Abundance and distribution of the multi-functional

root associated fungus *Meliniomyces variabilis*.

By
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Abstract

Meliniomyces variabilis Hambleton and Sigler is a fungal partner of the Ericaceae, forming ericoid mycorrhizal symbioses, and a member of the *Rhizoscyphus Ericae* Species aggregate. *M. variabilis* has been isolated from a range of non-ericaceous boreal plants where it has been observed to colonize roots endophytically. The present study derives species specific PCR primers to isolate *M. variabilis* from field samples of plant roots to investigate the host range of *M. variabilis*. Real-time PCR was utilized on samples from two sites to study factors that affect the abundance and distribution of *M. variabilis* on the host plants *Kalmia polifolia* and *Picea mariana*. This analysis expanded the host range of plants colonized by *M. variabilis* by five species, and one family. The above-ground plant community plays a significant factor in determining the host choice of *M. variabilis* by influencing it to either colonize a primary Ericaceous host or an alternate host.

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This one's for Carl!

Chapter 1 : Introduction to root associated fungi, ericoid mycorrhiza and molecular fungal ecology.

Introduction

The fungal kingdom contains a massive variety of species that fulfill a diverse range of ecological roles. The relative simplicity of the fungal physiology, compared to multicellular plants and animals, allows for members of the same genus to occupy completely different ecological niches and life histories. Furthermore, molecular biology and systematics are constantly shifting our understanding of the species concept as it relates to fungi, and where we place fungal species and species complexes on the phylogenetic tree of life. Like the plants and animals that fungi often rely on for nutrition, as hosts for symbiotic colonization, or for pathogenic infection, the life histories of fungi are diverse and often depend on the environmental context under which a fungus may exhibit different physiological responses.

Within this context of complex species diversity, functional groups of root-associated fungi arise that form different functional relationships with the roots of plants. The two most commonly thought-of root-associated fungal groups are the symbiotic mycorrhizal fungi and the fungal plant pathogens. Mycorrhizal fungi form relationships with approximately 90% of plant families, but only 5% of these fungal partners have been described (Bonfante and Genre, 2010; De Bellis et al. 2007; Smith and Read, 2008). There are three major types of mycorrhizal symbiosis: arbuscular mycorrhiza (AM), ectomycorrhiza (ECM), and ericoid mycorrhiza (ERM) (Smith and Read, 2008). For a more in-depth review of arbuscular mycorrhizae and ectomycorrhizae, see Bonfante and Genre (2010). A third group of common root-associated fungi are the fungal endophytes, which colonize plant tissue internally and asymptomatically (Jumpponen and Trappe, 1998; Smith and Read 2008). The most widely studied root endophytes are the dark septate endophytes (DSE), such as the *Phialacephola fortinii* s.l.- *Acephala applanta*

species complex (PAC) (Grünig et al., 2008). Endophytes can be pathogenic, asymptomatic, or beneficial to their plant hosts (Jumpponen and Trappe, 1998; Smith and Read 2008), though the function and effects of root colonization by fungal endophytes is often unclear (Mayerhofer et al., 2013). Further complicating the picture is the fact that several authors have reported finding fungal species that fulfill a certain functional role on one host plant (such as a mycorrhizal symbiosis) yet colonize other plants endophytically. These could be considered "multifunctional" root associates (Brundett, 2006).

Regardless of the hypothesized or observed function of a fungal root-associate, the largest determinants of classification for a plant-fungus relationship are the morphology of the interaction and the physiological response of the two species. In general, more emphasis has been placed on the former, as the latter typically involves the use of radiolabelled isotopes to demonstrate a reciprocal exchange of nutrients. Following morphological classification, the study of a mycorrhizal symbiosis usually turns to what nutrients are exchanged between the fungus and the host plant, or what services the two organisms provide for each other, and how the process occurs at a physiological level (Casieri et al., 2013; Fellbaum et al., 2012). This can take the form of cytological studies, nutrient exchange studies using radioisotope labeled nutrients, biochemical studies of secreted enzymes, or the study of fungal genomics and genes involved in the interaction. Finally, the third level at which a plant-fungus interaction is studied is typically at the ecological or evolutionary level, and studies of these topics are usually not mutually exclusive. The purpose of the current study is to observe the autecology of the ericoid mycorrhizal and endophyte forming *Meliniomyces variabilis* Hambleton and Sigler using molecular techniques to quantify its abundance and distribution on two host plants.

For a plant-fungus interaction to be classified as mycorrhizal, there are a number of factors that need to be demonstrated. Firstly, there must be the formation of specialized structures formed by the fungus within or around the cells of the plant root. An ECM association is characterized by the formation of a Hartig Net—an extracellular fungal network that grows between the root cells, but does not penetrate or colonize the intracellular spaces of the root cells (Smith and Read, 2008; Bonfante and Genre, 2010). ECM fungi are commonly associated with conifers and woody shrubs in temperate and boreal forests (Kernaghan, 2013; Reithmeier and Kernaghan, 2013; Vohník et al., 2013) and exchange soil nutrients, primarily N and P, for carbon sources generated by the plant host. Arbusucular mycorrhizal (AM) fungi, in contrast, penetrate the cell wall of their plant hosts and form extensive, branching tree-like (arbuscular) structures that are the primary source of nutrient exchange. AM fungi are less common on woody plants, and more common on herbaceous vegetation. AM fungi may also be found in the roots of deciduous trees in tropical environments (Rodriguez et al., 2009). The last of the three major mycorrhizal symbioses are the ERM, which are characterized by the formation of intracellular fungal coils formed by fungal hyphae in the hair roots of Ericaceous plants. ERM fungi are known to be active in the degradation and transportation of organic N and P from soils into the metabolically active hair roots in exchange for carbon (Smith and Read, 2008).

Fungal root endophytes are another type of ubiquitous and highly diverse plant-fungus interaction (Jumpponen and Trappe, 1998; Kernaghan and Patriquin, 2011; Rodriguez et al.2009; Schulz and Boyle, 2005). The classification of Rodriguez et al. (2009) recognizes two types and four major classes of fungal endophytes: Clavicipitaceous endophytes (C-endophytes) and Nonclavicipitaceous endophytes (NC-endophytes), and class 1-4 endophytes. Class 1 endophytes

are made up of C-endophytes and are exclusively systemic endophytes of grasses, they also generally provide their host plants with well defined benefits such as stress reduction, enhanced nutrient acquisition, and decreased herbivory through the secretion of secondary metabolites (Rodriguez et al., 2009). Class 2 through 4 are the NC-endophytes, and they possess a larger host range than Class 1 fungal endophytes. Class 2 endophytes can be found throughout a plant's roots, shoot, and surrounding rhizome; Class 3 endophytes are limited to the plant's shoot; Class 4 endophytes extensively colonize a plant's roots (Rodriguez et al., 2009). Class 4 endophytes can be from the Basidiomycota or the Ascomycota and are the focus this thesis.

Endophytes, taken literally, are simply organisms that exist within plants; though this description belies the complex interactions and subtle effects that fungal and microbial endophytes can have on a plant's physiology and the ecological community. Many attributes have been ascribed to fungal endophytes, from conferring heat resistance, decreasing herbivory, reducing plant-plant competition, enhancing nutrient uptake, and reducing the ability of pathogenic microorganisms to take hold (Maciá-Vicente et al, 2008; Mayerhofer et al., 2013; Rodriguez et al., 2009; Schulz and Boyle, 2005; Wagg et al., 2011). The fact remains that many of these changes are subtle and hard to observe, or else there is no apparent benefit or drawback to colonization of a plant by many fungal endophytes (Jumpponen and Trappe, 1998; Kernaghan and Patriquin, 2011; Mayerhofer et al., 2013; Rodriguez et al., 2009).

Given that the vast majority of land plant roots are colonized by specialized mycorrhizal fungi (Wang and Qiu, 2006; Smith and Read, 2008), and even some of the lower terrestrial plants such as liverworts exhibit some level of colonization by fungi (Field et al., 2015), it seems that mycorrhiza are or were an essential evolutionary step to the colonization and continued success of

plants in terrestrial environments (Wang et al., 2010). The prevalence of specialized plant-fungi root associations then makes the existence of an equally widespread but unspecialized, asymptomatic plant colonization somewhat puzzling. ECM and ERM fungi evolved later and form less specialized relationships than the AM fungi that make up the majority of all mycorrhizal relationships (Cullings, 1996; Wang and Qiu, 2006; Wang et al., 2010). As such, there have been many instances of ERM fungi being isolated from the roots of non-Ericaceous plants (Bergero et al, 2000; Bergero et al, 2003; Kernaghan and Patriquin, 2011; Reithmeier and Kernaghan, 2013; Vohník et al., 2013), which has lead to the working assumption that ERM fungi are colonizing these alternate hosts endophytically.

In an analysis by of 78 species of Ericaceous plants (Wang and Qui, 2006), all were found to form mycorrhizal symbioses. Seventy-four percent of these formed ericoid mycorrhiza, eight percent formed arbuscular and ericoid mycorrhiza, five percent ectendomycorrhiza, four percent were mycoheterotrophs, and nine percent formed other unspecified forms of mycorrhiza (Wang and Qiu, 2006). The hair roots of ericaceous plants colonized by ERM fungi are delicate and ephemeral, rarely lasting more than one season (Allaway and Ashford, 1996; Valenzuela-Estrada et al., 2008). This has given rise to the hypothesis that ERM fungi may form a quiescent state in the soil and senescent hair root cells of Ericaceous plants, or that they may colonize alternate hosts endophytically when no ericaceous plants are in the vicinity (Bergero et al., 2000; Bergero et al., 2003; Gavin Kernaghan and Patriquin, 2011; Vohník et al., 2013).

Wang and colleagues (2010) hypothesized that the three main genes from the Glomeromycota that underlie AM formation in plants (*doesn't make infection 1 [DMI1], doesn't make infection 3[DMI3], and interacting protein of DMI3 [IPD3]*) were co-opted for formation of other types of mycorrhiza. This seems likely, as gene duplications are common in multicellular

eukaryotes such as terrestrial plants (Lynch, 2007a). Furthermore, mutations in duplicated genes are common due to the gene being heavily methylated as the cell attempts to suppress the duplicate gene; this can lead to heritable mutations and a change of function for the gene by genetic drift (Lynch, 2007a and b; Gorelick and Laublicher, 2008). Little research has been done on the evolutionary ecology of endophytes (Clay and Schardl, 2002) and symbiotic fungi in general; such work would shed light on both the biology of mycorrhizal fungi and endophytes in particular, but also help determine the limitations of our current understanding of evolutionary biology.

Mycorrhizal fungi of all typess are defined by the formation of structures for nutrient exchange within the cells of plant roots. ERM fungi form hyphal coils inside of the short lived epidermal cells of ericaceous hair roots (Smith and Read, 2008). However, there is a continuum of morphologies within closely related species such as the *Rhizoscyphus ericea* aggregate (REA) (Vrålstad et al., 2000), wherein the species *Meliniomyces bicolor* is known to produce an ECM morphotype in the presence of spruce roots (Villarreal-Ruiz et al., 2004), while *M. variabilis* is known to produce the ERM morphotype in the presence of ericaceous hair roots (Grelet, et al., 2010), and both *Meliniomyces* species are known to colonize the roots of other hosts endophytically (Kernaghan and Patriquin, 2011; Vohník and Albrechtová, 2011; Zijlstra et al., 2005). The production of different mycorrhizal associations by members of closely related species within complexes such as REA and PAC, and the endophytic colonization of plants by these same fungi, has given rise to a number of theories regarding why fungi colonize some plants asymptomatically but form beneficial or deleterious relationships with others (Rodriguez et al., 2009; Schulz and Boyle, 2005). One hypothesis is that mycorrhizal fungi may be colonizing the

roots of alternate hosts in order to persist in an ecosystem (Bergero et al., 2003; Vohník et al., 2013).

The degree of fungal diversity in the rhizosphere and in plant roots is extensive, and thought to be a potential mechanism driving or enhancing the diversity of plant communities (Kernaghan, 2005; Vandenkoornhuyse et al., 2002; Wagg et al., 2011). Similarly, the assemblage of plant communities has been shown to impact the diversity and assemblage of soil communities of fungal root associates throughout stages of ecosystem development (Dickie et al., 2013; Dickie et al., 2012; Kernaghan et al., 2003; Yamasaki et al., 1998). Ecological and biogeographic studies into these phenomena often occur in greenhouse experiments, or from isolating DNA from field samples of soils or plant tissues from natural ecosystems. Surveys utilizing DNA technology, in particular clonal DNA libraries and next generation sequencing, have uncovered a previously unexpected diversity of fungi from plant roots and the rhizosphere (Jumpponen et al., 2010; Jumpponen and Trappe, 1998; Kernaghan and Patriquin, 2011; Tedersoo et al., 2012; Vandenkoornhuyse et al., 2002), although some of these technologies may be over-estimating diversity through sequencing errors (Dickie, 2010).

In the following two studies, the question of preferred host associations by *Meliniomyces variabilis* will be addressed by the development of species-specific polymerase chain reaction (PCR) DNA primers (Chapter 2) for detection of *M. variabilis* from field samples. The PCR primers will then be used to quantify *M. variabilis* on two plant hosts: Black Spruce (*Picea mariana*) and on the common bog-laurel, (*Kalmia polifolia*) an ericaceous shrub (Chapter 3). *M. variabilis* forms ERM morphotype associations with ericaceous plants (Hambleton and Sigler, 2005), and this ERM association has been demonstrated to exhibit reciprocal transfers of soil nutrients for photosynthetically-derived carbon sources (Grelet et al., 2009). By focusing on the

abundance and distribution of one species using a quantitative molecular technique (qPCR), I will attempt to examine the range of plants *M. varaibilis* colonizes and whether the fungus displays any preference for a particular host. We hope to gain a greater understanding the life history of *M. variabilis* to help clarify its relationship with its host plants and its role in ecological processes.

Real-time quantitative PCR has been used previously to quantify soil-inhabiting pathogens in an agricultural systems (Kernaghan et al., 2007; 2008), but the practice could be extended to forest and microbial ecology. The knowledge derived from such studies could also can be used to optimize soil inocula for green roofs, partially managed agriculture (such as blueberries), and for managed fields. Furthermore, it could potentially be used in biocontrol, in attempts to determine how man-made chemicals are degraded organically in soils over time, or in bioreactors where the initial input and proportions of microbes is important.

Ericaceous plants are an excellent choice of study plant to observe a multitude of interactions between host plant and a variety root-associated fungi. Similarly, *M. variabilis* is an exceptional, fungal partner, given the large range of plant hosts it has been isolated from. The genus *Meliniomyces*, which was recently erected (Hambleton and Sigler, 2005) and is part of the REA species complex (Vrålstad et al., 2000), has been observed to colonize numerous ericaceous and non-ericaceous plants (See Chapter 2) (Chambers et al., 2008; Gorzelak et al., 2012; Grelet et al., 2010; Kernaghan and Patriquin, 2011; Kohout et al., 2011; Vohník and Albrechtová, 2011; Wang and Qiu, 2006). In fact, as seen in Chapter 2, *M. variabilis* has more often been isolated from non-ericaceous plants than it has from ericaceous ones. This widespread distribution on numerous hosts makes *M. variabilis* an intriguing prospect to study at a species ecology level, to assess its abundance and distribution on host plants (Chapter 3), and to make inferences regarding its life history strategies.

References

Allaway, W. G., and Ashford, a E. (1996). Structure of hair roots in Lysinema ciliatum R. Br. and its implications for their water relations. *Annals of Botany*, *77*(4), 383–388. Retrieved from http://linkinghub.elsevier.com/retrieve/doi/10.1006/anbo.1996.0046

Bergero, R., Girlanda, M., Bello, F., Luppi, A. M., and Perotto, S. (2003). Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. *Mycorrhiza*, *13*(2), 69–75. doi:10.1007/s00572-002-0202-9

Bergero, R., Perotto, S., Girlanda, M., Vidano, G., and Luppi, a M. (2000). Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (Quercus ilex). *Molecular Ecology*, *9*(10), 1639–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11050558

Brundett, M. (2006). Understanding the Roles of Multifunctional Mycorrhizal and Endophytic Fungi. *Microbial Root Endophytes*, *9*, 281–298. doi:10.1007/3-540-33526-9

Bonfante, P., and Genre, A. (2010). Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nature Communications*, *1*, 48. doi:10.1038/ncomms1046

Casieri, L., Ait Lahmidi, N., Doidy, J., Veneault-Fourrey, C., Migeon, A., Bonneau, L., … Wipf, D. (2013). Biotrophic transportome in mutualistic plant-fungal interactions. *Mycorrhiza*, *23*(8), 597–625. doi:10.1007/s00572-013-0496-9

Chambers, S. M., Curlevski, N. J. a, and Cairney, J. W. G. (2008). Ericoid mycorrhizal fungi are common root inhabitants of non-Ericaceae plants in a south-eastern Australian sclerophyll forest. *FEMS Microbiology Ecology*, *65*(2), 263–70. doi:10.1111/j.1574-6941.2008.00481.x

Clay, K. and Schardl, C. (2002). Evolutionary Origins and Ecological Consequences of Endophyte Symbiosis with Grasses. *The American Naturalist*, *160(8)*

Cullings, K. W. (1996). Single phylogenetic origin of ericoid mycorrhizae within the Ericaceae. *Canadian Journal of Botany*, *74*(12), 1896–1909. doi:10.1139/b96-227

De Bellis, T., Kernaghan, G., and Widden, P. (2007). Plant community influences on soil microfungal assemblages in boreal mixed-wood forests. *Mycologia*, *99*(3), 356–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17883027

Dickie, I. a. (2010). Insidious effects of sequencing errors on perceived diversity in molecular surveys. *The New Phytologist*, *188*(4), 916–8. doi:10.1111/j.1469-8137.2010.03473.x

Dickie, I. a, Fukami, T., Wilkie, J. P., Allen, R. B., and Buchanan, P. K. (2012). Do assembly history effects attenuate from species to ecosystem properties? A field test with wood-inhabiting fungi. *Ecology Letters*, *15*(2), 133–41. doi:10.1111/j.1461-0248.2011.01722.x

Dickie, I. a, Martínez-García, L. B., Koele, N., Grelet, G. -a., Tylianakis, J. M., Peltzer, D. a., and Richardson, S. J. (2013). Mycorrhizas and mycorrhizal fungal communities throughout

ecosystem development. *Plant and Soil*, *367*(1-2), 11–39. doi:10.1007/s11104-013-1609-0

Fellbaum, C. R., Gachomo, E. W., Beesetty, Y., Choudhari, S., Strahan, G. D., Pfeffer, P. E., … Bucking, H. (2012). Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1118650109

Field, K. J., Rimington, W. R., Bidartondo, M. I., Allinson, K. E., Beerling, D. J., Cameron, D. D., … Pressel, S. (2015). First evidence of mutualism between ancient plant lineages (Haplomitriopsida liverworts) and Mucoromycotina fungi and its response to simulated Palaeozoic changes in atmospheric CO 2. *New Phytologist*, *205*, 743–756. doi:10.1111/nph.13024

Gorelick, R., Laubichler, M.D. (2008). Genetic = Heritable (Genetic ≠ DNA). *Biological Theory. 3(1):79-84*

Gorzelak, M. a, Hambleton, S., and Massicotte, H. B. (2012). Community structure of ericoid mycorrhizas and root-associated fungi of Vaccinium membranaceum across an elevation gradient in the Canadian Rocky Mountains. *Fungal Ecology*, *5*(1), 36–45. doi:10.1016/j.funeco.2011.08.008

Grelet, G.-A., Johnson, D., Paterson, E., Anderson, I. C., and Alexander, I. J. (2009). Reciprocal carbon and nitrogen transfer between an ericaceous dwarf shrub and fungi isolated from Piceirhiza bicolorata ectomycorrhizas. *The New Phytologist*, 359–366. doi:10.1111/j.1469-8137.2009.02813.x

Grelet, G.-A., Johnson, D., Vrålstad, T., Alexander, I. J., and Anderson, I. C. (2010). New insights into the mycorrhizal Rhizoscyphus ericae aggregate: spatial structure and co-colonization of ectomycorrhizal and ericoid roots. *The New Phytologist*, *188*(1), 210–22. doi:10.1111/j.1469-8137.2010.03353.x

Grünig, C. R., Queloz, V., Sieber, T. N., and Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the Phialocephala fortinii s.l. – Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany*, *86*(12), 1355–1369. doi:10.1139/B08-108

Hambleton, S., and Sigler, L. (2005). Meliniomyces, a new anamorph genus for root-associated fungi with phylogenetic affinities to Rhizoscyphus ericae (Hymenoscyphus ericae), Leotiomycetes. *Studies in Mycology*, *53*(1), 1–27. doi:10.3114/sim.53.1.1

Jumpponen, A., Jones, K. L., and Blair, J. (2010). Vertical distribution of fungal communities in tallgrass prairie soil. *Mycologia*, *102*(5), 1027–1041. doi:10.3852/09-316

Jumpponen, A., and Trappe, J. M. (1998). Dark septate endophytes: A review of facultative biotrophic root-colonizing fungi. *New Phytologist*, *140*(2), 295–310. doi:10.1046/j.1469-8137.1998.00265.x

Kernaghan, G., Sigler, L., and Khasa, D. (2003). Mycorrhizal and root endophytic fungi of containerized Picea glauca seedlings assessed by rDNA sequence analysis. *Microbial Ecology*, *45*(2), 128–36. doi:10.1007/s00248-002-1024-1

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. (2007). Quantification of *Cylindrocarpon destructans* f. sp. panacis in soils by real-time PCR. *Plant Pathology*, *56*(3), 508-516.

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. (2008). Quantification of *Pythium* populations in ginseng soils by culture dependent and real-time PCR methods. *applied soil ecology*, *40*(3), 447-455.

Kernaghan, G. (2005). Mycorrhizal diversity: Cause and effect? *Pedobiologia*, *49*(6), 511–520. doi:10.1016/j.pedobi.2005.05.007

Kernaghan, G. (2013). Functional diversity and resource partitioning in fungi associated with the fine feeder roots of forest trees. *Symbiosis*, *61*(3), 113–123. doi:10.1007/s13199-013-0265-8

Kernaghan, G., and Patriquin, G. (2011). Host associations between fungal root endophytes and boreal trees. *Microbial Ecology*, *62*(2), 460–73. doi:10.1007/s00248-011-9851-6

Kernaghan, G., Widden, P., Bergeron, Y., Le, S., and Pare, D. (2003). Biotic and abiotic factors affecting ectomycorrhizal diversity in boreal mixed-woods, 497–504.

Kohout, P., Sýkorová, Z., Bahram, M., Hadincová, V., Albrechtová, J., Tedersoo, L., and Vohník, M. (2011). Ericaceous dwarf shrubs affect ectomycorrhizal fungal community of the invasive Pinus strobus and native Pinus sylvestris in a pot experiment. *Mycorrhiza*, *21*(5), 403–12. doi:10.1007/s00572-010-0350-2

Lynch, M. (2007a). *The Origins of Genome Architecture*. Sunderland Massachusetts: Sineaur Associates ltd.

Lynch, M. (2007b). The evolution of genetic networks by non-adaptive processes. *Nature Reviews: Genetics*. 8:803-813

Maciá-Vicente, J. G., Jansson, H.-B., Mendgen, K., and Lopez-Llorca, L. V. (2008). Colonization of barley roots by endophytic fungi and their reduction of take-all caused by Gaeumannomyces graminis var. tritici. *Canadian Journal of Microbiology*, *54*(8), 600–609. doi:10.1139/w08-047

Mayerhofer, M. S., Kernaghan, G., and Harper, K. a. (2013). The effects of fungal root endophytes on plant growth: A meta-analysis. *Mycorrhiza*, *23*(2), 119–128. doi:10.1007/s00572-012-0456-9

Reithmeier, L., and Kernaghan, G. (2013). Availability of ectomycorrhizal fungi to black spruce above the present treeline in Eastern Labrador. *PloS One*, *8*(10), e77527. doi:10.1371/journal.pone.0077527

Rodriguez, R. J., White, J. F., Arnold, a. E., and Redman, R. S. (2009). Fungal endophytes:

Diversity and functional roles: Tansley review. *New Phytologist*, *182*(2), 314–330. doi:10.1111/j.1469-8137.2009.02773.x

Smith, S.E. and Read, D. (2008). Ericoid mycorrhizas. In *Mycorrhizal Symbiosis 3rd Edition* (ed. S.E. Smith and D. Read), pp. 389-418. Great Britain: Elsevier ltd.

Schulz, B., and Boyle, C. (2005). The endophytic continuum. *Mycological Research*, *109*(6), 661–686. doi:10.1017/S095375620500273X

Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., Bahram, M., Bechem, E., Chuyong, G., Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New Phytologist*, *188*(1), 291–301. doi:10.1111/j.1469-8137.2010.03373.x

Valenzuela-Estrada, L. R., Vera-Caraballo, V., Ruth, L. E., and Eissenstat, D. M. (2008). Root anatomy, morphology, and longevity among root orders in Vaccinium corymbosum (Ericaceae). *American Journal of Botany*, *95*(12), 1506–1514. doi:10.3732/ajb.0800092

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J., and Young, J. P. W. (2002). Extensive fungal diversity in plant roots. *Science (New York, N.Y.)*, *295*(5562), 2051. doi:10.1126/science.295.5562.2051

Villarreal-Ruiz, L., Anderson, I. C., and Alexander, I. J. (2004). Interaction between an isolate from the Hymenoscyphus ericae aggregate and roots of Pinus and Vaccinium. *New Phytologist*, *164*(1), 183–192. doi:10.1111/j.1469-8137.2004.01167.x

Vohník, M., and Albrechtová, J. (2011). The Co-occurrence and Morphological Continuum Between Ericoid Mycorrhiza and Dark Septate Endophytes in Roots of Six European Rhododendron Species. *Folia Geobotanica*, *46*(4), 373–386. doi:10.1007/s12224-011-9098-5

Vohník, M., Mrnka, L., Lukešová, T., Bruzone, M. C., Kohout, P., and Fehrer, J. (2013). The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal Meliniomyces variabilis. *Fungal Ecology*, *6*(4), 281–292. doi:10.1016/j.funeco.2013.03.006

Vrålstad, T., Fossheim, T., and Schumacher, T. (2000). Piceirhiza bicolorata - The ectomycorrhizal expression of the Hymenoscyphus ericae aggregate? *New Phytologist*, *145*(3), 549–563. doi:10.1046/j.1469-8137.2000.00605.x

Wagg, C., Jansa, J., Stadler, M., Schmid, B., and Van Der Heijden, M. G. a. (2011). Mycorrhizal fungal identity and diversity relaxes plant-plant competition. *Ecology*, *92*(6), 1303–1313. doi:10.1890/10-1915.1

Wang, B., and Qiu, Y.-L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, *16*(5), 299–363. doi:10.1007/s00572-005-0033-6

Wang, B., Yeun, L. H., Xue, J.-Y., Liu, Y., Ané, J.-M., and Qiu, Y.-L. (2010). Presence of three

mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. *New Phytologist*, *186*(2), 514–525. doi:10.1111/j.1469-8137.2009.03137.x

Wani, Z. A., Ashraf, N., Mohiuddin, T., and Riyaz-Ul-Hassan, S. (2015). Plant-endophyte symbiosis, an ecological perspective. *Applied Microbiology and Biotechnology*, 2955–2965. doi:10.1007/s00253-015-6487-3

Yamasaki, S. H., Fyles, J. W., Egger, K. N., and Titus, B. D. (1998). The effect of Kalmia angustifolia on the growth, nutrition, and ectomycorrhizal symbiont community of black spruce. *Forest Ecology and Management*, *105*(1-3), 197–207. doi:10.1016/S0378-1127(97)00285-5

Zijlstra, J. D., Van ’t Hof, P., Braakhekke, W. G., Berendse, F., Baar, J., Paradi, I., … Summerbell, R. C. (2005). Diversity of symbiotic root endophytes of the Helotiales in ericaceous plants and the grass, Deschampsia flexuosa. *Studies in Mycology*, *53*(1), 147–162. doi:10.3114/sim.53.1.147

Chapter 2 : Design of taxon-specific primers for quantification of *Meliniomyces variablis* from field collected roots.

Abstract

Methods of detecting microbial samples from natural environments have evolved greatly over the years, allowing for many different ways to explore microbial communities. Utilizing publicly accessible ITS data from GenBank, the present study constructed species-specific PCR primers for the multifunctional root associated fungus *M. variabilis.* These primers were then rigorously tested to ensure specificity, both *in silico* and against phylogenetically close fungal species. The primers were tested against field samples of known *M. variabilis* hosts, as well as plants that had not previously been reported to be colonized by *M. variabilis* in order to study the range of hosts colonized. Using these primers the host range of *M. variabilis* has been expanded by five plant species and one plant family.

Introduction

The ecology of root-associated fungi has been tackled in a variety of ways in the field; the most common methods usually involve the use of DNA technology to discern what the fungal species are. These include, but are not limited to, ARISA (automated intergenic spacer analysis)(Gorzelak et al., 2012), microsatellites or ISSR (intersimple sequence repeats)(Grelet et al., 2010), the creation of clone libraries from field samples (Kernaghan and Patriquin, 2011), species specific PCR primers (Filion et al., 2003; Kernaghan et al., 2006), and the pyrosequencing of whole communities (Margulies et al., 2005; Tedersoo et al., 2010). These DNA-based approaches to fungal ecology generally make use of the fungal ITS (internal transcribed spacer) region, and by primers originally designed by White *et al*. for phylogenetic and systematic analysis of the fungi (White *et al*., 1990; Burns et al., 1991; Gardes et al., 1991).

The ITS is composed of two highly conserved, non-coding regions of DNA found between three genes that code for ribosomal DNA (rDNA) (Figure 2). ITS1 is found between rDNA genes that code for the small ribosomal subunit and the 5.8 ribosomal subunit, and ITS2 between the 5.8 ribosomal subunit and the large ribosomal subunit (Burns et al., 1991). Briefly, fungal ITS sequences differ enough from one species to the next for them to act as distinct markers when the ITS is amplified, sequenced, and compared against a database of known type

sequences. For environmental samples, or for sequences derived from cultured but unidentified species, a rule of thumb used by biologists is that a three percent sequence divergence from known sequences is probably a novel species (Nilsson et al., 2008).

The newest of these techniques is pyrosequencing, or massively parallel PCR. The technique utilizes polymer beads in a gel emersion to bind individual template strands, and allows for the sequencing of any DNA or RNA template strand present in an environmental sample. Despite the ability of ecologists to sequence and identify nearly all fungal species in an environmental sample by way of pyrosequencing, PCR and real-time PCR (qPCR) remain valuable techniques for focusing on single microbial species *in situ* (Dickie, 2010; Filion et al., 2003; Kernaghan et al., 2006; Smith and Osborn, 2009). One of the benefits of working with a single species instead of a community is that the data sets generated by pyrosequencing can over-estimate the number of unique species and the relative abundance of each sequence may not reflect the actual abundances of species in the environment (Dickie, 2010; Tedersoo et al., 2010). Therefore, we choose one root associated fungus, *Meliniomyces variabilis*, that forms multiple types of relationships with a variety of plant hosts (Grelet et al., 2010; Hambleton and Sigler, 2005; Vohník etal., 2007; Vohník et al., 2007) as our study species.

Plants of the Ericaceae are known to form special ericoid mycorrhizal (ERM) symbiosis with certain species of fungi (Smith and Read, 2008). Some of these ericoid forming species, *M. variabilis* included, can simultaneously appear as endophytes, lacking the specialized structures that define an ERM association, sometimes even on the same plant as where it was forming ERM (Vohník and Albrechtová, 2011). This ability to form several types of associations at once with one or multiple plants makes it difficult to determine the relationship between a fungus and its

tentative plant host in the field. As shown in Figure 1, *M. variabilis* has been isolated from a variety of plant hosts, and even though it is known to form ERM with plants from the Ericaceae under laboratory and greenhouse conditions, the majority of field plant hosts are actually trees (*Betula*, *Tsuga*, *Picea*, *Pinus*, *Abies*), as opposed to shrubs from the Ericaceae. This may indicate a much wider host range than already explored, and may indicate that *M. variabilis* is interacting with plants outside of the Ericaceae in meaningful ways.

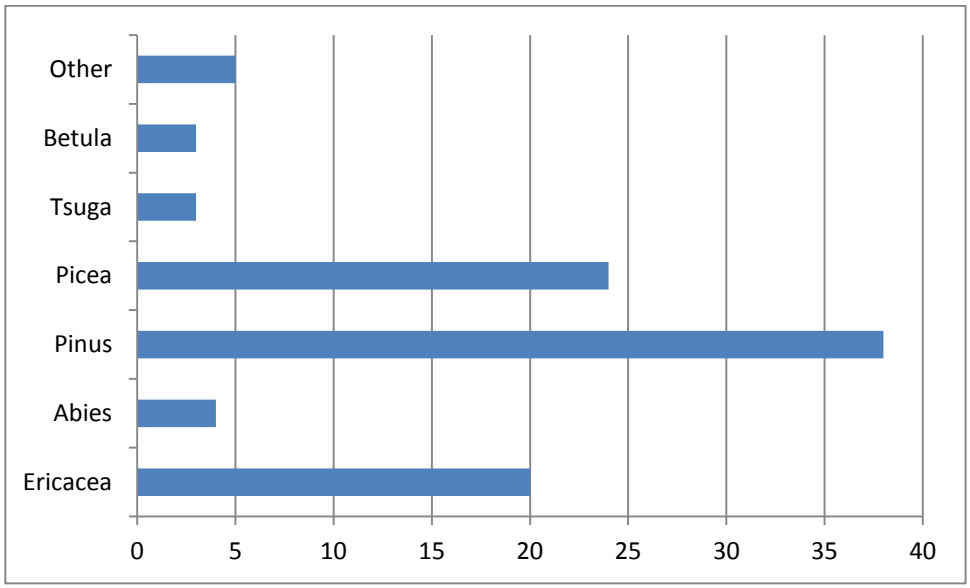

Figure 2-1: Literature reports of detection of *M. variabilis* ITS DNA grouped by plant genus. The majority of reports are from members of the Pinaceae.

Laboratory studies have been very useful for elucidating the physiological underpinning of the relationship between fungus and host. *M. variabilis* does not form relationships that are deleterious to its host in greenhouse or lab studies, nor does it exhibit strong saprophytic abilities (Mayerhofer et al., 2013). It also only seems to form hyphal coils (the hallmark of an ericoid symbiosis) in the hair roots of ericaceous plants (Hambleton and Sigler, 2005). Given these

factors, we set out to design species-specific DNA primers to amplify *M. variabilis* from field samples, which could be used to study the host range and preference of *M. variabilis* using both endpoint PCR and qPCR.

Developing single-species primers is not a new idea (Kernaghan et al., 2006), but using them to study the interaction between a fungus acting as an ERM associate and a fungal root endophyte simultaneously is novel. The requirements we sought from our primers were: i) the ability of the primers to amplify *M. variabilis* under conditions where primers would be exposed to genomic DNA from other fungal and plant species, and ii) optimization for qPCR in such a way that they would not produce false amplification positives in the form of primer-dimers.

Finally, as a way to field test the primers, plant species that *M. variabilis* had not previously been detected on would be sampled, as well as those it was known to colonize. This would potentially allow for further examination of the host range of *M. variabilis*. Using GenBank accessions as a proxy for host plant range, as of February 15, 2013, the Pinaceae and the Ericaceae are the most common families with which *M. variabilis* associates. However, as the overwhelming majority of studies that led to depositions in GenBank were from these two families (Figure 1) this may not reflect the true distribution of *M. variabilis.*

The majority of field studies that included *M. variabilis* in their GenBank depositions were conducted by a handful of research groups. These groups of researchers tended to study Scots Pine (*Pinus sylvestris*) and the communities around them. Other surveys were of ericaceous communities, which are of particular interest to researchers studying northern plant communities (Gorzelak et al., 2012). Therefore, they were not explicitly looking for *M. variabilis* in the field; it may have just happened to turn up frequently in their surveys of the trees and forest floor

communities. By including plants from outside the Ericaceae in the plant survey, the observed host range of *M. variabilis* will potentially be expanded.

Materials and Methods

Primers were designed utilizing publicly accessible nucleotide sequences from the National Center for Biotechnology Information (NCBI) database GenBank. The NCBI search engine Entrez was used to search for ITS sequences of *M. variabilis* and its close relatives. Two hundred and fifty-nine sequences, including all *M. variabilis*, *Meliniomyces*, *Rhizoscyphus*, *Cadophora* and several other species from closely related genera, were downloaded. Of these sequences, seven were removed for being too short (not including all of ITS1 or ITS2), an incorrect gene, or an unknown environmental sample. From the remaining sequences, 94 *M. variabilis*, including uncultured and environmental samples, belonged to the genus *Meliniomyces* and 158 sequences were from related species (See table 1 in Appendix 4). The sequences were aligned using the program MUSCLE (Edgar, 2004) and then inspected visually in BioEdit (Hall, 1999) for loci unique to *Meliniomyces variabilis*.

Two unique loci were identified in *M. variabilis*: one locus towards the 3' end of the ITS1 region and another locus towards the 3' end of the ITS2 region (Figure 2). From these two loci, a number of potential forward and reverse primer sequences were considered. Primers were amplified *in silico* using Amplify 3 (Engels, 2004) and the primers with the lowest potential for self-annealing and primer dimer formation were synthesized (Sigma). These primers were optimized to increase template affinity, harmonize melting temperatures, and minimize primer dimer formation by altering length and GC content. This included adding or removing bases from the 3' or 5' end to change the theoretical annealing temperature. Two forward and two reverse

primers were chosen and synthesized by Sigma Canada as salts. The final four primers are shown in Table 1.

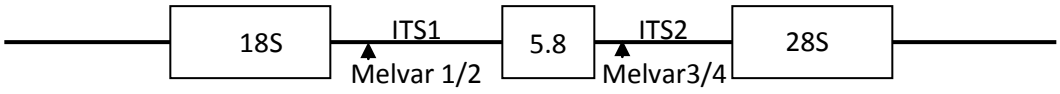

Figure 2-2: Annealing sites of *Meliniomyces variabilis* specific primers. Melvar1 and Melvar2 are forward primers that bind in the ITS1 region of *M. variabilis*. Mevlar3 and Melvar4 are reverse primers that bind in the ITS2 region. This allows for coverage of the highly conserved 5.8 subunit, as well as parts of the highly variable ITS1 and ITS2 regions.

Table 2-1: Sequences of the four species specific *Meliniomyces variabilis* PCR primers.

Forward Primers (5'→ 3')			Reverse Primers (5'→ 3')		
Primer	Sequence	T_m (^oC)	Primer	Sequence	T_m (^oC)
Melvar 1	CAT AAG AAT GGG TTC ATT CCC TT	52.5	Melvar 3	AGT AAC CAC CGG AAC CCT ATA	54.8
Melvar 2	AAA AAA AGA GAA ACG TCC CGT T	53.4	Melvar 4	GTT TTG GCA AGT AAC CAC GG	54.7

Primer combinations Melvar1+Melvar3 (Melvar1/3) and Melvar1+Melvar4 (Melvar1/4) were tested first using endpoint PCR to amplify template DNA extracted from pure cultures from Northern Quebec and the Cape Breton Highlands National Park, the identity of which had been verified by ITS sequencing (ARSL190907.5, ARSL190907.72, ARSL 220507.2, and ARSL 230507.30I)((Kernaghan and Patriquin, 2011). PCR was carried out on an Applied Biosystems Veriti 96 well thERMocycler (Applied Biosystems) using GoTaq Green Master Mix (Promega) following the manufacturer's instructions mixed to volumes of 25 *u*l. The reaction conditions in the thERMocycler were as follows: 95^{o}C for 10 minutes to ensure all template DNA was

dissociated, followed by thirty cycles of 95^{o}C for 1 minute melting, annealing temperatures ranging from 52^{O}C to 66^{O}C for 30 seconds, 72^{o}C for 2 minutes of extension, and a final extension at 72^{o}C for 10 minutes. Based on the initial temperature gradient, the optimal annealing temperature was detERMined to be 58^{o}C. A portion of PCR products were run on agarose gels to check that products of the expected size were being produced.

PCR products were analyzed by RFLP analysis of PCR products using the restriction enzymes Alu I, Hinf I, and Taq I (Invitrogen), to ensure that the primers were amplifying the same sequence from all cultured isolates. PCR products and digested fragments were then mixed with EZ vision DNA dye (Amresco) following the manufacturer's instruction and run on sodium borate agarose gels (SB gels) for imaging using an Alpha Imager EP (Alpha-Innotech).

In order to ensure that the Melvar1/3 and Melvar1/4 primer combinations were amplifying only *M. variabilis*, these primers were used in PCR reactions with the DNA from a number of closely-related species (within the genus *Meliniomyces*), as well as unrelated species, as templates (Table 3). Endpoint PCR was conducted as before, and PCR products were run on agarose gels to ensure they were of the expected size. Aplicons identities were verified with RFLP using the restriction enzymes AluI, HinfII, and TaqI (Figure 2-5).

To test the applicability of the primers to plant materials collected from the field, a number of species were collected from Taylor Head Provincial Park in Nova Scotia (Table 2). Roots were frozen in 600 *u*L of CTAB buffer and then homogenized using a mortar and pestle. Six hundred *u*L of CTAB was added to the homogenized mix, to remove any further DNA from the pestle. The supernatant was transferred to a 2.5 mL Eppendorf tube and incubated at 65^{o}C and 700 RPM in an Eppendorf ThERMomixer R (Eppendorf). Six hundred *u*L of chloroform: isoamyl

alcohol (24:1) was added and the mixture was vortexed until emulsified. Lipids, proteins and other organic PCR contaminants were then separated by centrifugation at 13,000 RPM for 15 minutes in an Eppendorf Centrigue 5424 (Eppendorf). The aqueous supernatant was transferred to a new 2.5 mL tube and mixed by inversion with 600 *u*L of cold isopropanol, and then frozen. The mixture was centrifuged again at 13,000 RPM for 10 minutes and the supernatant was discarded. The pellet was washed twice with 70% ethanol and then air dried. Once the sample was dry, it was re-suspended in 50 *u*L of nuclease free H_2O until needed. PCR was carried out on extracted DNA using the primer combination Melvar1/Melvar3. Aliquots of the PCR products were verified using RFLP as before, and another portion was sent to Genome Quebec at McGill University, Montreal, Quebec, Canada, for Sanger Sequencing. Resulting ITS sequences were then identified by comparison with GenBank accessions using Blast.

Table 2-2: Species sampled for primer specificity testing and information on *M. variabilis* host range.

Host plant	Location
Empetrum nigrum	Taylor Head, N.S.
Linnea borealis	Taylor Head, N.S.
Cornus canadensis	Taylor Head, N.S.
Kalmia polifolia	Taylor Head, West Mabou Beach, and Purcell's Cove, N.S.
Vaccinium vitis-idaea	Taylor Head, N.S.
Rhododendron canadensis	West Mabou Beach, N.S.
Rhododendron tomentosum	West Mabou Beach, N.S.
Abies balsamea	Mealy Mountains, Labrador
Picea mariana	West Mabou Beach, Purcell's Cove and Mealy Mountains, Labrador
Larix lacrina	West Mabou Beach, N.S. and Mealy Mountains, Labrador
Acer pseduoplanatus	Central Poland

The host range of *M. variabilis* was examined utilizing publicly available ITS sequences downloaded from Genbank (NCBI). Studies that included information on host plants from which *M. varaiabilis* had been isolated were compiled to assess the distribution of *M. variabilis*. The number of plants from which *M. variabilis* had been isolated was then compared to the number of studies that submitted sequence data. Sequences were downloaded several times from Genbank using the Entrez search engine, up to February 15, 2013.

Results

Primer combinations Melvar1/3 and Melvar1/4 successfully amplified M. *variabilis* from pure culture (Figure 3). The fragments were of the size expected based on *in silico* simulation in the program AMPLIFY. As seen in Figures 3 and 4, Melvar1/3 produced much cleaner amplification, while Melvar1/4 resulted in product "streaking" across the gel; Melvar1/4 also produced significantly more primer-dimers. To mitigate this, a temperature gradient ranging from 56^{O}C to 66^{O}C was used to find the optimal conditions for Melvar1/3 and Melvar1/4. A temperature range of 62-64^{o}C produced the most product, while limiting the amount of primer-dimers for the Melvar1/3 combination.

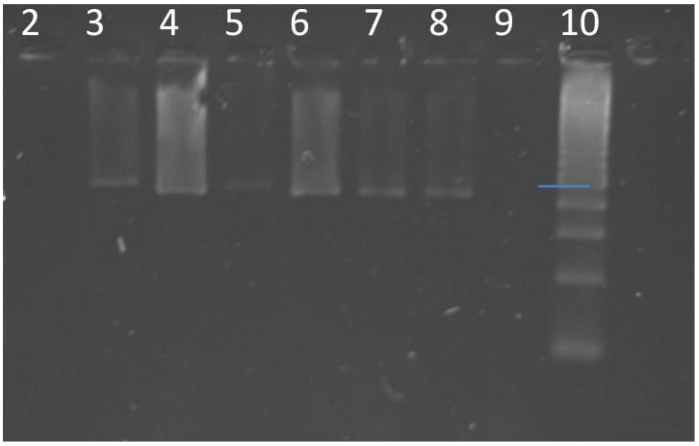

Figure 2-3: Gel image of *M. variabilis* strain ARSL 230507.30I amplified with Melvar1/3 primers and stained with EZ vision DNA dye (Mandel Scientific), expected product ~450bp. Lane number 10 contains a 100bp DNA ladder with 450bp indicated in blue, and lane 9 contains a negative control (dH_2O). Lanes three though eight are *M. variabilis*, where PCR annealing temperatures were $56^{O}C$, $58^{O}C$, $60^{O}C$, $62^{O}C$, $64^{O}C$ and $66^{O}C$.

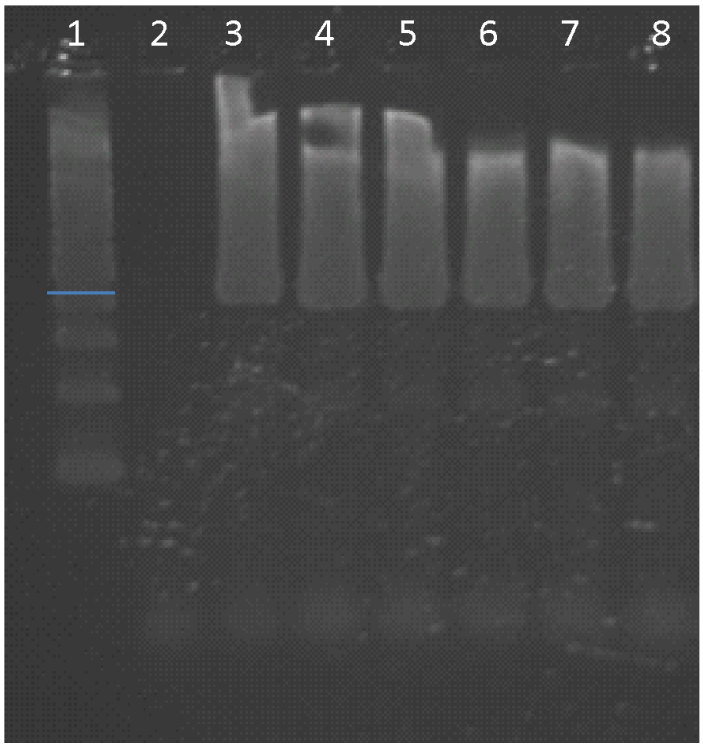

Figure 2-4: Gel image of *M. variabilis* strain ARSL 230507.30I ITS amplified with the primer combination of Melvar1/4 and stained with EZ vision DNA Dye, producing an expected product of ~450bp. The first well contains a 100bp DNA ladder, with 450bp marked in blue. The second lane contains a negative control (dH_2O). Lanes three through eight were *M. variabilis* samples, amplified at annealing temperature of $56^{O}C$, $58^{O}C$, $60^{O}C$, $62^{O}C$, $64^{O}C$ and $66^{O}C$. Aplicons are very streaky and a large amount of primer-dimers are visible at the end of the wells.

As some isolates produced double bands when amplified with Melvar1/4, Melvar1/3 was used for the remainder of experimental amplifications. Using an annealing temperature of $64^{o}C$ limited the streaking of products when using Melvar1/3. No such temperature was found to limit product streaking with Melvar1/4. Isolates ARSL 230507.30I and ARSL190907.5 were chosen as standard isolates to include with subsequent isolations and amplifications as a positive control for PCR experiments.

PCR products were digested with AluI (figure 5), HinfI and TaqI (not shown) and all primer set congruent digestion patterns within their primer combination. There is a slight, but

expected, difference in size between the products produced by Melvar1/3 and Melvar1/4, with the Melvar1/4 products being the smaller of the two. There were no significant differences in the size of the restriction fragments from the four isolates used in these digestions

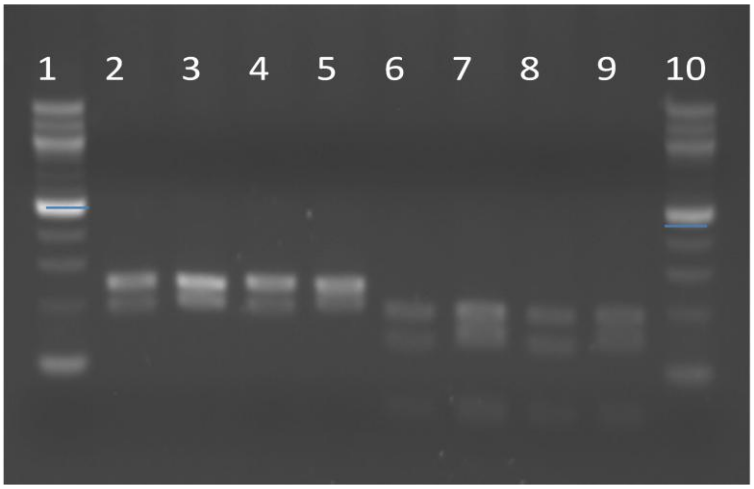

Figure 2-5:RFLP digestion of two isolates amplified with Melvar1/3 and Melvar1/4 using the restriction enyme AluI. PCR products amplified by Melvar1/3 are slightly larger than those produced by Melvar1/4, and have a brighter profile in gel imaging. Lanes 2 and 6 contain isolate ARSL190907.5, lanes 3 and 7 are ARSL190907.72, lanes 4 and 8 are ARSL 220507.2,, and lanes 5 and 9 are ARSL 230507.30I.

Table 3 indicates the results of Melvar1/3 amplification of *M. variabilis* and related species. The Melvar1/3 primer combination was tested against species within the *Rhizoscyphus ericae* species complex, a group containing the genus *Meliniomyces* and therefore closely related to *M. variabilis*. Melvar1/3 did not amplify the ITS of any species in this aggregate, while the non-species specific fungal primers NSI1/NLB4 (Martin and Rygiewicz 2005) did amplify the DNA of these species. Melvar1/3 was also unable to amplify DNA from other common root associates, including distantly related species, while NSI1/NLB4 amplified these successfully. Therefore, Melvar1/3 only amplified *M. variabilis* even when in the presence of amplifiable fungal DNA from other species.

Table 2-3: Results of attempted amplification of species phylogenetically related to *M. variabilis*, as well as unrelated but common root associates, using either the universal PCR primers NSI1-NLB4 or the *Melinomyces variabilis* specific primers Melvar1/3. Species that were successfully amplified by endpoint PCR using either NSI1-NLB4 fungal specific universal primers or Melvar1/3 species specific primers are designated with a plus (+). Those that did not amplify are designated with a minus (-) sign.

Species name:	NSI1/NLB4	Melvar1/3
Meliniomyces variabilis	+	+
Meliniomyces bicolour	+	-
Meliniomyces vraolstadiae	+	-
Meliniomyces. sp.	+	-
Rhizoscyphus ericae	+	-
Fusarium oxysporum	+	-
Fusarium solani	+	-
Phialacephala fortinii	+	-
Armillaria ostoyae	+	-
Saccharomyces cervisiae	+	-
Unknown sterile ascomycete	+	-

When attempting to amplify *M. variabilis* DNA from plants sampled from field conditions Melvar1/3 successfully amplified only *M. variabilis*. Table 4 shows the results of isolation and amplification with Melvar1/3. *M. variabilis* was found on every plant that was sampled, with the exception of *Larix laricina* and *Acer pseudoplanatus* from southern Poland. Positive amplifications were tested using RFLP and then verified by Sanger sequencing.

Table 2-4: Range of ericaceous and non-ericaceous plants tested for presence of *M. variabilis* with the Melvar1/3 primers. PCR product from successful amplification was then verified using RFLP analysis and Sanger sequencing.

Host plant:	Melvar1/3	Sequence Verified
Empetrum nigrum	+	+
Linnea borealis	+	+
Cornus Canadensis	+	+
Kalmia polifolia	+	+
Vaccinium vitis-idaea	+	+
Rhododendron Canadensis	+	-
R. tomentosum	+	-
Abies balsamea	+	+
Picea mariana	+	+
Larix laricina	-	NA
Acer pseudoplanatus	-	NA

From the 98 ITS sequences obtained from GenBank on February 15th, 2013, it is clear that many sequences were derived from samples taken from few species. On average, each study added one new host species for *M. variabilis*. The majority of samples were from *Pinus* and *Picea*, and the Ericaceae. Adding five new hosts for *M. variabilis* using Melvar1/3 brought the total number of known host plants to 24. Three new plants in the Ericaceae and two in Caprifoliaceae were detected using Melvar1/3 (Table 5). These 24 host species are representatives of seven plant families, the most common being the Pinaceae and Ericaceae. Members of the other five families, the Betulaceae, Orchidaceae, Cornaceae, Myrsinaceae, and Caprifoliaceae, have had *M. variabilis* detected on their roots substantially less often.

Table 2-5: Host plants from which *M. variabilis* has been detected on and verified by sequencing . Authors, GenBank accession numbers and the journal/title of the project are included for reference. New plant hosts from this study have not yet been submitted to GenBank and are marked with an asterisk.

Host	Host Family	Accession	Author
Cypripedium acaule Ait.	Orchidaceae	AY838792	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Phyllodoce empertiformis (sm.) D. Don	Ericaceae	AY838788	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Picea sitchensis (Bong.) Carr.	Pinaceae	AY838786	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Picea mariana (P. Mill.) B.S.P.	Pinaceae	AY838784	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Rhododendron albiforum Hook.	Ericaceae	AY762619	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Empetrum Nigrum L.	Ericaceae	AY838789	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Vaccinium membranaceum	Ericaceae	AY838787	Hambleton and Sigler. Stud. Mycol. 53, 1-27 (2005)
Rhododendron dauricum	Ericaceae	JQ088277	Yang and Yan. Unpublished.
Rhododendron sp.	Ericaceae	JQ272408	Baird. Unpublished.
Trientalis europaea	Myrsinaceae	HQ856903	Sauvola *et al. Unpublished.*
Picea glauca	Pinaceae	HQ157933	Kernaghan and Patriquin. Microb. Ecol. 62 (2), 460-473 (2011)
Abies balsamifera	Pinaceae	HQ157887	Kernaghan and Patriquin. Microb. Ecol. 62 (2), 460-473 (2011)
Betula papyrifera	Betulaceae	HQ157845	Kernaghan and Patriquin. Microb. Ecol. 62 (2), 460-473 (2011)

Picea abies	Pinaceae	EF093177	Vohnik *et al*. Unpublished
Pinus sylvestris	Pinaceae	HM190125	Bubner and Muenzenberger. Unpublished.
Pinus sylvestris	Pinaceae	FN678878	Grelet *et al.* Unpublished
Vaccinium vitis-idaea	Ericaceae	FN678876	Grelet *et al.* Unpublished
Tsuga heterophylla	Pinaceae	AY394898	Lim *et al*. Unpublished
Gaultheria shallon	Ericaceae	AF149083	Millar *et al*. Unpublished.
Rhododendron changii	Ericaceae	GU206875	Liu and Li. Unpublished
Linnea borealis	Caprifoliacea	NA	Present study.
Cornus canadensis	Caprifoliacea	NA	Present study.
Kalmia polifolia	Ericaceae	NA	Present study.
Rhododendron canadensis	Ericaceae	NA	Present study.
Rhododendron tomentosum	Ericaceae	NA	Present study.

Discussion

The species-specific primer combinations Melvar1/3 and Melvar1/4 successfully amplified *M. variabilis*. The primer combination Melvar1/3 was determined to be the most effective primer by *in silico* modeling, due to the potential production of fewer double bands than Melvar1/4. Furthermore, Melvar1/4 produced too many primer-dimers for accurate qPCR quantification of fungal DNA from soil samples (Figure 4). Therefore, in subsequent specificity tests, we focused on Melvar1/3. The primers were tested against many different *M. variabilis* isolates from different geographical locations and were found to amplify all isolates. Some

isolates did produce double bands when amplified with Melvar1/3 and this was thought to be due to the variant strains possessing two forms of ITS, with one form being slightly shorter than the other. As such, we isolates ARSL 230507.30I (from Northern Quebec) and ARSL190907.5 (from the Cape Breton Highlands) were chosen as positive controls in subsequent qPCR amplifications.

Melvar1/3 amplified only *M. variabilis* ITS and not that of the other fungi tested. This exemplifies the the specificity of the Melvar1/3 primers to *M. variabilis*, as Melvar1/3 was tested against all the currently known species from the *Rhizoscyphus ericae* species aggregate. Table 3 summarises the results of this experiment, and shows that for all isolates other than *M. variabilis*, PCR was successful only with the general fungal primers NSI1/NLB4.

Taxon specific primers, such as the Melvar primer sets derived for this study, can help to elucidate the workings of the microbial world by allowing us to examine the abundance and distribution of specific microbes from environments harbouring a multitude of other microbial species, and relate this information to local environmental factors. Using broader approaches, such as clone libraries or Next Generation Sequencing, can lead to errors in estimating the proportion and abundance of microbes due to preferential amplification of certain host DNA templates in the sample (Dickie, 2010). The potential for overestimation is also true for single taxon PCR primers, but quantification by this approach is likely to be more accurate.

Additional to the design of species specific primers, a significant outcome of this study was the increase in the range of plants that were associated with *M. variabilis*. *M. variabilis* was isolated, amplified, verified from five host plant species from which *M. variabilis* had not previously been associated. Although *M.variabilis* ITS DNA was successfully amplified from surface sterilized roots, nothing can be said about the nature of the plant-fungal relationship. Therefore, further histological studies should be done to elucidate the nature of the association

and to investigate whether *M. variabilis* is colonizing these plants pathologically, endophytically, or as mycorrhizae. We suspect that *M. variabilis* is forming relatively intimate associations with these plants as previous work has shown that it forms hyphal wefts on the surface of *P. abies* roots, as well as penetrating and growing through the cortical cells of the roots (Vohník et al., 2007).

Based on our PCR survey, *Larix laricina* and *Acer psuedoplanatus* did not appear to be associates of *M. variabilis*. *Larix* sampled from both Cape Breton and Labrador were not colonized by *M. variabilis* at levels detectable by PCR. *A. pseudoplanatus* was collected from southern Poland near Krakow, and also failed to produce any amplifiable *M. variabilis* PCR product. This may be an example of niche partitioning (Simard and Austin, 2010), in which *M. variabilis* is excluded from *L. lacrinia* and *A. pseudoplanatus* by competition from other mychorrizal fungi and endophytic fungi. It is possible that as an endophyte, *M. variabilis* does not provide any benefit to these hosts; or these hosts are more specialized in their recruitment of endophytes and are somehow excluding *M. variabilis*. Further research into the inoculation of sterile seedlings of these plants and histological studies would go a long way to determining if *M. variabilis* is indeed somehow excluded from colonizing these species; if there was colonization too low for the qPCR assay to detect, or if the roots sampled were simply not colonized. This information would be very interesting with respect to *Larix*, as it is a northern, ectomycorrhizal member of the Pinaceae. Our results, and previous studies, show that *M. variabilis* colonizes many similar hosts (*Abies, Tsuga, Picea* and *Pinus*) and an investigation of potential exclusionary mechanisms that *Larix* uses to prevent *M. variabilis* could be very useful for understand fungal endophytes and how their host ranges are created and maintained.

It is worth noting that the two species for which *M. variablis* colonization was not detected (*A. psuedoplanatus* and *L. laricina)* were also the only two deciduous tree species sampled. In fact, the majority of the hosts on which *M. variablis* has been detected are inhabitants of the boreal forest, and there are no records from more southerly habitats.

Never the less, the host association data from GenBank and our brief survey show that *M. variabilis* is a very widespread root associate, capable of forming both ericoid mycorrhizae and endophytic associations with a wide variety of hosts. The Ericaceae tolerate low quality soils, as do many of the plant species that *M. variabilis* colonizes (Figure 4). Depending on how *M. variabilis* is distributed on different hosts, it may be possible to gauge host preference based on the relative abundance of *M. variabilis* on its different host plants.

M. variablilis may either be primarily an ericoid mycorrhizal fungus, or it might exists primarily as an endophytic fungus. Given that *M. variabilis* forms hyphal coils when grown with a suitable ericaceous host, and not when colonizing other hosts (Hambleton and Sigler, 2005), it may be that it is primarily an ericoid mycorrhiza former, and that it forms endophytic associations as "accessory" colonisations. It has been suggested that due to the ephemeral nature of ericaceous hair roots, ERM may form associations with other plants in order to persist in the environment until more hair roots are present (Bergero et al., 2000; Bergero et al., 2003; Vohník et al., 2013).

Other members of the *Rhizoscyphus ericae* species aggregate have been known to form both ERM and ECM associations when suitable plant hosts are present (Vrålstad et al., 2000). A third option is that *M. variabilis* is ubiquitous in environments where the Ericaceae occur, and will colonize everything either endophytically or as an ERM, given the opportunity. Further studies are required to determine the host range *M. variabilis* and its relationships with hosts both within and outside the Ericaceae.

References

Bergero, R., Girlanda, M., Bello, F., Luppi, A. M., and Perotto, S. (2003). Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. *Mycorrhiza*, *13*(2), 69–75. doi:10.1007/s00572-002-0202-9

Bergero, R., Perotto, S., Girlanda, M., Vidano, G., and Luppi, a M. (2000). Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (Quercus ilex). *Molecular Ecology*, *9*(10), 1639–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11050558

Burns, T. D., White, T. J., and Taylor, J. W. (1991). Fungal Molecular Systematics. *Annual Review of Ecology and Systematics*, *22*, 525–564.

Dickie, I. a. (2010). Insidious effects of sequencing errors on perceived diversity in molecular surveys. *The New Phytologist*, *188*(4), 916–8. doi:10.1111/j.1469-8137.2010.03473.x

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–7. doi:10.1093/nar/gkh340

Filion, M., St-Arnaud, M., and Jabaji-Hare, S. H. (2003). Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods*, *53*(1), 67–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12609725

Gardes, M., White, T. J., Fortin, J. A., Bruns, T. D., and Taylor, J. W. (1991). Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Canadian Journal of Botany*, *69*, 180–190. Retrieved from http://www.nrcresearchpress.com/doi/pdf/10.1139/b91-026

Gorzelak, M. a, Hambleton, S., and Massicotte, H. B. (2012). Community structure of ericoid mycorrhizas and root-associated fungi of Vaccinium membranaceum across an elevation gradient in the Canadian Rocky Mountains. *Fungal Ecology*, *5*(1), 36–45. doi:10.1016/j.funeco.2011.08.008

Grelet, G.-A., Johnson, D., Vrålstad, T., Alexander, I. J., and Anderson, I. C. (2010). New insights into the mycorrhizal Rhizoscyphus ericae aggregate: spatial structure and co-colonization of ectomycorrhizal and ericoid roots. *The New Phytologist*, *188*(1), 210–22. doi:10.1111/j.1469-8137.2010.03353.x

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 95–98. Retrieved from http://jwbrown.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf

Hambleton, S., and Sigler, L. (2005). Meliniomyces, a new anamorph genus for root-associated fungi with phylogenetic affinities to Rhizoscyphus ericae (Hymenoscyphus ericae),

Leotiomycetes. *Studies in Mycology*, *53*(1), 1–27. doi:10.3114/sim.53.1.1

Kernaghan, G., and Patriquin, G. (2011). Host associations between fungal root endophytes and boreal trees. *Microbial Ecology*, *62*(2), 460–73. doi:10.1007/s00248-011-9851-6

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. (2006). Quantification of Cylindrocarpon destructans f. sp. panacis in soils by real-time PCR. *Plant Pathology*, *56*(3), 508–516. doi:10.1111/j.1365-3059.2006.01559.x

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. a, … Rothberg, J. M. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, *437*(7057), 376–80. doi:10.1038/nature03959

Martin, K. J., and Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC microbiology*, *5*(1), 1.

Mayerhofer, M. S., Kernaghan, G., and Harper, K. a. (2013). The effects of fungal root endophytes on plant growth: A meta-analysis. *Mycorrhiza*, *23*(2), 119–128. doi:10.1007/s00572-012-0456-9

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N., and Larsson, K. H. (2008). Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*, *2008*(4), 193–201. doi:10.4137/EBO.S653

Simard, S. W., and Austin, M. E. (2010). Climate change and variability, *1993*(Ipcc 2007).

Smith, S.E. and Read, D. (2008). Ericoid mycorrhizas. In *Mycorrhizal Symbiosis 3rd Edition* (ed. S.E. Smith and D. Read), pp. 389-418. Great Britain: Elsevier ltd.

Smith, C. J., and Osborn, a M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology*, *67*(1), 6–20. doi:10.1111/j.1574-6941.2008.00629.x

Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., Bahram, M., Bechem, E., Chuyong, G., Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New Phytologist*, *188*(1), 291–301. doi:10.1111/j.1469-8137.2010.03373.x

Vohník, M., and Albrechtová, J. (2011). The Co-occurrence and Morphological Continuum Between Ericoid Mycorrhiza and Dark Septate Endophytes in Roots of Six European Rhododendron Species. *Folia Geobotanica*, *46*(4), 373–386. doi:10.1007/s12224-011-9098-5

Vohník, M., Fendrych, M., Albrechtová, J., and Vosátka, M. (2007). Intracellular colonization of Rhododendron and Vaccinium roots by Cenococcum geophilum, Geomyces pannorum and Meliniomyces variabilis. *Folia Microbiologica*, *52*(4), 407–414. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18062190

Vohník, M., Fendrych, M., Kolařík, M., Gryndler, M., Hršelová, H., Albrechtová, J., and Vosátka, M. (2007). The ascomycete Meliniomyces variabilis isolated from a sporocarp of Hydnotrya tulasnei (Pezizales) intracellularly colonises roots of ecto- and ericoid mycorrhizal host plants. *Czech Mycology*, *59*(2), 215–226.

Vohník, M., Mrnka, L., Lukešová, T., Bruzone, M. C., Kohout, P., and Fehrer, J. (2013). The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal Meliniomyces variabilis. *Fungal Ecology*, *6*(4), 281–292. doi:10.1016/j.funeco.2013.03.006

Vrålstad, T., Fossheim, T., and Schumacher, T. (2000). Piceirhiza bicolorata - The ectomycorrhizal expression of the Hymenoscyphus ericae aggregate? *New Phytologist*, *145*(3), 549–563. doi:10.1046/j.1469-8137.2000.00605.x

Chapter 3 : Quantification of M. variabilis by quantitative PCR: the abundance and distribution on two host plants, K. polifolia and P. mariana.

Abstract

Meliniomyces variabilis is a root-associated fungus with a broad distribution throughout the northern hemisphere, and is commonly isolated from Boreal plant species. Using species-specific PCR primers, the present study attempts to elucidate factors that influenced the colonization of two plants known to associate with *M. variabilis* – the ericaceous shrub *Kalmia polifolia* and the dominant Boreal tree *Picea mariana*. By sampling along a gradient of ericaceous ground cover in two similar sites in Nova Scotia, I sought to isolate certain environmental factors that might affect the distribution of *M. variabilis* on these two hosts over a wide geographic range. Real-time PCR determination of *M. variabilis* abundance on the two hosts was found to be heavily influenced by the composition of the plant community, and less influenced by soil factors of the sites.

Introduction

Meliniomyces variabilis has been isolated from a variety of host plants, and forms ericoid mycorrhizal symbioses when grown in the presence of ericaceous hair roots (Grelet et al., 2009; Hambleton and Sigler, 2005). *M. variabilis* also grows endophytically on hosts other than ericaceous plants, in a manner similar to that of the dark septate endophytes (DSE) described by Jumpponen and Trappe (1998).

With the observations that *M. variabilis* is incredibly widespread over a large number of plant families and species (Chapter 2), it is expected that *M. variabilis* will be found in nearly any temperate or boreal environment surveyed. Grelet et al. (2009) demonstrated reciprocal transfer of nutrients between an ericaceous host plant and *M. variabilis*, and the fungus forms hyphal coils in the cells of ericaceous plants (Hambleton and Sigler, 2005). These two findings suggest that *M. variabilis* would preferentially colonize an ericaceous plant when ericaceous plants are present and may only colonize non-ericaceous plants as "accessory" hosts. . Regardless of the preferred host, plant community structure is likey to have an important role in determining the distribution of a root associated fungus such as *M. variabilis* (De Bellis et al. 2007; Kernaghan and Patriquin

2011).Ericoid, ectomycorrhizal and arbuscular mycorrhizal fungi all enhance plant uptake of important mineral nutrients such as N, P and K. It is therefore expected that when *M. variabilis* is colonizing an Ericaceous host, it will be in a region where *M. variabilis* is able to assist its host by assimilating nutrients that would be otherwise unavailable to the plant host. For this reason, the distribution of soil nutrients may be an important factor influencing the distribution of *M. variabilis.*

Following the successful synthesis of species-specific PCR primers for the ericoid mycorrhiza (ErM) *Meliniomyces variabilis*, a survey of two host plants *Picea mariana* and *Kalmia polifolia* was undertaken to examine the influence of ecological factors on the abundance and distribution of *M. variabilis* colonization on the two plants. The species specific PCR primer set was demonstrated in Chapter 2 to be able to detect *M. variabilis* from plant tissues that were collected from field samples. Quantification of *M. variabilis* on field samples of the two host plants by quantitative real-time PCR (qPCR), and collection of soil data and environmental data in the form of ericaceous ground cover and the basal area of over story trees, will allow for an analysis of factors influencing the distribution of *M. variabilis*. The abundance and distribution of *M. variabilis* on the two host plants will be used as a measure of host preference by the ubiquitous root-associated fungus.

qPCR was used to examine this the distribution of *M. variabilis* on both *Kalmia* and *Picea* at two *Picea mariana* dominated sites in Nova Scotia. These two sites were chosen because of the abundance of ericaceous plants making up the understory, and because of the dominance of the boreal tree *P. mariana*. The boreal forest occupies a massive proportion of Canada's land mass, and ericaceous plants are common throughout Canada, from the southern provinces to the

northern tundra. *Kalmia polifolia* is a common ericaceous plant found in the understory of many forest ecosystems and around bodies of water throughout eastern Canada. Therefore, the study of a ubiquitous root-associated fungus on these two hosts means that the results of this study should be broadly applicable to many northern ecosystems.

Materials and Methods

Two study sites, Purcell's Cove near Halifax, Nova Scotia and West Mabou Beach Provincial Park on the southwestern side of Cape Breton Island in Nova Scotia, were chosen for their relatively high abundance of *P. mariana*. On average, the plots from West Mabou Beach had higher *P. mariana* biomass than the plots from Purcell's Cove. The sites from Purcell's Cove were less densely populated by large trees in general, and there was slightly more ground foliage.

Ten plots were set up at each of the two study sites for a total of twenty plots. Plots were selected in such a way as to minimize tree species other than *P. mariana* in order to avoid confounding factors that may result from the presence of multiple canopy tree species. The ten plots at each site were also chosen such that the ground cover provided by ericaceous plants ranged from 0% to ~100% of the interior 2x2m quadrats.

Each plot was composed of two concentric quadrats. First, a 4x4m quadrat was established, in which the diameter at breast height (DBH) was measured as a proxy of tree biomass for all trees in the quadrat. A second 2x2m quadrat, interior to the 4x4m quadrat, was used to collect four 10x10cm soil monoliths and four *K. polifolia* plants (one monolith and one *k. polifolia* per $1m^2$)(Figure 1). Plant tissues and soil were stored in coolers and transported to Halifax, where they were stored at -4^oC until processed. The plant species abundance was estimated within each

interior 2x2m quadrat. Table 1 shows the location of each plot and the abundance of key plants that were used in site selection.

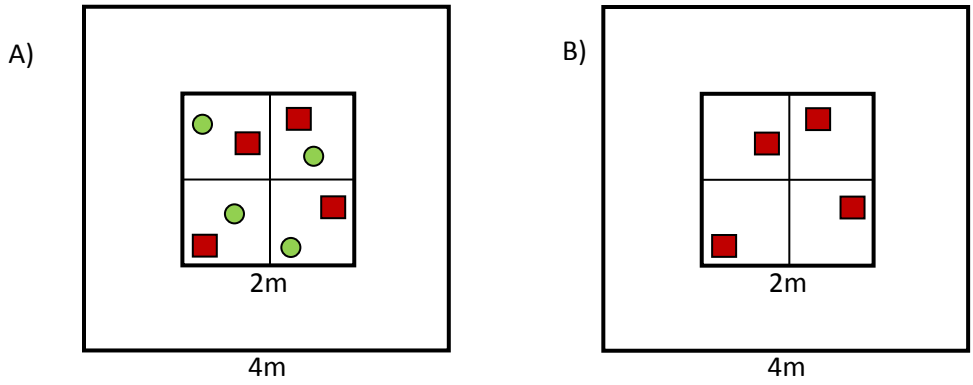

Figure 3-1: Experimental plot design composed of two concentric quadrats. Red squares denote 10cm X 10cm soil monoliths that that were sampled from each plot; green circles represent *K. polifolia* plants that were sampled. On sites with zero *K. polifolia*, there are no green circles as there was no *Kalmia* roots to be sampled. Four soil monoliths and four plants were taken from each plot. Ground cover of ericaceous plants was estimated in the interior quadrat. Tree biomass was measured as DBH throughout the larger 4X4m quadrat. A) A study plot sampling *K. polifolia* and soil monoliths; B) A study plot with no *K. polifolia*.

Table 3-1: The 20 plots at Purcell's Cove and West Mabou Beach Provincial Park. Each plot was numbered and selected according to the abundance of ericaceous shrubs and *Kalmia* on the sites. Plots were chosen such that they were dominated by were *P. mariana* and *K. polifolia*. An asterisk (*) denotes that the study site had more than ten *P. mariana* saplings.

				Diameter at breast height (inches)		Ground Cover (%)	
Site	E (m)	N	W	Total *P. mariana*	Total *A. balsamea*	Total ericaceous	*K. polifolia*
PC01	23	44°36.396'	063°34.389'	34	12	0	0
PC02	26	44°36.412`	063°34.48`	57.5*	0	0.225	0.21575
PC03	28	44°36.306`	063°34.331	16	0	0.225	0.1625
PC04	9	44°36.217`	063°34.294`	15	23	0.62	0.56875
PC05	35	44°36.151`	063°34.172`	2	4	0.9	0.9
PC06	23	44°36.327	063°34.412`	87.5	0	0.545	0.5325
PC07	15	44°36.010'	063°33.929'	30.5	0	0.2625	0.225
PC08	29	44°36.147`	063°34.321`	65	0	1	1
PC09	22	44°36.428`	063°34.542`	108	5	0.375	0.375
PC10	28	44°36.463`	063°34.524`	153.5	7	0	0
MB01	23	46°04.719`	061°27.829`	88	0	0.26375	0.26375
MB02	-17	46°04.641`	061°27.945`	157	0	0	0
MB03	3	46°04.719`	061°28.133`	99.25	0	0.0375	0.0375
MB04	-3	46°04.678`	061°28.221`	40.5*	0	0.2975	0.29
MB05	-8	46°04.717`	061°28.279	99.25*	0	0.47125	0.24375
MB06	-15	46°04.702`	061°28.315`	79.75*	0	0.70875	0.37875
MB07	-12	46°04.789`	061°28.323`	50	0	0.055625	0.0425
MB08	-17	46°04.761`	061°28.404`	70.5	0	0.18875	0.1675
MB09	-10	46°04.725`	061°28.459`	77	0	0.37	0.145
MB10	-13	46°04.689`	061°28.506`	51*	0	0.6325	0.6325

A portion of the soil monoliths were pooled from within each interior quadrat. Pooled 250g and 200g soil conglomerates from the four soil monoliths from each plot were sent to the University of Guelph and to the Faculty of Agriculture of Dalhousie University, respectively, for analysis of organic and mineral content. Ground cover estimates, tree DBH, and soil characteristics were then used as regression variables for analyzing distribution of *M. variabilis* across the twenty plots.

P. mariana roots were extracted from soil monoliths and washed under running tap water. Two centimeter sections of second order roots, with an average length of 2.0 cm and diameter of 1.0 mm, were collected from each quadrat, for a total of 80 root segments. This resulted in an average dry weight of 4.0mg (0.004g) of spruce tissue. Each root segment was then homogenized using an OmniBead Ruptor in 2ml tubes containing a 1:1 mixture of glass and zirconium beads, suspended in 600ml of AP1 buffer from Qiagen DNEasy plant extraction kits. The OmniBead Ruptor was run for ten cycles of one minute on and 55 seconds off at 4500 RPM. The supernatant was collected and the rest of the DNA extraction followed the Qiagen DNEasy protocols.

Sections of *K. polifolia* hair roots were picked off each plant (Valenzuela-Estrada et al. 2008) and weighed such that a total of 1.0mg of frozen (not dried) hair roots was sampled per DNA extraction. This was found to be the equivalent of 4 cm of uncoiled hair root, or 1.5 cm of highly coiled hair roots. This resulted in a 4:1 ratio of tissue mass. One segment from each quadrat was homogenized in 600ml of AP1 buffer from the Qiagen DNEasy extraction kit using a mortar and pestle, for a total of 80 extractions. Following the homogenization, each extraction was carried out using the Qiagen DNEasy extraction kit following the manufacturer's protocols. DNA extractions from both *P. mariana* and *K. polifolia* were diluted to $1/20^{th}$ of their original concentration to avoid over saturating qPCR reagents with excessive template DNA, and to dilute any PCR inhibitors that may have been contained in the soil.

qPCR was carried out in triplicate on all 160 root samples using an Applied BioSystems StepOne Plus RT PCR system (Applied BioSystems). Thermocycling conditions were as follows: a 10 minute melting phase at 95^0C, followed by 60 cycles of a 95^0C melting phase for 15 seconds, a 30-second annealing phase at 64^oC, and extension at 72^OC for 10 seconds. The cycle ended with

a melt curve, which was analyzed for the presence of primer-dimers or failed qPCR runs. An average fluorescence value for each triplicate run was then used for further analysis and standardization. qPCR reactions that failed to produce any product, or were outside the two standard deviations of the average, were excluded from subsequent analyses. qPCR fluorescence values that were anomalously low were assumed to be from failed amplifications, and were counted as 0 ng DNA * mg root^{-1} if all three qPCR attempts failed to amplify *M. variabilis* from the root tissue.

To obtain an absolute number for comparisons between sites and between plants, a standard curve of purified calf thymus DNA and *M. variabilis* DNA from pure culture ARSL 220507.2 was constructed. Calf Thymus DNA of a known quantity and *M. variabilis* DNA with Quant-iT PicoGreen dsDNA reagent (Invitrogen) following the manufacturer's instructions. The fluorescence of a gradient of *M. variabilis* dilution was taken using a BioTek Synergy HT with a Take3 nanodrop adaptor for small quantities of DNA (Biotek). Figure 2 shows an average fluorescence from the quantified Calf Thymus DNA; both axes have been log transformed. The equation of the line of best fit was used to convert fluorescence units (RFU) acquired from qPCR to ng of DNA per ml. This was then compared to the average fluorescence from a dilution series of three cultures of (ARSL 230507.30I, ARSL190907.72, and ARSL 220507.2) *M. variabilis*. This allowed for a more accurate estimation of the colonization of each plant root.

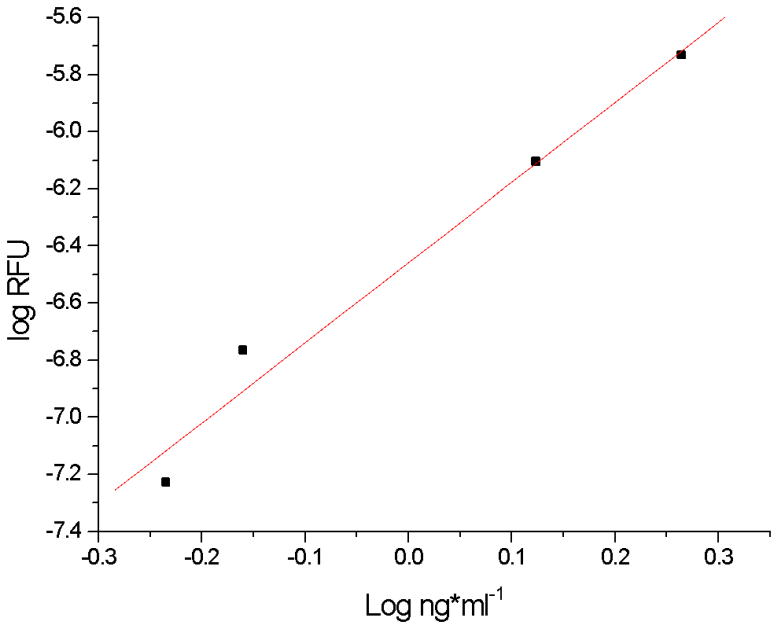

Figure 3-2: Calf Thymus DNA was used to calibrate the line of best fit, and convert *M. variabilis* culture ARSL 220507.2 RFU to ng DNA ml^{-1}. The equation of best fit was y = 2.807x – 6.4591, p = 0.012.

Following Kernaghan *et al.* (2006), LinReg was used to estimate the initial fluorescence (N_0) in each root sample from the qPCR data. LinReg uses assumption-free algorithms to estimate the initial fluorescence, and therefore initial DNA concentration, in a sample by extrapolating from the exponential portion of a PCR amplification curve (Figure 3) (Ramakers et al.2003). This makes LinReg a useful tool for samples that will have a PCR efficiency of less than two, which occurs due to the presence of PCR inhibitors in roots and soil samples (Ramakers et al., 2003). The value of N_0 and the conversion factor obtained from the PicoGreen standard curve were used to determine the amount of *M. variabilis* template DNA in each plant root sample.

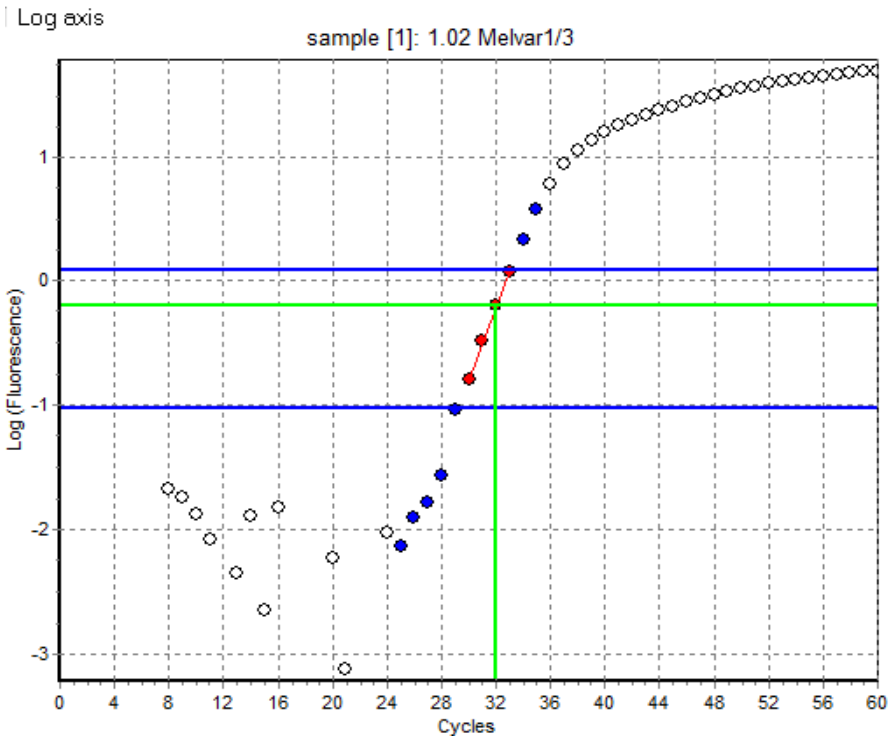

Figure 3-3: Window of linearity during exponential PCR amplification as detERMined by LinReg. Using this information, LinReg detERMines the individual PCR efficiency and estimates the initial concentration of each sample in fluorescent units. Using a conversion factor, the corresponding DNA concentration (ng DNA ml^{-1} extract) was obtained for each sample.

An index of soil nutrient quality for each site was generated for each site by ranking the sites from one to twenty on the basis of mineral nutrient content, excluding nitrogen as it is a good indicator of soil productivity, and summing the ranks. A higher total indicates higher total soil nutrient content. Plant biomass and ground cover estimates were then plotted against the abundance and distribution of *M. variabilis* DNA. Data on plant abundance and soil characteristics were compiled into a correlation matrix along with the concentrations of *M. variabilis* DNA found in the roots of *K. polifolia* and *P. mariana* using SPSS. Plant and soil

factors that were correlated (Pearson correlation $p < 0.05$) with the amount of *M. variabilis* on either host plant at each plot were retained, while the rest were not used in any further analyses.

Canonical correspondence analysis was carried out using the program PAST. Trends in environmental variance were compared across both study sites. Eigenvalue estimates were checked using permutation tests N = 999. Factors that were correlated with M. *variabilis* were further analyzed by multiple regression using MyStat software.

Results

The data output from the StepOne Plus RT PCR system is in relative fluorescence units (RFU) rather than ng DNA. After analyzing the RFU data in LinReg to obtain the initial fluorescence (N_0), the equation of the calibration curve of the calf thymus DNA against *M. variabilis* DNA from pure culture ($y = 2.807x – 6.4591$, $p = 0.012$) was used to determine the initial concentration of *M. variabilis* template DNA (ng) in the root tissue samples.

Amplifications were excluded from analysis if they were outside of one standard deviation from their triplicate N_0 mean. This was done because using two standard deviations did not exclude any amplifications. However, amplifications that did not amplify when the other two other replicates from the same sample did (particularly those that were close to but non-zero when N_0 was determined) were obvious. Therefore, it was determined that one standard deviation was conservative enough to exclude failed amplifications, but lenient enough to allow for sites that actually did not contain *M. variabilis* to be included. This allowed for a much more precise quantification of N_0 DNA values. There was only one plot from which there was no *M. variabilis*

found on *P. mariana*. This was taken to be actual absences as all three PCR amplifications from the plot had failed to amplify any DNA (Table 2).

Table 3-2: Average amount of *M. variabilis* DNA (ng) within root samples of each of the host plants collected from the plots at the two study sites (ng Melvar DNA per sample). NA denotes sites where *K. polifolia* was absent from the sampling area. An asterisk (*) denotes that the study site had more than ten *P. mariana* saplings.

Site	*P. mariana*	*K. polifolia*	Total
MB-01	2.28	*NA*	2.28
MB-02	1.58	*NA*	1.58
MB-03	3.03	5.90	8.93
MB-04*	4.33	6.09	10.42
MB-05*	1.81	7.08	8.88
MB-06*	1.26	6.59	7.86
MB-07	3.48	5.52	9.00
MB-08	3.33	5.25	8.59
MB-09	2.30	6.21	8.51
MB-10*	2.21	3.07	5.27
PC-01	1.48	*NA*	1.48
PC-02*	0.81	2.50	3.31
PC-03	0.20	1.82	2.02
PC-04	2.44	3.64	6.08
PC-05	1.57	3.03	4.60
PC-06	1.61	2.55	4.16
PC-07	0.00	1.65	1.65
PC-08	1.22	3.97	5.20
PC-09	0.88	4.72	5.59
PC-10	1.80	*NA*	1.80

Soil analysis (Appendix 1) indicated that West Mabou Beach and Purcell's Cove were similar in tERMs of organic matter and soil mineral composition. Within study sites, variations in soil factors were larger than the variation between the soil factors of the two study sites. The range of variance for soil factors from both sites was similar between sites, with the greatest variations occurring within sites (between study plots – raw data in Appendix 1 Table 1 and 2).

Both study sites are near-coastal environments, receiving a similar amount of precipitation and wind. Both sites also contain fairly large bodies of water in the forms of a pond in West Mabou Beach Provincial Park, and a bog, small lakes, and streams in Purcell's Cove conservation area.

There was a slight difference in the canopies of the two sites, which could potentially reflect a different disturbance regime. West Mabou Beach contained a higher number of larger *P. mariana* trees than Purcell's Cove, but after excluding seedlings, the difference in the mean DBH was not significantly different. (Figure 4). The plots at Purcell's Cove contained a greater number of seedlings than those at West Mabou Beach, lowering the average DBH of Purcell's Cove.

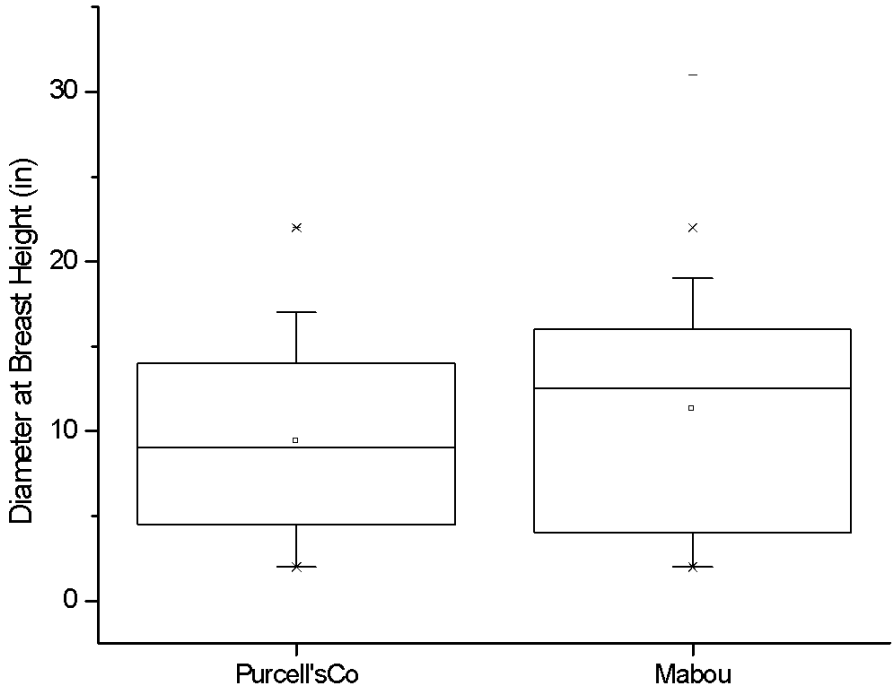

Figure 3-4: Average size of *P. mariana* (DBH) at the West Mabou Beach Provincial Park plots was not statistically different from those at the Purcell's Cove plots after excluding seedlings from the analysis. However, there were a greater number of *P. mariana* trees at West Mabou Beach than at Purcell's Cove, where sites included more seedlings.

Across both study sites potassium (as KO) and magnesium were highly correlated with the colonization of both host plants (*P. mariana* and *K. polifolia)* by *M. variabilis* (KO; 2-tail Pearson Correlation = .0649, p = 0.002, n =20; Mg; 2-tail Pearson Correlation = 0.616, p = 0 .004, n=20). Sodium was positively correlated with the colonization of *P. mariana* (2-tail Pearson Correlation = 0.573, p = 0.008, n=20) and aluminum was negatively correlated with the colonization of *P. mariana* (2-tail Pearson Correlation = -0.493 p = 0 .027, n=20), although neither of these micronutrients were correlated with the colonization of *K. polifolia* by *M. variabilis.* No other soil factors had significant correlations at this scale.

When examining the distribution of *M. variabilis* on both host plants across the two study sites, *M. variabilis* was found in higher absolute amounts (ng DNA) on *K. polifolia* (Figure 5). In every site where both host plants occurred, *M. variabilis* was found in a higher amount on *K. polifolia* than it was on *P. mariana* (Table 2). As seen in Figure 5, the range of host colonization for both hosts overlapped between the highest levels of colonization of *P. mariana* and the lowest levels of *K. polifolia* colonization. The highest detected amount of *M. variabilis* on *P. mariana* is roughly equivalent to the median of the colonization on *K. polifolia*. The lowest level of *K. polifolia* colonization coincides roughly with the average amount of *M. variabilis* DNA detected on *P. mariana*.

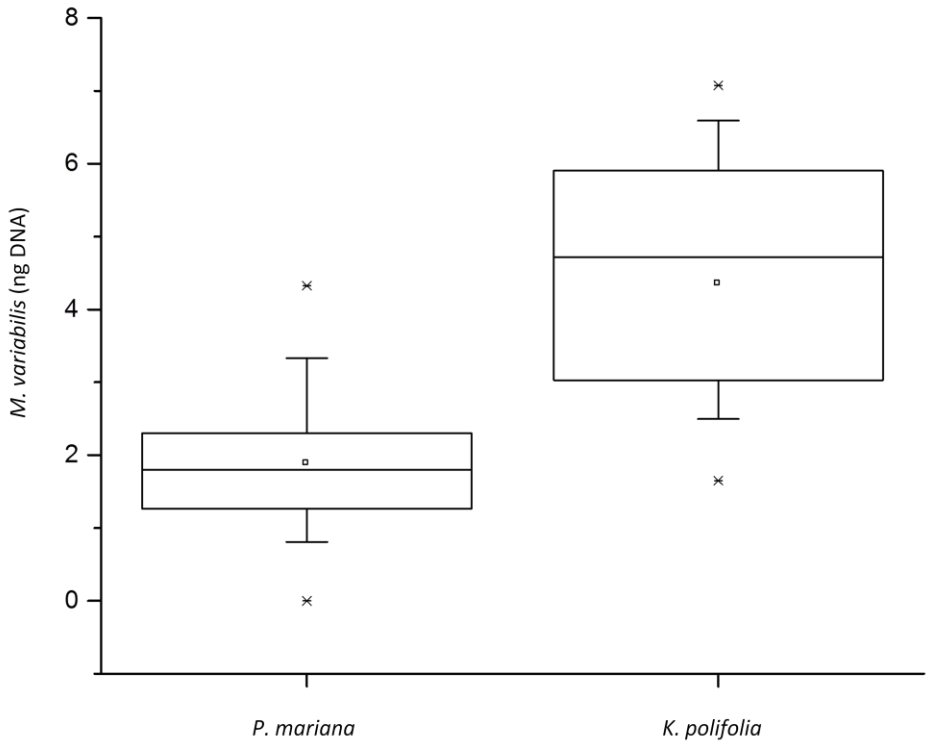

Figure 3-5: Colonization of *P. mariana* and *K. polifolia* by *M. variabilis* across West Mabou Beach Provincial Park and Purcell's Cove. There is only a slight overlap between the lowest levels on *K. polifolia* and the highest levels on *P. mariana* over the 20 study plots. Means and medians are significantly different.

On sites where no *K. polifolia* was present, there were medium-to-high levels of *M. variabilis* DNA detected on *P. mariana*, when compared to the colonization of *P. mariana* on plots with *K. polifolia* (Figure 6). The range in which the colonization of *P. mariana* by *M. variabilis* on sites lacking *K. polifolia* is contained within the range of colonization of *P. mariana* by *M. variabilis* on sites with *K. polifolia* and the mean is not different between the two groups of *P. mariana* (Student's T-test = 0.17748) Though only four of 20 plots are shown on the graph, it should be noted that each of these four points is an average of four plants from each plot.

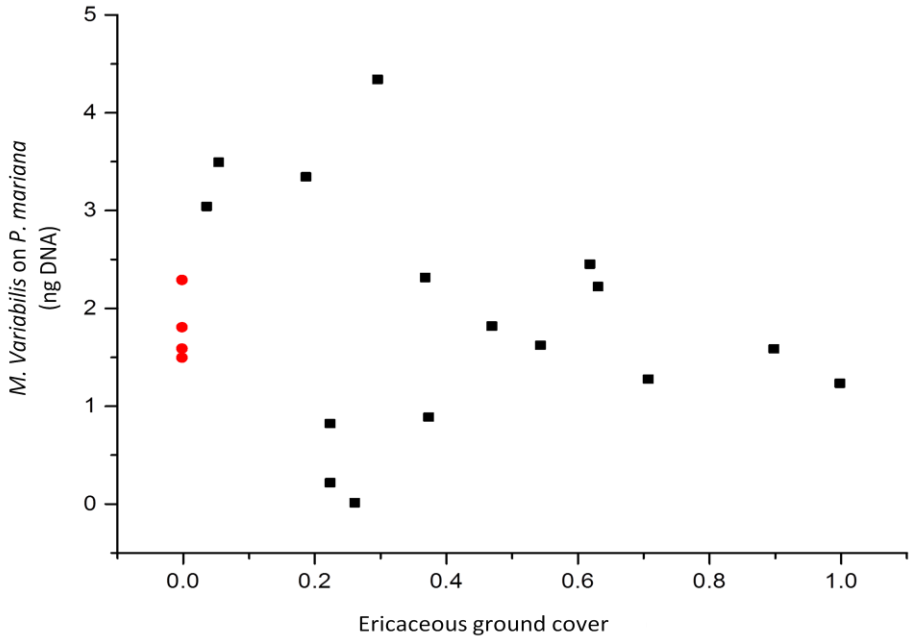

Figure 3-6: Amounts of *M. variablis* DNA on *P. mariana* roots at varying levels of ericaceous ground cover. Plots in which *K. polifolia* is absent are in red.

When comparing the colonization by *M. variabilis* on one host plant as it relates to the abundance of the other across both study sites, one significant correlation and three non-significant correlations were detected (Figure 7). The amount of *M. variabilis* colonizing roots is apparently related to the abundance of each host plant. *M. variabilis* abundance on *K. polifolia* was strongly positively correlated with the biomass (measured as DBH) of the conifers (*P. mariana* and *A. balsamea*) on each plot. Conversely, the amount of *M. variabilis* on *P. mariana* roots was only slightly positively associated with the biomass of conifers, though this association was not significant. Finally, the amount of ericaceous plants on a plot seemed to have a weakly negative correlation between the amounts of *M. variabilis* isolated from both host plants; though, again, these weak correlations were not significant.

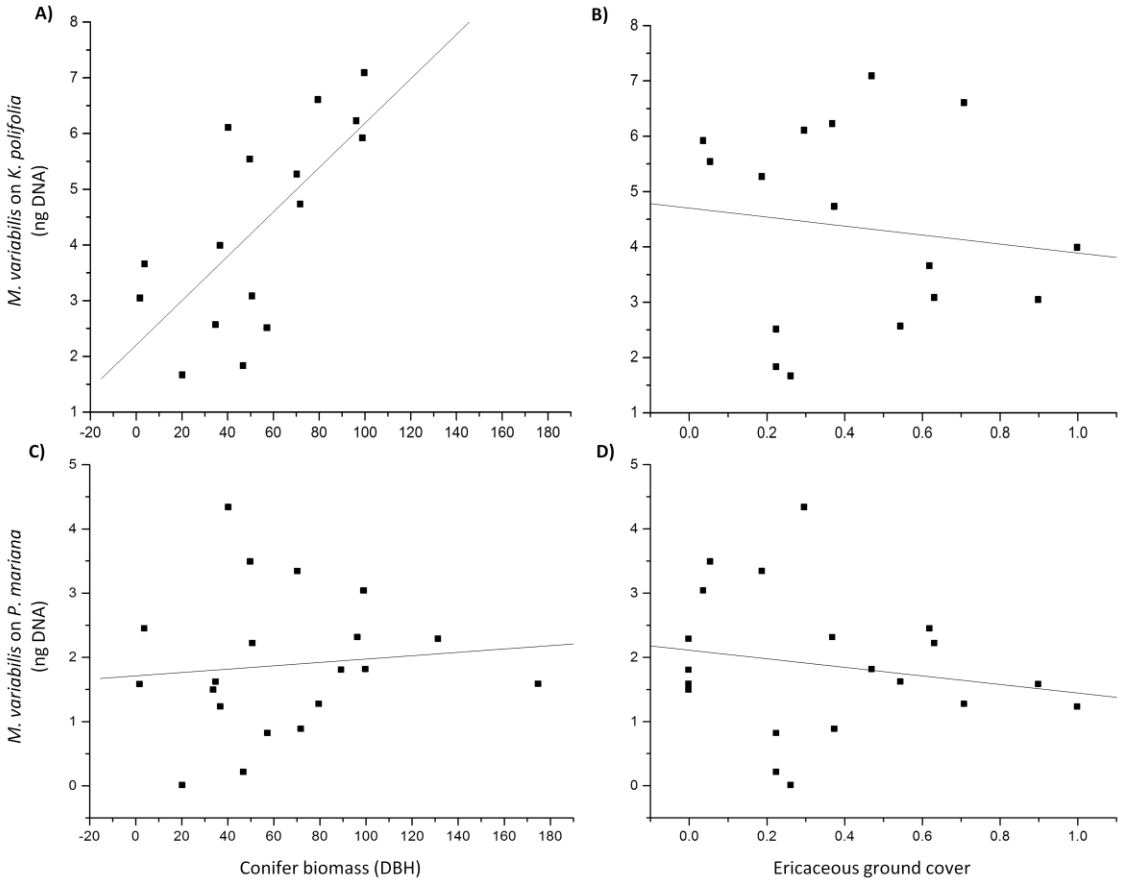

Figure 3-7: The amount of *M. variabilis* DNA onthe two host plants as affected by the abundance of each host plant. **A)** Conifer biomass was significantly correlated with the amount of *M. variabilis* isolated from *K. polifolia* (r^2 = 0.476, p = **0.003**). **B)** *M. variabilis* abundance on *K. polifolia* is weakly, and not significantly negatively correlated with the amount of ericaceous ground cover on a plot (r^2 = -0.017, p = 0.635). **C)** Conifer biomass had a slightly positive association with the amount of *M. variabilis*, though the correlation was not significant (r^2 = 0.011, p = 0.667). **D)** Ericaceous ground cover had a weak, non-significant, negative correlation with the amount of *M. variabilis* from *P. mariana* (r^2 = 0.036, p = 0.452).

There was a strong positive correlation between the amount of *M. varaibilis* on *P. mariana* and on *K. polifolia*. When sampled across all 20 plots, the correlation between the amount of *M. variabilis* isolated from *K. polifolia* and *P. mariana* were correlated, but not statistically significant (Pearson correlation = 0.441, p = 0.051, n = 20). When the sites that did

not include *K. polifolia* were excluded, the correlation between *M. variabilis* and *K. polofiolia* and *M. variabilis* on *P. mariana* became significant (Pearson correlation = 0.605, p =0.013 n = 16).

Colonization of the two host plants correlated differently with pH at the two sites, and pH was not significantly related to the colonization of either plant when calculated in aggregate across both sites (Figure 8). At Purcell's Cove an increase in soil pH (less acidic soil) was negatively associated with the abundance of *M. variabilis* colonization (*P. mariana*, r^2 = -0.80166, p = 0.00529; *K. polifolia*, r^2 = -0.7151, p = 0.04616; total *M. variabilis*, r^2 = 0.2761, p = 0.32276). At West Mabou Beach, the relationship between soil pH and *M. variabilis* colonization was less linear, with the highest amount of *M. variabilis* occurring in host plants with a pH around 4.2 (*P. mariana*, r^2 = 0.72364, p = 0.0111; *K. polofolia*, r^2 = 0.20464, p = 0.56418; total *M. variabilis*, r^2 = 0.60922, p = 0.0373). The range of pH values collected from West Mabou Beach was lower than that of Purcell's Cove, with West Mabou Beach ranging from 4.0 to 4.4 and Purcell's Cove ranging from 3.7 to 4.6.

When data from both sites were pooled, pH did not have a clear effect on the colonization of either plant by *M. variabilis* (*P. mariana*, r^2 = 0.17405, p = 0.19683; *K. polifolia*, r^2 = 0.23734 p = 0.17185). However, the total abundance of *M. variabilis* at the sites may have been influenced by soil pH, although the effect was not significant (total *M. variabilis*, r^2 = 0.27739, p = 0.0632).

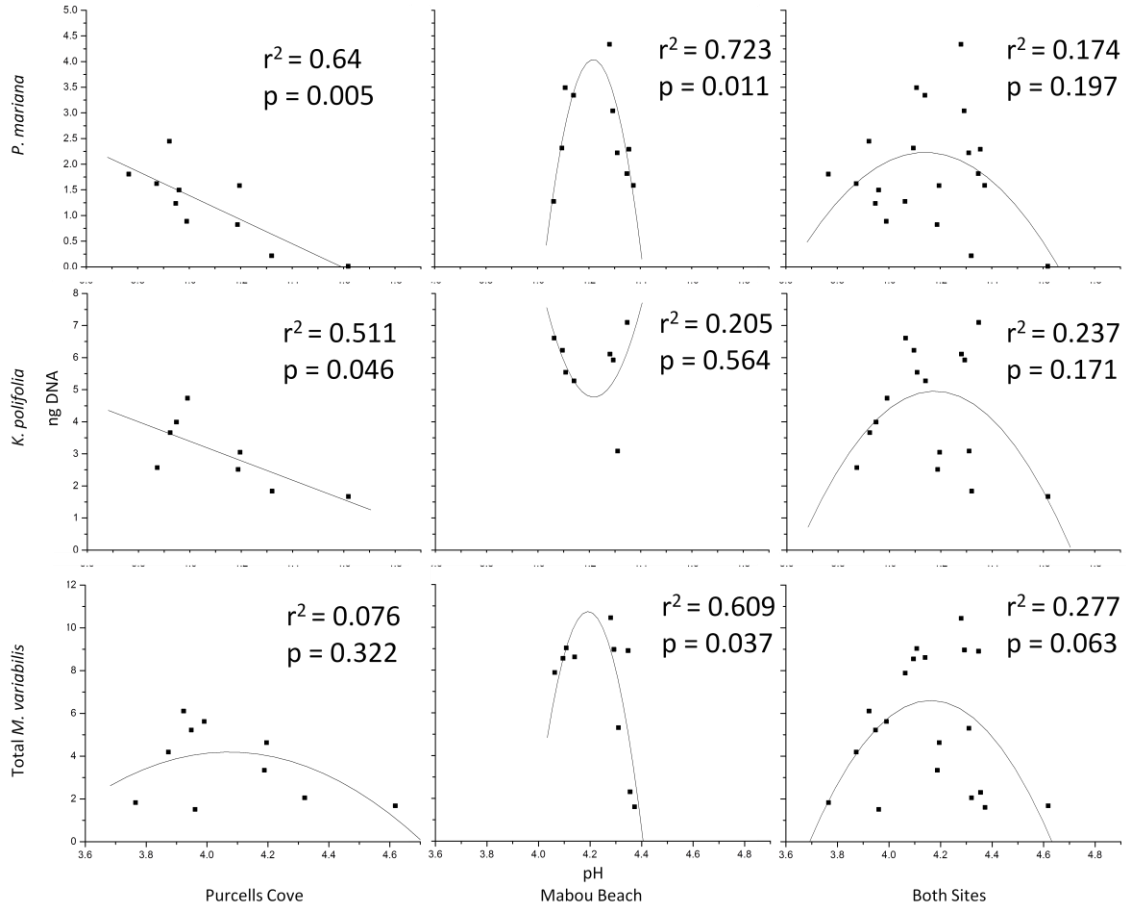

Figure 3-8: Influence of pH on the colonization of host plants by *M. variabilis* at each site and for both sites combined. pH did not have a strong, universal effect on the colonization by *M. variabilis* across host plants or study sites.

The regression coefficients shown in table 4 were selected from the constellation of equations shown in table 3. Equations were selected on the basis of significance, R^2, and AIC. Each system of equations for host colonization had the same general trends, though some predictors were better than others when accounting for variance in the system. As with Table 3, the only significant predictors of *M. variabilis* on its host plants are *A. balsamea* and the abundance of Ericaceae being either negatively or positively related to its colonization of *K. polifolia*. The abundance of *P. mariana* at a site negatively affected the colonization of *P.*

mariana by *M. variabilis*. The effect of Ericaceae was significantly positive on the colonization of *P. mariana*, and pH (increasingly acidic soils) had a negative effect on *M. variabilis*'s ability to colonize *P. mariana*.

Table 3-3: Regression equations tested for overall model significance, R^2, and AIC. Significant models with the best ability to explain variance and least amount of information loss were selected for Table 4.

		Mabou Beach Provincial Park	Adjusted R^2	Sig.	AIC
Kalmia	1)	*M. variabilis* DNA = 5.620 - 0.128(Fir)	0.445	**0.021**	45.64
	2)	*M. variabilis* DNA = 4.938 - 0.113(Fir) + 2.007(Ericaceous)	0.408	0.066	46.95
Spruce	3)	*M. variabilis* DNA = 6.868 - 0.022(Spruce) - 3.229(Ericaceous) - 0.011 (Soil)	0.586	**0.041**	25.29
	4)	*M. variabilis* DNA = 5.639 - 0.025(Spruce) - 2.683(Ericaceous) - 0.002 (Ammonium)	0.513	0.65	26.91
	5)	*M. variabilis* DNA = 5.467 - 0.024(Spruce) - 2.614(Ericaceous)	0.579	**0.02**	24.98
		Purcell's Cove Conservation Area	Adjusted R^2	Sig.	AIC
Kalmia	6)	*M. variabilis* DNA = 1.086 + 3.007(Ericaceous)	0.472	**0.017**	34.56
	7)	*M. variabilis* DNA = 0.574 + 3.652(Ericaceous) + 0.010(Spruce)	0.429	0.058	36.0
	8)	*M. variabilis* DNA = -1.641 + 3.296(Ericaceous) + 0.666(pH)	0.412	0.065	36.31
Spruce	9)	*M. variabilis* DNA = 12.788 - 3.006(pH) - 0.011(Spruce) + 0.007(Soil) + 0.006(Ammonium)	0.765	**0.006**	5.41
	10)	*M. variabilis* DNA = 12.659 - 2.991(pH) - 0.011(Spruce) + 0.007(Soil) + 0.006(Ammonium) + 0.92(Ericeacous)	0.827	**0.024**	7.24

Table 4 displays the statistically significant multiple regressions analyzing the effect of environmental factors on the colonization of *K. polifolia* and *P. mariana* by *M. variabilis* at West Mabou Beach Provincial Park and Purcell's Cove conservation area. Analysis of West Mabou Beach showed that *Abies balsamea* negativley influenced the colonization of *K. polifolia* by *M. variabilis* and that the abundance of Ericaceae and *P. mariana* on each site negatively affected the colonization of *P. mariana* by *M. variabilis*. At Purcell's Cove, colonization of *K. polifolia* was positively influenced by the abundance of Ericaceae and colonization of *P. mariana* was negatively influenced by pH.

Table 3-4: Regression coefficients (Beta values) and p values for individual factors, and adjusted R^2, over all p values and AIC values for statistically significant multiple regression equations relating *M. variabilis* abundance on *K. polifolia* and *P. mariana* to environmental factors measured from West Mabou Beach and Purcell's Cove Conservation Area.

Host	Fir		Ericaceae		Soil Index		pH		Ammonium		Spruce		R^2	Overall p	AIC
	B	**p**	**β**	**p**	**β**	**p**	**β**	**p**	**β**	**p**	**β**	**p**			
K. polifolia [a]	-0.13	**0.02**											0.45	**0.02**	45.6
P. mariana [a]			-3.23	**0.02**	-0.01	0.33					-0.02	**0.03**	0.59	**0.04**	25.2
K. polifolia [b]			3.01	**0.02**									0.47	**0.02**	34.5
P. mariana [b]			0.92	0.81	0.01	0.16	-2.99	**0.00**	0.01	0.12	-0.01	0.07	0.83	**0.02**	7.24

[a]West Mabou Beach Provincial Park

[b]Purcell's Cove

Canonical correspondence analysis did not yield statistically significant eigenvalues, though axis one was nearly significant with a p value of 0.057 (Table 4). Axes one and two were the best approximations of variance amongst the sites, and were chosen to create the ordination. Sites from West Mabou Beach and Purcell's Cove separated along axis one with the soil nutrient index having a greater effect on sites from Mabou Beach, and soil nitrogen, pH, and organic matter having more of an effect on sites from Purcell's Cove (Figure 9). Maple, Birch, and Fir all

have a large effect on the abundance of *M. variabilis*, while *P. mariana* and *K. polifolia* have less of an effect. This can be explained by the appearance of Maple, Birch and Fir on study plots being random, whereas the sites were picked specifically to include *P. mariana* and *K. polifolia*.

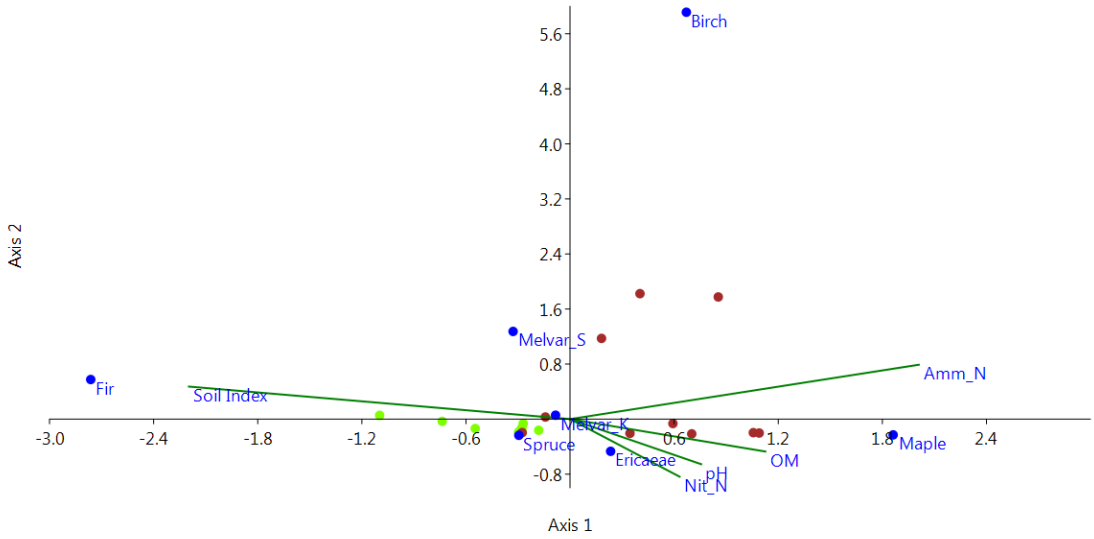

Figure 3-9: Ordination based on axis 1 and 2 of a canonical correspondence analysis of both sites. Sites from West Mabou Beach are coloured in green, sites from Purcell's Cove are in brown. Trends in environmental variables are plotted in green, species scores are plotted as blue points.

Table 3-5: Axis' generated by CCA and the proportion of variance explained by each axis, as an eigenvalue, and the significance of each CCA axis.

Axis	Eigenvalue	%	p
1	2.031×10^{-1}	81.4	0.057
2	4.19×10^{-2}	16.83	0.461
3	4.37×10^{-3}	1.752	0.852
4	4.04×10^{-5}	1.61×10^{-2}	0.989
5	5.59×10^{-8}	2.24×10^{-5}	0.752

Discussion

Mycorrhiza and fungal root endophytes are a major component of soil communities and an essential part of a healthy plant's microbiome (Casieri et al., 2013; Rodriguez et al. 2009). Of the fungal endophytes, the Class 4, non-claviceipitaceous (NC-endophytes) root associated fungi are the least well studied in form, function, ecology, and evolutionary biology (Rodriguez et al., 2009). While many studies of root endophytes, in particular the dark septate endophytes (DSE), have been conducted, their role in ecosystem processes and their effects on plant health remain obscure (Grünig et al. 2008; Jumpponen and Trappe, 1998; Mayerhofer et al. 2013). Furthermore, the picture is often confounded by the wide range of hosts that DSE and other endophytic fungi are isolated from during plant assays (Bergero et al., 2000; Bergero et al., 2003; Grelet et al., 2010; Kernaghan et al., 2003; Kernaghan and Patriquin, 2011; Kernaghan, 2013; Kohout et al., 2011; Vohník and Albrechtová, 2011; Vohník et al., 2013). These fungi may also form either ectomycorrhiza (ECM) or ericoid mycorrhiza (ERM) when colonizing an appropriate host, and may colonize a host endophytically when in the presence of an alternate host (Grelet et al., 2010; Kernaghan and Patriquin, 2011; Reithmeier and Kernaghan, 2013; Vohník and Albrechtová, 2011; Vohník et al., 2013).

In the present study, species-specific PCR primers based upon unique regions in the *Meliniomyces variabilis* ITS sequence were synthesized (chapter 2), and used to investigate host associations of *M. variabilis* and ecological factors that influenced the colonization of two host plant species (chapter 3). This single-species approach was undertaken in order to reduce the complexity of the ecological analysis, while increasing the accuracy of the study of a multifunctional root-associated species that has been documented forming ERM associations and colonizing many other species endophytically (Bergero et al., 2000; Gorzelak et al., 2012; Grelet

et al., 2009; Hambleton and Sigler, 2005; Kernaghan and Patriquin, 2011; Ohtaka and Narisawa, 2008; Vohník et al., 2007, 2013). These methods have successfully been used before to quantify the abundance of the root pathogen *Cylindrocarpon destructans* from soil samples (Kernaghan et al., 2006).

This method of single species study is possible for any root associated fungus with a sequenced ITS region. Fungal ITS regions differ by approximately three percent across species , and thus must have unique differences somewhere in their ITS regions (Burns et al., 1991). Utilizing any sequence, it should be possible to design a set of DNA primers with at least one species-specific sequence for amplification and quantification of template DNA. Contemporary DNA technology is sensitive enough to distinguish between a mixed sample containing DNA templates from many different species, and to amplify the correct sequence, although care must be taken to ensure there is no cross contamination by PCR products into an original DNA extract, and that proper annealing temperatures are used to maximize the yield from specific primers while preventing the propagation of primer-dimers and mispriming. The use of DNA binding probes such as TaqMan® could also be used in this type of study, if the unique ITS sequences are very small or very close together.

During the development and testing of the species-specific PCR primers *Melvar1* and *Mevlar3* (Melvar1/3)(Chapter 2, Figure 2 and Table 2) was tested against 10 other fungal species, four of which were other *Meliniomyces* species, and could not successfully amplify DNA from those species. Melvar1/3 was then tested on DNA extracted from the roots of a number of species that had not been surveyed before for colonization by *M. variabilis*. Table 4 in chapter 2 lists the species of plant hosts that *M. variabilis* has been isolated from by sequencing as of February 15th, 2013. Through our survey, we isolated *M. variabilis* from five novel hosts; two of which belonged

to the Caprifoliaceae, a family of plants *M. variabilis* had not been observed to associate with before. *M. variabilis* was found on all but two plants that were sampled, *Larix* (larch) and *Acer* (maple), both of which lose their foliage in winter.

M. variabilis typically forms ERM morphotypes when colonizing Ericaceous plants (Hambleton and Sigler, 2005), and reciprocal nutrient exchange has been demonstrated between *M. variabilis* and the ericaceous shrub *Vaccinium vitis-ideae* (Grelet et al., 2009). Through the quantification of *M. variabilis* using the Melvar1/3 primers, we were able to demonstrate that *M. variabilis* is present in significant amounts on *P. mariana*, though always in higher amounts on *K. polifolia*, demonstrating some degree of host preference (chapter 3). This is a technique that could be applied to the study of other root associated fungi in order to better elucidate their host preferences, and examine their life history strategies.

Analysis of the patterns of *M. variabilis* host colonization from both study sites in Purcell's Cove and West Mabou Beach Provincial Park showed a large amount of variance with respect to the factors driving the distribution of *M. variabilis*. Between the two sites, ericaceous ground cover was observed to negatively influence the colonization of *K. polifolia*, while at Purcell's Cove it was positively correlated with the colonization of *K. polifolia*. While the negative predictor was not statistically significant, it does suggest that there were site differences that could not be accounted for using the variables measured in our study. The history of *M. variabilis* colonization at each area and the method of dispersion of *M. variabilis* are not yet fully understood, but would go a long way towards understanding the drivers of colonization of host plants by a generalist fungus.

The study of host associations is an emerging field in fungal ecology, and the wide range of life histories complicates matters. Since a fungus may exhibit characteristics ranging from

pathogenic, saprotrophic, mutualistic to endophytic under different conditions, and also when grown in the presence of different hosts, the ability to isolate one species and study it in greater detail in natural ecosystems is quite useful. Since *M. variabilis* is an ascomycete, it does not form conspicuous above-soil reproductive structures like many of the basidiomycete forming ECM fungi. This can make it harder to study in natural ecosystems, as many traditional approaches to fungal ecology involve sampling above-ground sporocarps (Dahlberg, 1997). Molecular techniques based on PCR technologies, such as clone libraries, and next-generation massively parallel sequencing allow for studies of ascomycetes in natural ecosystems. However, these methods are not without pitfalls, and are more useful for studying the ecology of root associated ascomycetes at the community level (Dickie, 2010; Kernaghan and Patriquin, 2011).The datasets generated by such techniques are not suitable to the pursuit of the type of study described in chapter 3. Clone libraries and next-generation sequencing are more suitable for the study of community composition, and the proportions of those communities that are made up of certain species, but not for quantifying individual species from environmental samples. The application of these species-specific primers and qPCR allowed for a more complete study of particular root associated fungi in a natural ecosystem.

Another avenue of research open to the use of species specific primers and qPCR data is that of evolutionary ecology. Class 4 fungal endophytes are often ECM or ERM fungi that are living on an alternate host. The technique described in chapter 3 is well suited to generating host colonization data, and the variation in such data sets could be used to study bet-hedging life history strategies. Some of the most common examples include studies of seed germination and drought, or variations in growing seasons. Given that there is variation in colonization data under otherwise optimal conditions, such as plentiful ericaceous hosts, the host association of endophyte

forming fungi such as *M. variabilis* would seem to be suitable subjects for the study of bet-hedging life history characteristics.

References

Bergero, R., Girlanda, M., Bello, F., Luppi, A. M., and Perotto, S. (2003). Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. *Mycorrhiza*, *13*(2), 69–75. doi:10.1007/s00572-002-0202-9

Bergero, R., Perotto, S., Girlanda, M., Vidano, G., and Luppi, a M. (2000). Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (Quercus ilex). *Molecular Ecology*, *9*(10), 1639–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11050558

Burns, T. D., White, T. J., and Taylor, J. W. (1991). Fungal Molecular Systematics. *Annual Review of Ecology and Systematics*, *22*, 525–564.

Casieri, L., Ait Lahmidi, N., Doidy, J., Veneault-Fourrey, C., Migeon, A., Bonneau, L., … Wipf, D. (2013). Biotrophic transportome in mutualistic plant-fungal interactions. *Mycorrhiza*, *23*(8), 597–625. doi:10.1007/s00572-013-0496-9

Dahlberg, A. (1997). Population ecology of Suillus variegatus in old Swedish Scots pine forests. *Mycological Research*, *101*(1), 47–54. doi:10.1017/S0953756296002110

Dickie, I. a, Fukami, T., Wilkie, J. P., Allen, R. B., and Buchanan, P. K. (2012). Do assembly history effects attenuate from species to ecosystem properties? A field test with wood-inhabiting fungi. *Ecology Letters*, *15*(2), 133–41. doi:10.1111/j.1461-0248.2011.01722.x

Dickie, I. a, Martínez-García, L. B., Koele, N., Grelet, G. -a., Tylianakis, J. M., Peltzer, D. a., and Richardson, S. J. (2013). Mycorrhizas and mycorrhizal fungal communities throughout ecosystem development. *Plant and Soil*, *367*(1-2), 11–39. doi:10.1007/s11104-013-1609-0

De Bellis, T., Kernaghan, G., and Widden, P. (2007). Plant community influences on soil microfungal assemblages in boreal mixed-wood forests. *Mycologia*, *99*(3), 356–67.

Grelet, G.-A., Johnson, D., Paterson, E., Anderson, I. C., and Alexander, I. J. (2009). Reciprocal carbon and nitrogen transfer between an ericaceous dwarf shrub and fungi isolated from Piceirhiza bicolorata ectomycorrhizas. *The New Phytologist*, 359–366. doi:10.1111/j.1469-8137.2009.02813.x

Grelet, G.-A., Johnson, D., Vrålstad, T., Alexander, I. J., and Anderson, I. C. (2010). New

insights into the mycorrhizal Rhizoscyphus ericae aggregate: spatial structure and co-colonization of ectomycorrhizal and ericoid roots. *The New Phytologist*, *188*(1), 210–22. doi:10.1111/j.1469-8137.2010.03353.x

Grünig, C. R., Queloz, V., Sieber, T. N., and Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the Phialocephala fortinii s.l. – Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany*, *86*(12), 1355–1369. doi:10.1139/B08-108

Hambleton, S., and Sigler, L. (2005). Meliniomyces, a new anamorph genus for root-associated fungi with phylogenetic affinities to Rhizoscyphus ericae (Hymenoscyphus ericae), Leotiomycetes. *Studies in Mycology*, *53*(1), 1–27. doi:10.3114/sim.53.1.1

Jumpponen, A., and Trappe, J. M. (1998). Dark septate endophytes: A review of facultative biotrophic root-colonizing fungi. *New Phytologist*, *140*(2), 295–310. doi:10.1046/j.1469-8137.1998.00265.x

Kernaghan, G. (2005). Mycorrhizal diversity: Cause and effect? *Pedobiologia*, *49*(6), 511–520. doi:10.1016/j.pedobi.2005.05.007

Kernaghan, G. (2013). Functional diversity and resource partitioning in fungi associated with the fine feeder roots of forest trees. *Symbiosis*, *61*(3), 113–123. doi:10.1007/s13199-013-0265-8

Kernaghan, G., and Patriquin, G. (2011). Host associations between fungal root endophytes and boreal trees. *Microbial Ecology*, *62*(2), 460–73. doi:10.1007/s00248-011-9851-6

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. (2006). Quantification of Cylindrocarpon destructans f. sp. panacis in soils by real-time PCR. *Plant Pathology*, *56*(3), 508–516. doi:10.1111/j.1365-3059.2006.01559.x

Kohout, P., Sýkorová, Z., Bahram, M., Hadincová, V., Albrechtová, J., Tedersoo, L., and Vohník, M. (2011). Ericaceous dwarf shrubs affect ectomycorrhizal fungal community of the invasive Pinus strobus and native Pinus sylvestris in a pot experiment. *Mycorrhiza*, *21*(5), 403–12. doi:10.1007/s00572-010-0350-2

Mayerhofer, M. S., Kernaghan, G., and Harper, K. a. (2013). The effects of fungal root endophytes on plant growth: A meta-analysis. *Mycorrhiza*, *23*(2), 119–128. doi:10.1007/s00572-012-0456-9

Ohtaka, N., and Narisawa, K. (2008). Molecular characterization and endophytic nature of the root-associated fungus Meliniomyces variabilis (LtVB3). *Journal of General Plant Pathology*, *74*(1), 24–31. doi:10.1007/s10327-007-0046-4

Ramakers, C., Ruijter, J. M., Deprez, R. H. L., and Moorman, A. F. . (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters*, *339*(1), 62–66. doi:10.1016/S0304-3940(02)01423-4

Reithmeier, L., and Kernaghan, G. (2013). Availability of ectomycorrhizal fungi to black spruce above the present treeline in Eastern Labrador. *PloS One*, *8*(10), e77527. doi:10.1371/journal.pone.0077527

Rodriguez, R. J., White, J. F., Arnold, a. E., and Redman, R. S. (2009). Fungal endophytes: Diversity and functional roles: Tansley review. *New Phytologist*, *182*(2), 314–330. doi:10.1111/j.1469-8137.2009.02773.x

Smith, S.E. and Read, D. (2008). Ericoid mycorrhizas. In *Mycorrhizal Symbiosis 3rd Edition* (ed. S.E. Smith and D. Read), pp. 389-418. Great Britain: Elsevier ltd.

Simons, A. M. (2011). Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proceedings. Biological Sciences / The Royal Society*, *278*(1712), 1601–1609. doi:10.1098/rspb.2011.0176

Simons, A. M. (2014). Playing smart vs. playing safe: The joint expression of phenotypic plasticity and potential bet hedging across and within thermal environments. *Journal of Evolutionary Biology*, *27*(6), 1047–1056. doi:10.1111/jeb.12378

Smith, S.E. and Read, D. (2008). Ericoid mycorrhizas. In *Mycorrhizal Symbiosis 3rd Edition* (ed. S.E. Smith and D. Read), pp. 389-418. Great Britain: Elsevier ltd.

Valenzuela-Estrada, L. R., Vera-Caraballo, V., Ruth, L. E., and Eissenstat, D. M. (2008). Root anatomy, morphology, and longevity among root orders in Vaccinium corymbosum (Ericaceae). *American Journal of Botany*, *95*(12), 1506–1514. doi:10.3732/ajb.0800092

Vohník, M., and Albrechtová, J. (2011). The Co-occurrence and Morphological Continuum Between Ericoid Mycorrhiza and Dark Septate Endophytes in Roots of Six European Rhododendron Species. *Folia Geobotanica*, *46*(4), 373–386. doi:10.1007/s12224-011-9098-5

Vohník, M., Fendrych, M., Kolařík, M., Gryndler, M., Hršelová, H., Albrechtová, J., and Vosátka, M. (2007). The ascomycete Meliniomyces variabilis isolated from a sporocarp of Hydnotrya tulasnei (Pezizales) intracellularly colonises roots of ecto- and ericoid mycorrhizal host plants. *Czech Mycology*, *59*(2), 215–226.

Yamasaki, S. H., Fyles, J. W., Egger, K. N., and Titus, B. D. (1998). The effect of Kalmia angustifolia on the growth, nutrition, and ectomycorrhizal symbiont community of black spruce. *Forest Ecology and Management*, *105*(1-3), 197–207. doi:10.1016/S0378-1127(97)00285-5

Chapter 4 : Applications and Concluding Discussion

Applications and Concluding Discussion

Mycorrhiza and fungal root endophytes are a major component of soil communities and an essential part of a healthy plant's microbiome (Casieri et al., 2013; Rodriguez et al., 2009). Of the fungal endophytes, the Class 4, non-claviceipitaceous (NC-endophytes) root associated fungi are the least well studied in form, function, ecology, and evolutionary biology (Rodriguez et al., 2009). While many studies of the root endophytes, in particular the dark septate endophytes (DSE), have been conducted, their role in ecosystem processes and their effects on plant health remain obscure (Grünig et al., 2008; Jumpponen and Trappe, 1998; Mayerhofer, Kernaghan, and Harper, 2013). Furthermore, the picture is often confounded by the wide range of hosts that DSE and endophytic fungi are isolated from environmental samples and plant assays (Bergero et al., 2000; Bergero et al., 2003; Grelet et al., 2010; Kernaghan et al., 2003; Kernaghan and Patriquin, 2011; Kernaghan, 2013; Kohout et al., 2011; Vohník and Albrechtová, 2011; Vohník et al., 2013). These fungi may be known to form either ectomycorrhiza (ECM) or ericoid mycorrhiza (ERM) when colonizing an appropriate host, and may appear to colonize a host endophytically when in the presence of an alternate host (Grelet et al., 2010; Kernaghan and Patriquin, 2011; Reithmeier and Kernaghan, 2013; Vohník and Albrechtová, 2011; Vohník et al., 2013).

In the studies discussed here, species-specific PCR primers based upon unique regions in the *Meliniomyces variabilis* ITS sequence were synthesized (chapter 2), and used to investigate host associations of *M. variabilis* and ecological factors that influenced the colonization of two host plant species (chapter 3). This single-species approach was undertaken in order to reduce the complexity of the ecological analysis, while increasing the accuracy of the study of a multifunctional root-associated species that has been documented forming ERM associations and colonizing many other species endophytically (Bergero et al., 2000; Gorzelak et al., 2012; Grelet

et al., 2009; Hambleton and Sigler, 2005; Kernaghan and Patriquin, 2011; Ohtaka and Narisawa, 2008; Vohník et al., 2007, 2013). These methods have successfully been used before to isolate and quantify the abundance of the root pathogen *Cylindrocarpon destructans* from soil samples (Kernaghan et al., 2006).

During the development and testing of the species-specific PCR primers *Melvar1* and *Mevlar3* (henceforth *Melvar1/3*; chapter 2, figure 2 and table 2), Melvar1/3 was tested against 10 other fungal species, four of which were other *Meliniomyces* species, and could not successfully amplify DNA from those species. Melvar1/3 was then tested on DNA extracted from the roots of a number of species that had not been surveyed before for colonization by *M. variabilis*. Table 4 in chapter 2 lists the species of plant hosts that *M. variabilis* has been isolated from by sequencing as of February 15th, 2013. Through our survey, we isolated *M. variabilis* from five novel hosts; two of which belonged to the Caprifoliaceae, a family of plants *M. variabilis* had not been observed to associate with before. *M. variabilis* was found on all but two plants that were sampled, both of which were trees that lose their foliage in winters, and *Acer pseudoplanatus* is known to typically form AM associations.

M. variabilis typically forms ERM morphotypes when colonizing Ericaceous plants (Hambleton and Sigler, 2005), and reciprocal nutrient exchange has been demonstrated between *M. variabilis* and the ericaceous shrub *Vaccinium vitis-ideae* (Grelet et al., 2009). Through the quantification of *M. variabilis* using the Melvar1/3 primers, we were able to demonstrate that *M. variabilis* is present in significant amounts on *P. mariana*, though always in higher amounts on *K. polifolia*, demonstrating some degree of host preference (chapter 3). By sampling soil monoliths, we were able to examine the effects of natural soil concentrations on the distribution of *M. variabilis* between the two host plants. This is a technique that could be applied to the study of

other root associated fungi in order to better elucidate their host preferences, and examine their life history strategies.

Analysis of the patterns of *M. variabilis* host colonization from both study sites in Purcell's Cove and West Mabou Beach Provincial Park showed a large amount of variance in what was driving the distribution of *M. variabilis*. Between the two sites, ericaceous ground cover was observed to negatively influence the colonization of *K. polifolia*, while at Purcell's Cove it was seen to increase the colonization of *K. polifolia*. While the negative predictor was not statistically significant, it does suggest that there were site differences that could not be accounted for using the variables measured in our study. The history of *M. variabilis* colonization of each area and method of dispersion of *M. variabilis* are not yet fully understood and would go a long way to understanding what drives colonization of host plants by a generalist fungus.

The study of host associations is an emerging field in fungal ecology, and the wide range of life histories complicates matters. Since a fungus may exhibit different characteristics under different environments and when grown in the presence of different hosts, ranging from pathogenic, saprotrophic, mutualistic to endophytic, the ability to isolate one species and study it in greater detail in natural ecosystems is quite useful. Since *M. variabilis* is an ascomycete, it does not form conspicuous above-soil reproductive structures like many of the basidiomycete forming ECM fungi do. This can make it harder to study in natural ecosystems, as many traditional approaches to fungal ecology involve sampling above-ground sporocarps (Dahlberg, 1997). Molecular techniques based on PCR technologies, such as clonal libraries (cDNA), and next-generation massively parallel sequencing allow for studies of ascomycetes in natural ecosystems. However, these methods are not without pitfalls, and are more useful for studying the ecology of root associated ascomycetes at the community level (Dickie, 2010; Kernaghan and Patriquin,

2011).The datasets generated by such techniques is not suitable to the pursuit of the type of study described in chapter 3. cDNA libraries and next-generation sequencing are more suitable for the study of community composition, and the proportions of those communities that are made up of certain species, but not for quantifying the amount of DNA of those species from environmental samples. The application of these species-specific primers and qPCR would allow for a more complete study of particular root associated fungi natural ecosystems, and is based on very well used and heavily relied upon molecular techniques.

Another avenue of research open to the use of species specific primers and qPCR data is that of evolutionary ecology. Class 4 fungal endophytes are often ECM or ERM fungi that are living on an alternate host. The technique described in chapter 3 would be well suited to generating host colonization data, and the variation of such data sets could be used to study bet-hedging life history strategies. In brief, bet-hedging traits have evolved as a mechanism for a species to cope with varying environmental conditions. Some of the most common examples include studies of seed germination and drought, or variations in growing seasons. Given that there is variation in colonization data under otherwise optimal conditions, such as plentiful ericaceous hosts, the host association of endophyte forming fungi such as *M. variabilis* seem like suitable subjects for the study of bet-hedging life history characteristics.

References

Bergero, R., Girlanda, M., Bello, F., Luppi, A. M., and Perotto, S. (2003). Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. *Mycorrhiza*, *13*(2), 69–75. doi:10.1007/s00572-002-0202-9

Bergero, R., Perotto, S., Girlanda, M., Vidano, G., and Luppi, a M. (2000). Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (Quercus ilex). *Molecular Ecology*, *9*(10), 1639–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11050558

Casieri, L., Ait Lahmidi, N., Doidy, J., Veneault-Fourrey, C., Migeon, A., Bonneau, L., … Wipf,

D. (2013). Biotrophic transportome in mutualistic plant-fungal interactions. *Mycorrhiza*, *23*(8), 597–625. doi:10.1007/s00572-013-0496-9

Dahlberg, A. (1997). Population ecology of Suillus variegatus in old Swedish Scots pine forests. *Mycological Research*, *101*(1), 47–54. doi:10.1017/S0953756296002110

Dickie, I. a. (2010). Insidious effects of sequencing errors on perceived diversity in molecular surveys. *The New Phytologist*, *188*(4), 916–8. doi:10.1111/j.1469-8137.2010.03473.x

Gorzelak, M. a, Hambleton, S., and Massicotte, H. B. (2012). Community structure of ericoid mycorrhizas and root-associated fungi of Vaccinium membranaceum across an elevation gradient in the Canadian Rocky Mountains. *Fungal Ecology*, *5*(1), 36–45. doi:10.1016/j.funeco.2011.08.008

Grelet, G.-A., Johnson, D., Paterson, E., Anderson, I. C., and Alexander, I. J. (2009). Reciprocal carbon and nitrogen transfer between an ericaceous dwarf shrub and fungi isolated from Piceirhiza bicolorata ectomycorrhizas. *The New Phytologist*, 359–366. doi:10.1111/j.1469-8137.2009.02813.x

Grelet, G.-A., Johnson, D., Vrålstad, T., Alexander, I. J., and Anderson, I. C. (2010). New insights into the mycorrhizal Rhizoscyphus ericae aggregate: spatial structure and co-colonization of ectomycorrhizal and ericoid roots. *The New Phytologist*, *188*(1), 210–22. doi:10.1111/j.1469-8137.2010.03353.x

Grünig, C. R., Queloz, V., Sieber, T. N., and Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the Phialocephala fortinii s.l. – Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany*, *86*(12), 1355–1369. doi:10.1139/B08-108

Hambleton, S., and Sigler, L. (2005). Meliniomyces, a new anamorph genus for root-associated fungi with phylogenetic affinities to Rhizoscyphus ericae (Hymenoscyphus ericae), Leotiomycetes. *Studies in Mycology*, *53*(1), 1–27. doi:10.3114/sim.53.1.1

Jumpponen, A., and Trappe, J. M. (1998). Dark septate endophytes: A review of facultative biotrophic root-colonizing fungi. *New Phytologist*, *140*(2), 295–310. doi:10.1046/j.1469-8137.1998.00265.x

Kernaghan, G. (2013). Functional diversity and resource partitioning in fungi associated with the fine feeder roots of forest trees. *Symbiosis*, *61*(3), 113–123. doi:10.1007/s13199-013-0265-8

Kernaghan, G., and Patriquin, G. (2011). Host associations between fungal root endophytes and boreal trees. *Microbial Ecology*, *62*(2), 460–73. doi:10.1007/s00248-011-9851-6

Kernaghan, G., Reeleder, R. D., and Hoke, S. M. T. (2006). Quantification of Cylindrocarpon destructans f. sp. panacis in soils by real-time PCR. *Plant Pathology*, *56*(3), 508–516. doi:10.1111/j.1365-3059.2006.01559.x

Kernaghan, G., Sigler, L., and Khasa, D. (2003). Mycorrhizal and root endophytic fungi of

containerized Picea glauca seedlings assessed by rDNA sequence analysis. *Microbial Ecology*, *45*(2), 128–36. doi:10.1007/s00248-002-1024-1

Kohout, P., Sýkorová, Z., Bahram, M., Hadincová, V., Albrechtová, J., Tedersoo, L., and Vohník, M. (2011). Ericaceous dwarf shrubs affect ectomycorrhizal fungal community of the invasive Pinus strobus and native Pinus sylvestris in a pot experiment. *Mycorrhiza*, *21*(5), 403–12. doi:10.1007/s00572-010-0350-2

Mayerhofer, M. S., Kernaghan, G., and Harper, K. a. (2013). The effects of fungal root endophytes on plant growth: A meta-analysis. *Mycorrhiza*, *23*(2), 119–128. doi:10.1007/s00572-012-0456-9

Mrnka, L., Kohout, P., Fehrer, J., Republic, C., and Universitario, C. R. (n.d.). Ericoid mycorrhizal Meliniomyces variabilis and dark septate endophytes commonly associate with Norway spruce ectomycorrhizae in a montane temperate forest, 1–24.

Ohtaka, N., and Narisawa, K. (2008). Molecular characterization and endophytic nature of the root-associated fungus Meliniomyces variabilis (LtVB3). *Journal of General Plant Pathology*, *74*(1), 24–31. doi:10.1007/s10327-007-0046-4

Reithmeier, L., and Kernaghan, G. (2013). Availability of ectomycorrhizal fungi to black spruce above the present treeline in Eastern Labrador. *PloS One*, *8*(10), e77527. doi:10.1371/journal.pone.0077527

Rodriguez, R. J., White, J. F., Arnold, a. E., and Redman, R. S. (2009). Fungal endophytes: Diversity and functional roles: Tansley review. *New Phytologist*, *182*(2), 314–330. doi:10.1111/j.1469-8137.2009.02773.x

Smith, S.E. and Read, D. (2008). Ericoid mycorrhizas. In *Mycorrhizal Symbiosis 3rd Edition* (ed. S.E. Smith and D. Read), pp. 389-418. Great Britain: Elsevier ltd.

Vohník, M., and Albrechtová, J. (2011). The Co-occurrence and Morphological Continuum Between Ericoid Mycorrhiza and Dark Septate Endophytes in Roots of Six European Rhododendron Species. *Folia Geobotanica*, *46*(4), 373–386. doi:10.1007/s12224-011-9098-5

Vohník, M., Fendrych, M., Kolařík, M., Gryndler, M., Hršelová, H., Albrechtová, J., and Vosátka, M. (2007). The ascomycete Meliniomyces variabilis isolated from a sporocarp of Hydnotrya tulasnei (Pezizales) intracellularly colonises roots of ecto- and ericoid mycorrhizal host plants. *Czech Mycology*, *59*(2), 215–226.

Vohník, M., Mrnka, L., Lukešová, T., Bruzone, M. C., Kohout, P., and Fehrer, J. (2013). The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal Meliniomyces variabilis. *Fungal Ecology*, *6*(4), 281–292. doi:10.1016/j.funeco.2013.03.006

Appendix: Soil Data

Table A-1: Purcell's Cove soil data.

	PC-01	PC-02	PC-03	PC-04	PC-05	PC-06	PC-07	PC-08	PC-09	PC-10
pH	3.5	3.6	3.9	3.5	3.7	3.5	4.2	3.6	3.7	3.3
Organic Matter (%)	46.3	42.0	40.1	50.1	30.4	45.6	56.4	49.9	47.2	41.2
P_2O_5 (kg/ha)	46	32	30	39	54	11	32	13	17	14
K_2O	192	290	232	311	313	180	54	179	241	176
Ca (kg/Ha)	407	425	149	421	632	185	145	483	437	184
Mg (kg/ha)	241	386	99	439	230	285	38	394	272	290
Na (kg/ha)	123	72	62	66	59	67	48	84	107	137
Sulfur (kg/ha)	13	19	26	18	16	11	28	15	13	19
Al (ppm)	78.46	143.41	990.25	123.70	195.47	105.41	769.19	166.58	141.41	193.54
Fe (ppm)	44	61	214	55	77	52	68	95	68	78
Mn (ppm)	4	4	2	3	7	2	1	2	6	2
Cu (ppm)	0.32	0.45	0.55	0.81	0.57	0.40	0.21	0.33	0.62	0.46
Zn (ppm)	6.0	8.8	3.2	8.6	6.0	4.2	0.9	3.4	5.7	4.9
B (ppm)	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50	< = 0.50	<= 0.50	<= 0.50	<= 0.50
CEC (meq/100gm)	17.1	19.9	16.4	19.0	17.6	19.3	7.4	17.8	16.0	23.9
Base Sat.										
K (%)	1.2	1.5	1.5	1.7	1.9	1.0	0.8	1.1	1.6	0.8
Ca (%)	6.0	5.3	2.3	5.5	9.0	2.4	4.9	6.8	6.8	1.9
Mg (%)	5.9	8.1	2.5	9.6	5.5	6.1	2.1	9.2	7.1	5.1
Na (%)	1.6	0.8	0.8	0.8	0.7	0.8	1.4	1.0	1.5	1.2
H (%)	85.4	84.3	92.9	82.4	82.9	89.7	90.8	81.9	83.0	91.0
% Soil Moisture (% dry)	199.24	314.38	415.81	329.22	332.75	304.00	1365.49	425.59	306.61	157.71
Ammonium-N (mg/kg)	61.8	75.5	36.7	93.7	57.0	34.9	146.0	13.4	46.6	74.3
Nitrate-N (mg/kg)	0.515	0.675	0.941	0.569	0.857	0.632	3.030	0.849	0.768	0.589
Ammonium:Nitrate	120.0	111.9	39.0	164.7	66.5	55.2	48.2	15.8	60.7	126.1

Table A-2: West Mabou Beach Provincial Park soil data.

	MB-01	MB-02	MB-03	MB-04	MB-05	MB-06	MB-07	MB-08	MB-09	MB-10
pH	3.9	3.6	3.7	3.5	3.7	3.7	3.7	3.8	3.7	3.7
Organic Matter (%)	32.2	41.3	49.4	47.1	23.1	48.7	47.3	38.9	47.9	44.5
P_2O_5 (kg/ha)	58	33	16	72	35	24	26	14	14	21
K_2O	389	223	383	356	286	323	402	340	316	270
Ca (kg/Ha)	1019	598	546	595	186	336	363	388	289	630
Mg (kg/ha)	333	366	417	436	494	418	279	359	498	248
Na (kg/ha)	251	281	260	168	206	181	184	244	344	151
Sulfur (kg/ha)	32	23	32	21	23	21	30	22	26	16
Al (ppm)	241.48	327.48	99.84	81.63	312.38	140.90	251.32	67.15	194.14	72.32
Fe (ppm)	120	148	63	44	113	71	134	38	68	36
Mn (ppm)	59	8	12	19	5	21	8	7	3	6
Cu (ppm)	0.34	0.29	0.30	0.22	0.28	0.28	0.27	0.29	0.24	0.27
Zn (ppm)	5.7	3.4	2.7	6.1	2.3	4.2	2.5	2.1	1.6	1.7
B (ppm)	<= 0.50	<= 0.05	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50	<= 0.50
CEC (meq/100gm)	16.9	19.5	16.5	20.8	15.0	17.6	13.5	13.6	16.6	17.4
Base Sat.										
K (%)	2.4	1.2	2.5	1.8	2.0	1.9	3.2	2.6	2.0	1.6
Ca (%)	15.1	7.6	8.3	7.2	3.1	4.8	6.7	7.1	4.4	9.1
Mg (%)	8.2	7.8	10.5	8.7	13.7	9.9	8.6	11.0	12.5	5.9
Na (%)	3.2	3.1	3.4	1.8	3.0	2.2	3.0	3.9	4.5	1.9
H (%)	71.0	80.2	75.3	80.5	78.2	81.2	78.5	75.3	76.6	81.5
% Soil Moisture (% dry)	279.0	232.1	514.92	321.72	383.96	287.50	385.80	450.21	361.83	236.21
Ammonium-N (mg/kg)	12.2	18.2	19.7	31.7	12.9	22.0	108.0	99.9	5.37	70.8
Nitrate-N (mg/kg)	1.09	0.569	3.30	0.841	1.02	0.940	0.712	0.820	0.307	0.780
Ammonium:Nitrate	11.2	32.0	6.0	37.7	12.6	23.4	151.7	121.8	17.5	90.8