an important part in the regulation of soil temperature and moisture levels.

The temperature of the forest soil is the primary determinant of plant growth rates and microbial activity (Viro, 1974). However, with a thick, wet humus layer it takes longer for the soils to heat during the spring, which limits tree growth in northern climates. During the fall, the dry humus acts as a good insulator, allowing soils to remain warm longer. Therefore soils tend to be cold during spring, when solar and nutrient conditions are optimum for vegetation development (Viro, 1974). When fire destroys the humus layer the effects can be either beneficial or detrimental. Low intensity burns result in partial destruction of the humus layer, leaving behind a thinner humus layer covered with ash and charcoal. The combination of the increased absorption of solar radiation by the black ash and the insulation properties of the humus allow for soils to heat sooner in spring. This heat increase can cause explosions of microbial and plant activity within a short time after the fire (Viro, 1974; Sims, 1975). Prescribed fires that burn at high intensities tend to volatilize the organic material covering the soil. Although the initial

release of nutrients into the soil will be greater, this is a short-term effect and positive impacts tend to be out weighed by the effects of more extreme temperature fluctuations. Neal *et al.* (1965) found that burned soils reach temperatures 11°C higher than unburned soils during hot summer months. During the winter these soils, without the insulating effects of humus, become frozen much sooner and to greater depths. The combined effect of drying in the summer and early freezing slows decomposition, microbial and chemical activity, the effect of which can be worse than having too thick of a humus layer.

Besides regulating the soil temperature, the humus layer plays a major role in controlling soil moisture levels. When humus is severely dry it can act as a hydrophobic layer that prevents the penetration of rain to soils, unless the rains are heavy. However, during dry periods this covering of dry humus prevents the loss of moisture from the soil by breaking the capillary connection to the surface (Viro, 1974). As in the above situation, a low intensity burn, leaving some of the humus layer, will allow for the entrance of more moisture into the soil and upon drying will slow the loss through evaporation. A high intensity burn would remove this water retaining feature, therefore altering the soil conditions and possibly allowing for the introduction of new flora and fauna. Feller (1982) indicates that the moisture holding capacity of soil usually decreases with burning. However, this effect will depend upon such factors as: intensity of the burn, amount of organic material present in the soil, and the degree of incorporation in the soil, and the moisture content of the soil during the burn.

Studies have shown that surface conditions after burning will vary depending on the intensity of the fire, thus the degree of erosion will vary as well (Feller, 1982). A vegetative cover slows or prevents the degradation and removal of soil and rock. However, when this cover is removed it does not take long for weathering to become apparent. Ash and charcoal provide little protection against rain and wind thus, depending on the climate, the parent material of the soil, soil porosity and depth, slope of the land, and the rate of revegetation, erosional effects can be significant (Feller, 1982). Low intensity fires only remove a portion of the vegetation, usually the above ground portion and they may create patches of burnt/unburnt regions. As such, the roots of shrubs and trees can remain in the soil for several years before being degraded. These roots maintain the porosity of the soil, allowing for aeration and drainage of surface water. They are also responsible for preventing the compaction and separation of soils (Viro, 1974). Roots of some species are regenerative in nature, and will sprout new shoots once conditions become favorable in the post-fire environment. This rate of revegetation will vary depending on the plant species that previously occupied the site and the surrounding vegetation.

Whether prescribed burning is used to clear a section of decaying forest, or to remove the slash left on a clear-cut, studies have made some general observations of the chemical changes which result both above and below ground. The amount of change to the organic material in the soil depends on the intensity of the fire and its duration. However, in general, at least part of the litter and humus covering the forest floor becomes volatilized, releasing large amounts of nitrogen and smaller amounts of other elements, while less-

volatile compounds are transformed to soluble mineral forms which are more susceptible to leaching (Wells *et al.*, 1979).

One element released by burning is nitrogen. A well aged forest or a clear-cut, containing slash, holds tons of nitrogen in the form of living and dead plant materials. The amount of nitrogen present in this organic material will depend on the fertility of that particular site (Viro, 1974). During a fire there is a significant reduction in the overall mass of the surface vegetation. Grier's (1975) study on the Entiat fire in north central Washington showed that only about 3% of the nitrogen originally in the forest floor remained after burning. Most of this nitrogen is lost to the atmosphere during volatilization and some via ash-fly. However, upon combustion of the vegetation there is an increase in the amount of usable nitrogen in the form of ammonium within the ash (Boyle, 1973; Grier, 1975; Neal *et al.*, 1965; Viro, 1974; Vold, 1982).

After a fire the amount of ammonium nitrogen available within the ash layer will depend on how much nitrogen was lost to volatilization. Thus the more intense a fire, the less usable nitrogen there is left behind. This is evident in Boyle's (1973) jack pine plantation study in which he found no significant increase in available nitrogen after burning off the slash and heavy litter layer.

Two other important nutrients found in charred humus are calcium (Ca) and magnesium (Mg). These usually exist as oxides and carbonates and small amounts as phosphates. Due to the solubility of oxides in water, their maintenance in this state is dependent upon the first rains (Viro, 1974). Water in the form of rain or melting snow dissolves Ca and Mg compounds and carries them from the ash to the soil below. The

level of each nutrient in the ash layer is dependent on the vegetation that existed prior to the fire. [1]Alhgren (1974) found that after burning a spruce dominated forest, the level of Ca in the charred humus was three times the amount on the control sites and the level of Mg was twice that found in the controls.

Phosphorus (P) and potassium (K) are two essential nutrients which become available within the ash layer. Phosphorus usually exists as alkali phosphates and K occurs both as an oxide and carbonate. The oxide of K is very water soluble and is quickly converted to the carbonate form ([2]Alhgren, 1974). Like Ca and Mg, the amounts of K and P found in the ash will depend on the amount and types of vegetation.

The amount of each nutrient present in the ash layer is dependent on the previous vegetation cover as well as the fertility of the soil. However, investigations have shown (Boyle, 1973; Grier, 1975; [2]Alhgren, 1974) that the amounts of Ca, Mg , K, and P present after a fire vary from those amounts existing prior to the fire. This variation can be attributed to the vaporization temperatures of each element. K vaporizes at 760°C, P at 280°C, whereas Ca and Mg vaporize at 1240°C and 1107°C, respectively (Grier, 1975). The lower vaporizing temperatures of K and P mean that larger volumes of these essential elements will be lost to the atmosphere than that of Ca and Mg.

The previously mentioned nutrients all exist within the ash layer, but over time become dissolved in water and are leached into the mineral soil. Bivalent elements displace monovalent elements from the surface of soil particles (e.g. bivalent elements like Ca displaces monovalent elements such as Mg). However, if there is an over abundance of a monovalent element such as K, then it can displace a bivalent element to

some degree (Grier, 1975; [2]Alhgren, 1974). The movement of cations through the ash/soil column is important since the cationic and anionic charges will become balanced within the soil. The movement of elements through the soil column will vary depending on such factors as: the amount of precipitation that area receives, slope, ash layer thickness, soil composition (whether sandy, rocky, clay based, etc.), amount of external cation input from surrounding areas.

1.3: Fire and Micro-organisms

Boreal forests are characterized by podzolic soil conditions. Cooler summer temperatures and a short growing season are characteristic of temperate boreal forests. It is these conditions that slow the microbial breakdown of vegetative matter thus producing a thick humus layer overlying the parent soil. The build up of plant tissue and the slow release of humic and fluvic compounds during decomposition, gives podzolic soils their characteristically high acidity (Pietikainen and Fritze, 1993). As previously mentioned, the burning of boreal soils results in the reduction of the humus layer thus leading to a number of physical and chemical changes to these soils. Fire also directly and indirectly affects the microfauna existing within these soils.

The effects of wild and prescribed fires upon ecosystems are complex and intricate, thus providing researchers with a multitude of questions. However, over the past several decades researchers such as Ahlgren (1974), Wells *et al.* (1979), Klopatek *et al.* (1988) have attempted to determine the effects that fire disturbances have on soil residents, in particular bacteria and fungi.

Klopatek *et al.* (unpublished and 1988) have shown that vesicular-arbuscular mycorrhizae (VAM) colonization and propagule survival are negatively affected by an increase in soil disturbance. The 1988 study showed that VAM fungal propagules were moderately affected when soil temperatures were less than 50°C. However, once temperatures rose above 60°C there was a noticeable negative effect upon spore survival. Within this experiment Klopatek *et al.* (1988) noticed that the moisture content of the soil helped prevent soil temperatures from reaching levels that would be seen in dry soils. Soils supporting VAM propagules are more likely to be recolonized by VAM dependent plants if the soil had a high moisture content prior to burning as compared to the same soil burned dry. A follow up study done by Klopatek *et al.* (unpublished) confirmed the previous results, as well as indicated that the fuel load prior to a burn can drastically affect future VAM colonization. The researchers found that soils with a good canopy cover had a higher litter cover than interspace zones (areas between adjacent trees, i.e. no canopy cover), which in turn provided more fuel for burning. Higher fuel loads allowed fires to burn longer and at much higher temperatures than fires over interspaces. Since VAM spores and propagules are temperature sensitive, it was not surprising that the total number of propagules decreased significantly under canopies as compared to that of interspaces. This study also showed how fire can drastically alter the species distribution of VAM fungi. Prior to burning VA mycorrhizal fungal diversity was greater under pinyon and juniper canopies, however after burning both the canopy and interspace zones had a more homogenous species distribution. Zak and Wicklow (1979) also looked at the heat sensitivity of fungi, in particular carbonicolous ascomycetes. In their study they

were concerned with the effects that temperature, as well as ash and subsurface soils, had upon the structure of post-fire ascomycete communities. They noticed that soils heated to either 55°C or 70°C contained high occurrences of carbonicolous ascomycetes. They attributed this to two factors: 1) high temperatures stimulated the germination of ascospores, and 2) high temperatures reduced the levels of other soil microbes, which prior to the fire were possibly releasing fungistatic compounds which were inhibiting growth and fruiting of carbonicolous fungi. They also noticed that the addition of an ash layer to steam-sterilized soil altered the community structure from the steam treatment alone. This suggests that although carbonicolous fungi can thrive in post-fire habitats, the amount of ash left behind can alter the pH of the subsurface soil, thus ensuring the growth of only those fungi that can tolerate higher alkalinity levels. As well, toxic elements, or allelopatic compounds can bind with the carbon in the ash thus providing a less favorable environment for certain species of fungi. As mentioned earlier, a fire's intensity and duration is directly dependent upon the amount of fuel existing on that site. Thus a lower fuel load may mean that only the first few centimeters of topsoil will become sterilized. Soil layers below this sterile soil still contain microbial populations that could provide inoculum for surface colonization. Zak and Wicklow (1979) noticed that when they added untreated soil to the steamed soil there was a significant decrease in the number of ascocarps developing as compared to the steamed soil alone. This would support the idea that either the untreated soil contained some type of fungistatic property or that the microbial population out competed the carbonicolous fungi for available resources within the steamed soil.

From the previous studies it appears that the temperature and duration of a fire on a site has the greatest influence on future microfloral community structure. The more intense the fire then the more cellular destruction to vegetation and microbial life. This in effect will sterilize the upper portion of the soil and mineralize any accumulated organic matter (Gochenaur, 1981).

The sterilization of soils after burning is a short-lived event that is usually followed by a bloom of bacterial and fungal growth. An intense burn will result in combustion of all surface materials thus enriching the surface with a variety of oxides, hydroxides and salts (Petersen, 1970; Gochenaur, 1981; Neal *et al.*, 1965). Rebuilding of bacterial and fungal communities after such an intense burn will vary with time. In some cases populations may remain well below pre-fire conditions anywhere from a few months (Neal *et al.*, 1965) up to several years (Bissett and Parkinson, 1980). Usually the addition of moisture from rains allows for an increase in the rate of microfloral recolonization. Less intense fires leave behind a matrix of charred material and soils penetrated with roots of previous plants. The blackened surface absorbs incoming solar radiation much more easily than the white ash commonly found after very intense fires, thus altering soil temperatures from that of pre-fire conditions. In both cases, the result is a change in the structure of the microbial and fungal communities. Many fungi are unable to survive once soil temperatures rise above 50°C (Bissett and Parkinson, 1980). The overall effect is a reduction in the species diversity, but the severity of this species reduction will depend on the intensity of the fire (Pietikainen and Fritze, 1993; Gochenaur, 1981).

1.4: Mycorrhizal fungi

Estimates have been made that at least over 90 percent of the world's higher plants have some type of mycorrhizal association including arbuscular (VAM), ecto-(ECM), or ericoid (EM) (Kendrick, 1985). There have been numerous studies to determine the nature of this complex association. A consensus opinion is that this association is mutualistic, a beneficial partnership in which the fungus and the plant each obtain nutrients from the other.

Mycorrhizae occur in many different habitats, but for this review I will mostly limit the discussion to temperate forest types. Forest soils, especially coniferous types, are relatively acidic, thus providing an environment in which nitrification processes are slow and the main form of ionic nitrogen exists as ammonium (France and Reid, 1983). Within the soil, ammonium and nitrate are absorbed by the plant roots via metabolically active carrier systems. The rates at which these nitrogen-based molecules are taken up depends on their concentrations within the soil, plant demand, and the soil moisture levels. Preference for one or the other, by the plant, is not only environmentally dependent but also plant dependent. Since nitrate reduction is energy consuming, then the plant's energy status, and the induction of nitrate reductase will limit its use of soil nitrates (France and Reid, 1983). The nitrogen obtained from the absorbed nitrates, ammonium, and organic N are essential for plant growth and productivity.

Once mycorrhizal fungi infect a host root system there is generally an increase in the plant's growth (Marschner and Dell, 1994). These fungi act as root extensions that move beyond the nutrient depletion zones that generally surround most plant root systems. The

plant on its own usually obtains its nitrogen from inorganic sources but this will depend on many of the environmental conditions within that area. Depending on the fungus inhabiting the plant, the nitrogen source can vary. VAM fungi rely on inorganic nitrogen sources such as NH_4-N or NO_3-N, similar to that of plant roots. ECM and EM fungi can use inorganic N as well as make use of organic N (Chalot and Brun, 1998). These fungi are able to release acid proteinases which can release N that normally would be inaccessible to non-mycorrhizal plants, thus giving an edge to those plants growing in areas low in inorganic N and high in organic N (Marschner and Dell, 1994).

Phosphorus is another nutrient that can become limiting within ecosystems. Phosphorous limitations tend not to be as serious as nitrogen limitations, although most plants have a P depletion zone around their root systems. Again the association of hyphae with the roots allows for the exploitation of unattainable P sources. Li *et al.* (1991) found that VAM hyphae could draw upon P resources that extended over 10 cm beyond the root surface of white clover. ECM and EM fungal hyphae also extend into the soil, which may make more P sources available to them. However, few studies have been conducted on P transport and P acquisition by ECM and EM fungi. One of the ways in which these fungi gain access to P that normally would be unavailable to the plant is with the release of acid phosphatases, which free P from organic compounds within the soil (Ho, 1989). Some studies have shown that VAM fungi also release acid phosphatases but more work is necessary before definitive conclusions can be drawn (Marschner and Dell, 1994).

Aside from providing the host plant access to new macro- and micro- nutrient resources, the fungal symbiont can also provide physical and chemical protection. The thick mantle created by many ECM fungi may reduce the grazing on roots by many soil nematodes. As well many mycorrhizal fungi release chemical compounds which can inhibit bacteria as well as parasitic fungi (St. John and Coleman, 1983). A study by Garcia-Garrido and Ocampo (1989) showed how mycorrhizal tomato plants were better protected against the bacterium *Pseudomonas syringae*, than non-mycorrhizal plants. Not all mycorrhizal fungi reduce the incidence of infection by parasites upon their host plant but they can increase the plant's tolerance to these external or internal attacks, so one must not assume that all mycorrhizal associations will increase protection for the host plant (Fitter and Garbaye, 1994). Such things as climate, soil conditions, and floral and fauna populations associated with the plant/fungal symbionts will all affect the plant/fungus relationship. More studies need to be performed before firm conclusions can be made.

The extension of plant roots systems by mycorrhizal fungi not only allows for the uptake of soil nutrients but soil water as well (St. John and Coleman, 1983; Boyle and Hellenbrand, 1991). The existence of mycorrhizal fungi on roots of plants inhabiting xerophytic environments indicates the importance of such associations. Stutz and Morton (1996) showed that the species richness of VAM fungi in arid systems was equivalent to that of most other plant communities. These fungi permit the plants to exist in soils that experience extreme physical and chemical stress due the low availability of water. Boyle and Hellenbrand (1991) were interested in finding a mycorrhizal fungus that would be

appropriate for inoculating conifer seedlings that were to be planted in drought-stressed areas. Their study showed that different mycorrhizal fungi vary with respect to their response to imposed water stresses. It was found that fungi that formed rhizomorphs, which can penetrate into soil horizons containing water, provided host plants with more abundant water supplies than fungi that do not form rhizomorphs or rhizomorphs which only extend into the upper reaches of the soil. This study was done under controlled laboratory conditions and thus may not apply in the field.

So far I have only discussed the benefits which the fungus provides for the host plant. In order for this to be considered a mutualistic relationship there must be an exchange between both partners. On its own, the fungus may exists as a saprotrophic organism extracting necessary carbohydrates from dead or dying organic matter or it may remain as a dormant spore in the soil column. However, once the mycorrhizal fungus encounters a host plant and associates with the root system, it begins to draw upon the plant's store of photosynthates. There are many difficulties associated with looking at plant/fungal carbon exchanges in the field, thus laboratory studies have been used as simplified models. Most studies looked at what compounds the fungus could breakdown. These ranged from monosaccharides, disaccharides, and trisaccharides to more complex polymeric carbohydrates and organic acid structures (France and Reid, 1983). The exact process of carbon exchange from plant to fungus is still being investigated and is believed to vary from one mycorrhizal type to another. VAM fungi have structures, arbuscules, which exist within the host's root cells. Here fungal and plant membranes come in contact and enzymes such as succinate dehydrogenase are concentrated, which may aid in

carbon transfer (Allen, 1996). ECM fungi lack the delicately structured arbuscules of VAM fungi but the hartig net common to this group provides broad contact between the hyphae and cortical root cells. The fungus commonly hydrolyzes sucrose into glucose and fructose and after uptake these sugars become converted to trehalose, glycogen, and mannitol, respectively (France and Reid, 1983; Kendrick, 1985). These new carbohydrates are unusable by the plant, thus a gradient from host to fungus is created, making the fungus a metabolic sink (France and Reid, 1983).

It would appear that the union of mycorrhizal fungi with plants can only bring prosperity to both partners, however this is not always the case. Studies have shown that as the concentration of P around VAM colonized plants increases, the growth enhancement effects conferred by the fungus decreases. Thus there may be a halt or even a decrease in the productivity of that plant (Marschner and Dell, 1994). The mutualistic relationship has now switched to that of a parasitic one with the fungus being the sole benefactor. It has also been shown that mycorrhizal fungi can be used to combat certain diseases whether they are viral, fungal, or animal related. However, a study by Toth *et al.* (1990) indicated that there may be a positive correlation between disease susceptibility and mycorrhizal susceptibility. This clearly shows that more field based experiments are required if we wish to better understand the complex relationship between plant and fungus.

1.5: Biodiversity and mycorrhizal fungi

Biodiversity is defined as the measure of the extent of all biological variation on Earth (Hawksworth, 1991). It has been estimated that there are over 5000 ecto/ectendo-

mycorrhizal fungal species (Allen *et al.*, 1995; Molina and Trappe, 1982). Much of this diversity of ECM species is in the higher latitudes or at higher elevations where the diversity of plant species is much lower than that of tropical latitudes (Allen *et al.*, 1995). Some of these temperate and boreal coniferous tree species , such as Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco), have been estimated to associate with over 2000 ECM fungi (Trappe, 1977). It has been argued that with such a high number of fungal associations there must be some degree of functional redundancy (Hawksworth, 1991).

Some have argued that within a given ecosystem there are numerous species that perform the same function, suggesting that species diversity is not related to functional diversity (Leps *et al.*, 1982; MacGillivary *et al.*, 1995). Therefore, the removal of some of these redundant species would not affect the overall productivity of that ecosystem. A recent paper by Tilman *et al.* (1997) has shown that the functional component of diversity is much more important to ecosystem processes than the species component. They argue that the number of functional groups in an ecosystem is more important than the total number of species. However, Tilman and his colleagues do realize that functional diversity is dependent to a certain extent upon species diversity. Their findings support conclusions drawn by Hawksworth (1991) and Bond (1994), that there are certain 'keystone species' within all ecosystems. Keystone species are species that once removed would lead to major, and in some cases drastic, changes to that particular ecosystem (Hawksworth, 1991; Bond, 1994; Ehrlich, 1994). Thus, many agree that removal of

redundant species from any given ecosystem may have no significant effect upon the overall 'productivity' of each functional group.

The debate over the significance of species eradication from an ecosystem is an age old one. Since the existence of each species within an ecosystem influences the existence of many other species in that community, it is impossible to predict all of the consequences when a species is removed. Thus, the debate over the importance of maintaining biodiversity within an ecosystem continues!

1.6: Morphological vs Molecular

1.6.1: Molecular Characterization

Mycologists have relied upon macroscopic and microscopic observation as a means of identifying mycorrhizal fungi. This involves noting such things as color, presence of emanating hyphae, presence of rhizomorphs, and characterizing cystidia. Agerer (1987) put together an impressive list of characters one should note during mycorrhizal identifications. Even with a few samples this process can take an experienced mycologist an extensive amount of time. When one is faced with surveying a large population of mycorrhizas, morphological classification is not feasible (Egger, 1995; Henrion *et al.* 1994; Lutzoni and Vilgalys, 1995; Mehmann *et al.* 1995; Nylund *et al.* 1995; Sanders *et al.* 1995).

Due to sample numbers, the assessment of mycorrhizal diversity within and between communities requires the use of techniques that are efficient, reproducible, and relatively inexpensive. The advent of the Polymerase Chain Reaction (PCR) method has made this

possible (Mullis and Faloona, 1987). During the early stages of molecular biology, chemical and equipment expenses were high, however today companies are offering lower prices for higher quality materials, thus molecular analysis is rapidly becoming an important method for assessing biodiversity (Gardes *et al.* 1991; [2]Gardes and Bruns, 1996).

1.6.2: Why Ribosomal DNA?

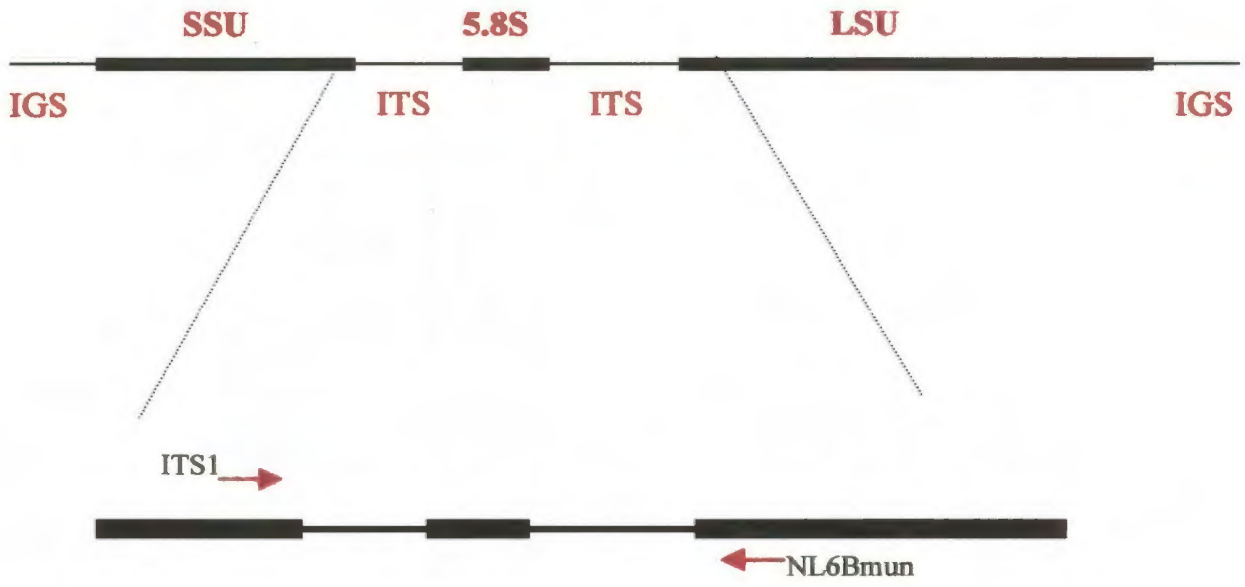
Mycological investigations that rely on molecular techniques to clarify fungal classification must have an appropriate molecular marker. This marker must be universally conserved amongst the various organisms but at the same time show maximum differentiation between species and minimum differentiation within species (Egger, 1995). As a result the nuclear ribosomal RNA gene region (rDNA) was adopted as a molecular marker for my fungal studies. Specifically, an 1100 bp fragment within the rRNA region was amplified using the universal primer ITS1, and the fungal specific primer NL6Bmun.

The nuclear rDNA is composed of three gene coding regions: the 18S subunit, also called the small subunit (SSU); the 5.8S subunit, and the 28S subunit, also called the large subunit (LSU) (Figure 1). Located on either side of the 5.8S subunit are two noncoding but transcribed spacer DNA segments called **Internal transcribed spacers (ITS)**. They have been designated as ITS1 and ITS2. ITS1 lies between the SSU and the 5.8S subunit; ITS2 lies between the 5.8S subunit and the LSU. Between each coding region is a largely non-transcribed region referred to as the **Intergenic spacer (IGS)** (Singer and Berg, 1991).

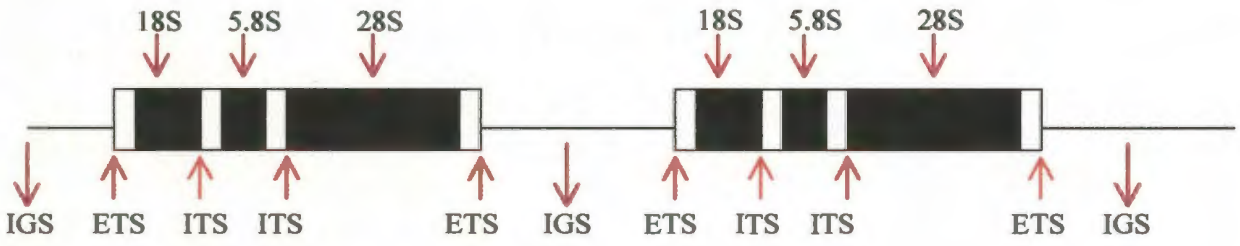
The nuclear rDNA usually occurs as long clusters of tandem repeats (Figure 1). The relatively high copy number of the rDNA provides an adequate amount of template during the PCR process. Another advantage of using rDNA is that there is a high degree of variability within each repeat. The coding regions are the most highly conserved, followed by the moderately conserved ITS regions, and the IGS region, which has the highest degree of variability. These different degrees of variability have been utilized by a variety of researchers in order to determine the degree of variation within a species (Hausner *et al.*, 1993; Hintz *et al.*, 1988), or between species (Egger and Fortin, 1989; Egger and Sigler, 1993) and has become the standard for PCR-RFLP studies of mycorrhizae (Gardes *et al.*, 1991; Erland, 1995;; Kårén and Nylund, 1996; [1]Kårén *et al.*, 1997; [2]Kårén and Nylund, 1997).

Figure 1: (a) rDNA structure and primer sites utilized in this study. (b) an example of rDNA organized as tandem repeats [**IGS** (intergenic spacer), **ETS** (external transcribed spacer), **ITS1** and **ITS2** (internal transcribed spacers), **SSU** (small subunit), and **LSU** (large subunit)].

(A)


SSU
5.8S
LSU
IGS
ITS
ITS
IGS
ITS1
NL6Bmun

(B)


18S
5.8S
28S
18S
5.8S
28S
IGS
ETS
ITS
ITS
ETS
IGS
ETS
ITS
ITS
ETS
IGS

1.7: What is a species?

The first question that must be asked is whether PCR-RFLP genotypes correspond to a taxonomic level, such as genus or species, or encompasses intraspecific variation. Most PCR-RFLP studies of the ITS region assume that PCR-RFLP genotypes correspond roughly to species ([1]Gardes and Bruns, 1996). [2]Kårén *et al.* (1997) indicate that sometimes a single RFLP-type may represent more than one species due to the low interspecific variation of the ITS of closely related species. This could lead to an underestimation of overall species diversity. It is also known that certain fungal species exhibit intraspecific variation within the ITS region, albeit occurring at a relatively low frequency (Gardes *et al.*, 1991; [2]Kårén *et al.*,1997; Lee and Taylor, 1992). This would tend to lead to an overestimate of species diversity. In order to assess the degree of interspecific versus intraspecific variation, a more intensive analysis using such techniques as DNA sequencing would be required. However, due to the high number of isolates and the lack of an effective morphological characterization of the morphotypes, it would require extensive research to infer which isolates were intraspecific variants and which were species or groups of closely-related species. Since my study was concerned with using PCR-RFLP as a method of assessing ectomycorrhizal diversity, for calculating the Shannon-Wiener and the Simpson diversity indices I made the assumption that each unique RFLP pattern represented individual species. If considerable intraspecific variation was present in my samples, then following this assumption could lead to an overestimation of the true number of mycorrhizal fungal species present in the study site. However, this would be balanced somewhat by closely-related species that share the

same RFLP pattern. When estimating the Phi Index (see later discussion), it is not necessary to make the assumption that each RFLP genotype is a different species.

1.8: Root endophytes: More than mycorrhizas!

As previous sections will support, the rhizosphere is rich in organisms such as arthropods, annelids and bacteria. There are also saprophytic and pathogenic fungi residing near or on plant roots. This is not the only realm of activity, as tissues within the plant may also harbor organisms. Within the root, fungi and bacteria can inhabit the intercellular spaces, or they can occur within cells.

Many researchers have devised terminology to describe organisms that live within plant tissues. The most common is "endophyte". Petrini (1991) and Chanway (1996) use the term to encompass all organisms that grow inside plant tissues without causing disease symptoms. Others view them as 'latent pathogens' (Wilcox, 1983; Egger and Paden, 1986), or consider them 'pseudomycorrhizas' (Melin, 1923) As the terminology suggests, it is not clear if they are pathogens, mutualists, or saprotrophs. Regardless, the one characteristic that these fungi share is that they spend most, if not all, of their life within plant tissues.

These fungi include species of ascomycetes, deuteromycetes, a few basidiomycetes and a few oömycetes (Isaac, 1992). They are found in most, if not all plants (Isaac, 1992; Carroll, 1995). They can be found in most conifers, several *Quercus* species, other members of Fagaceae, *Coprinus*, *Eucalyptus*, and many woody species (Petrini *et al.*, 1992). Host plants can carry multiple endophyte species (Carroll, 1992), although they

tend to occupy limited domains within plant tissues (often in lumens and single cells) (Carroll, 1986; Carroll, 1995). It has also been found that these endophytes often differ in frequency among organs or even parts of organs within host plants (Rodrigues, 1994; Carroll, 1995).

Surprisingly, even though these endophytic fungi are prevalent, little is known about them, perhaps because many of them do not cause severe morphological distortions, or diseases or their 'mutualistic' behaviour is not as obvious as that of mycorrhizal fungi. Regardless of the reasons for our lack of knowledge, these fungi must be considered when we are investigating rhizosphere activities.

Melin's 1923 study showed pine and spruce trees growing on drained peat bogs were deficient in mycorrhizae, or that they were unable to proliferate. However, hyphae of non-mycorrhizal fungi did exist intracellularly within the host's lateral roots. Melin proposed that these 'pseudomycorrhizas' occurred in 'mother' roots and older portions of mycorrhizae but were excluded from short roots by true mycorrhizas. If the short root was uncolonized or the mycorrhizal fungus became inactive, there was an opportunity for the pseudomycorrhizal fungi to move intracellularly. He also isolated a dark brown pseudomycorrhiza that he assigned to the genus *Mycelium radicis atrovirens* (M.r.a.). A study by Richard and Fortin (1974) showed that this endophyte could exist, as different strains, within roots of *Picea mariana* (Mill) B.S.P., but as a pathogen.

1.9: Objectives of this study

1. To measure mycorrhizal composition on clear-cut (unburned) and prescribe burned (low and high intensity burned) plots

2. To determine whether fire or fire intensity causes changes in mycorrhizal diversity and/or community structure.

   The null hypothesis was that fire would not alter mycorrhizal diversity or community structure.

3. To develop an appropriate diversity measure for molecular (PCR-RFLP) data, and compare this measure against other diversity measures on the molecular data collected in the field study.

Chapter 2:

Methods and Materials

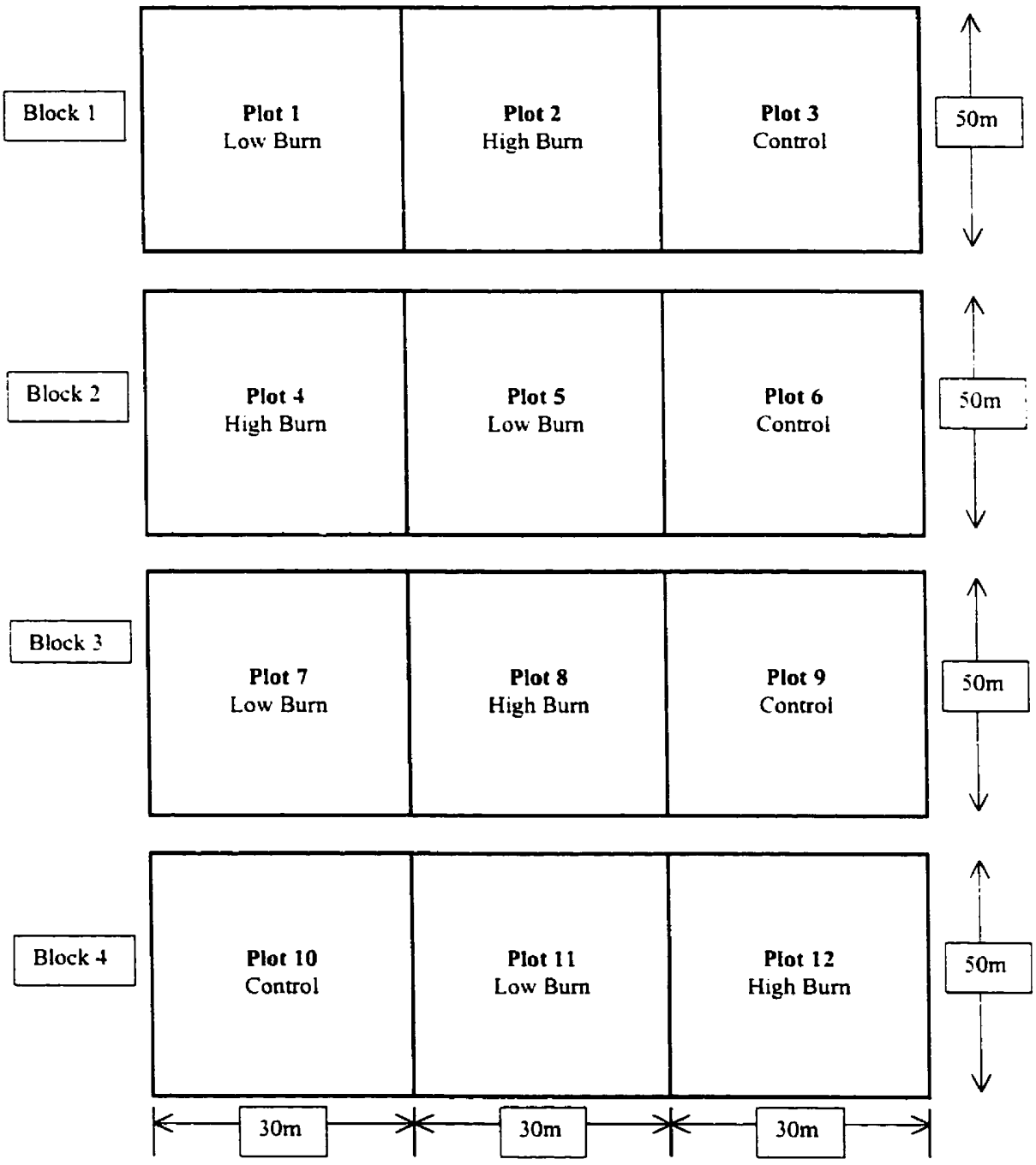
2.1: Study site and Specimen Collection

Twelve (12) treatments were situated on a southwest facing slope located a few hundred meters from Glide Lake (57°18"N and 49°8"E). Glide Lake is located 20-25 kilometers northeast of the town of Pasadena, Newfoundland. The site is situated within a clear-cut on the edge of a mixed coniferous forest, the dominant trees being balsam fir [*Abies balsamea* (L.) Mill.] and black spruce [*Picea mariana* (Mill.) B.S.P.].

The Glide Lake site was clear-cut during the winter of 1990 and summer of 1991. A randomized complete block design, consisting of 4 blocks with 3 randomly allocated burn treatments (control, low intensity, high intensity), was applied to the site in the fall of 1992 (Figure 2). In 1993 burn treatments were initiated for the 12 plots. Two weeks after burning the plots were planted with 25 week old container-grown black spruce. Each plot (30 m x 50 m) received 364 seedlings at 2 m spacing. Another 217 seedlings were planted at 1 m spacing between the 2 m rows. These seedlings served for destructive sampling (Wells, 1994).

This project has been broken down into two studies. The first study was initiated in the fall of 1993 and will be referred to as "Regen 1" (refer to Appendix I for details). The second started in the fall of 1995 and will be referred to as "Planted 2". Each study will be referred to as such from this point on.

Figure 2: Experimental design of Glide Lake research site


Block 1
Plot 1
Low Burn
Plot 2
High Burn
Plot 3
Control
50m
Block 2
Plot 4
High Burn
Plot 5
Low Burn
Plot 6
Control
50m
Block 3
Plot 7
Low Burn
Plot 8
High Burn
Plot 9
Control
50m
Block 4
Plot 10
Control
Plot 11
Low Burn
Plot 12
High Burn
50m
30m
30m
30m

2.2: Sampling and Collection

2.2.1: Planted 2

During the middle of August, 1995, a total of 120 black spruce seedlings were collected from the twelve experimental plots. Ten seedlings were randomly selected from the 217 destructible samples. Each seedling had approximately two and a half years of growth. A 60 cm diameter rootball was excavated for each seedling. Excess soil was shaken from the rootball in order to reduce the carrying load. Extreme care was taken to ensure that roots weren't lost or damaged during the transport back to the laboratory.

2.3: Mycorrhizal Recovery and Processing

2.3.1: Planted 2

The rootball of each seedling was soaked for approximately half an hour and then gently washed to ensure minimal destruction of the finer roots. In many cases the root plug ,characteristic of containerize greenhouse seedlings, was still intact which provided a reference point from which specific roots could be selected. It is not uncommon for greenhouse grown seedlings to contain certain mycorrhizal fungi. One of the more common forest nursery fungi is *Telephora terrestris* Ehrh.:Fr.. It flourishes in the high moisture and nutrient rich greenhouse environment (Browning and Whitney, 1993). Since the objective of this project was to investigate the mycorrhizal biodiversity of the treatments, steps were taken to avoid collecting these greenhouse fungi; only roots that extended 5 cm or more from the root plug were selected. It has been shown that greenhouse mycorrhizas cannot compete with wild mycorrhizal fungi (Dahlberg and

Stenström, 1991), thus the further from the plug then the less chance there would be of collecting these introduced mycorrhizas.

Roots were collected from each tree seedling. A root in this case refers to larger primary roots (holding secondary and tertiary roots) that were quite noticeably extending out from the root plug. The number of roots collected ranged from 2-24 per seedling. The roots for each tree were numbered and then a 50% sample was randomly selected (unpublished data suggested that a 50% sampling of a population provides a good estimate of diversity). These selected roots were then cut into 2 cm sections from which ten (10) sections were randomly selected. After selection the roots were blotted dry and stored at -20°C. Another ten (10) sections were collected and lyophilized as a backup precaution.

During collection roots were examined microscopically in order to gain morphological data on the types of mycorrhizal fungi being collected. Such things as color, mantle type (if any), hyphal density and structure and overall root appearance allowed for the description of several morphotypes which will be referred to in more detail later.

In early august of 1996 another thirty-six (36) black spruce (3 per plot) seedlings were collected from the treatment plots. These roots were analyzed more thoroughly for mycorrhizal morphotypes (Tables 1a and 1b).

Percentage abundance data had been obtained from a previous study, within this site, carried out during the summer of 1993. Collection of root tips is described by Baldwin (1994). Ten, 2 cm root segments/seedling (spruce and balsam fir) were surveyed, with a stereomicroscope, for the presence of several noticeable mycorrhizal types (types: 1, 3, 6,

8, and 10). Percentage abundance of these morphotypes (corresponding to those of the present study) were estimated (Appendix Va and Vb).

Table 1 : Brief descriptions of mycorrhizal types found on *Picea mariana* root tips. These types were noted during the 1995 summer collection as well as during the 1996 collection. EH= emanating hyphae; R= rhizomorph. Appendix II contains several plates of some of the morphotypes mentioned below.

Mycorrhizal type	Color	EH/R	Description
Type 1 (*Cenococcum geophilum*)	Black (dull appearance)	+/-	Dense mantle, very stiff EH (brown/black), Stellar pattern on mantle
Type 2	Tawny Brown	+ (sparse)/-	Loose mantle, hyaline, EH are clamped
Type 3	**young:** Coal Black (shiny) **old:** Black-brown	+ (sparse)/-	Dense mantle, stiff EH
Type 4	White	+ (sparse)/-	Loose mantle
Type 5	Coal Black	-	Dense mantle
Type 6	**young:** light brown **old:** Dark red-brown	-	Tight fitting mantle, roots take on twisted deformation
Type 7	**young:** White **old:** Golden Brown	+/-	Dense mantle, matted EH, clamped EH, concentric patterns on mantle
Type 8	Hyaline	-	Thin mantle, roots are inflated (2-3X normal size)
Type 9	Brown-Black	+/-	Dense mantle, roots inflated (2X), clamped EH
Type A	Smoky Gray	+/-	Very loose mantle, EH have a cottony appearance
Type B	Creamy Yellow	R	Very loose and patchy mantle, elaborate R
***Type E**	Brown	+/-	Dense mantle, coarse EH
***Type G**	Greenish-Brown	-	Dense mantle
***Type L**	Yellow	-	Dense mantle

* These types were described during the harvesting of tips for extraction, i.e. after being frozen. Due to the freezing, color and other descriptive details may not be accurate.

Table 2: These mycorrhizal types, classified as unknown (UK), are types that were not found during the 1995 collection. They were found only on the 1996 summer collections.

Mycorrhizal type	Color	EH/R	Description
UK1	Hyaline	+ (sparse)/-	Sheathing mantle, smooth, translucent, meandering EH at base of roots
UK2	**young:** Beige-yellow **old:** Beige	-	Sheathing, balloons over root tip, root is inflated (2X)
UK3	Patchy yellow-green and black-brown.	-	Dense patches of mantle,
UK4	**young:** light brown **old:** black	-	Dense mantle, smooth and shiny
UK5	Yellow-white	+/-	Loose mantle, hyaline EH, clamped EH
UK6 (possibly Type A)	Black-gray	+/-	Loose mantle, EH have cottony appearance
UK7	**young:** Hyaline **old:** golden brown	-	Sheathing mantle
UK8	**young:** yellow-white **old:** tawny to dark brown	+ (sparse)/-	Dense sheathing mantle, inflated mantle hyphae
UK9	**young:** Tawny brown **old:** Reddish brown	+/-	Loose mantle, EH are clamped
UK10	White	+/-	Dense mantle, EH have cottony appearance, H junctions and tips anastamosing
UK11	**young:** whitish-yellow **old:** brownish-yellow	+ (sparse)/-	Patchy but dense mantle regions, inflated mantle hyphae
UK12	**young:** light brown **old:** black-brown	+/-	Loose mantle, clamped EH, EH exist as clusters
UK13	**young:** Brownish-black **old:** Coal black	+ (sparse)/-	Dense sheathing mantle, clamped EH, large angular synenchyma cells
UK14	**young:** Whitish-yellow **old:** Deep Yellow	R	Dense mantle, EH have cottony appearance, densely clamped EH
UK15	Hayline	R	Dense mantle, clamped EH

2.4: Genomic DNA Extraction

2.4.1: Sporocarps

Genomic DNA was extracted via the CTAB PCR miniprep procedure (Zolan and Pukkila, 1986). Approximately 0.02 to 0.04 grams of air dried sporocarp tissue, taken from the cap to ensure a good supply of DNA, was used in each extraction. Each tissue sample was deposited in a 1.5 ml microcentrifuge tube and then crushed with a tube pestle. Approximately 700 ul of 1X CTAB extraction buffer (700 mM NaCl, 50 mM Tris-pH 8, 10 mM EDTA, 1% CTAB, 0.2% mercaptoethanol) was added to the tubes containing the air dried tissue. Tissues were then incubated in a 60°C water bath for approximately forty-five (45) minutes.

After incubation, 700 ul of a Chloroform:Isoamyl alcohol (24:1) mixture was added to each tube. Samples were emulsified and then centrifuged at 13,000 rpm for 10 minutes at room temperature. Most of the upper aqueous phase was removed with a pipette, being careful not to take up any of the cellular phase, and transferred to a clean 1.5 ml microcentrifuge tube.

Precipitation of the genomic DNA was facilitated by dispensing 700 ul of cold isopropanol (Propan-2-ol) into the aqueous phase and then inverting the tubes repeatedly to ensure mixing. Usually a nucleic acid rope precipitated out immediately. However, if this did not occur then the tubes were placed at -15°C for 5 minutes and then repeatedly inverted at room temperature for another 3-4 minutes.

The nucleic acids were pelleted by centrifuging (13,000 rpm) the tubes for 2 minutes at room temperature. The supernatant was decanted off and pellets were washed twice

with two 500 ul aliquots of cold, 70% ethanol. After each wash the pellets were centrifuged (13,000 rpm) for 2 minutes at room temperature.

Pellets were dried in a vacuum desiccator and then resuspended in 50 ul of autoclaved, nanopure filtered (ANF) water. The pellets were either allowed to dissolve overnight at 4°C or they were dissolved by incubating the tubes at 60°C over a 10 minute period. The latter method was used when the DNA was required immediately. All DNA extractions were stored at -20°C.

Horizontal gel electrophoresis was used to determine the presence and quality of DNA from the sporocarp extractions. About 5.0 ul of the extraction was mixed with 3.0 ul of loading buffer (0.25% bromophenol blue, 30% glycerol) and then loaded into an agarose gel (0.7% GTG grade agarose in 0.5X TBE buffer). Gels were run in 0.5X TBE buffer (89 mM Trisborate, 89 mM boric acid, 2 mM EDTA pH 8.0) at 70-90 volts for approximately 20 minutes. The gel was soaked in ethidium bromide solution (0.5 mg/ml) for 15 minutes in order to stain any DNA within the gel. Bands were visualized using the Biophotonics Gel Print 2000*i* (BioCan Scientific) system and photographed with the Mitsubishi P67UA processor.

2.4.2: Root tips

Root tips were collected from the naturally regenerating seedlings (fir and spruce) and from the outplanted black spruce seedlings. Recovery of all root tips was done on ice to slow the enzymatic breakdown of the genomic DNA.

Approximately 2-3 mm of a root apex was severed from the parent root and placed into a cold micromortar (Mandel Scientific Inc.). Root tips and mortars were cooled to -80°C for 15-30 minutes. Afterwards the tips were crushed with micropestles. Freezing the tips increased the amount of cellular fracturing during crushing which allowed for the release of more genomic DNA from fungal cells..

Micromortars, containing the crushed tissue, received 175 ul of a 2X CTAB extraction buffer (1.4 mM NaCl, 100 mM Tris-pH 8, 20 mM EDTA, 2% CTAB, 0.2 % mercaptoethanol). The contents of each micromortar were homogenized with a micropestle and transferred to their respective 1.5 ml microcentrifuge tubes. Another 175 ul of 2X CTAB buffer was used to rinse the micromortars and this solution transferred to their respective tubes. Samples were incubated in a 60°C water bath for approximately forty-five (45) minutes.

After incubation, 350 ul of a Chloroform:Isoamyl alcohol (24:1) mixture was added to each tube. Samples were emulsified and then centrifuged at 13,000 rpm for 10 minutes at room temperature. Most of the upper aqueous phase was pipetted off, being careful not to pick up any of the cellular phase, and transferred to a clean 1.5 ml microcentrifuge tube.

Precipitation of the genomic DNA was facilitated by dispensing 320 ul of cold isopropanol (Propan-2-ol) into the aqueous phase and then inverting the tubes repeatedly to ensure mixing. Unlike with the sporocarp extractions the amount of DNA being extracted was too minute to be seen thus the alcohol/DNA mixtures were set at -15°C for

five (5) minutes to ensure maximum precipitation. Afterwards the tubes were repeatedly inverted at room temperature for another 3-4 minutes.

The nucleic acids were pelleted by centrifuging (13,000 rpm) the tubes for five (5) minutes at room temperature. However, since the quantity of DNA being extracted from a single tip was so little precautions were taken to ensure that none was lost during the recovery. The supernatant was removed by suction until approximately 50 ul was left. The pellets were washed twice, the suction procedure being used each time between washes, with two 175 ul aliquots of cold ethanol (70%). After each wash the DNA solutions were centrifuged (13,000) rpm for five (5) minutes at room temperature.

After a final suctioning, leaving a 50 ul volume, the samples were dried in a desiccater and then resuspended in 50 ul of sodium hydroxide solution (8mM NaOH). The pellets were either allowed to dissolve overnight at 4°C or they were dissolved by incubating the tubes at 60°C over a 10 minute period. The latter method was used when the DNA was required immediately. All DNA extractions were stored at -20°C.

The amount of DNA present in the root tip extractions was too minute for gel detection. Due to time constrains, resulting from the optimization of the protocol design for mycorrhizal root tips, only nine of the twelve sites (collected from in 1995) were analyzed.

2.5: DNA Amplification

2.5.1: Sporocarps

The Polymerase Chain Reaction (PCR; Mullis and Faloona, 1987) was used to amplify a specific region of the ribosomal DNA (rDNA) from the genomic DNA extracts, using two rDNA specific primers, ITS1 and NL6Bmun. NL6Bmun is a primer designed specifically for fungal amplifications (Egger, 1995).

The sporocarp extractions were diluted (1:50) in order to lower the concentrations of phenolic compounds and other inhibitors which might have interfered with the amplification. Dilutions would have decreased the chances of amplifying from root tips thus the undiluted extractions were used for PCR.

The amplification of the rDNA segment from the sporocarp tissue involved the preparation of a PCR premix (0.2 mM dNTPs, 1X Promega thermo buffer, 1.5 mM $MgCl_2$, 0.4 uM NL6Bmun, 0.4 uM ITS1, 0.5 units Taq DNA polymerase, ANF water). All mixtures and stock solutions were kept on ice to prevent premature annealing of primers to other regions within the genomic DNA. Approximately 2.0 ul of diluted, genomic DNA was then mixed into 48.0 ul of the PCR premix. Two control tubes were also prepared with the reaction tubes. The positive control, was used to ensure that the amplification was working properly while the negative control ensured that none of the stock solutions were contaminated. One drop of light, white mineral oil (Sigma Chemical Co.) was used as an overlay to prevent the evaporation of the mixture. Prepared tubes were centrifuged (800 rpm) at room temperature for a few seconds to ensure good mixing of the premix/DNA mixture. All reaction samples and controls were

loaded onto the preheated (94°C) block of a Perkin-Elmer Cetus DNA thermocycler (model 480). All sporocarp samples were amplified using the same parameters.

The initial denaturing step maintains the block at 94°C for two minutes. This step is performed only once during the entire amplification. All denaturing steps afterwards were held at 94°C for 45 seconds. The block was cooled to 42°C, the annealing temperature, at maximum speed and maintained at this temperature for 45 seconds. A ramp time of 1 minute was used to heat the block from 42°C up to 72°C, the extension temperature. The block remained at this temperature for a period of 1 minute and 30 seconds. A total of 35 cycles were performed. As the program proceeded through each cycle the final extension time was increased by one second for each new cycle, therefore the final cycle had an extension time of 2 minutes and 5 seconds. Once the thirty five cycles had been completed, the block remained at 72°C for a further 5 minutes then cooled to 5°C until the tubes were removed.

Horizontal gel electrophoresis was used to verify the success of the amplification. Approximately 4.0 ul of PCR product was mixed with 3.0 ul of loading buffer and then pipetted into a 0.7% TBE agarose gel. One half a microgram of a one kilobase DNA ladder (1 ug/ul; Life Technologies) was run with the samples as a standard to determine the band sizes. The gel was run at 80 volts for 25 minutes. It was then stained in ethidium bromide (0.5 mg/ml) for 15-20 minutes. DNA bands were illuminated and controls checked to ensure that there was no contamination of the PCR premix. Gels were then photographed for future reference.

2.5.2: ECM tip amplifications

Due to the lower concentrations of DNA within the root tip extractions, premix concentrations and amplification parameters had to be altered from the previously mention protocol.

The premix contained the following solutions: 0.21 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia); 1X thermo buffer (BioCan Scientific); 2.1 mM $MgCl_2$ (BioCan Scientific); 0.43 uM each of ITS1 and NL6Bmun; and 0.007 units of Ultra Therm Taq DNA Polymerase (Bio Can Scientific). Again all solutions were on ice and the final reactions mixtures were kept on ice. Approximately 4.0 ul of root tip extraction was added to each 30.0 ul volume reaction and then topped with a drop of oil.

Reactions were amplified using either the Perkin-Elmer Cetus 480 or the Delta II (Idaho Inc.) thermocyclers. Both thermocyclers, used the following parameters: preheated block (not possible for the Delta II); initial denaturing step was 94°C for one minute; all denaturing afterwards consisted of 94°C for 45 seconds; the annealing took place at 48°C over 45 seconds; a ramp time of 55 seconds was used to reach the extension temperature of 72°C, where it remained for 2 minutes and 10 seconds. This was repeated for 35 cycles. After every cycle the extension time was increased by one second thus taking 2 minutes and 45 seconds to finish the last extension.

The final extension and gel electrophoresis follow the same as previously mentioned. Any root tip samples that did not amplify the first time were used in a second attempt in which the amount of Ultra Therm (Bio Can Scientific) was boosted to 0.014 units. If this attempt was unsuccessful then it was assumed that there was no DNA present in the

extraction. Whether this was due to effects of inhibitors or lack of DNA was not determined.

2.6: Restriction Fragment Length Polymorphisms (RFLPs)

Once the ITS1/NL6Bmun fragment had been successfully amplified for all sporocarps and root tips, samples were then cleaved by three endonucleases: *Alu* I (AG/CT), *Rsa* I (GT/AC), and *Hinf* I (G/ANTC, with 'N' referring to any nucleotide; Pharmacia).

Approximately 5.0 ul of ANF water was added to a 200 ul microcentrifuge tube, along with 2.0 ul of a 10X One-Phor-All buffer (Pharmacia). Approximately 7.0 ul of PCR product, whether fruitbody or root tip, was added into the reaction. Two (2) units of one of the restriction enzymes was added to the mixture. The mixture was "finger vortexed", centrifuged for a few seconds, and then incubated at 37°C over a 5 hour period.

After incubation approximately 4.0 ul of loading buffer was mixed with the digested sample, which was then loaded into a 2.5% horizontal agarose gel (1.5% Nusieve: 1% Agarose GTG in 0.5X TBE buffer). During the casting of the gel, 0.23 ug of ethidium bromide was incorporated into the gel in order to facilitate band visualization after electrophoresis. The gel was immersed in 0.5X TBE buffer and run at 105 volts. The current was maintained until the dye front was one centimeter from the end of the gel (run time was approximately 4 hours). The DNA bands were visualized and digitally photographed using the 2000 *i* imaging system (Bio Can Scientific).

2.7: Data Analysis

2.7.1: Regen1

Analysis of the percent abundance data involved employing nonparametric techniques since standard transformation formulas could not normalize the data. The Kruskal and Wallis test (Kruskal and Wallis, 1952; Sokal and Rohlf, 1995) was used to rank the mean percent cover displayed for each morphotype/tree species. This test was used to determine whether the percentage abundance of each ectomycorrhizal morphotype was affected by: that particular morphotype in question, or by the location of the seedling with respect to the forest. The Mann-Whitney U-test was used when there was a comparison between two samples (Sokal and Rohlf, 1995). Again I tested to see if the location of the seedling would affect the overall ectomycorrhizal percent coverage, as well I was interested in whether the species of the host tree affected coverage.

2.7.2: Planted 2

Restriction fragment patterns, from the gels, were saved as digital images and analyzed with RFLPscan (v3.0; Scanalytics). The program allowed us to set up two tolerance levels in order to determine the percent similarity between bands from different samples. The first tolerance level was set within each gel. Each individual gel had a tolerance level of 2%. This is to say that if a band was weighted at 1000 basepairs (bp) then any band falling within the range of 980-1020 bp would be considered the same as that 1000 bp band. This tolerance level was determined by comparing known fragment sizes to that of molecular standards (in this case, 1 Kb ladder [Life Technologies]). The

other tolerance level was set when comparing samples between gels. A tolerance level of 6% was used to compare samples in order to compensate for uncontrollable errors such as: slight changes in gel density, and fluctuations in buffer concentrations which would alter DNA migration. It was found that after running a range (1-12 %) of tolerance levels, against a known database of molecular genotypes, the level which produced the most accurate depiction of the total number of RFLP genotypes was that of 6%.

RFLP data collected from the Regen 1 mycorrhizal samples was used to determine: whether there were any molecular types common to both naturally regenerating spruce and fir seedlings, did a particular tree species host unique molecular types, were there any differences between the molecular types on trees found in or near the forest verses those collected from within the clear-cut, and could any of the molecular types be matched up with the RFLP data of collected sporocarps.

The RFLP data collected from the 'planted2' study was divided two sub-sets (9 of 12 treatment plots x 2; time constraints prevented us from analyzing plots 10,11 and 12). It was noticed that when the RFLP data was separated on the basis of presence or absence of a 'whole' mantle, data interpretation was easier. Thus, each plot has RFLP data separated into two sub-sets; that which was obtained from root tips harboring fungi with a complete or partial fungal mantle (e.g. *Cenococcum geophilum*) verses that which was obtained from root tips which were presumed to harbor no fungi (i.e. non-mycorrhizal). These 18 mini-databases were then merged in a variety of ways to compare samples. The combinations are as follows: all control samples (plots 3, 6, and 9), all low burn samples (plots 1, 5, and 7), all high burn samples (plots 2, 4, and 8), all samples in block 1 (plots

1, 2, and 3), all samples in block 2 (plots 4, 5, and 6), all samples in block 3 (plots 7, 8, and 9). Comparisons were made between plots with different treatments to determine if they shared any similar ectomycorrhizal types and to determine if there were any unique types found within a particular treatment. The main interest was assessing the biodiversity within each treatment to determine if high or low intensity fires affect the overall diversity of ECM fungi. Minidatabases were also compared with the sporocarp databases to try and link fruitbodies with mycorrhizal types.

During August of 1996 another 36 outplanted, spruce seedlings were collected from the twelve plots (4 blocks). The mycorrhizal fungi associated with these seedlings were morphologically characterized (to a much greater extent than that of the 1995 collections), and several samples of each morphotype were collected. RFLP patterns were obtained for each 1996 morphotype and recorded into a reference database. This reference database was compared with each of the 18 mini-databases (1995 data). This was an attempt to try and match more of the 1995 unknown RFLP genotypes to the better described 1996 morphotypes. Comparisons were also made between data from "Regen 1" and "Planted 2" to determine whether fungi that were occurring on the roots of naturally regenerating seedlings (fir and spruce) were colonizing the roots of the outplanted spruce.

Database comparisons were accomplished using the "pairwise function" within RFLPscan (Scanalytics) software. This "pairwise function" takes each lane in the database and matches it, pair-wise, against every other lane. A lane refers a single RFLP pattern produced from a single restriction enzyme. This study used 3 restriction enzymes, thus each sample had three lanes of patterns. All comparisons were stored in a

"matched pairs" table listing the paired samples, the number of shared bands between the two, the number of polymorphic bands and the total number of bands. The default procedure in this program generates a distance matrix based upon total number of polymorphic bands and inputs this into the UPGMA cluster analysis module in the phylogenetic analysis package PHYLIP (Felsenstein, 1993). However, I wished to use a different index, Dice's Index (Appendix III), which corresponds better to the distribution of restriction site changes between two isolates (Egger, unpublished):

Dice Index = 2x common bands/2x common bands + polymorphic bands

In order to output the data to PHYLIP, it had to be converted into a distance value. Thus the distance for each pair of samples was calculated using a modified version of Dice's index which is mathematically equivalent to: 1 - Dice Index.

Modified Dice Index = **sum[(polymorphic bands)/(shared bands + total bands)]/3**.

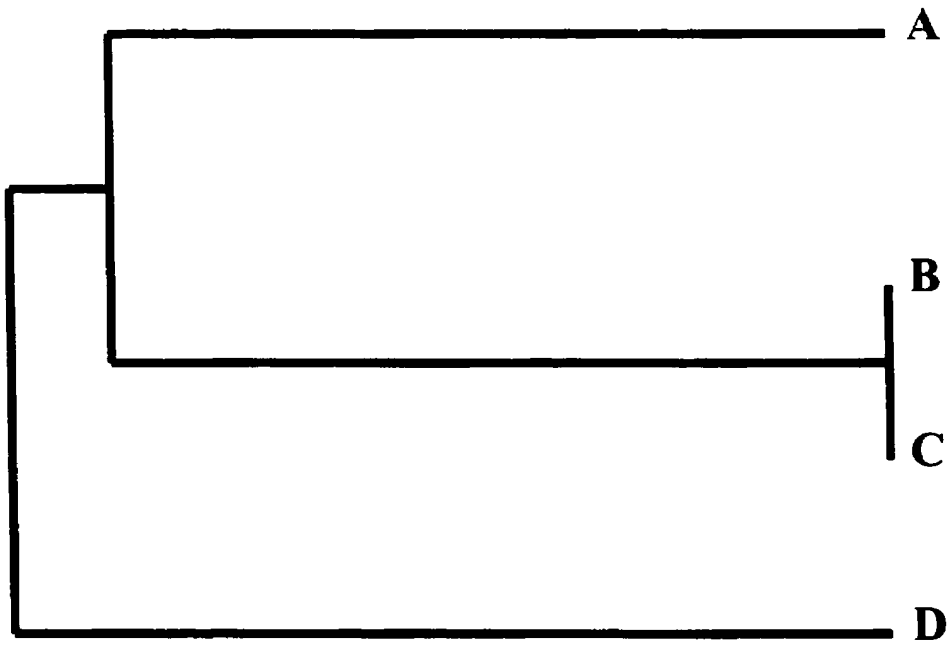
Division by '3' was needed since three restriction endonucleases were used. The resulting value ranges between 0 and 1, with 0 representing identity, and 1 representing maximum distance (i.e. no shared bands).

I then used cluster analysis (UPGMA) to create a phylogram. The Neighbor program from PHYLIP (Felsenstein, 1993) was used for UPGMA cluster analysis. All phylograms were visualized using Treeview95 (version 1.5.0) (Page, 1996). The tree was used to visually identify identical types, rather than to infer phylogenetic relationships.

The phylograms provided data on both RFLP genotype number and the number of individuals within each RFLP genotype. To achieve this it was assumed that every sample that grouped alone, on a phylogram, was an individual genotype. Figure 3

explains in more detail these assumptions. Both the number of genotypes and the number of individuals per genotype were recorded for each treatment. Both the Simpson and Shannon-Wiener indices were used to determine the amount of diversity present in each treatment. Appendix IV contains the equations for each index as well as their assumptions. An analysis of variance (ANOVA) was implemented to determine whether the differences existing between diversity values, for each treatment, were significant.

Figure 3: A simple phylogram which will be used to explain our assumptions. The above phylogenetic tree shows the similarity of four individuals, with respect to RFLP data. '**A**' and '**D**' are unique in their RFLP patterns, however, individuals '**B**' and '**C**' have the same RFLP pattern. Thus, this tree shows three species; two unique species and one species with two individuals.


A
B
C
D

2.8: Root staining

Several sections of root, which appeared to harbor no ectomycorrhizal fungi, were selected for observation. The objective of this procedure was to determine the source of positive PCR amplifications derived from root tips that were thought to be uncolonized.

Root tips were placed in a test tube containing a few milliliters of a 10% sodium hydroxide (NaOH) solution. Several test tubes were set up in such a manner and a variety of cellular stains added to each. One stain was phenol cotton blue (30 ml of 80% phenol with 5 mg of cotton blue). Another was phenol red (in 50% glycerol) and another was cotton blue in lactic acid (5mg/30 ml of lactic acid).

Each KOH/stain mixture was gently heated over an open flame to boiling. Gentle boiling continued for approximately five minutes and then the solution was set aside to cool. Roots were removed and rinsed several times with distilled water to remove excess stain. Root tips were viewed and photographed, using bright field, with the Olympus BX50 compound microscope.

Chapter 3:

Results

3.1: Preliminary Data

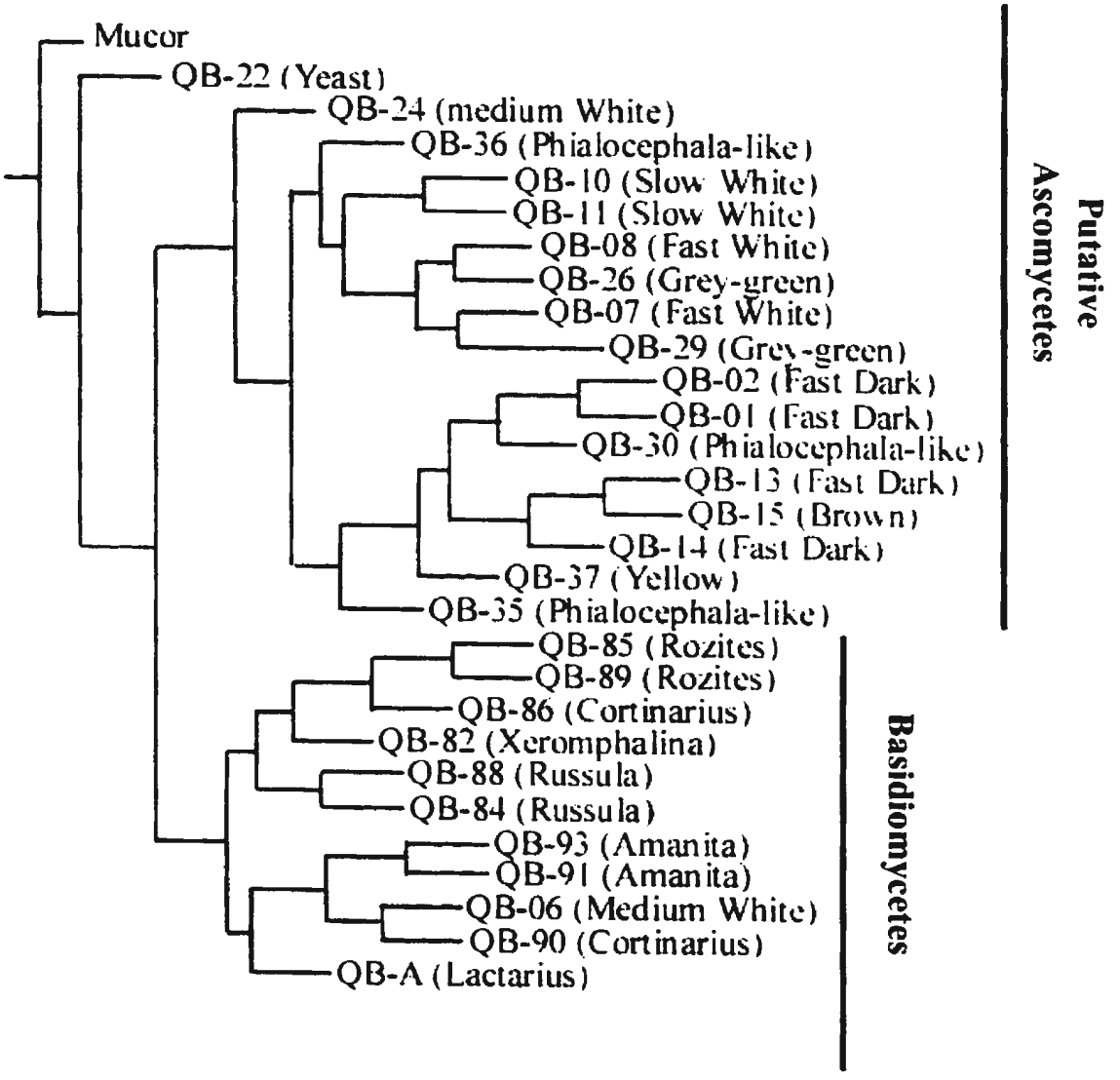
During the summer of 1993, three year old, naturally regenerating black spruce and balsam fir seedlings were harvested from a clear-cut in western Newfoundland. A total of six sites were investigated: three sites were within 10 m of the boundary line of the forest and clear-cut, the other three sites were located more than 20 m from the forest boundary, well within the clear-cut. The objective was to determine whether there was a difference between the types of mycorrhizal fungi inhabiting the rhizosphere of naturally regenerating fir and spruce seedlings in relation to the clear-cut boundaries (Baldwin, 1994)

Fungal cultures were grown, on E-strain agar (Egger and Fortin, 1989), from individual root tips that were known to harbor ectomycorrhizal fungi. Once enough tissue was obtained from the cultures the genomic DNA was extracted and subjected to the PCR method. The combination of a universal primer (ITS1) and a fungal specific primer (NL6Bmun) were used to amplify an approximately 1100 bp fragment within the rDNA region. Four restriction endonucleases (*Alu* I, *Hinf* I, *Hha* I, and *Rsa* I) were used to generate restriction fragment patterns for each fungal isolate. These fragment patterns were compared among isolates to determine proportion of shared restriction fragments. Also fragment patterns from root tips were cross-referenced with patterns derived from basidiocarps collected from the clear-cut site.

RFLP data indicated that of the twenty (20) isolates, 18 apparently belonged to ascomycotina (Figure 4). One of the remaining two isolates fell in with the

basidiomycete group, which consisted of sporocarps collected from the site, and the other isolate was identified as a yeast contaminant (Figure 4).

Figure 4: Classification based on neighbor-joining analysis of RFLP patterns from digestion of partial rDNA sequences of 18 fungal isolates and 10 basidiocarps. Specimen numbers are given in the diagram along with either a brief culture description or the genus name for the basidiocarps.


Mucor
QB-22 (Yeast)
QB-24 (medium White)
QB-36 (Phialocephala-like)
QB-10 (Slow White)
QB-11 (Slow White)
QB-08 (Fast White)
QB-26 (Grey-green)
QB-07 (Fast White)
QB-29 (Grey-green)
QB-02 (Fast Dark)
QB-01 (Fast Dark)
QB-30 (Phialocephala-like)
QB-13 (Fast Dark)
QB-15 (Brown)
QB-14 (Fast Dark)
QB-37 (Yellow)
QB-35 (Phialocephala-like)
QB-85 (Rozites)
QB-89 (Rozites)
QB-86 (Cortinarius)
QB-82 (Xeromphalina)
QB-88 (Russula)
QB-84 (Russula)
QB-93 (Amanita)
QB-91 (Amanita)
QB-06 (Medium White)
QB-90 (Cortinarius)
QB-A (Lactarius)
Putative Ascomycetes
Basidiomycetes

Sporocarp collections made by Keith Egger and Dirk Krüger were subjected to PCR/RFLP analysis in order to build a basidiomycete database for which to compare with ectomycorrhizal root tip fragment patterns. Table 3 provides a listing of basidiocarps collected during the summers of 1993 and 1994.

Table 3: Sporocarps collected in and around the Glide Lake study site, during the summers of 1993 and 1994 (molecular code is 'GL'). Other sporocarps were collected from St. John's area (molecular code is 'SJ'). Letter within the collection codes refers to sub-sites and can be located on figure A-1, Appendix I (Glide Lake area only).

Species	Collection code (s)	Molecular code(s)	Mycorrhizal	Author
Amanita muscaria	QFB93	GLsp93qb	yes	(LIN. ex FR.)PERS.
Amanita prophyria	A 62, QFB91	GLA62, GLsp91qb	yes	(ALB.etSCHW.ex FR) SECR.
Armillaria ostoyae	L 102, CC 142	GLL102, GLCC142	no	ROMAGN.
Bisporella citrina	A4	GLITS1-4 , GLITSV-4	no	(BATSCHexFR.) KORFetCARPENTER
Clavulina cristata	S 40	GLS40	no	(FR.)SCHRÖT
Clitocybe clavipes	CC 53	GLCC53	no	(PERS.exFR.)KUMMER
Collybia acervata	A8, CC 110	GLA8, GLCC110	no	(FR.)KUMMER
Cortinarius (Leprocybe) spp	G 21	GLG21	yes	
Cortinarius (Myxacium) spp	B1 76	GLB176	yes	
Cortinarius (Phlegmacium) spp	B1 69, Q 82	GLB169, GLQ82	yes	
Cortinarius (Sericeocybe) lilacinus cf.	Q 79	GLQ79	yes	PECK
Cortinarius (Sericeocybe) spp	A 57	GLA57	possibly	
Cortinarius (Sericeocybe) traganus	Q 80	GLQ80	yes	FR.
Cortinarius (Sericeocybe) violaceus	Q 31	GLQ31	yes	(FR.)S.F.GRAY

Cortinarius (Telamonia) spp	B1 11, Q 32, B1 67, E2 89, E2 92, E1 96, E1 98, R 115, R 116	GLB111, GLQ32, GLB167, GLE289, GLE292, GLE196, GLE198, GLR115, GLR116	possibly	
Cortinarius lilacinus	QFB90	GLsp90qb	yes	PK.
Cortinarius spp.	QFB86	GLsp86qb	possibly	
Cortinarius spp.	QFB83	GLsp83qb	possibly	
Dacrymyces palmatus	CC 83	GLQ83	no	(SCHW.)BRES.
Dentinum repandum	Q 38	GLQ38	no	(FR.)S.F.GRAY
Dermocybe spp	A 58, E2 91, CC 132	GLA58, GLE291, GLCC132		
Entoloma spp	CC 86	GLCC86	possibly	
Fuscoboletinus spectabilis	F 123	GLF123	yes	(PECK)POMERLEAUet A.H.SMITH
Galerina spp	Q 34, CC 55, CC 138, A 63	GLQ34, GLCC55, GLCC138, GLA63	no	
Heboloma spp	B1 77, Q 81	GLB177, GLQ81	possibly	
Hygrophoropsis aurantiaca	CC 128, SJ@2, ITSI@1, SJITSV@1, SJgama	GLCC128, SJ@2, ITSI@1, SJITSV@1, Sjgama	possibly	(WULF.exFR.)MRE.
Hygrophorus spp	B1 78	GLB178	yes	
Hypholoma capnoides	CC 54, CC 106	GLCC54, GLCC106	no	(FR.exFR.)KUMMER
Hypholoma polytrichi group	B1 101	GLB1101	no	(FR.)SINGER
Inocybe geophylla s.l.	K 141	GLK141	yes	(SOW.exFR.)KUMM.
Inocybe spp	B1 68, E1 94	GLB168, GLE194	yes	

Laccaria bicolor	E1 19, QFBB, QFBC	GLE119, GLspBqb, GLspCqb	no	(MRE.)ORTON
Laccaria deceptivus	B1 70	GLB170	no	PECK
Laccaria laccata	CC 49, A 60, CC 66, SJIXXX	GLCC49, GLA60, GLCC66, SJIXXX	no	(SCOP.exFR.)FR.
Lactarius camphoratus	K 137, QFBD	GLK137, GLspDqb	yes	(BULL.exFR.)FR.
Lactarius hibbaradae	K 136	GLK136	yes	PECK
Lactarius helvus	QFBA	GLspAqb	yes	FR.
Lactarius vinaceorufescens	R 39, R 117	GLR39, GLR117	yes	A.H.SMITH
Limacium erubescens	R 112, K 135	GLR112, GLK135		(FR.)WÜNSCHE
Lycoperdon pyriforme	B1 16, CC 51	GLB116, GLCC51	no	SCHAEFF.exFR.
Mycena or *Collybia* spp	A7	GLA7	no	
Mycena spp	B1 10, CC 111	GLB110, CLCC111	no	
Paxillus involutus	CC 127, SJPax.in	GLCC127, SJPax.in	yes	(FR.)FR.
Peziza badia	CC 103, CC 120	GLCC103, GLCC120	no	PERS.exMERAT
Pluteus atricapillus	CC 48, BN#2 118	GLCC48, GLBN2118	no	(SECR.)SINGER
Psathyrella spp	CC 44, CC 46, CC 143	GLCC44, GLCC46, GLCC143	no	
Psilocybe tenax s.l.	BN#8 87	GLBN887	no	(FR.)KÜHNERet ROMAGN
Rickenella fibula	CC 139	GLCC139	no	(BULL.exFR.)RA ITH
Rozites caperata	A 65, QFB85, QFB89	GLA65, GLsp85qb, GLsp89qb	yes	(FR.)KARSTEN
Russula spp	QFB88	GLsp88qb	yes	
Russula spp	QFB84	GLsp84qb	yes	

Russula spp	A5, A6, B1 72, R 113, K 134	GLA5, GLA6, GLB172, GLR113, GLK134	yes	
Stropharia hornemannii	L 114, CC 129	GLL114, GLCC129	no	(FR.exFR.)LUND .et NANNF.
Suillus spp.	QFB92	GLsp92qb	yes	
Tricholoma davisiae c.f.	E1 122	GLE1122	yes	PECK
Tricholoma fulvum	A 64	GLA64	yes	(DC.exFR.)SACC .
Tricholoma portentosum	A 61	GLA61	yes	(FR.)QUEL.
Tricholoma virgatum	B1 73, R 108	GLB173, GLR108	yes	(FR.exFR.)KUM MER
Tricholoma spp.	SJ beta	SJ beta	yes	
unknown	CC 52	GLCC52		
unknown	CC 99	GLCC99		
unknown	CC 100	GLCC100		
unknown	BN#2 105	GLBN2105		
unknown	BN#2 119	GLBN2119		
unknown	SJIXXXX	SJIXXXX		
unknown	G 125	GLG125		
Xeromphalina campanella	B1 71, QFB82	GLB171, GLsp82qb	no	(BAT.exFR.)KÜH NERetMRE.

3.1.1: Percent Abundance of Mycorrhizal Fungi

Root tips collected for the preliminary study (above, section 3.1) were further surveyed to determine the percent abundance of several different fungal morphotypes on naturally-regenerating seedlings. Six sites were surveyed in total; three bordered the edge of the clear-cut/forest and three were located within the Glide Lake clear-cut. See appendix I for a visual layout of sites. Table 4 gives a brief description of the morphotypes. Appendix Va and Vb contains raw data for the percent abundance of each morphotype

upon each tree species. Tables 5 and 6 show the mean percentage abundance of each fungal morphotype.

An attempt was made to do an analysis of variance for various aspects of the data, however, an inspection of the residuals indicated that the data were not normally distributed. Parametric analysis had to make way for nonparametric techniques in the form of either a Kruskal and Wallis (Kruskal and Wallis, 1952; Sokal and Rohlf, 1995) or a Mann-Whitney test (Sokal and Rohlf, 1995).

Table 4: A brief description of five mycorrhizal types discovered on naturally regenerating balsam fir and black spruce seedlings. Seedlings were collected during the summer of 1993, near Glide Lake (Pasadena, Newfoundland). EH= emanating hyphae, R= rhizomorphs.

Mycorrhizal type	Color	EH/R	Description
Type 1 (*Cenococcum geophilum*)	Black (dull appearance)	+/-	Dense mantle, very stiff EH (brown/black), Stellar pattern on mantle
Type 3	**young:** Coal Black (shiny) **old:** Black-brown	+ (sparse)/-	Dense mantle, stiff EH
Type 6	Hayline	+/-	Thin mantle, short EH
Type 8	White	+/-	Roots take on a twisted appearance, EH are cottony in appearance
Type A	Smoky Gray	+/+	Very loose mantle, EH have a cottony appearance

Table 5: Mean percentage abundance of mycorrhizal fungi on naturally regenerating balsam fir seedlings collected from Glide Lake. Root systems were analyzed from three seedlings (T), per site.

Type	Mean percent abundance/site/fir seedling								
	site 1			site 2			site 3		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
1	0.7	2.7	-	-	-	-	-	-	-
3	-	-	1.1	-	5.4	0.2	11.1	2.2	1.3
6	-	0.1	-	-	0.2	0.2	-	-	-
8	6.1	30.5	0.2	-	11.1	-	-	-	-
A	-	-	-	-	-	-	10.5	-	-
	site 4			site 5			site 6		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
1	-	-	-	8.6	-	-	-	-	-
3	6.3	19.1	0.6	-	2.0	14.7	6.3	6.7	1.8
6	-	-	-	-	-	-	-	-	-
8	5.6	9.6	4.9	3.1	1.3	1.1	6.1	1.0	-
A	-	-	-	-	-	-	-	-	-

Table 6: Mean percentage abundance of mycorrhizal fungi on naturally regenerating black spruce seedlings collected from Glide Lake. Root systems were analyzed from three seedlings (T), per site.

Type	Mean percent abundance/site/fir seedling								
	site 1			site 2			site 3		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
1	-	5.5	-	-	-	1.1	20.1	-	-
3	-	-	19.4	6.1	14.3	-	-	2.3	10.1
6	0.2	1.4	0.1	-	-	-	8.1	-	-
8	25.0	-	22.5	0.1	3.3	-	-	-	-
A	-	-	-	-	-	-	-	-	0.8
	site 4			site 5			site 6		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
1	-	-	9.3	-	-	-	-	-	16.5
3	6.4	6.3	-	4.5	4.8	26.1	18.7	18.7	-
6	-	-	-	-	-	-	-	-	-
8	-	-	7.7	2.5	4.1	-	18.5	-	-
A	0.8	-	-	-	-	-	-	-	-

Table 7: An average of the mean percent abundance values for each morphotype displayed in tables 4 and 5.

Morphotype	Sample Size	Mean % abundance (Black Spruce)	Mean % abundance (Balsam Fir)
1	18	2.92	0.667
3	18	7.65	4.38
6	18	0.544	0.0278
8	18	4.65	4.48
A	18	0.0889	0.583

Averages of the mean percent abundance of each morphotype for each tree species are shown in table 7. The Kruskal and Wallis test was used to determine if there was significant difference between the average percent mycorrhizal coverage for each morphotype, per tree (table 8).

Table 8: Results of the Kruskal and Wallis test to determine whether there was a significant difference between the morphotype and its percent abundance on a particular tree species. Tests were also performed to determine whether or not the mean differences based on seedling location were significant. "*" indicates significance. H= Kruskal and Wallis statistic, D= Correction factor due to ties, H_a= Adjusted Kruskal and Wallis statistic using the correction factor, a= sample size, χ^2= Chi-squared statistic.

Tree species	Scenario	H	D	H_a	a	$\chi^2_{.005\,[a-1]}$
Picea mariana	Morphotype vs % abundance	13.26	0.718310	**18.46 ***	5	14.860
Picea mariana	location vs % cover	0.21	0.724138	0.29	2	7.879
Abies balsamea	Morphotype vs % abundance	25.83	0.745885	**34.63 ***	5	14.860
Abies balsamea	location vs % abundance	0.05	0.714285	0.07	2	7.879

Results from the Kruskal and Wallis test indicate that there is a significant difference between the % ectomycorrhizal abundance based upon the morphotype for both tree species. Thus, morphotypes 1, 3 and 8 appear to be the dominant ectomycorrhizas on collected tips in comparison to types 6 and A, at least with respect to black spruce seedlings. With respect to balsam fir (Table 7) it appears that this is the case only for types 3 and 8. This raised the question of whether or not differences for the mean abundance, for the same morphotype, between tree species, were significant.

Mann-Whitney U-tests were performed on morphotypes 1, 3, and 8. All three cases showed no significant difference (α=0.05) between tree species, based upon the mean percent abundance of each morphotype. Appendix VI contains the output information, in Minitab (Minitab Inc.) format, for the three Mann-Whitney comparisons. I was also concerned with whether or not the location of a seedling, with respect to the surrounding forest, affected the overall ectomycorrhizal percent abundance (Table 9).

Table 9: An average of the mean percent abundance values based upon location of mycorrhizal seedlings. Seedlings belonging to sites 1,3, and 6 were either in the forest or near its edge (within 3m). Seedlings of sites 2,4 and 5 ranged from 40-100m from the forest edge, inside the clear-cut.

		Mean % abundance	
Location	**Sample Size**	**Black Spruce**	**Balsam Fir**
Near Forest edge	45	4.18	1.964
In Clear-cut	45	2.16	2.089

The generic null hypothesis would state that there was no difference between the mean percent abundance of ectomycorrhizal fungi growing in the clear-cut and the nearby

forest. This hypothesis was applied and tested for both tree species. Table 8 shows the results obtained from a Kruskal and Wallis test. In both cases the null hypothesis was not rejected. The Mann-Whitney U-test was also employed and confirmed these findings (Appendix VII).

Lastly the Mann-Whitney U-test was used to determine if there was an overall difference between the mean total ectomycorrhizal % abundance occupying spruce and fir seedlings. Again there was no significant differences between each tree species' mean percent abundances (Appendix VIII).

3.2 Molecular analysis of ECMs

3.2.1: Sampling of Root Tips

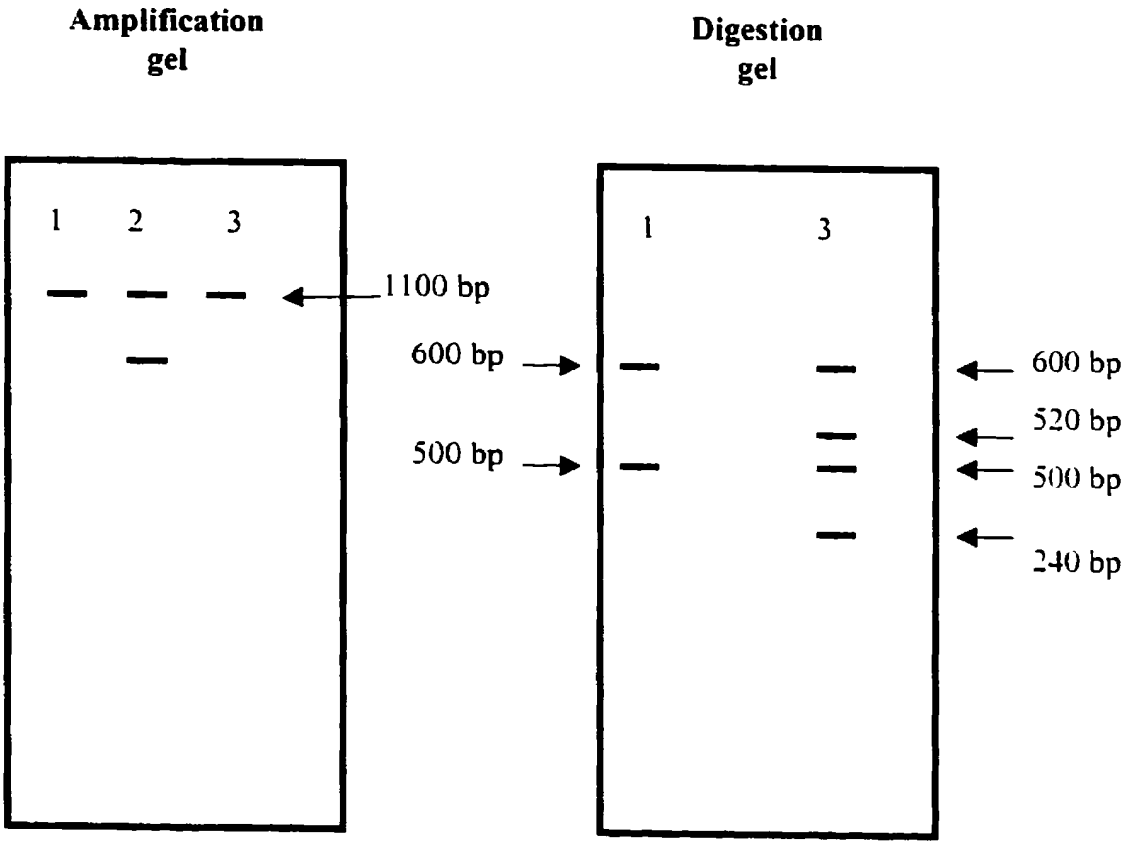
Root tips were collected in late summer of 1995 and stored at -20°C until analyzed. Three replicates of each treatment, for a total of nine plots, were studied: three controls, three high intensity burns and three low intensity burns (Plots 1-9; Plots 10-12, although sampled were not included in the analysis due to time constraints). A total of 100 root tips were collected from ten spruce seedlings per plot. Table 10 shows the success rate for the amplification of fungal rDNA from each of the 100 root tips. An average amplification rate of 76 percent indicates that the modified CTAB extraction (see previous section on methods) proved to be quite reliable for the extraction of the minute quantities of fungal DNA found on these root tips.

Table 10: Information concerning the success of PCR amplifications for each plot. One hundred root tips were examined from each plot. Note: the values in the 'Total Mean' column were calculated from actual values and not from the percentages presented in this table.

	Control			High Burn			Low Burn			
	Plot 3	**Plot 6**	**Plot 9**	**Plot 2**	**Plot 4**	**Plot 8**	**Plot 1**	**Plot 5**	**Plot 7**	**Total Mean**
% amplified	78	88	69	80	80	63	74	72	78	**75.8**
% noticeable db after amplification	5.13	1.14	0	1.25	1.25	0	1.35	1.39	2.56	**1.61**
% digested singlets	100	77.3	92.8	88.8	72.5	81.0	94.6	73.6	87.2	**85.2**
% db after digestion of singlets	3.85	1.14	0	8.75	5.00	9.52	2.70	9.72	0	**4.40**

Normally with an amplification of a fragment of rDNA one sees only one florescent band on the gel, however, one of two scenarios can occur to produce different results. First, one might obtain two separate bands for a single sample. Second, the amplification gel shows only one band for the sample but restriction endonuclease digestion reveals two PCR products within this one band. Figure 5 provides a visual explanation of these two scenarios.

Figure 5: In the amplification gel, used as a check to determine whether or not the amplification was successful, sample 2 shows a typical 'double amplification' pattern. Samples 1 and 3 show there seems to be only one product, i.e. only one fungal species was amplified. However, upon digestion we see that this is not always the case. The sum of sample one's digested fragments equals that of the original band size, thus suggesting only one species was amplified. The sum of the digestion fragments for sample three is much greater than the original band size. Thus two fungal species were amplified originally. Both had an equal base pair size thus making it appear as if there was a single amplification product.


Amplification gel
Digestion gel
1
2
3
1100 bp
600 bp
500 bp
1
3
600 bp
520 bp
500 bp
240 bp

A double amplification is the result of two fungal species being present on one root tip, and where both are amplified by PCR. It is not unusual to see two or more fungal species present on a root tip. It is assumed that both fungal species provided DNA template of roughly equal quantity (no PCR competition) and of equal annealing compatibility with the primer. However, one of the fungal species has either a deletion or insertion within its ITS regions, which produces a PCR product that is either larger or smaller than the expected size (figure 5). However, more frequently both PCR products are of the same molecular size, thus producing a single band on the amplification gel (sample 3, figure 5). We are only aware of this scenario after endonuclease digestion, when the sum of the fragments adds to larger than the original amplification size.

On average at least 1.6 percent of the amplified products displayed double or multiple bands (multiple bands, i.e. more than two, were very rare) when visualized on a gel (Table 10). In the case of double bands that were only detectable after digestion, this number jumped to 4.4 percent (table 10). Overall, approximately 6 percent of the successful amplifications displayed double PCR products.

These double amplifications were not included in this study since it would have required separating the two bands, extracting each band from the agarose gel and doing a re-amplification from the extracted bands. A future project is being planned to look more closely at these double bands to determine if the species being amplified together form a particular association.

Although there was a relatively high success of amplification (76 %) this does not mean that all amplified products were suitable for digestion. Some amplifications

produced relatively weak bands when stained with ethidium bromide. Thus, if these samples had been digested they would have been difficult to visualize on the agarose gels. Several attempts were made to generate a better amplification product for these 'weak amplifications' but I was not always successful. Table 10 indicates that approximately 85 % of the amplified products were successfully digested.

Extraction of mycorrhizal DNA required using a single root tip measuring approximately 3mm in length. Since I was dealing with such minute quantities of fungal material (ectomycorrhizal mantles usually covered 35-70 % of the root tip), dilution of the template before PCR amplification proved unsuccessful. When trying to amplify from fungal material associated with plant tissue one has to deal with such things as phenolic compounds and other substances that can inhibit the PCR reaction. Overcoming these inhibiting compounds can be achieved by either diluting the template DNA or ensuring that when extracting from the target tissue there is a relatively large quantity of starting material used so that there is sufficient template DNA for amplification.

My experimental design was set up such that not only were 'ectomycorrhizal' root tips selected, but also root tips that did not show obvious signs of ectomycorrhizal colonization. Since I was using a fungal specific primer it was expected that non-ectomycorrhizal (NM) root tips would not produce PCR products. However, table 11 indicates that approximately 66.3 % of the NM root tips produced PCR product, indicating that some fungal material was associated with these root tips and was amplified. Theoretically, the PCR reaction only requires one copy of the template DNA

in order for it to produce successful amplification, however, in reality this low a template concentration would be difficult to visualize on the amplification gels.

Table 11: Percentage of non-ectomycorrhizal root tips that had successfully amplified.

	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	Plot 7	Plot 8	Plot 9	Total Mean
% of tips not mycorrhizal	37	33	30	40	54	35	52	49	34	**40.4**
% of non-mycorrhizal tips that amplified	62.2	69.7	66.7	67.5	61.1	74.3	75.0	67.3	52.9	**66.3**

Successful amplification from NM root tips suggests that enough fungal material was present either on the outside or within the root tip to be amplified by PCR. Each tip was individually viewed with a stereo-microscope, thus those tips classified as NM could probably not have contained enough visible fungal material on their exterior to have provided a template for the PCR reaction. This suggests that some internal fungus, or endophyte, must have provided enough template DNA which had been amplified. In order to test this hypothesis, several non-ectomycorrhizal root tips were cleared stained and studied using a stereomicroscope. Figure 6 shows that tips which lacked ectomycorrhizal hyphae harbored endophytic hyphae. A study by Ahlich and Sieber (1996) showed there to be a wide variety of endophytic fungi inhabiting non-ectomycorrhizal roots of *Abies alba, Picea abies,* and *Pinus sylvestris*, thus providing support for my findings. As well, Richard and Fortin (1974) found MRA to be common in the roots of black spruce.

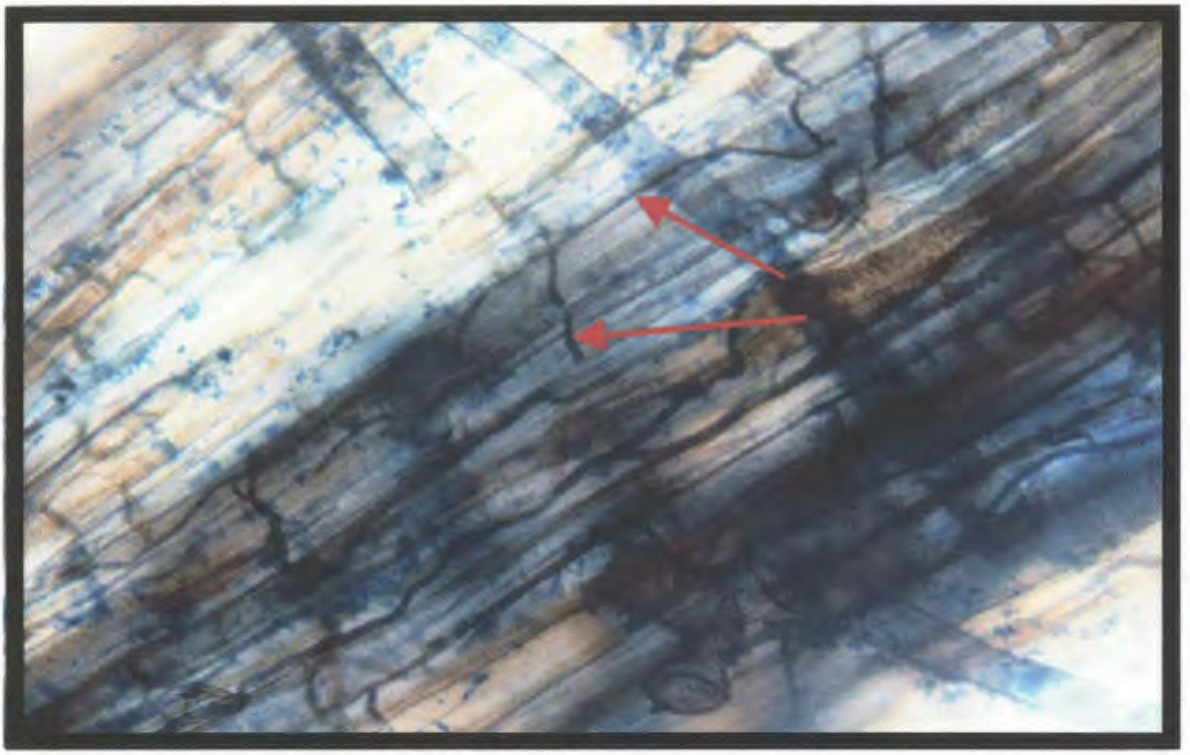
Further investigations found that some of the NM tips showed no visible signs of mycorrhization but after staining a very thin hyphal mantle did surround the tip. Thus,

my database of NM tips could contain molecular types of these thin, hyaline ectomycorrhizas. Figure 7 provides a few pictures of these NM tips with their hyaline ECMs.

Several tips harboring noticeable ectomycorrhizal fungi, mainly *Cenococcum*, were also stained to determine whether endophytes were present. Again, some tips did show that the intracellular spaces of the ECM tips did support endophytic fungi. Figure 8 shows what appears to be MRA fungi running between plant cells with several *Cenococcum geophilum* hyphae on the exterior of the root.

Figure 6: (A) MRA-like hyphae within a black spruce root tip classified as non-ectomycorrhizal. Stained with cotton blue. 200X (B) Another MRA-like hyphal network. Stained with phenol blue. 400X.

A)



B)

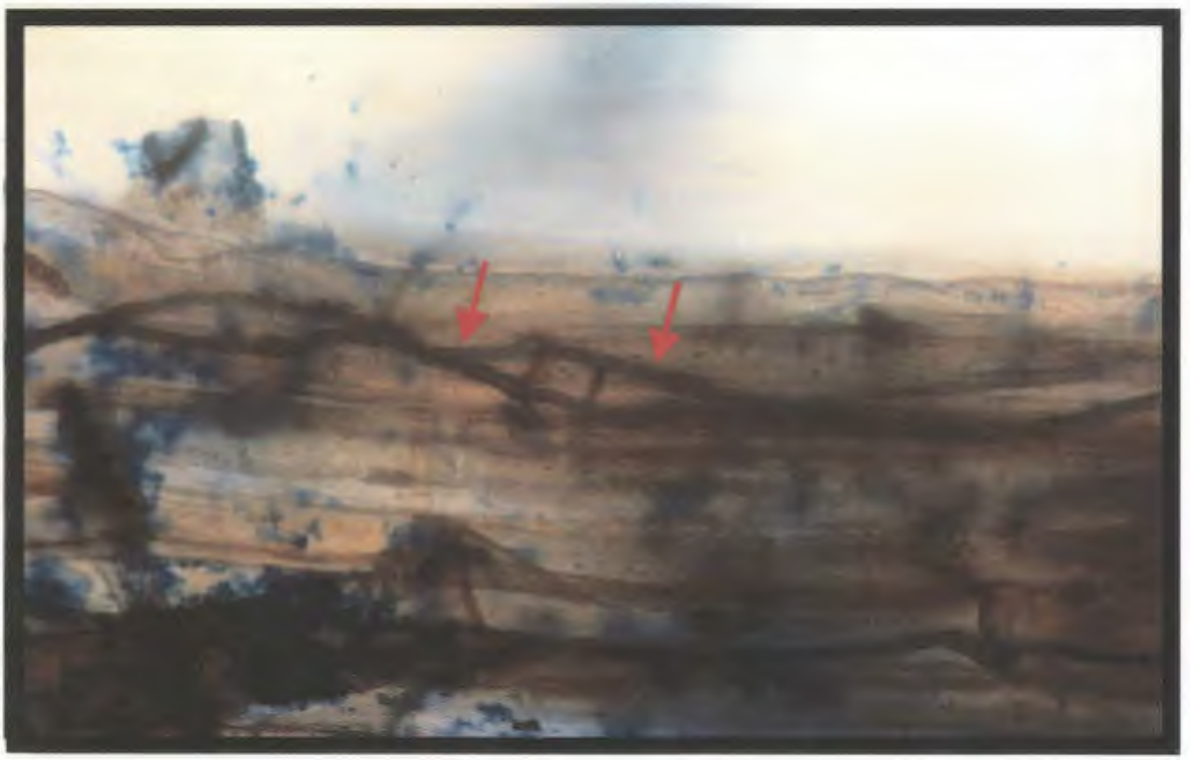
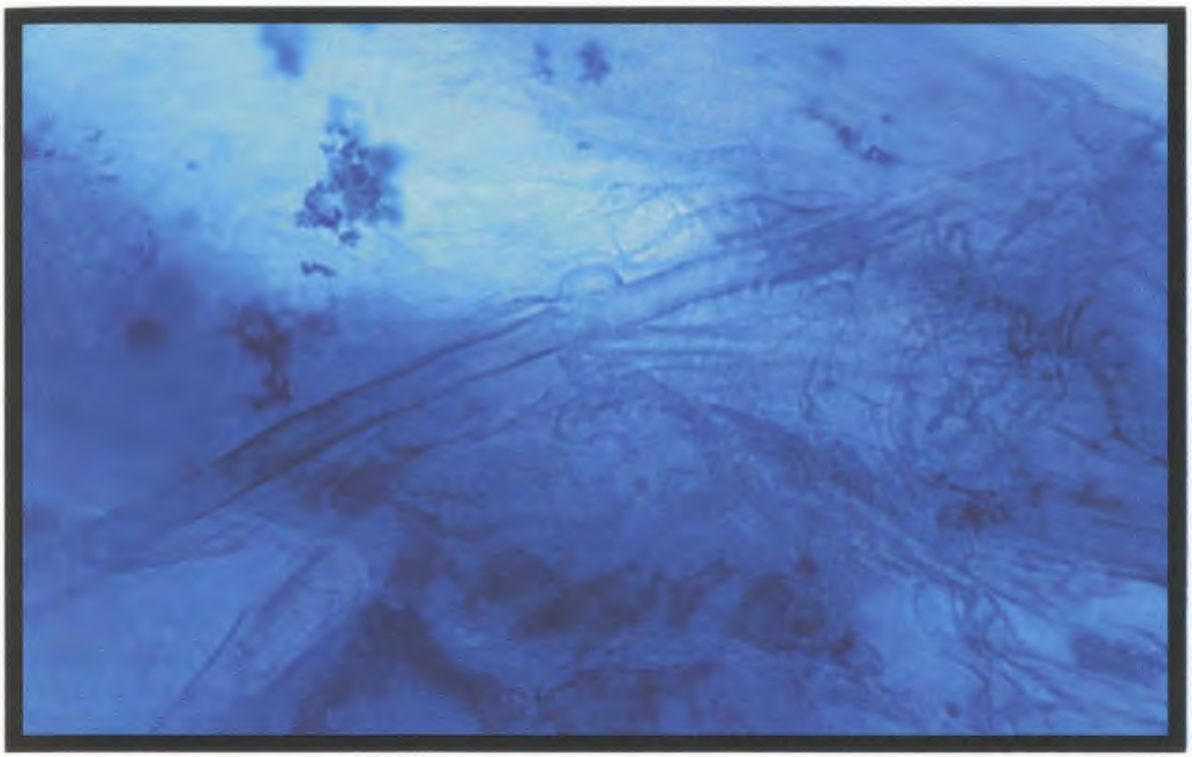


Figure 7: (A) A very loose, hayline hyphal mantle covering what was assumed to be A non-ectomycorrhizal (NM) tip. Stained with Phenol blue. 1000X. (B) What appears to be a semi-loose mantle covering another presumed NM tip. Stained with phenol red. 400X.

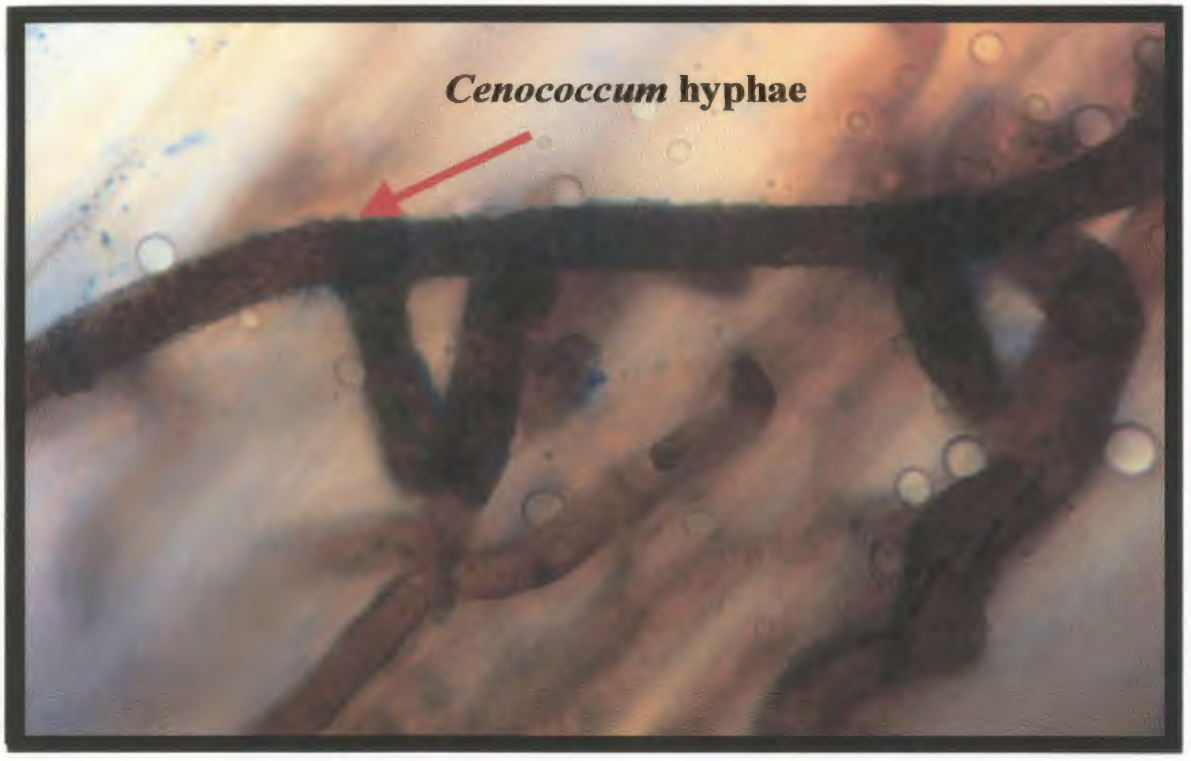
A)



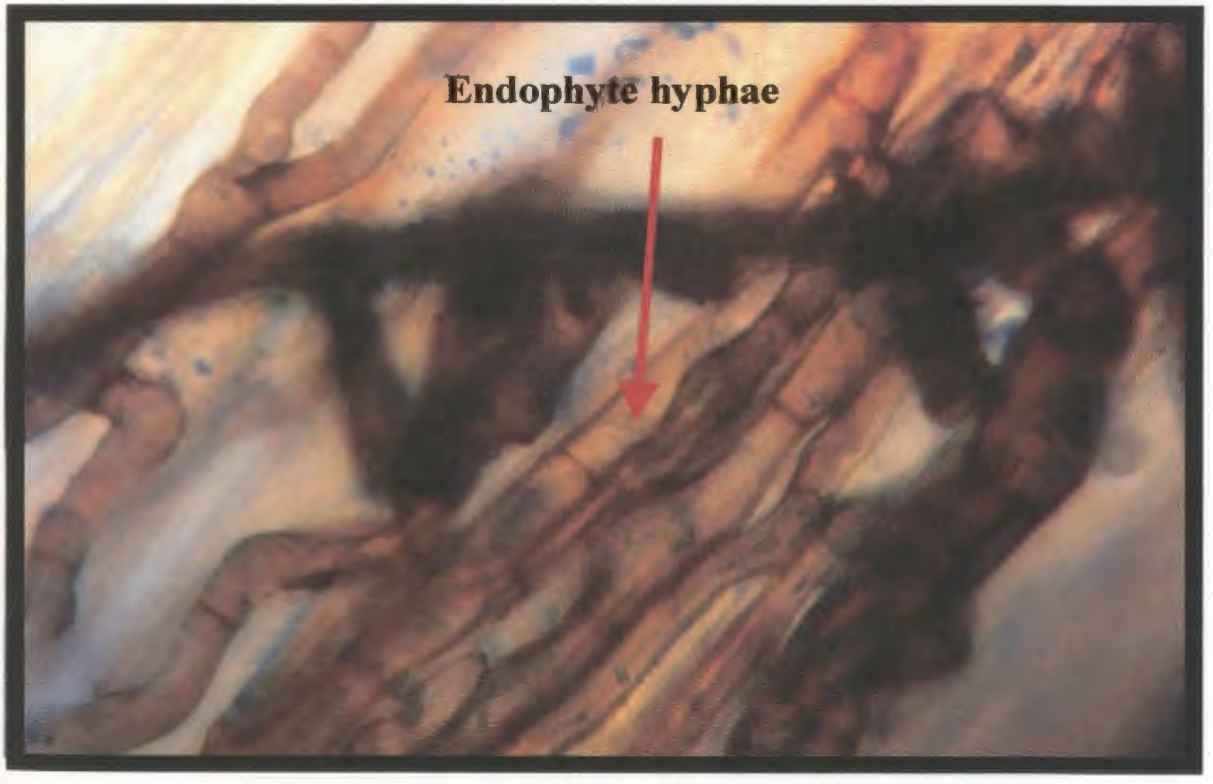
B)

Figure 8: (A) Hyphae of *Cenococcum geophilum*. This is a perimeter shot since internal plant cells could not be stained due to the thickness of *C. geophilum*'s thick mantle. (B) Endophytic hyphal cells occupying root intracellular spaces, just below the mantle of *C. geophilum*

A)



B)

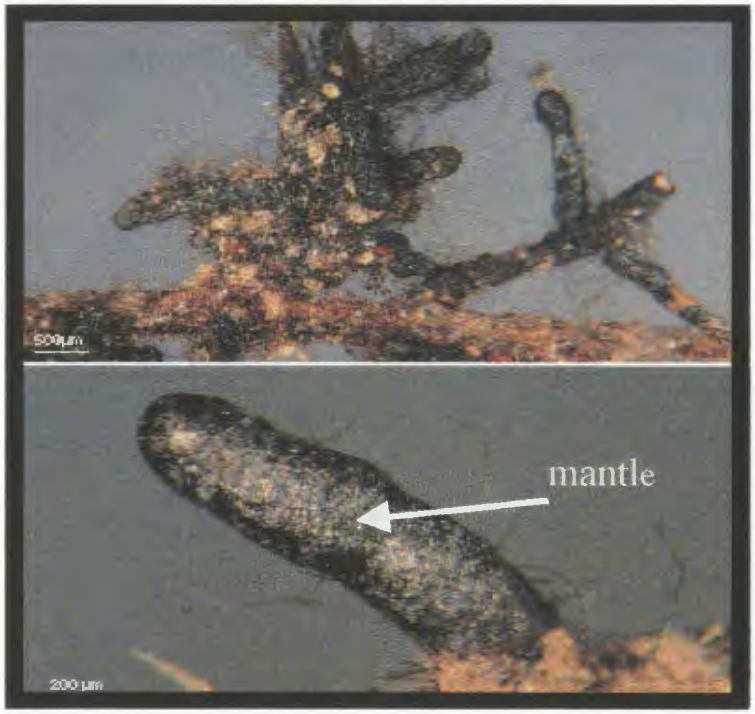


3.2.2: Prescribed burning and biodiversity

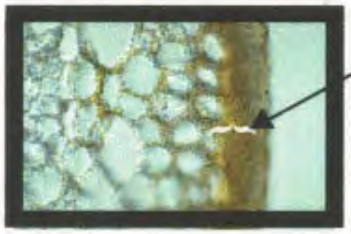
Since the main objective of this study was to look at the effects of prescribed burning on the molecular diversity of ectomycorrhizal (ECM) fungi, I decided to remove PCR amplifications from the data suspected of representing non-target fungi. As such, all data obtained from NM tips were separated from the ECM tips data [ECM tips refer to those tips harboring distinct mantles (figure 9) and those harboring patchy mantles (figure 10)]. It was noticed that within the ECM-tip RFLP data there were several samples that matched to the RFLP patterns of NM tips. See figures A-3 to A-11 in appendix IX for more detail. As a result these ECM samples were also removed from the ECM databases. In the previous section it was shown that endophytic hyphae do exist within NM and *Cenococcum* ECM tips, suggesting the possibility that endophytic hyphae could have been present within other ECM tips.

Figure 9: (A) Overview of a root tip harboring a ectomycorrhizal fungus, *Cenococcum geophilum*. Photo taken by Harniman and Durall (1996). Notice the extensive mantle. (B) and (C) A cross section of an ectomycorrhizal root tip. Notice that the mantle is several cell layers thick.

A)



B)



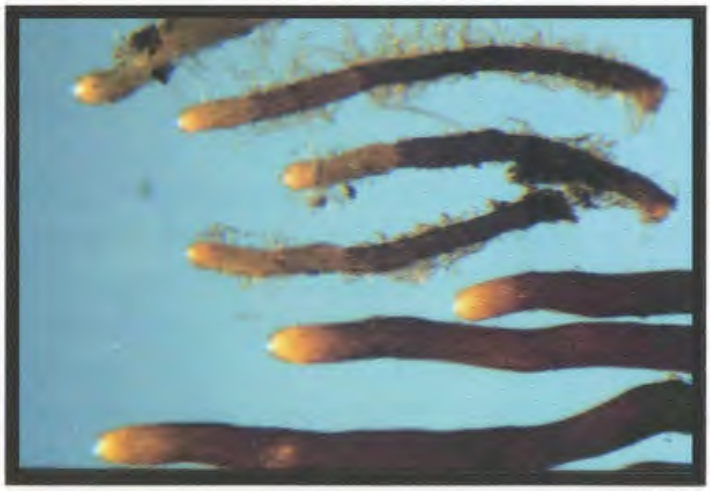
C)

Figure 10: Overview of a black spruce root tips harboring an ectomycorrhizal fungus, E-strain. (a) Notice the loosely developed mantle. Notice that the mantle is a single cell layer thick. (b) A loosely colonized root tip. (c) Colonized, with emanating hyphae, and uncolonized roots.

(A)

(B)

(C)



3.2.3: Diversity of Ectomycorrhizal fungi

After the removal of the NM samples from each of the nine databases, their respective RFLP data were analyzed using RFLPscan software (Scanalytics). Using a modified Dice's index (Appendix III) and Neighbor joining (UPGMA clustering) software (Felsenstein, 1993) phylograms were created to show similarities between samples for each database (Figures 11 to 19). The phylograms that are to follow all contain sample code names that describe various facts about that particular root tip. Each code contains 8 characters. The first two, "GL", refer to the study site (Glide Lake). The third character refers to the plot from which the sample was taken (there were a total of nine plots). The fourth, fifth, and sixth characters refer to the tree (within that plot) from which the root was sampled. There were a total of 217 trees that had the potential of being selected, thus the reason for the three digits in the code. The seventh character refers to the crude morphotype number assigned to the fungus on that particular root tip (e.g., "'1" refers to type 1 = *C. geophilum*; "0" refers to the non-mycorrhizal tips; "'?" refers to the uncertainty of the mycorrhizal status of a tip). The last character of the code refers to the root tip number of that particular seedling (a total of 10 tips were selected from each seedling so an alphanumeric coding, 1-A, was used). This is probably best explained with an example. Sample "GL11273A" was taken from study plot 1, seedling number 127. The mycorrhizal fungus on the tip appeared to be a "type 3". Finally, this sample was the tenth root tip ("A") from that particular seedling. In order to make it easier to talk about certain trends associated with a particular morphotypes, code names

were colored. Each phylogram will denote a particular morphotype with a color. For example, in figure 11 all samples denoted as "Type 1" have been color coded in red.

Figure 11: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 1 (low burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

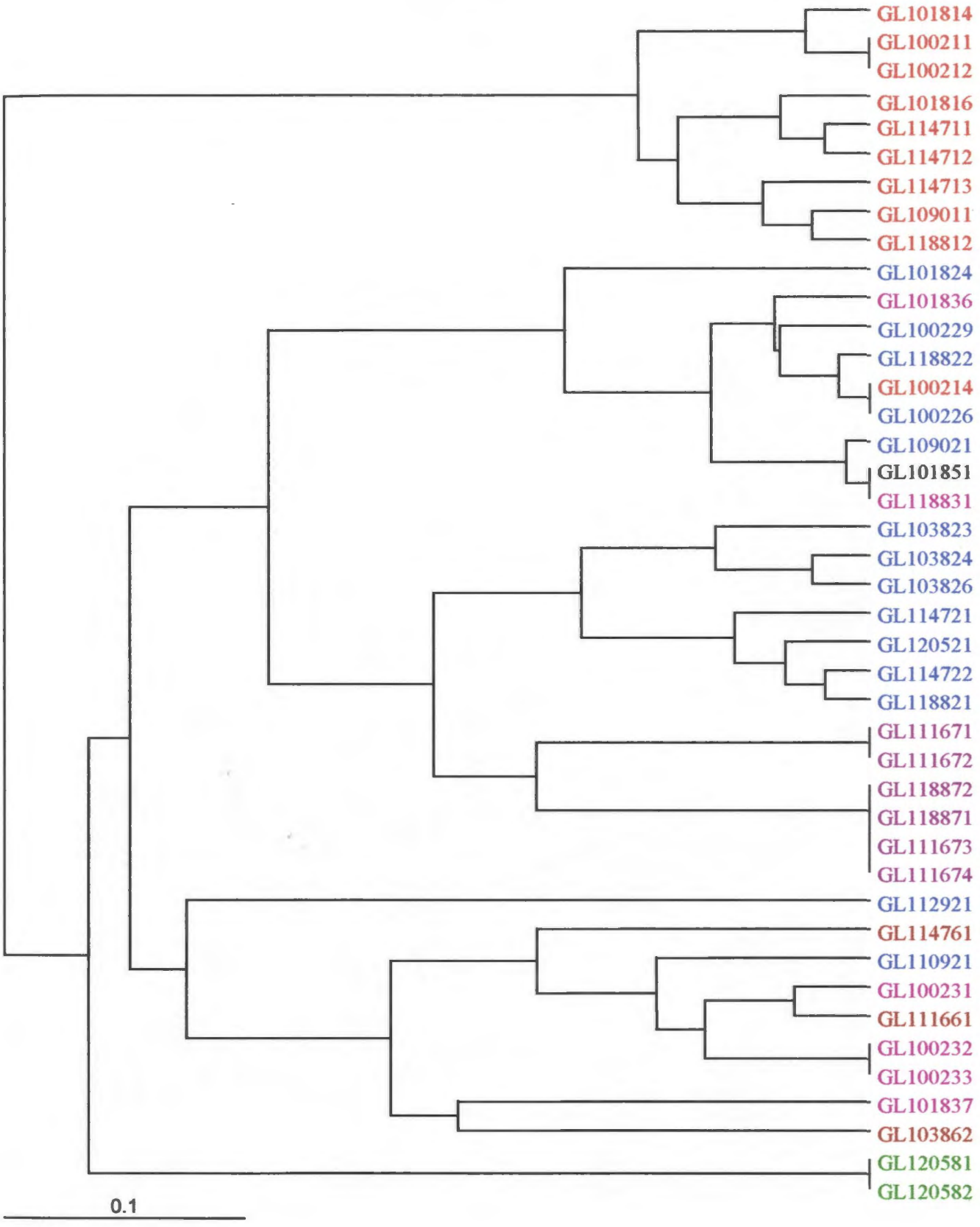

GL101814
GL100211
GL100212
GL101816
GL114711
GL114712
GL114713
GL109011
GL118812
GL101824
GL101836
GL100229
GL118822
GL100214
GL100226
GL109021
GL101851
GL118831
GL103823
GL103824
GL103826
GL114721
GL120521
GL114722
GL118821
GL111671
GL111672
GL118872
GL118871
GL111673
GL111674
GL112921
GL114761
GL110921
GL100231
GL111661
GL100232
GL100233
GL101837
GL103862
GL120581
GL120582
0.1

Figure 12: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 2 (high burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

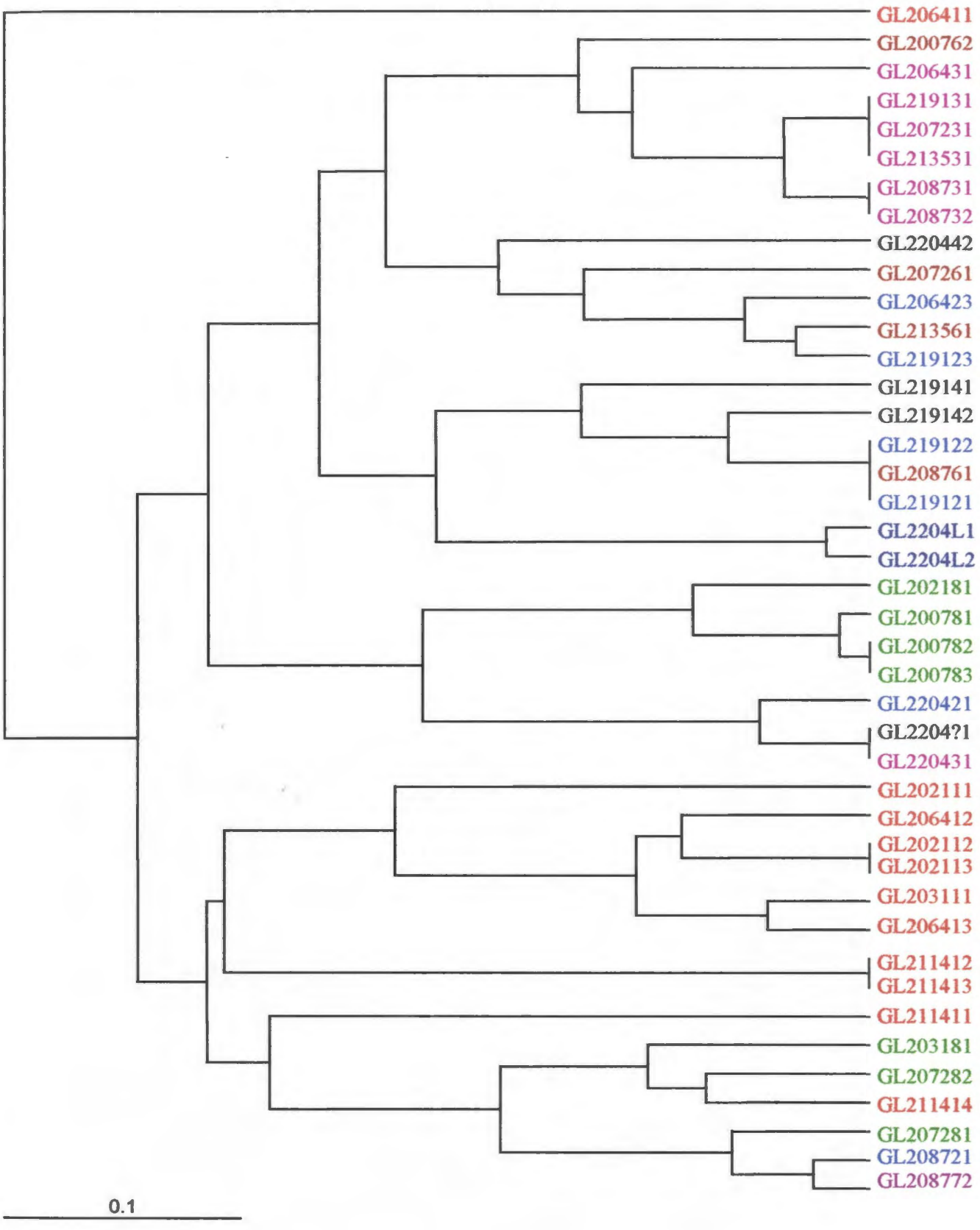

GL206411
GL200762
GL206431
GL219131
GL207231
GL213531
GL208731
GL208732
GL220442
GL207261
GL206423
GL213561
GL219123
GL219141
GL219142
GL219122
GL208761
GL219121
GL2204L1
GL2204L2
GL202181
GL200781
GL200782
GL200783
GL220421
GL2204?1
GL220431
GL202111
GL206412
GL202112
GL202113
GL203111
GL206413
GL211412
GL211413
GL211411
GL203181
GL207282
GL211414
GL207281
GL208721
GL208772
0.1

Figure 13: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 3 (control). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

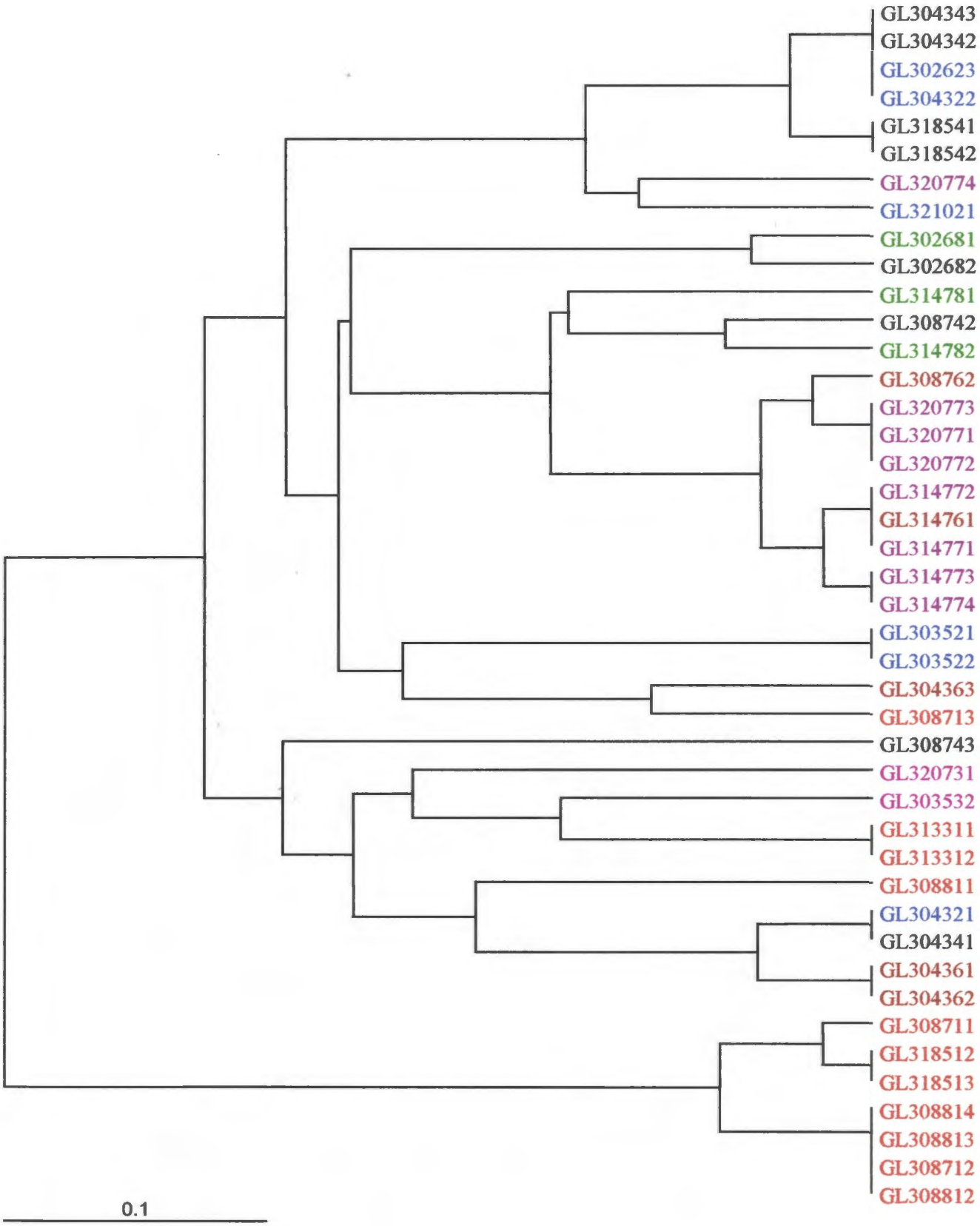

GL304343
GL304342
GL302623
GL304322
GL318541
GL318542
GL320774
GL321021
GL302681
GL302682
GL314781
GL308742
GL314782
GL308762
GL320773
GL320771
GL320772
GL314772
GL314761
GL314771
GL314773
GL314774
GL303521
GL303522
GL304363
GL308713
GL308743
GL320731
GL303532
GL313311
GL313312
GL308811
GL304321
GL304341
GL304361
GL304362
GL308711
GL318512
GL318513
GL308814
GL308813
GL308712
GL308812
0.1

Figure 14: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 4 (high burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

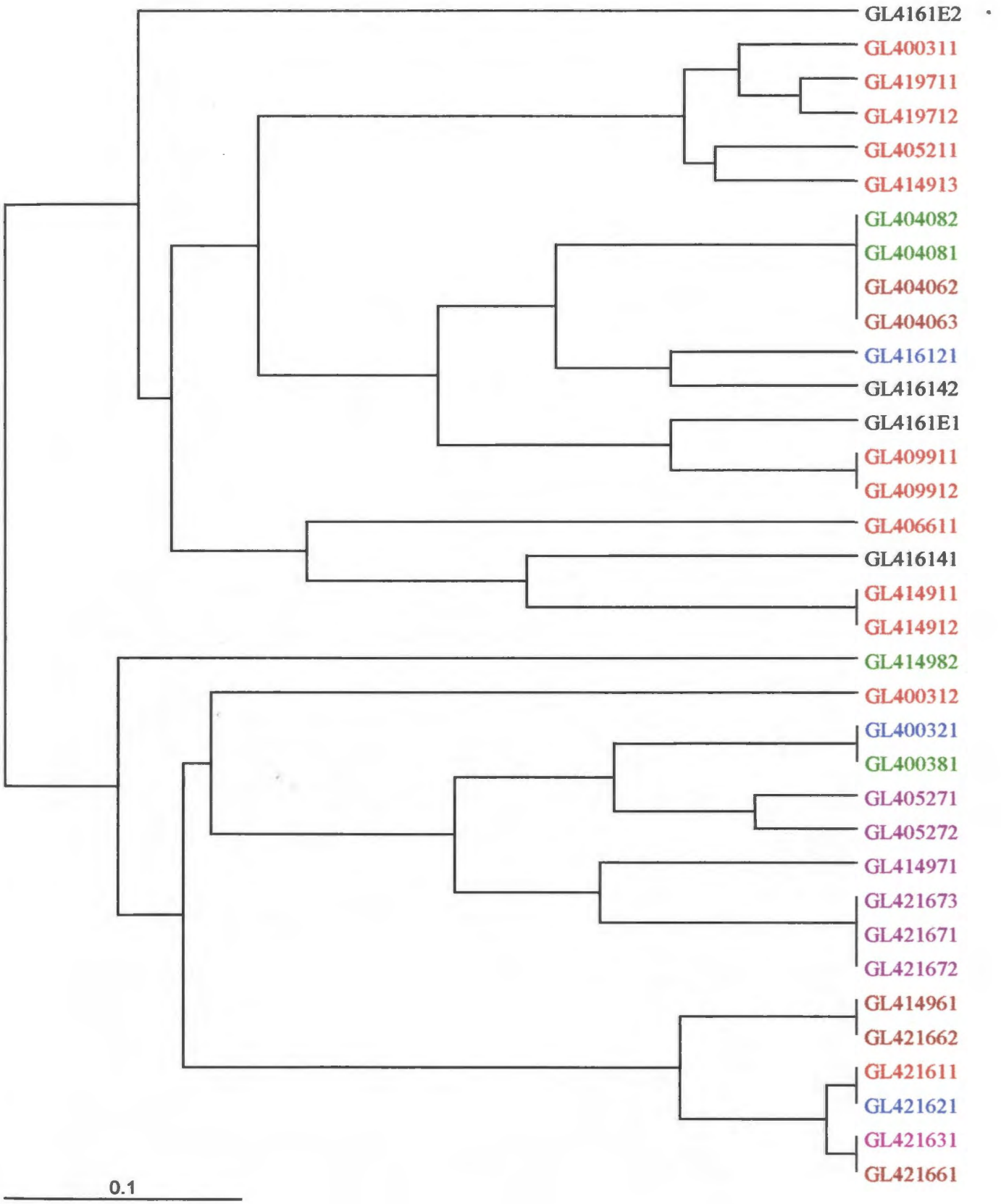

GL4161E2
GL400311
GL419711
GL419712
GL405211
GL414913
GL404082
GL404081
GL404062
GL404063
GL416121
GL416142
GL4161E1
GL409911
GL409912
GL406611
GL416141
GL414911
GL414912
GL414982
GL400312
GL400321
GL400381
GL405271
GL405272
GL414971
GL421673
GL421671
GL421672
GL414961
GL421662
GL421611
GL421621
GL421631
GL421661
0.1

Figure 15: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 5 (low burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

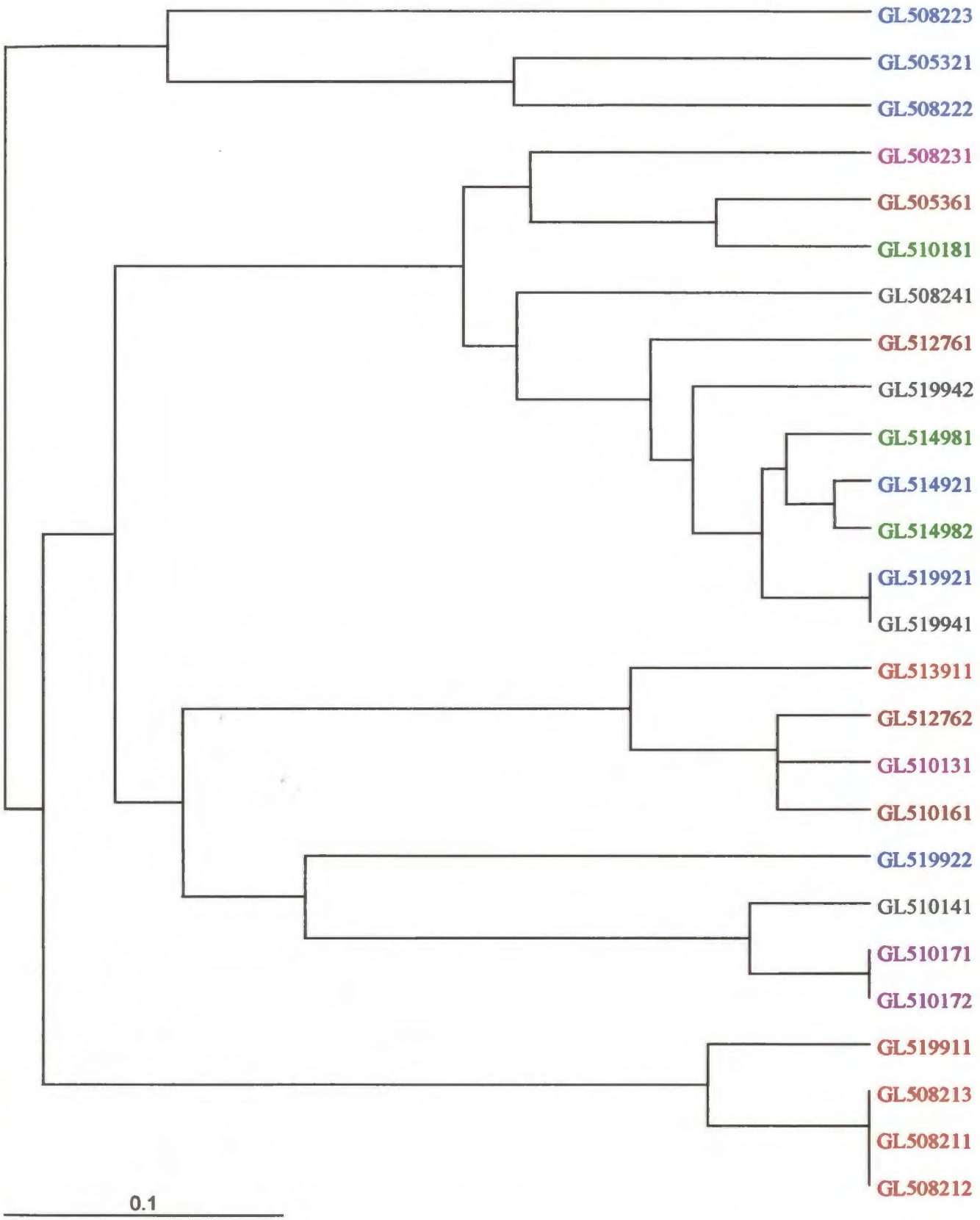

GL508223
GL505321
GL508222
GL508231
GL505361
GL510181
GL508241
GL512761
GL519942
GL514981
GL514921
GL514982
GL519921
GL519941
GL513911
GL512762
GL510131
GL510161
GL519922
GL510141
GL510171
GL510172
GL519911
GL508213
GL508211
GL508212
0.1

Figure 16: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 6 (control). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

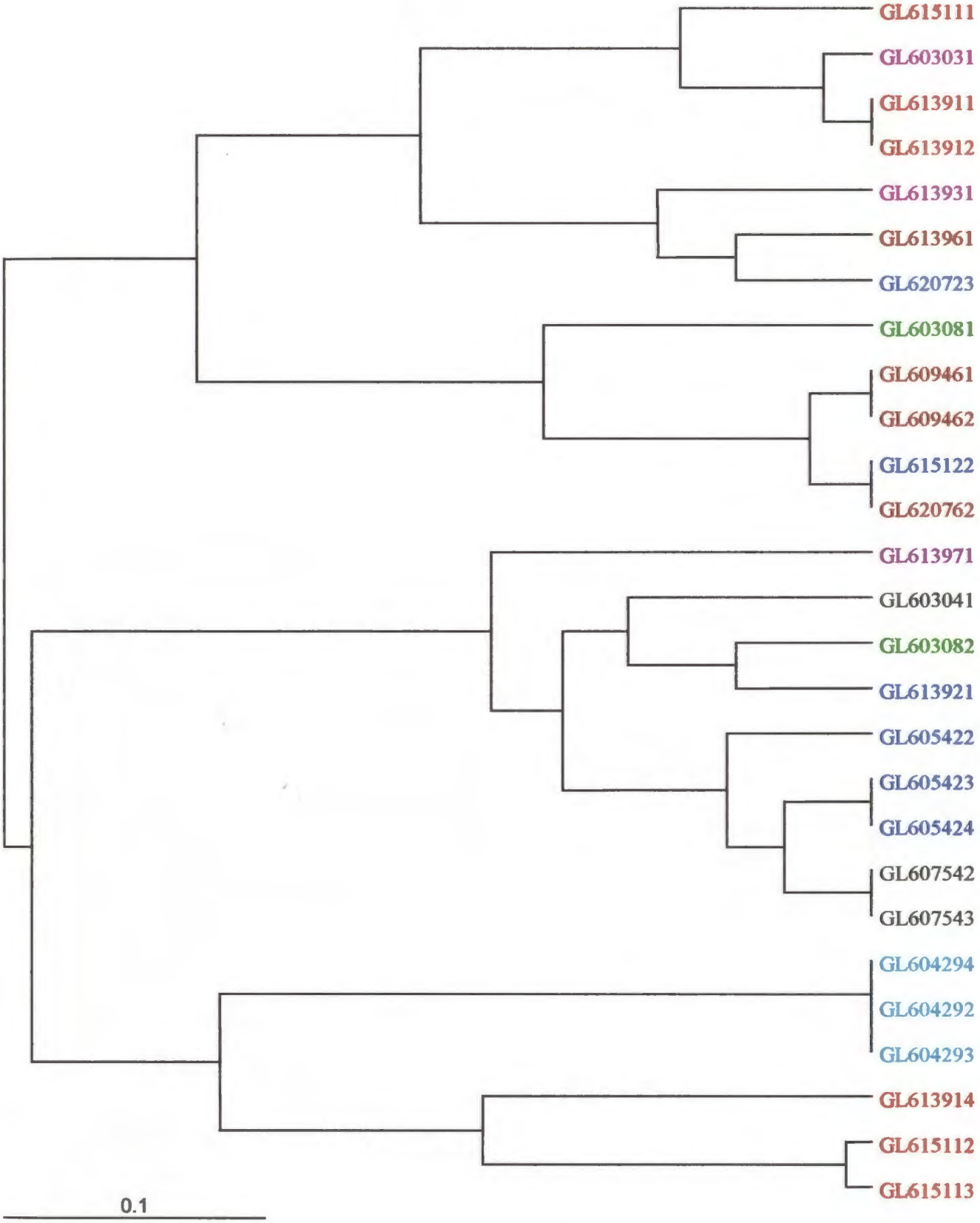

GL615111
GL603031
GL613911
GL613912
GL613931
GL613961
GL620723
GL603081
GL609461
GL609462
GL615122
GL620762
GL613971
GL603041
GL603082
GL613921
GL605422
GL605423
GL605424
GL607542
GL607543
GL604294
GL604292
GL604293
GL613914
GL615112
GL615113
0.1

Figure 17: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 7 (low burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

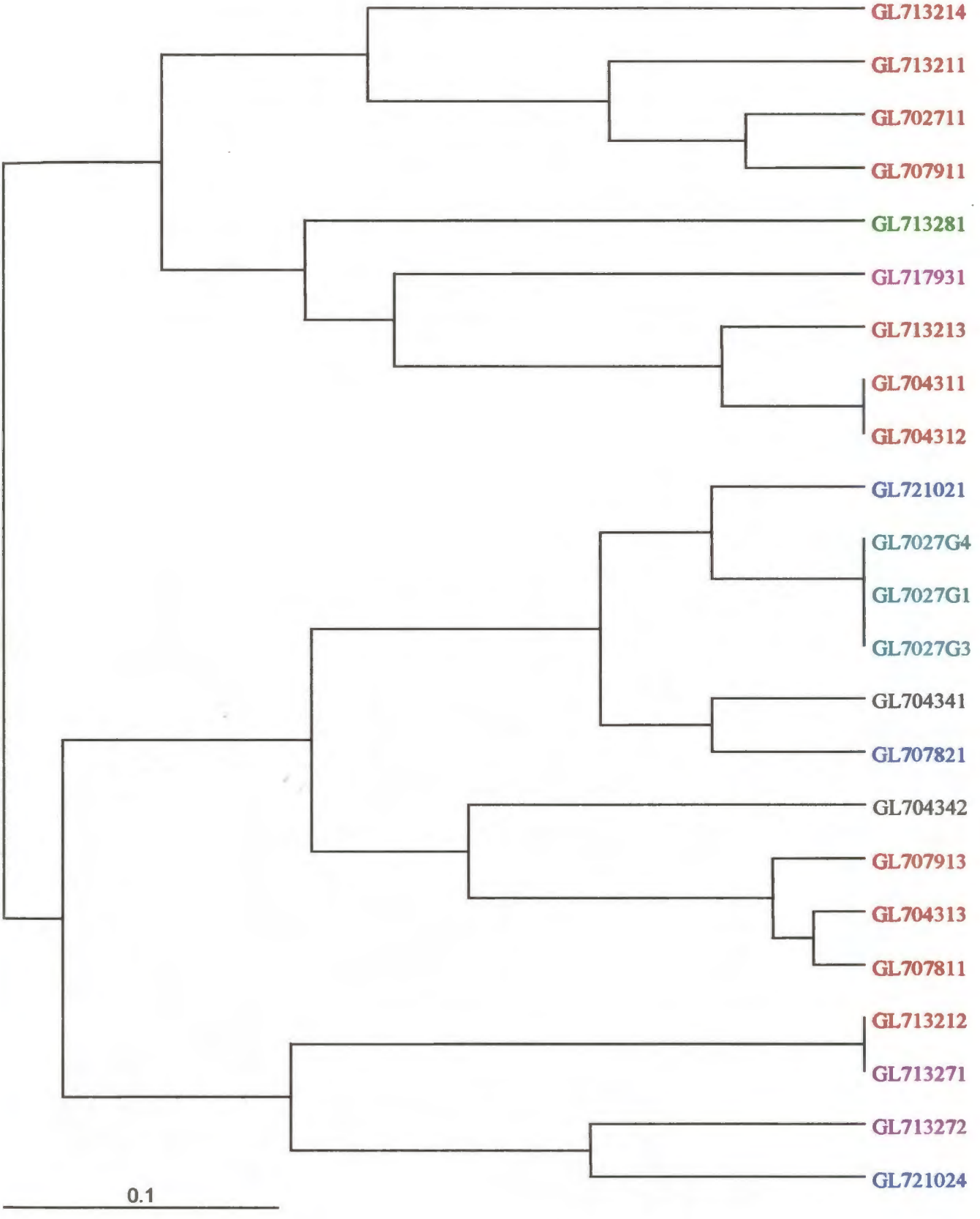

GL713214
GL713211
GL702711
GL707911
GL713281
GL717931
GL713213
GL704311
GL704312
GL721021
GL7027G4
GL7027G1
GL7027G3
GL704341
GL707821
GL704342
GL707913
GL704313
GL707811
GL713212
GL713271
GL713272
GL721024
0.1

Figure 18: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 8 (high burn). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.

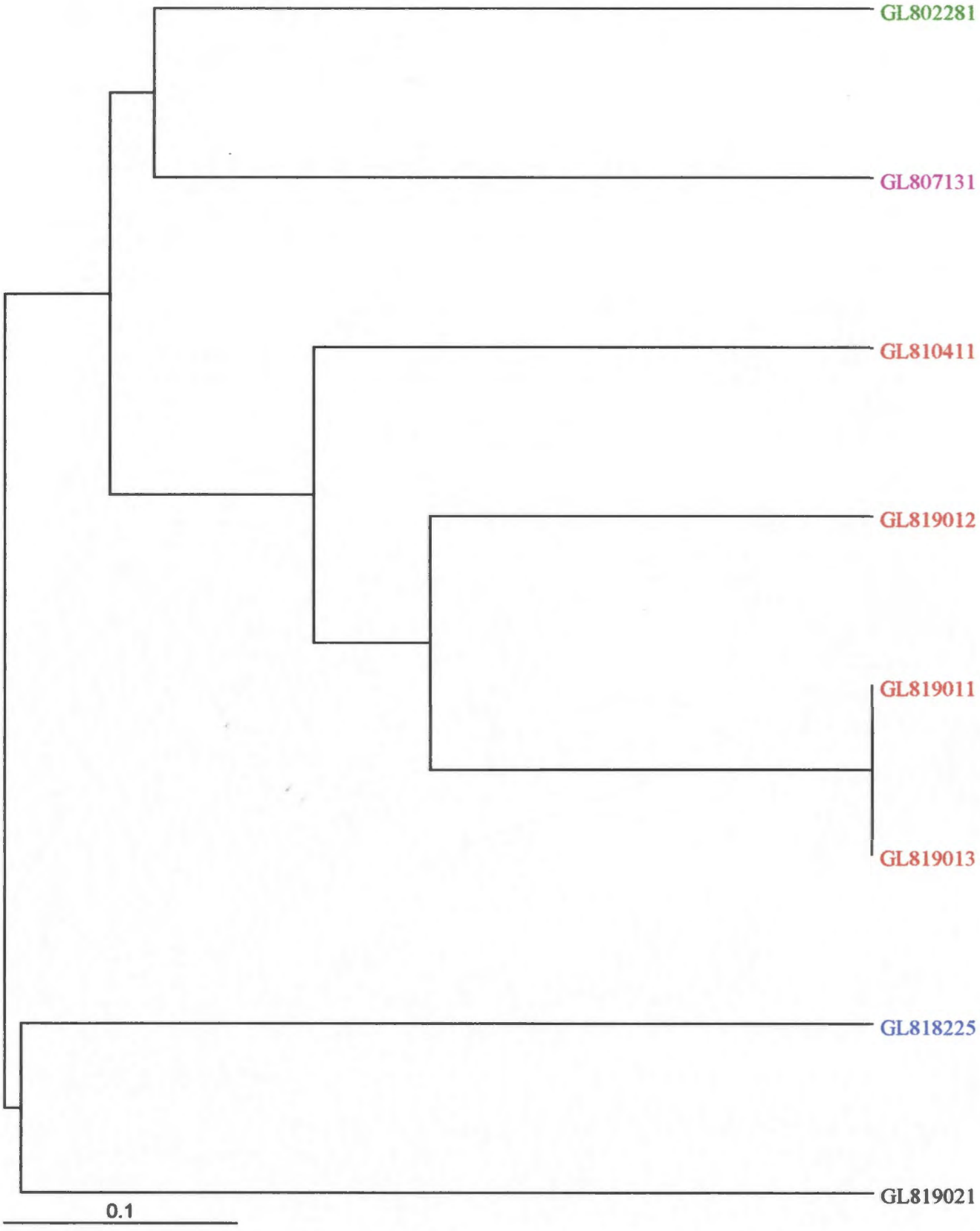
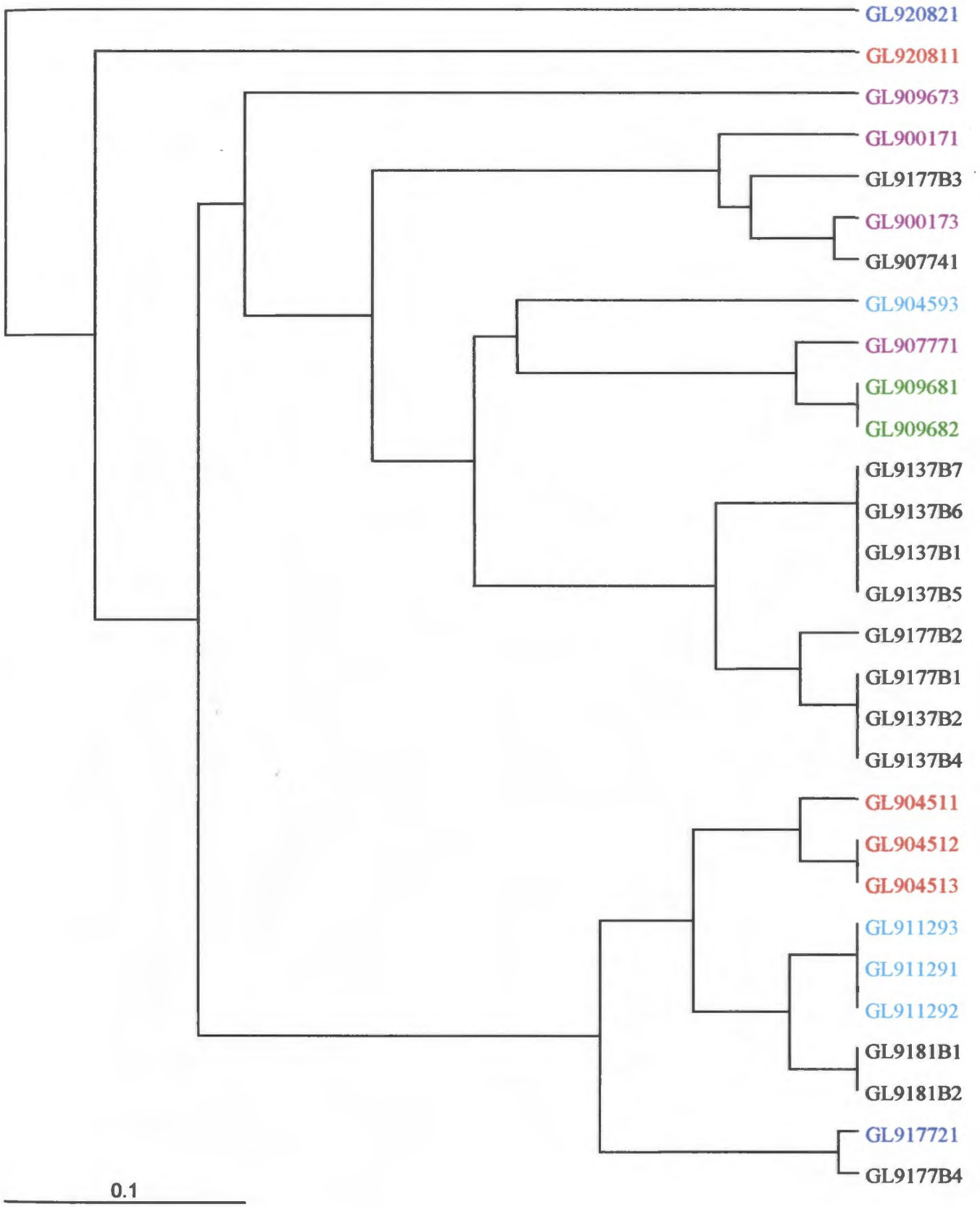

GL802281
GL807131
GL810411
GL819012
GL819011
GL819013
GL818225
GL819021
0.1

Figure 19: Phylogram showing the similarity among ectomycorrhizal fungi collected from plot 9 (control). Each sample has been color coded according to a crude morphotyping. Morphotype is coded as the second last number of the sample name. See table 1a for morphotype descriptions.


GL920821
GL920811
GL909673
GL900171
GL9177B3
GL900173
GL907741
GL904593
GL907771
GL909681
GL909682
GL9137B7
GL9137B6
GL9137B1
GL9137B5
GL9177B2
GL9177B1
GL9137B2
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GL904513
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GL911291
GL911292
GL9181B1
GL9181B2
GL917721
GL9177B4
0.1

Table 12 shows that each plot has quite a wide range with respect to the number of molecular "species" and individuals. Both the Shannon-Wiener and Simpson diversity indices were calculated using these data (Table 13). These two indices were calculated in order to determine whether the differences that were found were a result of differences in the data and not a factor of the index being used. Each index factors in all of the individuals within that sample, however, they each place a different value on the importance of unique species. The Simpson index puts less weight on those species represented by only one individual as compared to that of the Shannon-Wiener index. Therefore the Simpson index places a greater emphasis on species dominance, while the Shannon-Wiener index emphasizes species richness. See appendix IV for comparison of the two indices.

It is assumed that diversity indices are normally distributed (Magurran, 1988), thus a one-way analysis of variance (ANOVA) was used to look at treatment relationships. My analysis looked at three possible treatment comparisons; Control verses Low intensity burns, Control verses High intensity burns, and Low verses High intensity burns. All comparisons, using both indices, showed that there were no significant differences across any of the treatments. Calculated ANOVAs can be viewed in appendix X.

Table 12: Summary of the number of molecular species and individuals/species for each research plot. LB= low burn intensity; HB= high burn intensity; Control= unburned.

Plot	# of species	# of individuals
1 (LB)	33	42
2 (HB)	33	42
3 (Control)	26	43
4 (HB)	24	35
5 (LB)	22	26
6 (Control)	20	27
7 (LB)	19	23
8 (HB)	7	8
9 (Control)	19	29

Table 13: Calculated Shannon-Wiener and Simpson indices for each treatment.

Treatment	Plot	Shannon index (H')	Simpson index (1-D)
Control	**3**	3.1263	0.9725
	6	2.9161	0.9775
	9	2.8065	0.9629
Low Burn	**1**	3.4073	0.9858
	5	3.0264	0.9846
	7	2.8728	0.9801
High Burn	**2**	3.4155	0.987
	4	3.0667	0.9747
	8	1.906	0.9643

Table 14 shows the F statistic taken from each calculated ANOVA. According to my analysis the overall molecular diversity, of ectomycorrhizal fungi, is not significantly changed when one burns a clear-cut using either a high or low intensity burning protocol.

Table 14: F statistics for each ANOVA comparison using both the Shannon-Wiener and Simpson indices. The expected F value was $F_{0.05[1,4]}= 7.71$. Since all values are less than the expected F value then there is no significant difference between treatments.

ANOVA comparisons	Shannon-Wiener	Simpson
Control vs Low	0.08	7.35
Control vs High	0.11	0.31
Low vs High	0.00	1.45

Upon viewing individual phylograms I found that some of my crude morphotypes formed strong molecular groupings while others seemed to hold no structure whatsoever. Across all nine plots it was found that types 1 (*C. geophilum*), 3, 6, and 7 tended to form strong molecular groups. In each case there were individuals that strayed from the groups and were dispersed throughout the phylograms. Morphotypes B (plot 9) and 9 (plots 6 and 9) also clustered into groups. These six ectomycorrhizal morphotypes all represented fungi that had very distinct and thick mantles. It was found that morphotypes which displayed very thin and non-continuous mantles tended to produce molecular patterns that did not form nicely clustering groups similarly to those previously mentioned. Mainly I am speaking of morphotypes 2 and 8. In most phylograms it was seen that they were randomly scattered and only forming clusters if they happened to have been sampled from the same seedling (forming a genet).

Another area of concern was to determine whether or not there was any sharing of species across similar treatments as well as across different treatments within the same block. Figures 20, 21 and 22 show phylograms, each constructed from three plots, for each treatment type. Comparisons within repeat treatments showed that there was

minimal sharing of species. Within the three control plots only two species were shared, and even then they were not shared across all three plots. One species was shared between plots 6 and 9, the other was shared between plots 3 and 6 (figure 20). The low burn plots showed that two species were shared between plots 1 and 5, and none with plot 7 (figure 21). The high burn plots had only one species from plots 2 and 4 that matched band patterns (figure 22). The low number of ECM samples for plot 8 may explain why there were no matches (figure 18).

Figure 20: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across the three control treatments (plots 3, 6 and 9). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse grouping.

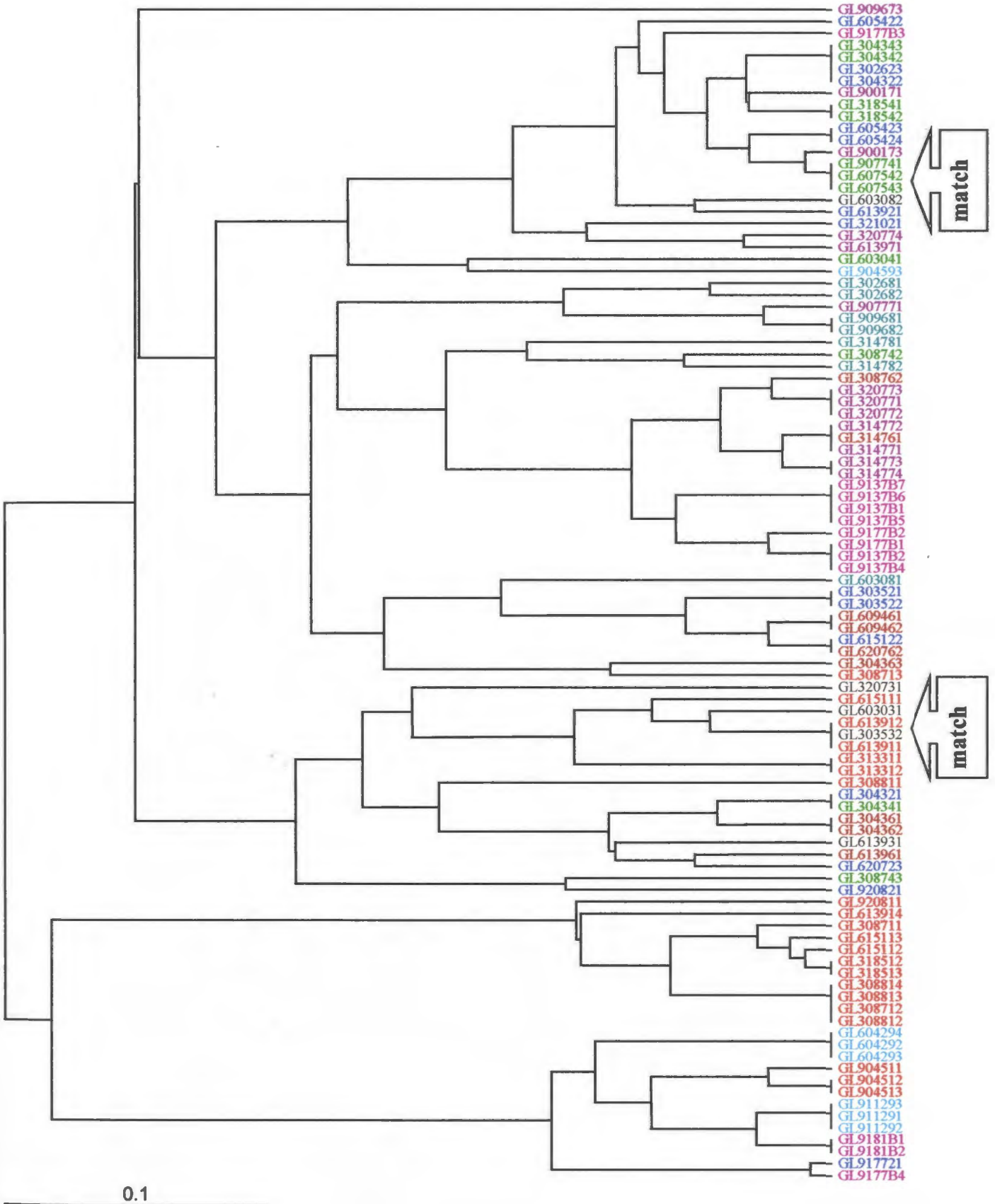

GL909673
GL605422
GL9177B3
GL304343
GL304342
GL302623
GL304322
GL900171
GL318541
GL318542
GL605423
GL605424
GL900173
GL907741
GL607542
GL607543
GL603082
GL613921
GL321021
GL320774
GL613971
GL603041
GL904593
GL302681
GL302682
GL907771
GL909681
GL909682
GL314781
GL308742
GL314782
GL308762
GL320773
GL320771
GL320772
GL314772
GL314761
GL314771
GL314773
GL314774
GL9137B7
GL9137B6
GL9137B1
GL9137B5
GL9177B2
GL9177B1
GL9137B2
GL9137B4
GL603081
GL303521
GL303522
GL609461
GL609462
GL615122
GL620762
GL304363
GL308713
GL320731
GL615111
GL603031
GL613912
GL303532
GL613911
GL313311
GL313312
GL308811
GL304321
GL304341
GL304361
GL304362
GL613931
GL613961
GL620723
GL308743
GL920821
GL920811
GL613914
GL308711
GL615113
GL615112
GL318512
GL318513
GL308814
GL308813
GL308712
GL308812
GL604294
GL604292
GL604293
GL904511
GL904512
GL904513
GL911293
GL911291
GL911292
GL9181B1
GL9181B2
GL917721
GL9177B4
match
match
0.1

Figure 21: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across the three low burn treatments (plots 1, 5 and 7). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse grouping.

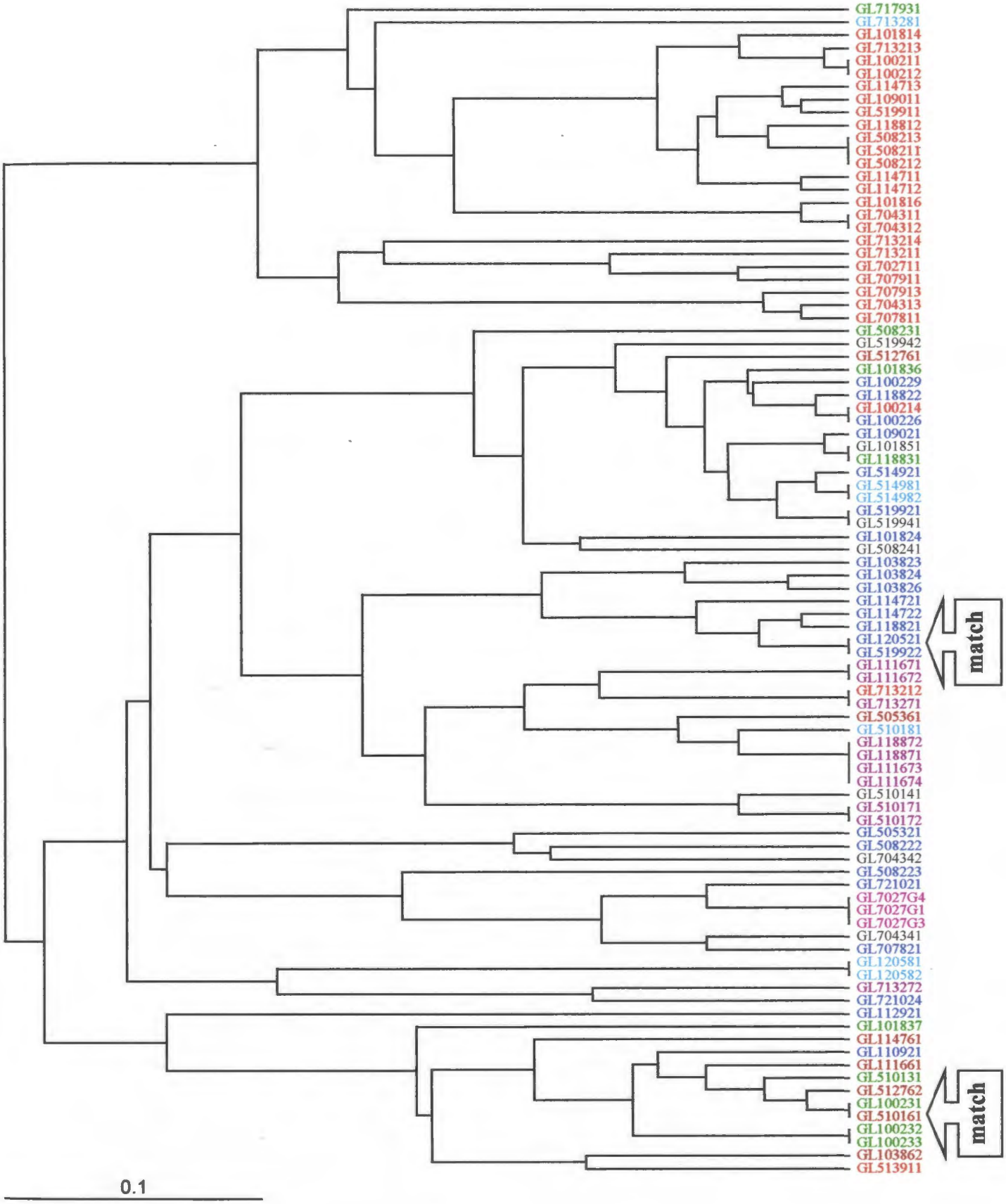
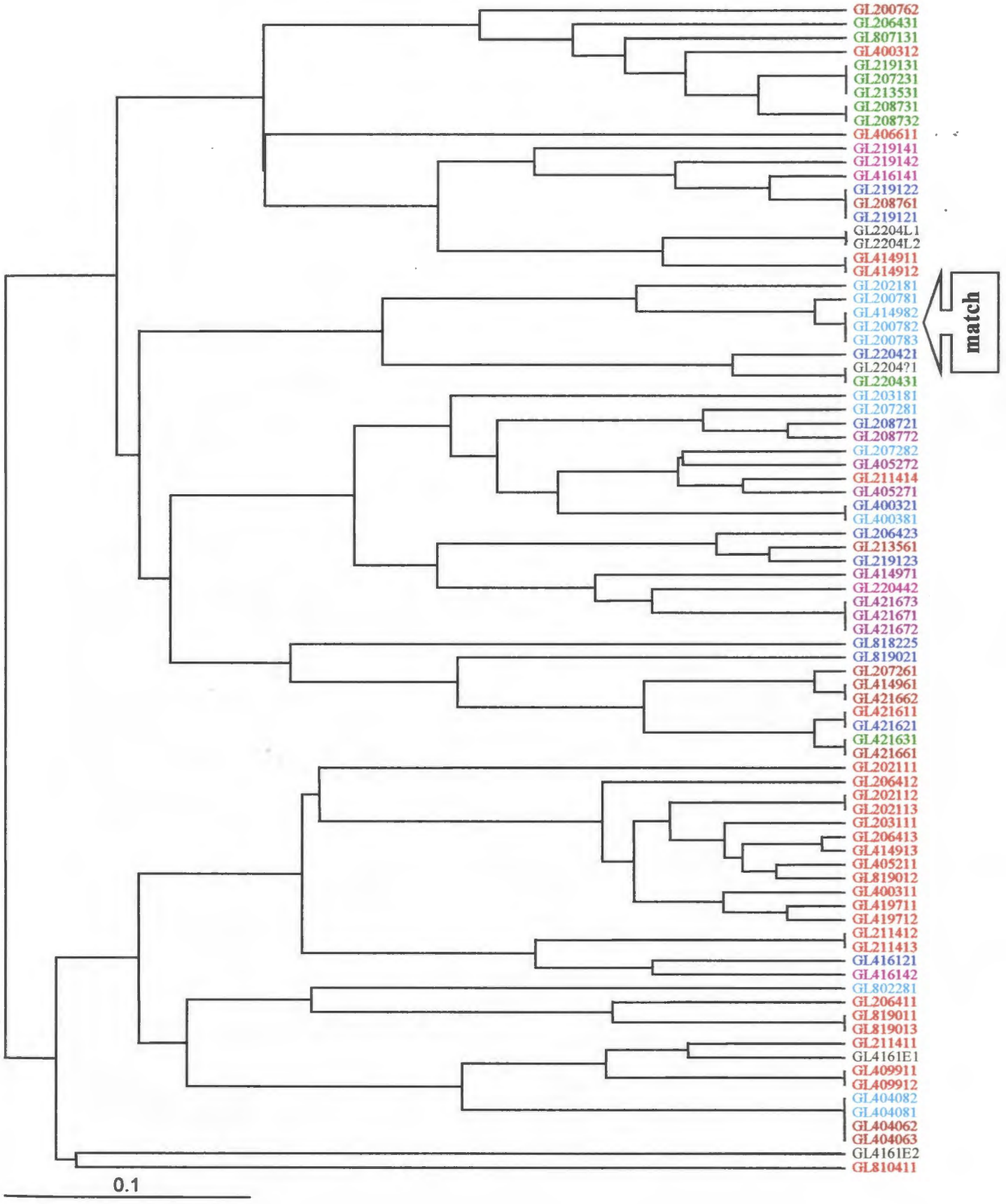

GL717931
GL713281
GL101814
GL713213
GL100211
GL100212
GL114713
GL109011
GL519911
GL118812
GL508213
GL508211
GL508212
GL114711
GL114712
GL101816
GL704311
GL704312
GL713214
GL713211
GL702711
GL707911
GL707913
GL704313
GL707811
GL508231
GL519942
GL512761
GL101836
GL100229
GL118822
GL100214
GL100226
GL109021
GL101851
GL118831
GL514921
GL514981
GL514982
GL519921
GL519941
GL101824
GL508241
GL103823
GL103824
GL103826
GL114721
GL114722
GL118821
GL120521
GL519922
GL111671
GL111672
GL713212
GL713271
GL505361
GL510181
GL118872
GL118871
GL111673
GL111674
GL510141
GL510171
GL510172
GL505321
GL508222
GL704342
GL508223
GL721021
GL7027G4
GL7027G1
GL7027G3
GL704341
GL707821
GL120581
GL120582
GL713272
GL721024
GL112921
GL101837
GL114761
GL110921
GL111661
GL510131
GL512762
GL100231
GL510161
GL100232
GL100233
GL103862
GL513911
match
match
0.1

Figure 22: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across the three high burn treatments (plots 2, 4 and 8). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse grouping.


GL200762
GL206431
GL807131
GL400312
GL219131
GL207231
GL213531
GL208731
GL208732
GL406611
GL219141
GL219142
GL416141
GL219122
GL208761
GL219121
GL2204L1
GL2204L2
GL414911
GL414912
GL202181
GL200781
GL414982
GL200782
GL200783
GL220421
GL2204?1
GL220431
GL203181
GL207281
GL208721
GL208772
GL207282
GL405272
GL211414
GL405271
GL400321
GL400381
GL206423
GL213561
GL219123
GL414971
GL220442
GL421673
GL421671
GL421672
GL818225
GL819021
GL207261
GL414961
GL421662
GL421611
GL421621
GL421631
GL421661
GL202111
GL206412
GL202112
GL202113
GL203111
GL206413
GL414913
GL405211
GL819012
GL400311
GL419711
GL419712
GL211412
GL211413
GL416121
GL416142
GL802281
GL206411
GL819011
GL819013
GL211411
GL4161E1
GL409911
GL409912
GL404082
GL404081
GL404062
GL404063
GL4161E2
GL810411
match
0.1

Previously I had looked at the clustering of individual morphotypes within each of the nine plots. When I merged the data from each treatment (three plots/treatment) it was found that some of the morphotypes/molecular groups held together very strongly, namely types 1, 7, 9, and B. Thus suggesting morphotype variants were shared across all treatment plots. Types 3 and 6 broke down into smaller, yet distinct clusters. Suggesting that each type may contain two or three distinct molecular groups which exist across all plots. Those types that had thin, non-continuous mantles (types 2 and 8) showed no consistent groupings across all plots.

Comparisons were also made between different treatments, arranged according to the blocks each plot fell into (see figure 2, Section 2.1). It was seen that those morphotypes/ molecular groups that had previously shown strong cohesion began to break apart. For example, within blocks 2 and 3 (figures 24 and 25, respectively) it was seen that type 1s formed a much looser association than previously viewed. Block 1 is an exception. It was noticed that most of the type 1 samples still clustered relatively tightly (figure 23).

There was minimal sharing of molecular species between different treatments. There were no cases in which one species was found in all three plots. Within block 1 there were only three matches (figure 23). Two were shared between plots 1 and 2 (low and high burns, respectively), and one with plots 2 and 3 (high burn and control, respectively). Block 2 had only one match between plots 5 and 6 (low burn and control, respectively) (figure 24). Block 3 did not have any sharing of species between different treatments (figure 25).

Figure 23: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across block 1 (containing plots 1, 2 and 3). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse grouping.

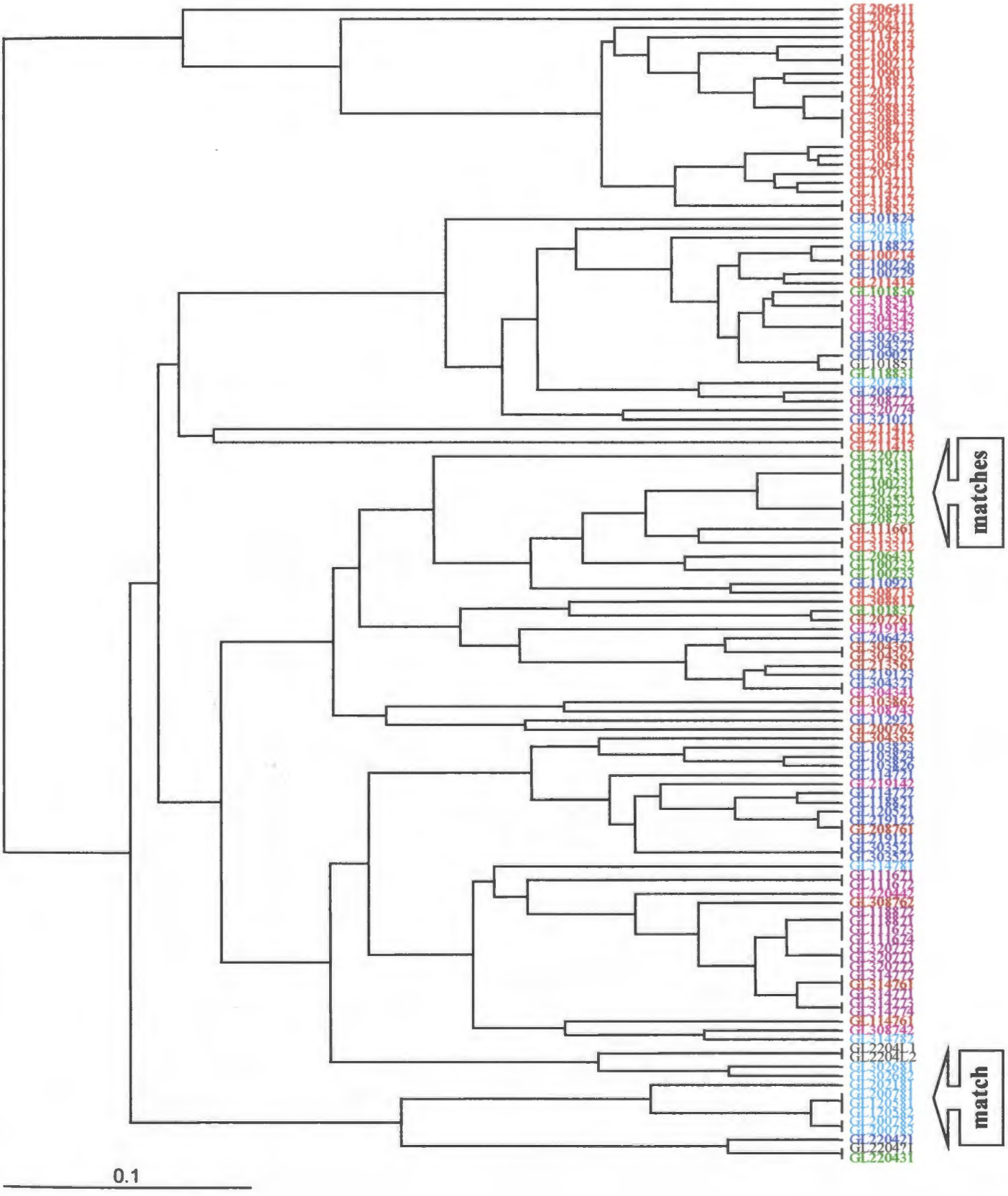

matches
match
0.1

Figure 24: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across block 2 (containing plots 4, 5 and 6). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse grouping.

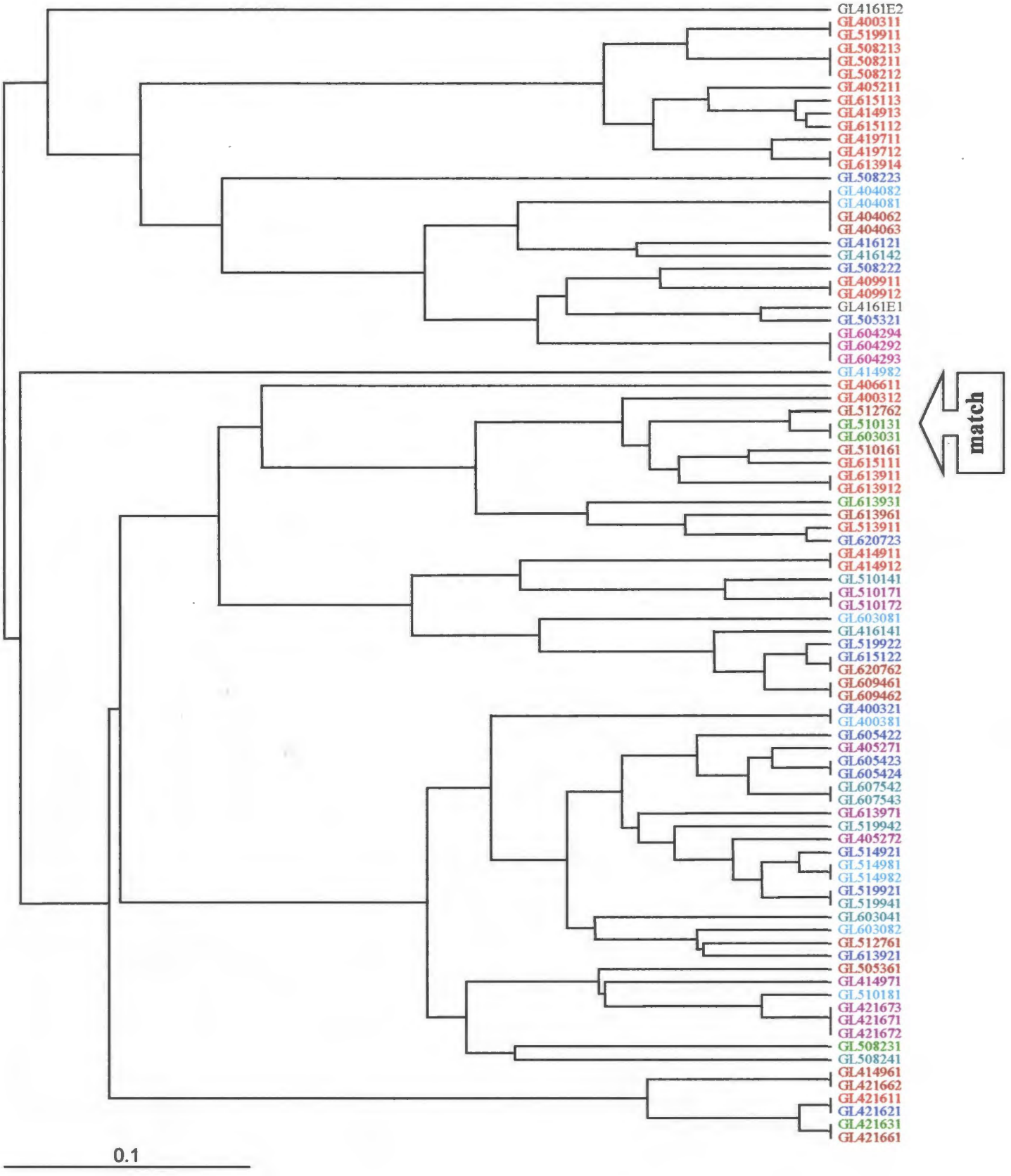
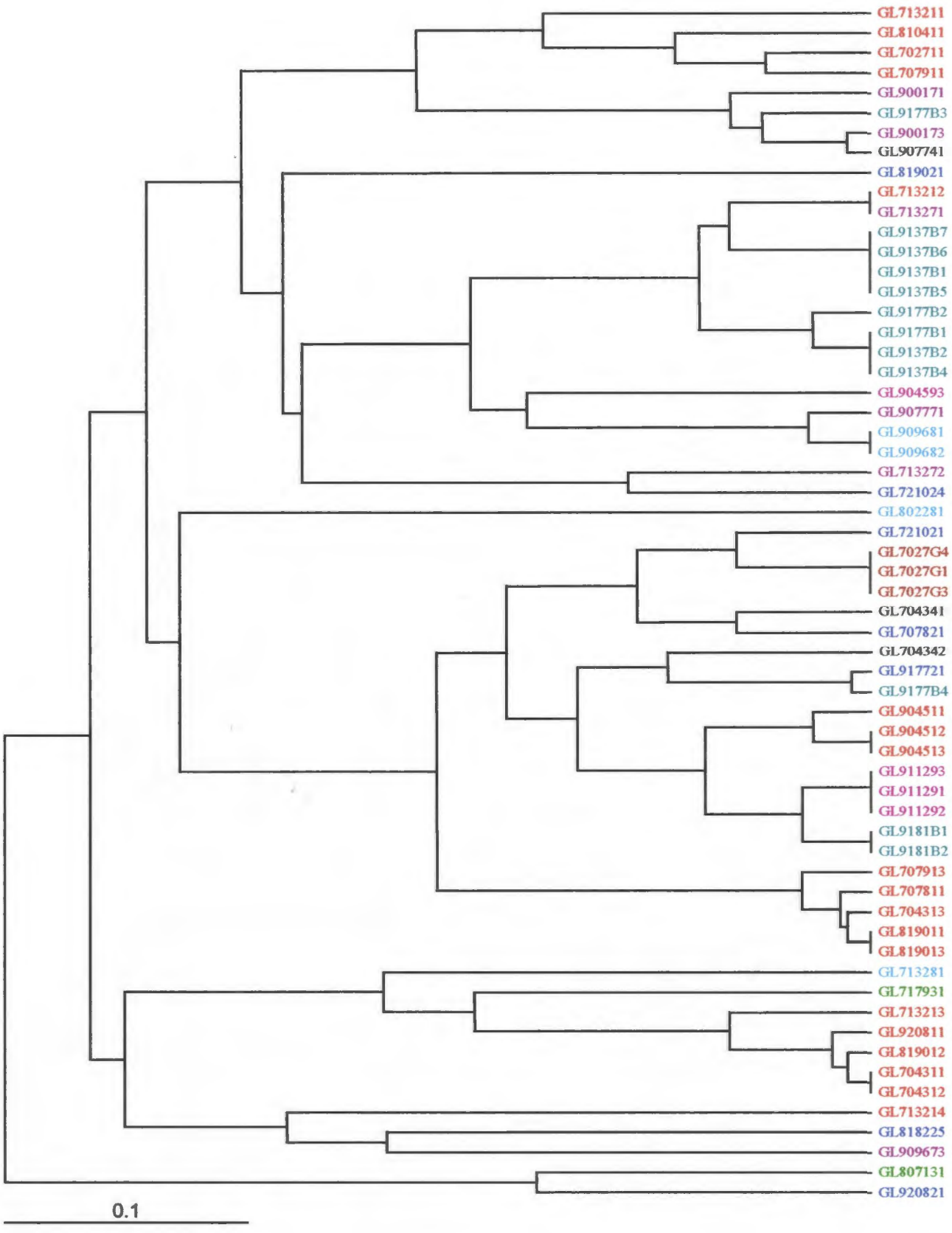

GL4161E2
GL400311
GL519911
GL508213
GL508211
GL508212
GL405211
GL615113
GL414913
GL615112
GL419711
GL419712
GL613914
GL508223
GL404082
GL404081
GL404062
GL404063
GL416121
GL416142
GL508222
GL409911
GL409912
GL4161E1
GL505321
GL604294
GL604292
GL604293
GL414982
GL406611
GL400312
GL512762
GL510131
GL603031
GL510161
GL615111
GL613911
GL613912
GL613931
GL613961
GL513911
GL620723
GL414911
GL414912
GL510141
GL510171
GL510172
GL603081
GL416141
GL519922
GL615122
GL620762
GL609461
GL609462
GL400321
GL400381
GL605422
GL405271
GL605423
GL605424
GL607542
GL607543
GL613971
GL519942
GL405272
GL514921
GL514981
GL514982
GL519921
GL519941
GL603041
GL603082
GL512761
GL613921
GL505361
GL414971
GL510181
GL421673
GL421671
GL421672
GL508231
GL508241
GL414961
GL421662
GL421611
GL421621
GL421631
GL421661
match
0.1

Figure 25: Phylogram showing the molecular diversity of ectomycorrhizal fungi found across block 3 (containing plots 7, 8 and 9). "match" refers to the matching of two or more RFLP genotypes that have originated from different replication plots. As can be seen the distances are very large indicating a very diverse.


GL713211
GL810411
GL702711
GL707911
GL900171
GL9177B3
GL900173
GL907741
GL819021
GL713212
GL713271
GL9137B7
GL9137B6
GL9137B1
GL9137B5
GL9177B2
GL9177B1
GL9137B2
GL9137B4
GL904593
GL907771
GL909681
GL909682
GL713272
GL721024
GL802281
GL721021
GL7027G4
GL7027G1
GL7027G3
GL704341
GL707821
GL704342
GL917721
GL9177B4
GL904511
GL904512
GL904513
GL911293
GL911291
GL911292
GL9181B1
GL9181B2
GL707913
GL707811
GL704313
GL819011
GL819013
GL713281
GL717931
GL713213
GL920811
GL819012
GL704311
GL704312
GL713214
GL818225
GL909673
GL807131
GL920821
0.1

3.2.4: Diversity of Presumed Endophytic Fungi

As mentioned earlier the RFLP data obtained from the 'non-mycorrhizal' (NM) root tips were kept separate from the ectomycorrhizal (ECM) root tip data. Initially when both NM and ECM data were combined it was found that some of the ECM tips fell in with the NM tips, suggesting that there was no amplification of the ECM fungus. Instead non-target DNA was amplified by the PCR reaction. Figures 26 to 34 show the relationships, using UPCMA clustering, found between the non-target amplifications (presumably root endophytes) within each study plot.

Figure 26: Phylogram showing the similarity among non-target DNAs, discovered in plot 1 (low burn).

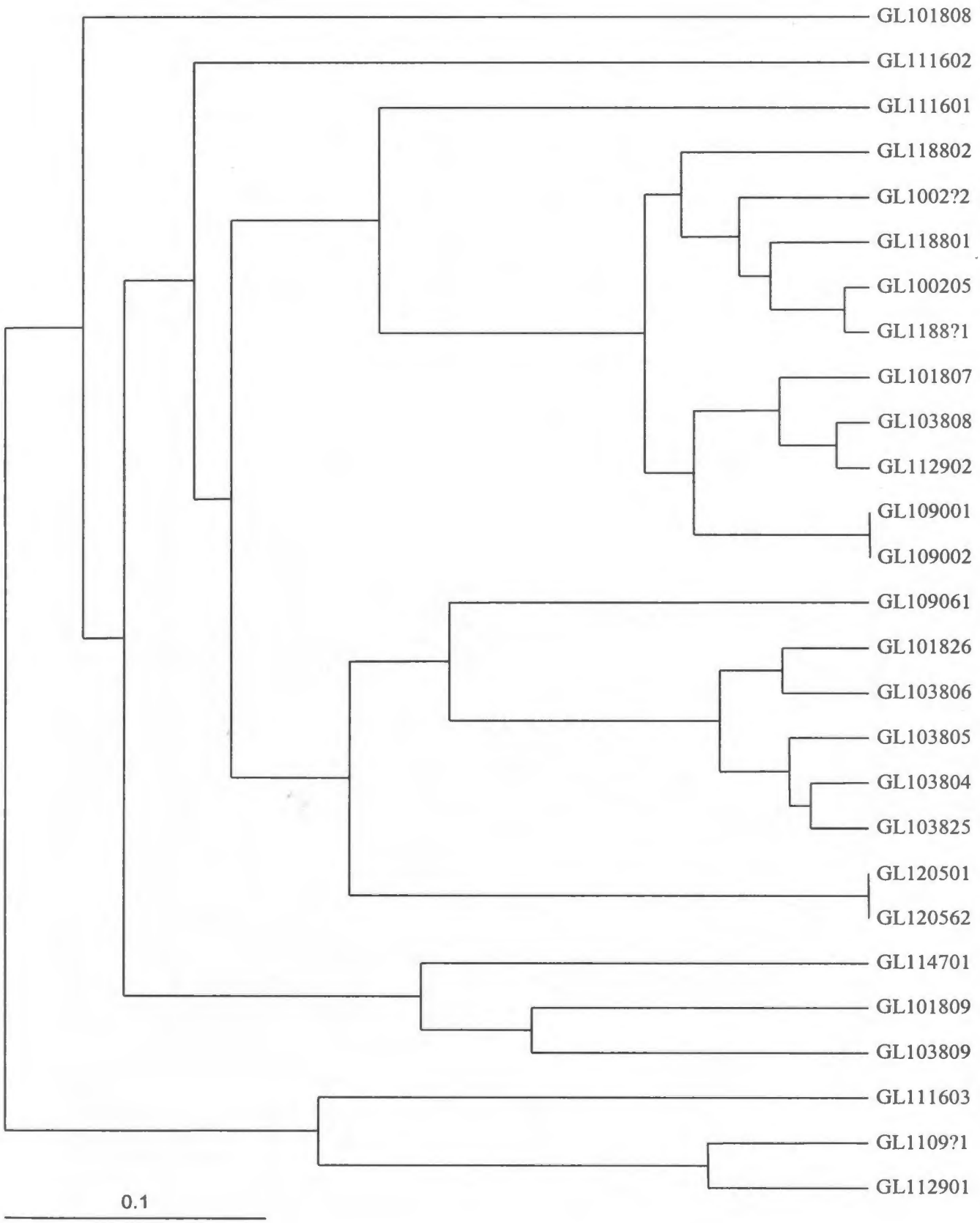

GL101808
GL111602
GL111601
GL118802
GL1002?2
GL118801
GL100205
GL1188?1
GL101807
GL103808
GL112902
GL109001
GL109002
GL109061
GL101826
GL103806
GL103805
GL103804
GL103825
GL120501
GL120562
GL114701
GL101809
GL103809
GL111603
GL1109?1
GL112901
0.1

Figure 27: Phylogram showing the similarity among non-target DNAs, discovered in plot 2 (high burn).

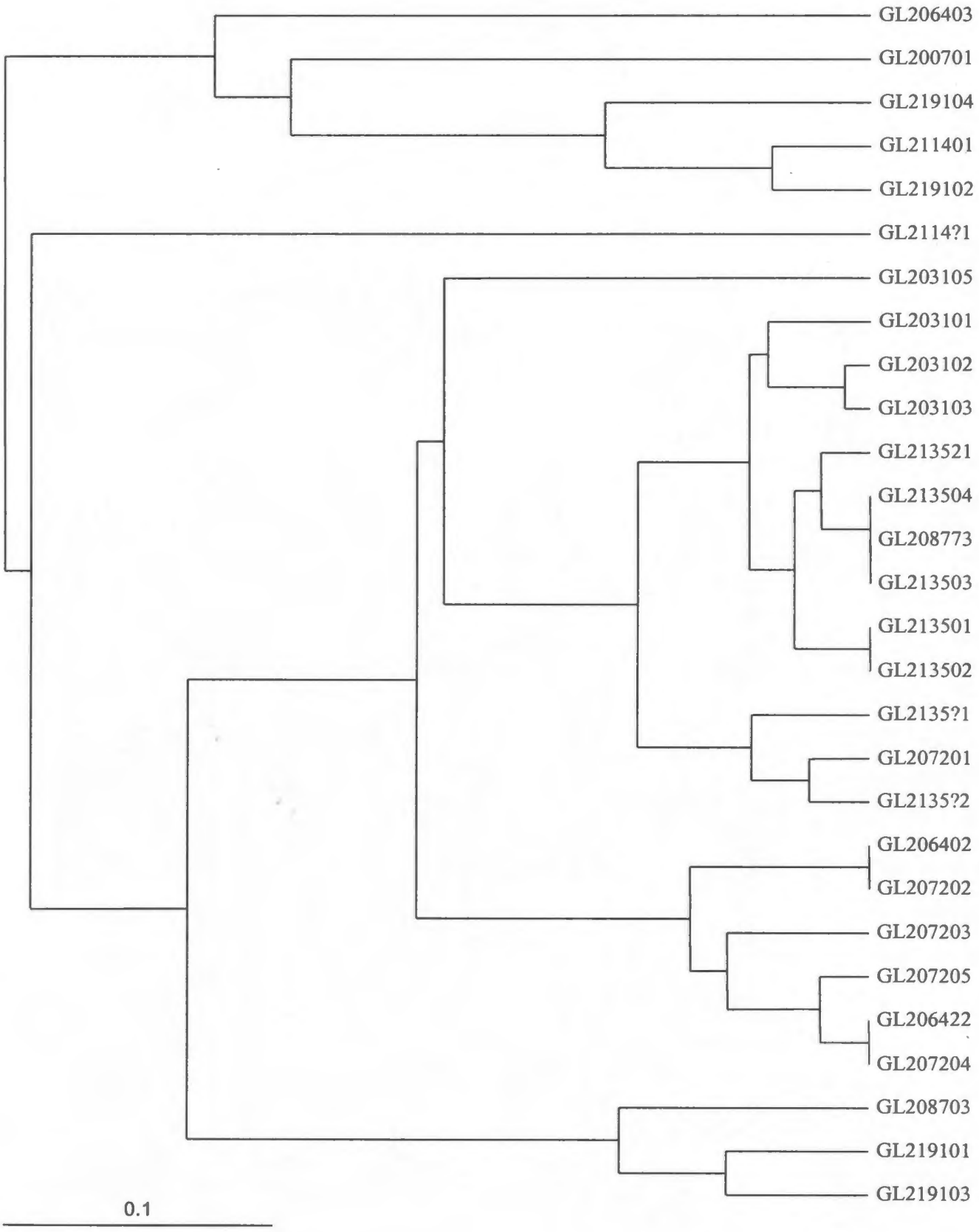

GL206403
GL200701
GL219104
GL211401
GL219102
GL2114?1
GL203105
GL203101
GL203102
GL203103
GL213521
GL213504
GL208773
GL213503
GL213501
GL213502
GL2135?1
GL207201
GL2135?2
GL206402
GL207202
GL207203
GL207205
GL206422
GL207204
GL208703
GL219101
GL219103
0.1

Figure 28: Phylogram showing the similarity among non-target DNAs, discovered in plot 3 (control).

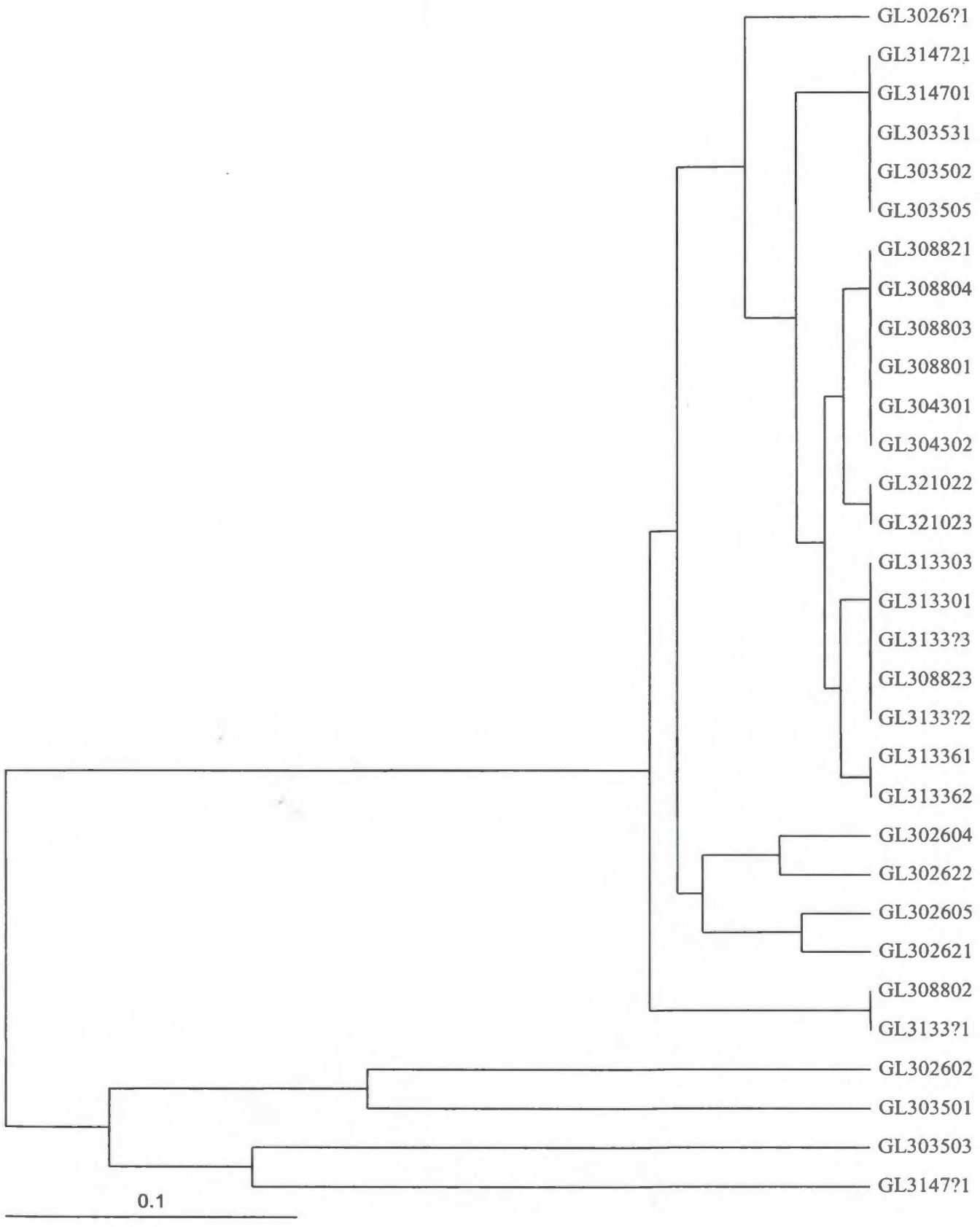

GL3026?1
GL314721
GL314701
GL303531
GL303502
GL303505
GL308821
GL308804
GL308803
GL308801
GL304301
GL304302
GL321022
GL321023
GL313303
GL313301
GL3133?3
GL308823
GL3133?2
GL313361
GL313362
GL302604
GL302622
GL302605
GL302621
GL308802
GL3133?1
GL302602
GL303501
GL303503
GL3147?1
0.1

Figure 29: Phylogram showing the similarity among non-target DNAs, discovered in plot 4 (high burn).

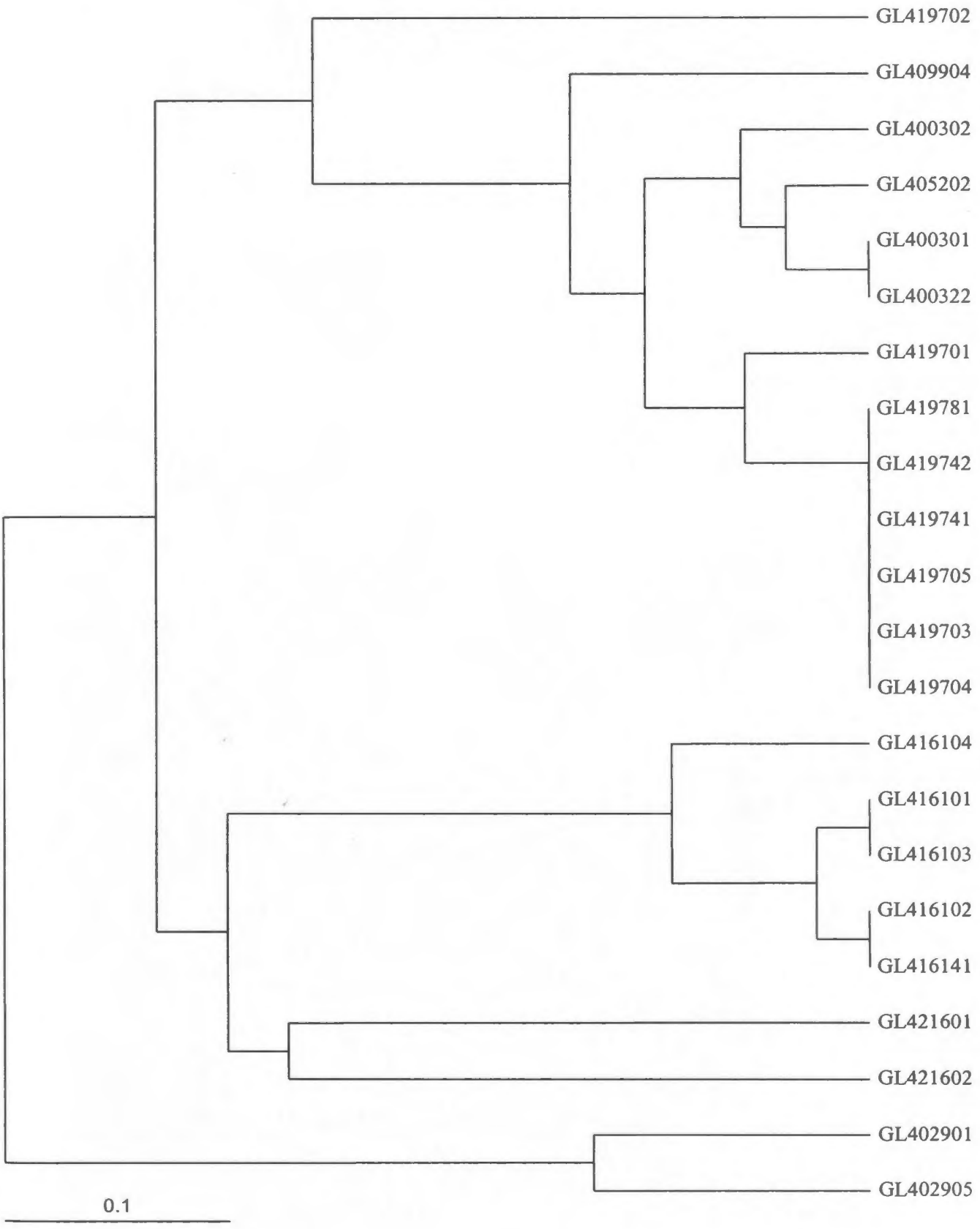

GL419702
GL409904
GL400302
GL405202
GL400301
GL400322
GL419701
GL419781
GL419742
GL419741
GL419705
GL419703
GL419704
GL416104
GL416101
GL416103
GL416102
GL416141
GL421601
GL421602
GL402901
GL402905
0.1

Figure 30: Phylogram showing the similarity among non-target DNAs, discovered in plot 5 (low burn).

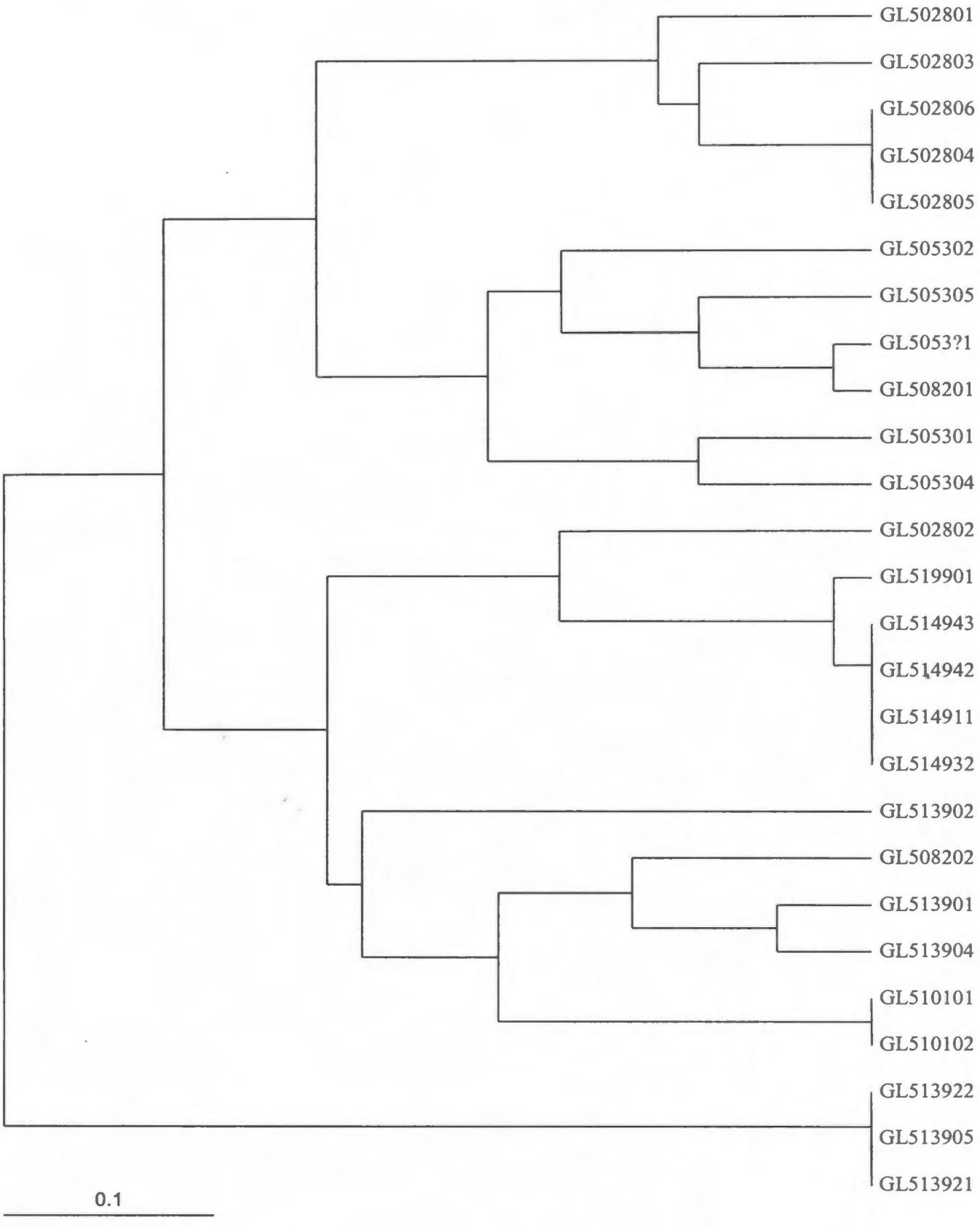

GL502801
GL502803
GL502806
GL502804
GL502805
GL505302
GL505305
GL5053?1
GL508201
GL505301
GL505304
GL502802
GL519901
GL514943
GL514942
GL514911
GL514932
GL513902
GL508202
GL513901
GL513904
GL510101
GL510102
GL513922
GL513905
GL513921
0.1

Figure 31: Phylogram showing the similarity among non-target DNAs, discovered in plot 6 (control). **Genotype I** is an example of a "genet", i.e. an example where a single RFLP genotype appears to have dominated the roots of a single seedling.

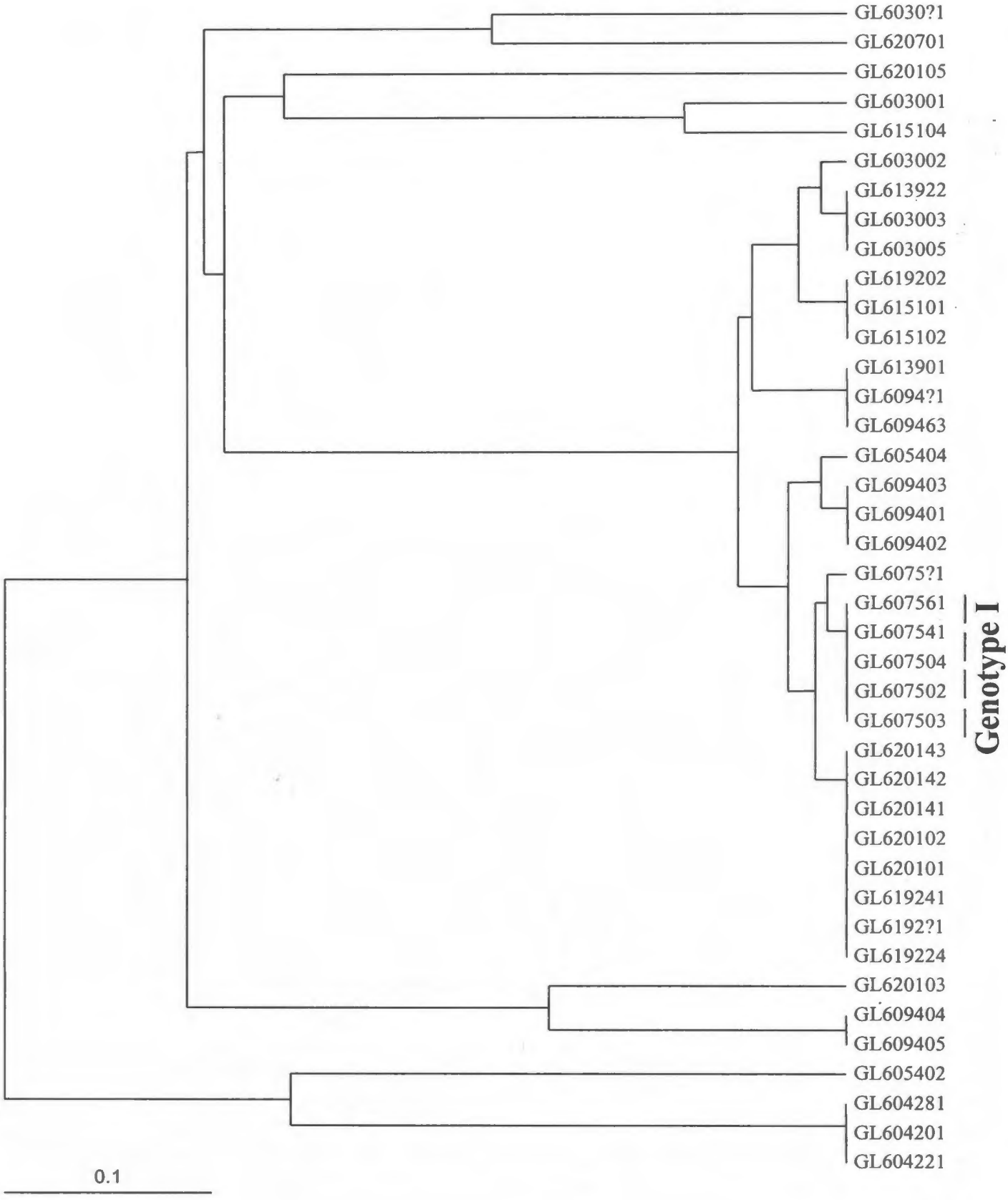

GL6030?1
GL620701
GL620105
GL603001
GL615104
GL603002
GL613922
GL603003
GL603005
GL619202
GL615101
GL615102
GL613901
GL6094?1
GL609463
GL605404
GL609403
GL609401
GL609402
GL6075?1
GL607561
GL607541
GL607504
GL607502
GL607503
GL620143
GL620142
GL620141
GL620102
GL620101
GL619241
GL6192?1
GL619224
GL620103
GL609404
GL609405
GL605402
GL604281
GL604201
GL604221
Genotype I
0.1

Figure 32: Phylogram showing the similarity among non-target DNAs, discovered in plot 7 (low burn).

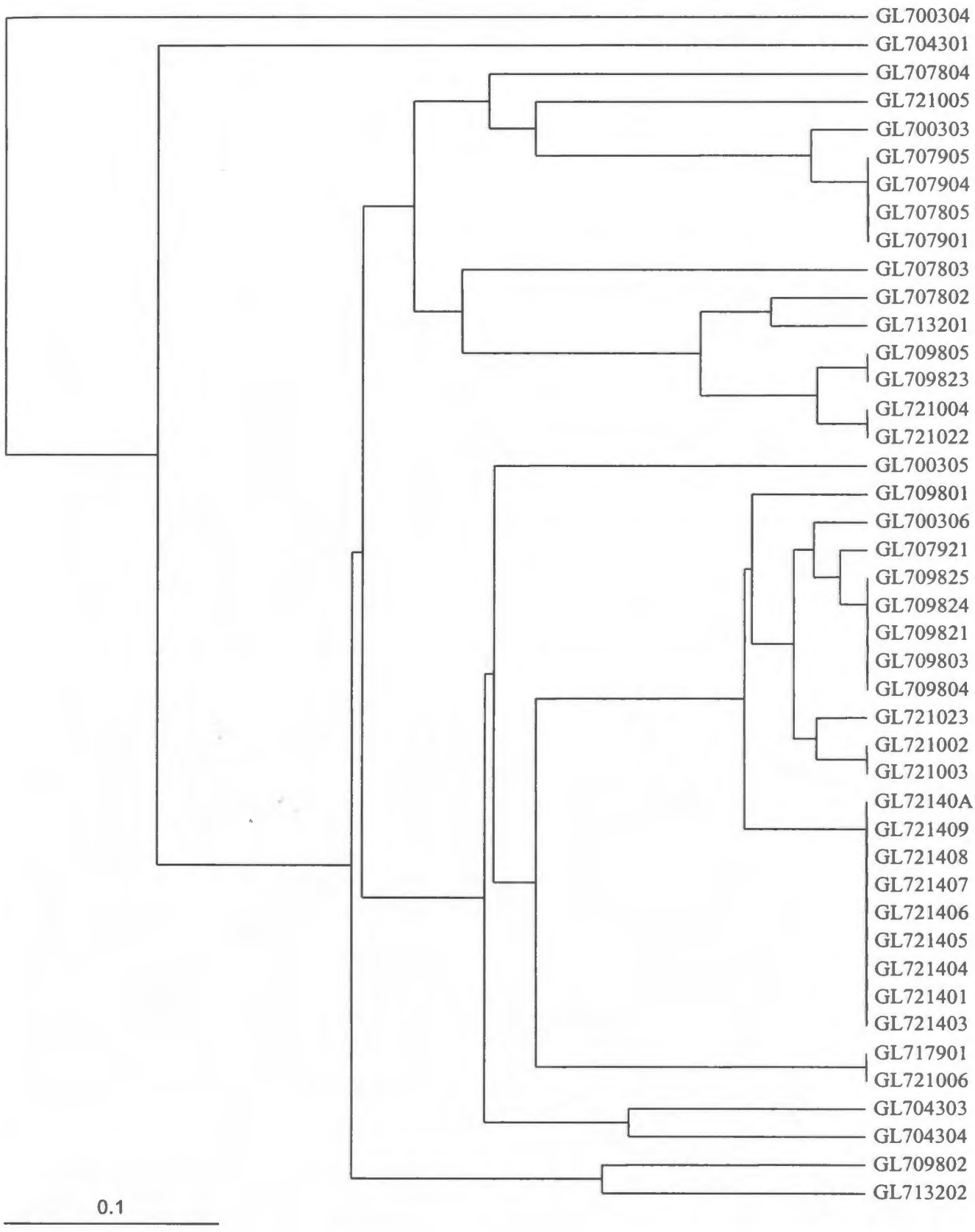

GL700304
GL704301
GL707804
GL721005
GL700303
GL707905
GL707904
GL707805
GL707901
GL707803
GL707802
GL713201
GL709805
GL709823
GL721004
GL721022
GL700305
GL709801
GL700306
GL707921
GL709825
GL709824
GL709821
GL709803
GL709804
GL721023
GL721002
GL721003
GL72140A
GL721409
GL721408
GL721407
GL721406
GL721405
GL721404
GL721401
GL721403
GL717901
GL721006
GL704303
GL704304
GL709802
GL713202
0.1

Figure 33: Phylogram showing the similarity among non-target DNAs, discovered in plot 8 (high burn).

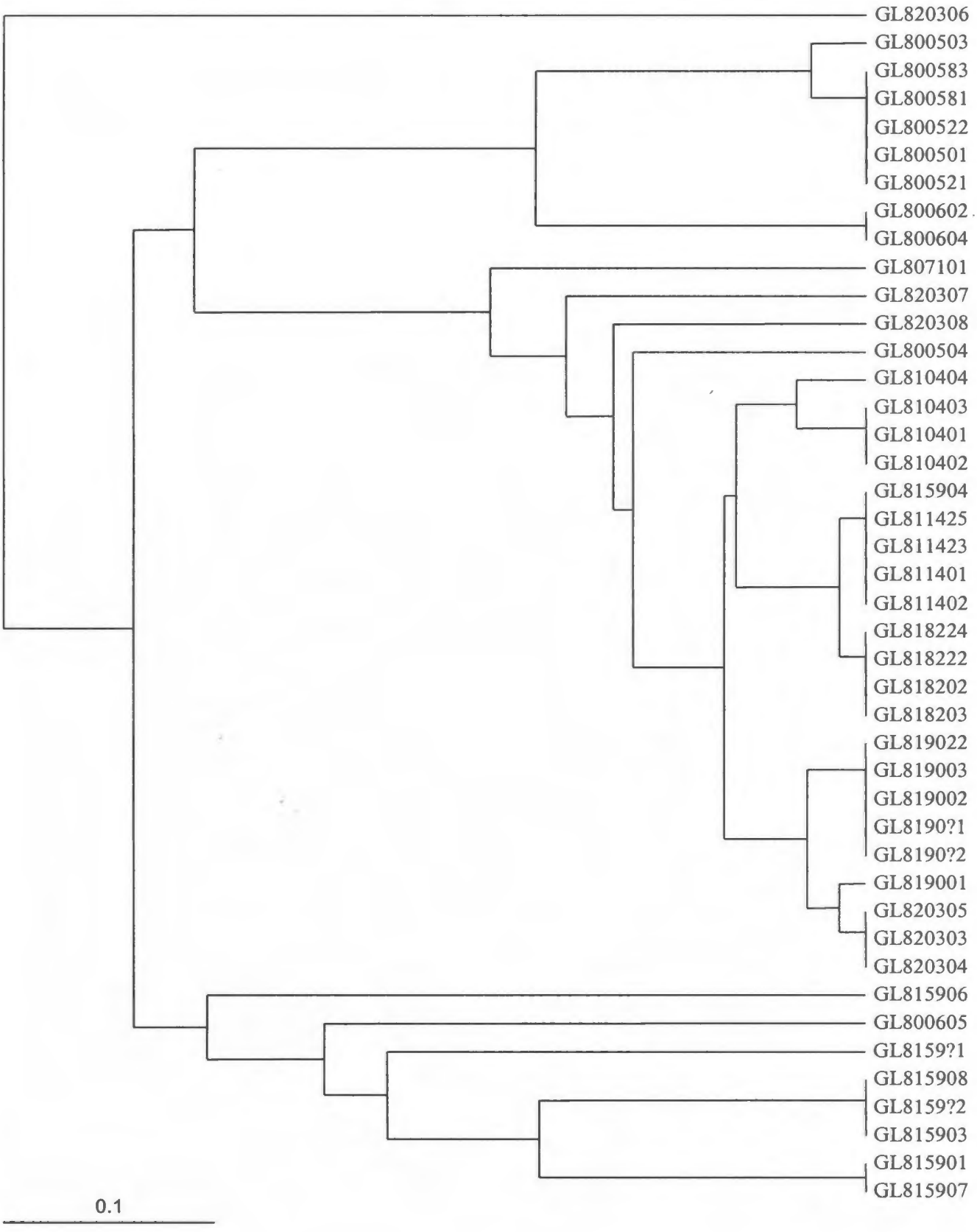
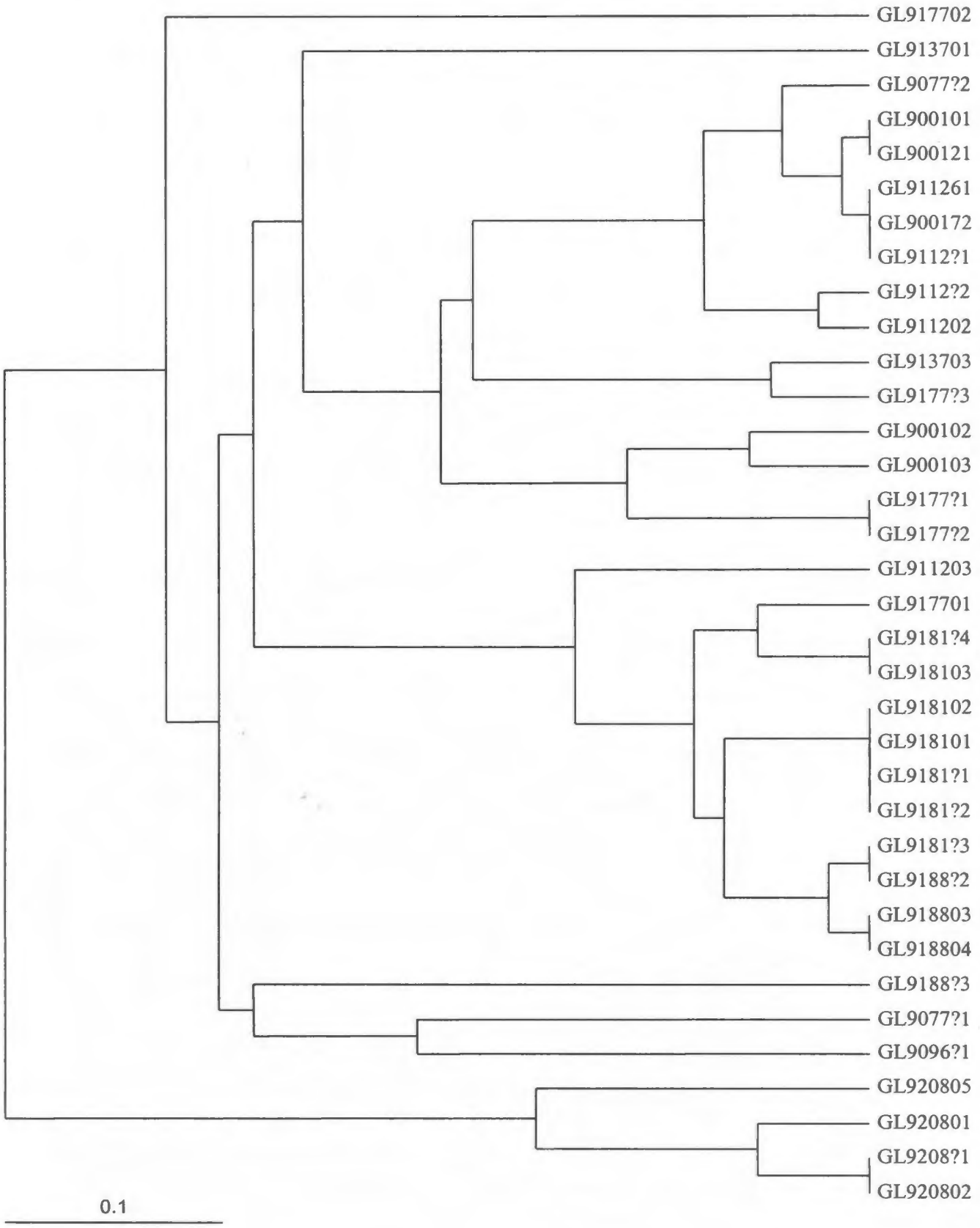

GL820306
GL800503
GL800583
GL800581
GL800522
GL800501
GL800521
GL800602
GL800604
GL807101
GL820307
GL820308
GL800504
GL810404
GL810403
GL810401
GL810402
GL815904
GL811425
GL811423
GL811401
GL811402
GL818224
GL818222
GL818202
GL818203
GL819022
GL819003
GL819002
GL8190?1
GL8190?2
GL819001
GL820305
GL820303
GL820304
GL815906
GL800605
GL8159?1
GL815908
GL8159?2
GL815903
GL815901
GL815907
0.1

Figure 34: Phylogram showing the similarity among non-target DNAs, discovered in plot 9 (control).


GL917702
GL913701
GL9077?2
GL900101
GL900121
GL911261
GL900172
GL9112?1
GL9112?2
GL911202
GL913703
GL9177?3
GL900102
GL900103
GL9177?1
GL9177?2
GL911203
GL917701
GL9181?4
GL918103
GL918102
GL918101
GL9181?1
GL9181?2
GL9181?3
GL9188?2
GL918803
GL918804
GL9188?3
GL9077?1
GL9096?1
GL920805
GL920801
GL9208?1
GL920802
0.1

A quick review of each phylogram shows that there is considerable diversity among endophytes within each plot. Distances in most cases are quite large, usually greater than 10%. However, a few plots do show some patterns. Control plots 3 and 6 reveal a lower number of unique RFLP genotypes. Instead there are more closely related clusters containing several representatives of an individual genotype. This, to a lesser degree, can also be seen for plots 7 (low burn) and 8 (high burn).

Frequently most of the individuals that form that cluster originate from the same tree. For example, within plot 6 I saw that 'genotype I' contains 5 individuals all originating from tree #75. The only other sample from tree #75 is situated within a 2% difference from the clade. This suggests that the entire tree could possibly be harboring only one RFLP genotype, or even genet of endophytic fungi, i.e. endophytic growth is clonal. Observation of other clusters and other plots shows that this type of genet organization to be a common occurrence. Thus within individual plots there tends to be a large degree of endophytic fungal diversity between trees, however, individual trees contain a very low degree of diversity. It would appear an individual tree usually contains only one or two dominant endophytic RFLP genotypes.

Comparisons were made across each of the treatments. Data from each of the replicate plots were combined for each treatments and analyzed for distinguishable patterns. Figures 35 to 36 show that the endophytic diversity is quite high for both the low and high burn treatments. Distances are quite large indicating that there is little sharing of genotypes across these two treatments. In fact there is only one example

where the same RFLP genotype is found across two replicate plots ("Genotype II", Figure 36). 'Genotype II' is found in plots 2 and 8.

Figure 37 illustrates the data for the three control plots. The most noticeable factor is the decrease in the distance between RFLP genotypes, in comparison to the previous two treatment phylograms. This indicates that the control plots contain genotypes that are more closely related than genotypes in the other two treatments. Another thing to note is that there is an increase in the number of endophytic genotypes shared between plots. At least four different genotypes were found to be shared amongst control plots. There was no case in which one genotype was shared across all three plots.

Figure 35: Phylogram showing the molecular diversity of endophytic fungi found across the three low burn treatments. As can be seen the distances are very large indicating a very diverse group.

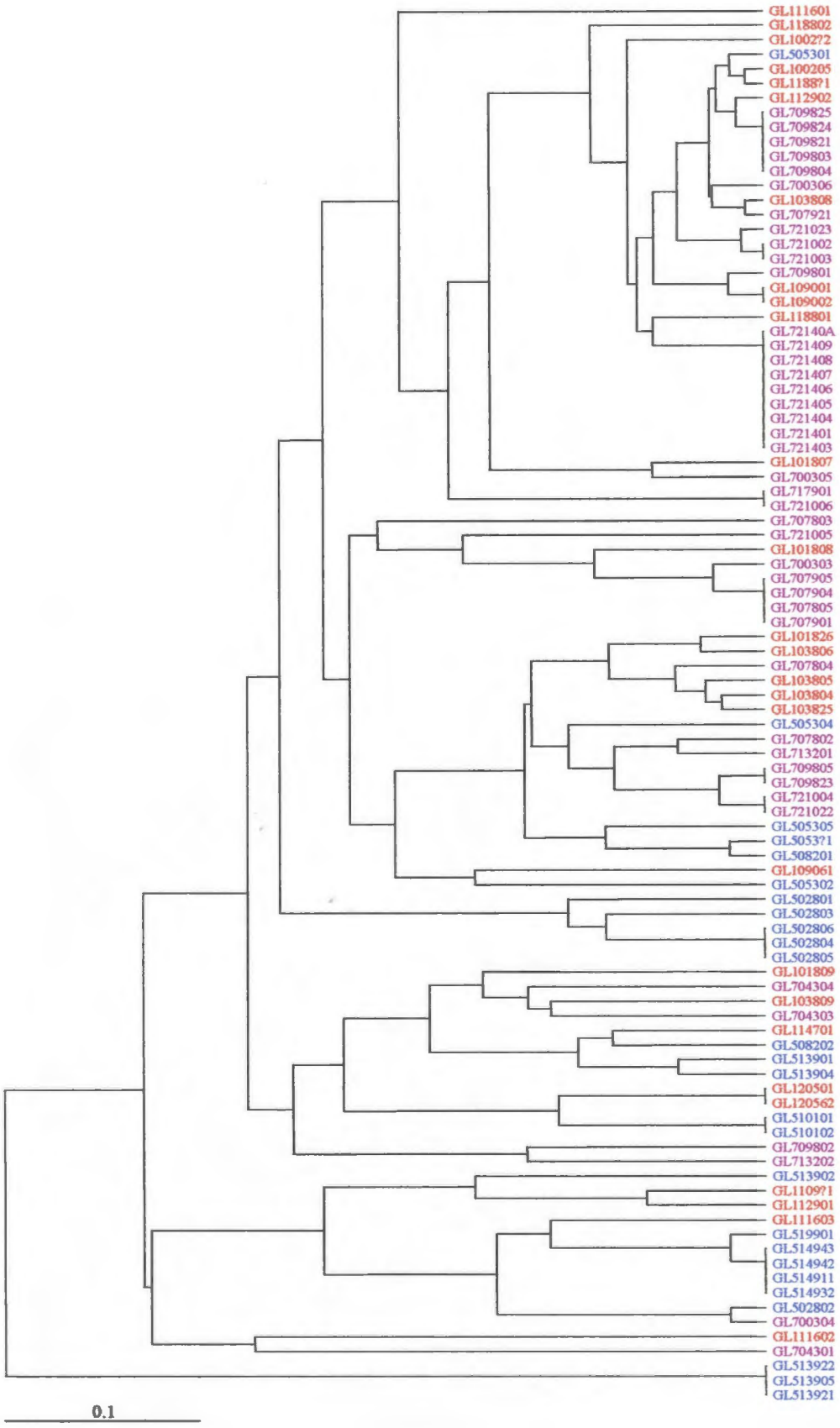

GL111601
GL118802
GL100272
GL505301
GL100205
GL118871
GL112902
GL709825
GL709824
GL709821
GL709803
GL709804
GL700306
GL103808
GL707921
GL721023
GL721002
GL721003
GL709801
GL109001
GL109002
GL118801
GL72140A
GL721409
GL721408
GL721407
GL721406
GL721405
GL721404
GL721401
GL721403
GL101807
GL700305
GL717901
GL721006
GL707803
GL721005
GL101808
GL700303
GL707905
GL707904
GL707805
GL707901
GL101826
GL103806
GL707804
GL103805
GL103804
GL103825
GL505304
GL707802
GL713201
GL709805
GL709823
GL721004
GL721022
GL505305
GL505371
GL508201
GL109061
GL505302
GL502801
GL502803
GL502806
GL502804
GL502805
GL101809
GL704304
GL103809
GL704303
GL114701
GL508202
GL513901
GL513904
GL120501
GL120562
GL510101
GL510102
GL709802
GL713202
GL513902
GL110971
GL112901
GL111603
GL519901
GL514943
GL514942
GL514911
GL514932
GL502802
GL700304
GL111602
GL704301
GL513922
GL513905
GL513921
0.1

Figure 36: Phylogram showing the molecular diversity of endophytic fungi found across the three high burn treatments. As can be seen the distances are very large indicating a very diverse group. **Genotype II** refers to a single RFLP genotype that was found in two different treatment plots (in this case plots 2 and 8).

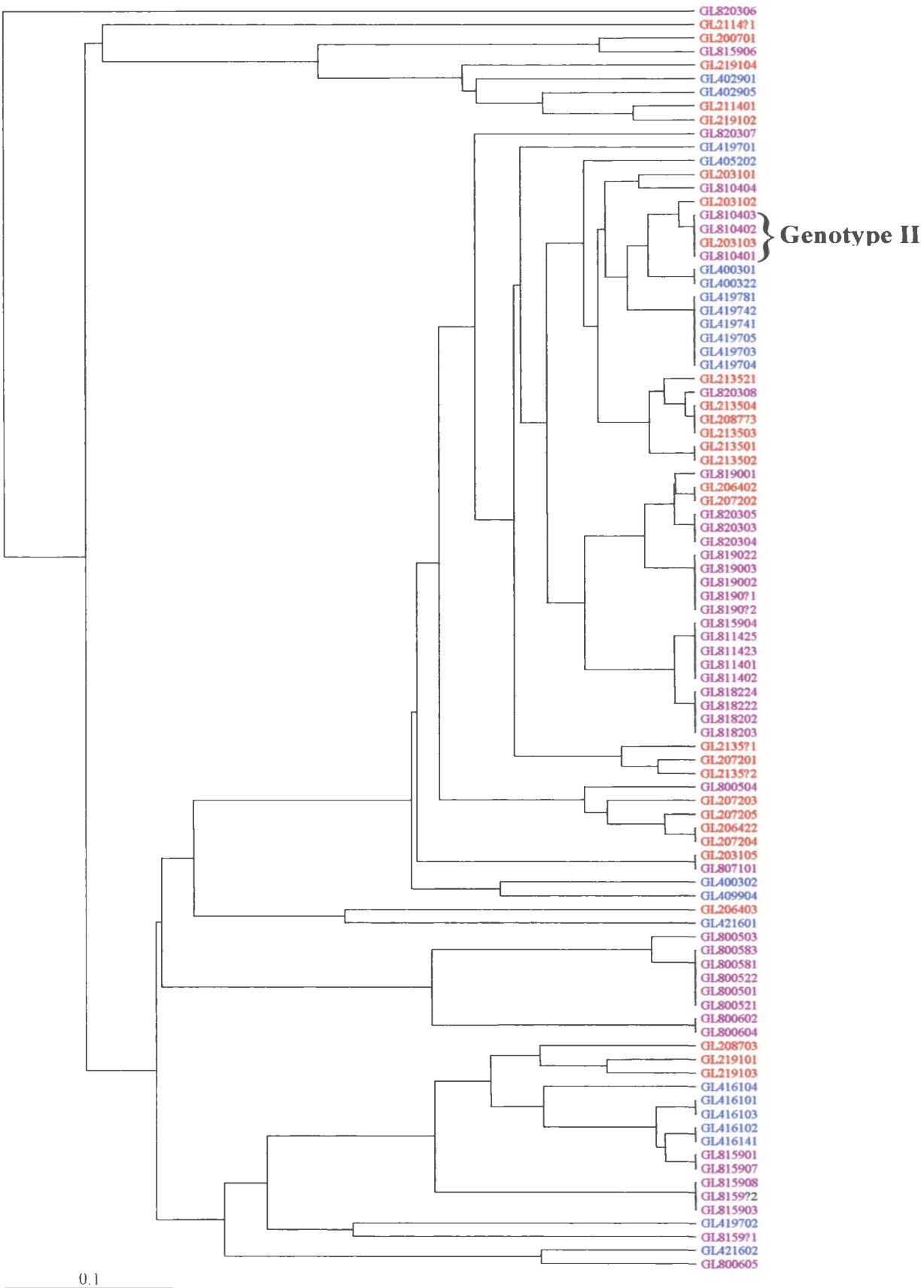
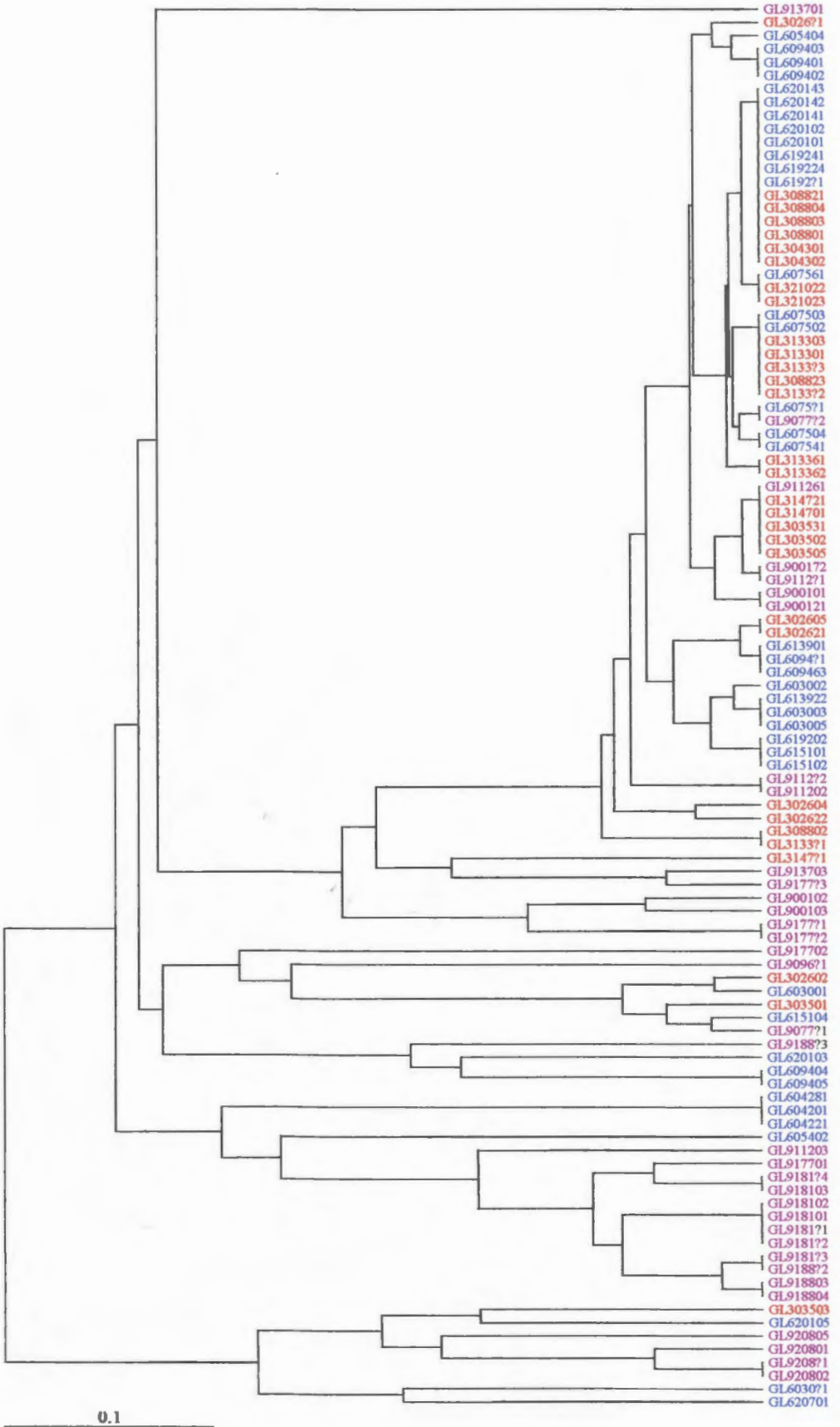

GL820306
GL2114?1
GL200701
GL815906
GL219104
GL402901
GL402905
GL211401
GL219102
GL820307
GL419701
GL405202
GL203101
GL810404
GL203102
GL810403
GL810402
GL203103
GL810401
Genotype II
GL400301
GL400322
GL419781
GL419742
GL419741
GL419705
GL419703
GL419704
GL213521
GL820308
GL213504
GL208773
GL213503
GL213501
GL213502
GL819001
GL206402
GL207202
GL820305
GL820303
GL820304
GL819022
GL819003
GL819002
GL8190?1
GL8190?2
GL815904
GL811425
GL811423
GL811401
GL811402
GL818224
GL818222
GL818202
GL818203
GL2135?1
GL207201
GL2135?2
GL800504
GL207203
GL207205
GL206422
GL207204
GL203105
GL807101
GL400302
GL409904
GL206403
GL421601
GL800503
GL800583
GL800581
GL800522
GL800501
GL800521
GL800602
GL800604
GL208703
GL219101
GL219103
GL416104
GL416101
GL416103
GL416102
GL416141
GL815901
GL815907
GL815908
GL8159?2
GL815903
GL419702
GL8159?1
GL421602
GL800605
0.1

Figure 37: Phylogram showing the molecular diversity of endophytic fungi found across the three control treatments. Distances for a large portion of the samples is greatly reduced in comparison to the previous two phylograms.


GL913701
GL3026?1
GL605404
GL609403
GL609401
GL609402
GL620143
GL620142
GL620141
GL620102
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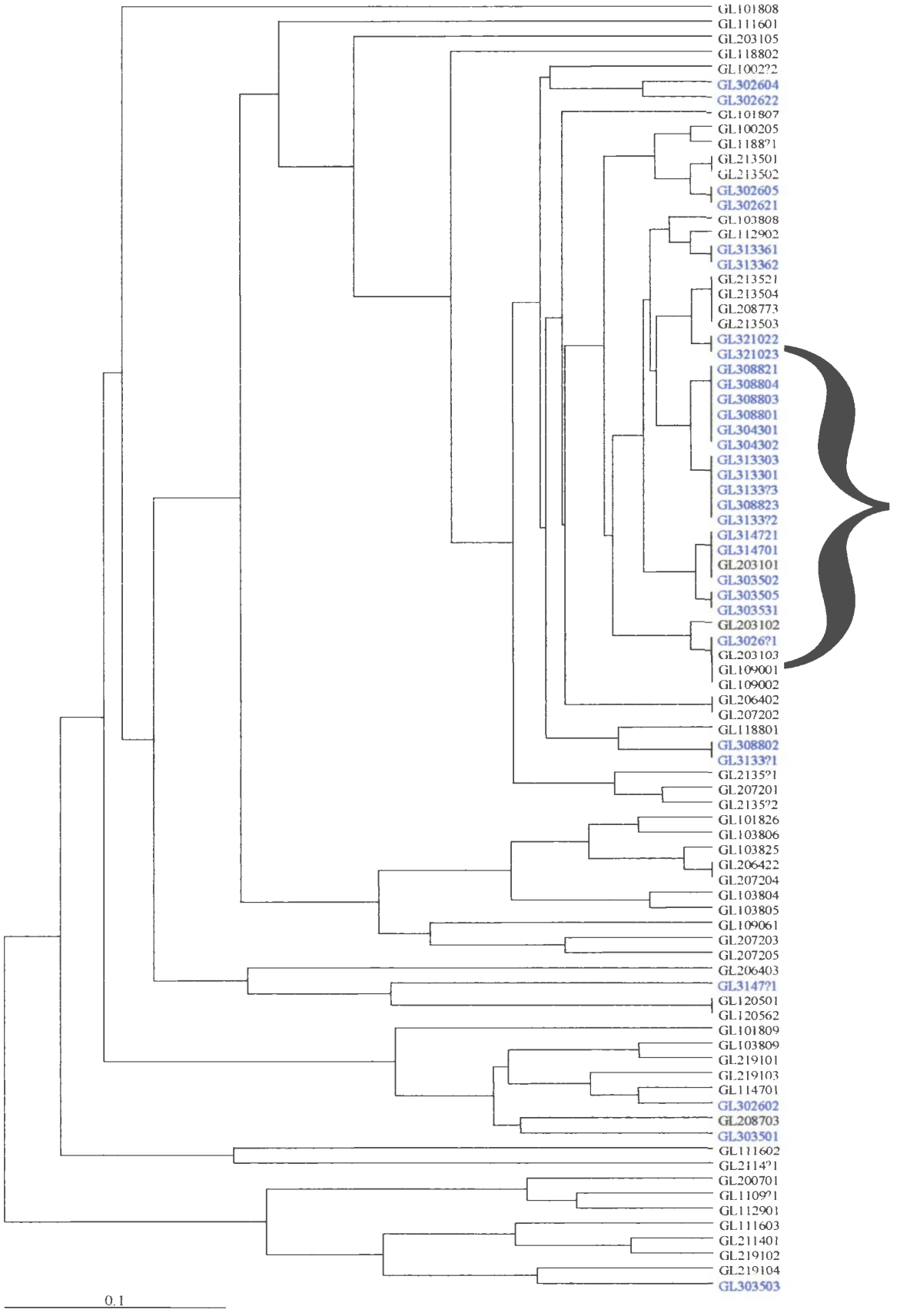
GL619241
GL619224
GL6192?1
GL308821
GL308804
GL308803
GL308801
GL304301
GL304302
GL607561
GL321022
GL321023
GL607503
GL607502
GL313303
GL313301
GL3133?3
GL308823
GL3133?2
GL6075?1
GL9077?2
GL607504
GL607541
GL313361
GL313362
GL911261
GL314721
GL314701
GL303531
GL303502
GL303505
GL900172
GL9112?1
GL900101
GL900121
GL302605
GL302621
GL613901
GL6094?1
GL609463
GL603002
GL613922
GL603003
GL603005
GL619202
GL615101
GL615102
GL9112?2
GL911202
GL302604
GL302622
GL308802
GL3133?1
GL3147?1
GL913703
GL9177?3
GL900102
GL900103
GL9177?1
GL9177?2
GL917702
GL9096?1
GL302602
GL603001
GL303501
GL615104
GL9077?1
GL9188?3
GL620103
GL609404
GL609405
GL604281
GL604201
GL604221
GL605402
GL911203
GL917701
GL9181?4
GL918103
GL918102
GL918101
GL9181?1
GL9181?2
GL9181?3
GL9188?2
GL918803
GL918804
GL303503
GL620105
GL920805
GL920801
GL9208?1
GL920802
GL6030?1
GL620701
0.1

Molecular data from each block (each block contains three plots, representing each of the treatments) was analyzed using UPGMA clustering. Figures 38 to 40 show the results. Blocks 1 and 2 show that those samples from the control plots (3 and 6) tend to group quite tightly and with smaller average distances between isolates as compared to those from the other treatments. There is some sharing of RFLP genotypes across treatments.

Figure 40 shows that plot 9, although a control plot, is more diverse than plots 3 and 6. Although a control, it appears to be more characteristic of the burned plots.

Figure 38: Phylogram displaying the molecular relationships between fungal endophytes located within Block 1 (plots 1, 2, and 3). Notice how tightly clustered many of those samples from the control treatment (plot 3) in comparison to those samples from either the high or low burned treatments (plots 2 and 1, respectively).


GL101808
GL111601
GL203105
GL118802
GL100272
GL302604
GL302622
GL101807
GL100205
GL118871
GL213501
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GL302605
GL302621
GL103808
GL112902
GL313361
GL313362
GL213521
GL213504
GL208773
GL213503
GL321022
GL321023
GL308821
GL308804
GL308803
GL308801
GL304301
GL304302
GL313303
GL313301
GL313373
GL308823
GL313372
GL314721
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GL203101
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GL206402
GL207202
GL118801
GL308802
GL313371
GL213571
GL207201
GL213572
GL101826
GL103806
GL103825
GL206422
GL207204
GL103804
GL103805
GL109061
GL207203
GL207205
GL206403
GL314771
GL120501
GL120562
GL101809
GL103809
GL219101
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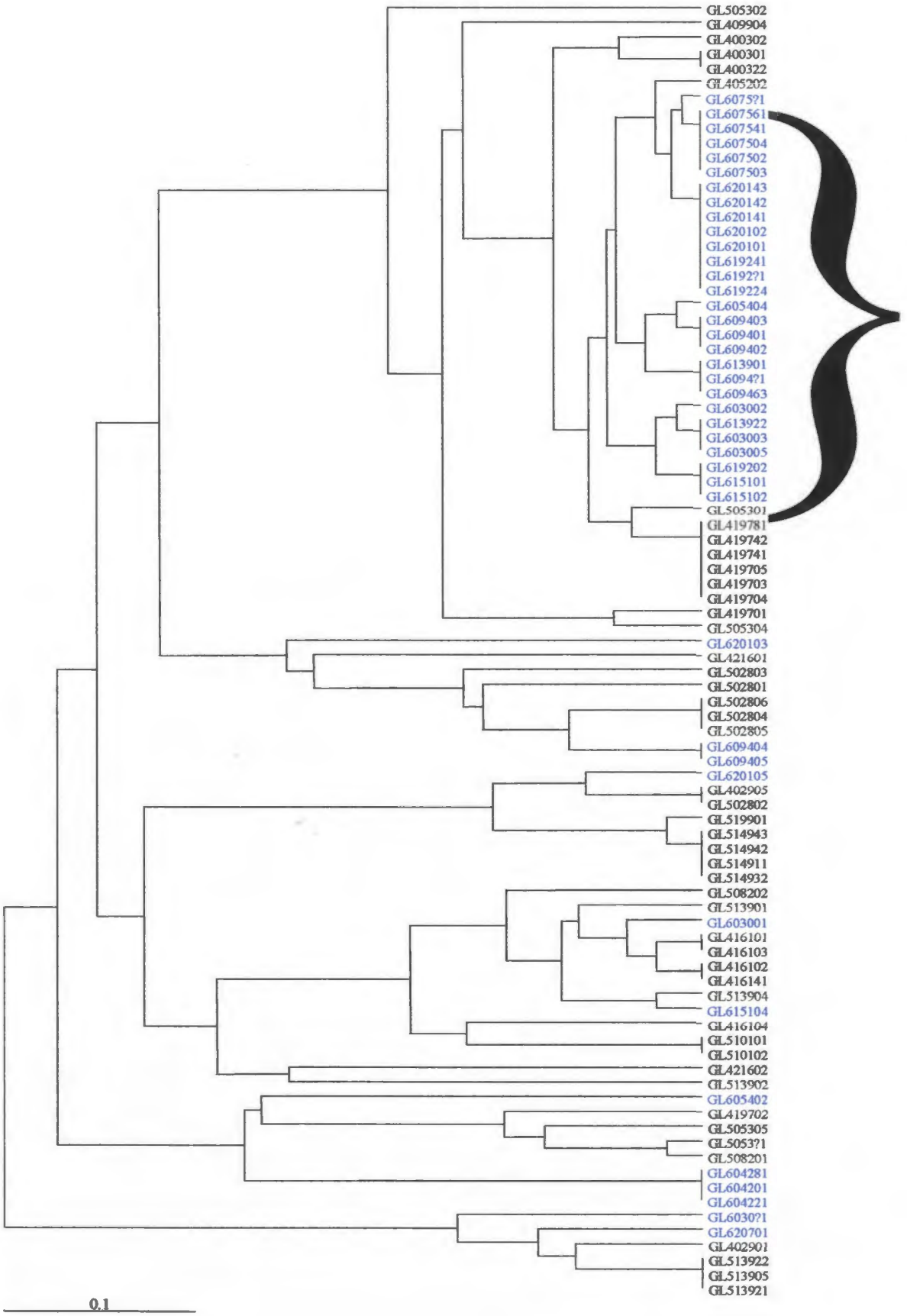
GL211471
GL200701
GL110971
GL112901
GL111603
GL211401
GL219102
GL219104
GL303503
0.1

Figure 39: Phylogram displaying the molecular relationships between fungal endophytes located within Block 2 (plots 4, 5, and 6). Notice how tightly clustered many of those samples from the control block (plot 6) in comparison to those samples from either the high or low burned treatments (plots 4 and 5, respectively).


GL505302
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GL400302
GL400301
GL400322
GL405202
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GL607561
GL607541
GL607504
GL607502
GL607503
GL620143
GL620142
GL620141
GL620102
GL620101
GL619241
GL6192?1
GL619224
GL605404
GL609403
GL609401
GL609402
GL613901
GL6094?1
GL609463
GL603002
GL613922
GL603003
GL603005
GL619202
GL615101
GL615102
GL505301
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GL419741
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GL416141
GL513904
GL615104
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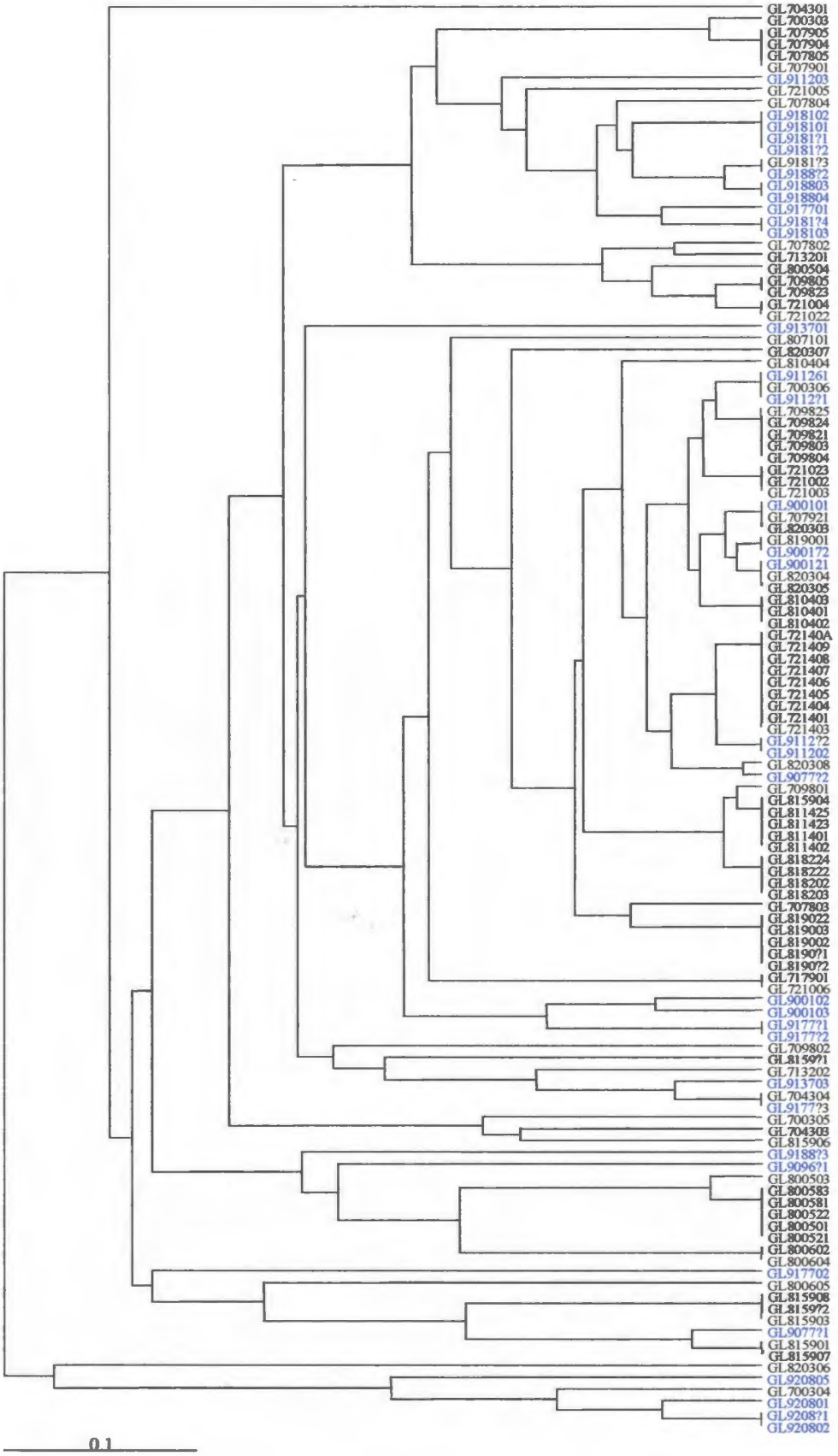
GL510102
GL421602
GL513902
GL605402
GL419702
GL505305
GL5053?1
GL508201
GL604281
GL604201
GL604221
GL6030?1
GL620701
GL402901
GL513922
GL513905
GL513921
0.1

Figure 40: Phylogram displaying the molecular relationships between fungal endophytes located within Block 3 (plots 7, 8, and 9). Samples from control plot #9 are much more scattered throughout the phylogram as compared to the control plots from the two figures previously.


GL704301
GL700303
GL707905
GL707904
GL707805
GL707901
GL911203
GL721005
GL707804
GL918102
GL918101
GL9181?1
GL9181?2
GL9181?3
GL9188?2
GL918803
GL918804
GL917701
GL9181?4
GL918103
GL707802
GL713201
GL800504
GL709805
GL709823
GL721004
GL721022
GL913701
GL807101
GL820307
GL810404
GL911261
GL700306
GL9112?1
GL709825
GL709824
GL709821
GL709803
GL709804
GL721023
GL721002
GL721003
GL900101
GL707921
GL820303
GL819001
GL900172
GL900121
GL820304
GL820305
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GL721401
GL721403
GL9112?2
GL911202
GL820308
GL907?2
GL709801
GL815904
GL811425
GL811423
GL811401
GL811402
GL818224
GL818222
GL818202
GL818203
GL707803
GL819022
GL819003
GL819002
GL8190?1
GL8190?2
GL717901
GL721006
GL900102
GL900103
GL9177?1
GL9177?2
GL709802
GL8159?1
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GL913703
GL704304
GL9177?3
GL700305
GL704303
GL815906
GL9188?3
GL9096?1
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GL800521
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GL815903
GL907?1
GL815901
GL815907
GL820306
GL920805
GL700304
GL920801
GL9208?1
GL920802
0.1

3.2.5: Ectomycorrhizal identification

Sporocarps that were collected from the study site, for two consecutive summers, were subjected to molecular analysis in order to determine the identity with any of the mycorrhizal root tips. As well other sporocarps were collected from various regions in St. John's, Newfoundland. Sporocarp specimens were also obtained from the National Mycological Herbarium of Canada and used as another means of trying to identify my unknown ectomycorrhizal fungi. Tables 4 and 15 list the details of each specimen.

Table 15: Fungal sporocarps obtained from the Mycological Herbarium of Canada.

Species	Collection code(s)	Molecular code(s)	Author
Cortinarius (Leprocybe) *limonius*	DAOM 187547	SR187547	(FR.exFR.)FR.
Entoloma abortivum	DAOM 190387	SR190387	(BERK.etCURT.)DONK
Inocybe geophylla var. *lilacina*	DAOM 190876	SR190876	QUEL.
Russula olivacea	DAOM 213247	SR213247	FR.
Thelephora terrestris	DAOM 196056	SR196056	(BULL.exFR.)KARST.

A total of 122 sporocarps, encompassing 33 different fungal genera, were used to identify my molecular mycorrhizal types. Forty-three of these sporocarps were identified to the species level. In the previous sections I determined that other fungal DNA, besides ectomycorrhizal, had been amplified. Thus, I used mycorrhizal and non-mycorrhizal sporocarps in the identification process. However, even with this large number of sporocarp RFLP patterns my success at identifying tips was very poor. Only two of the 310 (0.6%) molecular patterns (presumed to be ectomycorrhizal) could be associated with

the sporocarps. Even then, these were not perfect matches, suggesting that the sporocarp and ECM tip could be closely-related species, or genotypic variants of a particular species. Table 16 lists the ECM tips and the sporocarp fragment sizes. The main base pair discrepancies occur with *Alu* I and *Rsa* I. ECM tip GL613971, is approximately 89% similar to the sporocarp, while the other (GL320774) is 83% similar. Each ECM tip arises from a different study plot, plots 6 and 3 respectively. Two points of interest do arise. Both plots belong to the control treatment, and both fungi were classed as a 'type 7', a white ECM type (table 1). Upon viewing the phylograms for each plot (figures 13 and 16) it can be seen that both samples fell out as 'unique' molecular species. The sporocarp was identified as *Lactarius deceptivus*.

Table 16: Sporocarp and EM tip fragment lengths (bps) after endonuclease digestion using *Alu* and *Rsa* I. Digested PCR product contained the ITS region belonging to fungal rDNA. All numbers represent base-pairs.

Sample	Fragment Molecular weights (bps)				Total	
B170 (*Lactarius deceptivus*)	466	229	188	112	**995**	
GL613971	471	219	148	125	**963**	**Alu I**
GL320774	485	224	186	115	**1010**	
B170 (*Lactarius deceptivus*)	396	322	166	150	**1034**	
GL613971	390	321	172	157	**1040**	**Hinf I**
GL320774	399	325	167	151	**1042**	
B170 (*Lactarius deceptivus*)	816	189			**1005**	
GL613971	795	200			**995**	**Rsa I**
GL320774	831	203			**1034**	

As previously mentioned, only 0.6% of the ECM tips were linked to a sporocarp, at least to the species level. This percentage may rise if I were to look only at the generic level, however due to a RFLPscan software problem I was unable to produce phylograms for my 'sporocarp/ECM tip' comparisons. Without these phylograms it proved to be too difficult to decipher the similarity tables in order to look at sister clusters, which may suggest generic relationships.

3.2.6: Molecular patterns and unique species

Although there were no significant differences in diversity between treatments this does not mean that they shared similar RFLP genotypes. A total of 14 different morphotypes were identified during the harvesting of the root tips (see methods section, table 1). The molecular data indicates that this was a large underestimation of the diversity. However, the phylograms (figures 11 to 19) do show that some of the morphotypes do form molecular clusters that hold together for individual plots as well as when the data for each plot is merged. This section will look into some of the major molecular groups and their molecular patterns.

One of the more prominent morphotypes was identified as *Cenococcum geophilum* (type 1). A total of 75 samples, presumed to be 'type 1', made it to the final analysis stage. These were spread across all nine plots but had a higher occurrence in plots 1, 2, 3, 4, 7, and 8 (see table 17). From figures 11 to 19 it can be seen that a majority of the samples classified as 'type 1' group into a distinct cluster, suggesting that the molecular similarity within this group is high. Other samples that were identified as 'type 1' showed highly variable RFLP genotypes, thus placing these samples well outside the

'*Cenococcum* cluster'. These highly variable RFLP genotypes may have resulted from two types of errors: 1) the sample appeared to be 'type 1' but was another fungal species, or 2) the sample is a 'type 1' but another fungus, either an endophyte or some other ectomycorrhizal fungus associated with the ECM in question, was amplified instead of the target DNA.

Table 17: Percentage occurrence of certain collected morphotypes, from individual treatment plots.

Morphotype	Percentage occurrence per plot									
	1	2	3	4	5	6	7	8	9	Plots
1	12	12	16	14	7	9	14	10	4	
7	8	5	10	9	3	3	2	2	7	

Within the main 'type 1' cluster it can be seen that there is more than one molecular type. In fact there appears to be at least four polymorphic types. These four polymorphic types are spread across plots 1, 2, 3, 4, 5, 6, and 7. Plots 8 and 9 both have 'type 1' samples but the RFLP patterns are very different from those in the other plots. Table 18 presents the average molecular weights for each polymorphic 'type 1'. However, since the morphotyping was a crude method for identifying the mycorrhizal fungi, some of these 'polymorphic types' may be different species of fungi.

Table 18: Fragment lengths after endonuclease digestion using *Alu* I, *Hinf* I, and *Rsa* I. Digested PCR product contained the ITS region belonging to fungal rDNA. All numbers represent base-pairs.

Molecular type	*Alu I*					Sum of *Alu I*	*Hinf I*						Sum of *Hinf I*	*Rsa I*		Sum of *Rsa I*.
Polymorphic																
Type 1a	434	152	114			**700**	271	180	164	130		93	**838**	904		**904**
Type 1b	439	159	120	96		**814**	275	185	168	135	98		**861**	870		**870**
Type 1c	431	154	117			**702**	273		167	135	104		**679**	888		**888**
Type 1d	437	152	116			**705**	276		164	130	98	93	**761**	910		**910**
Type 1_8	430	149	112			**691**	278		161	127		87	**653**	991		**991**
Type 1_9	420	184	131	123		**858**	355	324	165	150			**994**	993		**993**
Type $7_{1,3}$	665	192		112		**969**	321	279	165	152			**917**	730	178	**908**
Type 7_3	663	187	142	114		**1106**	318	276	165	151	75		**985**	747	176	**923**
Type 9_6	419	177	110	98		**804**	318	216		164	151		**849**	970		**970**
Type 9_9	422	182	124	115		**843**		219	180	161	145	108	**813**	988		**988**
Non-polymorphic																
Type L	377	186	124	119	100	**906**	356	324					**680**	830	176	**1006**
Type G	566	183	116	111		**976**	316	259	163	150	103		**991**	1055		**1055**
Type Ba	573	191		118	98	**980**	316	286	163	150			**915**	749	178	**927**
Type Bb	422	188	149	123	115	**997**	322	222	168	153	107		**972**	1031		**1031**

subscripts refer to the research plot from which the molecular type belonged.

'Type 7' is another ectomycorrhizae that had very distinct morphological characteristics. A total of 30 samples, presumed to be type 7, made it to the analysis stage. Table 17 indicates that plots 1, 3, 4, and 9 harbored the highest proportions of this type. However, RFLP patterns indicate that plots 4 and 9 show no molecular types like those from plots 1 and 3. Almost every sample seems to be a different molecular species. Plots 1 and 3 do share similar molecular types with plot 3 possibly holding two polymorphic types (table 18).

Other morphotypes (2, 3, 4, and 8) were shared across all plots, however these types showed numerous molecular fragment patterns. Aside from 'type 3' the other three types possessed mantles that were very thin, and non-continuous (patchy). These mycorrhizal types would have fewer fungal cells, thus less DNA available for PCR. Therefore, it is likely that if there were other fungi, endophytic or ectomycorrhizal, in the vicinity then these non-target types could compete with the target template for amplification. Thus it is difficult to determine the molecular pattern for each of these morphotypes. As for 'type 3', it looked very similar to *C. geophilum* but it is evident from the many molecular patterns that it is more diverse than 'type 1'. A total of 20 samples, presumed to be type 3, made it to the final analysis. Earlier explanations regarding competitive PCR, primer affinity, and how viable the fungus was upon collection can explain why 'type 3' has such a diversity of molecular patterns.

Aside from molecular types that are shared throughout treatments and plots, there were four morphotypes that were restricted to single plots or treatments. Two of these morphotypes had low representation in terms of occurrence per plot; 2% for 'type L' and

4% for 'type G'. When the RFLP fragment patterns were investigated it was found that a single, distinct, molecular pattern existed for each morphotype (table 18). 'Type L' was found only in plot 2, high burn, and 'type G' was discovered in plot 7, low burn. Since these molecular types did not occur on any of the other treatment plots, it is impossible to determine whether or not these fungi prefer burned soils, for these EM fungi. It is possible that these ECM fungi are of the carbonicolous type, that is types that prefer post-fire regimes.

Morphotypes '9' and 'B' had higher occurrence than the previous two types; 10% and 16% respectively (plots 6 and 9). 'Type 9' was found on plots 6 and 9, and 'type B' was restricted to plot 9. Both plots were control treatments. Each morphotype appeared to be visually distinct thus increasing the accuracy of my morphotyping in these cases (see table 1 for descriptions). However, a closer inspection of their RFLP fragment patterns revealed that each morphotype had at least two major molecular types.

Figures 16 and 19 show that within each plot those classified as 'type 9' form a distinct molecular type. However, fragment patterns for *Hinf* I indicate that those 'type 9' samples from plot 6 (hereafter referred to as type 9_6) have a 318 bp fragment which is not found in the samples from plot 9. Instead those samples in plot 9 (referred to as type 9_9) have two smaller bands (180 bp and 108 bp) which may indicate extra restriction sites not found on those samples in plot 6. These two smaller bands would only produce a fragment of 288 bp, which is 30 bp shorter than the 318 bp fragment found in type 9_6. One of two possibilities explain this 30 bp loss: 1) measurement errors associated with the software package and standards, or 2) there were two *Hinf* I restriction sites within the

318 bp fragment. This would give us the 180 bp and 108 bp bands, which are quite visible on the agarose gel, as well as a 30 bp fragment which would not have been visible. Table 18 shows that fragment patterns for *Alu* I and *Rsa* I were very similar but there were some length differences. Again these differences may be measurement errors incurred when comparing samples from different gels. I conclude that type 9_6 and 9_9 are polymorphs.

A close inspection of 'type B' suggests that there are two possibilities: 1) there are two variants, or 2) these are two closely related species. The first possibility is remote since within each restriction set there is considerable length variability between respective fragments. These length variances cannot be attributed to measurement error since they are so large. The second possibility that seems more likely given the restriction fragment differences is that these are two closely related species, which share similar macroscopic characters. This example stresses the importance of molecular characterization so that such confusion can be avoided.

3.2.7: Traditional verses New Diversity Measures

Diversity is a concept that seems intuitively simple. Even without the complex equations which set a value for the index of diversity within a given area, most people are able to grasp the concept of one area being more biologically diverse than another (Magurran, 1988). However, in order to discuss the diversity of one community in comparison to others, researchers need consistent and comparable values for which to quantify this diversity. Over time numerous diversity indices have been created to help

with this quantification. The main reason that so many indices exist is that diversity takes into consideration both species evenness and abundance (Magurran, 1988).

Three types of diversity measures can be described. The first is *species richness indices*. This type of index measures the number of species in a sampling unit (*e.g.* Margalef index). Secondly there are *species abundance models* which describe distributions of species abundance (*e.g.* Log series). Lastly are those indices that emphasize *proportional abundance of species*. These indices try to put richness and evenness into a single value (*e.g.* Shannon-Wiener and Simpson). These types of indices are the most commonly used.

Indices based upon the proportional abundance of species can be broken down into two categories. First are those that measure diversity in a similar manner to the way one would get information from a code or message. These indices are called the *information theory indices*. The Shannon-Wiener index is one such example. This index assumes that sampling of individuals is from an 'infinitely large' population. It takes into consideration all individuals within the sample ($p_i = n_i/N$; n_i is the number of individuals within the i^{th} species and N being the total number of individuals within the sample). However, this proportion will always be biased since one can never really obtain the true proportion from a sampling. Therefore, the error will increase as sampling intensity decreases.

The second category of abundance indices is referred to as *dominance measures*. These indices are more concerned with the abundance of the commonest species instead of species richness. For example, the Simpson index (D) estimates the probability of any

two individuals drawn at random from an infinitely large community belong to different species, *i.e.* $D=\sum p_i^2$. One problem with this index is that it is heavily weighted towards the most abundant species and less sensitive to rare species.

Within this study both the Shannon-Wiener and Simpson indices were used to attempt to determine the diversity of mycorrhizal fungi based upon molecular data. Both indices rely upon the fact that we are already aware of the number of species within a given sample. Intraspecific variation within a given species may not be noticeable or may be de-emphasized in identification keys, at the phenotypic level, thus making diversity analysis relatively easy. However, at the molecular level the presence of intraspecific variation can have major repercussions for traditional diversity measures.

The amount of intraspecific variation within a species will vary. Such things as the geographic location of that species, whether it reproduces sexually or asexually, and past genetic history are some of the factors that determine the proportion of intraspecific variation residing within a species. Restriction fragment length polymorphisms (RFLPs) can be very sensitive to these changes. As well RFLP analysis can detect error associated with PCR-RFLP analysis. These errors can factor in those patterns resulting from incomplete digestion, phantom PCR bands resulting from multiple binding sites, and fragment size calculation errors resulting from comparing samples across gels. As a result a single fungal species may have more than one molecular pattern.

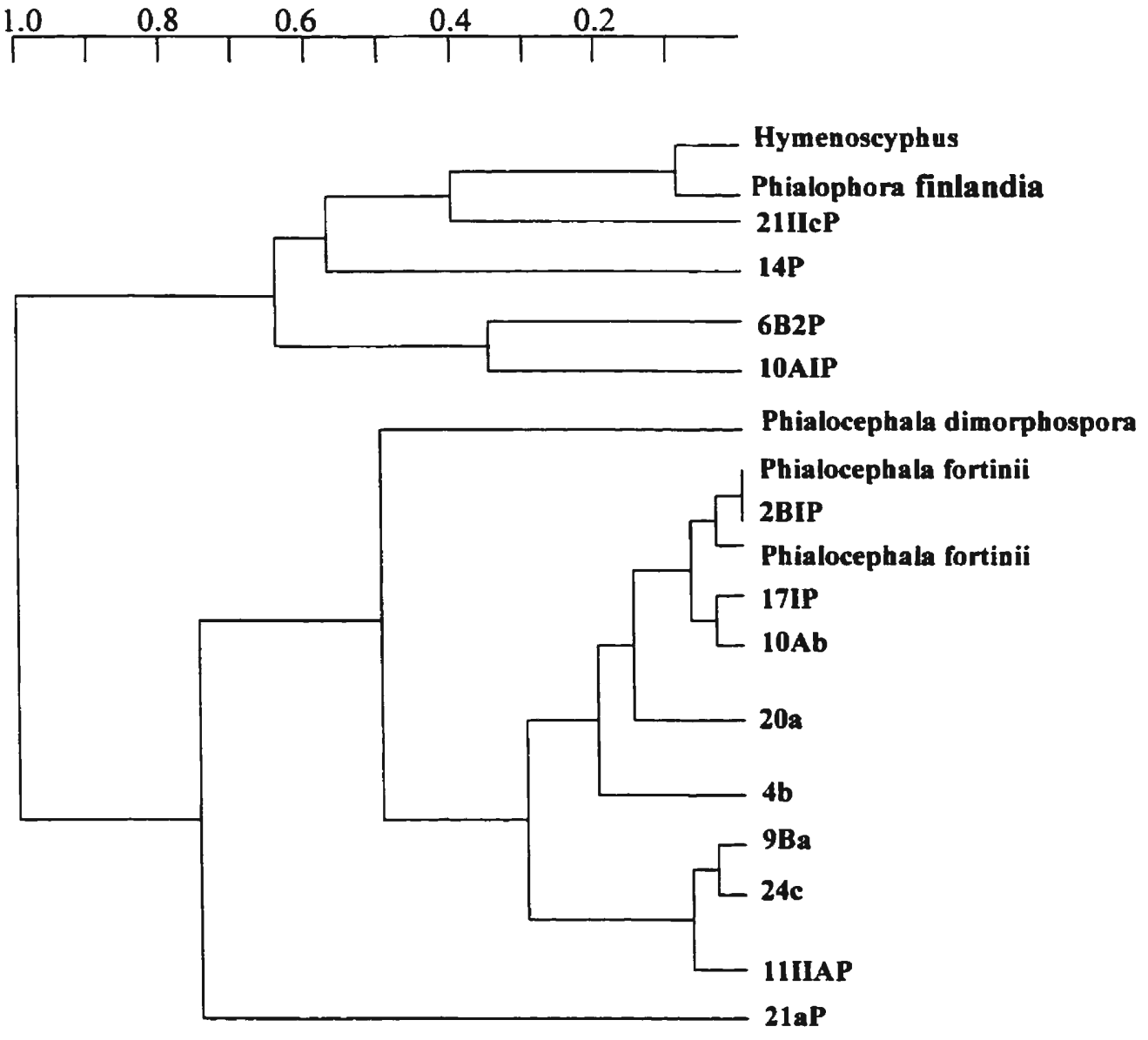
An easier way to view the effects of intraspecific variation upon traditional diversity measure is via an example. Figure 41, taken from Stoyke *et al.* (1992), shows the diversity within a *Phialocephala fortinii* clade. This example will proceed to increase the

amount of intraspecific variation within this phylogram in order to see the effects it will have upon traditional diversity indices. At 0%, that is no intraspecific variation, we see that there is one species with two individuals and fifteen species with one individual. At 5% there are nine species with one individual, one species with five individuals, and one species with three individuals. At 10% there are eight species with one individual, one species with six individuals, and one species with three individuals. Lastly, at 15% there are seven species with one individual and one species with ten individuals. Table 19 shows the calculated Shannon-Wiener and Simpson values.

Table 19: Varying levels of intraspecific variation applied to figure 41and the associated values for two traditional diversity indices.

% intraspecific variation	Shannon-Wiener (H')	Simpson value (1-D)
0	2.75	0.993
5	2.17	0.9
10	2.01	0.868
15	1.48	0.669

Figure 41: Phylogram showing the diversity within the *Phialocephala fortinii* clade (Stoyke *et al.*, 1992).


1.0
0.8
0.6
0.4
0.2
Hymenoscyphus
Phialophora finlandia
21IIcP
14P
6B2P
10AIP
Phialocephala dimorphospora
Phialocephala fortinii
2BIP
Phialocephala fortinii
17IP
10Ab
20a
4b
9Ba
24c
11IIAP
21aP

From these calculations it can be seen that species richness indices, such as Shannon-Wiener, are least affected by intraspecific variation whereas species dominance indices (Simpson) are greatly affected. Regardless, all traditional indices that are based upon proportional abundance of individuals within species are all sensitive to molecular intraspecific variation to some degree.

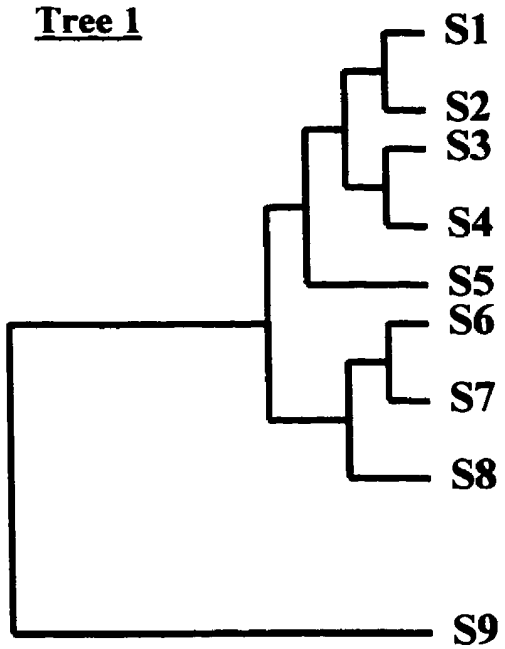
Although I used traditional diversity indices to analyze my molecular data, it was soon obvious that they may lead to misleading interpretations, thus a more reliable index was needed. There are numerous indices that look at the levels of genetic variation within and between populations (e.g. Lynch, 1990). However, these indices are based upon the assumption that variation is occurring 'within' a single species, not between different species. There did not appear to be any population diversity index that would be appropriate for measuring diversity of a sample of different species. Indices based upon phylogenetic data have been developed which are more appropriate for measuring diversity of a sample of species (Krajewski, 1994). However, these indices were designed to examine diversity of monophyletic groups of organisms, and to take extinction rates into account in the estimate. Since the ecological samples I obtained would not conform to the requirement for monophyly, and extinction rates were unknown, I felt that these indices were also not appropriate for ecological samples. Therefore, a new index was invented. Since I was obtaining species richness and abundance data from the phylograms, I decided that a more sensitive measure should take into consideration the actual distances used to create the phylograms themselves. This is intuitively satisfactory since groups of similar species would contribute smaller distances,

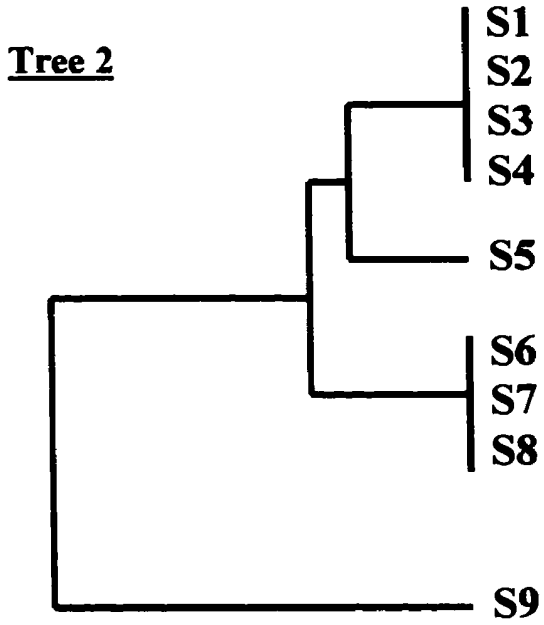
even factoring in the intraspecific variation that exists within a species. To illustrate this, figure 42 shows three phylograms which contain the same number of species, but all with varying degrees of similarity. Tree 1 shows that there are small distances separating most of the species indicating that the relationships are high among them. Tree 2 has collapsed many of the shorter branches that were viewed in tree 1, thus increasing the similarity among several species. However, tree 3 shows a similar grouping of species as that of tree 2, but larger distances separate each cluster. Both the Shannon and Simpson indices were calculated for each of the trees, and the values are in table 20.

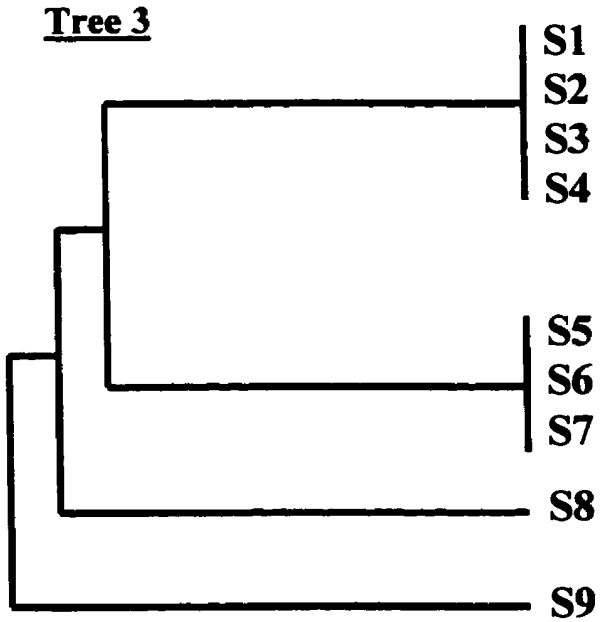
Table 20: Shannon-Wiener, Simpson and *Phi* index values calculated for the three hypothetical phylograms (Figure 42).

	Diversity Values		
Phylogram	**Shannon (H')**	**Simpson (D)**	**Phi (P)**
Tree 1	2.20	0	0.150
Tree 2	1.21	0.25	0.146
Tree 3	1.21	0.25	0.291

Figure 42 Three hypothetical phylograms to demonstrate the Shannon-Wiener, Simpson, and Phi indices.


Tree 1
S1
S2
S3
S4
S5
S6
S7
S8
S9


Tree 2
S1
S2
S3
S4
S5
S6
S7
S8
S9


Tree 3
S1
S2
S3
S4
S5
S6
S7
S8
S9

The Shannon index suggests that tree 1 has a more diverse community than the other two, while trees 2 and 3 have the same values. However, from looking at the distances separating each cluster it is quite apparent that tree 3 would be the most diverse at the phylogenetic level. Appendix XI contains the matrix used for the creation of each of the three trees . Also included are the calculations for my "*Phi index*" (Appendices XII) . The *Phi index* is calculated using the phylogenetic distances obtained from the distance matrix created during my PHYLIP analysis (Felsenstein, 1993). All the distances (d) for an individual are squared and summed (Σd^2) to give D. This D value is then divided by "N-1", that is "D/(N-1)". "N" being the total number of individuals for that sample. These two steps were done for each individual within the matrix. All "D/(N-1)" values were summed and then divided by "N", that is "Σ[D/(N-1)]/N". Table 20 shows that trees 1 and 2 are much closer in diversity using the *Phi index*, whereas tree 3 shows a greater difference and a much greater diversity. This would suggest that my *Phi index* is less sensitive to the problems intraspecific variation introduces, and thus may be more reliable than traditional diversity indices.

Previously the Shannon, Simpson and *Phi* indices were calculated for the Glide Lake data. Table 21 summarizes the index values.

Table 21: Shannon-Wiener (H'), Simpson (1-D) and *Phi index* (P) values calculated for the nine experimental plots near Glide Lake. There are two values for each plot based upon the separation of non-target data from ectomycorrhizal data. LB= low burn, HB= high burn.

	'Mantled tips'			'Non-mantled tips'		
Plot	H'	1-D	P	H'	1-D	P
1 (LB)	3.41	0.986	0.363	3.19	0.994	0.278
2 (HB)	3.42	0.987	0.324	3.07	0.984	0.239
3 (Control)	3.13	0.973	0.268	2.43	0.918	0.096
4 (HB)	3.07	0.975	0.400	2.41	0.922	0.312
5 (LB)	3.03	0.985	0.282	2.74	0.96	0.417
6 (Control)	2.92	0.978	0.349	2.63	0.931	0.255
7 (LB)	2.87	0.980	0.316	2.86	0.938	0.194
8 (HB)	1.91	0.964	0.440	2.78	0.948	0.302
9 (Control)	2.81	0.963	0.236	3.12	0.973	0.339

The values for the Simpson index shows very little difference between plots. Since this index is sensitive to the abundance of individuals, and due to the fact that most RFLP genotypes in a plot only consisted of one sample, it is not surprising that the diversity was so uniform in each plot. The Shannon-Wiener index relies more upon species richness, thus explaining the high values (which were very close to the maximum index value based upon the sample size. For example: plot 1 had 33 RFLP genotypes in only 42 samples, giving a H'= 3.41. If plot 1 had 42 genotypes in 42 samples then H'= 3.50. The two are quite close, thus suggesting that plot 1 was very close to having the maximum possible diversity). The greater the number of genotypes (distinct molecular patterns) then the greater the index value, that is to say this index is sensitive to the genotype number and to the number of samples. For example, plot 8 (mantled tips) has a total of 7 molecular species, the lowest of all the plots, thus explaining its low index value. The *Phi index* does not depend upon species abundance nor species richness. Instead it relies

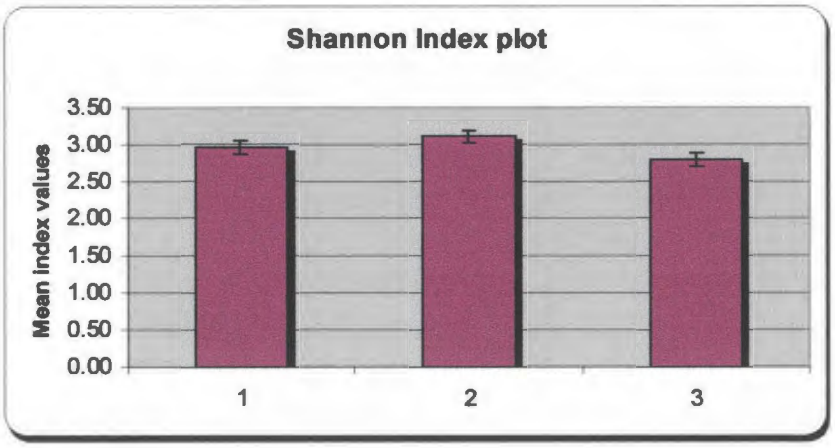
upon the distance data thus producing values that are more sensitive to changes in the phylogenetic diversity of the samples.

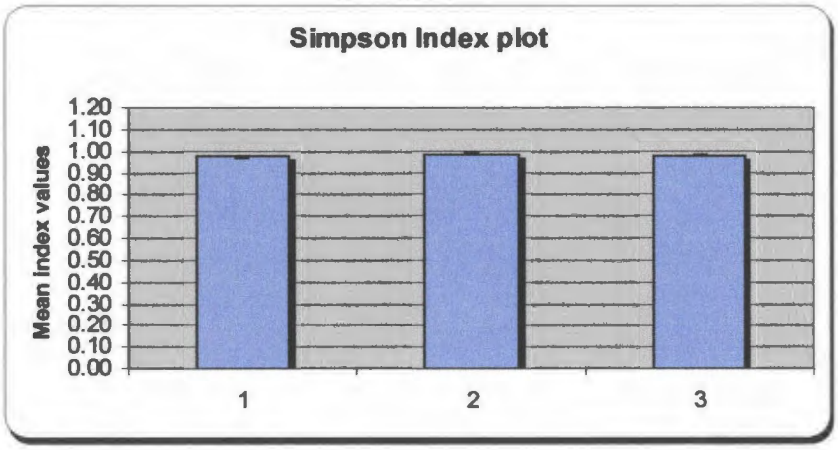
If we were to look at bar graphs of the means for each of the above mentioned indices certain trends can be noticed. Figure 43 shows that within the ectomycorrhizal root tips there is a different trend for each index. Looking at the Shannon-Wiener values it appears that the low burns have the highest diversity, followed by the controls and then the high burns. The Simpson shows that all three treatments seem to be almost equal with respect to diversity. On the other hand the *Phi index* shows an increase in ECM diversity as one moves from control, to low, to high treatments. In the previous section (3.2.3) an ANOVA was performed upon both the Shannon and Simpson values and it was found that neither were statistically significant. An ANOVA performed upon the *Phi index* values for ECM root tips showed that there was no significant difference between treatments with respect to diversity (Appendix XIII).

ANOVAs performed on the three index values (table 21) calculated with the non-target data (non-mantled tips) indicated that all possible comparisons showed no significant differences between treatments, with respect to molecular diversity (Appendix XIV). Looking at a plot of the means, of each index, for the non-target DNAs data it was seen that they also showed different trends. The Shannon-Wiener index had the low burns showing the highest diversity, followed by the control and the high burns having about equal mean diversity values. The Simpson index showed very little difference between the three treatments. The *Phi index* showed a trend different from that which

was viewed for the ectomycorrhizal index. The low burn had the greatest degree of diversity. This was followed by the high burn and finally the control (figure 44).

Figure 43: Graphic representations of the means of each index (Shannon-Wiener, Simpson, and Phi) obtained for the three treatment plots. Indices were calculated using the data collected for ectomycorrhizal root tips only. Bars in the center of each bar represent the standard error. (1= Control treatments; 2= Low burn treatments; 3= High burn treatments).


Shannon Index plot
Mean index values
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3


Simpson Index plot
Mean index values
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
3

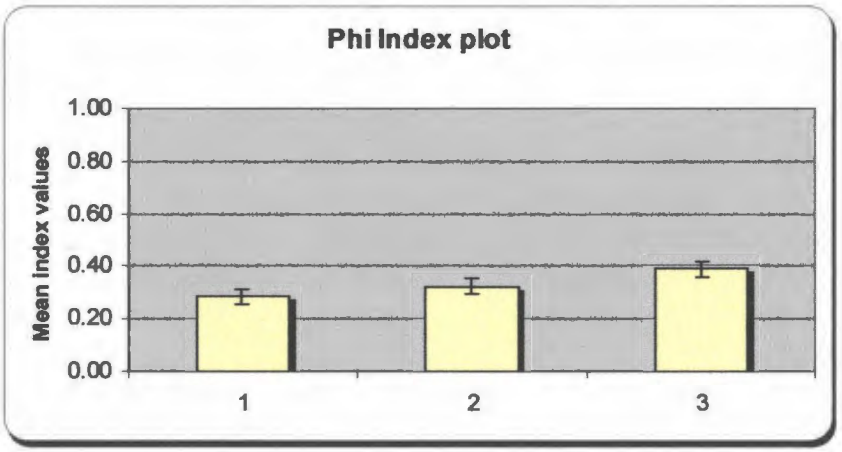
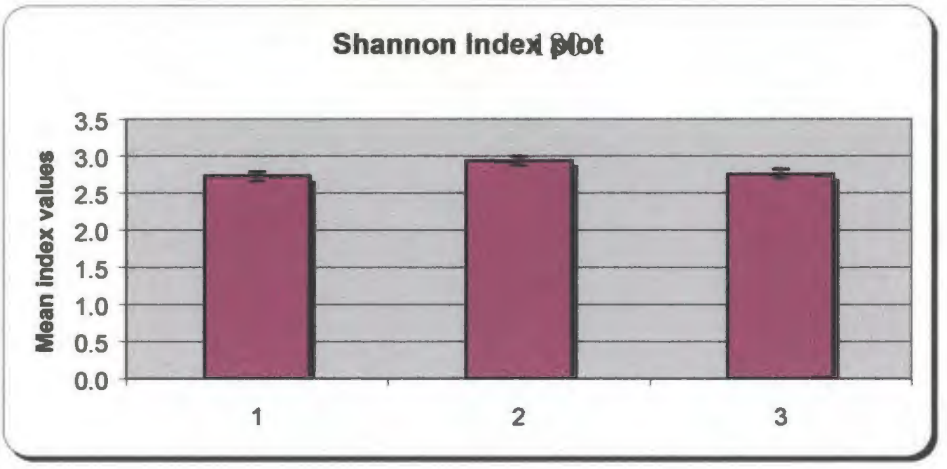
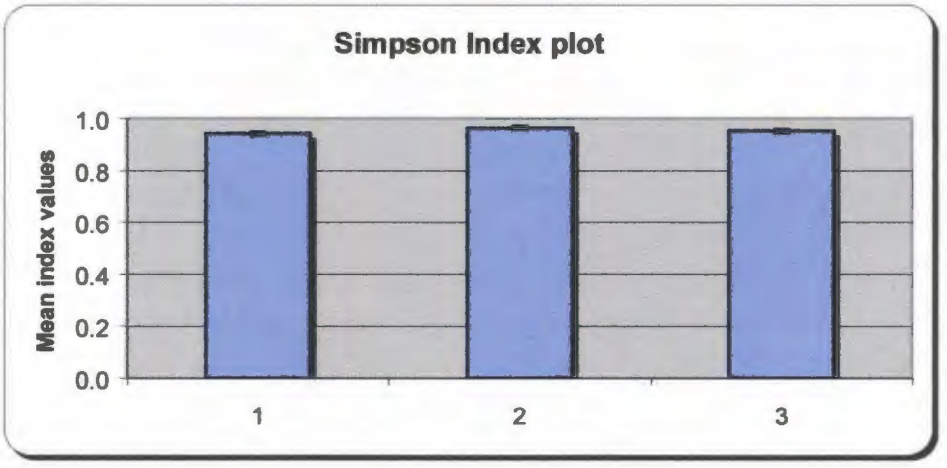
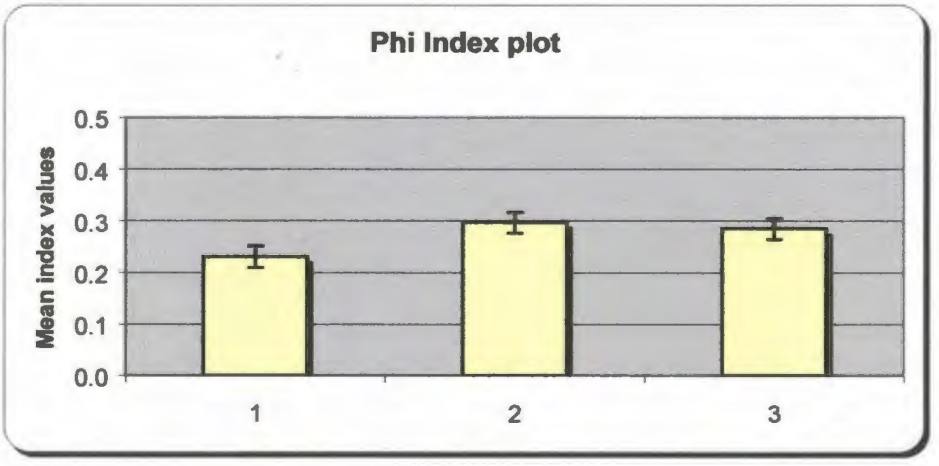

Phi Index plot
Mean index values
1.00
0.80
0.60
0.40
0.20
0.00
1
2
3

Figure 44: Graphic representations of the means of each index (Shannon-Wiener, Simpson, and Phi) obtained for the three treatment plots. Indices were calculated using the data collected for non-mantled root tips only. Bars in the center of each bar represent the standard error. (1= Control treatments; 2= Low burn treatments; 3= High burn treatments).


Shannon Index plot
Mean index values
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3


Simpson Index plot
Mean index values
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3


Phi Index plot
Mean index values
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3

Chapter 4:

Discussion

4.1: Ectomycorrhizal Abundance

4.1.1: Preliminary Analysis

The preliminary study allowed me to draw several conclusions concerning the rhizosphere diversity of naturally regenerating spruce and fir seedlings within the Glide Lake clear-cut. First, it was evident that roots of naturally-regenerating seedlings were colonized by ectomycorrhizal fungi, as evidenced by the presence of roots with developed fungal mantles. Second, the endophyte diversity within this site was potentially quite large. Fungal cultures indicated that there was a large population of ascomycete fungi inhabiting the root rhizosphere. Since the root tips were sterilized before cultures were isolated, I believe that most of the cultures obtained were root endophytes rather than surface contaminants. Finally, it was clear from collections of basidiocarps that there was a high diversity of potential mycorrhizal fungi in the surrounding forest, although I was unable to confirm that mycorrhizal basidiocarps represented in the sample were forming mycorrhizae on seedlings in the clear-cut.

4.1.2: Percent Abundance of Mycorrhizal Fungi

The new analysis of data from the preliminary study indicated that there were no statistically-significant differences between percent abundance of individual ectomycorrhizal fungal species verses location, for mycorrhizas found on black spruce or balsam fir seedlings. The colonization potential of these five morphological types were similar, regardless whether the seedlings were planted near the forest edge or in the clear-cut. Of course this can only be said for the five fungal types that were investigated.

My investigation showed that there was a significant difference, for both tree species, in the types of fungi colonizing roots. Both tree species had morphotypes 3 (shiny, black fungus) and 8 (white, cottony fungus) being the most dominant. Type 3 covered almost twice as much of the root systems of black spruce as compared to that of balsam fir. This would suggest that spruce seems to be the preferred host for this fungus. It was found that type 1 (*C. geophilum*) was another dominant fungus on spruce roots, but not on the fir. In fact it was five times more prevalent on the spruce than the fir. This may suggest that spruce, instead of fir, may be the preferred host for *C. geophilum* within my study area. A study by Doudrick *et al.* (1990) showed that aseptic synthesis of ectomycorrhizas between black spruce and *Cenococcum* sp. were accomplished quite easily. It appeared that overall ectomycorrhizal abundance, regardless of species, was greater on black spruce instead of on fir, however, I found that this was not statistically the case.

I saw that both naturally regenerating black spruce and balsam fir harbor a variety of ectomycorrhizal fungi. Overall both tree species have an equivalent amount of ectomycorrhizal abundance. However, does this mean that the greater ectomycorrhizal abundance a particular host has, then the better it's chances of survival? This is an ongoing debate. Some believe that it is not the frequency of mycorrhizal species which affects the host's fitness, but the particular species of fungi that form these associations. That is to say that some fungal species have more important functional roles than others (MacGillivary *et al.*, 1995; Tilman *et al.*, 1997). If this is true then one would expect to see more of certain species occupying root systems of a host than that of other fungal

species. In the case of my data this would seem to hold true. Maybe the host is not always the determining factor in what fungal species will occupy root systems. Other factors such as soil type, moisture content, fauna composition could have more of an influence than host type. In my study it was apparent that both fir and spruce seedlings held the same frequency and types of ectomycorrhizal fungi. However, this speculation must be taken carefully since my study did have a limited sample size and limited morphotyping capabilities. Although my five fungal types were quite distinct, this does not mean that some individual types could not have actually been two or more species. For example, my type 3 (shiny, black mantle) when analyzed at the molecular level (section 3.2.6) revealed that many different molecular patterns arose from it. This suggests that many fungal types can adopt a similar morphological appearance, which could have easily affected the analysis of percent coverage. Thus, molecular analysis allows one to formulate a better estimate of the 'true' species diversity which would enable one to perform more accurate assessments of things like functional and species diversity.

4.2: Molecular analysis of ectomycorrhizal fungi

4.2.1: Percent PCR amplification and presence of doublets

The mean success rate of the PCR amplifications was estimated to be 76% (684 out of 900), ranging from 88% to 63%. Kårén and Nylund (1997) had a success rate of 89% (94 out of 106). Erland *et al.* (unpublished) had an amplification success rate of 86% and 82% at their two study sites. The main reason for the difference between my success rate

and that of the others is that I was not just dealing with ectomycorrhizal roots. Our study also included 'non-mycorrhizal' root tips. If I were to just look at the amplification rate of ectomycorrhizal tips, I saw that I had 440 out of 536 successful amplifications (82%). This is much closer to the success rates of the other studies. Also we must factor in the amount of material used in each study. My study and that of Erland *et al.* (unpublished) extracted from individual root tips. Kårén and Nylund (1997) used multiple tips within a single extraction. The increase in the amount of fungal material used by these researchers may explain their higher amplification rate.

My overall percentage of double amplifications (doublets), noticeable on detection gels and those within digestion gels, equaled 6%. Kårén and Nylund (1997) had approximately 38% of their amplifications containing doublets. Their high value may be attributed to the use of several tips within a single extraction, thus increasing the chances of obtaining two fungal species within the same extraction. Kårén and Nylund (1997) also suggest the possibility of the co-existence of mycorrhizal or saprophytic fungi on single root tips as another reason for the double amplification. My data also suggests that there is yet another possibility for this double amplification. Since endophytic fungi were so prevalent throughout our study site, it is possible that the Kårén and Nylund study site also harbored a healthy population of endophytes, which would provide another source for double amplifications. Erland *et al.* (unpublished) found that their percentage of doublets were 11% and 21% for each study site. The higher degree of doublets compared to my study could be a result of numerous factors. Different sites contain different fungal species which may form different associations with roots, thus my site may have root

systems that contain fewer dual occupied tips. Also another factor may have to deal with the primer sets used in each study, and the specificity regime of each set. I used ITS1/NL6Bmun and Erland *et al.* (unpublished) used ITS1/ITS4.

Therefore, one must be wary of the potential for dsDNA being amplified from some mycorrhizal and non-mycorrhizal root tips to belong to that of non-mycorrhizal fungi (e.g. endophytes). There are several scenarios possible when amplifying from a root tip: (1) a living ectomycorrhizal fungus has taken up residence on the tip and has excluded both invading soil fungi as well as endophytic fungi. Thus, amplified DNA will belong to this particular fungus. (2) The ectomycorrhizal tip has either senesced or become damaged thus exposing root epidermal cells through the mantle. As a result endophytic fungi may have invaded the root tip. Thus, the amplified DNA may belong to the endophyte(s) or the ectomycorrhizal fungus (if it has senesced then the time of death will affect the condition of its DNA, which in turn will affect the PCR outcome). (3) a non-mycorrhizal root tip may appear uninfected, however it is being colonized by one or more endophytic fungi. Thus, a positive amplification would result from the PCR. (4) the root is inhabited by an ectomycorrhizal fungus that supports a relatively thin mantle, thus preventing the fungus from excluding endophytes. Again the PCR could contain the DNA of either the mycorrhiza or the endophyte. In the case of scenarios 2 and 4, another possibility can arise. Both the mycorrhizal and endophytic fungal DNAs have equal affinity for the primers. The result is equal amplification of both thus producing a **'double amplification'**. In some cases there is a significant size difference between amplified products, and when run on a horizontal agarose gel they separate to indicate a

double amplification. However, in other cases, when both have equivalent base numbers. double amplifications will not be realized until after endonuclease digestion. In this case one, or more, endonuclease band patterns will sum much greater than the expected base size.

4.2.2: What was responsible for amplification from "non-mycorrhizal" roots?

A high proportion of roots that were classified as non-mycorrhizal when sorted under a dissecting microscope, did produce amplification products. There were three possible sources for the template DNA: 1) surface contaminants, 2) ectomycorrhizal fungi that produce a thin, hyaline mantle, and 3) root endophytes.

Endophytic fungi are not newcomers to the mycological field. Melin (1923) makes reference to 'pseudomycorrhizas' which were considered to be non-mycorrhizal fungi that occurred in mother roots and older portions of mycorrhizas found on pine and spruce trees. One particularly dark pigmented pseudomycorrhiza was named *Mycelium radicis atrovirens* (M.r.a.). Richard and Fortin (1974) found this endophyte to be quite common in the roots of *Pica mariana* (Mill) B.S.P. I as well saw similar hyphal structures within my spruce root tips (Figures 6 and 8). After clearing and staining numerous spruce roots, both non-mycorrhizal and mycorrhizal tips contained extensive fungal hyphae within the intra- and intercelluar spaces of these roots. This staining process was implemented because I witnessed, initially, a very diverse set of RFLP patterns for what were presumed 'non-mycorrhizal' (NM) root tips

Given the observations of endophytic fungi in cleared and stained roots showed only sparse coverings of surface fungi, but abundant endophytic fungi, I consider it most likely

that the largest proportion of amplification products recovered from non-mantled roots were endophytes. A study by Ahlich and Sieber (1996) showed there to be a wide variety of endophytic fungi inhabiting non-ectomycorrhizal roots of *Abies alba*, *Picea abies*, and *Pinus sylvestris*, thus providing support for my findings.

In some cases I was able to link roots that were classified as non-mycorrhizal to roots that were classified as ectomycorrhizal. This probably occurred because some collections represented ECM in early stages of development, before the mantle was fully formed. In these cases, the thin mantled ECM type was transferred to the ECM database. The remaining types were transferred to a separate endophyte database. The results of the analysis of these two databases is discussed below.

4.2.3: Why did we amplify non-target DNA?

There have been numerous studies which have looked at the molecular diversity of endophytic fungi (Stoyke *et al.*, 1992; Wetzel III *et al.*, 1996; Harney *et al.*, 1997), as well as that of ectomycorrhizal fungi ([1]Gardes and Bruns, 1996; Kårén and Nylund, 1996; Kårén and Nylund, 1997; Erland *et al.*, unpublished; Dalhberg *et al.*, 1997). However, there appear to be no studies which have looked at both fungal types within the same host. This was surprising since both are common to most plant systems.

The main purpose of this study was to look at the molecular diversity of ECM fungi, however, it is sometimes easy to overlook ECM fungi that are in the early stages of development, thus healthy, 'non-mycorrhizal' root tips were included in my sampling [By 'non-mycorrhizal', I refer to those root tips that did not show any signs of infection such as color change, distortion of the tip (like swelling, twisting, etc.), or harboring of

hyphae]. By doing this I discovered that there was a very diverse endophytic community within my study area, and that this community existed along with the ECMs!

The presence of these endophytes with ECMs indicates that any study dealing with direct amplification from ECM roots must be careful with the assumptions made. No matter how distinct the ECM fungus observed, there is the potential that the RFLP pattern may belong to any underlying endophyte. Kårén and Nylund (1997) indicated that they were aware, from double amplifications, that there were other non-target fungi present with ECMs. They refer to the possibility that these fungi were external saprophytes, however my study indicates that these non-target fungi could exist within the roots, underneath the ECM mantle.

Another reason that my study has identified so many endophytic RFLPs has to deal with the primer set used in the PCR. The study used the universal primer **ITS1** along with **NL6Bmun** (fungal specific). Although the latter was designed to favor amplification of basidiomycete rDNA, extensive tests show that, at the annealing temperature used in this experiment, it readily amplifies most ascomycete rDNA as well (unpublished data). Other studies have used primers that favor the amplification of basidomycetes, ITS1F/ITS4B ([1]Gardes and Bruns, 1996), or ascomycetes, ITS1/ITS4 (Kårén and Nylund, 1997). Although ITS1/ITS4 are considered universal primers and thus should recognize both ascomycete and basidiomycete DNAs, a study by Gardes and Bruns (1993) showed that this primer set has a higher affinity for ascomycetes.

Therefore, the types of primers used in a study can greatly affect the diversity of endophytic species amplified from root tips. [1]Gardes and Bruns (1996) found that they

could not identify five RFLP types from ECM roots. It may be possible that some of these are basidiomycete endophytes. Since only a few endophyte species belong to the Basidiomycota (Isaac, 1992) there is probably more of a likelihood that these unknown molecular types are ECM types. Many endophyte species belong to the Ascomycota group (Isaac, 1992), which in part may explain the higher number of unknown RFLP patterns (12 of 21) that Kårén and Nylund (1997) encountered while looking at ECM fungi on Scots pine and Norway spruce. Since my primer set readily amplifies ascomycete DNA then this may explain why there were more molecular patterns than morphotypes and why I had amplification from NM root tips.

Our observations of endophytic fungi in ECM roots would agree with the findings of Summerbell (1989), who was able to isolate *Phialocephala fortinii*, a dark septate endophytic fungus, from serially washed ectomycorrhizal roots of black spruce. Others have also found various conifers that harbor a variety of these DSE fungi within their roots (Holdenrieder and Sieber, 1992; Harney *et al.*, 1997).

There are four possible explanations to explain why the DNA of these endophytes amplified instead of that of the target ectomycorrhizal fungus which was observed upon the root tip. First, although a mantle was present the ECM fungus may not have been viable. This is especially a problem for ECM with darkly pigmented hyphae such as *Cenococcum geophilum* (type 1) and type 3. After death DNA is quickly broken down and would yield little to no amplifiable ECM DNA. If there were endophytic fungi present below the old mantle then this could produce a positive amplification of non-target DNA. A second scenario is that competitive PCR was occurring, that is

competition between DNA templates belonging to two or more fungal species. Not all DNA amplifies equally efficiently. Therefore, rDNA from an ECM and an endophytic fungus may have been present in the extraction solution, however during the PCR reaction the endophyte's DNA had a higher affinity for the primers thus being preferentially amplified over the ECM template. A third potential problem is differential concentration of DNA template for each fungus. Some ECM fungi do not produce uniform, thick mantles (figure 9). Instead their mantles are thin, and patchy (figure 10). In the case of those ECM fungi with thin and patchy mantles the amount of DNA present in the hyphae is likely to be lower than that of ECM fungi with thick mantles. Therefore, an endophyte inhabiting a root tip that was host to a thin, patchy mantled ECM fungus, may provide relatively more DNA for the PCR reaction than that of the ECM fungus. A final possible cause of differential amplification is the presence of inhibitory compounds naturally occurring in the DNA extracts. These compounds can interfere with the normal operation of the PCR reaction. They may bind to the DNA polymerase used in the reaction or they may interact with the template DNA and prevent amplification. The latter is dependent on the characteristics of the template. Just as some DNAs have higher affinities towards primer annealing, some are more resistant to inhibitory compounds. Therefore, in the presence of a PCR inhibiting compound, some ECM DNAs may not have been able to take part in the amplification reaction, but an endophyte's DNA may not have been affected.

It is quite apparent that trying to amplify DNA from an ECM fungus, on an individual root tip, is no easy task! There will always be the presence of other potential DNA

templates, whether they come from other ECMs, surface non-mycorrhizal fungi, or endophytic fungi. Thus, if we wish to better understand mycorrhizal communities, we must gain a better knowledge of how; our primers behave *in situ*, how we can better protect DNAs from inhibitory compounds, how different DNAs interact during the amplification process, and how to improve screening techniques so that we can reduce the chances of including several fungal DNAs into a single extraction (if possible).

4.2.4: Non-Target DNAs: Now you see 'em, now you don't!

Many researchers have looked at the morphological and ecological characters of endophyte fungi (Peterson *et al.*, 1980; Wang and Wilcox, 1985; Wilcox and Wang, 1987; Stoyke and Currah, 1991; Holdenrieder, and Sieber, 1992; Stoyke *et al.*, 1992; Currah *et al.*, 1993; Currah and Tsuneda, 1993; Carroll, 1995; Ahlich and Sieber, 1996; Fernando and Currah, 1996; Bayman *et al.*, 1997). Researchers have found that these endophytes exist extensively throughout plant systems. They are quite numerous within the leaves of some trees (Carroll, 1995). They have been isolated form bark samples (Bissegger and Sieber, 1994). They are also quite often found throughout the roots of many plants (Stoyke *et al.*, 1992; Currah *et al.*, 1993; Harney *et al.*, 1997). Our data for the NM tips showed that: (1) every treatment had a very diverse set of molecular species, (2) some treatments showed sister taxa that originated from the same seedling, and (3) the treatment type may have affected the community structure, with respect to species composition.

The first observation is easily supported by the phylograms (figures 20 to 28), and from the calculated *Phi index* (table 21, section 3.2.7). Within each burn treatment the

diversity tends to be quite extensive, some plots more so than others. For example, plot 1 (low burn, Figure 11) shows at least 33 distinct molecular species. The distances are so large, greater than 5%, between most isolates, this is justification enough to support their claims as being distinct species. It was found that distances greater than 5% indicated that compared samples had all three enzyme patterns varying. According to Kårén *et al.* (1997), a three enzyme variation within the ITS region of rDNA tends to be a satisfactory criteria for separation of most mycorrhizal species.

However, when I looked at the control treatments, it was seen that two of the three plots (3 and 6) contained a large group of isolates with distances of 10% or less, between each other (figures 13 and 16). This suggests that samples were more closely related. The other control treatment, plot 9, showed a very high degree of diversity. The larger distances indicate that there are fewer closely related RFLP genotypes. As to why this control did not show the same trends as its sister treatments, no answer is yet available.

Our data suggests that burned soils contain a higher diversity of endophytic fungi than that of the controls. Endophytic fungi can be latent pathogens, mutualist, and/or saprobes (Carroll, 1995). Carroll (1995) observed that endophytic fungi tend to restrict themselves to a few cells within tissues. Since endophytes encompasses such a large group of fungal types, and the ability for several species to exist within a single host may explain the high degree of diversity found on the burned sites. After burning there was plenty of damaged plant material left behind which could have provided a multitude of host material necessary for pathogenic and saprophytic endophytes. Once the spruce

seedlings were planted then fresh plant tissue could possibly provide and attraction for many of these endophytes.

Within the control plots there is the possibility that the endophytic community is becoming more homogeneous. It could be a result of limited resources requiring specialized endophytic species which can tap these resources. An extended project looking at yearly, or even, seasonal, changes in the endophyte community would be in order before any conclusions could be made.

It was noticed that within many of the treatments there could be seen particular groupings of endophytes which had originated from the same seedling. For example, consider 'genotype I' within figure 31. This group contains one, possibly two (closely related), molecular type(s) for what was assumed to be at least three different fungal morphotypes (types 0, 4 and 6). It would appear that this group, or genet, was the predominant fungal type within that seedling since no other molecular types are shown to exist for that seedling. A total of ten root tips were randomly selected from each seedling, thus on a microscopic level, providing a vast amount of tissue material for potential endophyte colonization. Carroll (1995) found that within an individual Douglas-fir needle there existed at least five endophyte species. Carroll suggests that these fungi occupy extremely limited domains within plant tissues. Thus, given the amount of material I used and the vastness of the selection potential, it would seem that my findings do not agree with that of Carroll. It appears that my seedling's root system was taken over by either a single individual (a clone), or an individual species (a multitude of individuals). This is similar to what Liu *et. al.* (1998) found when they looked at ericoid

endophytes within *Woollsia pungens*. They found that some of their root systems seemed to contain only one endophyte genet. In support of Carroll's observations there are also numerous examples within my data in which a single seedling supports 3 or 4 distinct molecular species. These genets can be found in every treatment, but are most strongly supported in the control plots (plots 3 and 6; figures 13 and 16, respectively), thus explaining the lower Phi index values for these plots (table 21).

4.3: ECM Diversity

4.3.1: Identification of ECM root tips

It was not surprising that I was unable to identify such a large portion of my ECM molecular types, only 0.6%. It is becoming increasingly clear that above ground fruiting structures do not correlate well with below ground mycorrhizal fungi (Read, 1984; [1]Gardes and Bruns, 1996). Even though my sampling of fruitbodies took place over two seasons, it appears that I barely came close to collecting one percent of the mycorrhizal fruit bodies. As fungal fruiting is so dependent upon a variety of environment factors it is not inconceivable that many of my ECM fungi did not produce fruiting structures in either summer. It is quite likely that I missed the fruiting of many of the ectomycorrhizal fungi inhabiting the root tips. It is also possible that these fungi may have produced fruiting structures that were either too small or buried beneath litter and soil, thus preventing us from detecting them.

Although morphological and molecular data can provide insights into better understandings of mycorrhizal biodiversity, both techniques in unison give maximum

clarity. Without this union, scientists may over, or under estimate the true mycorrhizal biodiversity.

4.3.2: Why no change in ECM diversity?

Our study found that there was no significant difference in diversity between ECM fungal communities existing on control plots compared to that of burned plots, regardless of burn intensity. This would suggest that ectomycorrhizal fungi within the Glide Lake region could be composed of species that can tolerate the physical and chemical effects that accompany burning and/or have a high capability for recolonization after disturbance. If this is the case it would suggest that the site itself may have experienced past pressures, whether wildfire or some other type of disturbance, which caused the ECM community to contain only those species that could thrive after such disturbances. Therefore, with respect to the planting of black spruce it would seem that the intensity of the prescribed burn would have no harmful effects on the ectomycorrhizal populations inhabiting their root systems. However, this is not to say that these burning techniques would not alter the growing regime of the ectomycorrhizal community. Although the diversity of the ECM community may remain the same as the control, it does not mean that the uptake of nutrients and water will be equivalent. Water and nutrient availability for burned areas are drastically altered in comparison to unburned areas. With less vegetative cover there is an increased amount of moisture evaporating from burned soils. Ash left after burning will alter the pH of burned soils and allow N and P to be leached into the soils. Unburned soils tend to have lower concentrations of N and P due to their being tied up in above ground vegetative matter (Neal *et al.*, 1965; Boyle, 1973; Grier,

1975; Sims, 1975; Wells *et al.*, 1979; Feller, 1982; Vold, 1982). These alterations to the soil may not have been enough to change the species composition of the ECM community, but there could be major alterations to the types and amounts of nutrients extracted by the fungi. For example, one particular species of mycorrhizal fungus may have a slightly faster growth rate than that of another, thus allowing it to explore a larger volume of soil and have access to larger volumes of nutrients. This in turn could drastically alter the growth rates of the host, in this case black spruce.

Our assumption that ECM fungal diversity has not been affected by the prescribed burning treatments must be viewed in relation to black spruce. Since the site is situated within a mixed forest (balsam fir, paper birch, and black spruce), there is no doubt other ECM fungi exist within the surrounding soils. However, the out planted black spruce may not be the preferred host for some of these other ECM fungi, thus the survey would have missed them. As such a broader host range would need to be investigated in order to determine the overall effects of burning on the ECM fungal community.

Another factor to take into consideration concerns timing. This is making reference to the time of the burning of the sites and the collection of the root tips. The study sites were burned around mid august of 1993, however the collection of root tips did not occur until two years later. There is no doubt that burning has significant effects upon microbial soil populations, however it does allow for the release of oxides, hydroxides and salts which can promote bacterial and fungal, saprophytic and mycorrhizal, growth (Petersen, 1970; Gochenaur, 1981; Neal *et al.*, 1965). The time required for mycorrhizal populations to return to pre-fire conditions can vary from a few months to several years

(Neal *et al.*, 1965; Bissett and Parkinson, 1980). There might have been a different pattern if root tip collection had been implemented after the first year. It is possible, although unlikely, that ectomycorrhizal fungal populations may already have returned to pre-burn conditions over the two years prior to collection.

From my results it would appear that foresters need not be overly concerned with prescribed burning, nor the intensity of these burns, affecting the ectomycorrhizal diversity of outplanted black spruce. Seedlings inhabiting prescribed burned sites did not have significantly lower ECM diversity than those planted upon unburned sites.

4.4: ECMs and Endophytes: What is the connection?

Why did I amplify so many putative endophytic fungi? This is a question that does not have an easy answer. For decades researchers have been trying to understand the function and structure of ectomycorrhizal communities. The endophyte community, which had been ignored for quite a while, due to the difficulties associated with their culturing and characterization, is only just recently being explored.

Following are some hypotheses regarding the relationship between endophytic fungi and ECM fungi. (1) ECM may protect roots from infection by endophytes. It is known that certain ectomycorrhizal fungi can protect the host against pathogenic fungi. ECMs can present both physical and chemical barriers to invading fungi (Zak, 1964; Richard *et al.*, 1971). Many endophytes are thought to be pathogenic, although some appear to provide growth benefits under marginal conditions (Wilcox and Wang, 1987). Endophytes can also exist within plant tissues without causing damage to the host. Wilcox (1983) referred to these endophytes as latent pathogens. Therefore, endophytes

may represent a range of symbiotic associations, from parasitic to commensalistic or even mutualistic. Whatever the type of symbiosis, it is likely that endophytes represent more of a net carbon drain than the ECM fungi associated with the root. If so, I hypothesize that endophytic fungi would be reduced in frequency in root tips that are colonized by ECM fungi. Unfortunately, my experimental design did not allow us to test this hypothesis. I did observe fewer non-target amplifications from roots with an obvious ECM mantle. However, since I amplified from whole root tips, I could not determine whether endophytes are less frequent in ECM roots, or whether endophytes are less likely to be amplified from ECM roots because of the greater quantity of ECM template DNA available for PCR. In order to distinguish whether this is a difference in frequency of the fungi, rather than difference in relative quantity of template for PCR, we would have to remove the ECM mantles from the roots and amplify. Although, we must realize that removing the ECM mantle is only 'reducing' the amount of ECM hyphal material available in the reaction. The Hartig net formed by the ECM fungus will still provide a template. If the root sample with ECM mantles removed have fewer non-target amplifications than NM tips, it would suggest that ECM play a role in restricting infection by endophytic fungi. A second hypothesis is that both the ECM and endophytic fungi have some type of symbiotic, mutalistic relationship with one another. Not all ECMs provide a protective advantage for the host, however, it has been shown that some endophytic fungi demonstrate antagonism towards certain insects (Webber, 1981; Wilson, 1992). Maybe the ECM fungus is taking advantage of the endophyte's protective ability. The ECM may or may not be conferring some sort of benefit upon the

endophyte in return. It would require further experimentation to determine if there are such secondary associations.

4.5: Intraspecific variation and molecular species

Not all samples which display a different RFLP pattern are distinct species. For example, there were several morphotypes which displayed two or more similar but distinct RFLP patterns, which can be attributed to intraspecific variation. Intraspecific variation has been investigated by numerous researchers, such as LaChance *et al.* (1985), LoBuglio *et al.* (1991), Chew *et al.* (1997), Sequerra *et al.* (1997), and Karén *et al.* (1997). These and others have shown that the amount of intraspecific variation within fungi can vary from one species to another, depending upon the techniques used to assess the variation.

RFLP analysis of 'type 1', which on the basis of gross morphology was recognized as *Cenococcum geophilum*, revealed at least 4 (possibly 6) different molecular types. Table 16 shows that most of the variation was noticeable with the use of the restriction endonuclease, *Hinf* I. The presence of possibly 6 different polymorphs from 75 samples suggests that *C. geophilum*, although a morphologically distinct species, could actually be several species that display similar morphological traits. This would agree with the findings of LoBuglio *et al.* (1991) who found that from their 71 isolates of *C. geophilum*, 32 distinct restriction fragment length phenotypes arose. They suggest that *C. geophilum* may be a polyphyletic group that has undergone phenotypic morphological convergence. If this is the case, the question arises, how many other morphologically distinct mycorrhizal fungi actually consist of an aggregation of several molecular species? Such

findings could have important implementations. For instance, *C. geophilum* has been found on over 200 tree species (Trappe 1962; Trappe, 1964; Chilvers 1968; Molina and Trappe 1982) which indicates its ecological importance. Mycorrhizal fungi vary in their ability to increase nutrient and water uptake (Rousseau *et al.*, 1994; Boyd *et al.*, 1986; St. John and Coleman, 1983; Boyle and Hellenbrand, 1991), and release different enzymes into the soil thus allowing the host plant access to a variety of nutrient pools (Marschner and Dell, 1994; Li *et al.*, 1991; Ho, 1989). Therefore, if a mycorrhizal fungus such as *C. geophilum* is actually a complex of different species, each taxon may provide different benefits to the plant. This could have implications for reforestation, as inoculation with strains native to a site may produce better results. More research needs to be done to determine if strains that previously existed on a site are better suited for nutrient acquisition within that area, and thus allow new seedlings to establish more effectively.

Morphological characterization has always played an important role in species identification, however as we investigate organisms as variable as mycorrhizal fungi we are finding that this method of classification can be deceiving. Due to the variety of hosts and environments that one mycorrhizal fungal species can be associated with, it is not inconceivable that one species could take on a different morphological traits in different environments and on different hosts. As such we must draw upon other tools, such as molecular characterization, in order to escape the pitfalls imposed by traditional methods. This is not to say that molecular identification does not have its own problems. As previously mentioned, there may be numerous fungi inhabiting a single root tip and there

is no guarantee that the mycorrhizal fungus viewed on the root tip will be the one that gets amplified during the PCR process. Such factors as competitive PCR, template affinity to oligonucleotide primers, viability of the fungus in question, and the type of inhibitory compounds associated with the root tip all determine to which fungus the final PCR product will belong. Just as a single mycorrhizal fungal species can have a variety of physical characteristics that vary according to host and environment, the same can be said for its molecular characters. The previous paragraphs have shown that polymorphic types for a single species can cause confusion. Is it a polymorphism or is it another closely related species? This is a difficult question to answer since at the molecular level we are still unsure how to classify a species. As such, tools which have worked successfully for classification based upon morphology can not always be applied at the molecular level. For example, both the Shannon-Wiener and Simpson indices assume that one can distinguish individual species and count numbers of individuals. Neither of these properties can be easily evaluated from molecular data on mycorrhizal fungi. As indicated previously, molecular genotypes do not always correspond to morphological species, because molecular analyses capture some proportion of intraspecific as well as interspecific genetic variation. If the average proportion of intraspecific variation in a sample varies from plot to plot, this can have significant impacts upon diversity index estimates. Also, we can not assume that each root tip represents a distinct mycorrhizal 'individual'. Fungi have indeterminate growth that is clonal, thus a single clonal individual could infect multiple root tips on a single plant, but would be counted as multiple individuals. For these reasons molecular data from mycorrhizal fungal samples

are particularly unsuited to analysis using traditional diversity indices. This is why new indices, such as the Phi Index, which do not depend upon knowledge of species and individuals are important.

4.6: Effects of prescribed burning on ECM community structure

It has been in only the past four or five years that people have started to use mycorrhizal roots as a means of investigating mycorrhizal community structure (Erland, 1995; Mehmann *et al.*, 1995; [1]Gardes and Bruns, 1996; Dahlberg *et al.*, 1997; Kårén and Nylund, 1997; Erland *et al.*, unpublished). Many of these researchers have found that traditional methods, using sporocarp data, provide an inaccurate account of the mycorrhizal community structure on root-tips, which underlines the benefits of using PCR based methods to get a better understanding of local mycorrhizal populations.

Our study was concerned with how an invoked disturbance, fire, affected the structural composition of an ectomycorrhizal community, at the species level, inhabiting the roots of black spruce seedlings. With respect to the statistical analysis of the ECM molecular diversity, using traditional indices (Simpson and Shannon-Wiener), it was found that there were no significant differences between control sites and burned sites, both high and low intensity burns. I also found that there was no significant differences between the ECM molecular diversity when the two burned treatments were compared. The variation in the intensity of prescribed fire does not appear to change the overall species composition of ECM fungi that colonize black spruce seedlings. These results are similar to what Kårén *et al.* (1997) discovered when investigating the effects of different regenerating methods upon mycorrhizal community structure. They found that

the method of forest regeneration, shelterwood verses clear-cut and planting, only affected the abundance of one of forty-three RFLP mycorrhizal types. This mycorrhizal type was more prevalent in the planted sites, than compared to the shelterwood and old forest. Another study by Kårén and Nylund (1996) found that the application of nitrogen-free fertilizer to Norway spruce stands did not have any drastic effects on the abundance of major ECM morphotypes.

Although there were no significant differences concerning the diversity of mycorrhizal genotypes, there were subtle differences with respect to community structure in regards to control plots verses treatment plots. The most obvious difference concerns the occurrence of genets, molecular species occurring on the same seedling which share very similar or identical RFLP patterns. Control plots contained a larger number of these genets, with smaller phylogenetic distances, in comparison to the high and low burned plots. What would cause these genets to be more infrequent, and more phylogenetically distant within the burned treatments? One reason may have to deal with the availability of mineral nutrients. Gibson and Deacon (1990) found that the types of mineral nutrients available to a fungus will affect its ability to colonize host roots. Burning releases usable mineral nitrogen, previously less available in the form of plant material, into the soil (Boyle, 1973; Grier, 1975; Neal *et al.*, 1965; Viro, 1974; Vold, 1982). Therefore, the mycorrhizal species previously residing on clear-cut plots may not have been destroyed by the fire but their numbers may have been reduced enough to allow other fungal species to move in that needed, or could utilize, this excess nitrogen which was previously unavailable. This hypothesis is supported by Arnebrant and Söderström

(1992), and Kårén and Nylund (1997) who showed that nitrogen fertilization did affect ECM community structure.

Another explanation for the higher frequency of genets within the control sites may have to do with competition effects. Some species of fungi may be better suited for a particular environment. Such species may have a similar genotype. Thus these closely related species may colonize new root tips and prevent different mycorrhizal genotypes, with a greater degree of variation, from moving into the community. This would reduce the degree of variation among genotypes, thus producing genets. However, after a disturbance such as fire or clear-cutting, the habitat has changed and new fungal colonists move in, or recolonize from soil propagules, thus displaying fewer genets.

4.7: Diversity index dilemmas

This study emphasizes the major problems associated with the use of traditional diversity indices to analyze species data at the molecular level. Both the Shannon-Wiener and Simpson indices make the major assumption that the investigator is clear on what is a species. The species concept is still being debated at the molecular level, and to some degree at the phenotypic level (Harrison, 1991). My study appeared to have several cases of intraspecific variation, which in turn showed that the traditional diversity indices could not handle such scenarios. I was relatively sure that these samples were the same genotype, but due to the design of the Shannon and Simpson indices, I had to count them as separate RFLP genotypes. As a result I was introducing errors into the statistical analysis which in turn may have affected the final outcome.

Due to the difficulties associated with these traditional diversity indices, I realized that a new index needed to be created in order to provide a better assessment of molecular data. I realized that the one reliable factor which would take into consideration the problem of intraspecific variation was the phylogenetic distance. This value is a measure of how closely related one individual is to another. The greater the phylogenetic distance between two individuals, then the greater the chance that they are different species. Based upon this assumption, I developed the *Phi index*. The example using hypothetical phylograms (section 3.2.7) showed that the use of the phylogenetic distance does seem to be a good estimator of diversity. When I used the *Phi index* against my data, I found that there was still no significant difference between control and treatment ECM diversities. I also found that the values obtained for each treatment (pertaining to the mycorrhizal data) showed a trend (control having the lowest diversity and high intensity burns having the greatest diversity), much different than those observed for the traditional indices, that seemed to be more explainable.

Since traditional (Shannon-Wiener and Simpson) indices requires one to know what is a species, their use in the analysis of molecular data is very limited. Their inability to decipher closely related individuals from that of intraspecific individuals, increases the chance of overestimating the diversity. Our newly formulated *Phi index* seemed to handle this problem without any difficulty, but I had limited the testing of this index to only a few scenarios. There is also the case in which interspecific variation may exist, but is not detected using the RFLP data. For example, Kårén *et al.* (1997) found that there were several *Cortinarius* species that could not be separated using three restriction

endonuclease enzymes, yet these samples were considered distinct species. On an evolutionary scale this would suggest that these are recent divergences. The Phi index would register this as being a low diversity value. This would in essence bias the reading as an underestimation of the overall diversity. However, an overestimation error would seem much more common than that of an underestimation. Before I can declare this as an alternative index more rigorous testing must be done.

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Appendices:

Appendix I

I.1: Regen 1: Seedling and sporocarp collections

In late August of 1993 mycorrhizal specimens were collected from 3 year old, naturally regenerating balsam fir and black spruce seedlings. Three seedling samples of each type were collected from six different locations within the study site. Three collection sites were situated near or within a 70 year old forest. Spruce seedlings of the required age were easily found under the dense canopy, however I was unsuccessful in finding fir seedlings within the forest boundary. I collected the fir seedlings within a three (3) meter distance of the forest/clear-cut boundary. The other three collection sites were located towards the central portion of the clear-cut region, distances ranging from 40-100m. Basidiocarps were collected from the clear-cut and the surrounding forest in order to provide information on the diversity of basidiomycetes within the surrounding area. Also they were to be used as references for the collected mycorrhizal samples. These basidiocarps were stored in brown paper bags and allowed to air dry for preservation.

In the fall of 1994 another 24 seedlings, 12 of each species, were collected. Figure A-1 shows where the seedlings were collected. Dirk Krüger collected sporocarps from these sites which would be used as references in order to try and identify ectomycorrhizal types found on the study site.

Figure A-1: Schematic layout of collection area near Glide Lake. Refer to table 1 for a more detailed description of each site. Three tree species were common to this area: black spruce (), balsam fir (), and paper birch ()

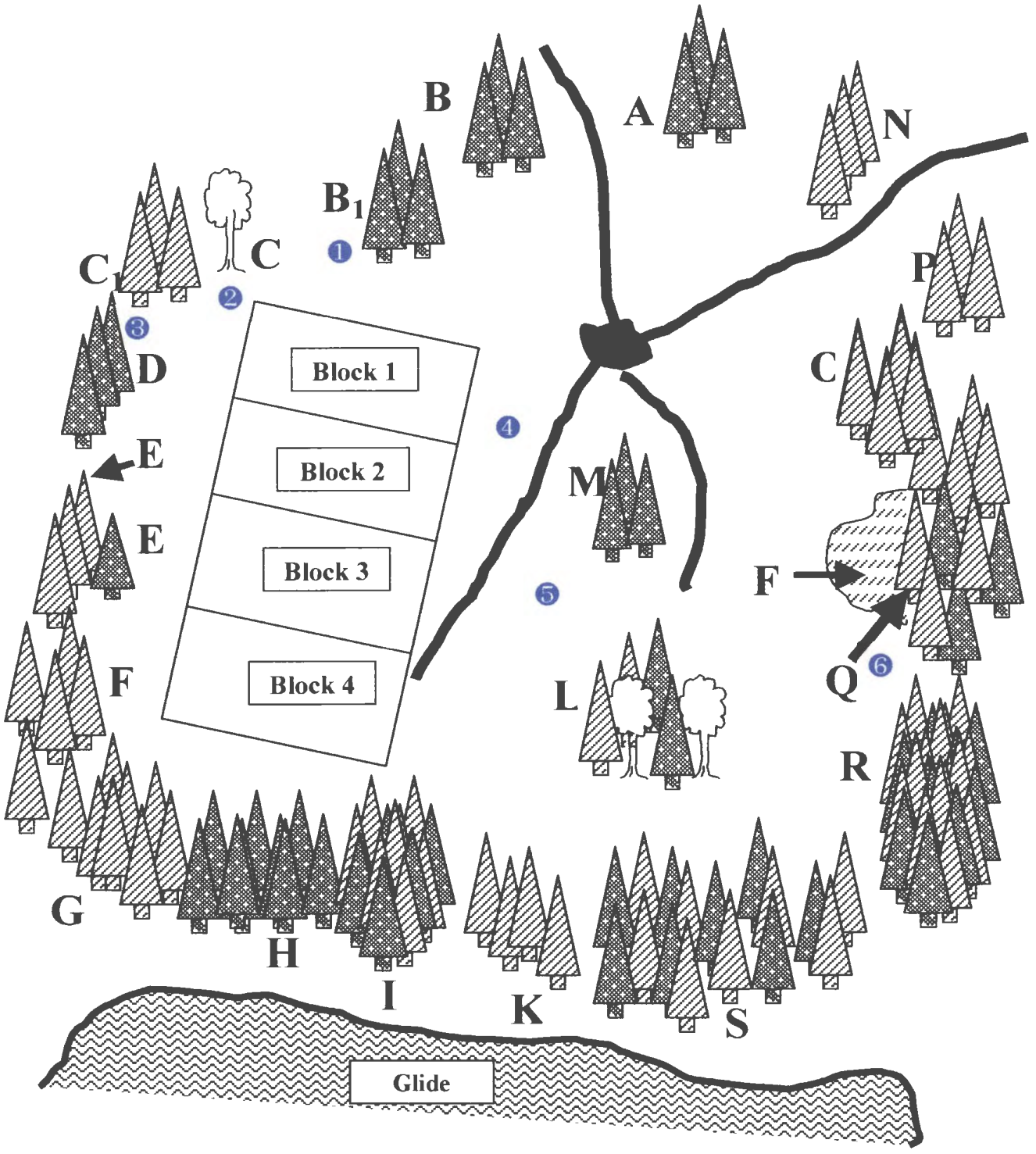
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Table 1: Brief site descriptions within the Glide Lake study area. All site names are taken from Figure 3 on the previous page.

Site	Description
A	*Picea* dominant, soil very moist and carpeted with mosses
B_1/B_2	*Picea* predominant, some *Juniperus sp* and mosses
C_1	*Abies* predominant with grasses, *Equisetum spp*, and water puddles present
C_2	Small group of *Betulla papyrifera* Marsh, and mosses
D	Scattered group of *Picea*, moss carpet
E_1	*Abies* predominant with grasses, ferns, *Equisetum spp*, extremely wet (open water)
E_2	*Picea* predominant, moss carpet
F	Scattered *Abies* and *Larix laricina* (Du Roi) K. Koch with grasses and water puddles
F_C	Fresh clear-cut, less than a year old
G	*Abies* predominant with grasses, ferns, *Equisetum spp*, extremely wet (open water)
H	*Picea* predominant, moss carpet
I	Mixed *Abies* and *Picea*, moss and *Lycopodium spp* ground cover
K	*Abies* predominant with grasses, ferns, *Equisetum spp*, extremely wet (open water)
L	Island of *Picea/Abies/Betula*, *Lycopodium spp* and *Vaccinium spp*
M	*Picea* predominant, moss carpet
N	*Abies* predominant
O	*Abies* and *Larix laricina* (Du Roi) K. Koch with grasses and water puddles
P	*Abies* predominant with grasses, ferns, *Equisetum spp*, extremely wet (open water)
Q	Mixed *Picea/Abies*, mossy ground cover
R	*Abies* and *Picea*, mixed grasses and ferns
S	Mixed *Picea/Abies*, mossy ground cover

I.2: Mycorrhizal recovery and processing

Roots collected in august 1993 were soaked in tap water for about half an hour and then washed under a slow stream of water to remove excess debris and to expose colonized roots. Only the external portions of the roots were examined, therefore only those roots with ectomycorrhizas were recovered. Various types of mycorrhizas were described during the inspection of the roots. These types were classified on the basis of

hyphal pigmentation, hyphal density within the mantle, and the overall appearance of the hyphal strands. Each mycorrhizal type was collected by severing the root tip from the parent root. Root tips were then washed with distilled water, blotted dry and then frozen (-15°C). Later these root tips were lyophilized to ensure that freeze/thawing did not occur.

Ten, 2 cm root sections were randomly collected from each of the thirty-six root systems (eighteen of each tree species). Each 2 cm root section was surveyed with compound microscope and the percentage cover of five ectomycorrhizal types was recorded.

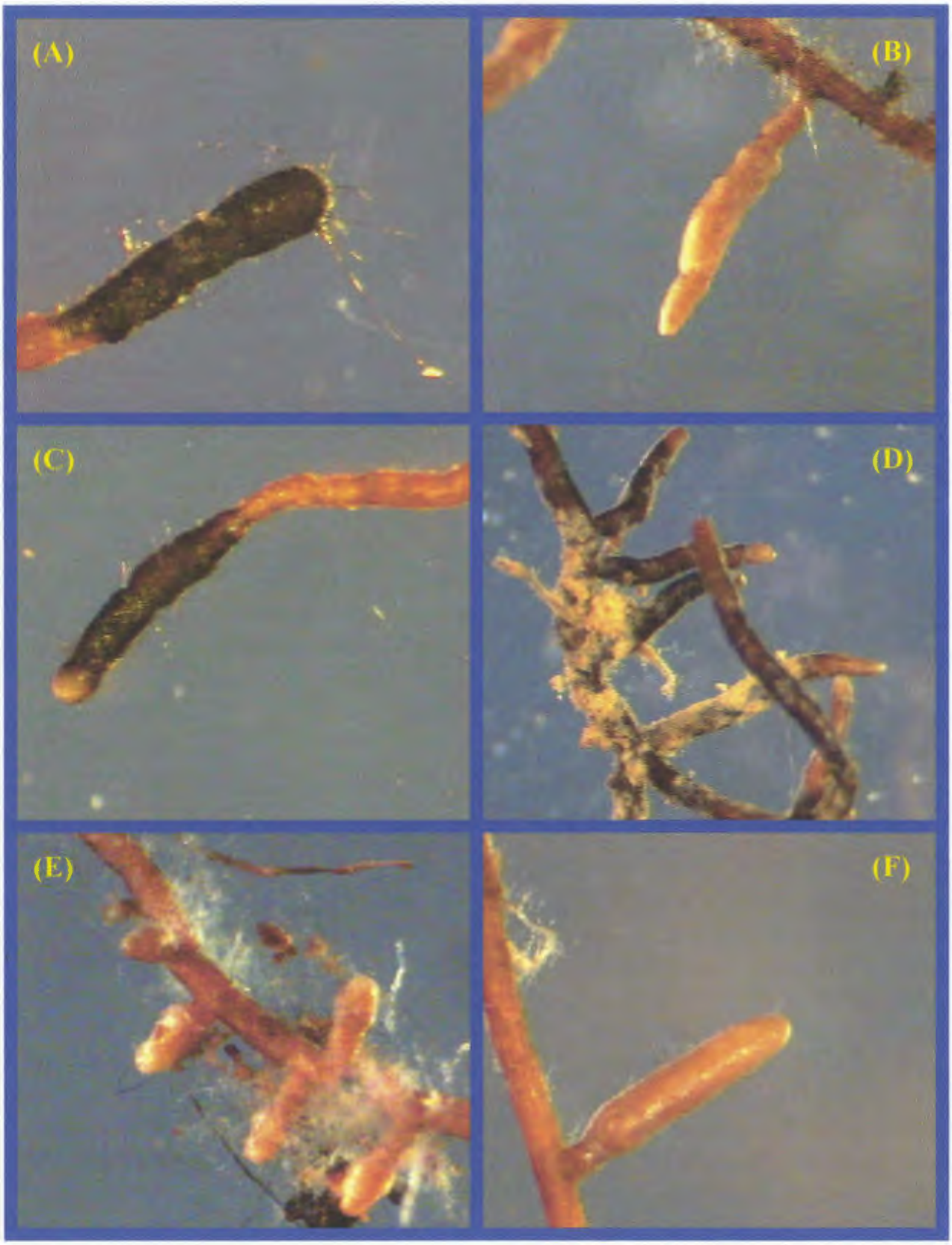
Seedlings, collected in august of 1994, containing intact rootballs were transported from the study site to the laboratory at 5°C. The roots were washed and prepared similarly as in the previous few paragraphs.

Root tip recovery and PCR procedures follow the same as those found in the Methods and Materials section. The same is true for sporocarp amplifications.

Appendix II

II.1: Morphotype plates

Figure A-2: Several photographs showing some of the morphotypes that were encountered in our study. **(A)** Type 1 (*Cenococcum geophilum*). **(B)** Type 2; notice the swelling and pointing of the tip. **(C)** Type 3; mantle is thinner and shiner than Type 1. Emanating hyphae are shorter and fewer than that of Type 1. **(D)** Type 6; no emanating hyphae and light brown in color. **(E)** Type 7; matted emanating hyphae with a cottony appearance. **(F)** Type 8; root tip is very swollen and more rounded than that of Type 2.


(A)
(B)
(C)
(D)
(E)
(F)

Appendix III

III.1: Dices index

Dice's similarity coefficient follows the formula:

$$2a/(2a+b+c)$$

This formula can be best explained using Figure IIIa. The coefficient is calculated by dividing two times the number of shared bands (since there are two samples) by the sum of the number of polymorphic bands for each sample (b and c) and twice the number of shared bands. Thus, the similarity coefficient for these two samples would be: (2*2)/(2*2+3+2) = 4/9. If we were to convert this coefficient into a distance value it would require subtracting the coefficient from one, ie. 1 - 4/9 = 5/9.

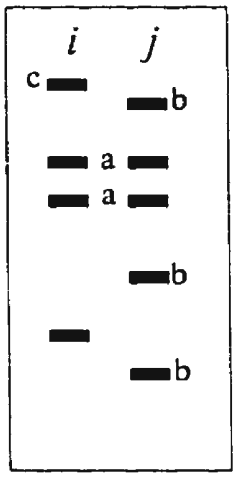


Figure IIIa: A hypothetical gel showing RFLP patterns for two samples (*i* and *j*). Shared bands are denoted with 'a', polymorphic bands for sample *i* are denoted with 'c', polymorphic bands for sample *j* are denoted with 'b'

Appendix IV

IV.1: Diversity comparisons

Shannon-Wiener

$H' = -\sum p_i \ln p_i$

p_i is the proportion of individuals found in the *i*th species, which is estimated as n_i/N. n_i= the number of individuals in the *i*th species, and N= the total number of individuals (Magurran, 1988).

This index assumes that the individuals are randomly sampled from an 'indefinitely large' population. It also assumes that all species are represented in the sample.

Simpson

$D = \sum p_i^2 = \sum [n_i(n_i - 1)/N (N - 1)]$

p_i is the proportion of individuals found in the *i*th species. n_i= the number of individuals in the *i*th species, and N= the total number of individuals.

Less weight is put on the 'unique' species, i.e. species that have only one individual.

Appendix Va

Va.1: Percentage abundance data for black spruce

Table 1: Percentage abundance of mycorrhizal fungi on Black Spruce (*Picea mariana*) roots.

Site	Tree #	Morphotype	% abundance				
1	1	6	0	0	0	1	1
			0	0	0	0	0
1	1	8	50	60	15	25	50
			5	10	25	10	0
1	2	1	10	10	0	5	0
			5	15	5	0	5
1	2	6	1	1	0	0	1
			1	0	5	0	5
1	3	3	1	1	50	30	20
			60	1	1	30	0
1	3	6	0	0	0	0	1
			0	0	0	0	0
1	3	8	10	10	15	20	50
			10	70	30	10	0
2	1	3	20	5	0	1	5
			10	5	0	5	10
2	1	8	0	0	0	0	0
			1	0	0	0	0
2	2	3	1	1	10	1	5
			5	40	30	50	0
2	2	8	5	0	1	1	10
			0	0	10	1	5
2	3	1	0	0	0	1	1
			1	5	1	1	1
3	1	1	30	1	50	10	5
			5	20	50	20	10
3	1	6	20	20	10	1	5
			0	5	5	10	5
3	2	3	5	1	0	5	10
			1	1	0	0	0
3	3	3	0	5	5	5	10
			40	5	1	0	30
3	3	10	0	0	0	1	5
			0	1	1	0	0

			0	0	0	0	0

Site	Tree #	Morphotype	% abundance				
4	1	3	1	5	0	1	40
			10	1	1	0	5
4	1	10	0	0	0	1	5
			1	0	1	0	0
4	2	3	10	5	1	5	1
			1	5	5	20	10
4	3	1	20	5	5	40	1
			10	5	1	5	1
4	3	8	10	1	0	0	15
			5	5	5	35	1
4	3	yellow	5	0	0	0	0
5	1	3	10	5	0	15	0
			10	5	0	0	0
5	1	8	5	0	0	5	0
			0	5	10	0	0
5	2	3	0	20	5	1	5
			1	1	5	5	5
5	2	8	5	5	10	0	5
			1	0	0	5	10
5	3	3	5	5	10	40	30
			50	60	1	30	30
6	1	3	1	60	5	40	15
			20	1	10	30	5
6	1	8	5	10	20	5	30
			10	0	40	5	60
6	2	3	0	50	1	20	1
			15	50	30	15	5
6	3	1	5	10	10	10	5
			20	10	5	30	60

Appendix Vb

Vb.1: Percentage abundance data for balsam fir

Table 2: Percentage abundance of mycorrhizal fungi on randomly selected Balsam Fir (*Abies balsamea*) roots.

Site	Tree #	Morphotype	% abundance				
1	1	1	0	1	0	0	5
			1	0	0	0	0
1	1	8	0	0	0	0	0
			50	5	0	6	0
1	2	1	1	1	0	1	4
			0	5	0	15	0
1	2	6	0	0	0	0	0
			0	0	1	0	0
1	2	8	5	40	7	40	60
			5	80	3	60	5
1	3	3	1	1	1	5	0
			0	1	1	0	1
1	3	8	0	1	0	0	1
			0	0	0	0	0
2	1	none					
2	2	3	2	2	10	3	2
			15	5	6	9	0
2	2	8	0	0	15	10	40
			8	10	12	6	10
2	3	6	0	0	0	1	1
			0	0	0	0	0
2	3	3	2	0	0	0	0
			0	0	0	0	0
3	1	3	0	0	10	1	5
			50	10	0	30	5
3	1	10	0	0	0	0	0
			15	40	0	40	10
3	2	3	1	1	1	1	1
			1	5	1	5	5
3	3	3	0	0	5	5	0
			1	1	0	1	0

Site	Tree #	Morphotype	% abundance				
4	1	3	0	5	0	0	5
			20	1	15	12	5
4	1	8	3	5	3	5	10
			3	15	10	1	1
4	2	3	10	5	5	5	40
			70	30	1	15	10
4	2	8	5	0	0	20	15
			0	5	20	1	30
4	3	3	0	0	0	0	0
			0	0	1	0	5
4	3	8	0	1	10	1	1
			0	0	1	5	30
5	1	1	0	1	50	10	5
			0	10	10	0	0
5	1	8	0	0	0	0	0
			0	0	0	30	1
5	2	3	1	0	1	0	10
			1	0	5	1	1
5	2	8	1	1	0	0	5
			0	0	0	5	1
5	3	3	20	10	20	1	1
			30	5	10	50	0
5	3	8	1	1	0	5	0
			0	1	1	1	1
6	1	3	0	5	5	40	5
			5	1	1	1	0
6	1	8	0	0	0	5	0
			1	50	5	0	0
6	2	3	0	0	50	1	5
			0	0	5	1	5
6	2	8	0	0	0	0	0
			10	0	0	0	0
6	3	3	0	1	0	5	0
			1	0	1	10	0

Appendix VI

VI.1: Mann-Whitney U-tests for morphotype comparisons

Mann-Whitney U-test preformed on (% abundance of a morphotype x tree species). Three morphotypes were investigated based upon data collected from the 1994 Glide Lake study.

Spr-x = Black spruce **Fir-x = Balsam fir** **-x = morphotype #**

Mann-Whitney Confidence Interval and Test

1) Morphotype 1

Spr-1 N = 18 Median = 0.000
Fir-1 N = 18 Median = 0.000
Point estimate for ETA1-ETA2 is 0.000
95.2 Percent C.I. for ETA1-ETA2 is (-0.000,-0.002)
W = 355.5
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.4864
The test is significant at 0.3389 (adjusted for ties)

Cannot reject at alpha = 0.05

2) Morphotype 3

Spr-3 N = 18 Median = 5.450
Fir-3 N = 18 Median = 1.900
Point estimate for ETA1-ETA2 is 1.100
95.2 Percent C.I. for ETA1-ETA2 is (-1.100,6.298)
W = 356.0
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.4765
The test is significant at 0.4717 (adjusted for ties)

Cannot reject at alpha = 0.05

3) Morphotype 8

Spr-8 N = 18 Median = 0.00
Fir-8 N = 18 Median = 1.20
Point estimate for ETA1-ETA2 is -0.00
95.2 Percent C.I. for ETA1-ETA2 is (-3.10,0.10)
W = 305.0
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.3843
The test is significant at 0.3624 (adjusted for ties)

Cannot reject at alpha = 0.05

Appendix VII

VII.1: Mann-Whitney U-tests for location comparisons

Black Spruce

Mann-Whitney *U*-Test (Raw results)
-location vs % abundance
Near For N = 45 Median = 0.000
In Clear N = 45 Median = 0.000
Point estimate for ETA1-ETA2 is 0.000
95.0 Percent C.I. for ETA1-ETA2 is (0.001,-0.001)
W = 2104.0
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.6513
The test is significant at 0.5939 (adjusted for ties)

Cannot reject at alpha = 0.05

-

Balsam Fir

Mann-Whitney Confidence Interval and Test

Near For N = 45 Median = 0.000
In Clear N = 45 Median = 0.000
Point estimate for ETA1-ETA2 is -0.000
95.0 Percent C.I. for ETA1-ETA2 is (-0.000,-0.001)
W = 2019.5
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.8244
The test is significant at 0.7972 (adjusted for ties)

Cannot reject at alpha = 0.05

Appendix VIII

VIII.1: Mann-Whitney U-tests for complete percent coverage comparisons

Fir %abundance N = 90 Median = 0.000
Spr %abundance N = 90 Median = 0.000
Point estimate for ETA1-ETA2 is -0.000
95.0 Percent C.I. for ETA1-ETA2 is (0.000,0.000)
W = 8105.5
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.9112
The test is significant at 0.8963 (adjusted for ties)

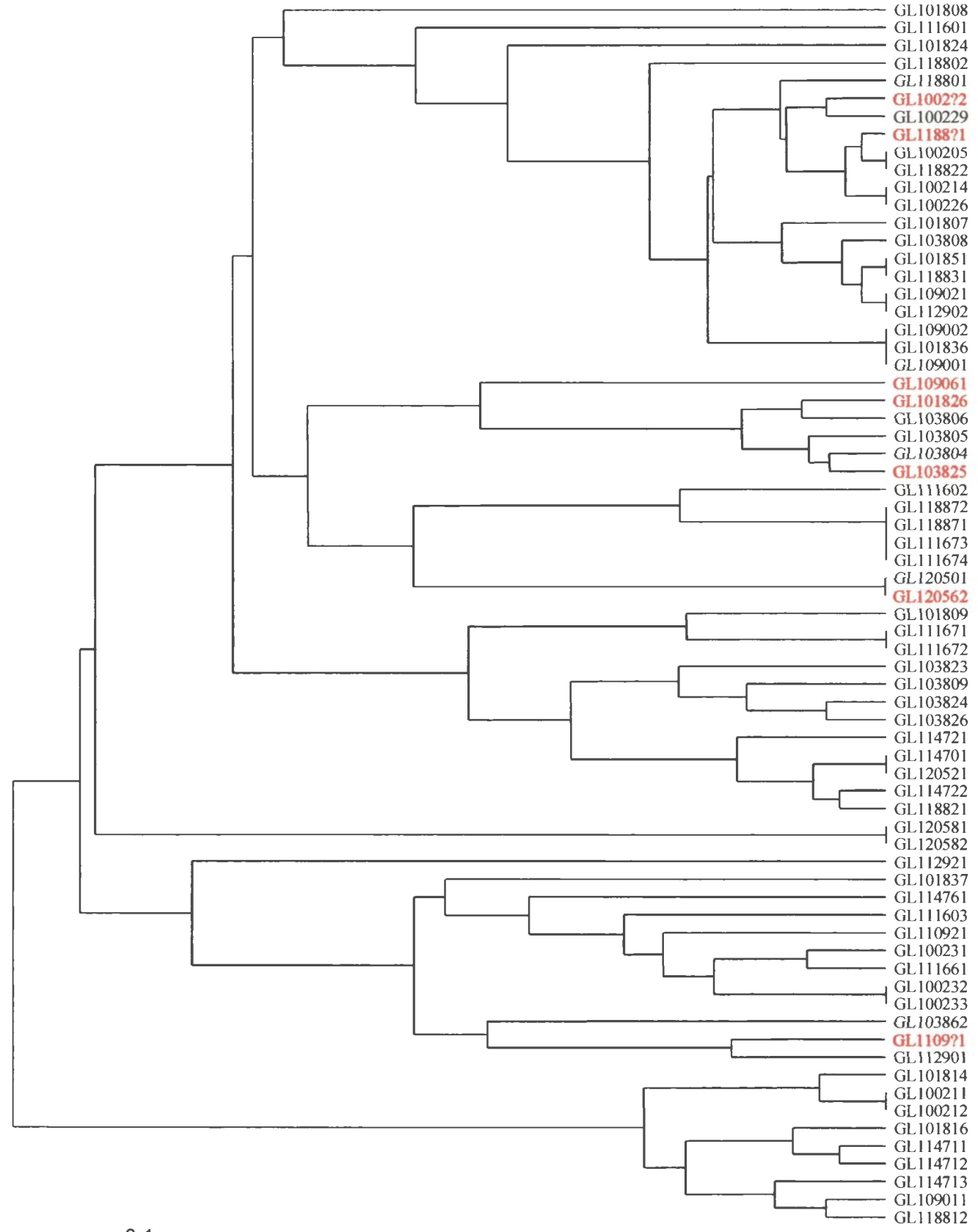
Cannot reject at alpha = 0.05

Appendix IX

IX.1: Relationships between mantled and non-mantled tips

The following figures show that some of the "mantled tips" are more closely related to the "Non-mantled tips" thus justifying their removal from the analysis of "mantled tips" only.

Figure A-3: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 1. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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GL109061
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GL111674
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GL120562
GL101809
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GL111672
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GL103824
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GL114722
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GL111661
GL100232
GL100233
GL103862
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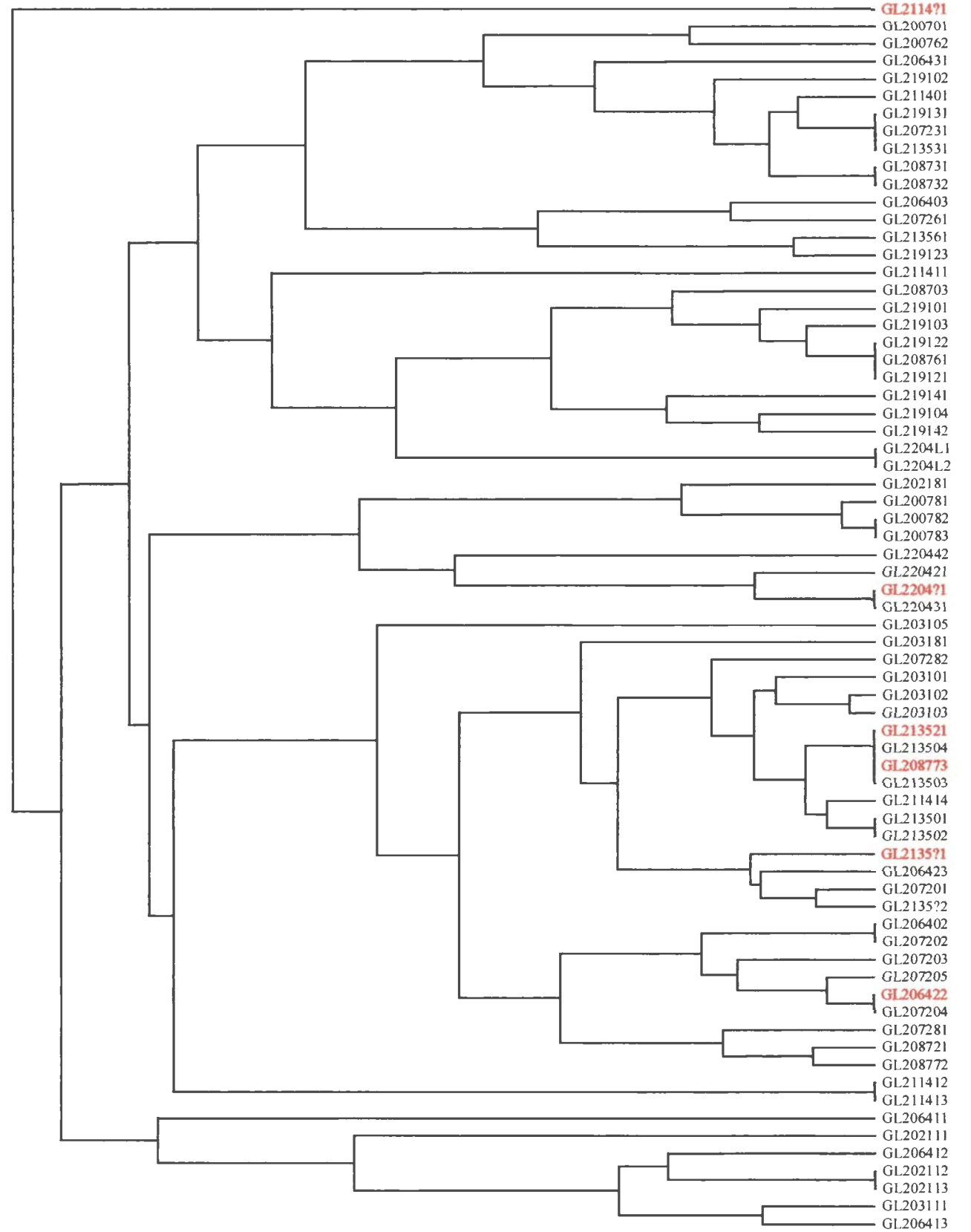
GL112901
GL101814
GL100211
GL100212
GL101816
GL114711
GL114712
GL114713
GL109011
GL118812
0.1

Figure A-4: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 2. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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GL208703
GL219101
GL219103
GL219122
GL208761
GL219121
GL219141
GL219104
GL219142
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GL2204L2
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GL200782
GL200783
GL220442
GL220421
GL2204?1
GL220431
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GL203181
GL207282
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GL203102
GL203103
GL213521
GL213504
GL208773
GL213503
GL211414
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GL213502
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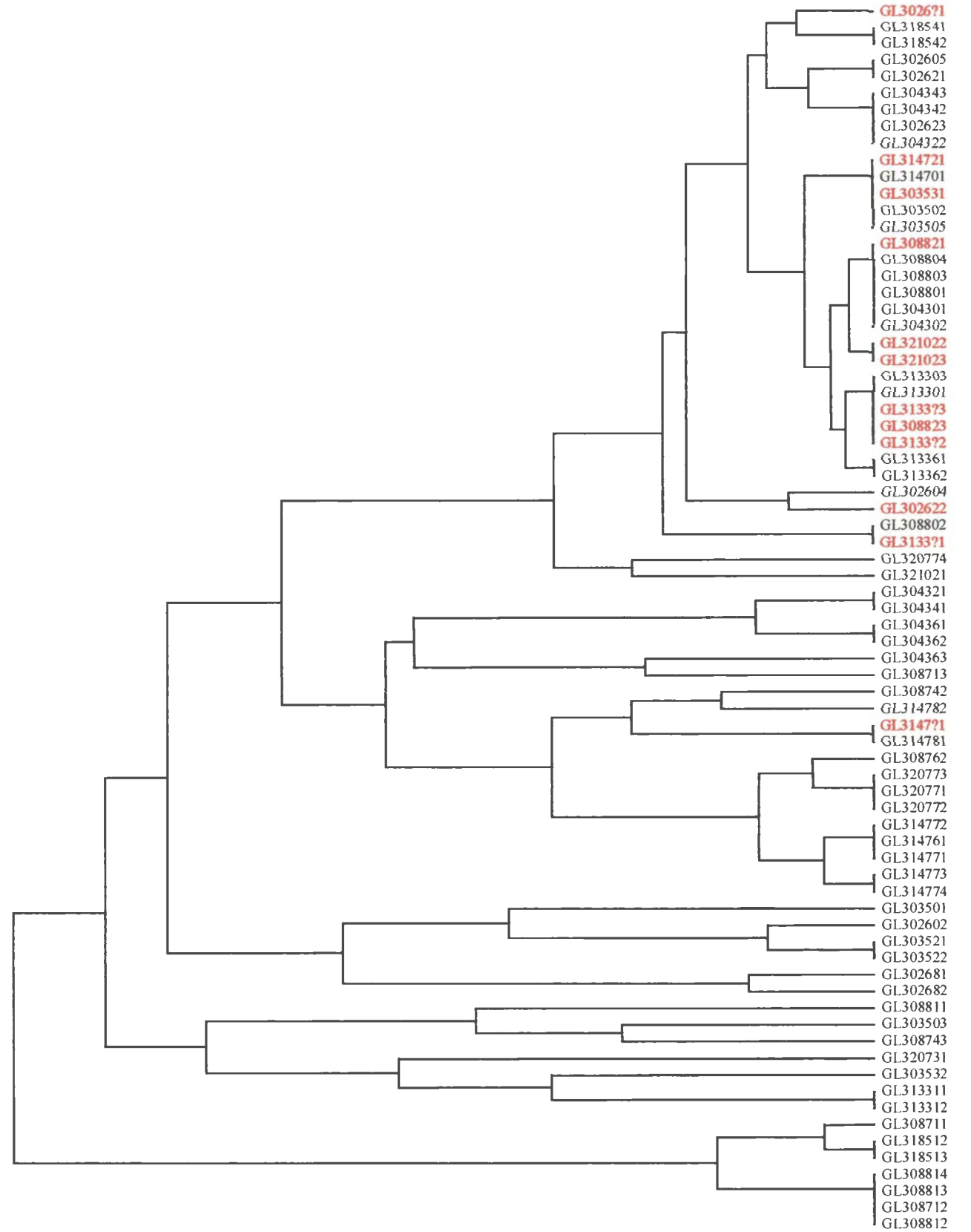
GL206412
GL202112
GL202113
GL203111
GL206413
0.1

Figure A-5: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 3. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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GL308821
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GL308803
GL308801
GL304301
GL304302
GL321022
GL321023
GL313303
GL313301
GL3133?3
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GL3133?2
GL313361
GL313362
GL302604
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GL308802
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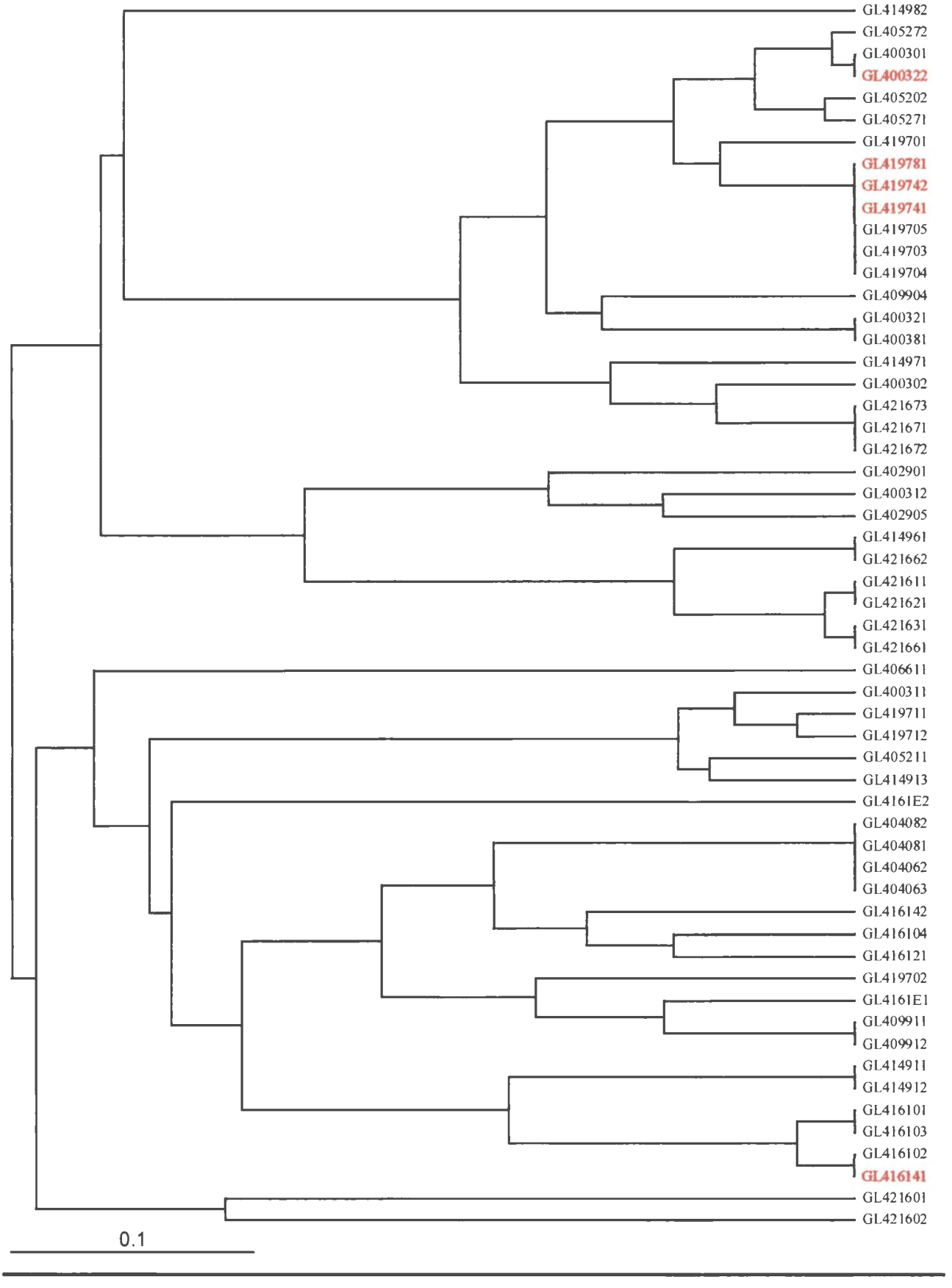
GL313312
GL308711
GL318512
GL318513
GL308814
GL308813
GL308712
GL308812
0.1

Figure A-6: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 4. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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GL414912
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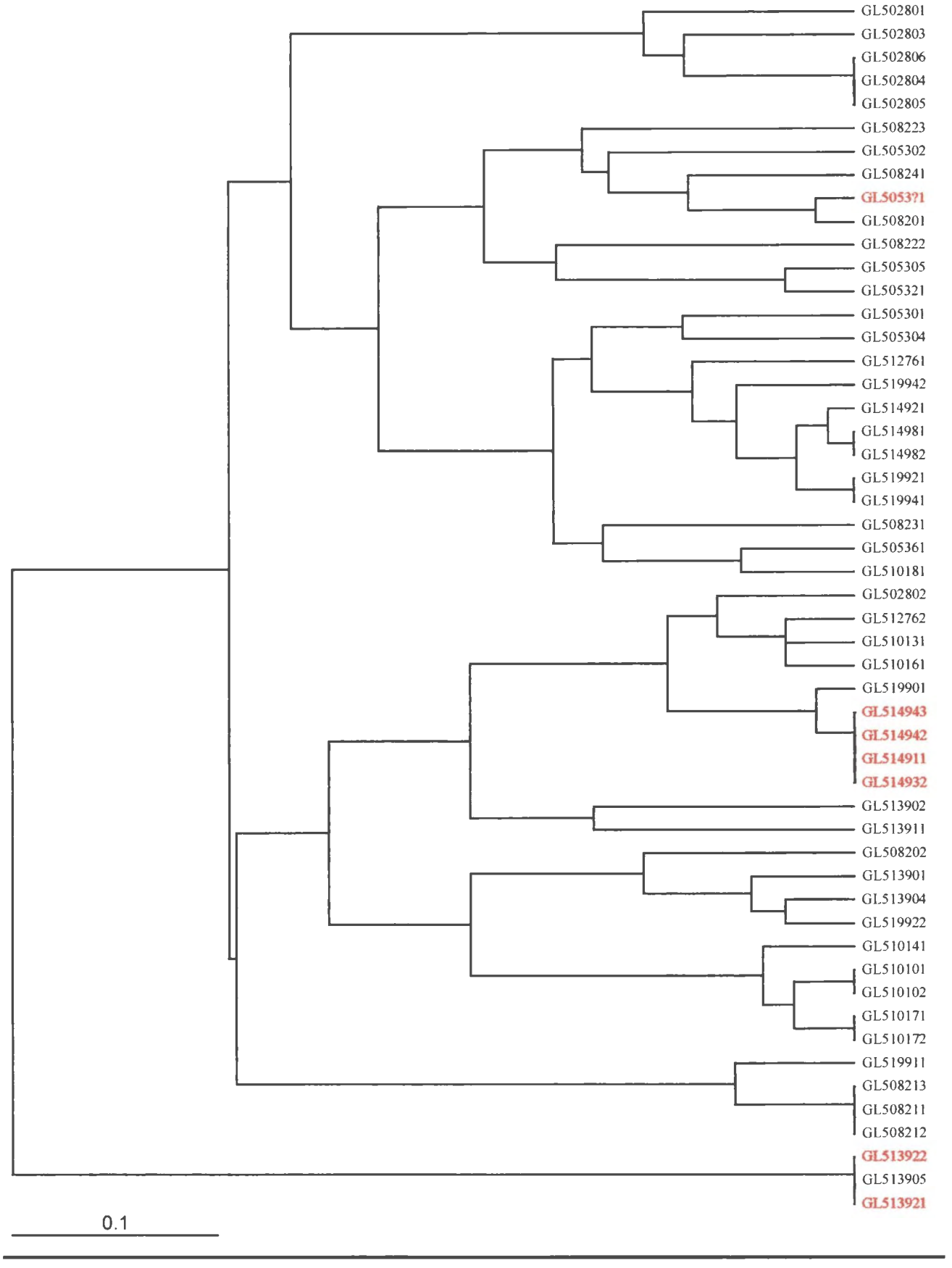
GL421602
0.1

Figure A-7: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 5. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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0.1

Figure A-8: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 6. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.

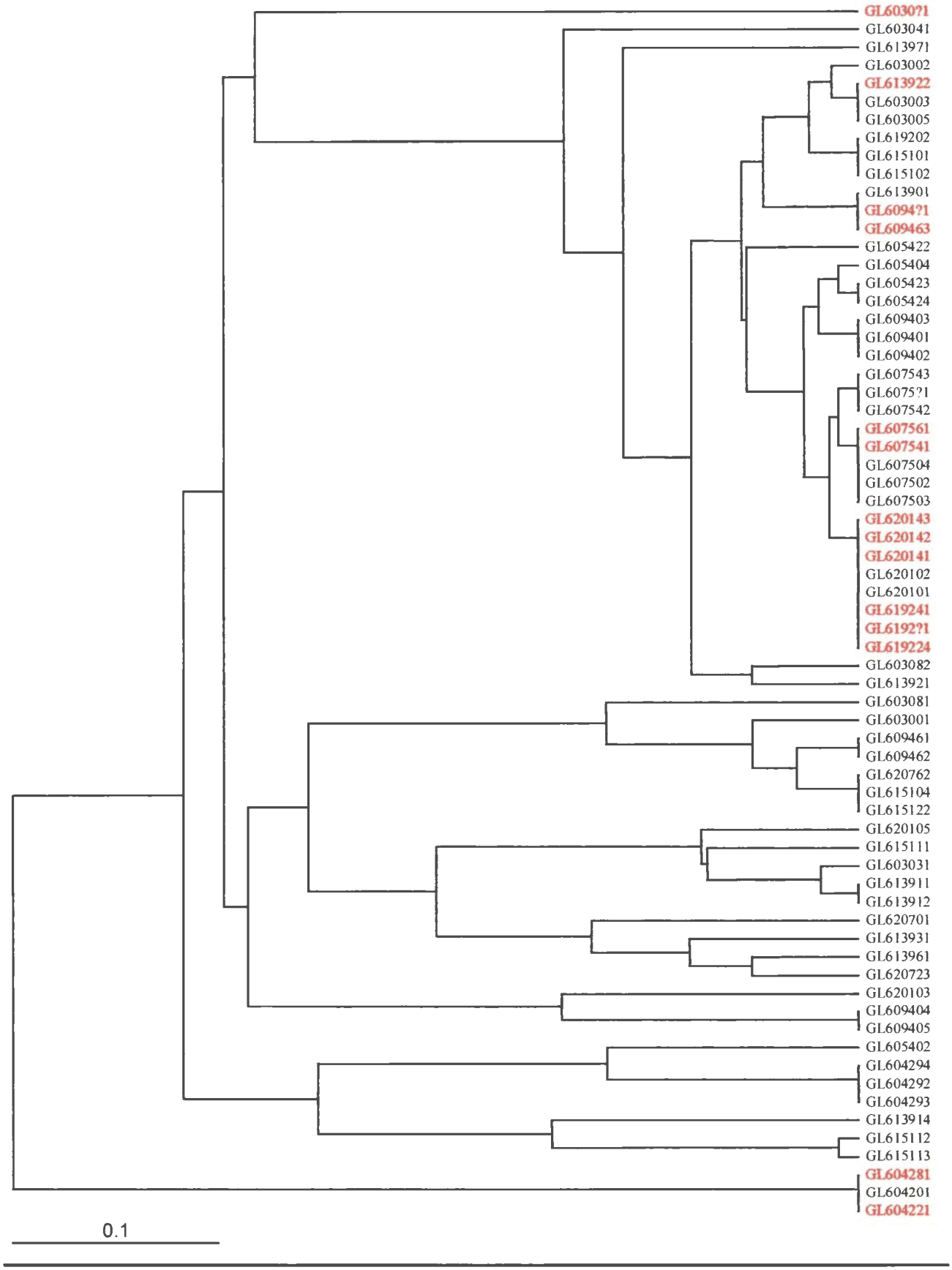
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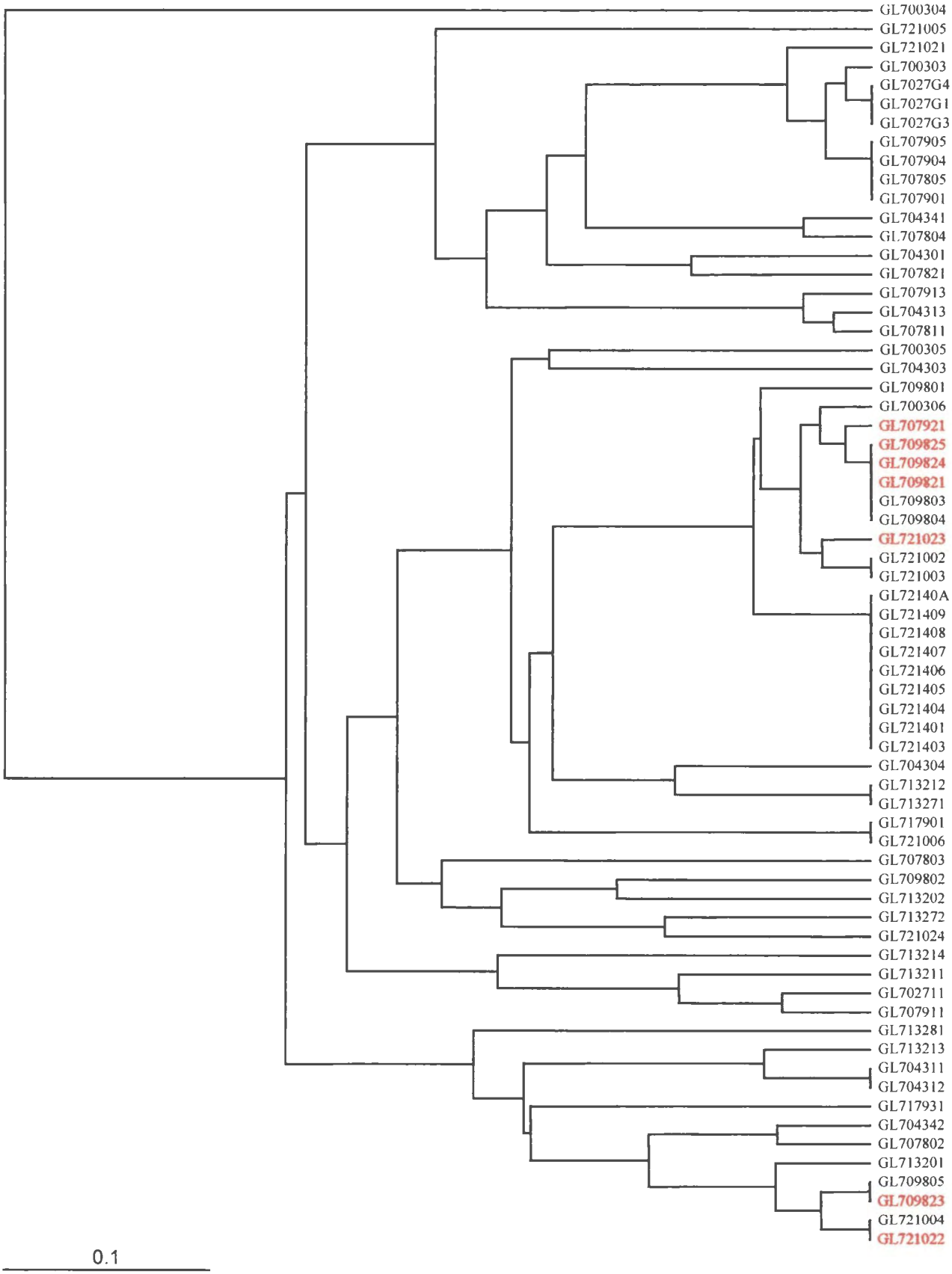
Figure A-9: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 7. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.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GL709824
GL709821
GL709803
GL709804
GL721023
GL721002
GL721003
GL72140A
GL721409
GL721408
GL721407
GL721406
GL721405
GL721404
GL721401
GL721403
GL704304
GL713212
GL713271
GL717901
GL721006
GL707803
GL709802
GL713202
GL713272
GL721024
GL713214
GL713211
GL702711
GL707911
GL713281
GL713213
GL704311
GL704312
GL717931
GL704342
GL707802
GL713201
GL709805
GL709823
GL721004
GL721022
0.1

Figure A-10: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 8. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.

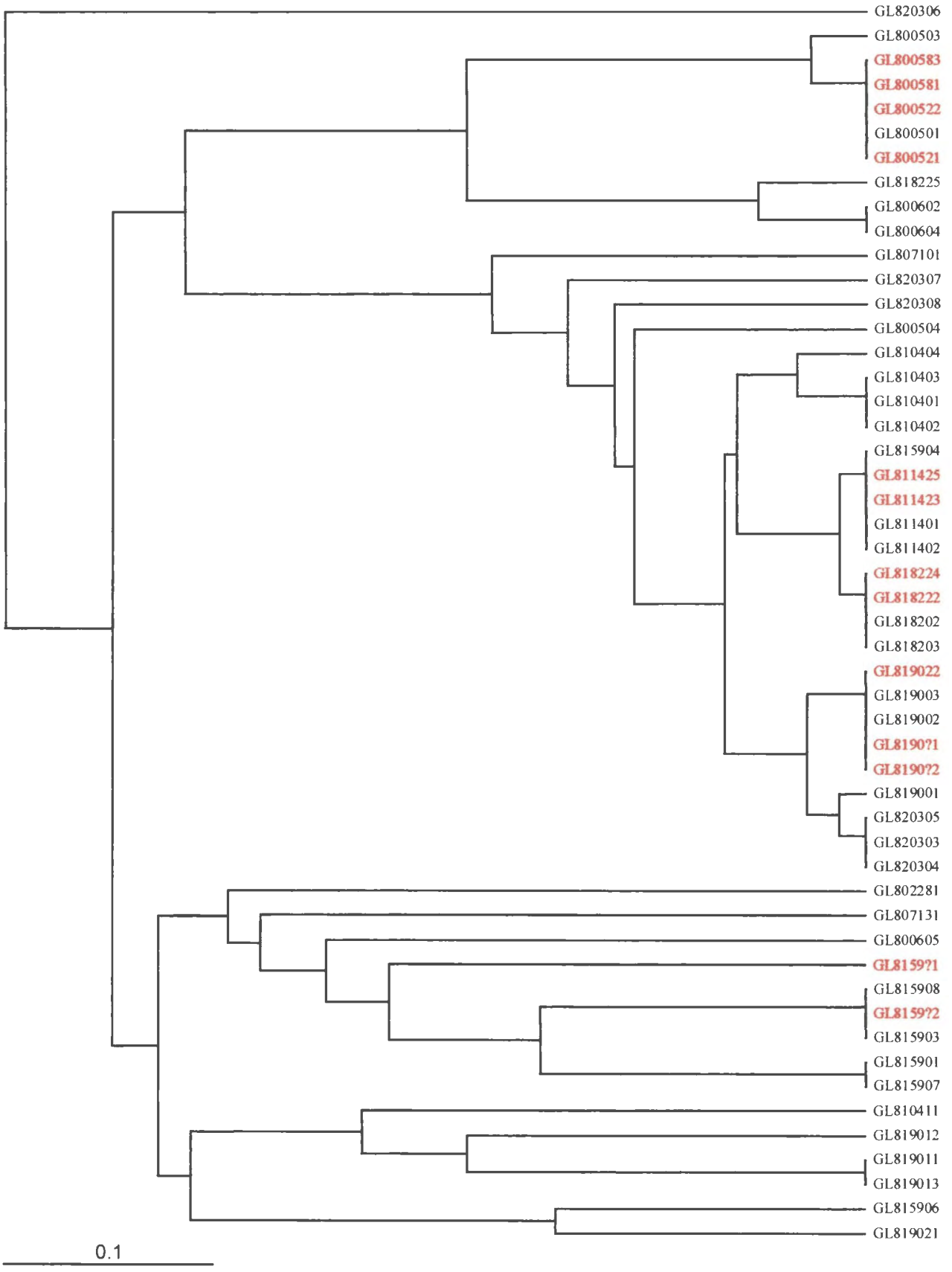
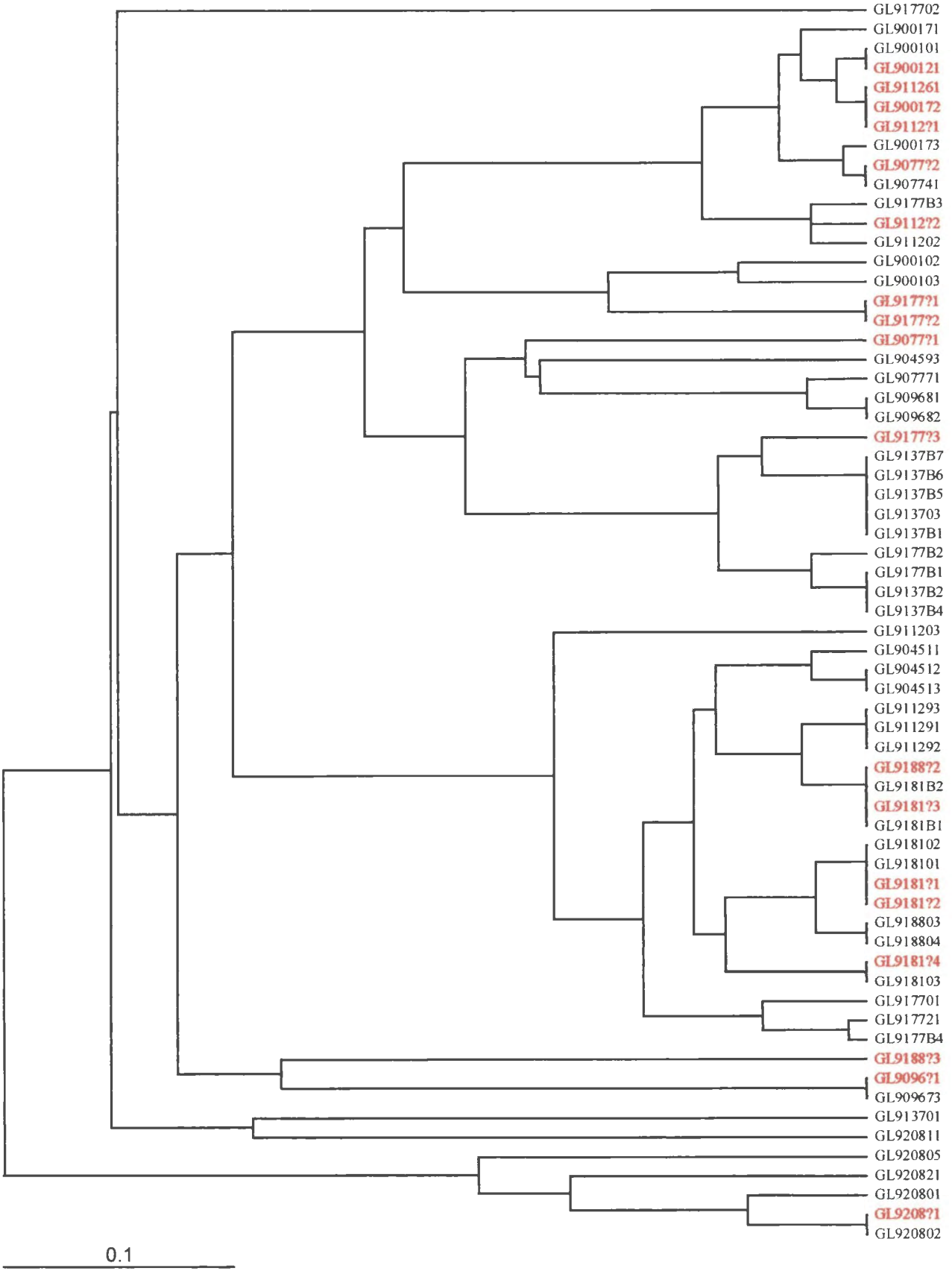

GL820306
GL800503
GL800583
GL800581
GL800522
GL800501
GL800521
GL818225
GL800602
GL800604
GL807101
GL820307
GL820308
GL800504
GL810404
GL810403
GL810401
GL810402
GL815904
GL811425
GL811423
GL811401
GL811402
GL818224
GL818222
GL818202
GL818203
GL819022
GL819003
GL819002
GL8190?1
GL8190?2
GL819001
GL820305
GL820303
GL820304
GL802281
GL807131
GL800605
GL8159?1
GL815908
GL8159?2
GL815903
GL815901
GL815907
GL810411
GL819012
GL819011
GL819013
GL815906
GL819021
0.1

Figure A-11: Phylogram showing the similarity of some 'mantled tips' towards some 'non-mantled tips' within plot 9. Highlighted samples were removed from the 'mantled tips' database and put with the 'non-mantled tips' database.


GL917702
GL900171
GL900101
GL900121
GL911261
GL900172
GL9112?1
GL900173
GL9077?2
GL907741
GL9177B3
GL9112?2
GL911202
GL900102
GL900103
GL9177?1
GL9177?2
GL9077?1
GL904593
GL907771
GL909681
GL909682
GL9177?3
GL9137B7
GL9137B6
GL9137B5
GL913703
GL9137B1
GL9177B2
GL9177B1
GL9137B2
GL9137B4
GL911203
GL904511
GL904512
GL904513
GL911293
GL911291
GL911292
GL9188?2
GL9181B2
GL9181?3
GL9181B1
GL918102
GL918101
GL9181?1
GL9181?2
GL918803
GL918804
GL9181?4
GL918103
GL917701
GL917721
GL9177B4
GL9188?3
GL9096?1
GL909673
GL913701
GL920811
GL920805
GL920821
GL920801
GL9208?1
GL920802
0.1

Appendix X

X.1: Calculated ANOVAs for treatment comparisons:

SW= Shannon Wiener **Sim= Simpson**
C= Control, L= Low intensity burn, and H= High intensity burn

ANALYSIS OF VARIANCE ON SW-CvsL

SOURCE	DF	SS	MS	F	p
treat	1	0.020	0.020	0.08	0.793
ERROR	4	0.998	0.249		
TOTAL	5	1.017			

LEVEL	N	MEAN	STDEV
1	3	2.9496	0.1625
2	3	2.8355	0.6874

POOLED STDEV = 0.4995

INDIVIDUAL 95% CI'S FOR MEAN
BASED ON POOLED STDEV

```
----------+---------+---------+------
   (---------------*---------------)
 (---------------*---------------)
----------+---------+---------+------
         2.50      3.00      3.50
```

MTB > Oneway 'SW-CvsH' 'treat'.

ANALYSIS OF VARIANCE ON SW-CvsH

SOURCE	DF	SS	MS	F	p
treat	1	0.035	0.035	0.11	0.758
ERROR	4	1.302	0.325		
TOTAL	5	1.337			

LEVEL	N	MEAN	STDEV
1	3	2.9496	0.1625
2	3	2.7961	0.7903

POOLED STDEV = 0.5705

INDIVIDUAL 95% CI'S FOR MEAN
BASED ON POOLED STDEV

```
---------+---------+---------+-------
   (---------------*---------------)
(---------------*---------------)
---------+---------+---------+-------
        2.40      3.00      3.60
```

```
MTB > Oneway 'SW-LvsH' 'treat'.

ANALYSIS OF VARIANCE ON SW-LvsH
SOURCE      DF         SS          MS          F          p
treat        1      0.002       0.002       0.00      0.951
ERROR        4      2.194       0.549
TOTAL        5      2.197

                                          INDIVIDUAL 95% CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV      ---------+---------+---------+---------
     1       3     2.8355     0.6874        (-----------------*----------------)
     2       3     2.7961     0.7903       (-----------------*----------------)
                                          ---------+---------+---------+---------
POOLED STDEV =     0.7407                      2.10      2.80      3.50


MTB > Oneway 'Sim-CvsL' 'treat'.

ANALYSIS OF VARIANCE ON Sim-CvsL
SOURCE      DF         SS          MS          F          p
treat        1  0.0002356   0.0002356       7.35      0.053
ERROR        4  0.0001282   0.0000320
TOTAL        5  0.0003638

                                          INDIVIDUAL 95% CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV      ---------+---------+---------+---------
     1       3    0.97097    0.00742       (---------*---------)
     2       3    0.98350    0.00301                 (---------*---------)
                                          ---------+---------+---------+---------
POOLED STDEV =    0.00566                     0.970     0.980     0.990
```

```
MTB > Oneway 'Sim-CvsH' 'treat'.

ANALYSIS OF VARIANCE ON Sim-CvsH
SOURCE      DF         SS          MS         F        p
treat        1  0.0000286   0.0000286      0.31    0.607
ERROR        4  0.0003684   0.0000921
TOTAL        5  0.0003970

                                          INDIVIDUAL 95% CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV      -----+---------+---------+---------+-
     1       3    0.97097    0.00742       (---------------*---------------)
     2       3    0.97533    0.01136            (---------------*---------------)
                                          -----+---------+---------+---------+-
POOLED STDEV =    0.00960                  0.960     0.970     0.980     0.990


MTB > Oneway 'Sim-LvsH' 'treat'.

ANALYSIS OF VARIANCE ON Sim-LvsH
SOURCE      DF         SS          MS         F        p
treat        1  0.0001000   0.0001000      1.45    0.295
ERROR        4  0.0002763   0.0000691
TOTAL        5  0.0003763

                                          INDIVIDUAL 95% CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV      --------+---------+---------+--------
     1       3    0.98350    0.00301              (--------------*-------------)
     2       3    0.97533    0.01136    (-------------*--------------)
                                          --------+---------+---------+--------
POOLED STDEV =    0.00831                       0.970     0.980     0.990
```

Appendix XI

XI.1: Distance matrices for hypothetical phylograms

Distance matrices for each of the hypothetical phylograms created to demonstrate the attributes of the Phi index, in comparison to traditional diversity indices.

Table A-4: Distance matrix for hypothetical phylogram #1.

Tree 1	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	0	0.07	0.14	0.14	0.21	0.28	0.28	0.28	0.7
S2	0.07	0	0.14	0.14	0.21	0.28	0.28	0.28	0.7
S3	0.14	0.14	0	0.07	0.21	0.28	0.28	0.28	0.7
S4	0.14	0.14	0.07	0	0.21	0.28	0.28	0.28	0.7
S5	0.21	0.21	0.21	0.21	0	0.28	0.28	0.28	0.7
S6	0.28	0.28	0.28	0.28	0.28	0	0.07	0.14	0.7
S7	0.28	0.28	0.28	0.28	0.28	0.07	0	0.14	0.7
S8	0.28	0.28	0.28	0.28	0.28	0.14	0.14	0	0.7
S9	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0

Table A-5: Distance matrix for hypothetical phylogram #2.

Tree 2	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	0	0	0	0	0.21	0.28	0.28	0.28	0.7
S2	0	0	0	0	0.21	0.28	0.28	0.28	0.7
S3	0	0	0	0	0.21	0.28	0.28	0.28	0.7
S4	0	0	0	0	0.21	0.28	0.28	0.28	0.7
S5	0.21	0.21	0.21	0.21	0	0.28	0.28	0.28	0.7
S6	0.28	0.28	0.28	0.28	0.28	0	0	0	0.7
S7	0.28	0.28	0.28	0.28	0.28	0	0	0	0.7
S8	0.28	0.28	0.28	0.28	0.28	0	0	0	0.7
S9	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0

Table A-6: Distance matrix for hypothetical phylogram #3.

Tree 3	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	0	0	0	0	0.56	0.56	0.56	0.63	0.7
S2	0	0	0	0	0.56	0.56	0.56	0.63	0.7
S3	0	0	0	0	0.56	0.56	0.56	0.63	0.7
S4	0	0	0	0	0.56	0.56	0.56	0.63	0.7
S5	0.56	0.56	0.56	0.56	0	0	0	0.63	0.7
S6	0.56	0.56	0.56	0.56	0	0	0	0.63	0.7
S7	0.56	0.56	0.56	0.56	0	0	0	0.63	0.7
S8	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0	0.7
S9	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0

Appendix XII

XII.1: Phi index calculations for hypothetical phylograms

Phi index calculations for the three hypothetical phylograms created to demonstrate the attributes of the Phi index, in comparison to traditional diversity indices. All distances (d) have been squared. Unaltered distances can be viewed in Appendix IX.

Table A-7: Phi-index calculations for hypothetical phylogram #1.

Tree 1									
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**
S1	0	0.0049	0.0196	0.0196	0.0441	0.0784	0.0784	0.0784	0.49
S2	0.0049	0	0.0196	0.0196	0.0441	0.0784	0.0784	0.0784	0.49
S3	0.0196	0.0196	0	0.0049	0.0441	0.0784	0.0784	0.0784	0.49
S4	0.0196	0.0196	0.0049	0	0.0441	0.0784	0.0784	0.0784	0.49
S5	0.0441	0.0441	0.0441	0.0441	0	0.0784	0.0784	0.0784	0.49
S6	0.0784	0.0784	0.0784	0.0784	0.0784	0	0.0049	0.0196	0.49
S7	0.0784	0.0784	0.0784	0.0784	0.0784	0.0049	0	0.0196	0.49
S8	0.0784	0.0784	0.0784	0.0784	0.0784	0.0196	0.0196	0	0.49
S9	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0
D	**0.8134**	**0.8134**	**0.8134**	**0.8134**	**0.9016**	**0.9065**	**0.9065**	**0.9212**	**3.92**
D/(N-1)=	0.10168	0.1017	0.1017	0.1017	0.1127	0.1133	0.1133	0.1152	0.49
Σ [D/(N-1)]/N	0.15013								

Table A-8: Phi-index calculations for hypothetical phylogram #2.

Tree 2									
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**
S1	0	0	0	0	0.0441	0.0784	0.0784	0.0784	0.49
S2	0	0	0	0	0.0441	0.0784	0.0784	0.0784	0.49
S3	0	0	0	0	0.0441	0.0784	0.0784	0.0784	0.49
S4	0	0	0	0	0.0441	0.0784	0.0784	0.0784	0.49
S5	0.0441	0.0441	0.0441	0.0441	0	0.0784	0.0784	0.0784	0.49
S6	0.0784	0.0784	0.0784	0.0784	0.0784	0	0	0	0.49
S7	0.0784	0.0784	0.0784	0.0784	0.0784	0	0	0	0.49
S8	0.0784	0.0784	0.0784	0.0784	0.0784	0	0	0	0.49
S9	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0
D	**0.7693**	**0.7693**	**0.7693**	**0.7693**	**0.9016**	**0.882**	**0.882**	**0.882**	**3.92**
D/(N-1)=	0.09616	0.0962	0.0962	0.0962	0.1127	0.1103	0.1103	0.1103	0.49
Σ [D/(N-1)]/N	0.14646								

Table A-9: Phi-index calculations for hypothetical phylogram #3.

Tree 3	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	0	0	0	0	0.3136	0.3136	0.3136	0.3969	0.49
S2	0	0	0	0	0.3136	0.3136	0.3136	0.3969	0.49
S3	0	0	0	0	0.3136	0.3136	0.3136	0.3969	0.49
S4	0	0	0	0	0.3136	0.3136	0.3136	0.3969	0.49
S5	0.3136	0.3136	0.3136	0.3136	0	0	0	0.3969	0.49
S6	0.3136	0.3136	0.3136	0.3136	0	0	0	0.3969	0.49
S7	0.3136	0.3136	0.3136	0.3136	0	0	0	0.3969	0.49
S8	0.3969	0.3969	0.3969	0.3969	0.3969	0.3969	0.3969	0	0.49
S9	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0
D	**1.8277**	**1.8277**	**1.8277**	**1.8277**	**2.1413**	**2.1413**	**2.1413**	**3.2683**	**3.92**
D/(N-1)=	0.22846	0.2285	0.2285	0.2285	0.2677	0.2677	0.2677	0.4085	0.49
Σ D/(N-1))/N	0.2906								

Appendix XIII

XIII.1: ANOVAs for Phi index

ANOVAs performed on the Phi index. Index was calculated with data collected from endophyte populations on nine study plots near Glide Lake.

```
MTB > Oneway 'ECM-CvL' 'subs'.

ANALYSIS OF VARIANCE ON ECM-Control verses Low Burn
SOURCE      DF         SS         MS         F         p
subs         1    0.00194    0.00194      0.77     0.430
ERROR        4    0.01009    0.00252
TOTAL        5    0.01204

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV    -------+---------+---------+---------
     1       3    0.28433    0.05824     (------------*-------------)
     2       3    0.32033    0.04067           (------------*-------------)
                                        -------+---------+---------+---------
POOLED STDEV =    0.05023                  0.240     0.300     0.360
-------------------------------------------------------------------------------

MTB > Oneway 'ECM-CvH' 'subs'.

ANALYSIS OF VARIANCE ON ECM-Control verses High Burn
SOURCE      DF         SS         MS         F         p
subs         1    0.01612    0.01612      4.70     0.096
ERROR        4    0.01373    0.00343
TOTAL        5    0.02985

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV    -------+---------+---------+---------
     1       3    0.28433    0.05824     (-----------*----------)
     2       3    0.38800    0.05892                (----------*-----------)
                                        -------+---------+---------+---------
POOLED STDEV =    0.05858                  0.240     0.320     0.400
-------------------------------------------------------------------------------
```

MTB > Oneway 'ECM-LvH' 'subs'.

ANALYSIS OF VARIANCE ON ECM-Low Burn verses High Burn

SOURCE	DF	SS	MS	F	p
subs	1	0.00687	0.00687	2.68	0.177
ERROR	4	0.01025	0.00256		
TOTAL	5	0.01712			

LEVEL	N	MEAN	STDEV
1	3	0.32033	0.04067
2	3	0.38800	0.05892

POOLED STDEV = 0.05063

INDIVIDUAL 95% CI'S FOR MEAN
BASED ON POOLED STDEV

```
------+---------+---------+---------+
(-----------*----------)
          (----------*----------)
------+---------+---------+---------+
    0.280     0.350     0.420     0.490
```

NOTE: p_s=0.05, $F_{0.05[1,4]}$=7.71; Since "p" for every comparison is greater than the statistical "p" then there is no significant difference between each comparison *Phi index*.

Appendix XIV

XIV.1: ANOVAs for Shannon, Simpson and Phi indices

ANOVAs performed on the Shannon, Simpson and Phi indices. Indicies were calculated with data collected from endophyte populations on nine study plots near Glide Lake.

Shannon-Wiener Index

```
MTB > Oneway 'HNM-CvL' 'treats'.

ANALYSIS OF VARIANCE ON NM-Control verses Low Burn
SOURCE      DF         SS         MS         F         p
treats       1     0.0620     0.0620      0.69     0.454
ERROR        4     0.3607     0.0902
TOTAL        5     0.4227

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   ------+---------+---------+---------+
     1       3     2.7267     0.3550   (-------------*-------------)
     2       3     2.9300     0.2330         (-------------*------------)
                                        ------+---------+---------+---------+
POOLED STDEV =    0.3003                  2.45      2.80      3.15      3.50
-------------------------------------------------------------------------------
```

```
MTB > Oneway 'HNM-CvH' 'treats'.

ANALYSIS OF VARIANCE ON NM-Control verses High Burn
SOURCE      DF         SS         MS         F         p
treats       1      0.001      0.001      0.01     0.929
ERROR        4      0.471      0.118
TOTAL        5      0.472

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   ---------+---------+---------+--------
     1       3     2.7267     0.3550   (----------------*----------------)
     2       3     2.7533     0.3308    (----------------*----------------)
                                        ---------+---------+---------+--------
POOLED STDEV =    0.3431                     2.45      2.80      3.15
-------------------------------------------------------------------------------

MTB > Oneway 'HNM-LvH' 'treats'.

ANALYSIS OF VARIANCE ON NM-Low Burn verses High Burn
SOURCE      DF         SS         MS         F         p
treats       1     0.0468     0.0468      0.57     0.492
ERROR        4     0.3275     0.0819
TOTAL        5     0.3743

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   ----+---------+---------+---------+--
     1       3     2.9300     0.2330        (----------------*----------------)
     2       3     2.7533     0.3308   (----------------*----------------)
                                        ----+---------+---------+---------+--
POOLED STDEV =    0.2861               2.40      2.70      3.00      3.30
---------------------------------------------------------------
```

Simpson index

```
MTB > Oneway 'DNM-CvL' 'treats'.

ANALYSIS OF VARIANCE ON NM-Control verses Low Burn
SOURCE      DF         SS         MS         F         p
treats       1   0.000817   0.000817      1.01     0.372
ERROR        4   0.003245   0.000811
TOTAL        5   0.004061

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   -----+---------+---------+---------+-
     1       3     0.9407     0.0287    (-------------*------------)
     2       3     0.9640     0.0282          (-------------*------------)
                                        -----+---------+---------+---------+-
POOLED STDEV =    0.0285                 0.910     0.945     0.980     1.015
-------------------------------------------------------------------------------
```

```
MTB > Oneway 'DNM-CvH' 'treats'.

ANALYSIS OF VARIANCE ON NM-Control verses High Burn
SOURCE      DF         SS         MS         F         p
treats       1   0.000171   0.000171      0.19     0.685
ERROR        4   0.003591   0.000898
TOTAL        5   0.003762

                                       INDIVIDUAL 95% CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   ---+---------+---------+---------+---
     1       3    0.94067    0.02875    (---------------*---------------)
     2       3    0.95133    0.03113        (---------------*---------------)
                                       ---+---------+---------+---------+---
POOLED STDEV =    0.02996               0.900     0.930     0.960     0.990
------------------------------------------------------------------------------
MTB > Oneway 'DNM-LvH' 'treats'.

ANALYSIS OF VARIANCE ON NM-Low Burn verses High Burn
SOURCE      DF         SS         MS         F         p
treats       1   0.000241   0.000241      0.27     0.629
ERROR        4   0.003531   0.000883
TOTAL        5   0.003771

                                       INDIVIDUAL 95% CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   ---------+---------+---------+-------
     1       3     0.9640     0.0282        (---------------*---------------)
     2       3     0.9513     0.0311   (---------------*---------------)
                                       ---------+---------+---------+-------
POOLED STDEV =     0.0297              0.930     0.960     0.990
MTB >
```

Phi index

```
MTB > Oneway 'NM-CvL' 'subs'.

ANALYSIS OF VARIANCE ON NM-Control verses Low Burn
SOURCE      DF         SS         MS         F         p
subs         1     0.0066     0.0066      0.47     0.529
ERROR        4     0.0558     0.0140
TOTAL        5     0.0624

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV   --------+---------+---------+--------
     1       3     0.2300     0.1234    (-----------*-----------)
     2       3     0.2963     0.1126         (-----------*-----------)
                                        --------+---------+---------+--------
POOLED STDEV =    0.1181                    0.15      0.30      0.45
```

```
MTB > Oneway 'NM-CvH' 'subs'.

ANALYSIS OF VARIANCE ON NM-Control verses High Burn
SOURCE      DF        SS         MS         F         p
subs         1   0.00427    0.00427      0.50     0.517
ERROR        4   0.03387    0.00847
TOTAL        5   0.03814

                                       INDIVIDUAL 95% CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL       N      MEAN      STDEV   --+---------+---------+---------+----
     1       3   0.23000    0.12341   (--------------*--------------)
     2       3   0.28333    0.04130         (--------------*--------------)
                                       --+---------+---------+---------+----
POOLED STDEV =   0.09202               0.10      0.20      0.30      0.40
-------------------------------------------------------------------------------
MTB > Oneway 'NM-LvH' 'subs'.
ANALYSIS OF VARIANCE ON NM-Low Burn verses High Burn
SOURCE      DF        SS         MS         F         p
subs         1   0.00025    0.00025      0.04     0.860
ERROR        4   0.02878    0.00719
TOTAL        5   0.02903

                                       INDIVIDUAL 95% CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL       N      MEAN      STDEV   --+---------+---------+---------+----
     1       3   0.29633    0.11262     (----------------*----------------)
     2       3   0.28333    0.04130   (----------------*----------------)
                                       --+---------+---------+---------+----
POOLED STDEV =   0.08482               0.160     0.240     0.320     0.400
```

NOTE: p_s=0.05, $F_{0.05[1,4]}$=7.71; Since "p" for every comparison is greater than the statistical "p" then there is no significant difference between each comparison *Phi index*.