COMMUNITY STRUCTURE OF ARBUSCULAR MYCORRHIZAL FUNGI ALONG AN ALTITUDINAL GRADIENT

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requirements of the degree of Doctor of Philosophy.

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Abstract

A fundamental goal in ecology is to examine diversity patterns of naturally occurring communities and link these patterns to underlying structuring processes. Despite the importance of arbuscular mycorrhizal (AM) fungi in natural ecosystems, the majority of studies to date have focused on community structure in a restricted set of systems (mainly temperate grasslands, old fields, and agricultural ecosystems). This is highly limiting because it is well known that the functioning of mycorrhizal symbioses can be influenced by biotic and abiotic environmental factors, and thus our understanding of how mycorrhizas are structured and function across a wide variety of habitats is limited. Furthermore, with recent advances in next generation sequencing (NGS) technology, it is timely to begin a more thorough investigation of AM fungi across a wide variety of habitat types. The objectives of this thesis were to determine whether (1) modern NGS techniques accurately depict AM fungal communities using mock communities, (2) environmental filtering along an elevation gradient influences the phylogenetic structure of AM fungal communities in the soil, (3) habitat filtering or plant host selection is a stronger determinant of AM fungal communities along an elevation gradient, and (4) aerial dispersal of AM fungal spores varies among a wider variety of North American habitat types.

Examination of sequences generated from mock AM fungal communities revealed that taxonomic assignment of sequences within AM fungal families closely matched expected abundances, but accuracy decreased at lower taxonomic levels. However, analyses revealed that semi-quantitative metrics of sequence data still accurately inform on ecological patterns. Along elevation gradients AM fungal communities were observed to be more phylogenetically related than is expected by chance within all elevations sampled. In addition, AM fungal communities sampled from three co-occurring plant hosts revealed that communities are more strongly influenced by elevation sampled than host identity. Aerial dispersal also appears to be influenced by habitat type, where the highest proportion AM fungal spores in the air relative to in the soil was highest in more arid systems. Collectively, these studies indicate that local environmental conditions strongly influence assembly processes of AM fungal communities.

Preface

A version of Chapter 2 has been submitted for publication. Egan, C.P., Rummel, A., Kokkoris, V., Klironomos, J., Lekberg, Y., Hart, M. (2017) Using mock communities of arbuscular mycorrhizal fungi to evaluate fidelity associated with Illumina sequencing. Submitted to Fungal Ecology. Chapter 2 is work that emerged from a collaborative project conducted by myself, Dr. Miranda Hart, and Dr. Ylva Lekberg from MPG Ranch in Montana USA. For this Chapter, lab preparation of AM fungal mock communities from spore isolates was conducted by Alexii Rummel at MPG ranch's laboratory facility in Missoula Montana. Extraction of DNA from single spores of *Rhizophagus irregularis* (DAOM 197198) was conducted by Dr. Hart's PhD student, Vassilis Kokkoris, at UBC Okanagan with my supervision and guidance. With guidance from Dr. Matthew Settles from the University of California Davis Genome Center (formally from the IBEST Genomics Resources Core at the University of Idaho) I developed the polymerase chain reaction (PCR) protocol to amplify and prepare AM fungal DNA for Illumina MiSeq sequencing. I also prepared all mock community samples for sequencing, developed the bioinformatics pipeline to process MiSeq sequencing files, and conducted data analyses and interpretation. I wrote the manuscript with guidance and input from both Dr. Hart and Dr. Lekberg.

A version of chapter 3 has been published. Egan, C.P., Callaway, R.M., Hart, M.M., Pither, J., and Klironomos, J. (2016) Phylogenetic structure of arbuscular mycorrhizal fungal communities along an elevation gradient. *Mycorrhiza*. doi:10.1007/s00572-016-0752-x. Together with the guidance of my committee, Dr. John Klironomos, Dr. Ragan Callaway, Dr. Miranda Hart, and Dr. Jason Pither, I conducted the field work, collected the data, conducted the analyses, and wrote the manuscript.

For chapter 4, under the guidance of my committee, I conducted the field work, collected the data, conducted the analyses outlined in Chapter 4 and wrote the version presented in this thesis.

A version of chapter 5 has been published. Egan, C.P., Li, D.W., Klironomos, J. (2014) Detection of arbuscular mycorrhizal fungal spores in the air across different biomes and ecoregions. *Fungal Ecology*. http://dx.doi.org/10.1016/j.funeco.2014.06.004. For this Chapter, field work, sample collection, and data collection was undertaken by Jane Cullen, Vincent Emmerton, Alex Farkas, Edward Griffin, Benjamin Kitano, Hannah Kunz, Zhen Xu,

Dr. De-Wei Li, and Dr. John Klironomos. With guidance from Dr. Klironomos I conducted the data analysis, and wrote the manuscript.

Please see the first pages of these chapters to see footnotes with similar information.

Table of Contents

List of Tables

List of Figures

List of Symbols

α	Alpha diversity is the diversity of a local species pool, contained in either a sample or site.
β	Beta diversity is the variation in species composition among two (or more) samples.
$\chi 2$	Chi-squared is a statistical hypothesis test wherein the sampling distribution of the test statistic is a chi-squared distribution when the null hypothesis is true.

List of Abbreviations

AM	Arbuscular mycorrhiza is a type of mycorrhizal association in which the fungal partner (from the Glomeromycota phylum) penetrates the cortical cells of the roots of the vascular plant host.
MNTD	Mean nearest taxon distance is the average of the phylogenetic distance (branch length) joining each species with its nearest neighbour. This metric is more sensitive to phylogenetic patterns at the tips of phylogenetic trees.
MPD	Mean pairwise phylogenetic distance is the average of the phylogenetic branch length joining all pairs of species in a given sample (or community). This metric is more sensitive to tree-wide patterns of phylogenetic structure.
NGS	Next generation sequencing is a catch-all term used to encapsulate a number of different modern sequencing technologies. NGS technologies used in this thesis include both Illumina and Roche 454 sequencing.
OTU	Operational taxonomic unit is an operational term to define clusters of DNA sequences according to a pre-determined sequence similarity.
PD	Phylogenetic diversity is the sum of the phylogenetic branch lengths joining all species of a given sample (or community).
QIIME	Quantitative insights into microbial ecology is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data.
SSU rRNA	Small subunit ribosomal ribonucleic acid is a subunit of the RNA component of the ribosome, and is essential for protein synthesis. It is the most common marker choice used by arbuscular mycorrhizal fungal ecologists for examining molecular diversity of communities.
VT	Virtual taxa are phylogenetically defined arbuscular mycorrhizal fungal sequence groups which roughly correspond to species-level taxa developed by Maarja Öpik.

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Chapter 1 Introduction

1.1 Organization of Introduction

In this thesis, I investigate factors that influence the assembly of arbuscular mycorrhizal (AM) fungal communities in natural ecosystems. I begin this chapter by introducing AM fungi and discussing their function in terrestrial ecosystems. Next I discuss our current understanding of the diversity of AM fungi, as well as the molecular tools used for capturing that diversity, specifically next generation sequencing (NGS). I then discuss factors that structure AM fungal communities in natural systems, including our current understanding of AM fungal biogeography. In this section I outline environmental (both biotic and abiotic) determinants of AM fungal diversity, and then expand upon specific processes that were examined in this thesis including; community phylogenetic patterns (examined in chapter 3), patterns in the association with host plants (examined in chapter 4), and dispersal of AM fungi (examined in chapter 5). I then summarize our current understanding of AM fungal diversity along elevation gradients. In the final section I present the study objectives of this thesis, outlining specific goals of each chapter.

1.2 Arbuscular mycorrhizal fungi

Mycorrhizas, which are microscopic symbiotic associations between certain groups of soil fungi and the roots of plants, are arguably the most important symbioses in terrestrial ecosystems (Smith and Read 2008). The uniting feature among all mycorrhizas is the bi-directional exchange of nutrients between a plant host and fungal symbiont. The symbiosis is so prevalent, that it has been often stated that plants do not, strictly speaking, have roots; they have mycorrhizas (originally quoted by the European Bank of Glomeromycota committee

(BEG) in 1993, quoted here as it appears in Schüßler et al. 2007). Remaining relatively unchanged since their beginning, fossil records show some of the earliest land plants containing mycorrhizal structures (Giovannetti et al. 1999; Redecker et al. 2000), indicating that mycorrhizas aided plants in transitioning from aquatic to terrestrial ecosystems (Wang and Qiu 2006). Today, 90% of terrestrial plant families contain species that are mycorrhizal (Wang and Qiu 2006) and mycorrhizas are widely distributed across every major biome and ecoregion (Brundrett 1991; Read 1991).

Of all the mycorrhizal types, arbuscular mycorrhizas (AM) are the most ubiquitous, forming symbioses between obligate biotrophic fungi from the Phylum Glomeromycota (Schüβler et al. 2001) and a wide range of plant partners including members from the majority of families of angiosperms and gymnosperms (Smith and Read 2008). Phylogenetic classification of AM fungi is ever changing. Until recently, AM fungi were included in the order Glomales within the Phylum Zygomycota (Morton and Benny 1990). However, through morphological and sequencing of ribosomal genes (rRNA), they have been placed in a distinct Phylum Glomeromycota (Schüβler et al. 2001; Redecker et al. 2013), separate from the other four fungal phyla (Zygomycota, Chytridiomycota, Basidiomycota, and Ascomycota). Recently it has been proposed that AM fungi be grouped with other Zygomycetes, and be placed within a new subphylum Glomeromycotina within the Phylum Mucoromycota (Spatafora et al. 2016). Regardless of phylogenetic denomination, a unifying characteristic of AM fungi is their unique plant-fungal exchange interface (arbuscules), with two forms that are currently recognized, Paris type and Arum type (Armstrong and Peterson 2002). AM fungi are also distinguished by their multinucleate coenocytic mycelia, and soil-borne asexual spores that each contain hundreds to thousands of nuclei (Smith and Read

2008).

1.2.1 Functions of AM fungi

The most recognized function of the AM symbiosis is the take-up, translocation, and transmittance of soil nutrients to host plants in exchange for photosynthate. However, their function in terrestrial ecosystems extends beyond this bi-directional exchange. For example, AM fungi can alter the rate of water movement into, and throughout host plants, with consequences on tissue hydration and leaf physiology (Augé 2001). By improving water relations for host plants, stomatal conductance and water potential of hosts may be altered (Birhane et al. 2012), thereby enhancing host photosynthesis. Plants in arid and semi-arid ecosystems may benefit from the symbiosis by maintaining leaf turgor and conductance at greater water deficits, and lower leaf and soil water potentials, compared with non-mycorrhizal plants (Augé et al. 1986). Colonization of the soil by AM fungi may also improve plant-water relations, regardless whether the plant is directly colonized (Augé 2004), as AM fungi may improve moisture retention of the local soil environment (Augé 2001).

AM fungi may also benefit plant hosts by providing them with protection from soil-borne enemies (Sikes et al. 2009). AM fungi indirectly protect plant hosts by competing with pathogens for infection sites on host roots, or by competing with pathogens for plant-derived photosynthate (Whipps 2004). Indirect attack of AM fungi on soil pathogens may also occur, as the fungi can trigger plant hosts to release toxic compounds at the sites of infection (Gianinazzi-Pearson et al. 1994). AM fungi can also mitigate damage caused by pathogens, such as root feeding nematodes, by compensating for lost/damaged root biomass (Pinochet et al. 1998). Similar to other interactions with the plant host, the ability of AM fungi to enhance

resistance of plant roots is not equal for all fungi, and therefore interactions among AM fungi and plant pathogens is variable and depends on the host plant and environmental conditions (Azcón-Aguilar and Barea 1996).

Plant population dynamics may also be influenced by AM associations, largely through increased nutrition of host plants. Colonization by AM fungi can increase the uptake of phosphorus and thereby increase the rate of plant growth, thereby reducing time to flowering (Lu and Koide 1994). Therefore an infected host can potentially produce a higher abundance of flowers over its lifetime. For plants that rely on pollination, colonization by AM fungi has been show to increase duration of floral display, thereby increasing the frequency of pollinator visitations (Gange and Smith 2005; Wolfe et al. 2005). Pollen vigour has also been shown to be higher in pollen from colonized plants, as a result of increased phospohrous concentration within the pollen (Lau et al. 1995).

In addition to increasing the number and duration of plant sexual structures, AM fungi have been shown to stimulate the production of plant propagules. For example, mycorrhizal plants have been observed to produce more seeds when compared with non-mycorrhizal controls (Stanley et al. 1993). Higher seed production is of high ecological importance as it provides plants a competitive advantage by increasing the likelihood of more seedling establishment (Stanley et al. 1993). For clonal plants, the number and size of ramets produced may also be affected by different co-occurring AM fungal taxa (Streitwolf-Engel et al. 2001). In this instance, AM fungal taxa act synergistically, and can differentially influence the size and abundance of host ramets (Streitwolf-Engel et al. 2001).

Offspring of colonized parent plants may also receive benefits from the symbiosis.

Offspring of infected hosts have been shown to display faster growth rates (Koide and Lu 1992), which has been attributed to higher nutrient content of seeds prior to germination. This higher nutrient content in seed may result in a larger number of flowers in the developing seedlings (Shumway and Koide 1994). New seedling recruits may also receive mycorrhizal benefits quickly as they can attach to the established mycelium network below-ground (van der Heijden and Horton 2009). These seedlings may be "nursed" by parent plants via the common mycelium network by transferring and sharing nutrients.

The positive response of plant hosts to AM fungal infection has been shown to be inversely related to plant population density, where, as plant population density increases, the positive response of plants to colonization by AM fungi decreases (Allsopp and Stock 1992). This pattern is attributed to overlapping root depletion zones, which increase as plant density increases (Schroeder and Janos 2004). Dense root systems will result in the roots of multiple plants to overlap, causing nutrient poor root depletion zones to dominate the rhizosphere (Koide and Dickie 2002). As a consequence, colonization by AM fungi may have a less positive effect as there will be few non-depleted areas within the soil matrix to access nutrients. Greater plant density also increases competition above-ground for space and light, leading to a reduction in photosynthetic output from hosts. This reduction in photosynthesis may decrease host quality for AM fungi, resulting in less fixed carbon transferred to fungi in exchange for nutrients (Konvalinková and Jansa 2016).

At the community level, AM fungi may indirectly influence plant diversity by altering plant-plant interactions as well as by influencing the growth and reproduction of individual plants. As proposed by Tilman's resource ratio hypothesis, for two plants that compete for the same resource, the one with the lower resource requirements is considered

the better competitor (Tilman 1988). AM fungi may reduce the resource requirements of host plants by allowing them to access nutrients at levels below what they would be able to achieve without forming associations. The plant that is more efficient at acquiring resources should be the better competitor, and AM fungi have been linked to increased competitive ability of plants (Marler et al. 1999). AM fungi can also increase facilitative effects of plants through hyphal networks. Common mycorrhizal networks make it possible for nutrient and carbon transfer to occur between plants (Walder et al. 2012) as well as seedlings being nursed by already established plants in the same common mycorrhizal network (van der Heijden and Horton 2009). It is unresolved as to whether different AM fungal species play differing roles in plant-plant interactions.

1.3 Genetic investigation of AM fungal diversity

Despite the ecological importance of AM fungi, an understanding of their distribution patterns in the field is incomplete. This largely stems from the difficulty of examining the composition and abundance of AM fungi in natural ecosystems. Being microbial organisms, AM fungi are invisible from the naked human eye, requiring microscopic or molecular techniques to identify them.

AM fungal diversity has traditionally been studied by isolating AM fungal structures from the soil and identifying spores, hyphae, or internal root structures (Morton and Benny 1990; Morton and Redecker 2001). While morphological methods have contributed to our understanding of AM fungal diversity and functioning, the techniques are limited. For example, dimorphism has been observed in the spores of some species, which can confound diversity estimates (Morton and Redecker 2001; Rosendahl 2008). Also, taxonomic assignment is difficult beyond the family level when identifying AM fungi using intraradical

hyphal morphology (Morton and Redecker 2001; Rosendahl 2008). Through development of molecular tools, such as next generation sequencing (NGS), our ability to describe and classify AM fungi phylogenetically has improved (Redecker et al. 2013).

The use of DNA sequences to describe AM fungal diversity presents a separate set of challenges for AM fungal biologists. The Glomeromycota phylum presents a genetic rarity, in that, both, their tube-like coenocytic hyphae and spores harbour hundreds to thousands of nuclei within a common cytoplasm (Smith and Read 2008), with debate as to whether single spores contain genetically different nuclei (Kuhn et al. 2001) or polymorphic gene copies (Pawlowska and Taylor 2004; Lin et al. 2014). Therefore, environmental samples taken for sequencing may contain anywhere from hundreds to several thousand nuclei from a single AM fungal individual. Also, through parasexual activity, separate AM fungal individuals are able to exchange nuclei through fusion (anastomosis) of hyphae (Giovannetti et al. 2003; Croll et al. 2008), making the study of AM fungal populations difficult. Further complicating genetic investigation of AM fungal diversity is the observed high genetic diversity within single isolates (e.g. Sanders et al. 1995). Despite these challenges, use of sequencing has allowed us to characterize AM fungal diversity to levels previously unseen (Lee et al. 2013), with AM fungal communities being observed to be more diverse than originally thought (Fitter 2005).

When determining diversity through molecular techniques, the nuclear ribosomal operon (rRNA) gene has been the genetic target of choice. rRNA is amplified from the soil, capturing spore and hyphal DNA, and/or amplified from host roots capturing internal AM fungal structures. The rRNA gene is comprised of three regions; the small subunit (SSU), internal transcribed spacer (ITS), and long subunit (LSU), all of which have each been

utilized by AM fungal researchers. The ITS region has been primarily used as a barcoding marker (Schoch et al. 2012), while the SSU and LSU regions have been utilized for ecological studies of AM fungi. Currently, SSU is more widely used than LSU (Öpik et al. 2014). Popularity of this region can be attributed to several reasons, most notably of those being the length of the hypervariable region in the SSU being the suitable length for modern sequencing platforms (Lee et al. 2008), and the creation of the MaarjAM database (maarjam.botany.ut.ee), which is an expertly curated database of SSU Glomeromycotan sequences. While marker choice is still under debate (see Lindahl et al. 2013), no single marker to date has been able to capture the entire AM fungal lineage (Krüger et al. 2009).

Regardless of marker choice, the growing trend among researchers is to determine diversity in samples through NGS. Development of NGS technology has benefited AM fungal diversity studies by both increasing the number of sequences generated per sample, as well as reducing the cost per base pair (bp) sequenced when compared with previous sequencing technologies. With the first Roche 454-pyrosequencing platforms (454 Life Sciences, a Roche company, Branford, CT, USA) introduced in 2005, NGS technology has contributed to an unprecedented microbial richness being observed in environmental samples (Gibbons et al. 2013). Presently Roche 454 has the ability to sequence amplicon lengths of 400-600 bases, which is ideally suited for AM fungal studies, and has contributed enormously to our knowledge of AM fungal biodiversity patterns (e.g. Öpik et al. 2009; Lumini et al. 2010; Moora et al. 2011; Lekberg et al. 2012; Lekberg et al. 2013). However, support for the Roche 454 platform has been discontinued, and thus AM fungal researchers have been forced to transition to other NGS platforms; the most popular being Illumina platforms (Illumina Inc., San Diego, CA, USA). Historically, read lengths available with

Illumina platforms have been limited to short read lengths of 100-200 bases, which is too short to be appropriate for AM fungal diversity studies. However, recently developed protocols allowing for 250 paired end (PE) and 300 PE sequencing protocols, enable researchers to sequence regions 500-550 bps in length, the common amplicon length used in molecular diversity studies of AM fungi (Öpik et al. 2014).

Post-sequencing, sequences are phylogenetically grouped according to sequence similarity, with the goal being to roughly equate sequences to species level taxa. In the majority of AM studies sequences are first grouped into operational taxonomic units (OTUs; Schloss and Handelsman 2004) based on a pre-determined sequence similarity, often being 97% sequence similarity. OTUs can then be used to represent AM fungal species, or can be identified taxonomically against a curated database, the most common being Genbank (Benson et al. 2005), Silva (Quast et al. 2013), or MaarjAM (Öpik et al. 2010). For MaarjAM specifically, sequences are matched to virtual taxa (VT), which are phylogenetically defined sequence groups that represent species-level taxa (Öpik et al. 2014). Use of MaarjAM, and assigning identity to reads to VTs, has risen in recent years (Öpik et al. 2014) likely because; 1) VTs are associated with the MaarjAM database (Öpik et al. 2010), which is an AM fungal specific database containing sequences both cultured and uncultured AM fungal taxa, and 2) VTs present a standardized method of phylogroup designation, allowing for cross-study comparisons. Furthermore, VTs can be split and merged as new genetic information becomes available.

Although NGS has been unilaterally adopted by AM fungal ecologists, we do not know its accuracy and precision in representing AM fungal communities. Post-sequencing, AM fungal researchers implement ecological analyses under the assumption that sequencing

data accurately depicts the abundance and identity of species present in samples (Öpik et al. 2009; Dumbrell et al. 2010; Horn et al. 2014). However, the abundance of taxa, and consequently community composition, can be altered during amplicon library preparation and sequencing (Quince et al. 2009; Lee et al. 2012), bringing to question the validity of conclusions made from NGS studies (Brooks et al. 2015). Further complicating the interpretation of results post-sequencing, scarce information exists for the intra-specific variation of AM fungi at the molecular level. This brings to question whether grouping sequences at a similarity of 97%, a similarity threshold chosen by bacteriologists (Konstantinos and Tiedje, 2005), is sufficient to delimit individual species. This is also further complicated by high variation within the rRNA operon within some AM fungal species (Clapp et al. 1999; Rodriguez et al. 2004).

1.4 Assembly of AM fungal communities in natural systems

There has been a recent surge of research examining AM fungal diversity in natural systems (e.g. Öpik et al. 2006; Öpik et al. 2009; Öpik et al. 2010; Öpik et al. 2013; Yang et al. 2013; Horn et al. 2014; Li et al. 2014; Öpik et al. 2014; Davison et al. 2015), fueled in part by the increased feasibility of characterizing community diversity using NGS technology. Through these studies, AM fungal diversity has been shown to be greater than previously described, linked partly to our ability to sequence and phylogenetically place uncultured taxa (Ohsowski et al. 2014).

Local AM fungal communities are assembled through multiple processes. Recently, the filter model of community assembly of AM fungal communities has been proposed (Vályi et al. 2016), where AM fungal communities are initially structured by neutral processes (such as dispersal and chance), after which environmental and host filters are

imposed upon species. As AM fungi are obligate biotrophs, ability to form and maintain host associations is an especially important determinant of local community structure (Eom et al. 2000; Torrecillas et al. 2012a; Torrecillas et al. 2012b). However, several studies also highlight the importance of the abiotic environment (Rousk et al. 2010; Yang et al. 2013; Liu et al. 2015b). Therefore, both biotic and abiotic environmental factors should be considered when interpreting local diversity patterns.

1.4.1 Dispersal

AM fungi disperse via multiple mechanisms, at both local and long-distance scales. Locally, AM fungi primarily disperse belowground, where movement of propagules is accomplished through mycophagy by invertebrates such as earthworms (Gange 1993) and collembolans (Klironomos and Moutoglis 1999), or via mycelial spread (Friese et al. 1991). Long-distance dispersal of AM fungi is possible again through mycophagy of spores by mammals, such as rats (Vernes and Dunn 2009), or movement of sediments containing spores and other AM propagules (Harner et al. 2009). Even though AM fungi do not have epigeous structures allowing for direct release of propagules into the air, dispersal of AM fungal spores in the air has also been observed (Tommerup 1982; Allen 1987; Allen et al. 1989).

The mechanisms by which AM fungi disperse (Öpik et al. 2008), as well as environmental factors which can potentially influence AM fungal dispersal, should both be considered when interpreting diversity patterns. For example, in more productive habitats, with large plant cover, species able to disperse by mycelial growth would have larger dispersal ability than species with lower mycelial growth, as these species would be able to easily spread from host to host (Klironomos and Hart 2002). Conversely, in less productive

habitats, with more exposed surface soil, AM fungal species that produce more spores would have larger dispersal ability, as they would have an increased probability of having spores released to the air and moved via wind currents (Allen et al. 1989). Spore size, the number of spores produced (Bever et al. 2001), and mycelial growth (Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009; Maherali and Klironomos 2012) are all traits which are known to differ among phylogenetic groups of AM fungi. Therefore, phylogeny may be directly linked to propagation and dispersal characteristics of AM fungi in particular habitat types. Testing these concepts would greatly enhance our understanding of AM fungal diversity patterns.

1.4.2 Environmental determinants

Geographic patterns have been observed for AM fungi, with cosmopolitan taxa being found in most ecosystems surveyed, and specialist taxa being constrained to select habitats (Davison et al. 2015). There is some indication that specialist taxa are a result of local environmental factors. Both, AM fungal species diversity and abundance, have been shown to vary along gradients of soil nutrient availability (Egerton-Warburton and Allen 2000), soil physio-chemical composition (An et al. 2008; Mohamed and Martiny 2011), and temperature (Koske 1987). Indicating differential responses of AM fungal taxa to the abiotic environment. In addition, abiotic conditions can in turn influence AM fungal biological interactions, especially between AM fungi and plant hosts. For example, under low nutrient conditions, plants may allocate more resources through their roots to AM fungal partners that provide hosts with more nutrients from the soil (Johnson et al. 1997). This in turn can lead to a shift in AM fungal community structure (Johnson 1993), as shown through changes in composition and abundance with increased fertilization (Egerton-Warburton et al. 2007).

There is also indication that soil pH can influence AM fungal diversity (Rousk et al. 2010), where soils with different pH levels have been shown to differ in their community structure (Lekberg et al. 2011). AM fungal taxa have also been shown to respond differently to pH levels. For example, species of *Acaulospora*, *Paraglomus* and *Scutellospora* have been observed to produce more spores in acidic soils than alkaline soils (Klironomos et al. 1993; Lekberg et al. 2011), suggesting that genera differ in their physiological response to pH.

In addition to soil chemistry, soil structure may influence the composition of AM fungal communities. Size and aggregation of soil particles can directly influence plant available nutrients and carbon storage in the soil (Rillig 2004) which can have direct impact on how host plants associate with AM fungi. Soil structure may also have a direct influence on AM fungal community structure. For example, members of the *Glomeraceae* family have been shown to dominate in clay soils whereas members from the *Gigasporaceae* family are dominant in sandy soils (Lekberg et al. 2007). Lekberg et al. (2007) determined that the differences in dominance is linked to both sporulation rates as well as the number of vesicles formed in different soil types. Meaning that the distribution of AM fungi is influenced by the soil aggregation/characteristics, which may also be altered by the fungi themselves (Rillig and Mummey 2006).

1.4.3 Spatial distribution of AM fungi

Soil is a heterogeneous environment where chemical, physical, and biological factors vary at small spatial scales (Klironomos and Kendrick 1995; Whitcomb and Stutz 2007). AM fungal communities have been shown to be spatially structured at scales of less than 1 meter, and hyphal abundances have been shown to vary at distances less than 30 centimeters (Mummey and Rillig 2008). As the distance increase between communities, AM fungi

exhibit a decrease in compositional similarity (Lekberg et al. 2007; Davison et al. 2012). This indicates that deterministic interactions may be more important than random processes for structuring AM fungal communities at small spatial scales, while at larger distances stochastic processes (such as dispersal) may increase in importance.

1.4.4 Partner selection

AM fungi are reliant upon a plant host for survival. Therefore the ability for AM fungi to initiate, and maintain association with hosts, is of uppermost importance in determining diversity of AM fungi. AM fungi have differential effects on host performance, where the amount of benefit a plant host receives from forming association can be determined by the amount of nutrient benefit received from forming those associations minus the carbon cost of forming and maintaining those associations (Johnson et al. 1997). Differences in the amount of benefit received by hosts has been observed to differ even among closely related AM fungal taxa (Verbruggen et al. 2012). This can have a direct consequence on taxa, as hosts have been shown to preferentially allocate carbon to more mutualistic AM fungi while decreasing allocation of resources to less mutualistic fungi (Bever et al. 2009; Kiers et al. 2011). The result of increasing carbon allocation by hosts has been shown to reciprocally increase phosphorus transfer from the select AM fungus (Bücking and Shachar-Hill 2005). Therefore, in natural communities AM fungi and AM plants are expected to associate with the partner that increases benefit.

AM fungi may be highly mutualistic, parasitic, or anywhere in between (Johnson et al. 1997). The location of AM fungi on this spectrum of mutualism to parasitism has been shown to be influenced by environmental conditions. For example, high nutrient environments, such as agricultural fields, contain a higher abundance of parasitic AM fungal

taxa (Johnson et al. 1997). This likely occurs because the carbon cost of maintaining AM fungal associations for host plants is greater than the nutrient benefit received from associated fungi. As nutrient levels decrease, the relative benefit is expected to increase as AM fungi will be more efficient at uptake of scarce nutrients (Smith and Read 2008). Thus, plants found in low soil nutrient conditions may benefit from any AM fungal associations by their increased ability to access nutrients (Thrall et al. 2006).

Studies in grassland systems have shown that plant hosts and AM fungal communities may become co-adapted. Host plants have been observed to receive increased benefit from local AM fungi relative to foreign AM fungi when grown under identical conditions (Klironomos 2003). Reciprocal transplant experiments in grasslands and host plants and AM fungal communities have also supported increased benefit to local AM fungi relative to foreign fungi (Johnson et al. 2010). Co-adaptation to extreme environments, such as acidic soils, has also been observed among plants and AM fungal communities (Lekberg et al. 2011). The strength and direction of association (i.e. parasitic or mutualistic) is likely determined by the environment in which they are found, where mutualistic associations occur in nutrient limited environments, and parasitic associations are likely in high-fertility environments (Thrall et al. 2006; Johnson et al. 2010).

In natural systems, plant hosts are colonized by several fungal partners (Vandenkoornhuyse et al. 2007) suggesting that co-existence among AM fungal taxa is the normal state. It is largely in altered soil communities, such as in agriculture, where we observe dominance by one AM fungal taxon (Johnson et al. 1997). Therefore, natural AM fungal diversity may occur through occupation of different temporal or spatial niches (Koide 2000). As a result of competition for resources, AM fungal communities tend to be more

phylogenetically dispersed (species within a community are more distantly related than expected by chance) and decrease niche overlap (Maherali and Klironomos 2007). However, it has also been shown that phylogenetic relatedness among AM fungal species is associated with increased coexistence (Roger et al. 2013), suggesting that closely related species may interact synergistically. Also, natural AM fungal communities have been shown to be phylogenetically clustered (Kivlin et al. 2011; Horn et al. 2014; Liu et al. 2015b), indicating that local habitat filtering (Webb et al. 2002a; Stegen et al. 2012), or other local deterministic processes (Mayfield and Levine 2010) cause AM fungal communities to be more phylogenetically similar than is expected by chance.

While several studies show that abiotic conditions can alter the strength and direction of AM fungal associations with plant partners, knowledge of how abiotic conditions alter AM fungal diversity within the same host across a variety of abiotic environmental conditions is scarce. To better understand how association between plant hosts and AM fungi is influenced by the abiotic environment, and in turn how these association can alter AM fungal diversity, studies should focus on diversity of AM fungi associating with hosts along environmental gradients.

1.5 AM fungal diversity along elevation gradients

Elevation gradients in mountainous regions provide the most powerful natural system available to examine the ecological and evolutionary responses of living organisms to geophysical influences (Körner 2007). Abiotic factors such as temperature, precipitation, and solar radiation vary with elevation creating heterogeneous habitats along the gradient. As elevation increases, environmental stress also increases causing a reduction in productivity. Available space also decreases as elevation increases, reducing the spatial scale in which

species interact while being in stressed conditions (Rowe 2009). The relationship between changes in elevation and species diversity/richness has been examined using a variety of trophic groups, where the general trend is that species richness declines with elevation. This pattern is not always monotonic and can vary from species to species (Rahbek 1995). Patterns have also been shown to differ among groups, as supported by studies finding microbial communities to have differing diversity patterns from plant communities when sampled along the same gradient (Bryant et al. 2008; Fierer et al. 2011; Singh et al. 2012). Indicating that the forces structuring diversity along elevation gradients can differ among trophic groups (Bryant et al. 2008).

Diversity studies of AM fungi along elevation gradients to date have found contrasting patterns of community diversity with increasing elevation. AM fungal species richness has been observed to either peak at mid elevations (Gai et al. 2009), or decrease with increasing elevation (Gai et al. 2012; Li et al. 2014; Liu et al. 2015a). Underlying mechanisms that produce a unimodal peak in diversity remains contentious (Guo et al. 2013), as several possibilities exist (Graham and Duda 2011). However, with only a single study observing a mid-elevation peak in diversity, and several observing a monotonic decline in diversity with increasing elevation, the implication is that AM fungi respond to increased environmental constraints at higher altitudes such as a reduction in spatial area, a decrease in resources such as soil or plant hosts, and/or lower temperatures (Rahbek 1995; Sanders 2002). With differences in environmental conditions creating distinct AM fungal communities between elevations (Liu et al. 2015a), consisting of specialists constrained to single elevations, or generalists found along the entire breadth of the gradient (Li et al. 2014; Liu et al. 2015a).

AM fungi have been observed in some of the most extreme alpine environments, such as the Tibetan plateau in Asia (Gai et al. 2006; Gai et al. 2012; Liu et al. 2015a). This high elevation ecosystem has been shown to have well established AM fungal communities, with 72% of plant species being colonized by AM fungi despite (or because of) the extreme conditions (Gai et al. 2012). In these high elevation systems host density plays a large role in determining spatial distribution of AM fungal communities, where host plants act as resource islands for AM fungal communities. Such is the case in the high Andes where AM fungal colonization is low on isolated plants growing on bare ground patches, but high within cushion plants (Casanova-Katny et al. 2011). A high diversity of AM fungi has also observed in these high elevation systems where multiple AM fungal genera have been observed (Chaurasia et al. 2005; Gai et al. 2012). In addition, high elevations have been shown to harbour unique AM fungal species such as *Acaulospora alpina,* which is found only at elevations above 3000 m (Oehl et al. 2006).

Previous research on AM fungi along elevation gradients has largely examined colonization patterns of different soil fungi and host plants. Shifts between EM and AM fungal communities have been shown along elevation gradients, however this trend occurs regardless of elevation and is more strongly influenced by plant species than elevation per se (Becklin et al. 2012). Colonization by dark-septate endophytic (DSE) fungi is common at the higher elevations of the Andes and Rockies (Schmidt et al. 2008). DSE fungi can inhibit colonization levels of AM fungi as elevation increases, and is supported by coarse AM fungal structures decreasing at higher elevations where DSE are more prevalent (Schmidt et al. 2008). Colonization patterns along an elevation gradient will largely be moderated by the

identities of the studies host species rather than dispersal or environmental conditions (Newman and Reddell 1988; Fisher and Fule 2004; Gai et al. 2006).

While many good studies have been conducted examining AM fungal diversity along elevation gradients, they have been limited. Estimates of diversity have relied on either spore data (Gai et al. 2006; Lugo et al. 2008; Gai et al. 2009; Gai et al. 2012) biasing diversity estimates to sporulating species (Sýkorová et al. 2007), or cloning and sequencing (Li et al. 2014; Liu et al. 2015a) which can underestimate AM fungal diversity (Buée et al. 2009). Furthermore, by using traditional taxonomic methods phylogenetic relationships that exist among taxa within communities is neglected. While phylogenetic diversity patterns have been observed, such as increasing abundance of Acaulosporaceae species with increasing elevation (Gai et al. 2012; Liu et al. 2015a), and even observation of an *Acaulospora* species specialized to elevations above 1300 meters above sea level (MASL) in the Swiss Alps (Oehl et al. 2006), such patterns have yet to be formally tested. By integrating phylogenetic information on species co-occurring within AM fungal communities, improved interpretation of the relative importance of underlying evolutionary and ecological processes determining diversity patterns can be reached (Webb et al. 2002a; Webb et al. 2002b; Cavender-Bares et al. 2006; Kembel and Hubbell 2006; Kembel 2009). Also, diversity studies to date have been concentrated in the Tibetan plateau (Gai et al. 2006; Gai et al. 2009; Li et al. 2014; Liu et al. 2015a), with a single study along an elevation gradient in South America (Lugo et al. 2008), and no studies to my knowledge examining AM fungal diversity along North American elevation gradients.

1.6 Research outline and objectives

AM fungi are essential components of terrestrial ecosystems. While our knowledge of their patterns of diversity is growing, our understanding of the underlying processes that determine natural AM fungal diversity is limited. In this thesis, I examine the diversity of naturally occurring AM fungal communities across a variety of habitat types. My aim is to examine how AM fungal community structure varies across understudied habitat types, and to interpret diversity patterns to identify potential factors that influence the naturally occurring communities.

Before my examination of AM fungal communities in natural systems, I first assess the fidelity using NGS to determine AM fungal diversity. With the rapid advancement of next generation sequencing technologies, our understanding of AM fungal diversity has rapidly been growing. However, because of the rate at which new technologies are being developed, their ability to accurately capture AM fungal diversity has yet to be examined. Post-sequencing, presence/absence and abundance of AM fungal species sequences are used to determine AM fungal diversity patterns. While there has been an increasing number of molecular studies examining AM fungal diversity, none to date have verified whether such techniques are an accurate reflection of what is present in samples. In chapter 2 of this thesis I amplified and sequenced mock communities of known AM fungal species composition and abundance to evaluate whether NGS techniques are accurate.

In chapter 3, I examined AM fungal communities along an elevation gradient, and used a phylogenetic approach to assess the influence of environmental stress on determining phylogenetic structure of AM fungal communities. First, I predicted that phylogenetic diversity of AM fungal communities would decrease along the gradient, as a result of

increased environmental filtering as elevation increases. Second, I hypothesized that increased environmental filtering at high elevations would filter for a subset of AM fungal clades suited for such conditions, resulting in AM fungal communities that are more phylogenetically clustered at higher elevations. Third, I predicted that local environmental filters would select for unique AM fungal assemblages at each elevation sampled, resulting in communities from more distant elevations to be more phylogenetically dissimilar.

In chapter 4, another study along an elevation gradient was conducted to determine how allocation of biomass by AM fungi, and selection by host species vary among habitat types. Samples were collected along the same elevation gradient as chapter 3. In this study, I sampled AM fungal communities from the roots and soil beneath three co-occurring plant species. I predicted that fungal species that have been shown to allocate more biomass to structures in the soil would appear in higher abundances in soil samples, while those species that are known to allocate more biomass to intraradical structures would be more abundant in roots. I also predicted that allocation of biomass would determine which habitat species would be found in the highest abundance. For example, species that allocate more biomass to extraradical structures would be found in higher abundances in low stress/high productive habitats, and species that allocate more biomass to intraradical structures would have higher abundance in high stress/low productive habitats. I also predicted that host species would have a stronger influence on intraradical AM fungal communities, while habitat type would be a stronger determinant of extraradical AM fungal communities.

In my final study, I examine how dispersal of AM fungi can vary among habitat types. AM fungi are globally widespread, and have been shown to have low endemism (Davison et al. 2015), which is indicative of them having unconstrained dispersal

capabilities. An obvious dispersal method for fungi to achieve this ubiquity is through aerial dispersal. However, it is unknown whether AM fungi disperse readily through the air, and whether this ability is influenced by habitat type, and that habitats accompanying environmental conditions. Two chapters within this thesis examine AM fungal community structure in various habitat types, and work under the assumption that AM fungi are not limited by dispersal, but rather are limited by local abiotic constraints, or local biological interactions. However, if AM fungi have limited dispersal capabilities, interpretation of community patterns in natural ecosystems should take into account dispersal barriers that may exist. Because our current lack of knowledge about whether AM fungi readily use aerial dispersal, and whether this dispersal method is influenced by habitat type, I examine the occurrence of AM fungal spores in the air across several biomes and ecoregions. The goal of this study was to compare the abundance of AM fungal spores in the air across different habitat types, and to relate these abundances to the importance of aerial dispersal in the habitat types examined. Because AM fungi are globally prevalent, I predicted a high occurrence of AM fungal spores in the air for all ecoregions sampled. Furthermore, I hypothesized that ecosystems with large areas of exposed soil would have higher abundances of spores in the air than ecosystems with denser vegetation.

In chapter 6, the results from these chapters are synthesized and I present the overall conclusions drawn from these studies along with future areas of research.

Chapter 2 Using mock communities of arbuscular mycorrhizal fungi to evaluate fidelity associated with Illumina sequencing[1]

2.1 Background

Next generation sequencing (NGS) has been widely adopted and evaluated among microbial ecologists (Degnan and Ochman 2012; Egge et al. 2013; Fadrosh et al. 2014; Brooks et al. 2015; Nguyen et al. 2015; Ryberg 2015). While it has been similarly adopted by the AM fungal research community (e.g. Öpik et al. 2009; Dumbrell et al. 2011; Lekberg et al. 2012; Davison et al. 2015; Hart et al. 2015), its fidelity for representing AM fungal communities has not been rigorously tested. While ecological analyses are performed under the assumption that NGS, such as Illumina MiSeq, depicts the relative abundance and identity of AM fungal species (Öpik et al. 2009; Dumbrell et al. 2010; Dumbrell et al. 2011; Lekberg et al. 2012; Hart et al. 2015), how closely these data reflect AM fungal communities is not known. There are reasons to believe that NGS could introduce bias specific to AM fungi, which may confound accurate community description. In this sense, bias describes inherent aspects of sequencing technology and/or the analysis pipeline that results in certain taxa being preferentially, or erroneously reported. Two of the most pernicious biases for AM fungal community datasets are erroneous estimates of species richness/relative abundance and taxon misidentification.

Across platforms, NGS studies often report greater AM fungal richness compared with older techniques (e.g. Öpik et al. 2009; Dumbrell et al. 2011). While this may be attributed to increased sequence depth and a better detection of low abundance species

[1] A version of this chapter is under consideration for publication. Egan, C.P., Rummel, A., Kokkoris, V., Klironomos, J., Lekberg, Y., Hart, M. (2017) Using mock communities of arbuscular mycorrhizal fungi to evaluate fidelity associated with Illumina sequencing. Fungal Ecology.

(Sogin et al. 2006; Kunin et al. 2010), some of this richness may also reflect sequence polymorphisms among nuclei within hyphae and spores (Lin et al. 2014; Boon et al. 2015; Ropars and Corradi 2015; Wyss et al. 2016). Further, due to the higher incidence of sequencing errors associated with NGS, it is also possible that some of the increase in richness is artefactual (Reeder and Knight 2009). To guard against artefactual increases in species richness, a common practice is to remove operational taxonomic units (OTUs) containing only one (or very few) reads (Tedersoo et al. 2010; Lindahl et al. 2013). While this may be a valid approach, 'removing singletons' is an arbitrary solution that has not been tested. Also, with the order of magnitude increase in sequence output from Roche-454 to Illumina platforms, the question is whether the removal of singletons is stringent enough (Brown et al. 2015). No guidelines exist to guide abundance filtering of AM fungal OTUs, but mock communities may help guide post-sequencing analyses, such as adjusting the OTU clustering threshold to recover the same number of taxa present in the mock community (Nguyen et al. 2015).

Issues with NGS are further compounded by methods for taxon identification differing widely among researchers, which may result in datasets that are difficult to compare. The most common approach is to cluster sequences into OTUs of ≥ 97% identity. These OTUs are then typically compared to public databases to ascribe taxonomy (e.g. (Dumbrell et al. 2010; Dumbrell et al. 2011; Horn et al. 2014; Schlaeppi et al. 2016). However, as only 30% of the currently described 250 morphospecies have been sequenced and archived into public databases (Öpik et al. 2014), many OTUs fail to be identified using this approach. Additional variability may be introduced by the choice of database. The most comprehensive 18S database options for AM fungal researchers include the Glomeromycotan

specific MaarjAM (Öpik et al. 2014) and more general, SILVA (Quast et al. 2013). Due to inherent differences among databases in accessions, curation, and the organization of sequences into OTUs, the choice of database may affect taxon assignments, but this has not yet been tested.

In this study I examined how well Illumina MiSeq and associated bioinformatic analyses represent AM fungal communities. Specifically, I used mock AM fungal communities of known abundance and identity to assess 1) the fidelity and repeatability of Illumina MiSeq sequencing related to AM fungal species richness and relative abundance 2) the extent to which sequences vary within an individual isolate 3) the optimum threshold for filtering rare taxa from the dataset, and 4) the accuracy of taxon assignments (MaarjAM and SILVA) for identifying Glomeromycotan fungi.

2.2 Materials and Methods

2.2.1 AM fungal mock communities

Four mock community types were created (Table 2.1): the "even" community consisted of equal representation of all 16 morphospecies, the "Glomeraceae dominant" and "Glomeraceae less-dominant" communities were created as they represent typical AM fungal communities (e.g. Öpik et al. 2013), and the "Gigasporaceae dominant" community was included to assess the sensitivity of NGS when the dominance was switched from Glomeraceae to Gigasporaceae.

To construct mock communities, spores were extracted from single species cultures purchased from INVAM (www.invam.wvu.edu) using sucrose centrifugation (Brundrett et al. 1996). Spores were handpicked using Pasteur pipettes (70-1000 spores depending on the

size and availability), sonicated for 10 seconds to remove surface debris, rinsed twice in distilled water, and then crushed with a mini pestle. DNA was isolated and purified using the PowerPlant™ DNA isolation kit (MoBio Laboratories, USA). An initial polymerase chain reaction (PCR) was carried out with the general AM fungal primer AML1 (Lee et al. 2008) and the universal eukaryotic primer NS4 (White et al. 1990) that flank the target region, a 550 bp region in the small subunit (SSU) ribosomal RNA (rRNA) gene. Reaction conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final elongation for 10 min at 72°C. PCRs were carried out in 25µL reaction volumes containing 2µL of purified DNA template, 20pmol of each primer in 1 GoTaq® Green Master Mix [(Green GoTaq® Reaction Buffer, 200µM dATP, 200µM dGTP, 200µM dCTP, 200µMdTTP and 1.5mM $MgCl_2$) Promega, USA].

To confirm the presence of a single, ~1000 base pair (bp) PCR product, all reactions were analyzed by 1.5% agarose gel electrophoresis using a 100-1000 bp DNA ladder (O'GeneRuler DNA Ladder, Thermo Scientific, USA) as a size standard. PCR products were then purified with magnetic beads (Agencourt AMPure, Beckman Coulter Genomics, USA). DNA concentrations were then diluted to a final concentration of 2ng/µL, and dilutions were used to compose mock communities with compositions as described in Table 2.1. Each mock community was replicated three times.

Because the first PCR amplified a product outside the binding sites of the target primers, the inherent sequence variation of subsequent primer binding sites should have remained intact. This first PCR also ensured that relative abundance of reads would not be due to differences in gene copy numbers among morphospecies, as mock communities were

generated based on DNA concentrations measured on PCR products.

To test for bias due to primer efficiency and selectivity, I conducted an *in silico* analysis on the amplicon library primers, WANDA (Dumbrell *et al.*, 2011) and AML2 (Lee et al. 2008), on a subset of SSU rRNA sequences from MaarjAM that spanned the entire Glomeromycotan phylum using FastPCR (Kalendar et al. 2014). For primer efficiency I measured binding site complementarity (% complementarity) as well as annealing/melting point (Tm). For selectivity I measured the number of binding sites. These analyses showed that primer efficiency and primer selectivity did not differ among families, or among species within families (Table A.1). Thus, the primers chosen for this study should not contribute to any bias observed.

2.2.2 Amplification of single spores

To assess the extent of intra-isolate sequence variation, 16 individual spores of *Rhizophagus irregularis* (DAOM 197198) obtained from Premier Tech Agriculture (Premier Tech Ltd., Rivière-du-Loup, QC, Canada) were amplified and sequenced. Single spores were crushed with a pipette tip in the bottom of a microcentrifuge tube under a stereoscope to ensure that the spore wall had ruptured. Each ruptured spore was placed in 1µL of nuclease free water in an Eppendorf tube and amplified in 25 µl containing 5x GoTaq buffer, 25mM MgCl2, 20 mg/ml BSA, 10mM dNTP mix, 10µM NS31 (Simon et al. 1992), and 10µM AML2 (Lee et al. 2008). Thermocycler conditions were: initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, followed by final elongation at 72 °C for 10 min, using a Bio Rad C1000 thermo cycler. PCR products were examined by electrophoresis.

Table 2.1 Relative proportion (based on volume of standardized DNA concentrations) of morphospecies used in the four mock communities. All spores were purchased from INVAM (invam.wvu.edu) and extracted using sucrose centrifugation followed by DNA extraction and PCR amplification prior to mixing. Values in table are microliters (µl) of standardized DNA concentrations from each isolate.

Family	**Taxon**	**INVAM Isolate**	**Even**	**Glomeraceae dominant**	**Less dominant Glomeraceae**	**Gigasporaceae dominant**
Gigasporaceae	*Gigaspora gigantea*	NC150	1	1	1	10
	Gigaspora albida	CL151	1	1	1	10
	Gigaspora margarita	NC195	1	1	1	10
	Scutellospora calospora	MN414C	1	1	1	10
	Dentiscutata heterogama	IL203A	1	1	1	10
Claroideoglomeraceae	*Claroideoglomus etunicatum*	MT 109	1	4	4	4
	Claroideoglomus claroideum	UT 143B	1	4	4	4
Glomeraceae	*Rhizophagus sinuosum*	MD 126	1	50	10	1
	Rhizophagus intraradices	IN 220	1	50	10	1
	Funneliformis coronatum	MR 101	1	50	10	1
	Funneliformis mosseae	CA127	1	50	10	1
Acaulosporaceae	*Acaulospora colombiana*	BR100	1	10	5	5
	Acaulospora koskei	SC 705	1	10	5	5
Ambisporaceae	*Ambispora gerdemannii*	MT 106	1	1	1	1
	Ambispora leptoticha	IN218	1	1	1	1
Paraglomeraceae	*Paraglomus brasilianum*	FR120	1	3	3	3

2.2.3 Illumina library preparation for mock communities and single spores

I used a two-step PCR protocol to generate amplicon libraries. For PCR1, I targeted amplicons spanning ~550 bp of the informative region of AMF SSU rRNA (Lee et al. 2008). I used the Glomeromycota specific primer AML2 (Lee et al. 2008) as my reverse primer as it amplifies SSU rRNA gene sequences from each AMF phylogenetic group except for *Archaeospora trapeii* (Lee et al. 2008). For my forward target primer, I used the universal eukaryotic primer WANDA (SI from Dumbrell et al. 2011). WANDA is located 23 bp downstream from the start of NS31, bringing the informative region of AMF SSU rRNA closer to the start of each amplicon while shortening the target amplicon length (Dumbrell et al. 2011). Combined, WANDA and AML2 generates amplicons of ~550 bp in length. I chose the SSU region as it has been argued to have good resolution among species (with a couple of exceptions) and has support of an expertly curated database (Öpik et al. 2010). To date no other region of AMF rRNA is as well supported as the SSU.

Because low sequence diversity can be problematic on the Illumina MiSeq platform, I increased sequence diversity of my amplicon libraries by inserting a heterogeneity spacer region (0-6 nucleotides) between target primers and tags in our target primer complex for PCR1 (Fadrosh et al. 2014; yellow in Figure A.1). This generated seven unique forward and seven unique reverse target primer complexes, which were mixed and used for PCR reactions. External to the heterogeneity spacer region, 22 bp Fluidigm universal tags (Fluidigm Inc. San Francisco, CA, USA) were added, allowing adhesion of PCR2 primers to amplicons produced by PCR1 reactions. The universal tag CS1 was added to the forward primer complex, and the universal tag CS2 was added to the reverse primer complex (Figure A.1). Reactions were carried out in 50 µL volumes with 1 µL of DNA template, 5x GoTaq Buffer, 25 mM $MgCl_2$, 20 mg/ml BSA, 10mM

dNTP mix, 10 µM forward primer, and 10 µM reverse primer. Thermocycler conditions were: 95°C for 2 min; 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min; and 72°C for 10 min, on a Bio Rad C1000 thermo cycler.

In my PCR2 reaction, I flanked amplicons generated from PCR1 with barcodes and Illumina flowcell adapters. PCR2 primer complexes consisted of the same Fluidigm tags (CS1 or CS2) as PCR1, 8 bp Illumina Nextera barcodes (Illumina Inc., San Diego, CA, USA), and Illumina adapters (Figure A.1). CS1 and CS2 tags allowed for adhesion of PCR2 primer complexes to PCR1 amplicons. The advantage of adding barcodes and adapters in PCR2 is that they can be used in combination with other PCR 1 primers (as long as they have the CS1 and CS2), which significantly reduces the number of barcoded primers needed when targeting multiple organisms or regions. External to tags, 8 bp Illumina Nextera barcodes (Illumina Inc., San Diego, CA, USA) were added. To differentiate samples during sequence processing, and to further reduce the number of barcoded primers needed, I multiplexed unique forward and reverse barcodes, creating unique barcode combinations for each sample. External to tags, and barcodes were the flow cell binding site adapters (P5 and P7 in Figure A.1). P5 and P7 allow amplicons to attach to the Illumina MiSeq flow cell surface by annealing to complimentary oligos present on the flowcell. Amplicons generated during PCR1 were diluted 15 fold to be used as PCR2 template. PCR2 was carried out in 20 µL volume with 1 µL of diluted PCR1 product, 5x GoTaq Buffer, 25 mM MgCl2, 20 mg/ml BSA, 10mM dNTP mix, 2 µM forward primer, and 2 µM reverse primer. Thermocycler conditions were: 95°C for 1 min; 10 cycles of 95°C for 30 sec, 60°C for 30 sec, 68°C for 1 min; and 68°C for 5 min, on a Bio Rad C1000 thermo cycler. Products from PCR2 reactions were used as amplicon libraries for sequencing.

2.2.4 Sequencing

Sequencing of mock communities and individual spore amplicons was done at the Institute for Bioinformatics and Evolutionary Studies (iBEST) genomics resources core at the University of Idaho (http://www.ibest.uidaho.edu/; Moscow, ID, USA). Mock communities and spores were sequenced using two separate sequencing runs. Amplicon libraries were sequenced using 2 x 300 paired-end (PE) reads using 300 cycles on an Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA, USA). This paired-end sequencing allowed us to sequence the entire region flanked by WANDA and AML2, generating amplicons 550 bp (+/- 5 bp) in length. I also sequenced a negative control (PCR sample containing no spore template) to identify potential contamination. No sequences were observed in the negative control. Sequencing runs generated four *fastq* files; forward barcode, forward read, reverse barcode, and reverse read.

2.2.5 Bioinformatics

All bioinformatic analyses were conducted using 'quantitative insights into microbial ecology' (QIIME; Caporaso *et al.*, 2010). Forward and reverse barcode reads were combined using the *fastq-join* method in QIIME (Aronesty 2013) with a minimum 20 bp overlap. Primary quality filtering parameters recommended by Bokulich et al., (2013) were used during the 'splitting libraries' step. For mock communities; of the 2,652,423 reads that were joined, 102,411 did not contain a barcode present in the mapping file, 35,245 reads were too short after quality truncation (<420 bp), and 2,082,011 had one or more N characters. Those reads were excluded from further analyses. The remaining 432,756 sequences, ranging 7,370-24,622 sequences per sample, were checked for chimeras using usearch in QIIME (Edgar et al. 2011). After removing 5,483 sequences identified as chimeras, 427,273 sequences remained for downstream analysis. I applied the same quality filtering on 16 single spore samples, which resulted in 1,339-2,477

sequences per sample. After primary quality filtering one spore sample had only 2 sequences remaining and was excluded from downstream analyses. All paired-end sequences were sorted into OTUs using a cut-off threshold of 97% and the UCLUST algorithm (Edgar 2010), with the cluster seed being the representative sequence for each OTU cluster.

I used Sanger sequencing to obtain high quality reference sequences of the 16 mock community morphospecies to build the custom reference sequence database. Sequencing was done on PCR products amplified from individual isolates in mock communities using WANDA and AML2 (using the same PCR conditions as before) by the Fragment Analysis and DNA Sequencing Service (FADSS) at the University of British Columbia, Okanagan Campus (Kelowna, BC, CA). For each isolate, a single Sanger sequence trace file was generated. Sequence trace files were then viewed and edited using Sequence Scanner^{TM} Software 2 (Applied Biosystems©). For the majority, sequences contained high quality base calls, (quality value (QV) score >20). For base calls with QV <20, nucleotide selection was conducted manually by choosing the nucleotide with the highest peak. Regions of consistent low QV base calls present at the beginning and end of trace sequences were trimmed, resulting in final edited sequences ranging in 467-514 bp (mean 503 bp, SD 13) in size. Trimmed and edited sequences were then used as reference sequences to assign taxonomy. I used the BLAST algorithm (Altschul et al. 1990) to assign taxonomy to the OTU representative sequences against the custom reference database using an expected value (e-value) $\leq$ 1 e-9 as a minimal threshold for a match.

To determine the abundance threshold for rare OTUs, I tested relative abundance thresholds for excluding rare OTUs. I used the same method as outlined in Bokulich *et al.* (2013) and began trimming OTUs that contained $\leq$0.0001% of the total number of sequences in the dataset, and

then increased the abundance threshold in regular increments until 0.05%. At each threshold, I counted the number of observed OTUs, as well as matched OTUs with my custom database and counted the number of unique morphotypes that were identified. I chose the 0.01% threshold as it was the most stringent threshold where all OTUs matched with the16 morphospecies used in the created custom database. More stringent values resulted in loss of matches with morphospecies and less stringent values corresponded with an exponential increase in OTUs (Figure 2.1). This threshold value is on par with what has been suggested for other organisms (Degnan and Ochman 2012) and was also used for *R. irregularis* spore sequences. Overall, this threshold resulted in 11,440-22,576 sequences per mock community for taxonomic assignments

To assess accuracy of identification using databases available to all AM fungal researchers, I used BLAST to assign representative OTU sequences against the most recent downloadable version of MaarjAM (Öpik *et al.*, 2010; http://maarjam.botany.ut.ee/ accessed April 2015) and SILVA (Quast *et al.*, 2013; accessed March 2016). I used the same BLAST parameters as with the custom reference database to assign OTUs to virtual taxa (VT) in MaarjAM and sequence types in SILVA.

To determine how spore OTUs relate phylogenetically, I built a tree that included *R.irregularis* spore OTUs that remained after removing low abundance OTUs, the Sanger sequence of the morphospecies *R.intraradices* from the mock communities, a representative sequence of *R.irregularis* (DAOM197198) from GenBank, and VTX00105 (labelled as *Glomus intraradices* in MaarjAM). Sequences were first aligned using the multiple sequence comparison by log-expectation alignment algorithm (MUSCLE; Edgar, 2004), where evolutionary divergence is determined by constructing a global alignment of the pair of sequences and

determining the fractional identity. I then used the randomized axelerated maximum likelihood (RAxML; Stamatakis, 2006) to assemble a maximum-likelihood tree. I calculated 1000 bootstrap trees, and applied a maximum likelihood model to determine evolutionary relationships among sequences (Stamatakis et al. 2005). I visualized the tree, and abundances of OTUs within each spore sample, using the Phyloseq package (version 1.14.0) in R (McMurdie and Holmes 2013).

2.3 Statistical analyses

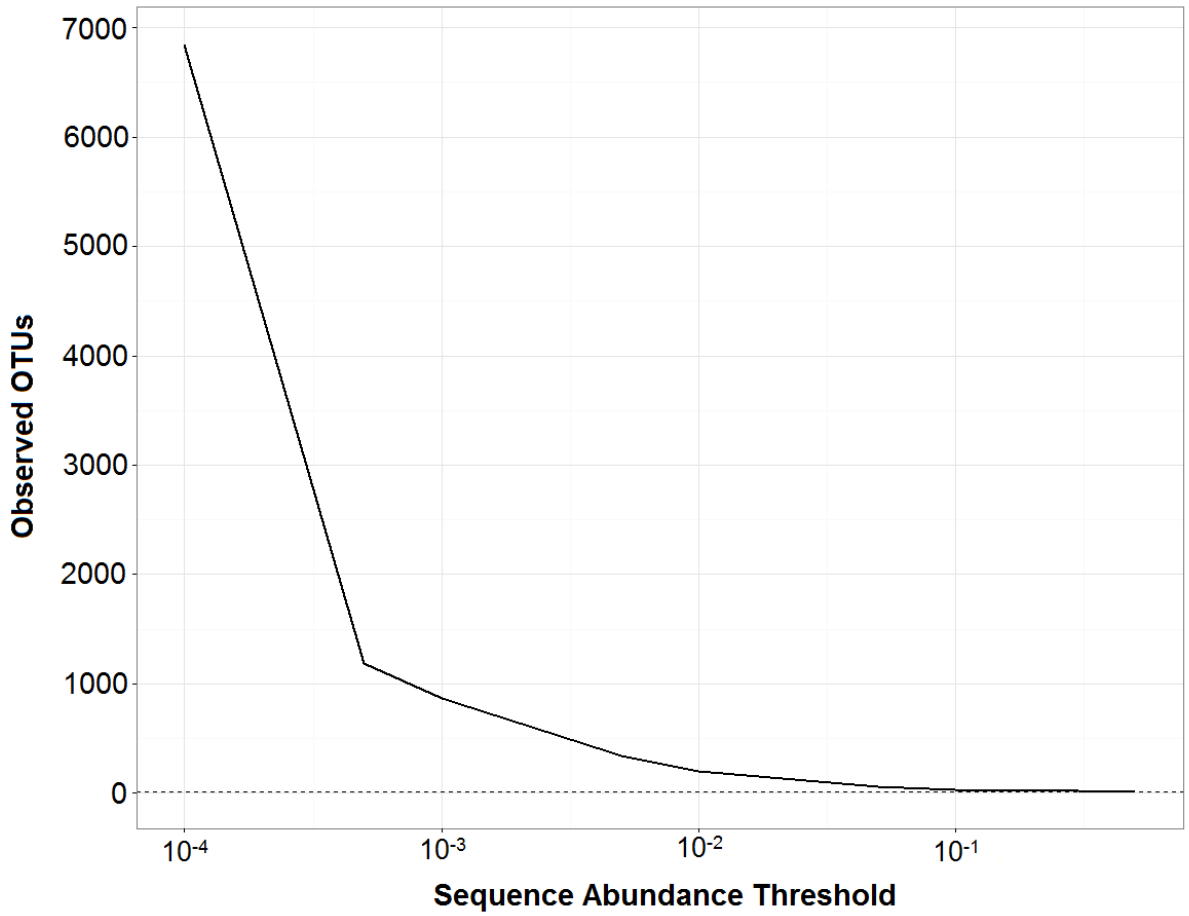
After mock community sequences were assigned to morphospecies (custom reference database), VTs (MaarjAM), or unique sequence types (SILVA), I generated three separate taxon by sample tables where samples included three replicates, each of the four mock communities. All tables were subsampled to 8000 sequences per sample, as OTU accumulation curves plateaued at this sequence depth (Figure A.3).

I used Pearson's χ^2 goodness of fit test (Chernoff and Lehmann 1954) to assess overall differences in expected and observed relative abundance of sequences when they were examined at the level of family, genus and species. To determine if biases were associated with specific families, genera and species, I used standardized residual values. Standardized residual values were calculated by determining the difference between the observed and expected proportion for a family in a sample, and then dividing that value by the square root of the estimated standard error (Agresti 2002). Standardized residuals measure the strength of differences between observed and expected values and are approximately normally distributed, with a mean of 0 and standard deviation of 1. Therefore standardized residuals +/- 1.96 from expected indicates a significance at an alpha of 0.05, and values +/- 2.58 indicate a significance at an alpha of 0.01.

Finally, I assessed if the taxon assignment method (custom reference database, MaarjAM, Silva) affected community composition of the mock communities based on Bray-Curtis

dissimilarities in PCoA space (Bray and Curtis 1957). Statistically significant differences among mock communities were evaluated within each method of taxonomic assignment using permutational multivariate analysis of variance using distance matrices (PERMANOVA; Anderson, 2001). PCoA plots depicting community composition patterns for taxon assignment methods were compared using Procrustes analysis (Peres-Net and Jackson, 2001; Caporaso *et al.*, 2012) in the Vegan package in R (Oksanen et al. 2015).

a.)


7000
6000
5000
4000
3000
2000
1000
0
Observed OTUs
10-4
10-3
10-2
10-1
Sequence Abundance Threshold

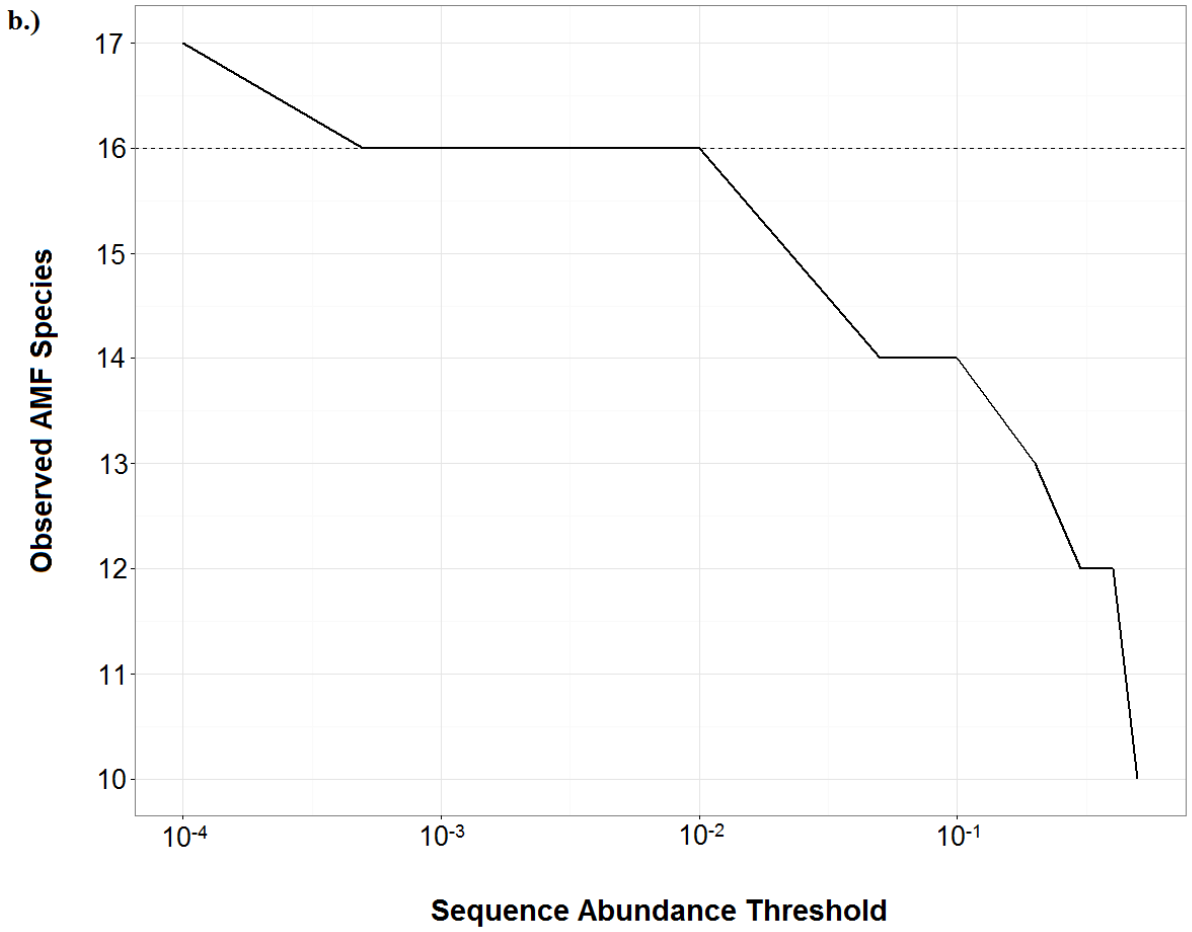


Figure 2.1 Observed taxa plotted against a series of sequence abundance thresholds. Taxa are represented as either OTUs clustered at 97% cluster similarity (a) or number of AM fungal species identified using our custom database (b). Solid lines represent observed number of OTUs/AMF species, dashed lines indicate the number of taxa I expected to observe based on how many isolates were used to construct our mock communities (16). Unidentified OTUs were classified taxonomically as 'unidentified' resulting in 17 AMF 'species' present at a sequence abundance threshold of 0.0001%.

2.4 Results

2.4.1 Fidelity and repeatability of Illumina MiSeq sequencing for AM fungi

Using the custom reference database, matches to Sanger sequences of all 16 morphospecies were recovered among mock communities sequenced using Illumina MiSeq. Sequences were clustered into 201 OTUs, with some morphospecies represented by a single OTU (*R. intraradices)*, whereas others comprised multiple (e.g. 43 *R. sinuosum)* OTUs (Figure A.3 and Table A.2). χ^2 tests revealed significant distortions in the depiction of AM fungal family relative abundances post-sequencing (Figure 2.2). Specifically, Ambisporaceae and Glomeraceae were less abundant than expected, whereas Claroideoglomeraceae and Paraglomeraceae were more abundant than expected (Figure 2.2 and table A.3a). For Acaulosporaceae and Gigasporaceae, the bias was dependent on the type of mock community; Acaulosporaceae appeared in significantly lower abundances than expected only in the Glomeraceae dominant community, whereas Gigasporaceae abundance was lower than expected only in the Gigasporaceae dominant community.

When I analysed the data using species-level taxonomy, differences between expected and observed communities became more pronounced (Table A.3b). For example, *Claroideoglomus claroideum* was under-represented whereas *C. etunicatum* was over-represented across mock communities. I observed the same pattern in Acaulosporaceae, where *Acaulospora colombiana* was over-represented and *A. koskei* was under-represented. In Gigasporaceae, *Gigaspora margarita* was over-represented while *Dentiscutata heterogama, Gigaspora albida,* and *Gigaspora gigantea* were under-represented. In Glomeraceae, *Funneliformis coronatum* was over-represented whereas the remaining species were under-represented. I observed high consistency among samples sourced from the same

mock community post sequencing. Samples clustered according to mock community post sequencing, and communities were shown to be significantly distinct according to Bray-Curtis dissimilarity (Figure 2.3, and table A.4). This consistency was supported by Chi^2 tests revealing no significant differences among samples from the same mock community at both the family and species level (Figure 2.2).

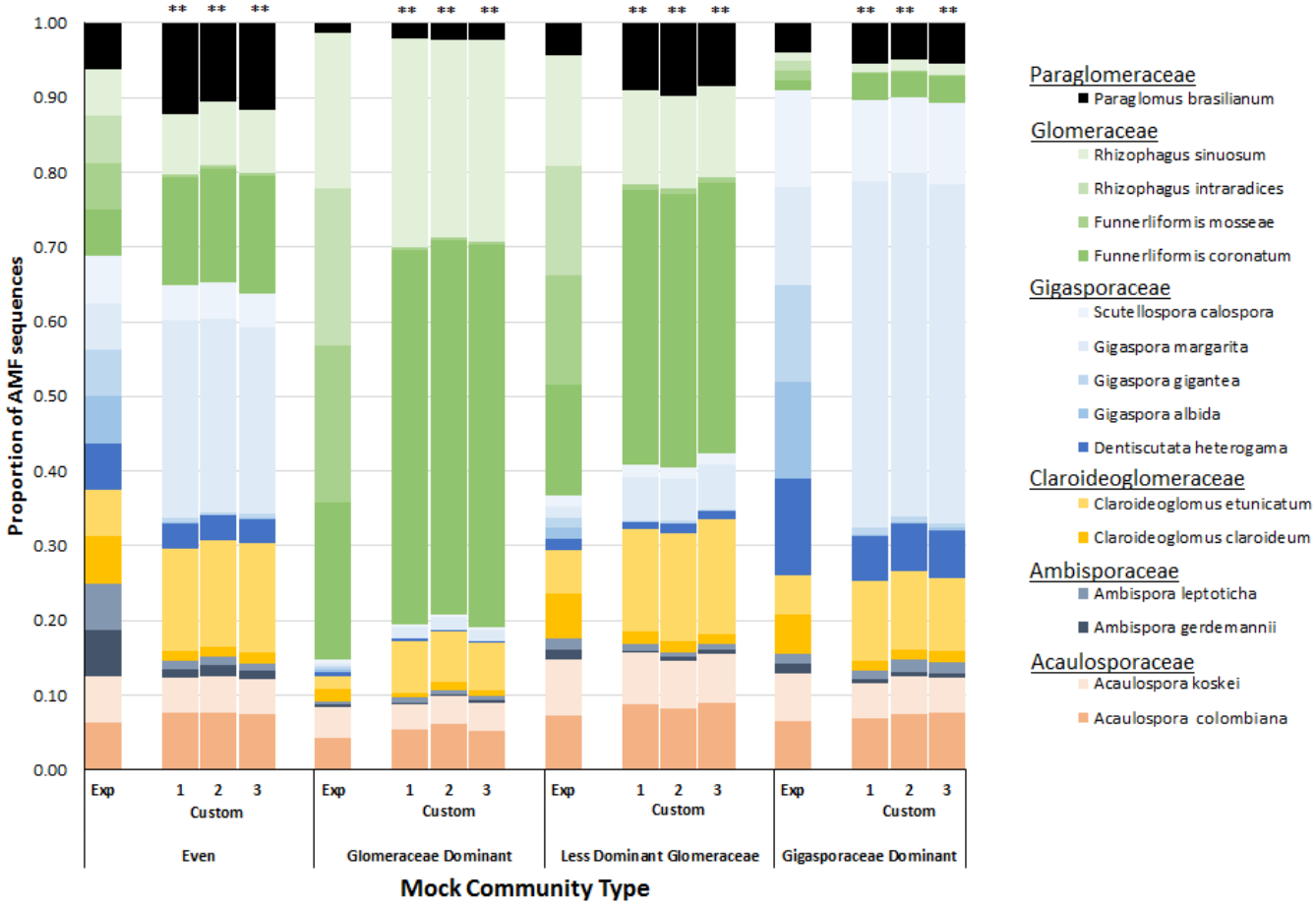


Figure 2.2 Stacked bar plot showing expected and observed sequence numbers of family and species across four mock community types and three replicates. Overall differences between observed and expected are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.001$). Comparisons among individual families and species are in Table S3a and S3b respectively. Replicates within mock communities did not differ significantly from each other.

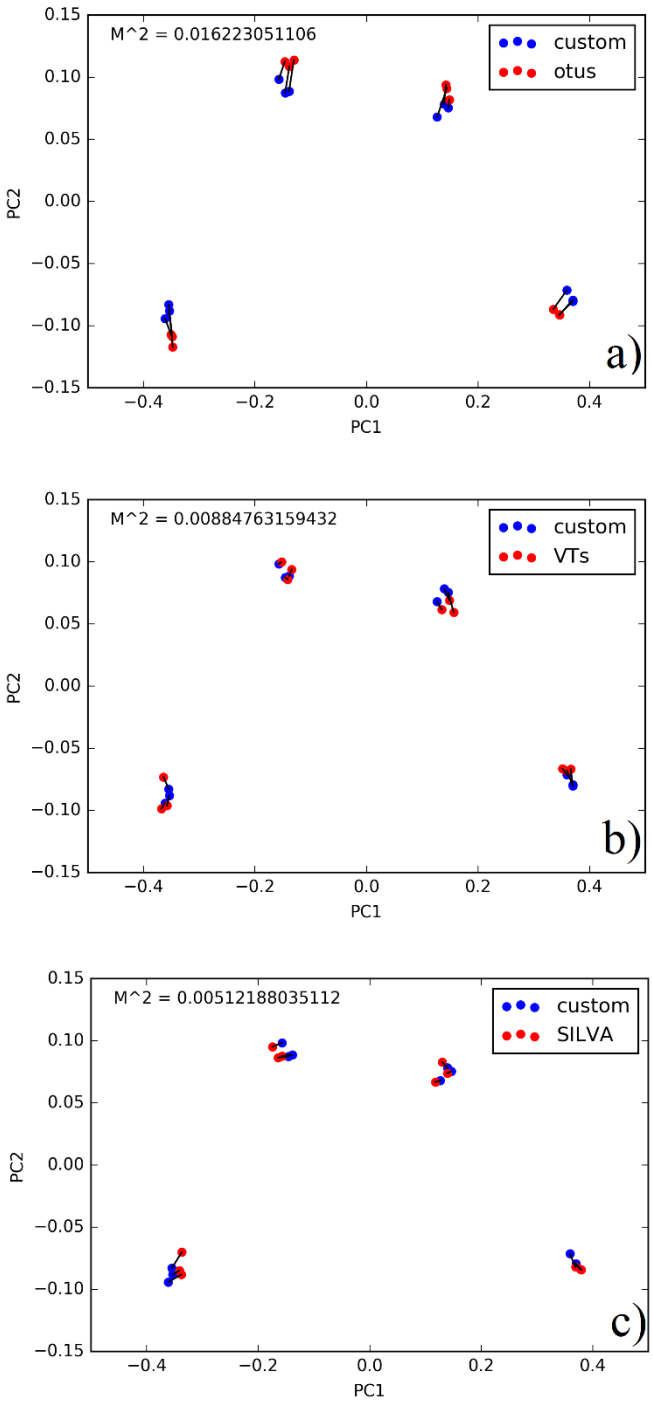


Figure 2.3 Procrustes analyses comparing taxonomic assignment approaches on the (1) Gigasporaceae dominant, (2) even, (3) less dominant Glomeraceae, and (4) Glomeraceae dominant communities. n=3 for all community types. Comparisons were made between a custom database comprised of Sanger Sequences from each of the morphotypes and OTUs (a), (b) VTs (from MaarjAM) (Öpik et al., 2010) and (c) SILVA (Quast *et al.*, 2013).

2.4.2 Sequence diversity among *R. irregularis* spores

Using a standard approach (97% similar OTUs) and discarding sequences occurring in lower abundances than 0.01% of all sequences, I obtained a total of 189 OTUs across all spores. Single spore libraries were represented by multiple OTUs, with a mean richness of 131 OTUs/spore (ranging between 98-151 OTUs). Of these, 41 OTUs matched with VTX00294 (Glomus sp.) in MaarjAM, while 7 were 100% similar to accessions in NCBI; four being *Rhizophagus irregulare* 181602, and three being unidentified *Glomus*. The remaining OTUs were uncategorized. Each sample contained many OTUs, but the same 4 OTUs dominated each sample, and rare OTUs were broadly distributed among spores (Figure 2.4).

2.4.3 Filtering threshold for low abundance OTUs

Varying the relative abundance threshold strongly influenced the number of OTUs and, to a lesser extent, matches with Sanger sequences of the 16 morphospecies (Figure 2.1). At the 0.01% minimum abundance threshold, all 16 morphospecies in the custom reference database were represented in the dataset. A more stringent minimum threshold resulted in a loss of morphospecies, while less stringent thresholds corresponded with an exponential increase in OTUs (Figure 2.1). Overall, this threshold resulted in 11,440-22,576 sequences for mock community samples, and 1332 – 2451 sequences per spore.

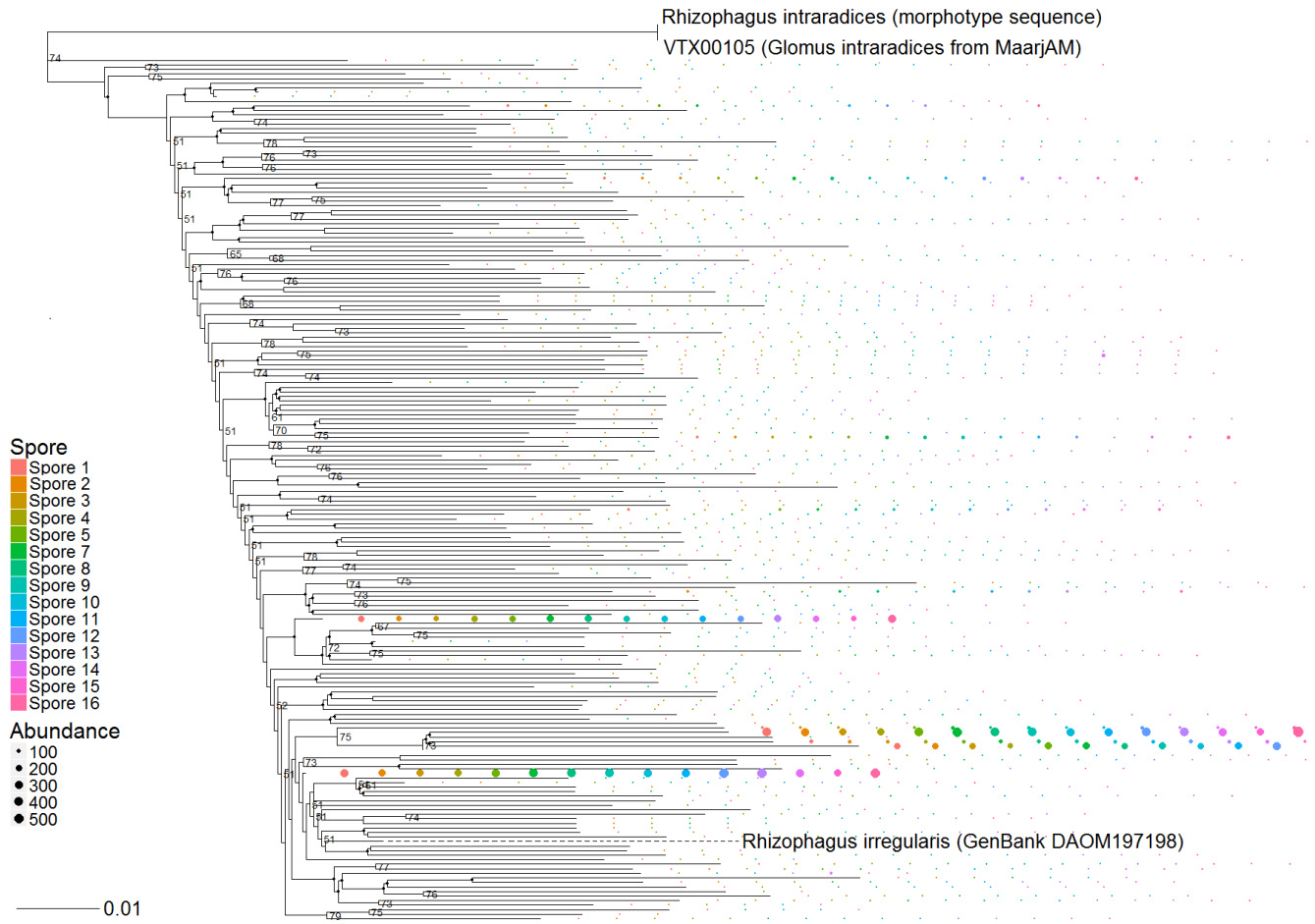


Figure 2.4 Maximum likelihood phylogenetic tree of 189 OTUs (represented by cluster centroid) obtained from amplifying and sequencing SSU rRNA from 16 *Rhizophagus irregularis* DAOM 197198 spores with Illumina MiSeq. Tree is complemented with a reference *R. irregularis* DAOM 197198 sequence from GenBank (Benson et al., 2005), and *Glomus/Rhizophagus intraradices* sequences for an outgroup (VTX00105 from MaarjAM (Öpik et al., 2010) and a Sanger sequences of the *Rhizophagus intraradices* morphotype used in the mock communities).

2.4.4 Accuracy of taxonomic assignment for identifying Glomeromycotan fungi

From an initial species richness of 16 morphospecies, the resulting 201 OTUs were matched with 28 VTs (MaarjAM) and 28 unique sequence types (SILVA) (Table A.5). Overall, family and genus level identifications were in agreement when using the custom database and MaarjAM as a reference, except for *Ambiopora* that identified as *Paraglomus* for 59% of the OTUs. The accuracy in species level identification varied among morphospecies. For example, when assigning taxonomy to OTUs using MaarjAM, assignments were in high agreement with the custom database for some species such as *Dentiscutata heterogama* (77%), *Scutellospora calospora* (77%), *Rhizophagus sinuosum* (91%), *Paraglomus brasilianum* (79%) and *Funneliformis mosseae* (83%), but not with species of *Gigaspora* and *Claroideoglomus* (Table A.4). Taxonomic assignment using SILVA was considerably less accurate as 1/3 of all sequence types were identified as non-Glomeromycotan, which was never the case with MaarjAM (Table A.5).

2.5 Discussion

In this study I assessed the ability of Illumina MiSeq amplicon sequencing and associated bioinformatic analyses to differentiate and correctly report on AM fungal species' identity and relative abundance. Overall, I found that the method used to delineate taxa resulted in disparate estimates of species richness, because the 16 morphospecies (spore morphology) separated into 201 OTUs (using a universal sequence-similarity threshold), which in turn matched with 28 VTs and unique sequence types using public databases. Further, there were differences in taxonomic assignment, with MaarjAM returning the most similar matches to original taxonomy. However, regardless of how sequences were organized and named,

repeatability within mock community was high and different mock community types were clearly distinguishable from each other.

2.5.1 Did Illumina MiSeq recreate expected AM fungal communities?

Using this approach, I was able to recapture all AM fungal taxa included in the mock communities, and broad differences among mock communities in relative abundances of AM fungal species were maintained (Figures. 2.2 and 2.3). This indicates that Illumina MiSeq can inform on quantitative differences within and among AM fungal communities, similar to what has been shown for other microorganisms (Bokulich et al. 2013; Nelson et al. 2014). However, while I observed clear separation among mock communities (Figure. 2.3, and Table A.4), some bias was apparent, even among families. For example, Paraglomeraceae sequences were more abundant than expected, whereas I found the opposite for sequences in the Ambisporaceae. Differences in observed and expected abundances of species are not uncommon in microbial studies using NGS (Degnan and Ochman 2012; Bokulich et al. 2013; Egge et al. 2013; Nelson et al. 2014) and can result from biases introduced during PCR amplification (Aird *et al.*, 2011; Nelson *et al.*, 2014; Brooks *et al.*, 2015), sequencing (Schirmer et al. 2015) and bioinformatic analyses (Bokulich et al. 2013). In this study, bias should not have been introduced during amplification, because I started with PCR products, and *in silico* analyses indicated no difference in primer affinity among AM fungal taxa (Table A.1). This may not apply to field samples amplified by other primers, however, as AM fungal taxa can differ in gene copy numbers (Sanders et al., 1995; Jansa *et al.*, 2008) and some primers can exhibit a strong bias toward Glomeraceae (Kohout et al. 2014) and/or fail to distinguish among taxa in the Gigasporaceae, Diversisporaceae, and

Claroideoglomeraceae (Öpik et al. 2013; Öpik et al. 2014). While barcodes can introduce bias by promoting template sequence-dependent selective amplification (Berry et al. 2011), I observed similar biases among communities receiving different barcodes, so this was unlikely a problem. Thus, while I cannot exclude potential biases prior to the bioinformatic analyses, I suspect that differences between expected and observed abundances were largely introduced during bioinformatic processing of sequences, OTU clustering (Schloss and Westcott 2011) and/or taxonomic assignments using the custom database. This may, to a large extent stem from the low resolution among some AM fungal species in the SSU region (Krüger et al. 2009). For example, all *Gigaspora* species match with a single VT in MaarjAM despite significant morphological and genetic differences among species (Bentivenga and Morton 1995; de Souza et al. 2004). Thus, my inability to separate these morphospecies is not unique to Illumina sequencing and associated bioinformatic analyses, but applies to all molecular approaches targeting this region. Currently, there is no marker for NGS that faithfully recapitulates known morphospecies taxon assignment. This is likely due to a combination of lack of sequences associated with morphospecies, and inherent inadequacies of marker regions (Hart et al. 2015).

2.5.2 Sequence variations within morphospecies and individual spores

Even when employing stringent OTU filtering thresholds, there was substantial sequence variation within morphospecies. High genetic diversity within species has been documented previously and may result from long-term asexual evolution (Sanders, 2002; Munkvold *et al.*, 2004; Koch *et al.*, 2006; Rosendahl, 2008; Stockinger *et al.*, 2009; Ropars et al., 2016), but how it is organized among and within AM fungal isolates is not resolved (Young 2015).

Large variations were also documented within single spores, with OTU numbers ranging from 98 to 151 (Figure 2.4). Some OTUs were rare, but consistently present in most spores, which give us more confidence that they represent real sequence variation and were not the result of sequencing errors. The results presented in this study support a growing body of literature that suggests that genetic variation can occur both within (Pawlowska and Taylor 2004) and between (Kuhn et al. 2001; Hijri and Sanders 2005) AM fungal spores. Due to the potential substantial genetic divergence within AM fungal isolates (Sanders 2002; Boon et al. 2015; Ropars and Corradi 2015) and because single populations can exhibit different genotypes and phenotypes (Koch et al. 2006), it is important to understand the 'ecologically relevant' level of genetic diversity of AM fungi in natural systems.

Given the potential for large intra-isolate variation, strict adherence to universal thresholds of species identity may over-estimate alpha diversity and may instead reflect sequencing variation within individuals. This may be magnified by clustering algorithm choice, as they can return different estimates of OTU richness (Kopylova et al. 2016). In this study, I chose UCLUST to cluster sequences as other methods have been shown to underestimate OTU richness (Kopylova et al. 2016). I encourage researchers to be aware of drawbacks of available clustering methods, and recognize these during analysis and interpretation of their data.

2.5.3 Filtering thresholds for low abundance OTUs

Quality filtering of Illumina reads is essential to retrieve reliable estimates of richness and taxonomic distribution within communities (Bokulich et al. 2013), but there is no clear guidance regarding appropriate threshold. Too liberal of a threshold may fail to remove

artefactual OTUs, whereas too stringent criteria may result in a loss of real sequence diversity. For example, when I increased the abundance threshold until only 16 OTUs were observed (i.e. 0.4%), I only recovered 12 of the morphospecies, whereas OTUs increase exponentially at a threshold <0.005% with most not matching any of the custom database sequences (Figure 2.1). At the 0.01% threshold (my chosen threshold for the bioinformatics analyses in this study), I was able to recover all morphospecies, and OTU numbers were not yet increasing exponentially. Importantly, this involved removing OTUs that contained <43 sequences, which goes far beyond the standard procedure of removing singletons. While this may have inadvertently removed some real sequence diversity, I recommend using more conservative filtering thresholds than what is currently done to reduce the risk of erroneously inflating alpha diversity. Similar suggestions have also been made for other organisms (Degnan & Ochman, 2012; Bokulich et al., 2013).

2.5.4 Taxonomic assignments

Based on the results presented herein, direct comparisons of alpha diversity among studies using different (molecular) species identification approaches will be difficult. Using highly curated databases, which cluster like-sequences into taxonomic units (i.e. VTs in MaarjAM) can help to mitigate some of these differences. In addition, phylogeny-based approaches, where sequences are grouped into clades, rather than taxonomic groups (Ross et al. 2008; Horn et al. 2014) can result in more reliable identification and transcend studies more easily (Lindahl et al. 2013, Hart et al. 2015).

While family level assignments were robust in my analysis, species identity varied across morphospecies and was often incorrect (Table A.5). This indicates that comparisons of

species across studies should be treated with caution. My results also showed that available databases were not equally robust at correctly identifying sequence types. For example, 1/3 of sequence types were reported as non-Glomeromycotan in SILVA. Thus, it is important to either verify sequence identity through alignments with existing phylogenies, or using expert curated databases (e.g. MaarjAM).

One main finding from this study is that regardless of how sequences were organized (OTUs, VTs), the observed communities did not cluster separately from mock communities that were assigned taxonomy using the custom database generated specifically for this study (Figure 2.3). Also, the different mock community types clearly separated from each other regardless of taxonomic assignment approach. Thus, our understanding of the organization of ecological communities may not depend on sequencing platform or bioinformatics, which supports previous findings (Powell et al. 2011; Lekberg et al. 2015). While fine scale information about community identity and absolute abundance remains technologically hindered, existing tools are capable of distinguishing among complex, and similar communities.

2.5.5 Recommendations

Based on the results herein, I can make three overall recommendations. First, due to the low variability among replicate mock communities, there may be little need to amplify and sequence the same sample multiple times. Instead, effort will be better spent by sequencing more samples. Second, I advocate for the inclusion of mock communities to guide the bioninformatic analyses, as have others before (Bokulich et al. 2013; Brooks et al. 2015; Nguyen et al. 2015), especially for determining filtering thresholds. In particular, the

standard method of removing singletons is likely inadequate to control for sequencing errors in most Illumina datasets. Third, given the substantial sequence variation observed within spores, and the known differences in gene copy numbers, let us use language that reflects what we measure; amplicon sequence data reflects gene abundance and diversity, which may or may not correlate with biomass and species diversity (Alkan et al. 2004; Vandenkoornhuyse et al. 2007; Jansa et al. 2008).

2.6 Summary

Results from this chapter show that NGS and associated bioinformatic analyses do not significantly distort the interpretation of AM fungal community composition, especially at higher taxonomic levels. However, this was not true when I considered communities at the level of species, which may be due to low sequence variability among closely related morphospecies. I also found that databases differ in their capacity to accurately identify taxa. Despite discrepancies at lower taxonomic levels, and discrepancies between databases when applying species names to sequences, different mock communities clearly separated from each other. Thus, community datasets generated from NGS approaches have the potential to inform on the organization of ecological communities. Because of this encouraging result, in chapters 3 and 4 of this thesis I apply NGS to characterize AM fungal communities in environmental samples. I then use sequencing results to examine different community assembly processes acting on AM fungal communities along an elevation gradient in the North American Rocky Mountains.

Chapter 3 Phylogenetic structure of arbuscular mycorrhizal fungal communities along an elevation gradient[2]

3.1 Background

Community composition of AM fungi has been the focus of many studies, from local (Dumbrell et al. 2010; Caruso et al. 2012; Davison et al. 2012; Horn et al. 2014) to global (Treseder and Cross 2006; Öpik et al. 2010; Kivlin et al. 2011; Moora et al. 2011; Öpik et al. 2013) scales. Studies focusing at the local scale have shown that assembly of AM fungal communities generally is driven by deterministic processes such as interactions with plant hosts (Klironomos 2003; Öpik et al. 2013), other AM fungi (Helgason et al. 1998), other fungal groups (Chilvers et al. 1987), bacteria (Artursson et al. 2006; Sabannavar and Lakshman 2011), soil fauna (Klironomos and Kendrick 1995; Klironomos and Ursic 1998), and local soil nutrients (Egerton-Warburton et al. 2007). Stochastic processes such as dispersal limitation also contribute to local AM fungal community assembly (Lekberg et al. 2007; Kivlin et al. 2011). While all of these factors are certainly important, their relative importance has been obscured by the majority of studies having taken place within a constrained set of conditions. Landscape-scale studies have revealed AM fungal communities may be primarily structured by local abiotic conditions rather than geographic distance (Hazard et al. 2013; Xiang et al. 2014; De Beenhouwer et al. 2015). However these regional-scale studies are largely underrepresented in the AM fungal literature. By studying patterns across environmental gradients we may be better able to identify determinants of community

[2] A version of Chapter 3 has been published. Egan, C.P., Callaway, R.M., Hart, M.M., Pither, J., Klironomos, J. (2016) Phylogenetic structure of arbuscular mycorrhizal fungal communities along an elevation gradient. Mycorrhiza doi:10.1007/s00572-016-0752-x

composition that do not emerge from locally-focused studies.

Elevation gradients are opportune systems for examining evolutionary and ecological processes that determine biodiversity. Plant and microbial communities have been shown to differ in composition along such gradients (Jankowski et al. 2009, Fierer et al. 2011, Kraft et al. 2011). In some cases, these changes are associated with changes in community phylogenetic structure (Bryant et al. 2008; Wang et al. 2012a). For example, Wang et al. (2012) observed biofilm bacterial communities to be more phylogenetically clustered (species are more phylogenetically related than expected by chance) at high elevations. They suggested that cooler water temperatures at higher elevations selected for closely-related species from phylogenetic clades that could tolerate lower temperatures. In other bacterial studies, there has been no observable change in phylogenetic structure with elevation. Bryant et al. (2008), in contrast to Wang et al. (2012), observed bacterial communities to be phylogenetically clustered throughout an elevation gradient. Again, they suggested that environmental filtering was the primary determinant of this structure, and that local abiotic conditions are unique at each habitat along the gradient. This also results in assemblages that are phylogenetically more closely related than expected by chance, an observation consistent with other microbial studies from a wide range of habitat types (Horner-Devine and Bohannan 2006).

Many studies have examined how AM fungal diversity changes along elevation gradients (Gai et al. 2012; Geml et al. 2014; Li et al. 2014; Liu et al. 2015 Altitudinal; Looby et al. 2016), but to date they have relied primarily on taxon-based approaches to examining diversity, omitting insights that phylogenetic relationships may provide when interpreting diversity patterns. This is unfortunate, as several studies show that AM fungal taxa have

phylogenetically conserved traits (Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009), and that the use of phylogenetic metrics to complement traditional taxonomic approaches in AM fungal community ecology is potentially powerful. For example, in a survey of DNA sequences available at GenBank sourced from 111 independent studies, Kivlin et al. (2011) reported that AM fungal communities were phylogenetically clustered in the majority of sites, and only two sites had communities that were phylogenetically dispersed (species being more distantly related than expected by chance). This indicates that habitat filtering or dispersal limitation may be the primary drivers of AM fungal community assembly.

At smaller spatial scales, AM fungal communities have been observed to be more phylogenetically clustered (Kivlin et al. 2011; Horn et al. 2014), indicating that closely related AM fungal taxa positively associate through biotic factors such as plant host selection and interactions with soil biota (Horn et al. 2014). Abiotic factors, such as atmospheric [CO_2] (Mueller and Bohannan 2015), and soil nutrients (Liu et al. 2015b) have also been linked to shifts in phylogenetic community structure. For example, AM fungal communities shift from being phylogenetically clustered under low nutrient conditions, to phylogenetically dispersed under high nutrient conditions, indicating that the primary ecological process structuring AM fungal communities can change along abiotic gradients (Liu et al. 2015b). Although interpretation of community phylogenetic structure is not always straightforward (Swenson et al. 2006; Swenson 2009; Mayfield and Levine 2010), studies examining AM fungal phylogenetic structure to date indicate that phylogenetic metrics can provide insight into deterministic factors that shape fungal community composition.

In this study I sampled along an elevation gradient in the North American Rocky

Mountains, and applied phylogenetic metrics to better understand how AM fungal communities may be structured with increasing elevation. I expected that AM fungal communities would be less phylogenetically diverse at high elevations (in the alpine), due to increased environmental filtering from stressful abiotic conditions (e.g. lower temperatures). I also expected that increased environmental filtering at high elevations would result in AM fungal communities that are more phylogenetically clustered compared to lower elevations. Third, I expected that local abiotic conditions would select unique AM fungal assemblages along the gradient, where communities from the more distant elevations (e.g. alpine compared with subalpine) would be more phylogenetically dissimilar.

3.2 Materials and Methods

3.2.1 Study site and sample collection

Soil samples were collected along a high elevation gradient at Glacier National Park, Montana, U.S.A. (48°28'N; 113°21'W). AM fungal communities were collected from the three major vegetation zones along the gradient; subalpine grassland, treeline, and alpine tundra. Plant communities were dominated by native grasses including *Festuca scabrella*, *F. idahoensis*, *Pseudoroegneria spicata*, herbaceous species including *Lupinus sericeus*, *Achillea millefolium*, *Dasiphora fruticosa*, and trees including *Abies lasiocarpa* and *Picea engelmannii*. At higher elevations both tree and shrub species developed a short stature, with no tree species found above 2064 meters above sea level (MASL). Above the treeline, plant communities occurred in clumped patches with large areas of bare soil present between plants. As *D. fruticosa* was dominant throughout all vegetation zones, and was easily identifiable, I used it to guide the limits of the elevation range sampled. I established my low

elevation site at the beginning of the subalpine grassland (1682 MASL), my mid elevation site at the treeline (2064 MASL) (which is the zone of transition between subalpine forest and alpine tundra), and my high elevation site well into the alpine tundra, above the treeline (at 2225 MASL).

To collect AM fungal communities, I established a 10 m x 10 m sampling plot within each site (subalpine grassland, treeline, and alpine). In each plot, 41 soil samples were collected in a predetermined sample pattern to control for environmental heterogeneity within elevations, using a soil core (5 cm diameter, 15 cm length) (Figure B.1). Nested within each plot, I established a smaller 1 x 1m nested plot, where samples were taken in the same pattern as the larger plot, where distances between samples ranged from 10 cm to 140 cm. Combined, the large plot and nested plot resulted in spatial distances between samples that range from 10 cm to 1414 cm. Within each plot 82 soil samples were collected, totaling 246 samples along the gradient. After collection, samples were placed in individual plastic bags and kept on ice until they were transferred to a -20˚C freezer (at the University of British Columbia, Okanagan Campus, Kelowna, BC, Canada), where they were stored until it was time for DNA extraction.

3.2.2 Molecular analyses

Roots and debris were removed from samples by passing soil through a 5 mm sieve, leaving fine soil only. Soil samples were then homogenized, and a 0.25 g subsample was used for DNA extraction (MoBio Laboratories Inc., Carlsbad, CA, USA). Amplicon libraries were generated for 454 sequencing by amplifying a fragment of the SSU region of the rRNA gene using the universal eukaryotic primer NS31 (Simon et al. 1992), and AM fungal

specific AM1 (Helgason et al. 1998). Forward primers consisted of the GS FLX – specific A-adaptor, a multiplex identifier (MID), and the sequencing primer NS31 (5'-CGTATCGCCTCCCTCGCGCCATCAG-(10 bp MID)-TTGGAGGGCAAGTCTGGTGCC-3'). Reverse primers consisted of AM1 only (5'-CGTATCGCCTCCCTCGCGCCATCAG-3'). Polymerase chain reactions (PCRs) were carried out in 25 µl volumes using; 1 µl of DNA template; 0.5 µl each of 2 mM dNTPs, 0.1 µM NS31, 0.1 µM AM1, 5 µL 5x GoTaq Buffer, 2 µL MgCl2, 1 µL BSA, and 0.25 µL GoTaq® DNA polymerase (Promega Biosciences LLC., San Luis Obispo, CA, USA). PCR conditions were: 95°C for 3 min; 44 cycles at 95°C for 0.5 min, 68.4°C for 0.5 min, and 72°C for 1 min; followed by 72°C for 10 min on a BioRad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Of the 246 samples collected, 132 samples failed to yield any PCR product, even after altering my amplification protocol (e.g., touchdown PCR, nested PCR, changing reagents, changing thermocycler conditions). As a result, these 132 samples were excluded from further analysis. Post-amplification PCR products from the 114 samples which did yield product were purified using QIA quick PCR Purification Kit (Qiagen Ltd. Crawley, UK) and were sent to the Vancouver Prostate Centre (http://www.prostatecentre.com/, Vancouver, BC, Canada), for 454-pyrosequencing using the GS-FLX platform (454 Life Sciences, a Roche company, Brandford, CT, USA).

3.2.3 Bioinformatics

Analysis of 454 sequencing reads was conducted using the open-source software QIIME (Caporaso et al. 2010). Post-sequencing, amplicon reads were de-noised and screened for chimeras using ampliconnoise (Quince et al. 2011), which removes 454 sequencing errors

and PCR single base errors, while using the Perseus chimera removal program to identify and remove chimeric sequences. After de-noising and chimera removal, sequences were screened for presence of the forward primer complex (A-adaptor, MID, and forward primer NS31), a quality score of ≥25, and sequence length of 400-700 bp. Sequences meeting these criteria were sorted into OTUs using the UCLUST algorithm (Edgar 2010). OTUs were grouped by a sequence similarity of 97%. Representative sequences were then selected for each OTU cluster, by choosing the most abundant sequence within each 97% similarity cluster.

OTUs were assigned taxonomy using the BLAST algorithm (Altschul et al. 1990) against type sequences downloaded from the MaarjAM database (Öpik et al. 2010; http://maarjam.botany.ut.ee/). Low abundance OTUs, appearing as single sequences were removed, as they may be false phylotypes generated during sequencing, which can overinflate diversity estimates in 454 datasets (Kunin et al. 2010). In addition, OTUs that were not identified taxonomically, or OTUs identified as non-Glomeromycota, were also excluded from further analysis. To control for differences in sequence reads generated for each sample, I sub-sampled to 3000 sequences per sample by rarefaction analysis, (Figure B.2). Excluding samples with fewer than 3000 sequencing reads resulted in 37 of the 114 sequenced samples remaining (subalpine n=11 (large sampling grid n=6, nested sampling grid n=5), treeline n=12 (large sampling grid n=6, nested sampling grid n=6), alpine n=14 (large sampling grid n=8, nested sampling grid n=6)). Identified OTUs and samples remaining after filtering were then used to generate an AM fungal VT by sample table, where VT abundances were represented by sequence reads.

3.2.4 Constructing the phylogenetic tree

I constructed a phylogenetic tree by first aligning representative OTUs using MUSCLE (Edgar 2004) in QIIME. Aligned sequences were then assembled into a maximum-likelihood tree using the randomized axelerated maximum likelihood algorithm (RAxML; Stamatakis 2006). One-thousand rapid bootstrap trees were built, and used to apply a maximum likelihood model to determine evolutionary relationships among sequences (Stamatakis et al. 2005). The tree was visualized and edited using FigTree (Rambaut 2012) resulting in a final phylogenetic tree (shown in Figure 3.1).

3.3 Statistical Analyses

3.3.1 Phylogenetic diversity (phylogenetic α-diversity)

To determine the phylogenetic diversity of AM fungal assemblages within samples (i.e. phylogenetic α-diversity), I used Faith's phylogenetic diversity (PD) metric (Faith 1992), using the Picante version 1.6-2 software package (Kembel et al. 2010). Due to violations of homogeneity, I used a non-parametric Kruskal-Wallis test (Kruskal and Wallis 1952) to determine significance of elevation on PD. Pairwise comparisons between elevations was done using Wilcoxon rank sum tests (Wilcoxon 1945) with Bonferroni corrections.

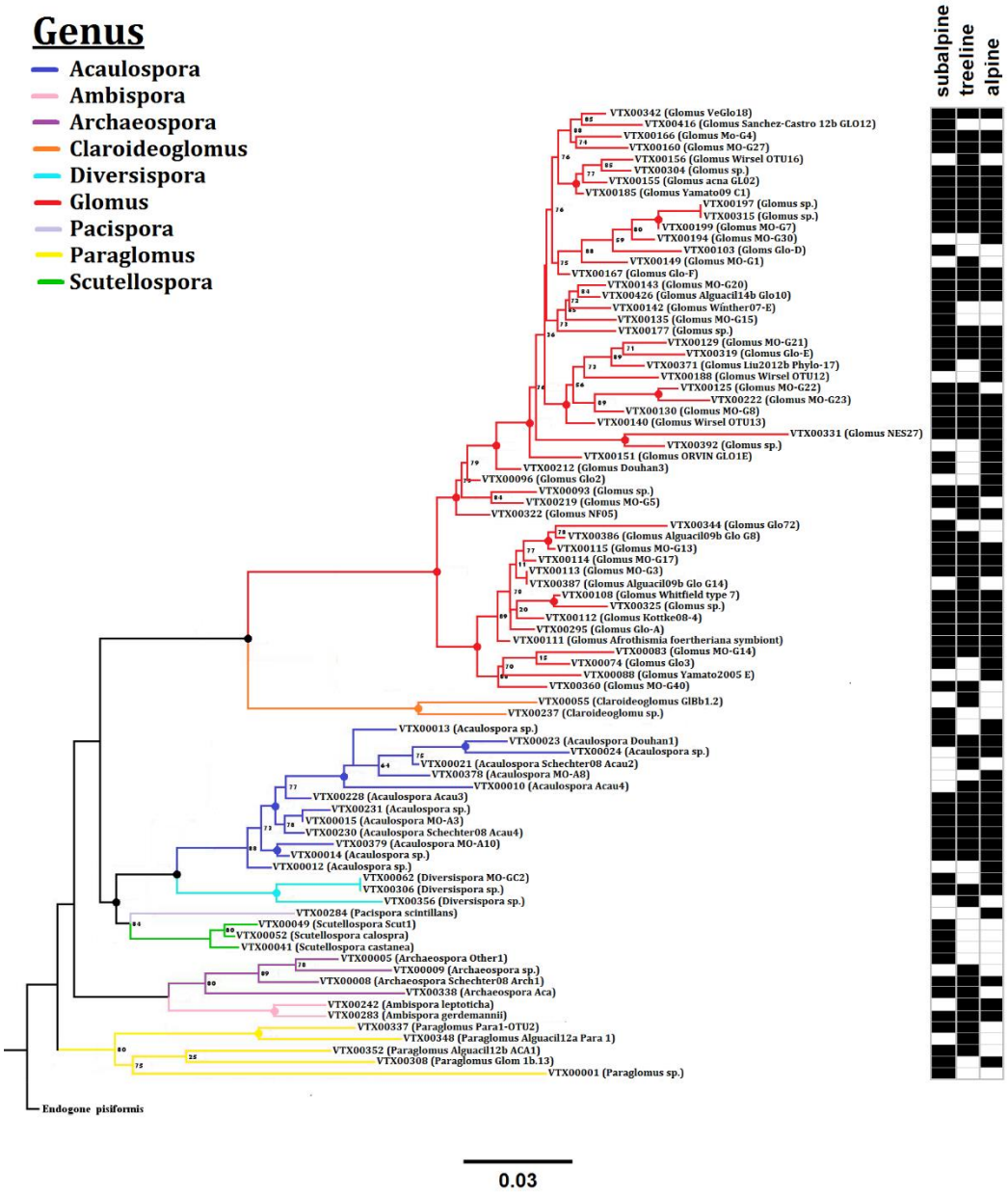


Figure 3.1 Maximum likelihood based phylogenetic tree and presence/absence matrix of 82 AM fungal virtual taxa (VT) found along the elevation gradient at Scenic Point, MT USA. The tree was built using randomized axelerated maximum likelihood (RAxML; Stamatakis et al., 2006) for sequential and parallel maximum likelihood-based inference of phylogenetic trees in QIIME. Node numbers represent Bootstrap values, node circles represent bootstrap values ≥ 90. Tips represent VT followed by AM fungal species names in brackets as they appear in the MaarjAM database. For the presence/absence matrix, rows represent AM fungal VT and columns represent elevation site. Subalpine n=11, treeline n=12, alpine n=14. Presence of an AM fungal VT is indicated by a dark square. Presence of an AM fungal VT at a site was determined by at least one sequence of that VT being present after bioinformatic filtering.

3.3.2 Phylogenetic structure within communities

Using null models I compared observed phylogenetic distances among species in samples to the distribution of phylogenetic distances generated in null communities (Kembel and Hubbell 2006), which allowed us to determine whether the assemblage of species within communities is non-random with respect to phylogeny (Webb et al. 2002a). Analyses of community phylogenetic structure were conducted using the Picante software package (Kembel et al. 2010). To examine phylogenetic structure within communities I used two metrics: mean pairwise phylogenetic distance (MPD) and mean nearest taxon distance (MNTD) (Webb et al. 2002). Each metric captures a different aspect of the phylogenetic relatedness of co-occurring species, where MPD is more sensitive to tree-wide patterns of phylogenetic structure, and MNTD is more sensitive to phylogenetic patterns of more recently evolved taxa (Webb 2000).

Three steps were taken to determine whether assemblages within communities were non-random with respect to phylogeny. First, I calculated observed phylogenetic distances among species occurring together in each sample using MPD and MNTD. MPD was calculated as the mean phylogenetic distance separating all pairwise combinations of species occurring within a sample, and MNTD was calculated as the mean phylogenetic distance to the nearest relative for all species occurring together in a sample (Kembel and Hubbell 2006). Next, I calculated the standardized effect size (SES) of observed MPD and MNTD distances using the "ses.mpd" and "ses.mntd" functions respectively in Picante (Kembel et al. 2010). Third, I compared SES values for each sample to null assemblages generated using 9999 randomizations of the "independentswap" null model in Picante. I chose this model as it is suited for communities that differ in species composition, accounting for variations in

diversity and richness (Gotelli 2000; Horn et al. 2014). When I compared SES values to null communities, values higher than zero indicated phylogenetic over-dispersion (species more distantly related than expected by chance), and values lower than zero indicated phylogenetic clustering (species more closely related than expected by chance) (Kembel 2009). Significant deviation of observed patterns from the null expectation of zero phylogenetic structure was determined using a two-tailed t test (Kembel 2009; Horn et al. 2014; Liu et al. 2015b) at a significance at $P < 0.05$. A Kruskal-Wallis analysis of variance by ranks test, followed by a Wilcoxon rank sum test were used to determine significance of elevation on phylogenetic structure (at $P < 0.05$).

3.3.3 Community composition (phylogenetic β-diversity)

I measured phylogenetic dissimilarity between samples (i.e. phylogenetic β-diversity) using unweighted and weighted UniFrac. Both phylogenetic β-diversity metrics were calculated using the "GUniFrac" function from the GUniFrac package (Chen 2015).

To evaluate the effect of elevation on phylogenetic community composition of AM fungi I used permutational multivariate analysis of variance using distance matrices (PERMANOVA; Anderson 2001). PERMANOVA was conducted using the adonis function from the Vegan version 2.3-3 software package (Oksanen et al. 2015). Pairwise comparisons between elevations were made using the "pairwise.perm.manova" function from the RVAideMemoire version 0.9-54 software package (Hervé 2015), which performs pairwise comparisons between group levels with corrections for multiple testing.

To evaluate changes in phylogenetic composition within elevations, I performed a permutation test of multivariate homogeneity of group dispersions, using the "betadisper"

function (Anderson 2006) in Vegan. Betadisper determines average distance of samples from a given group to the group centroid (Anderson 2006; Anderson et al. 2006), where groups more dissimilar in their phylogenetic composition will have larger distances to the group's centroid. Significance among group dispersions was determined by using the permutest function in Vegan using 9999 permutations. Pairwise comparisons between elevations was then performed using a t-test on mean group dispersions with a Bonferroni correction.

3.4 Results

3.4.1 Phylogenetic α-diversity

Phylogenetic diversity decreased with increasing elevation (Figure 3.2; $\chi^2 = 12.20$, df = 2, P = 0.002). Pairwise comparisons revealed that the subalpine communities had higher phylogenetic diversity than the alpine communities (P < 0.001). Treeline communities did not differ significantly from either subalpine (P = 0.35), or alpine communities (P = 0.39).

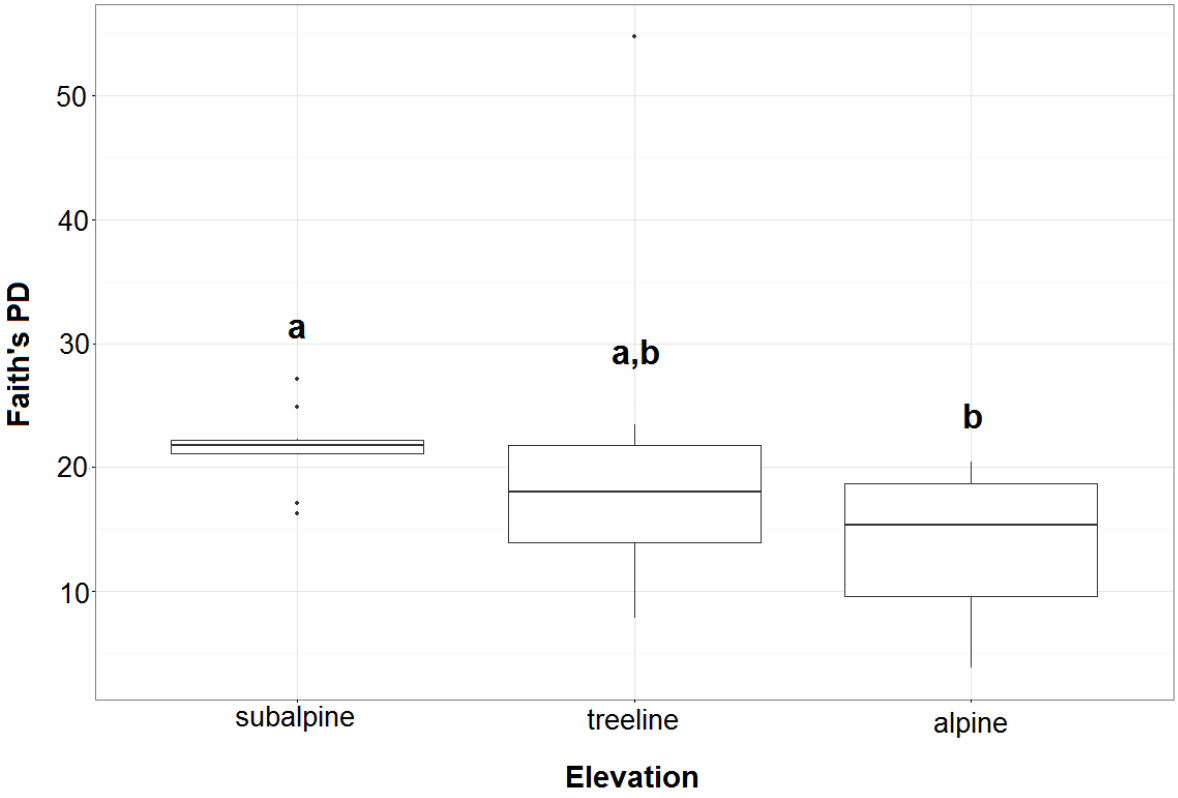


Figure 3.2 Phylogenetic diversity of AM fungal communities along the elevation gradient at Scenic Point, MT USA. Subalpine n=11, treeline n=12, alpine n=14. The bottom and the top of the boxes represent the first and third quartiles, the dark band inside boxes represents the median, the whiskers contain the upper and lower 1.5 interquartile range (IQR), and the dots represent outliers. Phylogenetic diversity (PD) was calculated as Faith's PD, and is quantified by calculating the total length of branches on a phylogenetic tree joining all species within a community (Faith 1992). Boxes topped by the same letter do not differ significantly at $P \leq 0.05$ using Wilcoxon rank sum tests.

3.4.2 Phylogenetic structure

AM fungal communities were phylogenetically clustered at all elevations (Figure 3.3). Both SES-MPD (Figure 3.3a) and SES-MNTD (Figure 3.3b) null model analyses showed that species co-occurring within communities were significantly phylogenetically clustered (AM fungal communities contain taxa that are phylogenetically more related than expected by chance), at all elevations (Table B.1). Comparisons among elevations sampled

revealed that elevation had no significant influence on this aspect of phylogenetic structure of the AM fungal communities for either MPD ($\chi 2 = 2.33$, df = 2, P = 0.31) or MNTD ($\chi 2 = 1.58$, df = 2, P = 0.45). This is supported by species from each elevation being phylogenetically clumped when plotted on the phylogenetic tree (Fig 3.1).

3.4.3 Phylogenetic β-diversity between elevations

PERMANOVA and NMDS ordination revealed that the phylogenetic composition of AM fungal communities was affected by elevation when unweighted-UniFrac distances were calculated between communities (Figure 3.4a, Table 3.1a). Pairwise comparisons revealed alpine communities to be significantly different in phylogenetic composition from both subalpine (P = 0.018) and treeline communities (P = 0.018), with no significant difference observed between subalpine and treeline communities (P =0.153). When I took species abundances into account I observed no significant difference in phylogenetic community composition among elevations (Figure 3.4b, Table 3.1b).

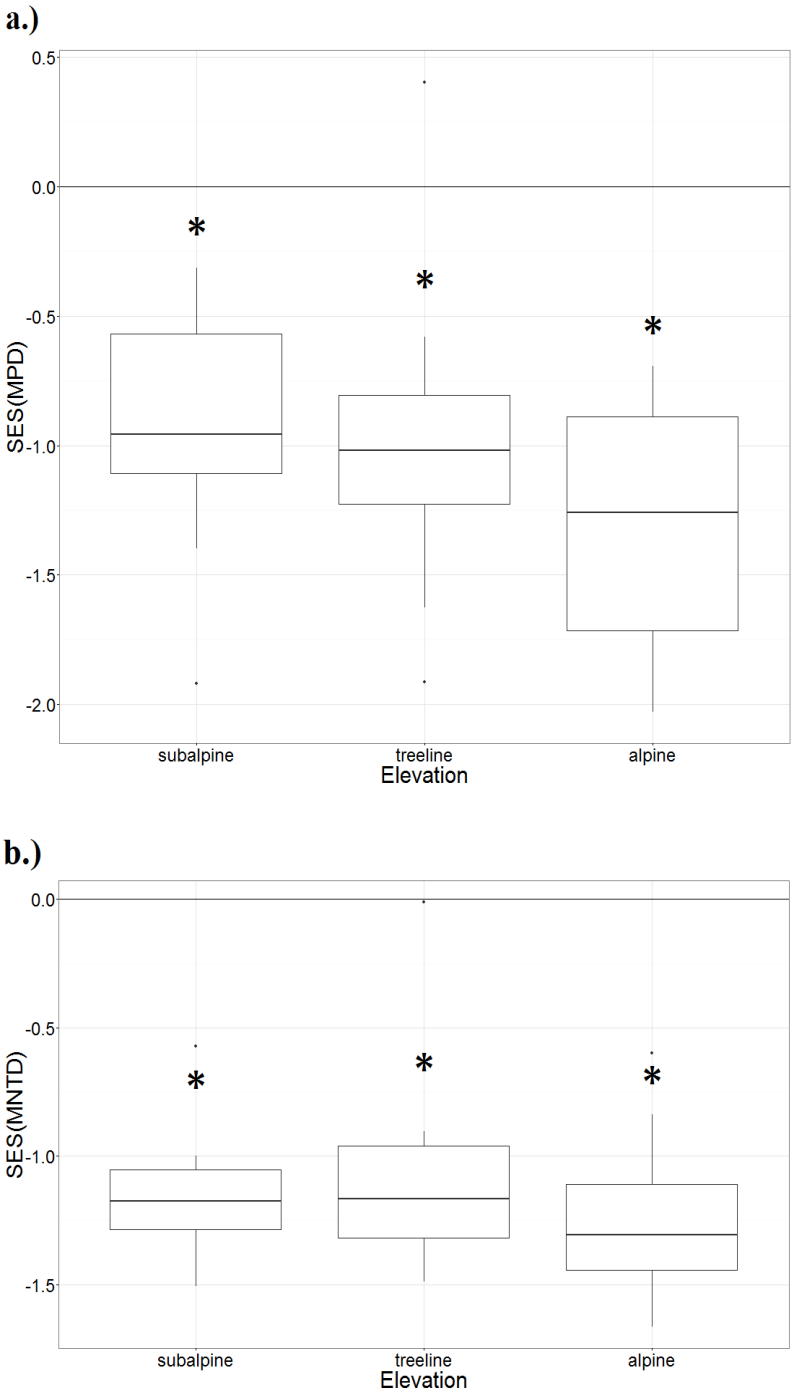


Figure 3.3 Boxplots (as described in Figure 3.2) showing phylogenetic structure within AM fungal communities from three elevations. Structure was determined by calculating standardized effect sizes (SES) of mean pairwise phylogenetic distance (MPD, a) and SES of mean nearest taxon distance (MNTD, b). Positive values indicate phylogenetic dispersion; negative values indicate phylogenetic clustering. Asterisks indicate deviation in phylogenetic structure from zero (null expectation) as determined by t-test (at a significance $P < 0.001$). Subalpine n=11, treeline n=12, alpine n=14.

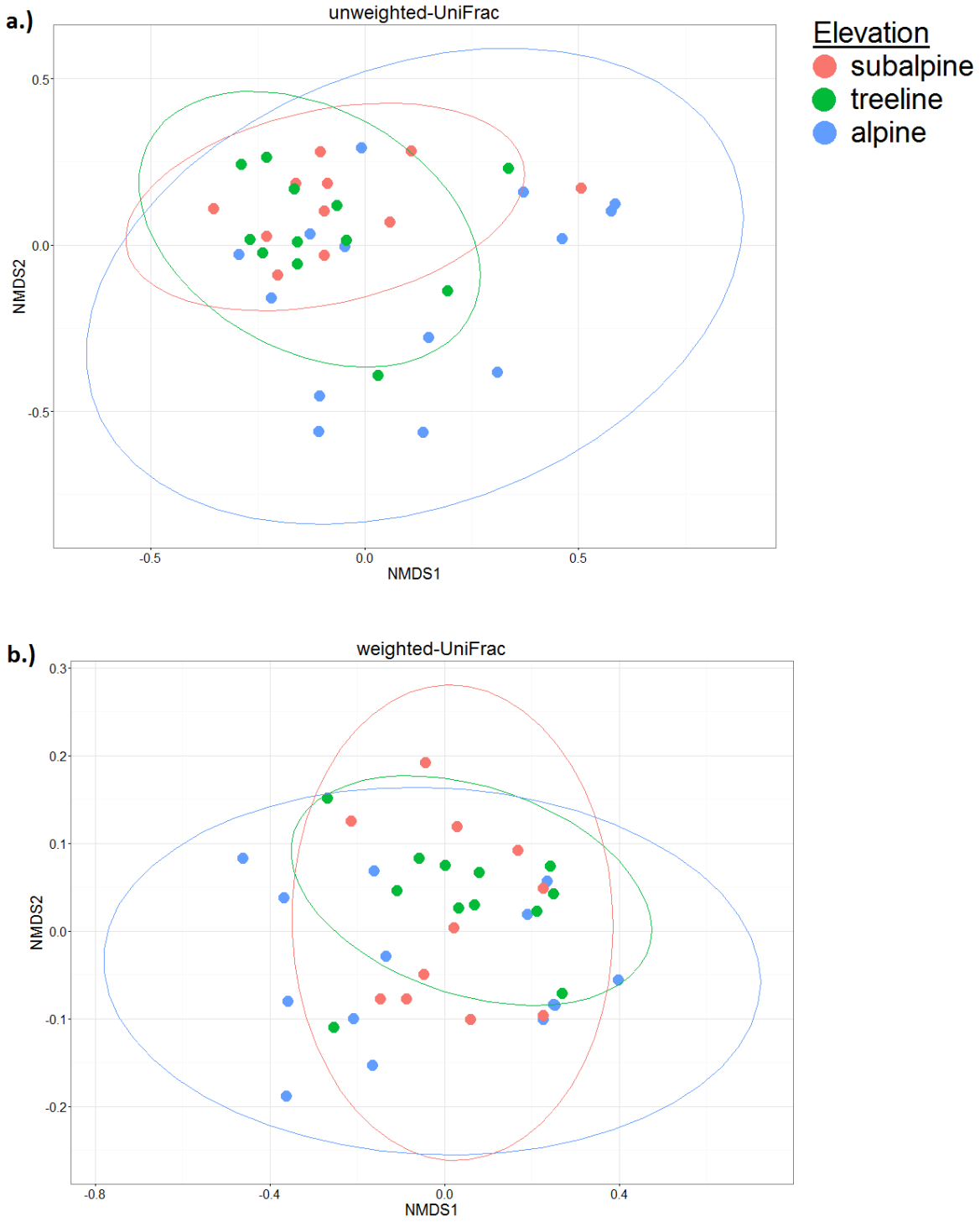


Figure 3.4 Non-metric multidimensional scaling (NMDS) ordination showing unweighted-UniFrac (a) and weighted-UniFrac (b) distances among AM fungal communities at Scenic Point, MT USA. Elevations are represented by different colours where; subalpine (n=11) samples are represented by red, treeline (n=12) samples are represented by green, and alpine (n=14) samples are represented by blue. Ellipses displaying 95% confidence are drawn around elevations.

3.4.4 Phylogenetic β-diversity within elevations

Samples in the alpine were more dissimilar to each other, relative to treeline and subalpine communities. However, this was only true for presence/absence data (Figure 3.4a, Table 3.2a), but not when relative abundance was considered (i.e. weighted-UniFrac; Figure 3.4b, Table 3.2b).

Table 3.1 Variation in AM fungal phylogenetic community composition with elevation, as determined by permutational multivariate analysis of variance (PERMANOVA) using distance matrices. PERMANOVA results shown for both unweighted (a) and weighted (b) UniFrac. Values shown include degrees of freedom (DF), sum of squares (SS), and mean squares (MS). Bold p-values indicate significance at $P < 0.05$.

β-diversity Metric	DF	SS	MS	PseudoF/F	R2	p
(a) Unweighted UniFrac						
Elevation	2	1.2326	0.6163	1.5393	0.0830	**0.007**
Residuals	34	13.6129	0.4004		0.9170	
Total	36	14.8455			1.0000	
(b) Weighted UniFrac						
Elevation	2	0.2075	0.1037	1.5078	0.0815	0.104
Residuals	34	2.3392	0.0688		0.9185	
Total	36	2.5467			1.0000	

Table 3.2 Variation in AM fungal phylogenetic community composition using unweighted (a) and weighted (b) UniFrac, as determined by multivariate dispersion analyses (Betadisper) using 9999 permutations. Bold p-values indicate significance at $P < 0.05$.

β-diversity Metric	DF	SS	MS	PseudoF/F	P
(a) Unweighted UniFrac					
Elevation	2	0.0211	0.0105	4.3837	**0.018**
Residuals	34	0.0817	0.0024		
(b) Weighted UniFrac					
Elevation	2	0.0143	0.0071	2.0511	0.14
Residuals	34	0.1184	0.0035		

3.5 Discussion

3.5.1 Phylogenetic α-diversity

In this study I clearly show that AM fungal communities differed along the elevation gradient that was sampled. Consistent with other studies of AM fungi along elevation gradients (Gai et al. 2012; Geml et al., 2014; Liu et al. 2015a; Looby et al., 2016), I observed a decline in species richness with increasing elevation (Figure B.3), but I also further observed a decline in phylogenetic diversity (Figure 3.2). This decline in phylogenetic diversity indicates that fungi in some clades are more sensitive than others to the environmental factors associated with increasing elevation, a pattern that has been observed in other studies of fungal diversity along elevation gradients (e.g. Looby et al. 2016). At high elevations, environmental factors such as suitable host abundance and productivity (i.e., available carbon from hosts), and abiotic factors such soil temperature, soil moisture and other adverse soil conditions (Bryant et al. 2008; Looby et al. 2016), may act as filters that select for AM fungal taxa with distinct traits. Specifically, I found links to mycelial biomass in determining species distribution patterns, supported by lack of any Gigasporaceae species in alpine samples, with a concurrent increase in the number of Acaulosporaceae species (Figure 3.1). This result is consistent with the observed phylogenetic trait conservatism within the Glomeromycota (Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009; Maherali and Klironomos 2012), as members of the Gigasporaceae produce the largest extraradical mycelial biomass, while taxa in the Acaulosporaceae produce the least amount of biomass (both intraradical and extraradical hyphae). Low biomass production would favour AM fungal taxa in two ways under stressful environmental conditions; 1) they would be less susceptible to environmental stress in the soil (i.e. cool temperatures, adverse

soil conditions), and 2) they would be less taxing for plant hosts to maintain the symbioses, thereby making them preferential partner choices in low productivity/high stress environments (Chagnon et al. 2013). These results are similar to other studies, where a higher abundance of taxa belonging to the Acaulosporaceae has been observed at higher elevations (Gai et al. 2012; Li et al. 2014; Liu et al. 2015a), and in one study, an Acaulosporaceae species was restricted to high elevation habitats (Oehl et al. 2006).

3.5.2 Phylogenetic Structure

Contrary to my expectations, I did not find that higher elevation communities were more phylogenetically clustered than lower elevation communities. Rather, I found AM fungal communities to be phylogenetically clustered at all elevations (Figure 3.3), a pattern also observed in bacterial communities along elevation gradients (Horner-Devine and Bohannan 2006; Bryant et al. 2008). Naturally occurring AM fungal communities have been shown to be phylogenetically clustered in multiple ecosystems (Kivlin et al. 2011; Horn et al. 2014), indicating that habitat filtering or dispersal limitation are the primary determinants of AM fungal community assembly. However, as highlighted in a recent editorial on use of phylogenetics in community assembly research, multiple processes may generate the same diversity patterns (Narwani et al. 2015). This is likely the case along the gradient examined, where habitats shift from being relatively low abiotic stress/high plant productivity at lower elevations, to areas with higher abiotic stress/lower plant productivity at higher elevations. In the alpine, where environmental conditions are harsh, AM fungal communities are likely primarily structured by abiotic conditions, where environmental conditions select for clades best able to withstand those local conditions. Conversely, at lower elevations, where there is

higher host productivity, higher host availability, and more favourable abiotic conditions, communities are more likely to be structured by factors other than abiotic filtering such as interactions with soil biota (Horn et al. 2014) or other AM fungi, (Goberna et al. 2014). The results from this study, together with other studies of AM fungal phylogenetic patterns, indicate that multiple determinants may result in observed phylogenetic patterns, and clearly more research is needed to better understand the driving factors under different environmental conditions.

3.5.3 Phylogenetic β-diversity

I was surprised to find high phylogenetic dissimilarity among samples within the alpine environment. I expected that the harsher environment at high elevations would result in the promotion of fungi from a restricted part of the phylogeny, resulting in assemblages being more similar in their phylogenetic composition. Rather, I observed the opposite pattern, where subalpine and treeline communities had relatively high phylogenetic compositional similarity, and alpine communities were more compositionally dissimilar (Figure 3.4a). Such a pattern may result from greater environmental heterogeneity in the alpine as vegetation in the alpine is more patchy compared to treeline and subalpine sites (Nagy and Grabherr 2009). Plant patchiness may also be contributing to the structure of AM fungal communities, a pattern which has been observed in other studies (Lekberg et al. 2010; Davison et al. 2012). In the alpine, AM fungal communities that form around plant patches may be subjected to local biotic and environmental conditions which may select for distinct, and phylogenetically similar AM fungal assemblages (e.g. Horn et al. 2014). However,

dispersal limitation among plant patches (Agnarsson et al. 2014), would enhance the persistence of phylogenetically distinct AM fungal communities in a patchy environment.

3.6 Summary

This study, along with previous studies of AM fungi along elevation gradients, show that AM fungi experience strong habitat filtering, as displayed by a universal loss/gain of AM fungal taxa with increasing elevation (Gai et al. 2012; Li et al. 2014; Liu et al. 2015 altitudinal), along with some species being found exclusively at high elevations (Oehl et al. 2006). Deterministic processes, such as habitat filtering result in phylogenetically structured AM fungal communities at local scales (this study, Kivlin et al. 2011, Horn et al. 2014). In addition, fungal communities seem to occur in distinct phylogenetic patches in the alpine environment, indicating that strong barriers to dispersal may exist in this habitat type.

NGS studies have the capability of unlocking diversity patterns previously missed by traditional methods for examining AM fungal diversity. Using NGS to examine assembly processes structuring fungal communities in poorly sampled areas, such as high elevations, is especially important. While I observed AM fungal communities to be strongly influenced by habitat type in this chapter, observations are restricted to soil communities, omitting a key component to AM fungal communities, their direct association with plant hosts. In the following chapter I use NGS to examine both root-associated, and soil-borne, AM fungal communities with three co-occurring plant hosts along the same elevation gradient. In the following chapter, my goal is to determine whether host selection or environmental filtering, has a stronger influence on AM fungal community structure along elevation gradients.

In addition to target sampling specific hosts, in the following chapter I also transition

to using Illumina sequencing platform from the Roche 454-pyrosequencer. The reason for this transition was two-fold 1.) Roche 454-sequencing will soon become obsolete, making Illumina the primary choice for studies examining AM fungal diversity, and 2.) Illumina sequences to depths magnitudes greater than Roche 454 (Harismendy et al. 2009), allowing samples with relatively low template concentrations to be potentially render sequences post-sequencing. In the current chapter I experienced low DNA issues both pre- and post-sequencing. Pre-sequencing many samples did not yield product, even after months of altering our amplification protocol in several ways (touchdown PCR, nested PCR, changing reagents, changing thermocycler conditions etc.). My conclusion was that those samples likely contained low concentrations AM fungal DNA, if any at all. Post-sequencing, I was again forced to discard samples with extremely low sequencing depths. The low DNA concentrations obtained from alpine system examined are not surprising given that alpine ecosystems experience low temperatures, which has been shown to reduce the abundance of AM fungal structures, especially hyphal length density (Zhang et al. 2016), making the transitioning from Roche 454 to Illumina when working in such systems pertinent.

Chapter 4 Arbuscular mycorrhizal fungal community structure in roots and soil of three co-occurring plant species along a high elevation gradient

4.1 Background

The arbuscular mycorrhizal (AM) association is complex and includes a suite of signals and morphological modifications between fungus and host (Gianinazzi-Pearson 1996; Armstrong and Peterson 2002; Bago et al. 2003; Smith and Read 2008). When an association is formed between an AM fungus and host, the fungal partner grows inside (intraradical) and outside (extraradical) the root. The intraradical phase includes the formation of hyphae and arbuscules, which are the sites of nutrient exchange between the plant and fungus. The extraradical phase includes an extensive mycelium that extends into the soil matrix, as well as spores. Since AM fungi occupy the soil and host roots, they must respond to two different environments, root cortical cells of the host, and soil. Studies have shown that AM fungal community composition is influenced by both host species (Husband et al. 2002; Öpik et al. 2009; Becklin et al. 2012; Torrecillas et al. 2012a; Torrecillas et al. 2012b; Varela-Cervero et al. 2015), and abiotic conditions (Jacobson 1997; Rousk et al. 2010; Yang et al. 2013; Liu et al. 2015b). This suggests that both host species and soil environment are highly influential in structuring AM fungal communities. However, the relative importance of host identity and the abiotic environment remains unclear.

How AM fungi allocate their biomass can help us to determine which structuring factor is more important. Field studies (Hempel et al. 2007; Yang et al. 2013; Saks et al. 2014; Shi et al. 2014), corroborated by controlled growth experiments (Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009; Maherali and Klironomos 2012)

show that AM fungal taxa preferentially allocate biomass to either intraradical or extra-radical growth. It has been suggested that allocation of biomass, intraradically vs extraradically, is linked to AM fungal functional traits (Chagnon et al. 2013). For instance, taxa that produce higher hyphal densities in the soil, such as species of Gigasporaceae, are able to transfer high rates of phosphorous to hosts (Jansa et al. 2005; Avio et al. 2006), thereby increasing the flow of plant carbon to the fungus (Kiers and van der Heijden 2006), allowing these fungi to be more competitive for host carbon. Under conditions where fungi receive low host carbon, fungi that produce less biomass, such as species of the Acaulosporaceae family, may have an advantage (Chagnon et al. 2013), as they require less fixed carbon from a host that is carbon-limited. In addition to host productivity, abiotic conditions directly acting upon fungi, may also determine what fungal traits are advantageous. For example, in habitats with adverse soil conditions, fungi that allocate more growth inside plant roots, would have a competitive advantage, as host roots can offer a space of refuge from harsh environmental conditions in the soil.

Because AM fungi are obligate biotrophs, host species identity has been credited as being influential on AM fungal community composition. This is supported by multiple studies. Torrecillas et al., (2012b) showed that plant hosts sampled from the same Mediterranean ecosystem harboured distinct AM fungal communities. Furthermore, they were able to show that species shared between species had different abundances, suggesting that AM fungi have host preferences. Eom et al. (2000) revealed that hosts harboured distinct AM fungal communities when plants were grown in the same soil. They planted host species in soil collected from the same tallgrass prairie system, and after 4 months examined the diversity of AM fungi beneath hosts. They observed plant species to host distinct AM fungal

communities, and attributed those differences to be directly controlled by the host. Both AM fungal growth rates, and relative dominance of AM fungal species within communities, have been linked to host identity (Bever et al. 2001; Vandenkoornhuyse et al. 2002; Mangan et al. 2010). Also, several studies have shown that AM fungal sporulation differs among host species (Sanders and Fitter 1992; Bever et al. 1996; Mangan et al. 2010), indicating that host identity can influence fungal processes such as propagation.

Overall, previous studies indicate that both host identity and environment can determine AM fungal community structure. However, it is still not clear which is more important. One approach to evaluate the relative contributions is to examine the same host species along a continuous environmental gradient. Elevation gradients provide ideal venues to examine this relationship. Over a relatively small spatial scale the same plant species may be found along with large variation in abiotic conditions (Körner 2007). However, not all studies at high elevation take advantage of environmental gradients. For example, Becklin et al. (2012) compared AM fungal diversity in the roots of three alpine plant species between two treeline habitat types on Pennsylvania Mountain in Colorado, and found that host identity was a stronger determinant of AM fungal community composition than habitat type. However, this study examined AM fungal diversity in plants at the treeline only, where abiotic conditions would be more similar than plants sourced from different altitudes. In another study Li et al., (2014) examined AM fungal communities in the roots of two host species, among three elevation habitats; montane temperate, subalpine, and alpine habitats along Mount Segrila, in the Tibetan Plateau. They observed AM fungal community composition differed significantly between their target hosts regardless of elevation (Li et al. 2014). They concluded that host identity was a stronger determinant than environment of root

AM fungal community composition. For one host species they also observed compositional dissimilarity to be significantly influenced by elevation from which samples were taken, indicating that environment also plays a role in determining root community composition. While both of these studies provide insight to how root communities are influenced by host species and environment, by studying root communities only, they are focusing on a subset of the total AM fungal community (Saks et al. 2014; Varela-Cervero et al. 2015).

In this study I examined AM fungal communities associated with the roots and soil directly beneath three plant species found in three different habitat types along a high elevation gradient in the North American Rocky Mountains. My aim was to examine how colonization strategy, host identity, and habitat type structure AM fungal communities. I predicted colonization patterns to mirror those previously observed in greenhouse studies (Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009; Maherali and Klironomos 2012), where taxa of Gigasporaceae and Acaulosporaceae would be more abundant in soil samples, and taxa of Glomeraceae would be more abundant in roots. Following the C-S-R framework of Chagnon et al., (2013), I also predicted Gigasporaceae taxa would more abundant in high productive/low stress habitats, while Acaulosporaceae will be more abundant in low productive/high stress habitats. I also predicted host identity would be a stronger determinant of AM fungal communities in roots, while habitat type would be a stronger determinant of AM fungal communities in the soil.

4.2 Materials and Methods

4.2.1 Study site and sample collection

Sampling was conducted along the same high elevation gradient as chapter 3, in

Glacier National Park, USA (48°28'N; 113°21'W). Here, I collected samples in August 2013 in three locations along the gradient; subalpine grassland (1682 MASL), treeline (2064 MASL) and alpine tundra (2330 MASL). When collecting samples, I targeted three plant species, each representing a different plant functional type (Díaz and Cabido 2001). Target species included *Dasiphora fruticosa* (shrub), *Achillea millefolium* (forb), and *Festuca idahoensis* (grass). These target species were chosen as they co-occurred in relatively close proximity along the entire gradient, were abundant in all habitats examined, and were easily identified in the field. To select individual plants I established a sampling transect running perpendicular to the gradient within each location. Along the transect I established five sampling points, each separated by 20 m. From each sampling point, the closest individual from each of the three target species was selected and sampled. For each plant I collected both fine root samples and bulk soil. Soil was collected directly underneath plant using two soil cores, which were combined in plastic bags and stored frozen until DNA extraction. Fine roots were identified by tracing them to larger roots directly beneath the plant. Root samples were also stored in plastic bags and kept frozen until DNA extraction.

4.2.2 Molecular analysis

Prior to DNA extraction, soil samples were passed through a 5 mm sieve, and then homogenized, after which a 0.25 g subsample was taken from each plant sampled for DNA extraction. For root extractions, fine roots were cleaned, dried, and then pulverized. Post-pulverization, a 0.25 g subsample was taken from each plant for DNA extraction. DNA was extracted using the FastDNA^{TM} spin kit for soil (MoBio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions. Two-step PCR reactions were conducted

post extraction. A two-step PCR protocol to generate amplicon libraries. For PCR1 reactions, 550 bp of AM fungal SSU rRNA was targeted. PCR 1 primer complexes consisted of the universal eukaryotic primer WANDA (SI from Dumbrell et al. 2010) with the Fluidigm tag CS1 attached to the 5' end (Fluidigm Inc., South San Francisco, CA, USA), and the Glomeromycota specific primer AML2 (Lee et al. 2008) with the Fluidigm tag CS2 attached to the 5' end. Reactions were carried out in 20 µL volumes using 1 µL of DNA template, 5 µL of 5x GoTaq Buffer, 1 µL of 25 mM $MgCl_2$, 0.25 µL of 20 $mgmL^{-1}$ BSA, and 0.5 µL each of 10mM dNTP mix, 10 µM AML2/CS2, 10 µM WANDA/CS1, and GoTaq polymerase® (Promega Biosciences LLC, San Luis Obispo, CA, USA). Thermocycler conditions were: 95°C for 2 min; 34 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 2 mins; and 72°C for 10 min, on a Bio Rad C1000 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). For PCR2 reactions, PCR 1 products were diluted 15-fold and were used to attach 8 bp Illumina Nextera barcodes and flowcell adapters (Illumina Inc., San Diego, CA, USA) to both ends of amplicon libraries. PCR2 primer complexes consisted of the same Fluidigm tags (CS1 or CS2) as PCR1, 8 bp Illumina Nextera barcodes, and Illumina adapters. To differentiate samples post-sequencing, I multiplexed unique forward and reverse barcode combinations for each sample. Reactions were carried out in 20 µL volume using 1 µL of diluted PCR1 product, 4 µL of 5x GoTaq Buffer, 3.6 µL of 25 mM MgCl2, 0.6 µL of 20 $mgmL^{-1}$ BSA, 10mM dNTP mix, 0.5 µL of 2 µM forward primer, 0.5 µL 2 µM reverse primer, and 0.2 µL of GoTaq polymerase. Thermocycler conditions were: 95°C for 1 min; 10 cycles of 95°C for 30 sec, 60°C for 30 sec, 68°C for 1 min; and 68°C for 5 min, on a Bio Rad C1000 thermocycler. PCR2 products were then sent to the Institute for Bioinformatics and Evolutionary Studies (iBEST) genomics resources core at the University of Idaho

(http://www.ibest.uidaho.edu/; Moscow, ID, USA), where PCR products were cleaned, standardized, and sequenced using 2 x 300 paired-end (PE) sequencing using 600 cycles on an Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA, USA).

4.2.3 Bioinformatics

As with the previous chapters, sequence analyses were conducted using QIIME (Caporaso et al. 2010). Post-sequencing, for *fastq* files were generated; forward barcode read, forward target read, reverse barcode read, and reverse target read. Barcode reads for each sample were combined and organized into a new *fastq* file. Reads were assigned to samples using the 'split libraries' step in QIIME, and were conducted separately for forward and reverse sequence reads, using the combined barcodes *fastq* file, along with primary quality filter parameters described by Bokulich et al., (2013). After primary filtering, chimeric sequences were subsequently removed using usearch (Edgar et al. 2011). Remaining reads were then grouped into operational taxonomic units (OTUs) using a sequence similarity of ≥97%, and cluster seeds as the representative sequences for each OTU. Low abundant OTUs were then filtered using the abundance threshold 0.01 %, meaning that for a sequence to be retained it must represent ≥ 0.01% of all sequence reads generated. Remaining OTUs were identified taxonomically by blasting (Altschul et al. 1990) representative sequences against type sequences downloaded from the MaarjAM database in April 2016 (Öpik et al. 2010); http://maarjam.botany.ut.ee/). For all analyses I used VT nomenclature of the MaarjAM database. To control for differences in sequence reads generated for each sample, I sub-sampled to 2000 sequences per sample determined by rarefaction analysis, (Figure C.1). I then generated a sample-by-VT table, where sequence abundances were used to represent VT

abundance in each sample. The sample by VT table, along with associated sample metadata were then exported from QIIME into the R console (R Development Core Team 2015) for downstream analyses.

4.3 Statistical Analyses

4.3.1 Effect of sample type and elevation on AM fungal genera abundances

To examine colonization patterns I compared abundance of genera, as determined by the number of sequence reads detected, in the roots vs soil for all elevations sampled. I chose to focus on genera since results from chapter 2 showed that genera level resolution produces more accurate approximations of AM fungal abundances than species in mixed communities. Abundance of a given AM fungal VT is defined as the number of sequences of that VT in a sample, and abundance of a given genus is the sum of abundance of all VTs belonging to that genus in a sample. Differences in abundance between roots and soil were compared for every genus detected at each elevation using a Welch two sample t-test to a significance level at $P < 0.05$.

To determine the effect of elevation on genera abundances I used generalized linear model-based analyses of multivariate abundance (multiGLM) using the "manyglm" function in the mvAbund package in R (Wang et al. 2012b). The multiGLM method uses generalized linear models (GLMs) to relate variation in abundance for each genus to a factor of interest, allowing for the interpretation of individual genera responses to that factor. Using a GLM with a negative binomial error distribution, I evaluated the effect of elevation on the abundance of each genus. I conducted multiGLM analyses for roots and soil in separate models. Significance was assessed using Bonferroni-adjusted P values to a significance level

at $P < 0.05$. Univariate P-values were adjusted for multiple testing using a step-down resampling procedure (Wang et al. 2012b).

4.3.2 Effect of elevation on AM fungal diversity in the soil and roots

To examine the effect of elevation on VT richness in the roots and soil, I used a GLM with a Poisson distribution and a log link function. A Poisson distribution was used as it best models species richness data (Fisher et al. 1943; Vincent and Hawthorn 1983). Assumptions of residuals being normally distributed, and homogeneity of variance and the linear relation were verified prior to running the GLM. Pairwise comparisons were then carried out by Tukey HSD multiple comparisons using the "glht" function in the multcomp package in R (Hothorn et al. 2008). Significance was assessed at $P < 0.05$.

Next I examined whether AM fungal community composition (i.e. β-diversity) varied between roots and soil. To do this I first calculated Bray-Curtis dissimilarity between samples (Bray and Curtis 1957), and then used permutational multivariate analysis of variance using distance matrices (PERMANOVA; Anderson 2001) to partition variation in AM fungal community composition in relation sample type. PERMANOVA was conducted using the "adonis" function in the vegan package in R (Oksanen et al. 2015). Non-metric multidimensional scaling (NMDS) ordinations were then used to visualize communities. It has been shown that PERMANOVA is influenced by differences in multivariate dispersion (i.e. within-group variability, PERMdisp) (Anderson 2006; Anderson et al. 2006), therefore I also examined differences in multivariate dispersion among factors using the "betadisper" function in the vegan package.

4.3.3 Effect of elevation and host species on AM fungal community composition

Finally, I examined the relative contribution of host species and habitat type on AM fungal diversity by examining AM fungal community composition in roots and soil, from the three elevations sampled, for the three target plant species. I evaluated the effect of host and elevation on AM fungal community composition, separately for roots and soil samples, again using Bray-Curtis dissimilarity. PERMANOVA was then used to partition variation in AM fungal community composition in relation to elevation and host. Multivariate dispersions for all groups were examined. Because both elevation and host have more than two factors, I followed PERMANOVA with pairwise comparisons using the "pairwise.perm.manova" function from the RVAideMemoire package in R (Hervé 2015). Again, PERMdisp analyses were used to determine if differences between factors was caused by differences in multivariate dispersion.

4.4 Results

4.4.1 Abundance of genera in roots vs soil

Acaulospora had higher abundance in soil than roots for all three elevations sampled (Figure 4.1; Table C.1). *Dentiscutata* species also had higher abundance in soil than roots, but only in subalpine and treeline habitats. Three genera: *Claroideoglomus*, *Diversispora*, and *Scutellospora*, had higher abundances in roots than soil, but only in the subalpine habitat. Elevation had a significant effect on the abundance of AM fungal genera in both roots (negative binomial multiGLM; Wald statistic = 11.3; df = 2,42; $P < 0.001$) and soil (Wald = 10.21; df = 2,42; $P < 0.001$). Post-hoc univariate tests revealed that in roots, both *Ambispora*

(Wald = 5.74, P = 0.001) and *Scutellospora* (Wald = 8.07, P = 0.001) abundances vary significantly with elevation, whereas *Diversispora* (Wald = 4.01, P = 0.05) and *Scutellospora* (Wald = 5.17, P = 0.004) abundances in soil vary significantly with elevation (Figure C.2).

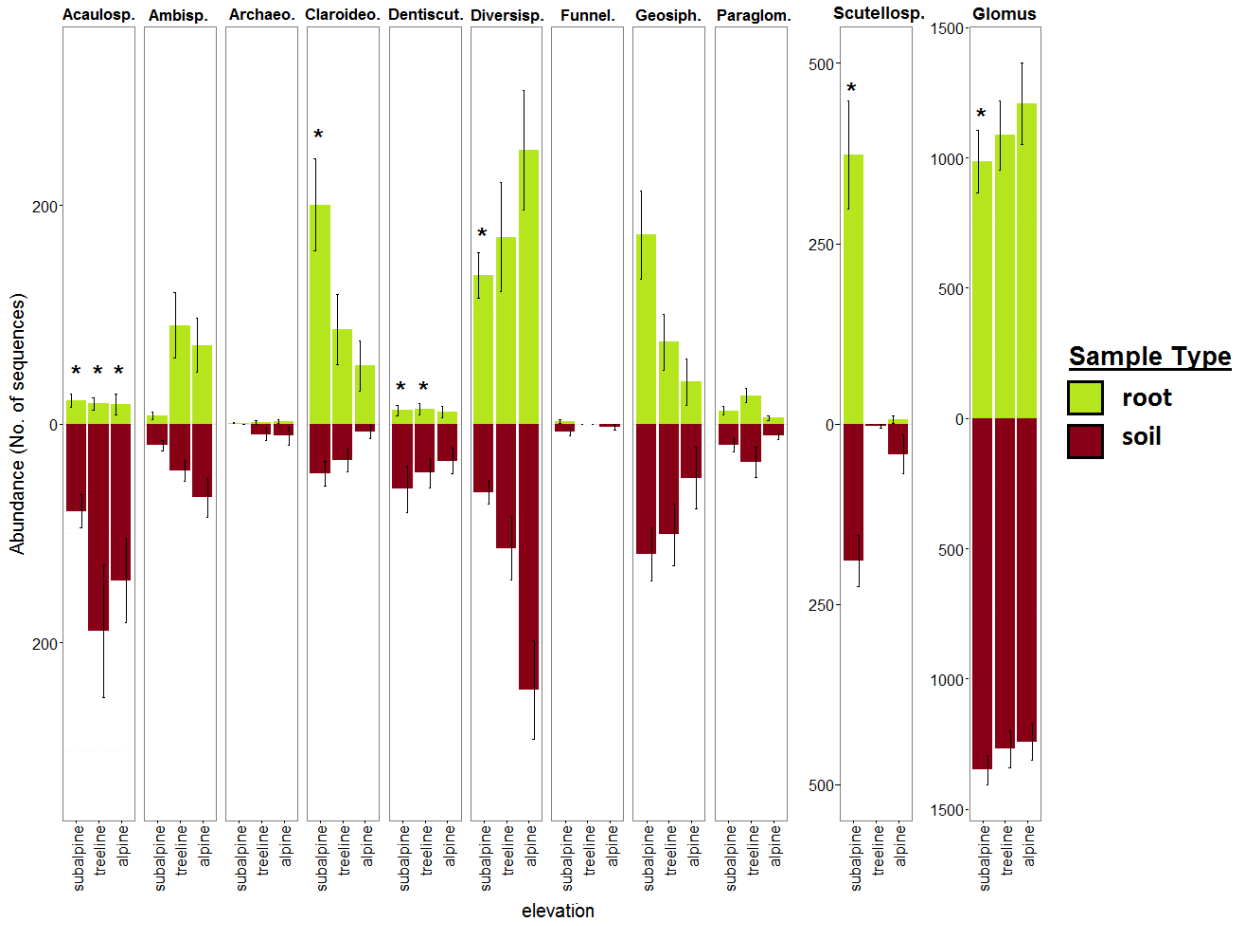


Figure 4.1 Bar plots showing sequence abundance of AM fungal genera in roots (green) and soil (brown), from the three elevations sampled. Subalpine root n=15, subalpine soil n=15, treeline root n=15, treeline soil n=15, alpine root n=15, treeline soil n=15. Results obtained after sub-sampling data to 2000 sequences per sample. Bars represent the mean abundance of a genus at each elevation, error bars represent standard errors of the means. Asterisks indicate significant differences in the mean abundance of a genus between roots and soil at

that elevation according to Welch two-sample t-test (at a significance of $P < 0.05$). Note the differences in y-axis scale for both, *Scutellospora* and *Glomus* relative to other genera.

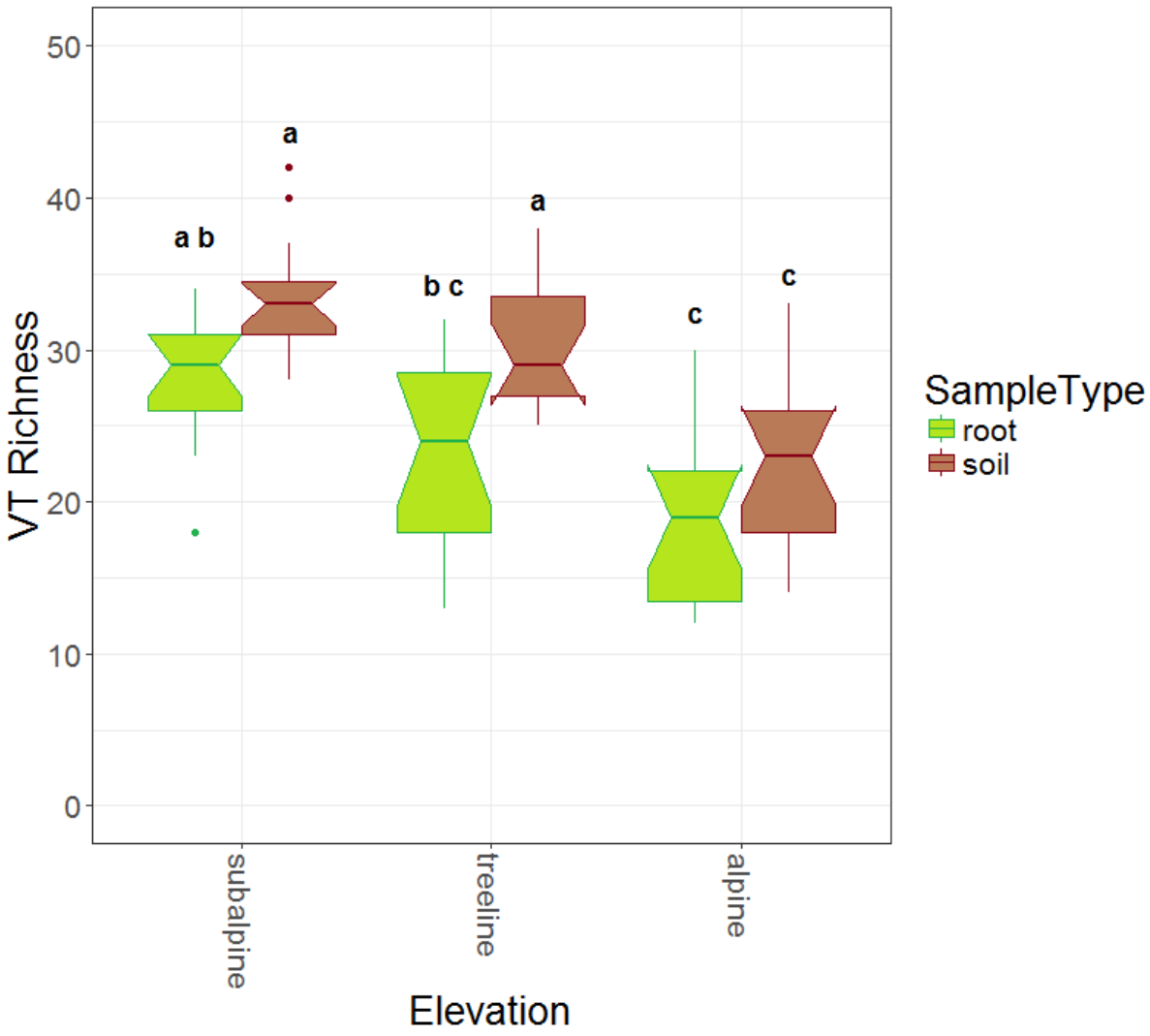


Figure 4.2 Notch boxplots showing AM fungal virtual taxa (VT) richness in roots (green) and soil (brown) along the gradient sampled elevation. Subalpine root n=15, subalpine soil n=15, treeline root n=15, treeline soil n=15, alpine root n=15, treeline soil n=15. The bottom and the top of the boxes represent the first and third quartiles, the dark band inside boxes represents the median, the whiskers contain the upper and lower 1.5 interquartile range (IQR), and the dots represent outliers. Boxes without shared letters indicate significant difference in VT richness as determined by Tukey Contrasts (at a significance of $P < 0.05$).

4.4.2 Effect of elevation on AM fungal diversity in the soil and roots

A total of 74 AM fungal VT from nine families, and eleven genera were detected along the elevation gradient. An average of 23 (6.7 SD) AM fungal VT were found in roots, while 29 (6.3 SD) were found in soil. GLM analysis revealed that AM fungal VT richness differed significantly with elevation (Figure 4.2; Poisson distribution GLM with log link function; $\chi^2 = 60.53$; df = 2; $P < 0.001$) for both soil and root AM fungal communities. Tukey HSD comparisons revealed that alpine soil AM fungal communities had significantly fewer VTs than both treeline and subalpine communities (at $P < 0.05$), while treeline and subalpine soil communities did not differ significantly. VT richness in roots decreased significantly between subalpine and alpine communities, while treeline root communities were not significantly different from root communities sampled from either elevation. VT richness was compared between soil and roots within elevations, I observed VT richness to be significantly lower in treeline roots vs treeline soil, and observed no significant difference between soil and root VT richness in either subalpine or alpine habitats.

PERMANOVA and NMDS ordination indicated that regional AM fungal community composition did not differ significantly between roots and soil (PERMANOVA; $F_{(2,88)} = 0.82$; $P = 0.55$; based on 9999 permutations; Figure 4.3).

4.4.3 Effect of elevation and host species on AM fungal community composition

PERMANOVA and NMDS ordination revealed root AM fungal communities to be significantly affected by elevation, host, and their interaction (Figure 4.4a, Table 4.1a). Pairwise comparisons revealed subalpine root communities to be significantly different from both treeline and alpine communities, with no significant difference observed between

treeline and alpine communities (Table C.2a). *A. millefolium* root communities shown to be significantly different from both *D. fruticosa* and *F. idahoensis* communities, with no significant difference found between *D. fruticosa* and *F. idahoensis* root communities (Table C.2a). PERMdisp analyses confirmed that differences between elevations and hosts were not caused by differences in multivariate dispersion (Table C.3a).

Soil AM fungal community composition was influenced by elevation only, with no significant difference observed between hosts (Figure 4.4B, Table 4.1B). Pairwise comparisons revealed soil AM fungal community composition to be significantly different among all three elevations (Table C.2b). PERMdisp analyses confirmed that differences among elevations were not caused by differences in multivariate dispersion (Table C.3b).

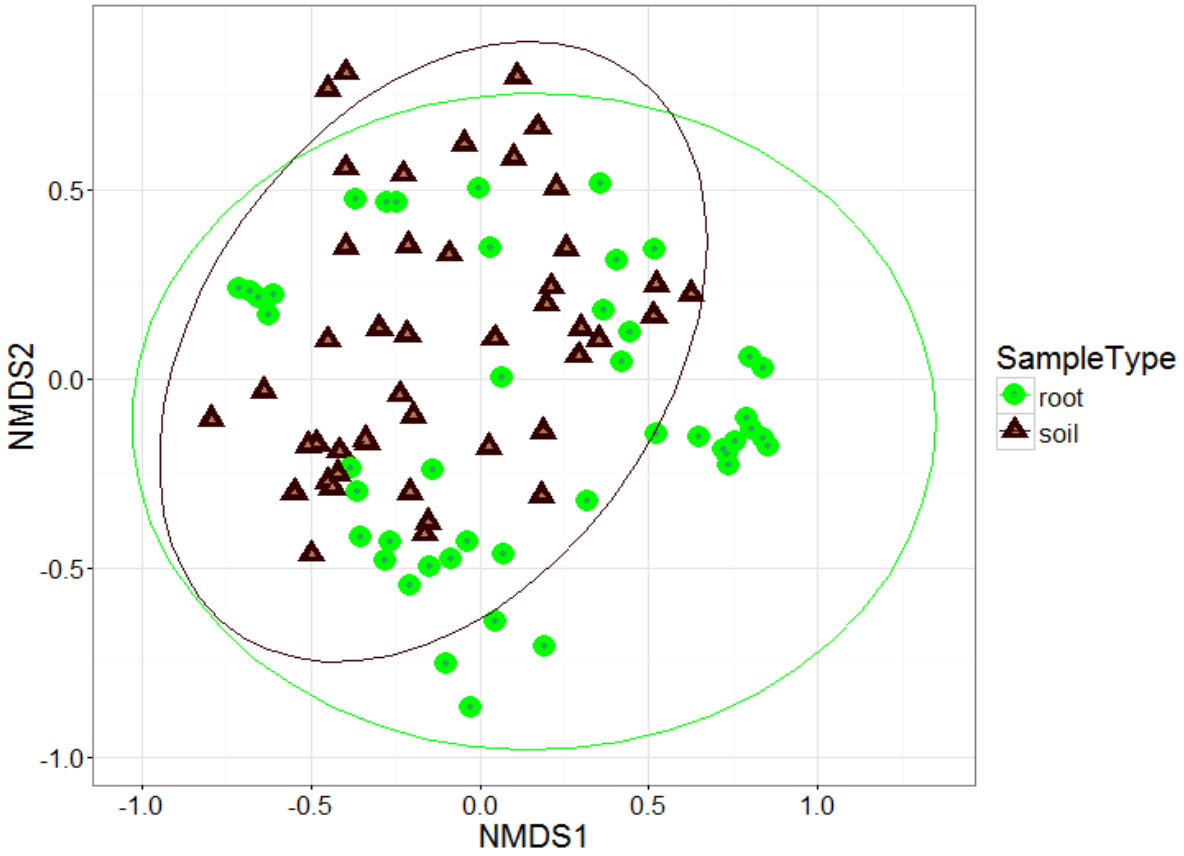


Figure 4.3 Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis distances among AM fungal communities in the soil (brown, n=45) and roots (green, n=45) collected along an elevation gradient.

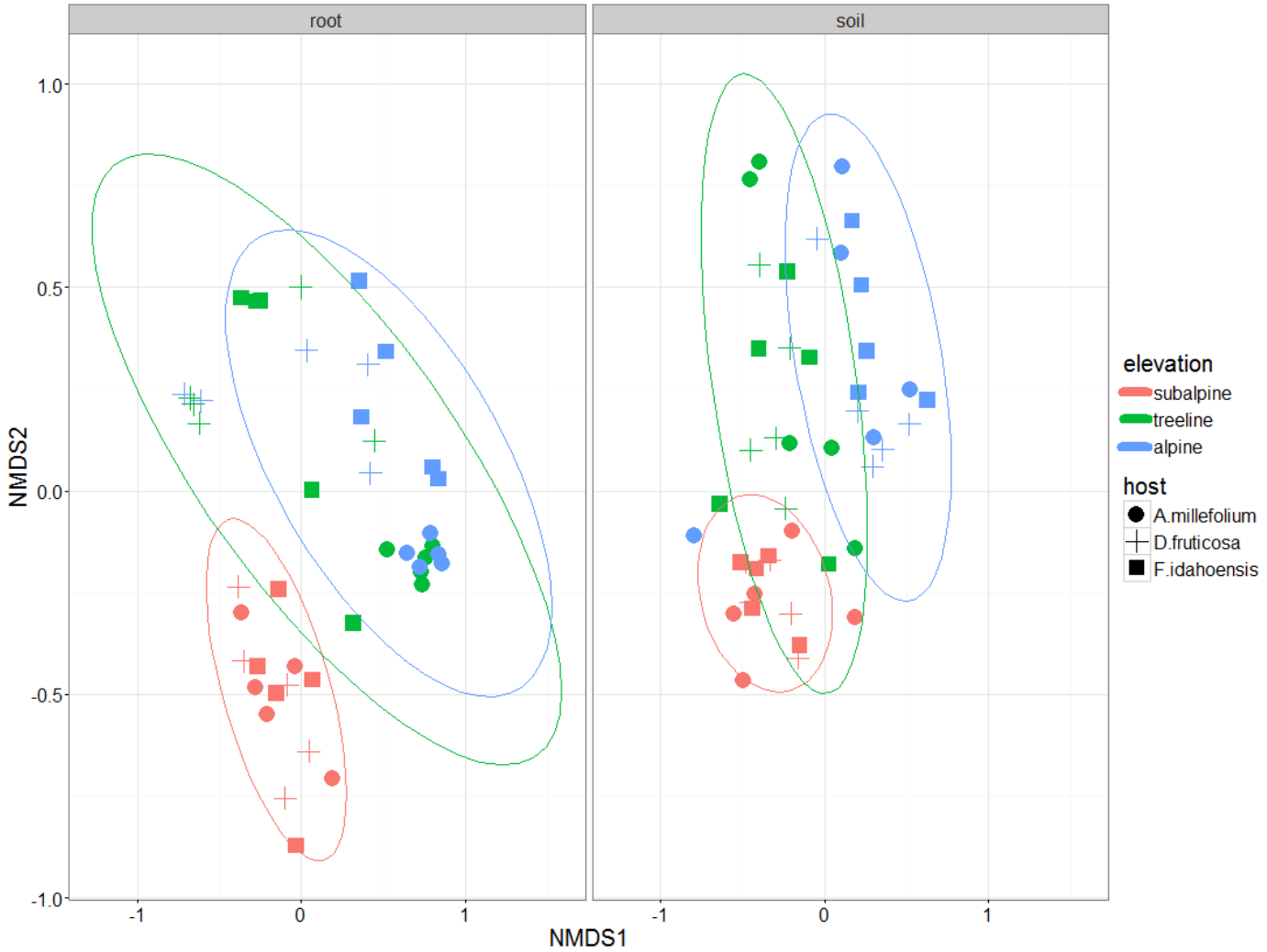


Figure 4.4 Non-metric multidimensional scaling (NMDS) ordination showing Bray-Curtis distances between AM fungal communities in roots (left) and soil (right), collected from three host species (n=5 for all host species) along an elevation gradient. Host species are represented by different shapes, and elevation is represented by different colours. Ellipses displaying 95% confidence are drawn around habitat types sampled, where colours correspond to elevation.

Table 4.1 Variation in AM fungal community composition, as determined by permutational multivariate analysis of variance using distance matrices (PERMANOVA) of AM fungal community composition in relation to elevation and host species, for both root (A) and soil (B) samples. Values shown include degrees of freedom (DF), sum of squares (SS), and mean squares (MS). Bold p-values indicate significance at $P < 0.05$.

	DF	SS	MS	PseudoF/F	R2	p
(a) Root PERMANOVA						
Elevation	2	2.5373	1.26865	10.1459	0.24197	**0.0001**
Host	2	2.3337	1.16684	9.3317	0.22255	**0.0001**
Elevation x Host	4	1.1137	0.27843	2.2267	0.10621	**0.0069**
Residuals	36	4.5015	0.12504		0.42928	
Total	44	10.4861			1	
(b) Soil PERMANOVA						
Elevation	2	1.6062	0.80309	5.6597	0.2122	**0.0001**
Host	2	0.2702	0.13512	0.9523	0.03554	0.4961
Elevation x Host	4	0.6198	0.15495	1.092	0.0815	0.3325
Residuals	36	5.1082	0.14189		0.67174	
Total	44	7.6044			1	

4.5 Discussion

In this study genera from the same family were observed to differ in their abundance patterns in roots vs soil. I observed genera from Acaulosporaceae to be more abundant in soil vs. roots (as predicted by Chagnon et al 2013), and similar to field studies (Hempel et al. 2007; Yang et al. 2013; Varela-Cervero et al. 2015). However, mixed results were observed in the Gigasporaceae, where I observed *Dentiscutata* taxa to appear in higher abundance in soil than roots, while *Scutellospora* taxa had higher abundance in roots than soil. This pattern suggests that phylogenetic trait conservatism may be constrained at lower taxonomic levels than AM fungal families as previously observed (Powell et al. 2009; Chagnon et al. 2013). Furthermore this indicates that genera from the same AM fungal families may contain different growth traits, and will respond differently to environmental conditions.

No difference in genera abundances were observed between root and soil in alpine

samples. Multiple studies have observed AM fungi to display preferences for where they allocate the majority of their biomass (e.g. Hart and Reader 2002; Maherali and Klironomos 2007; Powell et al. 2009; Maherali and Klironomos 2012). However, these studies have been conducted in laboratory conditions under relatively benign abiotic conditions. Results from the current study suggest that species colonization preference may be dulled by abiotic environmental stress, and that certain AM fungal phenotypes are filtered by their environment.

Previous studies have observed root AM fungal communities to be a subset of taxa present in soil communities (Saks et al. 2014), suggesting that hosts select for specific fungal taxa available in the source pool in the soil (Varela-Cervero et al. 2015). Along the elevation gradient studied here, I observed a high overlap between species found in soil and roots: 95% of VTs shared between the roots and soil, which is much higher that previous studies (e.g. 33% overlap in Varela-Cervero et al. (2015) and 36% overlap in Saks et al. (2014). I attribute the high overlap observed in the present study to be driven by high environmental stress that places a filter on the regional AM fungal species pool. This may result in plant hosts that are less "choosy" when forming association with AM fungi, forcing them to form partnerships with any local fungi that are available.

Clearly AM fungal community composition was significantly influenced by habitat, a pattern that was more pronounced in soil communities than root communities. Similar to my previous study along the same gradient (chapter 3 of this thesis), I observed a decrease in total AM fungal VT richness with increasing elevation in both roots and soil (Figure 4.2). These results imply that AM fungal taxa are responding to environmental conditions the gradient sampled. Even though I did not directly examine environmental drivers along the elevation gradient I attest the observed decrease in richness to the adverse environmental conditions

(caused by decreased availability of carbon from hosts, decreased temperatures, and adverse soil conditions) at higher elevation. These adverse conditions can reduce diversity in AM fungal communities in multiple ways including fewer available resources which means fewer species will be able to persist, or environmental filtering selecting for taxa with distinct traits.

Similar to other studies (Helgason et al. 1998; Öpik et al. 2006), AM fungal communities differed significantly among habitat types. This supports the hypothesis that plant identity is less important for determining AM fungal community structure, compared to environmental conditions (Lekberg and Waller 2016). However I also observed AM fungal communities in the roots to be significantly influenced by host identity, indicating that, to a degree, hosts are able to select which AM fungi they associate with (Bever et al. 2001; Bever et al. 2009). A pattern consistent with other studies examining root associated AM fungal communities across habitat types (e.g. Varela-Cervero et al. 2015). A limitation to the present study is that I am unable to address differences in intraspecific variation between hosts of the same species (ecotypes), a factor that could be driving the observed diversity patterns. Plant ecotypes have been identified along elevation gradients (Clausen et al. 1941), with ecotypes displaying differences in their response to environmental conditions and biotic interactions (Choler et al. 2001; Callaway et al. 2002). I observed patterns that suggest that species ecotypes may host similar AM fungal communities, as shown by treeline and alpine *A. millefolium* samples hosting significantly different communities from subalpine *A. millefolium*.

4.6 Summary

Even though AM fungi have been shown to exhibit low host specificity (e.g. Klironomos 2000), several studies have shown support for host preference existing within the AM symbiosis (Vandenkoornhuyse et al. 2002; Öpik et al. 2009; Martínez-García et al. 2015). Conversely,

other studies show that habitat type, and that habitat's coinciding environmental conditions, are a stronger determinant of AM fungal community structure that host identity per se (Öpik et al. 2006; Lekberg and Waller 2016). By analyzing AM fungal communities in the roots and surrounding soil of three co-occurring plant species along a common environmental gradient, I found that habitat types harboured distinct AM fungal communities, with little to no evidence for host species influencing community structure. This pattern was significant for both soil and root associated AM fungal communities. While I did observe a pattern of one plant species from different elevations containing highly similar communities of AM fungi, I consider this pattern to be the exception rather than the rule. I also observed some AM fungal genera displayed preferences for either the soil or the root environment, as shown by the abundance patterns in the respective sample types. However, this pattern was only observed in low elevation samples and disappeared at the highest elevation, suggesting that environmental stress may influence AM fungal growth strategies.

In both chapters 3 and 4 I observed that the distribution patterns, and community composition of AM fungi along an elevation gradient is strongly influenced by habitat type. This conclusion is consistent with niche theory, which emphasizes differences in species' responses to abiotic and biotic factors, and highlights the importance of deterministic processes such as environmental filtering and interspecific trade-offs in determining patterns of species diversity and composition (Chesson 2000). Alternatively, neutral theory emphasizes the importance of stochastic processes such as chance colonization, and assumes species to be ecologically equivalent in their demographic rates, such as dispersal ability (Chave 2004). Because AM fungi are widespread globally (Öpik et al. 2009; Kivlin et al. 2011), with the majority of taxa being found on multiple continents (Davison et al. 2015), it may be assumed that all species have

unlimited dispersal capabilities, and that dispersal ability will be unfettered by habitat type. However this has yet to be examined for AM fungi. In the following chapter I address this uncertainty by examining aerial dispersal ability of AM fungal spores across different habitat types. Rather than focusing on elevation gradients however, the study focuses on a broader scale, a comparison of spore abundance in air samples across sites form different ecoregions across North America.

Chapter 5 Detection of arbuscular mycorrhizal fungal spores in the air across different biomes and ecoregions

5.1 Background

Many fungi produce spore-bearing structures that extend from the substratum to the open atmosphere (e.g., ascomata, basidiomata, conidiophores, acervuli, sporangia) and can thus release their spores into the air (Kendrick 2001). This allows these organisms to use air currents for dispersal (Aylor 1986; Viljanen-Rollinson et al. 2007). Many other fungi do not have obvious structures associated with air dispersal, and are more likely dispersed by other means (e.g., hypogeous ascomata such as truffles that depend on mycophagy and animal dispersal, and slimy spores that depend on water, rain or insect dispersal).

One important and cosmopolitan fungal group is the Phylum Glomeromycota (Schüβler et al. 2001), whose members form symbiotic arbuscular mycorrhizal (AM) associations with plants. These fungi are hypogeous and produce hyphae and asexual chlamydospores in the soil. The spores are large, typically greater than 50 μm in diameter (up to 1mm in some species). Dispersal of these fungi is mostly local via invertebrates, such as collembola (Klironomos and Moutoglis 1999) and earthworms (Gange 1993). Over longer distances it has been shown that AM fungi are able to distribute their spores with the aid of mammal vectors (Vernes and Dunn 2009) or sediment movement (Harner et al. 2009).

Aerial dispersal of spores is not considered to be significant, although studies have shown that AM fungal spores can be moved by wind (Tommerup 1982; Allen 1987; Allen et al. 1989), assuming these spores are carried into air currents from the surface of soil. AM fungi are found in all of the continents (Öpik et al. 2013; Davison et al. 2015), and many species seem to be geographic generalists (Moora et al. 2011) with broad geographic distributions (Öpik et al.

2010). Such patterns of distribution indicate that aerial transport may be a significant mode of dispersal for this group of fungi.

The objective of this study was to determine the frequency of occurrence of AM fungal spores in the air at several North American locations, across different biomes: temperate broadleaf mixed forests, temperate grasslands/savannas/shrublands, Mediterranean forests/woodlands/shrublands, deserts and xeric shrublands, tropical and subtropical moist broadleaf forests, and tropical and subtropical dry broadleaf forests.

5.2 Materials and Methods

5.2.1 Data collection

Air samples were taken from 18 North American ecoregions within 6 biomes (Listed in Table 5.1). Within each ecoregion 10 different locations were typically sampled at 4 times throughout the year (April/May 2010, July/August 2010, October/November 2010, and January/February 2011). Sampling locations were a minimum 1 km apart from each other (Table 5.1).

At each location, three 10-minute air sub-samples were taken at a height of 1 m above ground. Each air sub-sample was taken using a Samplair-MK 1 particle sampler (Allergenco, 403-7834 Broadway, San Antonio, Texas). The three sub-samples were pooled during analysis. Each subsample drew 9 L of air min^{-1} and particles were trapped on a microscope slide containing a thin layer of a mixture of 90% Vaseline and 10% high melting point wax (w/w) (li and Kendrick 1994, 1995). The slide was then mounted with polyvinyl lactophenol under a coverslip, and AM fungal spores were counted at 400X magnification. Morphology was used to identify spores to AM fungal genus (*Acaulospora, Glomus, Gigaspora, and Scutellospora*).

Table 5.1 Number of air samples taken from each Ecoregion and grouped by Biome.

Biome	Ecoregion	Geographic Location	Number of sampling locations
Temperate broadleaf and mixed forests	Central US hardwood forests	Lower Missouri Ozarks, southern Missouri	4
		Shawanee Hills, southern Illinois	6
	Northern coastal forests	Cape May national wildlife refuge, southern New Jersey	6
		Great Bay wildlife refuge, southeastern New Hampshire	4
	Southeastern mixed forests	Uwharrie national forest, central North Carolina	3
		Bienville national forest, east central Mississippi	2
	Southern Great Lakes forests	Long Point provincial park, Ontario	3
		Rondeau provincial park, Ontario	3
		Point Pelee provincial park, Ontario	4
	Appalachian mixed mesophytic forests	Shawnee state forest, Ohio	5
		Monongahela national forest, West Virginia	5
Temperate grasslands, savannas, and shrublands	Central and southern mixed grasslands	Wichita mountains wildlife refuge, Oklahoma	5
		Great salt plains, Oklahoma	5
	Central tall grasslands	Loess hills, Iowa	10
	Western short grasslands	Pawnee, Colorado	5
		Commanche, Colorado	2
Temperate Mediterranean forests, woodlands, and scrub	California coastal sage	Sage scrub, southern California	10
	California montane chapparal and woodlands	Santa Lucia range, coastal California	10
Temperate deserts and xeric shrublands	Sonoran desert	Arizona uplands, Arizona	10
	Wyoming Basin shrub steppe	Central Wyoming	10
	Great Basin shrub steppe	Great Basin national park, Nevada	10
	Chihuahuan desert	southern New Mexico	10
	Mojave desert	Mohave national park, California	10
Tropical and subtropical moist broadleaf forests	Great Antillean moist forest - Puerto Rico	Puerto Rican moist forest, Puerto Rico	5
Tropical and subtropical dry broadleaf forests	Jalisco dry forests - Pacific Mexico near Puerto Vallarta	Jalisco coast, Jalisco Mexico	5
	Sierra de la Laguna dry forest - Baja, Mexico	southern Baja, California	6

In addition to the air samples, soil samples were also collected at each location and time interval (a paired soil/air sampling scheme). This was done to determine the abundance of AM fungal spores found in the source environment (soil), and to relate aerial abundance to source abundance. Soil samples were chosen at random within each site. Each sample consisted of two 15-cm deep subsamples that were taken using a 2-cm diameter soil corer. Abundance of AM fungal spores was determined following extraction from the soil using a wet-sieving technique (Klironomos et al., 1993). Spores were identified to morphotype as described above.

5.3 Statistical analyses

Abundances of spores in the air and the soil were compared among North American biomes using a Kruskal-Wallis one-way analysis of variance by ranks test (Kruskal and Wallis 1952) using the R beeswarm package. Pairwise comparisons were made between biomes using a Wilcoxon rank sum test (Wilcoxon 1945) with a Bonferroni correction to a significance level at $P < 0.05$. The ratios (number of *Glomus* spores in the air: number of *Glomus* spores in the soil) were compared among biomes using a Kruskal-Wallis rank sum test at a significance level of $P < 0.05$. Pairwise comparisons were made using a Wilcoxon rank sum test with a Bonferroni correction at a significance level of $P < 0.05$.

5.4 Results

In most biomes and ecoregions some AM fungal spores were found in the air samples that were collected (Figure 5.1). However, across all locations, only the *Glomus* morphotype was detected in the air, even though a much broader variety of morphotypes were found in

the soil.

Even though spores were found in the air, they were detected at very low frequency. Of the 622 air samples collected, only 83 contained AM fungal spores (0.13 success rate). This is in contrast to the high abundance of spores of other fungi that were seen in all samples (mainly anamorphic fungi from the Phylum Ascomycota, data not presented here).

In Figure 5.1, the abundance of AM fungal spores in the air is summarized by biome. In all biomes, many air samples yielded no AM fungal spores. The highest number of spores was seen in deserts and xeric shrublands, and the fewest in tropical and subtropical moist broadleaf forests. Although there were more spores found in some biomes than others there was no statistical difference of AM fungal spore abundance in the air among biomes.

Glomus spore abundance in the soil (Figure 5.2a) differed significantly by biome (Kruskal-Wallis rank sum test; $df = 5$; $\chi^2 = 254.8202$; $P < 0.001$). Where *Glomus* spore abundance was greatest in broadleaf mixed forests, temperate grassland/shrublands, tropical dry broadleaf forests, and tropical wet broadleaf forests. *Glomus* spores were least abundant in deserts/dry shrublands and Mediterranean forests/woodlands/shrublands ($P < 0.001$).

Acaulospora and *Entrophospora* spore abundances in soil (Figure 5.2b) also differed significantly by biome (Kruskal-Wallis rank sum test; $df = 5$; $\chi^2 = 263.0915$; $P < 0.001$). The lowest abundance of spores was in the deserts/dry shrublands and Mediterranean forests/woodlands/shrublands, with a higher abundance in broadleaf/mixed forests, and the highest abundance in temperate grassland/shrublands, tropical dry broadleaf forests, and tropical wet broadleaf forests ($P < 0.001$).

Gigaspora and *Scutellospora* spore abundances in the soil (Figure 5.2c) were also significantly influenced by biome (Kruskal-Wallis rank sum test; $df = 5$; $\chi^2 = 84.4086$; $P <$

0.01). The lowest abundance of spores was found in broadleaf/mixed forests, deserts/dry shrublands, and Mediterranean forests/woodlands/shrublands. A higher abundance of spores was found in temperate grass/shrubland as well as tropical dry broadleaf forests, and the highest abundance as in tropical wet broadleaf forests ($P < 0.01$).

Since the *Glomus* morphotype was found in the air and soil, the ratio of *Glomus* spores in the air vs in the soil was compared (Figure 5.3). This ratio differed significantly by biome (Kruskal-Wallis rank sum test; $df = 5$; $\chi^2 = 198.64$; $P < 0.001$). Deserts and dry shrublands had the highest ratio of spores in the air versus the soil, followed by Mediterranean forests, woodlands, and shrublands. The lowest ratio was in the temperate grasslands, savannas, and shrublands and broadleaf mixed forests. Tropical dry and tropical wet biomes also had low ratios, albeit higher than those in temperate grasslands, savannas and shrublands.

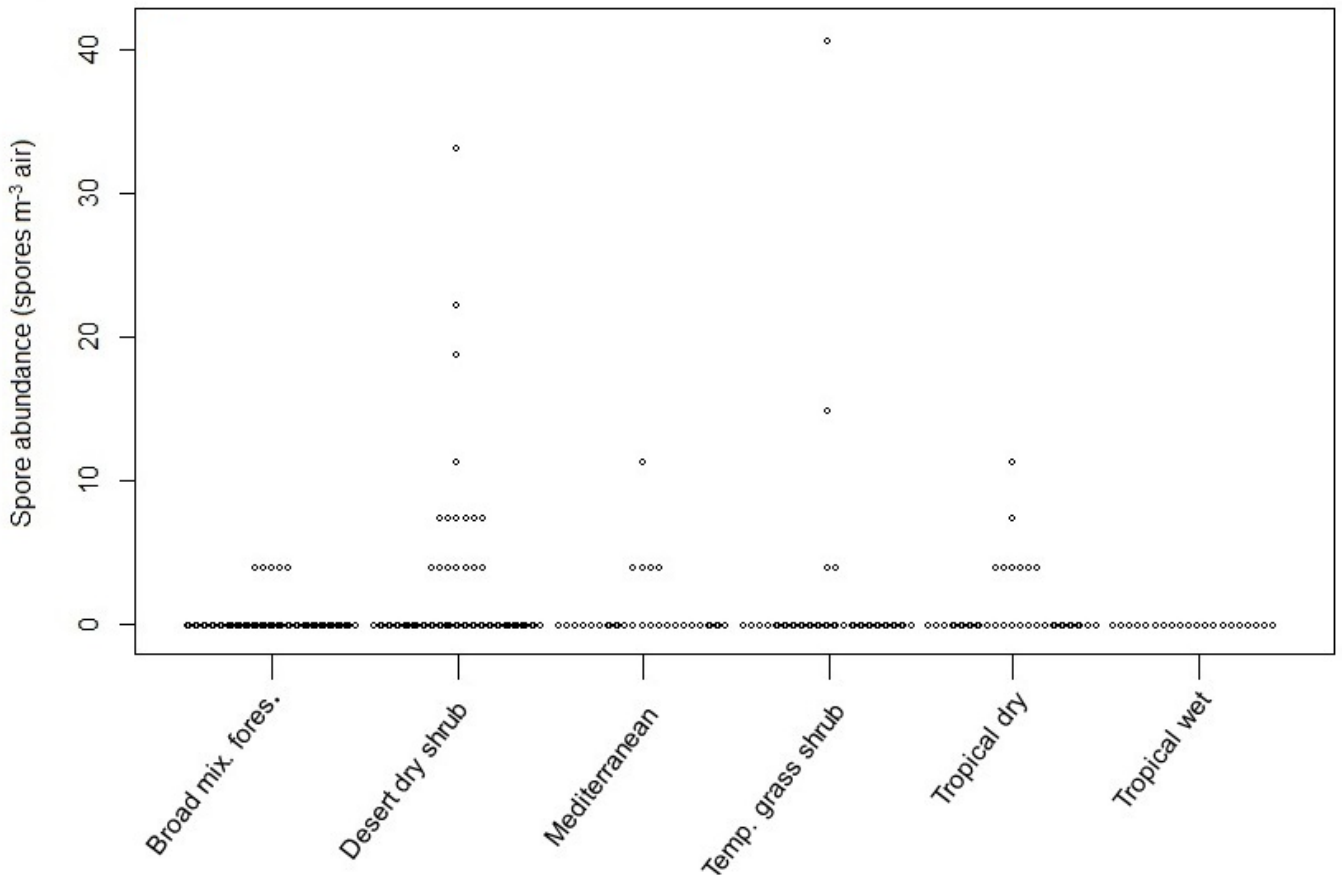
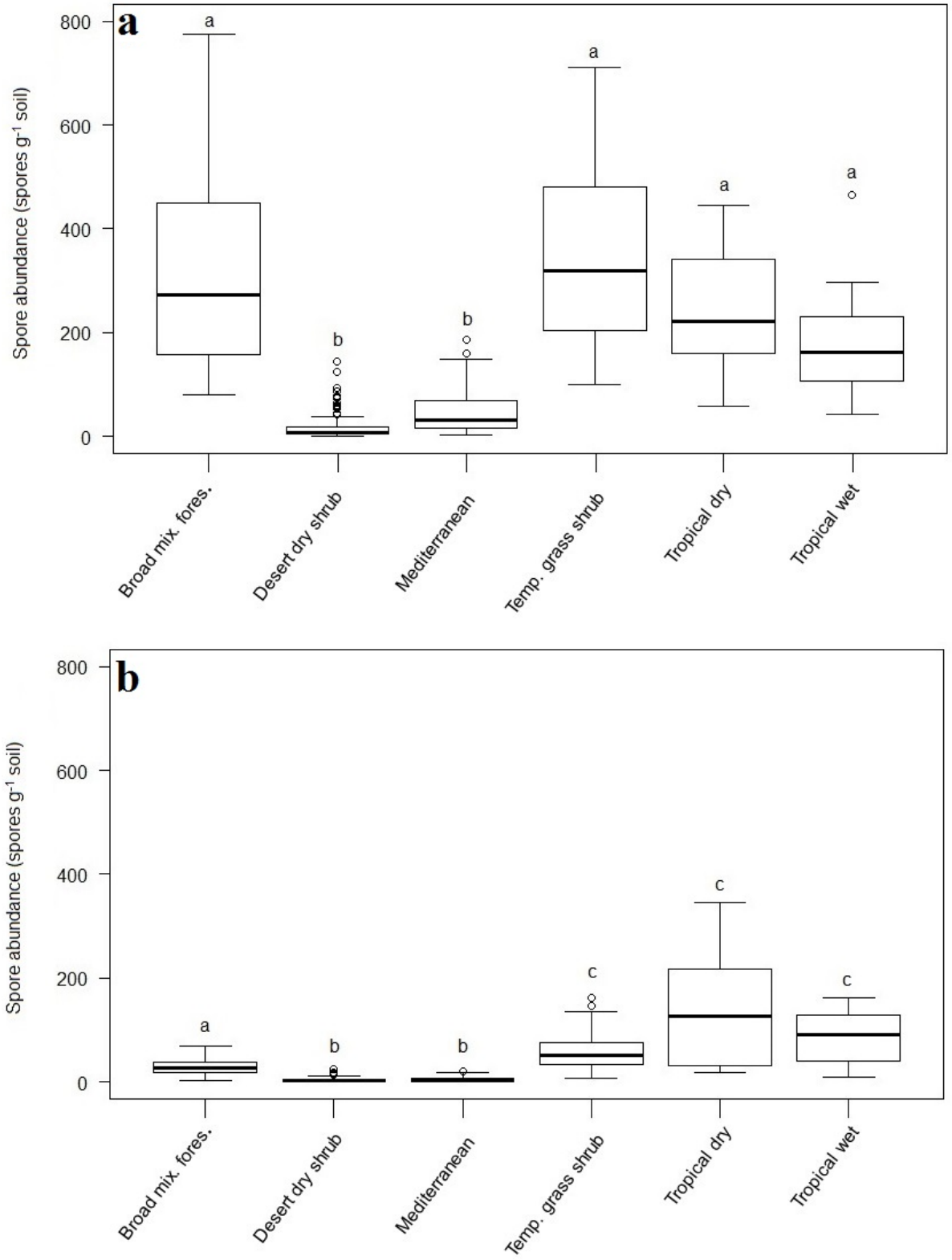


Figure 5.1 Dot plots showing the abundance of AM fungal spores in the air collected from six North American biomes; temperate broadleaf and mixed forests (n=45), temperate grasslands, savannas, and shrublands (n=27), temperate Mediterranean forests, woodlands, and scrub (n=20), temperate deserts and xeric shrublands (n=50), tropical and subtropical moist broadleaf forests (n=5), tropical and subtropical dry broadleaf forests (n=11). Abundances measured as the number of spores per m^3 of air. *Glomus* was the only morphotype detected in the air. Each dot represents a sample. No significant differences were found in spore abundance in the air.


a
b
Spore abundance (spores g-1 soil)
0
200
400
600
800
a
b
b
a
a
a
Broad mix. fores.
Desert dry shrub
Mediterranean
Temp. grass shrub
Tropical dry
Tropical wet
a
b
b
c
c
c

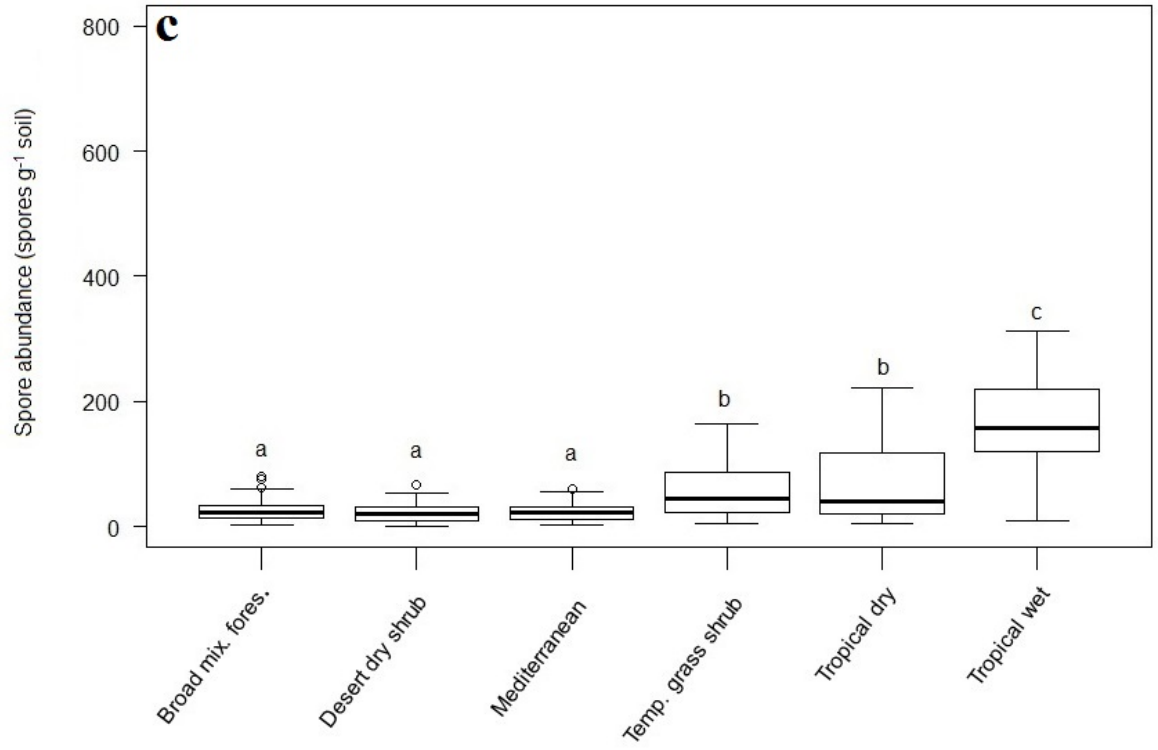


Figure 5.2 AM fungal spore abundances in the soil collected from six North American biomes; temperate broadleaf and mixed forests (n=45), temperate grasslands, savannas, and shrublands (n=27), temperate Mediterranean forests, woodlands, and scrub (n=20), temperate deserts and xeric shrublands (n=50), tropical and subtropical moist broadleaf forests (n=5), tropical and subtropical dry broadleaf forests (n=11). Boxplots display the abundances of *Glomus* spores (a), *Acaulospora* and *Entrophospora* spores (b), and *Gigaspora* and *Scutellospora* spores (c) in the soil. Abundance of spores in the soil were determined as number of spores per g of soil. The bottom and the top of the boxes represent the first and third quartiles, the dark band inside boxes represents the median, and the whiskers contain the upper and lower 1.5 interquartile range (IQR). A Kruskal Wallis test was used to detect significant differences among biomes for spore abundance of different AM fungal groups in the soil ($P < 0.001$). A post-hoc analysis using Wilcoxon rank sum tests with Bonferroni corrections was used to determine significant differences among biomes at $P < 0.01$ and are indicated by different letters above the boxes.

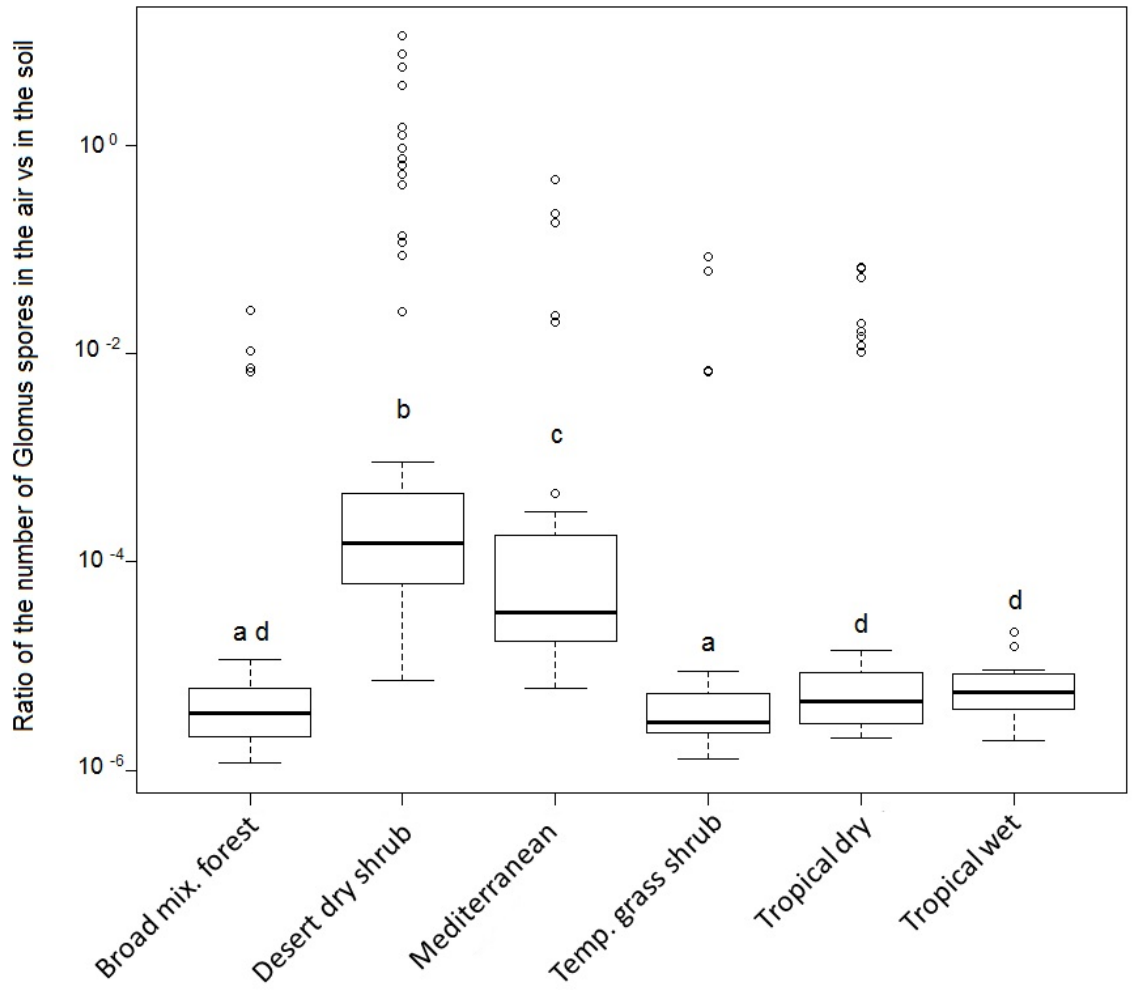


Figure 5.3 Ratio of the number of *Glomus* spores in the air versus in the soil among six North American biomes; temperate broadleaf and mixed forests (n=45), temperate grasslands, savannas, and shrublands (n=27), temperate Mediterranean forests, woodlands, and scrub (n=20), temperate deserts and xeric shrublands (n=50), tropical and subtropical moist broadleaf forests (n=5), tropical and subtropical dry broadleaf forests (n=11). Ratio values are log transformed to correct for the large skew in the data caused by a high number of small ratio values. A Kruskal Wallis rank sum test was used to detect significant differences among biomes ($P < 0.001$). Different letters indicate significant differences ($P < 0.05$) following Wilcoxon rank sum tests with a Bonferroni correction.

5.5 Summary

While AM fungal spores are present in the air, and are able to be dispersed over long distances by wind (Allen 1987), wind dispersal does not appear to be a major contributor to long range dispersal of AM fungal taxa. In the 168 m^3 of air sampled, only 165 AM fungal spores were found, a very low number compared to millions of Ascomycete and Basidiomycete spores that are typically observed with similar techniques (including in this study, data not presented) (Li and Kendrick 1994; Li and Kendrick 1995). AM fungal spores are typically much larger than ascomycete spores, and it is possible that some AM fungal spores did not stick to the thin layer of Vaseline/wax in the air sampler. Nonetheless, a large number of pollen grains were found in each sample that were as large as 80μm in diameter, and many other miscellaneous particles that were as large as 1mm.

Despite the results observed I remain cautious about making any inferences regarding the importance of aerial dispersal in AM fungi using the present data. Although many samples were taken across a wide variety of ecosystem types, the total volume of air sampled was still somewhat small. Sampling efforts consisted of three air samplers collecting air for 10-minute periods placed 1 m above the ground. It is difficult to know how the results would differ if samples were taken for shorter or longer times, or at different heights. Also, assuming that the observed low spore concentrations (during 10 minute sampling intervals) are consistent throughout the day, the total number of spores that may be moved by air over a longer time period may be quite large. For example, a concentration of 40 spores m^{-3} that was observed in one temperate grassland location is equivalent to 176,600 spores over a monthly period, which is a substantial number of spores that may travel by air across one spatial point. However, I do not have any information on how spore concentrations vary over time

throughout the day/week/month, so such extrapolations are not possible at this time.

A large number of AM fungal spores in the soil. Thus, I can dismiss the possibility that spores were not detected in the air because they were not present locally in the soil. In particular, *Glomus* spores were found in the highest abundances across all biomes sampled (Figure 5.2a) possibly contributing to their dominance in the air samples. However, I expected at least a few samples to contain some of the other morphotypes. That the other types were absent suggests that they are not as easily carried by wind as some species of *Glomus*. Interestingly, across all biomes, the pattern of abundance of *Glomus* spores in the air was not correlated to spore abundance in the soil. The lowest abundances in the soil were observed in the driest biomes (deserts and dry shrublands, and as well as Mediterranean forests/woodlands/shrublands (Figure 5.2a). However, those biomes contained relatively high concentrations of spores in the air (Figure 5.1). This indicates that spores are more likely to be lifted into the air in ecosystems where the surface soil is dry and may become more easily airborne.

Overall, it is clear from geographic patterns that AM fungi have dispersed over wide distance (e.g. Rosendahl et al. 2009). Certain AM fungal taxa, such as *Glomus intraradices/fasciculatum* group, *G. mosseae* and *G. hoi* have a broad global distribution (Öpik et al. 2006). The present study indicates that wind is one of many possible vectors for dispersal. However, unlike other fungal groups it appears that long-range dispersal by wind is not likely the primary method that has allowed these fungi to be ubiquitous globally (Öpik et al. 2010). Instead, these fungi have likely spread using multiple smaller dispersal moments such as soil movement or animal/invertebrate vectors. These fungi have been present since plants first colonized terrestrial environments (Remy et al. 1994) allowing for a large

timescale (at least 400 million years since they diverged from other fungi (Redecker and Raab 2006) in which small dispersal efforts could be combined to reach the current global distribution. Surprisingly, there is a scant amount of literature on investigations of AM fungal dispersal. I believe that this is an area of research deserving more attention considering that mycorrhizal fungal inocula is transported globally (Schwartz et al. 2006). We need a much better understanding of the fate of these fungi following release and their ability to spread to new locations.

Chapter 6 Conclusion

6.1 General Discussion

In this thesis I mainly focused on AM fungal communities along an elevation gradient. Such systems have not been well studied, and my objective was to use such a gradient to increase our understanding of how natural AM fungal communities are structured. Throughout the introduction I provided a background of several key factors that structure AM fungal communities including; dispersal, abiotic environmental determinants (temperature, soil chemistry, and soil structure), and partner selection. During my review of the literature I recognized that the majority of studies on AM fungal community structure have been conducted in a narrow set of habitat types, mainly temperate grasslands and old fields. While the frequency of studies has been increasing in other habitat types, especially with the advent of NGS, these studies still remain in the minority. I also recognized that while NGS is growing in popularity among AM fungal research, inference about species abundances operates under the assumption that sequence abundance accurately depicts what is present within samples. However, to date this concept has not formally been examined. In this thesis, I have attempted to fill some of these gaps in our understanding.

The use of sequence data to determine diversity in AM fungal communities has become routine (see Figure 1 in Öpik et al., 2014), therefore it is important to investigate the fidelity of NGS in capturing AM fungal diversity. While mock communities have been used with bacteria (Caporaso et al. 2011; Bokulich et al. 2013; Brooks et al. 2015) and other fungal groups (Nguyen et al. 2015) to determine accuracy of NGS and to improve bioinformatic analysis pipelines, their use with AM fungi has been lacking. In chapter 2, I used four mock communities, each composed of the same AM fungal species but differing in what species are dominant, and

examined the ability of NGS to reflect these differences in diversity. I found that the relative abundance and identity of OTUs, when identified to the family level, closely resemble expected abundances. However, this was not the case when sequences were identified to lower taxonomic levels. In addition to examining the ability of NGS to reflect sample diversity, I also examined the ability of two common AM fungal sequence databases, SILVA and MaarjAM, to accurately assign taxonomy to sequences. I observed taxon assignments using SILVA to be less accurate than MaarjAM. I also noted that NGS may introduce some bias in terms of relative abundance estimates and taxonomic identification. Also, multiple OTUs were detected within single spores, suggesting that universal thresholds may inflate richness and reflect sequence variation within individuals.

In chapters 3 and 4 I sampled along a high elevation gradient in the North American Rocky Mountains, to compare how AM fungal community structure is impacted by environmental changes along elevation gradients. Scenic Point provides a good location to conduct research on the impact of elevation on community structure, as plant communities are continuous along the slope, and climatic variables such as mean annual temperature and solar radiation change along the gradient, creating unique environmental filters within habitats found along the gradient. Furthermore, Scenic Point is located within the protected boundaries of Glacier National Park, reducing anthropogenic disturbances, and therefore making communities representative of naturally assembled AM fungi.

To begin exploring how AM fungal communities vary among habitat types, I investigated AM fungal phylogenetic community structure along an elevation gradient (chapter 3). I used NGS tools to characterize AM fungal taxa collected from the soil, and then determined phylogenetic relationships among sequences through bioinformatic analyses. I found that alpine

AM fungal communities had lower phylogenetic diversity relative to lower elevation communities. Alpine communities were also more heterogeneous in composition than treeline and subalpine communities. These patterns suggest that a reduced number of fungal clades are able to persist at higher elevations. I also found that AM fungal communities were phylogenetically clustered at all elevations, suggesting that environmental filtering (either selection by host plants or fungal niches) is the primary ecological process structuring communities along the gradient. This research provides novel insight into AM fungal community assembly in natural systems, as most published research has focused on taxon-based approaches to examine community structure (Gai et al. 2012; Geml et al. 2014; Liu et al. 2015a; Looby et al. 2016). Through this work I contribute to the growing body of literature showing that AM fungi are phylogenetically clustered at local scales (Kivlin et al. 2011; Horn et al. 2014; Liu et al. 2015b).

To further examine factors contributing to AM fungal community structure along environmental gradients, I conducted a study (chapter 4) that examined the influence of host selection along the same elevation gradient used in chapter 3. Debate exists as to whether AM fungi experience host selection, and whether host selection is a strong influence on AM fungal community structure. While some studies have found that plant species can have a strong effect on AM fungal community structure (e.g. Eom et al. 2000; Mangan et al. 2010; Martínez-García and Pugnaire 2011; Torrecillas et al. 2012b; Martínez-García et al. 2015), others have observed habitat filtering, and local environmental conditions, are a stronger determinant (Kivlin et al. 2011; Veresoglou and Rillig 2014; Lekberg and Waller 2016). To better disentangle the two contributing factors (habitat filtering versus host selection), I characterized the fungal communities associated with the same three hosts along an environmental gradient. Mainly, I

found that AM fungal communities, both in roots and soil, are strongly influenced by habitat type, a pattern that lends support for habitat type being more important for determining AM fungal community structure than plant hosts. Interestingly, the highest similarity among AM fungal communities was observed between treeline and alpine habitat types indicating that in extreme environments with high environmental stress (in this case decreased annual temperatures and adverse soil conditions) select for distinct fungal taxa. This notion is supported by an observed decrease in AM fungal richness at higher elevations, a pattern indicative of species being limited by physiological tolerances (Currie et al. 2004). Furthermore, this pattern matches those observed in chapter 3, and highlights that AM fungal taxa respond to environmental stress differently. While the literature addressing physiological tolerances of AM fungi is limited, there are a few studies that suggest that AM fungal taxa do experience environmental constraints (Gavito et al. 2000; Hawkes et al. 2008), and that abiotic conditions can influence AM fungal richness (Xiang et al. 2016).

In my final data chapter, I assessed the extent to which AM fungi can disperse through the air across different habitat types, as this would give an indication if AMF communities that are separated spatially can mix readily via aerial dispersal. I found AM fungal spores to appear in high abundances in the soil (hundreds of spores per gram of soil) in every ecoregion sampled. However, AM fungal spores were rarely found in the air (most samples contained no AM fungal spores). Furthermore, only the *Glomus* morphotype was found in the air, whereas spores in the soil were taxonomically more diverse (with *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora* morphotypes all being observed). I noted that the proportion of *Glomus* spores in the air relative to the soil was highest in more arid systems, indicating that AM fungi may be more likely to be dispersed in the air in such habitat types. The patterns observed in chapter 5 therefore appear to

capture a similar pattern to chapters 3 and 4, lending support for AM fungal community structure being primarily determined by habitat type. There is likely little mixing across elevations from aerial dispersal. In particular, this study highlights the importance of examining assembly processes of AM fungal communities across a variety of habitat types, as these processes can be context dependent.

6.2 Assumptions and Limitations

Despite the encouraging results obtained in the studies that constitute this thesis, there are limitations to the work described. First and foremost are the results obtained from my mock community analysis in chapter 2. While the patterns are interesting and provide insight, the scope of the study is limited. By using artificial communities composed of DNA templates, rather than beginning the analysis from mock communities composed of known identities and abundances of AM fungal spores, I bypassed any biases that could potentially be introduced during sample collection, sample preservation, and DNA extraction. This is important to note, as even small differences in sample handling (Hart et al. 2015), extraction (Lindahl et al. 2013), and choice of library preparation and primers (Reeder and Knight 2009; Schirmer et al. 2015) have been shown to introduce variation in sequence results among samples. To better understand what biases exist with NGS investigation of AM fungal diversity, a more comprehensive study should examine biases along the entire pipeline, which would include biases bypassed in my study.

In chapter 2, I also observed that single AM fungal spores contain multiple OTUs, indicating that OTUs represent both intra- (Pawlowska and Taylor 2004) and inter-species diversity (Kuhn et al. 2001; Hijri and Sanders 2005). This indicates that spores collected from the field have the potential to bias the results post sequencing. However, I found that all OTUs

from spores matched to the taxonomic identity of the isolate used to extract DNA (*Rhizophagus intraradices*). This highlights the importance of identifying AM fungal OTUs to a curated database prior to diversity analyses (Tedersoo et al. 2010; Öpik et al. 2014). Despite these limitations, results from Chapter 2 do indicate that abundance of AM fungal DNA templates within samples are reflected post-sequencing, allowing for quantitative analysis of NGS datasets.

A second limitation to the thesis was the restricted sampling carried out for chapter 3. Because I sampled within the protected boundaries of Glacier National Park, my sampling scheme was limited to specific areas within each elevation. In an attempt to control for this limited sampling distribution, I utilized a pre-determined sampling pattern before entering the field, with the goal being to reduce bias. Given that different AM fungal species are not distributed similarly in space (Wolfe et al. 2007), and species-area relationships show that species richness tends to increase with increasing sampling area (Drakare et al. 2006), I chose a sampling scheme that would optimize distances within my sampling area, allowing for sampling points to be separated by multiple distances. While this sampling scheme is enough to establish trends along the gradient examined, it is highly restricted to a few sites, making it difficult to make strong conclusions about general patterns of AM fungi along gradients.

A second difficulty I experienced with research conducted for chapter 3 was a high sample loss during molecular analysis. Many samples did not yield product, even after months of altering my amplification protocol in several ways; touchdown PCR (Korbie and Mattick 2008), nested PCR, changing reagents, changing thermocycler conditions. I therefore concluded that those samples likely contained low concentrations of AM fungal DNA, if any at all. Also, I note that my randomized sampling design resulted in taking samples from spots that were not near plant hosts, often on rocky substrates. Such substrates may have been inhospitable for AM fungi,

further reducing environmental template availability. I addressed that limitation when I sampled the same gradient for chapter 4, by using Illumina sequencing, which resulted in a 1,000-fold increase in the number of sequences (Caporaso et al. 2011). In addition, I sampled roots and the soil directly beneath plant hosts in chapter 4, increasing the possibility of unearthing AM fungal DNA in all samples.

6.3 Future Research Directions

If I had more time and resources, I would attempt to apply the techniques used in chapter 2 to artificially-assembled AM fungal spore communities in the soil environment. By sampling artificial communities in the soil, I would be better able to determine the efficacy of current techniques (sampling, DNA extraction, amplification, sequencing, and bioinformatics) in capturing AM fungal diversity. My analysis in chapter 2 is limited to statements of the accuracy of amplification and sequencing techniques for AM fungi, as the mock communities created post-extraction using different DNA concentrations. By applying the same techniques to AM fungal spore communities, I will be better able to address strengths and shortcoming of all stages of molecular investigation of AM fungal diversity: sample collection/preservation, DNA extraction, DNA amplification, sequencing, and bioinformatic processing. In addition, as I observed spores of *R. irregularis* to harbour multiple OTUs, I recommend that future research examine sequence diversity within single spores of other AM fungal species, to determine how sequence diversity within spores varies among AM fungal species, and how this might translate to sequence diversity.

Results from this thesis support the hypothesis that habitat type is a major determinant of AM fungal community structure, and influences AM fungal community assembly processes. In

this case, AM fungal community composition differed most when comparing a relatively low-stress/high productivity environment to a high-stress/low productivity environment. Future research should focus on the effects that stress gradients have on the functioning of AM fungi with host species. It has been well documented that plant-plant interactions vary with gradients of stress (Choler et al. 2001; Callaway et al. 2002). Facilitative (or positive) interactions are more frequent under high environmental stress (Callaway et al. 2002). Interactions between plants and AM fungi have also been shown to vary along environmental gradients, where interactions of AM fungi with hosts can be placed upon a continuum of parasitism to mutualism depending on environmental conditions (Johnson 1993). However, that research has been conducted primarily along gradients of soil fertility, omitting the influence of other environmental conditions on mycorrhizal functioning. I expect that, as per the stress-gradient hypothesis (Bertness and Callaway 1994), facilitative (i.e. mutualistic) interactions between hosts and fungi will be more frequent in conditions of high abiotic stress relative to more favourable abiotic conditions.

Finally, in chapter 3 I observed AM fungal communities to display community patterns at higher elevations indicative of AM fungal communities being shaped by limited dispersal or dispersal barriers. I observed in chapter 5 that aerial dispersal differs among habitat types, indicating that AM fungi likely disperse via other mechanisms in those environments. Further research should examine how AM fungal dispersal methods (air, animal vectors, extraradical mycelial movement etc.) varies across habitats. From an ecological perspective, dispersal method for AM fungi will have a significant effect on local, regional, and landscape scale diversity patterns of AM fungi. While previous studies have shown that AM fungi to disperse via multiple mechanisms (Klironomos and Hart 2002), how these dispersal mechanisms vary across environmental conditions remains to be explored.

With constant improvements to molecular techniques there is ample opportunity to examine biogeographic patterns of AM fungi in their natural environment. By pairing biogeographic studies with the examination of mycorrhizal functioning, our understanding of the AM symbiosis will continue to develop, advancing our understanding of AM fungal communities in natural ecosystems.

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Appendices

Appendix A - Supporting Materials for Chapter 2

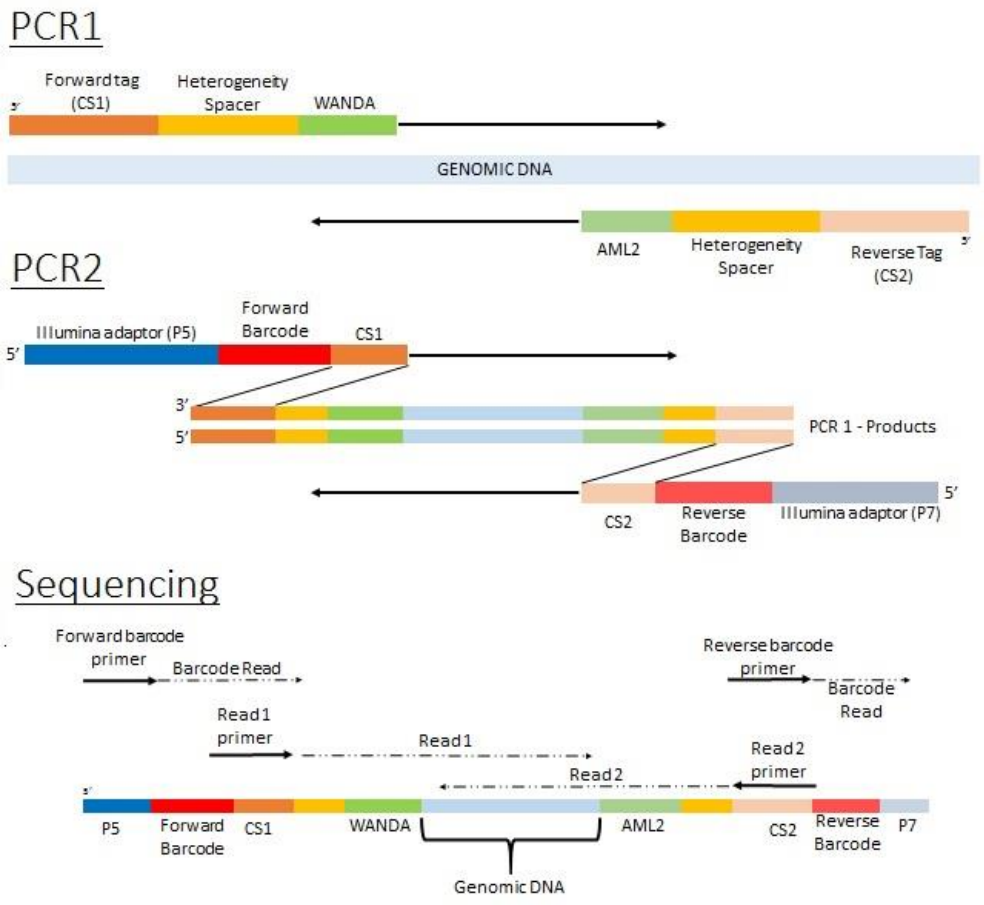

Figure A.1 PCR and sequencing workflow using the proposed dual index/paired end sequencing protocol on the Illumina MiSeq platform. During PCR1, the target region of extracted DNA is amplified using target specific primers WANDA (SI from Dumbrell et al. 2011) and AML2 (Lee et al. 2008). Attached to the target primers are the heterogeneity spacer regions (yellow) where nucleotides are added to increase heterogeneity of amplicon libraries being sequenced (Fadrosh et al., 2014). The universal Fluidigm tags (Fluidigm Inc. San Francisco, CA, USA) CS1 and CS2 act as sticky ends for PCR2 reaction. PCR2 reaction adheres Illumina adaptors P5 and P7 (Illumina Inc., San Diego, CA, USA), along with forward and reverse Nextera barcodes on PCR products created during the PCR1 reaction. PCR2 products are sequenced using 2 x 300 paired-end reads using 600 cycles on an Illumina MiSeq sequencing platform. Post-sequencing four sequence read files are generated; read 1, read 2, forward barcode read, and reverse barcode read.

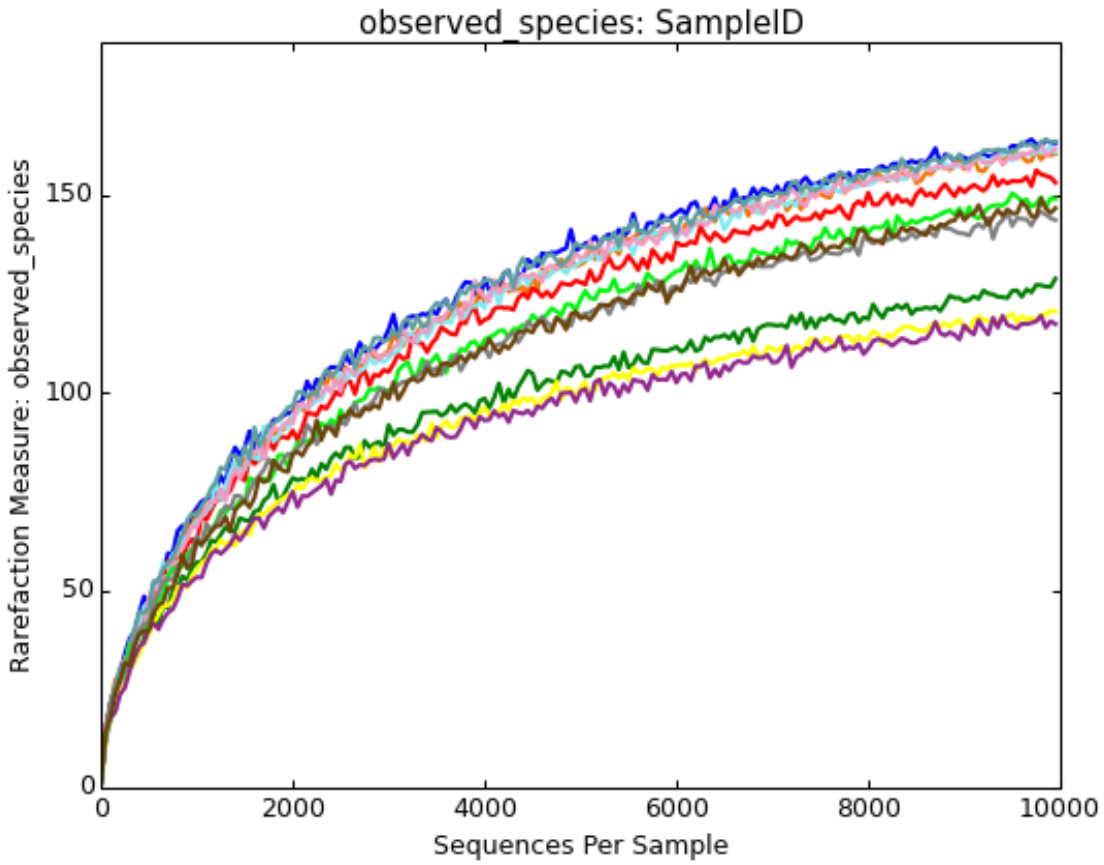

Figure A.2 Accumulation curve of operational taxonomic unit (OTU) richness of Glomeromycotan sequences from individual samples. Lines represent single samples (n=12). OTUs were created by clustering sequences ≥97% similarity.

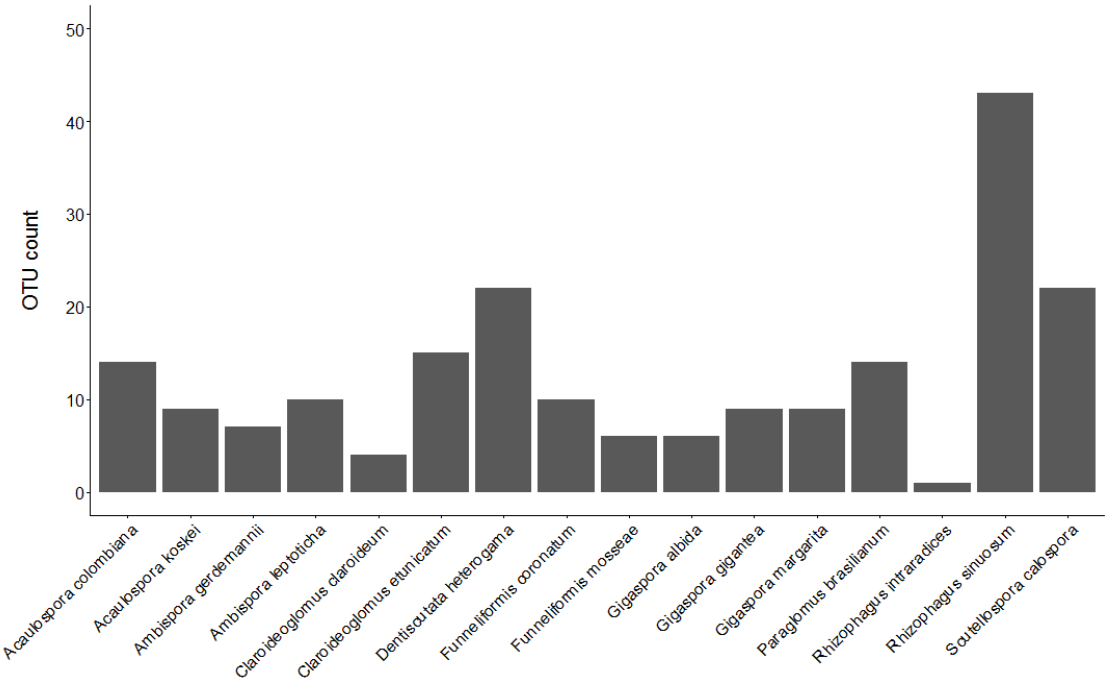

Figure A.3 Observed operational taxonomic unit (OTU) counts for each AMF isolate included in mock communities. Counts represent total number of OTUs identified to each isolate using the custom database of Sanger Sequences.

Table A.1 In silico PCR analysis of target primers against type sequences from MaarjAM (NCBI accession numbers shown in first column). For both target primers, PCR properties analyzed include; primer complementarity to binding sites (measured as a percentage of the total primer length), primer annealing/melting temperature (Tm), number of identifiable binding sites, and resulting fragment size, calculated as the number of total base pairs (bp).

NCBI Accession	Family	WANDA complementarity (%)	WANDA Tm (°C)	WANDA binding sites	AML2 complementarity (%)	AML2 Tm (°C)	AML2 binding sites	Fragment size (bp)
AY394664	Acaulosporaceae	100	59.7	1	99	55.3	1	544
JF414178	Acaulosporaceae	100	59.7	1	100	55.3	1	544
AB015052	Ambisporaceae	100	59.7	1	95	49.6	1	541
AM268193	Ambisporaceae	100	59.7	1	95	49.6	1	542
FN820273	Ambisporaceae	100	59.7	1	95	55.3	1	540
HQ424224	Ambisporaceae	100	59.7	1	100	55.3	1	540
FN869849	Archaeosporaceae	100	59.7	1	100	55.3	1	538
AJ276087	Claroideoglomeraceae	100	59.7	1	100	55.3	1	548
AF202280	Claroideoglomeraceae	100	59.7	1	100	55.3	1	547
HE615032	Claroideoglomeraceae	100	59.7	1	100	55.3	1	545
X86687	Diversisporaceae	100	59.7	1	100	55.3	1	544
Y17650	Diversisporaceae	100	59.7	1	100	55.3	1	544
FN869704	Diversisporaceae	100	59.7	1	100	52.3	1	544
JN252443	Diversisporaceae	100	59.7	1	91	55.3	1	544
FR865460	Diversisporaceae	100	59.7	1	100	39.6	1	543
AM418543	Diversisporaceae	100	59.7	1	89	49.6	1	543
Y15904	Geosiphonaceae	100	59.7	1	95	55.3	1	543
U96146	Gigasporaceae	100	59.7	1	100	55.3	1	539
Z14012	Gigasporaceae	100	59.7	1	100	55.3	1	538
AJ306436	Gigasporaceae	100	59.7	1	100	55.3	1	537

NCBI Accession	Family	WANDA complementarity (%)	WANDA Tm (°C)	WANDA binding sites	AML2 complementarity (%)	AML2 Tm (°C)	AML2 binding sites	Fragment size (bp)
AJ306434	Gigasporaceae	100	59.7	1	100	55.3	1	538
AJ242729	Gigasporaceae	100	59.7	1	100	55.3	1	540
AJ306437	Gigasporaceae	100	59.7	1	100	55.3	1	540
Y17635	Glomeraceae	100	59.7	1	100	55.3	1	543
AJ306438	Glomeraceae	100	59.7	1	100	55.3	1	543
AJ699070	Glomeraceae	100	59.7	1	100	55.3	1	542
DQ336496	Glomeraceae	100	59.7	1	100	55.3	1	543
Y17648	Glomeraceae	100	59.7	1	100	55.3	1	541
DQ336444	Glomeraceae	100	59.7	1	100	55.3	1	542
AF213462	Glomeraceae	100	59.7	1	100	55.3	1	542
AJ619948	Pacisporaceae	100	59.7	1	100	55.3	1	544
AF202299	Paraglomeraceae	100	59.7	1	100	55.3	1	553
AJ276081	Paraglomeraceae	100	59.7	1	100	55.3	1	539
AJ301862	Paraglomeraceae	100	59.7	1	100	55.3	1	540
AM295493	Paraglomeraceae	100	59.7	1	98	52.4	1	539
FN646035	Paraglomeraceae	100	59.7	1	100	55.3	1	542
FR821542	Paraglomeraceae	100	59.7	1	100	55.3	1	541

Table A.2 Number of operational taxonomic units (OTU) identified to each AMF isolate used to generate mock communities.

AMF isolate	OTU count
Paraglomus brasilianum	14
Rhizophagus sinuosum	43
Rhizophagus intraradices	1
Funneliformis mosseae	6
Funneliformis coronatum	10
Scutellospora calospora	22
Gigaspora margarita	9
Gigaspora gigantea	9
Gigaspora albida	6
Dentiscutata heterogama	22
Claroideoglomus etunicatum	15
Claroideoglomus claroideum	4
Ambispora leptoticha	10
Ambispora gerdemannii	7
Acaulospora koskei	9
Acaulospora colombiana	14

Table A.3 Deviation of observed proportions from expected when sequences were identified to the family (a) or species (b) level using our custom database. Cell values are average standardized residual values when observed sequence proportions were compared to expected proportions. Standardized residuals were calculated by subtracting expected proportions from observed proportions and dividing the difference by the square root of the estimated standard error (Agresti, 2013). Standardized residuals were then averaged across replicates from the same mock community. Negative values indicate smaller proportions than expected, positive values indicate greater proportions than expected. Standardized residuals differing significantly from expected proportions are indicated by asterisks beside values, where no asterisk indicates no significance, * indicates significance at 0.05, and ** indicate significance ≤ 0.01.

(a)	**Mock Community**							
AMF Family	**Even**		**Glomeraceae Dominant**		**Less Dominant Glomeraceae**		**Gigasporaceae Dominant**	
Acaulosporaceae	-0.3		1.8		1.02		-2.15	*
Ambiosporaceae	-24.6	**	-0.1		-7.82	**	-2.93	**
Claroideoglomeraceae	5.68	**	11.74	**	7.84	**	3.19	**
Gigasporaceae	4.13	**	0.27		3.25	**	-1.23	
Glomeraceae	-1.6		-9.81	**	-11.62	**	-0.61	
Paraglomeraceae	11.61	**	4.8	**	11.69	**	4.09	**

(b)		Mock Community							
AMF Family	**AMF Species**	**Even**		**Glomeraceae Dominant**		**Less Dominant Glomeraceae**		**Gigasporaceae Dominant**	
Acaulosporaceae	*Acaulospora colombiana*	3.19	**	4.08	**	3.1	**	2.06	*
	Acaulospora koskei	-3.75	**	-1.92		-1.87		-4.67	**
Ambiosporaceae	*Ambispora gerdemannii*	-15.96	**	-1.67		-6.7	**	-4.38	**
	Ambispora leptoticha	-16.43	**	1.16		-4.47	**	0.73	
Claroideoglomeraceae	*Claroideoglomus claroideum*	-15.51	**	-4.44	**	-14.78	**	-13.78	**
	Claroideoglomus etunicatum	16.07	**	15.86	**	18.11	**	12.25	**
Gigasporaceae	*Dentiscutata heterogama*	-8.31	**	-2.3	*	-2.39	*	-14.54	**
	Gigaspora albida	-20.85	**	-5.67	**	-10.49	**	-32.33	**
	Gigaspora gigantea	-19.06	**	-5.26	**	-8.62	**	-30.85	**
	Gigaspora margarita	31.6	**	7.19	**	14.7	**	45.76	**
Glomeraceae	*Scutellospora calospora*	-3.84	**	-0.75		0.57		-4.63	**
	Funneliformis coronatum	6.1	**	38.93	**	31.58	**	9.06	**
	Funneliformis mosseae	-12.39	**	-42.41	**	-32.97	**	-8.75	**
	Rhizophagus intraradices	-9.99	**	-43.23	**	-35.58	**	-10.18	**
	Rhizophagus sinuosum	-2.36	*	9.01	**	-4.23	**	0.65	
Paraglomeraceae	*Paraglomus brasilianum*	10.55	**	4.81	**	11.7	**	4.19	**

Table A.4 Variation in AM fungal community composition with elevation as determined by permutational multivariate analysis of variance using distance matrices (PERMANOVA; Anderson 2001), using Bray-Curtis (Bray and Curtis 1957) distances among samples. PERMANOVA results shown for when I identified OTUs using custom (a), MaarjAM (b), and Silva (c) databases. Values shown include degrees of freedom (DF), sum of squares (SS), and mean squares (MS).

	DF	SS	MS	PseudoF/F	R2	p
(a) Custom Database PERMANOVA						
Mock Community	3	0.87539	0.2918	1137.5	0.99766	3.00E-04
Residuals	8	0.00205	0.00026		0.00234	
Total	11	0.87744			1	
(b) MaarjAM PERMANOVA						
Mock Community	3	0.85263	0.28421	1149	0.99768	1.00E-04
Residuals	8	0.00198	0.00025	0.00232		
Total	11	0.85461	1			
(c) Silva PERMANOVA						
Mock Community	3	0.68429	0.2281	779.82	0.99659	1.00E-04
Residuals	8	0.00234	0.00029	0.00341		
Total	11	0.68663	1			

Table A.5 Species by sample tables depicting the number of sequences for each taxonomic assignment for each mock community. Operational taxonomic units (OTUs) were assigned taxonomically using; a custom database, MaarjAM, and Silva.

Taxonomy Assignment				Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
1423	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	0	0	0	0	0	0	1	0	1	7	5	3
1856	Acaulospora colombiana	VTX00328 (Acaulospora sp.)	Cystofilobasidium	0	0	1	3	1	4	2	0	2	0	0	1
3582	Acaulospora colombiana	VTX00328 (Acaulospora sp.)	Sclerocystis sinuosa	6	2	2	2	1	0	1	6	4	0	0	0
398	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	8	13	8	0	0	0	0	0	0	4	1	3
4157	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	14	11	8	1	0	2	1	5	4	24	34	34
4350	Acaulospora colombiana	VTX00402 (Claroideoglomus Torrecillas12b Glo G5)	Glomeromycetes sp.	1	1	1	5	3	1	3	3	1	1	0	0
4457	Acaulospora colombiana	VTX00328 (Acaulospora sp.)	Scutellospora projecturata	2	3	4	1	1	1	1	2	1	1	1	1

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
5324	Acaulospora colombiana	VTX00249 (Kuklospora PT6)	Acaulospora colombiana	2	1	3	7	8	8	2	3	4	0	0	0
5539	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	1	2	0	3	1	0	2	3	1	0	0	0
5710	Acaulospora colombiana	VTX00249 (Kuklospora PT6)	Acaulospora colombiana	14	18	13	1	0	1	1	3	2	3	6	7
5918	Acaulospora colombiana	VTX00102 (Acaulospora Acau16)	Gigaspora margarita	2	1	3	0	1	0	1	1	6	1	1	0
6332	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	0	0	0	0	0	0	2	1	3	5	1	2
6468	Acaulospora colombiana	VTX00249 (Acaulospora colombiana)	Acaulospora colombiana	1	0	2	3	3	1	1	1	1	1	0	0
6531	Acaulospora colombiana	VTX00328 (Acaulospora sp.)	Glomus sp. NBRrmc	2	1	2	2	0	2	1	2	4	1	0	1
2263	Acaulospora koskei	VTX00028 (Acaulospora Acau10)	Glomus sp. NBRrmc	0	0	1	1	0	0	0	1	1	3	3	2

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
2722	Acaulospora koskei	VTX00028 (Acaulospora Acau10)	Gigaspora margarita	0	1	1	2	1	4	11	8	7	85	68	73
337	Acaulospora koskei	VTX00028 (Acaulospora Acau10)	Glomeromycetes sp.	1	1	2	0	0	0	0	0	0	4	0	1
4507	Acaulospora koskei	VTX00328 (Acaulospora sp.)	Sclerocystis sinuosa	7	2	5	1	0	1	2	4	2	2	4	4
47	Acaulospora koskei	VTX00308 (Paraglomus Glom 1B.13)	Acaulospora spinosa	0	0	0	6	3	2	1	4	5	0	0	0
5302	Acaulospora koskei	VTX00024 (Acaulospora sp.)	Acaulospora spinosa	3	2	3	4	1	0	6	3	2	3	2	6
5335	Acaulospora koskei	VTX00028 (Acaulospora Acau10)	Gigaspora margarita	3	1	2	1	0	0	3	0	1	2	4	3
5862	Acaulospora koskei	VTX00328 (Acaulospora sp.)	Glomerales sp.	2	2	1	0	1	0	4	5	0	0	1	2
6491	Acaulospora koskei	VTX00028 (Acaulospora Acau10)	Glomus sp. NBR PP1	0	0	0	2	0	2	2	1	7	1	0	0

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
1390	Ambispora gerdemannii	VTX00283 (Ambispora fennica)	Ambispora fennica	41	73	58	5	2	4	8	16	12	18	19	8
3752	Ambispora gerdemannii	VTX00283 (Ambispora fennica)	Ambispora fennica	0	1	1	0	0	0	0	1	1	2	1	2
3933	Ambispora gerdemannii	VTX00308 (Paraglomus Glom 1B.13)	Piriformospora	5	4	3	6	6	5	20	7	14	2	0	0
4609	Ambispora gerdemannii	VTX00283 (Ambispora fennica)	Cystofilobasidium	0	0	0	0	0	0	0	0	0	0	2	1
4674	Ambispora gerdemannii	VTX00283 (Ambispora fennica)	Ambispora fennica	0	1	2	0	0	0	2	0	0	1	2	2
5317	Ambispora gerdemannii	VTX00308 (Paraglomus Glom 1B.13)	Cystofilobasidium	12	13	6	1	4	3	13	10	9	9	6	8
900	Ambispora gerdemannii	VTX00283 (Ambispora gerdemannii)	Ambispora fennica	6	8	9	0	0	0	1	2	2	1	1	1

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
2150	Ambispora leptoticha	VTX00255 (Dentiscutata heterogama)	Piriformospora	4	3	5	0	0	0	1	0	0	8	5	3
2206	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Piriformospora	0	0	1	3	1	3	3	0	3	0	0	1
2615	Ambispora leptoticha	VTX00352 (Paraglomus Alguacil12b ACA1)	Glomus sp. NBR PP1	0	0	0	0	0	0	0	0	0	1	0	2
3117	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Piriformospora	15	11	7	0	0	0	0	0	1	0	0	1
5308	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Gigaspora margarita	22	12	18	2	3	7	4	4	8	24	25	28
5351	Ambispora leptoticha	VTX00242 (Ambispora leptoticha)	Ambispora fennica	8	13	13	1	0	0	5	4	12	20	35	32
5799	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Glomus sp. NBR PP1	0	0	0	0	0	1	3	0	0	2	2	2

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
6281	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Glomus sp. NBRrmc	37	40	38	15	11	17	23	17	16	45	81	73
6679	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Piriformospora	3	2	1	6	3	5	5	1	6	1	3	2
957	Ambispora leptoticha	VTX00308 (Paraglomus Glom 1B.13)	Cryptococcus	8	0	3	12	11	7	4	5	10	1	0	1
4112	Claroideoglomus claroideum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	0	1	2	3	3	3	7	1	4	0	2	1
5097	Claroideoglomus claroideum	VTX00402 (Claroideoglomus Torrecillas12b Glo G5)	Glomeromycetes sp.	4	1	2	17	13	20	5	5	8	1	0	1
5645	Claroideoglomus claroideum	VTX00279 (Claroideoglomus sp.)	Sclerocystis sinuosa	1	0	0	1	1	3	3	2	3	0	0	0
6658	Claroideoglomus claroideum	VTX00402 (Claroideoglomus Torrecillas12b Glo G5)	Ambispora fennica	96	100	106	22	36	28	95	95	80	101	94	109

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
1349	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	0	0	0	1	0	1	8	12	6	2	2	4
139	Claroideoglomus etunicatum	VTX00402 (Claroideoglomus Torrecillas12b Glo G5)	Piriformospora indica	2	2	6	1	4	0	11	7	8	2	4	5
1901	Claroideoglomus etunicatum	VTX00279 (Claroideoglomus sp.)	Scutellospora projecturata	3	6	4	0	0	0	1	1	0	22	19	25
2136	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	5	6	0	0	0	0	4	2	1	1	8	9
2288	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	3	2	4	1	0	0	4	5	6	3	1	3
2418	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	2	2	0	10	12	14	4	4	4	1	0	1
2810	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	24	27	25	107	83	93	81	58	65	6	7	6

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
3663	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	0	1	1	1	0	0	1	5	1	19	16	9
3866	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	1	4	5	0	0	0	0	6	1	6	5	4
3955	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	2101	2051	1986	123	128	114	452	442	473	3691	3647	3621
4436	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	2	1	0	4	4	3	1	2	1	0	0	1
5358	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	0	2	1	0	0	0	0	0	0	5	4	2
6085	Claroideoglomus etunicatum	VTX00279 (Claroideoglomus sp.)	Glomus sp. NBRrmc	1	0	1	0	1	1	1	3	0	3	4	7
6523	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	3	6	9	12	12	11	21	18	23	1	3	2

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
6701	Claroideoglomus etunicatum	VTX00193 (Claroideoglomus lamellosum)	Glomus sp. NBR PP1	1076	1109	1134	523	533	486	1039	1093	1178	815	779	723
1282	Dentiscutata heterogama	VTX00318 (Scutellospora LER04)	Scutellospora dipapillosa	0	1	0	0	1	1	0	4	0	1	0	1
1343	Dentiscutata heterogama	VTX00308 (Paraglomus Glom 1B.13)	uncultured Boletaceae	3	4	0	0	0	0	0	1	0	3	2	0
140	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	36	41	28	0	0	0	2	0	0	9	3	5
1421	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	34	44	37	2	2	5	11	17	10	52	56	49
1527	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	0	0	0	0	0	0	1	0	0	5	5	2
1628	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	3	2	4	0	0	0	0	0	0	2	3	4

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
1641	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	0	0	0	0	0	0	0	1	1	2	0	1
1843	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	2	1	3	0	0	0	0	0	1	0	0	4
1908	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	2	4	5	0	1	0	1	1	1	1	1	0
2997	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	0	0	3	0	0	0	0	0	0	2	1	2
3231	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	2	1	0	0	0	0	2	1	1	3	6	10
3703	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	1	0	0	1	2	6	2	0	1	0	0	1
4059	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	0	1	0	2	3	5	4	3	5	0	0	0

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4216	Dentiscutata heterogama	VTX00308 (Paraglomus Glom 1B.13)	Piriformospora	17	11	19	1	0	1	1	2	2	6	9	3
4236	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	6	2	8	0	0	1	6	0	5	13	14	14
4427	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	4	3	0	0	0	0	5	3	2	1	1	1
4595	Dentiscutata heterogama	VTX00318 (Scutellospora LER04)	Scutellospora dipapillosa	1	0	0	0	0	0	0	2	2	0	0	0
4882	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	3	2	1	0	0	0	2	0	0	0	0	0
5290	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	2	2	1	2	0	0	2	1	2	2	1	1
5382	Dentiscutata heterogama	VTX00308 (Paraglomus Glom 1B.13)	Scutellospora dipapillosa	0	4	5	37	42	40	22	18	20	2	2	3

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
6208	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	2	1	2	1	0	0	0	0	0	0	2	1
64	Dentiscutata heterogama	VTX00255 (Dentiscutata heterogama)	Scutellospora dipapillosa	20	17	25	13	9	9	40	62	56	293	310	332
3292	Funneliformis coronatum	VTX00065 (Glomus caledonium)	Sclerocystis sinuosa	4	2	1	2	1	0	10	6	13	3	3	6
3916	Funneliformis coronatum	VTX00308 (Paraglomus Glom 1B.13)	Funneliformis geosporum	8	12	5	14	13	14	10	14	16	5	14	6
4088	Funneliformis coronatum	VTX00296 (Glomus Whitfield type 18)	Gigaspora margarita	2	1	2	2	0	3	9	6	4	2	6	4
4345	Funneliformis coronatum	VTX00067 (Funneliformis mosseae)	Glomus sp. NBR PP	1	2	2	0	0	0	0	0	0	3	4	2
5050	Funneliformis coronatum	VTX00067 (Funneliformis mosseae)	Gigaspora margarita	4	3	4	28	44	32	25	18	13	0	2	1
5272	Funneliformis coronatum	VTX00345 (Glomus sp.)	Liliopsida	118	86	94	1	0	0	4	5	2	22	15	20

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
5493	Funneliformis coronatum	VTX00067 (Funneliformis mosseae)	Mrakia frigida	455	462	420	327	374	315	481	455	536	392	413	447
5672	Funneliformis coronatum	VTX00067 (Funneliformis mosseae)	Glomus sp. NBR PP1	0	0	0	0	0	0	1	0	0	1	2	0
6466	Funneliformis coronatum	VTX00067 (Funneliformis mosseae)	Sclerocystis sinuosa	4	5	2	25	27	26	14	7	12	0	0	0
998	Funneliformis coronatum	VTX00296 (Glomus Whitfield type 18)	Glomeromycota sp. MIB 8848	5	3	5	7	9	11	15	16	12	2	0	2
1609	Funneliformis mosseae	VTX00067 (Funneliformis mosseae)	Gigaspora margarita	8	12	4	2	2	3	4	4	3	4	8	7
1642	Funneliformis mosseae	VTX00067 (Funneliformis mosseae)	Glomeromycota sp. MIB 8848	3	2	2	0	1	0	0	1	1	3	4	2
3975	Funneliformis mosseae	VTX00067 (Funneliformis mosseae)	Gigaspora margarita	0	1	0	3	5	2	0	3	5	0	0	0

	Taxonomy Assignment			Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4745	Funneliformis mosseae	VTX00067 (Funneliformis mosseae)	Cystofilobasidium macerans	907	779	871	140	155	154	618	689	583	373	340	379
6075	Funneliformis mosseae	VTX00067 (Funneliformis mosseae)	Glomeromycetes sp.	0	2	0	4	8	4	10	6	6	0	0	1
6520	Funneliformis mosseae	VTX00195 (Glomus Whitfield type 17)	Cantharellula umbonata	1	1	2	0	1	0	2	1	0	1	1	1
1682	Gigaspora albida	VTX00255 (Dentiscutata heterogama)	Gigaspora albida	5	6	6	42	52	45	20	17	27	4	1	5
3032	Gigaspora albida	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	3	5	6	8	8	12	21	10	15	1	2	2
3291	Gigaspora albida	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	0	0	0	1	0	2	3	0	3	0	0	1
5478	Gigaspora albida	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	1147	1209	1254	3955	3961	4036	2887	2878	2844	284	264	273

Taxonomy Assignment				Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
6103	Gigaspora albida	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	2	0	0	0	1	0	1	0	0	2	0	3
831	Gigaspora albida	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	6	7	8	0	0	0	0	2	3	9	16	11
1569	Gigaspora gigantea	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	0	4	5	0	0	0	2	1	0	3	2	1
3181	Gigaspora gigantea	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	0	0	0	1	1	2	5	10	6	2	4	0
3467	Gigaspora gigantea	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	48	49	56	8	12	11	0	4	4	0	0	1
3783	Gigaspora gigantea	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	1	1	1	0	0	0	1	0	0	1	1	3
4478	Gigaspora gigantea	VTX00039 (Gigaspora decipiens)	Gigaspora decipiens	1	2	1	0	1	0	1	4	2	0	0	2

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4555	Gigaspora gigantea	VTX00052 (Scutellospora calospora)	Gigaspora margarita	1	0	0	4	9	9	3	3	2	0	0	0
4659	Gigaspora gigantea	VTX00039 (Gigaspora albida)	Cystofilobasidium	6	6	0	0	0	1	0	0	1	0	0	0
5877	Gigaspora gigantea	VTX00318 (Scutellospora LER04)	Gigaspora margarita	5	2	3	0	0	1	0	1	3	0	0	1
6149	Gigaspora gigantea	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	21	15	20	0	0	0	8	12	8	30	28	20
145	Gigaspora margarita	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	4	3	6	0	0	0	3	2	5	8	7	7
2653	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora decipiens	367	380	364	258	290	301	524	497	492	361	395	352
3854	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	5	7	4	0	0	0	2	0	1	0	3	0
3963	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	17	12	17	15	16	20	49	45	52	3	3	3

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4348	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	0	0	0	0	0	1	0	1	0	3	2	3
4658	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	5	3	6	1	0	2	2	5	2	14	17	9
4809	Gigaspora margarita	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	2	1	2	0	0	0	1	1	0	1	2	2
5438	Gigaspora margarita	VTX00318 (Scutellospora LER04)	Gigaspora margarita	1	2	1	0	0	0	0	0	1	2	2	3
5614	Gigaspora margarita	VTX00039 (Gigaspora decipiens)	Gigaspora margarita	0	0	1	7	10	2	10	1	1	0	0	0
2760	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Scutellospora dipapillosa	0	0	0	6	4	3	1	2	2	1	0	0
3201	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Glomeromycetes sp.	0	4	4	0	0	0	0	0	1	1	2	8

Taxonomy Assignment				Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
3905	Paraglomus brasilianum	VTX00348 (Paraglomus Alguacil12a Para 1)	Paraglomus occultum	1	3	3	8	8	8	3	6	6	2	0	0
3954	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Cystofilobasidium macerans	0	2	2	4	0	1	3	2	4	0	0	0
4517	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Catenulostroma microsporum	29	20	19	133	123	140	58	65	45	5	14	3
4791	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Acaulospora colombiana	0	0	0	1	0	0	2	1	0	2	3	2
4841	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Scutellospora dipapillosa	1	1	1	0	0	0	0	0	1	3	3	2
4991	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Gigaspora margarita	1	0	0	0	2	1	2	1	7	1	0	0
5716	Paraglomus brasilianum	VTX00283 (Ambispora fennica)	Gigaspora margarita	0	0	0	0	0	1	1	0	0	1	2	2

Taxonomy Assignment				Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
5843	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Scutellospora projecturata	8	8	9	3	4	4	9	9	7	0	2	0
592	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Sclerocystis sinuosa	0	0	0	1	0	0	2	1	0	0	2	1
6792	Paraglomus brasilianum	VTX00238 (Paraglomus occultum)	Paraglomus occultum	1	0	2	0	0	3	9	6	4	0	0	0
6820	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Rhizophagus intraradices	1	3	5	3	1	4	2	5	4	0	0	0
704	Paraglomus brasilianum	VTX00239 (Paraglomus brasilianum)	Gigaspora margarita	32	35	29	7	6	5	27	30	27	44	31	45
6379	Rhizophagus intraradices	VTX00237 (Claroideoglomus sp.)	Glomus sp. NBR PP1	0	3	0	5	0	3	1	2	0	0	1	0
109	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	0	0	3	4	5	2	2	4	0	0	0

Taxonomy Assignment				Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
1183	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	0	0	1	4	6	2	4	1	0	0	0
1387	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	1	0	5	6	6	5	1	4	1	0	0
1459	Rhizophagus sinuosum	VTX00308 (Paraglomus Glom 1B.13)	Cystofilobasidium	0	1	2	11	4	12	4	7	2	0	3	1
158	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	7	1	8	9	4	2	2	5	0	1	1
1730	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	2	4	2	0	0	0	1	1	0	2	4	2
1895	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	3	2	0	1	0	2	1	0	0	2	4	2
2089	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	2	2	0	0	0	0	1	0	0	0	0

	Taxonomy Assignment			Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
2481	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	3	1	0	0	0	2	0	2	0	0	0
2508	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	4	16	6	2	0	2	4	3	3	6	5	2
2525	Rhizophagus sinuosum	VTX00328 (Acaulospora sp.)	Cystofilobasidium	15	20	18	11	8	13	16	16	22	24	25	29
2797	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	1	0	5	3	2	1	2	1	0	0	2
2853	Rhizophagus sinuosum	VTX00099 (Glomus proliferum)	Rhizophagus intraradices	0	0	0	0	0	0	0	0	0	2	6	1
2930	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	8	10	8	0	0	0	0	1	0	3	9	5
3055	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	466	473	494	1650	1561	1568	689	686	668	58	73	74
307	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	0	2	1	4	2	1	0	3	1	0	0

Taxonomy Assignment				Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
3529	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	4	1	4	3	1	2	1	2	2	0	2
3594	Rhizophagus sinuosum	VTX00069 (Glomus Glo3b)	Sclerocystis sinuosa	0	0	0	0	1	0	1	1	0	8	7	6
371	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	4	4	3	11	12	8	2	2	3	0	1	0
3710	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	2	0	1	2	1	1	0	1	0	1	2
3735	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	1	1	0	0	0	2	3	0	0	0	0
4400	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	1	0	1	0	1	4	2	1	1	3	1
4447	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	136	121	150	87	101	85	185	169	145	133	156	136
4527	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	2	1	0	0	0	0	2	0	4	1	7

	Taxonomy Assignment			Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4590	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	3	2	1	0	0	0	1	1	1	1	1
473	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	2	1	0	0	0	0	1	2	0	0	0
5255	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	2	1	3	5	0	2	3	1	0	1	2	4
5333	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	2	3	1	0	0	0	0	0	2	2	4	7
5419	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	7	1	7	1	1	1	4	2	7	3	5	3
5589	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	2	0	0	0	0	0	0	0	3	4	2
5868	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	7	17	7	14	12	9	12	7	8	5	4	9

Taxonomy Assignment				Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
601	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	1	0	6	4	5	1	4	1	1	0	0
6117	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	13	13	12	11	13	19	1	8	3	0	3	1
6290	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	3	1	4	3	0	0	3	1	2	2	1	0
63	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	2	1	5	6	7	0	3	3	1	0	0
6465	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	0	0	1	5	2	1	2	2	1	0	1
6603	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	0	2	1	12	14	13	8	9	1	0	1	2
6720	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	3	2	1	15	12	12	6	4	8	1	0	0

Taxonomy Assignment				Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
6733	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	6	4	2	7	15	8	14	14	16	0	1	1
784	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	2	0	4	3	4	1	1	0	0	0	0
836	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	4	5	2	49	39	43	14	18	15	0	1	0
891	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	1	1	1	1	1	1	3	2	2	0	0	0
93	Rhizophagus sinuosum	VTX00069 (Rhizophagus sinuosum)	Sclerocystis sinuosa	2	2	0	1	3	2	1	1	3	0	0	0
1065	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	1	0	0	0	0	0	0	0	0	3	1	4
2630	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	8	5	4	1	1	1	2	4	1	3	4	1

	Taxonomy Assignment			Mock Community											
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OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
2757	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Gigaspora margarita	1	1	3	10	6	8	17	14	20	0	1	6
2907	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Gigaspora margarita	1	0	0	2	8	3	1	2	1	0	0	1
3171	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Gigaspora margarita	7	1	3	0	0	0	2	1	1	9	5	8
3253	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	0	0	0	0	0	0	0	0	0	2	1	0
4322	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	0	1	1	4	6	2	4	7	5	1	0	1
4538	Scutellospora calospora	VTX00308 (Paraglomus Glom 1B.13)	Gigaspora margarita	5	0	0	0	0	0	2	1	2	2	1	0
4544	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	1	2	0	0	0	0	0	0	1	4	2	2

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
4545	Scutellospora calospora	VTX00255 (Dentiscutata heterogama)	Gigaspora margarita	288	325	296	27	19	24	99	102	86	702	642	679
4552	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	0	0	0	0	0	0	0	0	0	1	1	1
4817	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	10	3	4	2	0	2	3	2	3	2	4	2
5338	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	1	4	2	0	0	0	0	0	1	2	1	5
534	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	16	17	16	0	0	1	4	4	4	3	4	4
5707	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	3	8	5	4	2	1	12	11	5	2	4	3
5712	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	0	1	0	1	1	0	0	1	2	2	2	1

	Taxonomy Assignment			Mock Community											
				Even			Glom.dom			Less.Glom.dom			Gig.dom		
OTU	Custom	MaarjAM	Silva	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3	rep1	rep2	rep3
5803	Scutellospora calospora	VTX00318 (Scutellospora LER04)	Scutellospora projecturata	0	0	3	2	1	4	3	1	5	0	0	0
5926	Scutellospora calospora	VTX00318 (Scutellospora LER04)	Gigaspora decipiens	0	0	2	1	0	0	0	2	1	0	1	2
6262	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Gigaspora margarita	1	1	0	1	0	0	1	1	1	4	3	3
6348	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	4	3	5	0	0	0	0	0	0	0	1	1
6546	Scutellospora calospora	VTX00049 (Scutellospora Scut1)	Scutellospora projecturata	3	1	2	0	0	0	0	0	0	3	8	2
6791	Scutellospora calospora	VTX00052 (Scutellospora calospora)	Scutellospora projecturata	20	17	14	0	1	0	6	7	8	29	43	50

Appendix B - Supporting Materials for Chapter 3

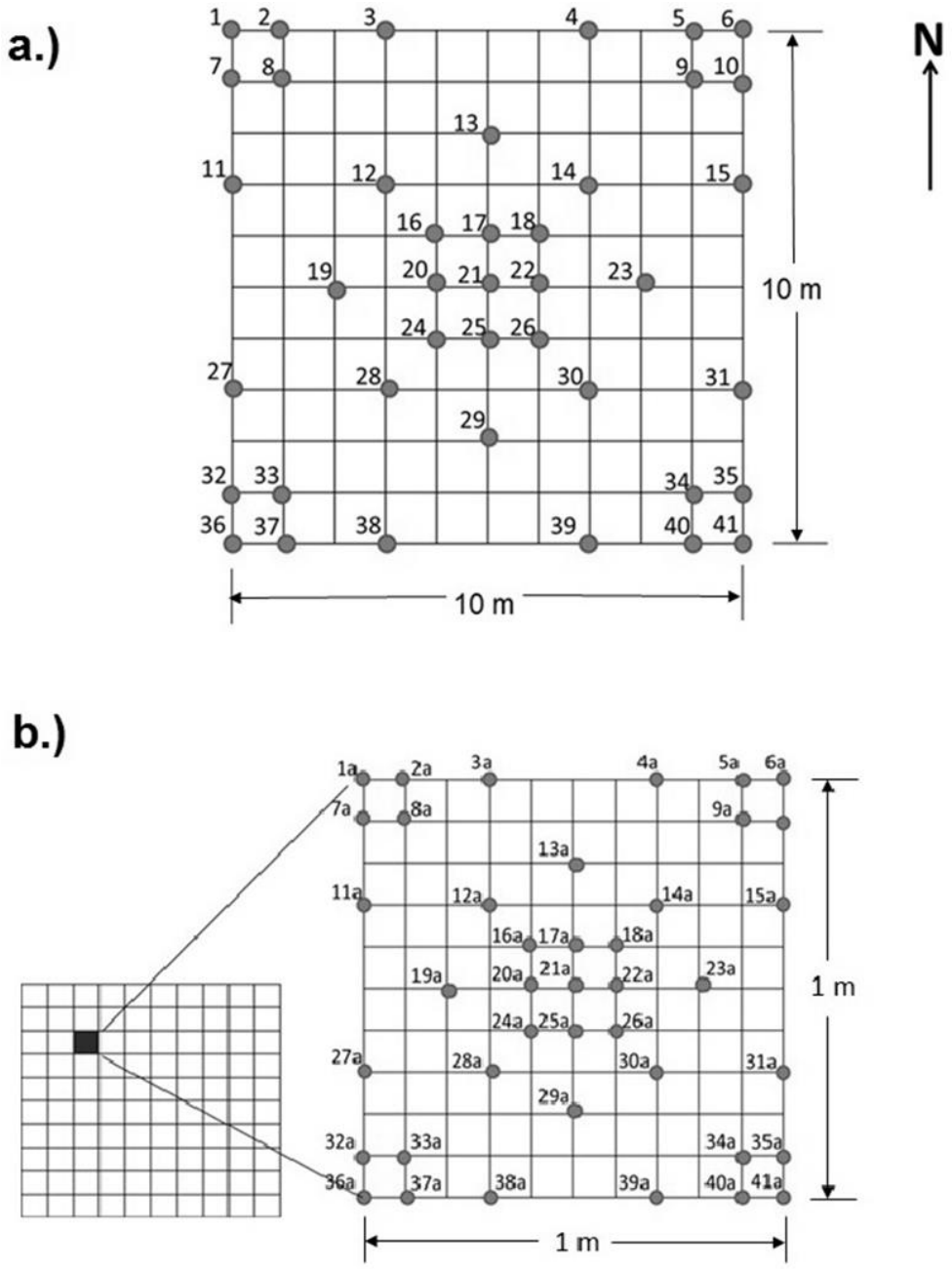


Figure B.1 Sampling design used for large sampling grid (a) and nested grid (b) (adapted from Lister et al. 2000). Dots at grid intersections indicate where soil cores were taken. Numbers indicate sample number that were denoted during sampling. Cell location of nested grid varied at each elevation.

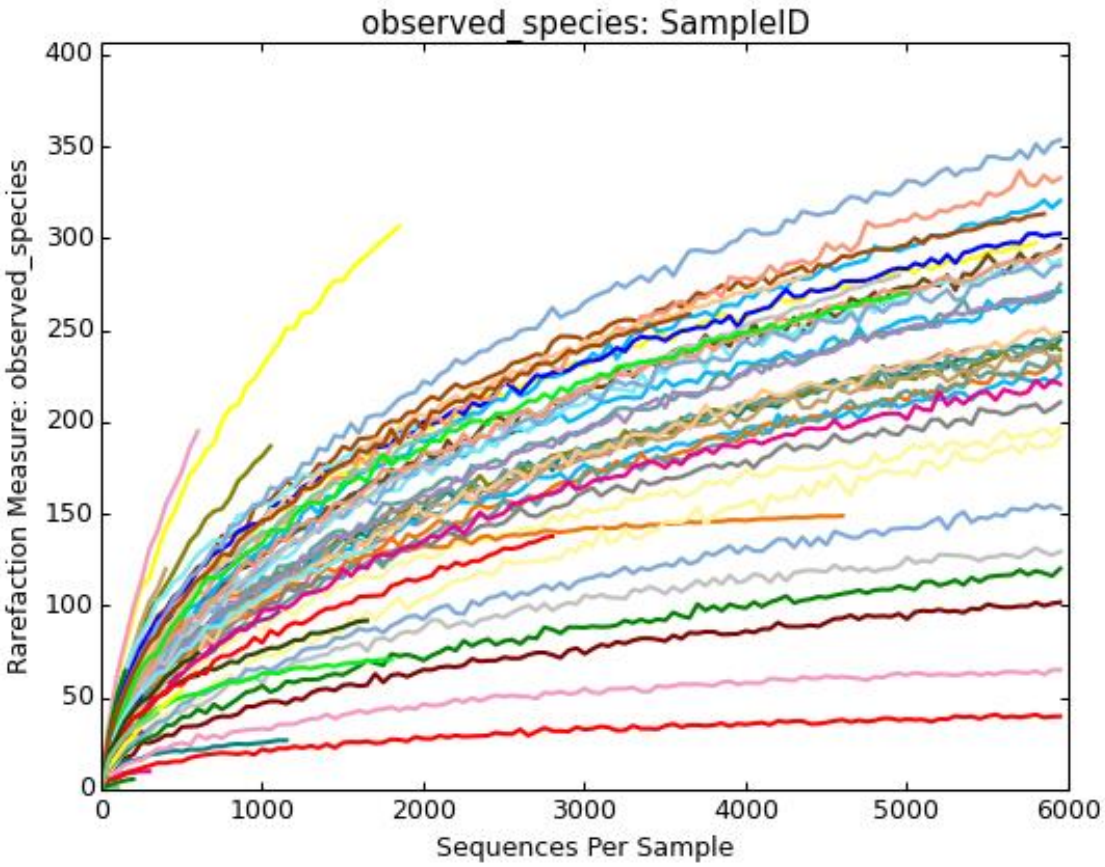


Figure B.2 Accumulation curve of operational taxonomic unit (OTU) richness of Glomeromycotan sequences from 114 samples that yielded PCR product. Sequence sampling curve was terminated at 3000 sequences.

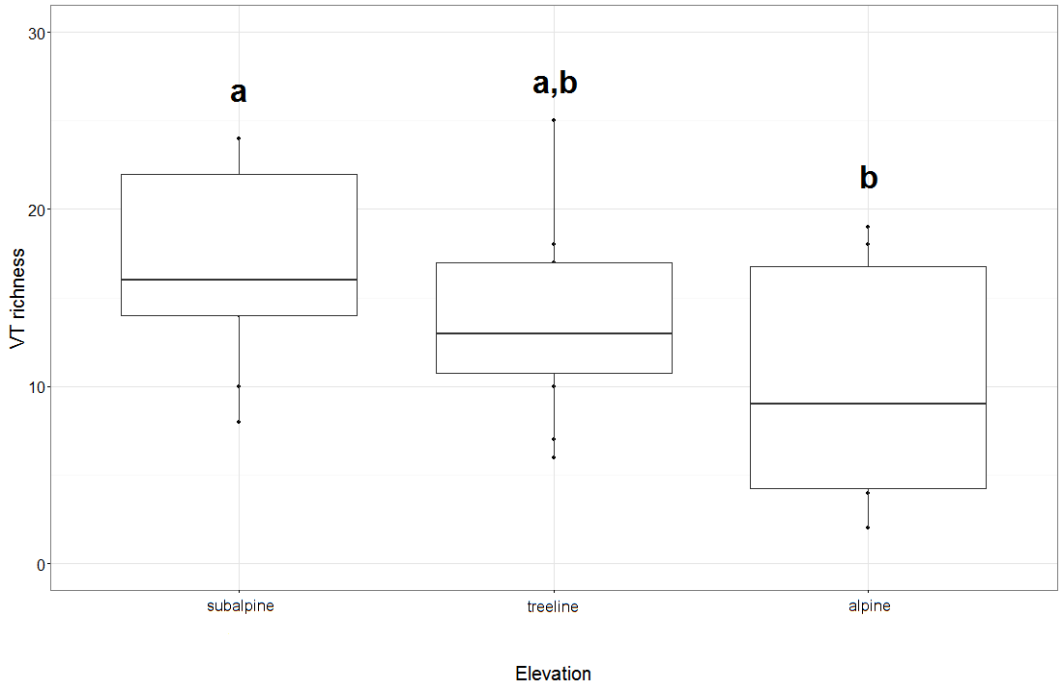


Figure B.3 Boxplots showing AM fungal virtual taxon (VT) richness per sample in relation to elevation. Subalpine n=11, treeline n=12, alpine n=14. VT richness significantly decreased with increasing elevation (Kruskal Wallis test; χ 2 = 5.72, df = 2, P = 0.05). Boxes topped by the same letter do not differ significantly as determined by a Wilcoxon rank sum test (at a significance of P < 0.05).

Table B.1 Deviation of observed mean pairwise phylogenetic distance (MPD, a) and mean nearest taxon distance (MNTD, b) from null expectation of zero phylogenetic structure for the three elevations sampled. Deviation from null expectation was determined using a two-tailed t-test at a significance at P <0.001.

	Elevation	**t**	**df**	**P-value**
(a) MPD	subalpine	-6.3656	10	8.19E-05
	treeline	-5.8556	11	0.00011
	alpine	-10.19	13	1.45E-07
(b) MNTD	subalpine	-15.881	10	2.02E-08
	treeline	-9.4849	11	1.25E-06
	alpine	-15.591	13	8.57E-10

Appendix C - Supporting Materials for Chapter 4

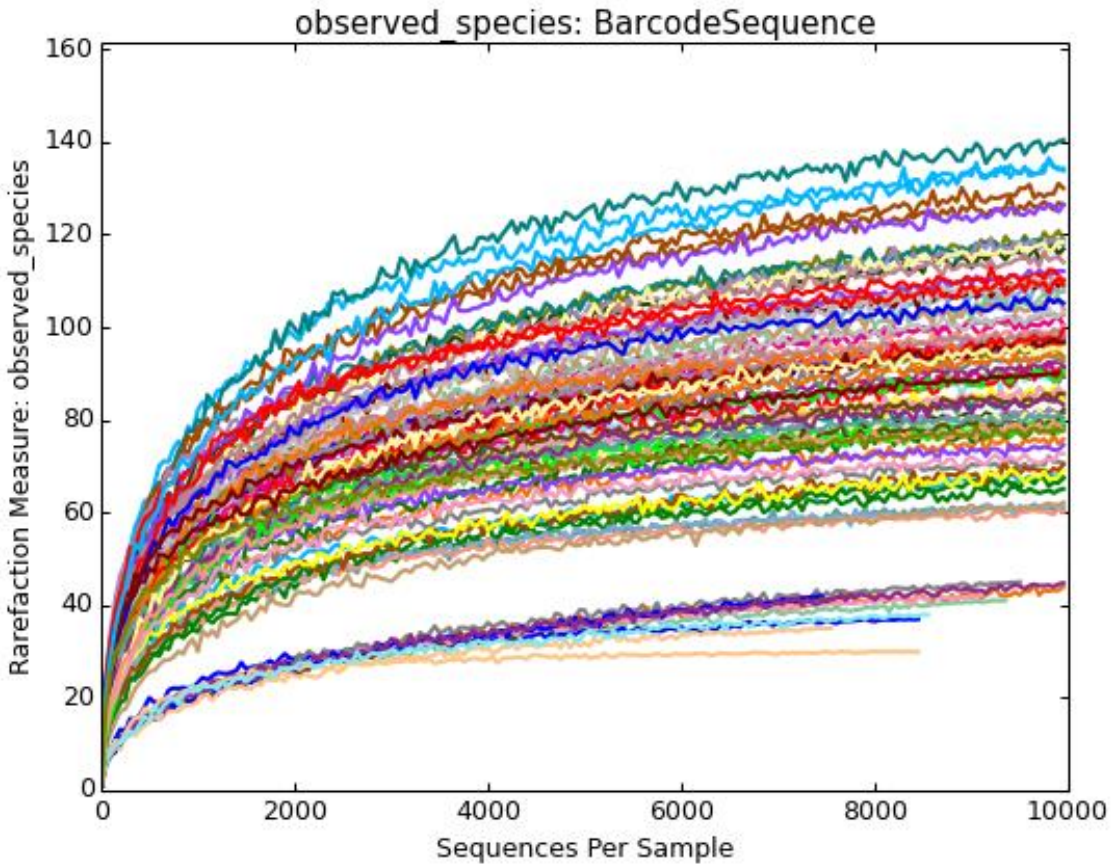
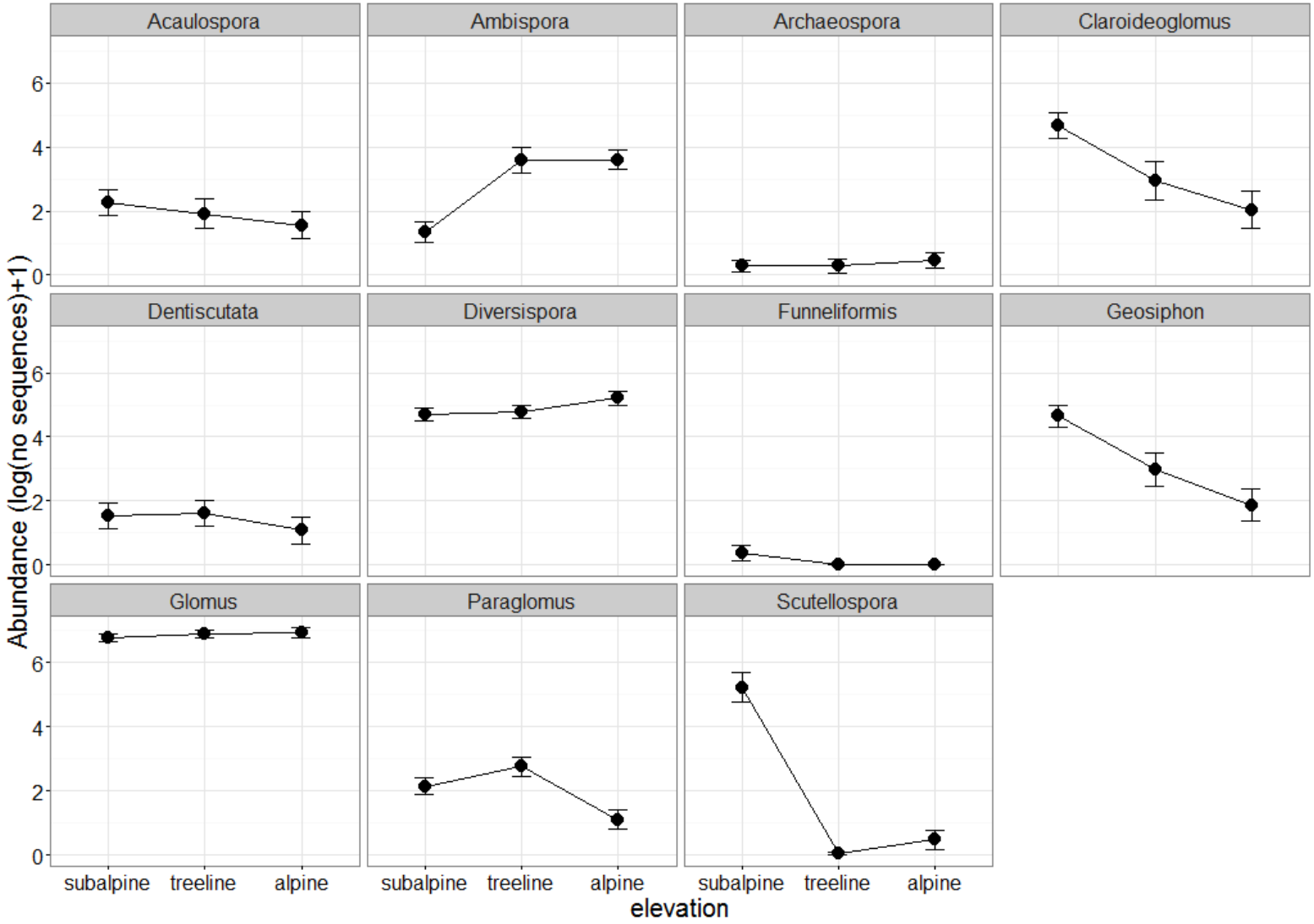


Figure C.1 Accumulation curve of operational taxonomic unit (OTU) richness of Glomeromycotan sequences from individual samples (n=90). OTUs were created by clustering sequences ≥97% similarity.

a.)


Acaulospora
Ambispora
Archaeospora
Claroideoglomus
Dentiscutata
Diversispora
Funneliformis
Geosiphon
Glomus
Paraglomus
Scutellospora
Abundance (log(no sequences)+1)
0
2
4
6
subalpine
treeline
alpine
elevation

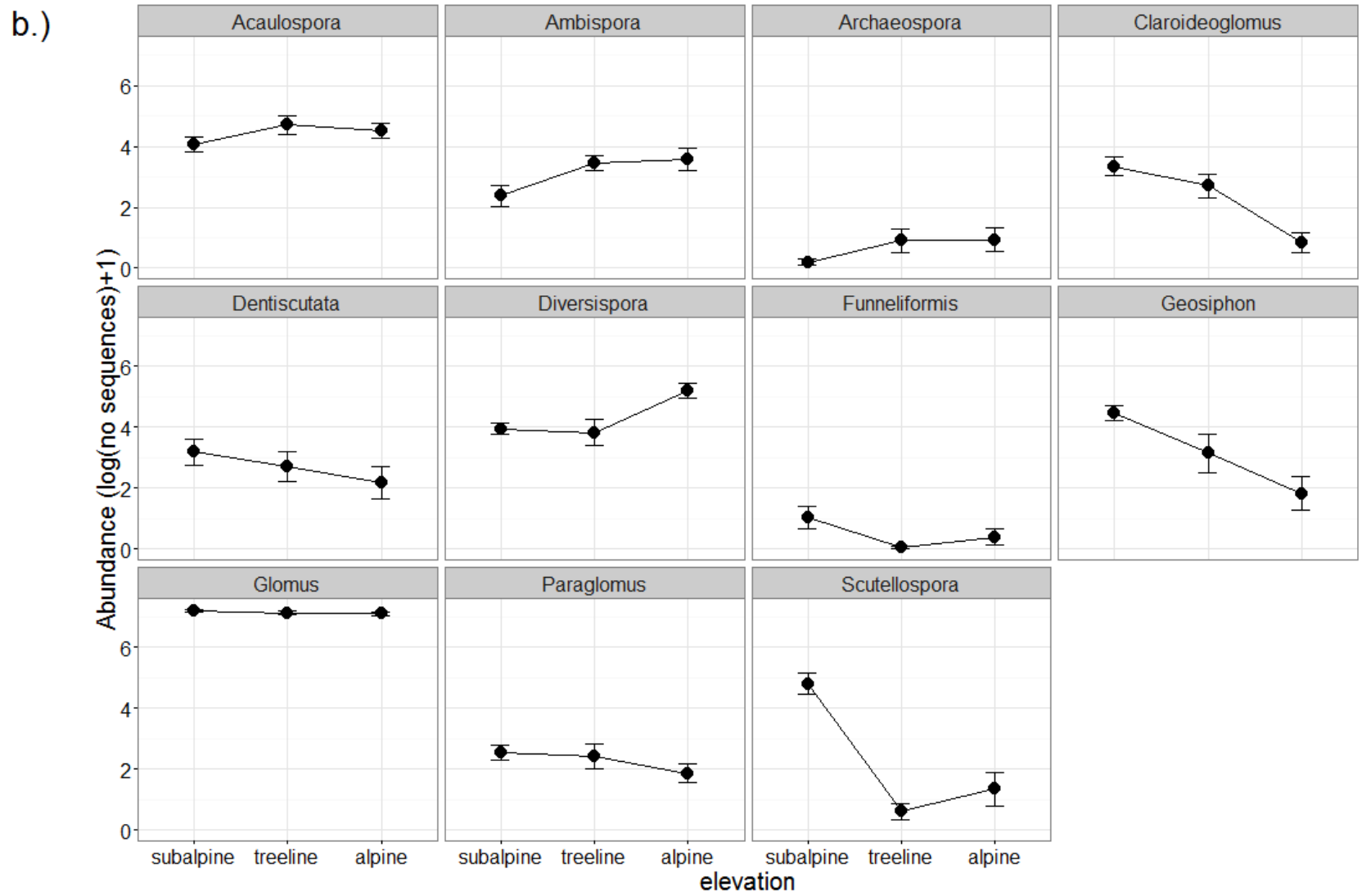


Figure C.2 Dot plots showing how abundances of AM fungal genera abundances in roots (a, n=45) and soil (b, n=45) vary with elevation. Log-scaled y-axis for graphing purposes only, multiGLM tests on abundance changes with elevation was done on non-transformed abundance data.

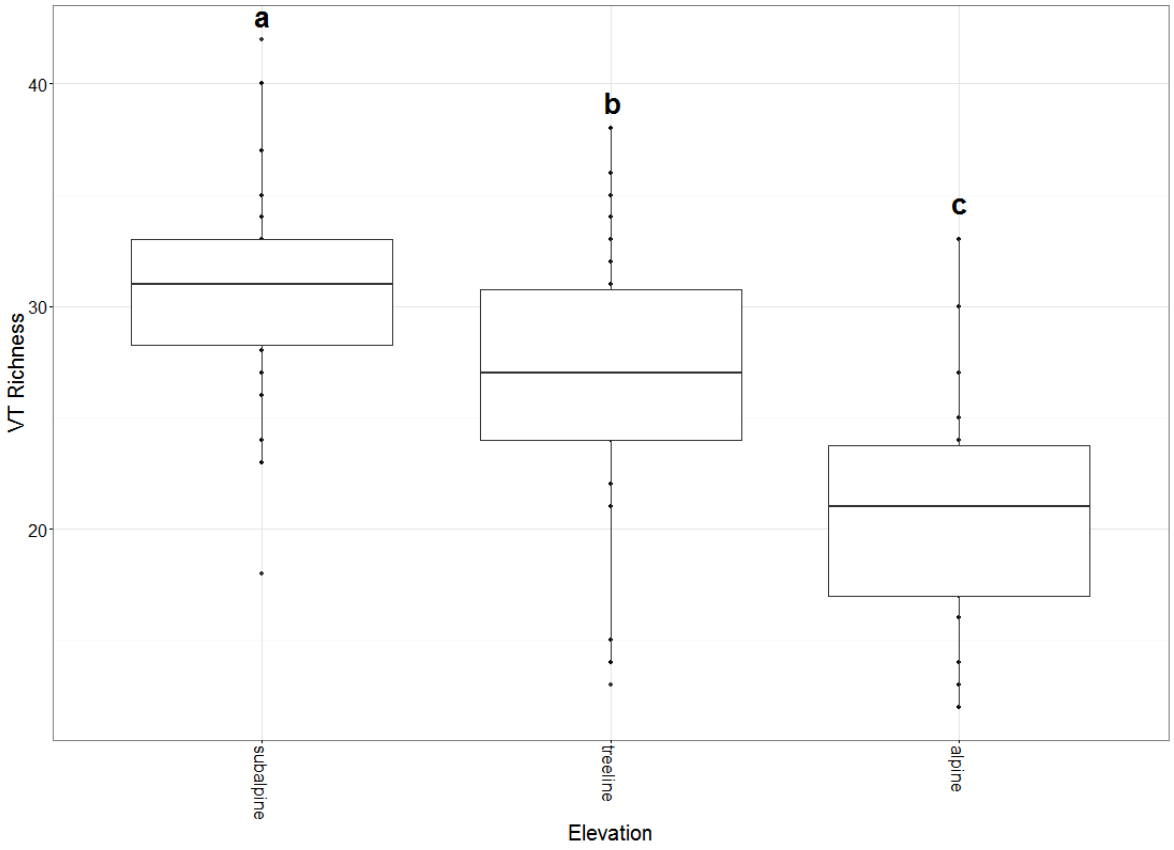


Figure C.3 Boxplots displaying overall AM fungal virtual taxa (VT) richness from three elevations sampled; subalpine n=30, treeline n=30, alpine n=30). Boxes without shared letters indicate significant difference in VT richness as determined by Wilcoxon rank sum tests (at a significance of $P < 0.05$).

Table C.1 Welch two-sample t-tests comparing abundance of genera in roots vs. soil for three elevations examined. Genera abundances obtained after standardizing samples to 2000 sequence reads. Bold p-values indicate significance at $P < 0.05$.

Elevation	Genus	t	df	p-value
Subalpine	Acaulospora	-3.58	18.82	**0.002**
	Ambispora	-2.00	25.30	0.057
	Archaeospora	0.83	16.55	0.419
	Claroideoglomus	3.55	16.00	**0.003**
	Dentiscutata	-2.15	15.57	**0.048**
	Diversispora	3.10	21.08	**0.005**
	Funneliformis	-1.20	20.44	0.243
	Geosiphon	1.15	22.94	0.263
	Glomus	-2.75	19.92	**0.012**
	Paraglomus	-1.05	23.39	0.306
	Scutellospora	2.22	20.09	**0.038**
Treeline	Acaulospora	-2.81	14.25	**0.014**
	Ambispora	1.51	16.82	0.149
	Archaeospora	-1.33	15.75	0.203
	Claroideoglomus	1.57	17.27	0.135
	Dentiscutata	-2.12	17.90	**0.048**
	Diversispora	1.00	22.67	0.327
	Funneliformis	-1.00	14.00	0.334
	Geosiphon	-0.69	27.80	0.499
	Glomus	-1.22	21.25	0.236
	Paraglomus	-0.57	19.55	0.574
	Scutellospora	-1.34	14.03	0.202
Alpine	Acaulospora	-3.15	15.61	**0.006**
	Ambispora	0.16	25.53	0.876
	Archaeospora	-1.04	14.98	0.316
	Claroideoglomus	1.92	15.79	0.073
	Dentiscutata	-1.79	20.05	0.088
	Diversispora	0.10	27.07	0.918
	Funneliformis	-1.27	14.00	0.224
	Geosiphon	-0.32	26.21	0.755
	Glomus	-0.21	19.53	0.839
	Paraglomus	-1.40	25.97	0.172
	Scutellospora	-1.32	15.11	0.208

Table C.2 Pairwise comparisons between factor levels for both roots (a) and soil (b) using permutational multivariate analysis of variance using distance matrices (PERMANOVA). Results obtained using 9999 permutations. Bolded p-values indicate significance at $P < 0.05$.

	Pairwise Comparison	p
(a) Roots		
	subalpine - treeline	**0.00015**
	subalpine - alpine	**0.00015**
	treeline - alpine	0.4526
	D.fruticosa - A.millefolium	**0.0003**
	D.fruticosa - F.idahoensis	0.0713
	A.millefolium - F.idahoensis	**0.0012**
(b) Soil		
	subalpine - treeline	**1.00E-04**
	subalpine - alpine	**1.00E-04**
	treeline - alpine	**1.00E-04**
	D.fruticosa - A.millefolium	0.77
	D.fruticosa - F.idahoensis	0.77
	A.millefolium - F.idahoensis	0.77

Table C.3 Variation in AM fungal community composition in roots (a) and soil (b) as determined by multivariate dispersion analyses using 9999 permutations**.**

	DF	SS	MS	PseudoF/F	R2	p
(a) Root Multivariate dispersions						
Elevation	2	0.02298	0.011492	0.7504	999	0.489
Residuals	42	0.64319	0.015314			
Host	2	0.01688	0.008438	0.8835	999	0.436
Residuals	42	0.40113	0.009551			
(b) Soil Multivariate dispersions						
Elevation	2	0.17852	0.089259	1.5953	999	0.227
Residuals	42	2.34989	0.05595			
Host	2	0.008756	0.004378	0.8253	999	0.459
Residuals	42	0.222804	0.005305			