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Fungi Associated with Common Buckthorn (Rhamnus cathartica) in Southern Ontario

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Abstract

Common buckthorn (*Rhamnus cathartica*) is a competitive Eurasian woody shrub currently invading North America. Buckthorn thickets reduce native diversity and may reduce mycorrhizal diversity through the release of allelochemicals. Two aspects of buckthorn's invasional biology are explored: 1) identifying fungi associating with buckthorn, and 2) determining buckthorn's allelochemical impacts on arbuscular mycorrhizae in forest soils and an open-greenhouse experiment.

Twenty-three fungi were found growing on buckthorn, including *Armillaria mellea* s.l., *Hypoxylon fuscum*, *H. perforatum*, *Nectria cinnabarina*, and *Cylindrobasidium evolvens*. Data from invaded and uninvaded sugar maple (*Acer saccharum*) soils revealed that arbuscular mycorrhizal fungi (AMF) diversity fluctuated as a function of season or potting disturbance, but the presence of buckthorn had little effect on AMF development in maple roots. Buckthorn may be a mycorrhizal generalist, and changes in AMF abundance may be more influenced by underlying stochastic soil processes and aboveground plant composition than by buckthorn and its allelochemicals.

Keywords: arbuscular mycorrhizal fungi, *Rhamnus cathartica*, *Acer saccharum*, allelochemicals, PCR, small-subunit RNA gene, Illumina MiSeq, bioinformatics, R programming, phylogenetics

Dedication

To Achchi.

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General Abbreviations

AMF	Arbuscular mycorrhizal fungi; Glomeromycota
BH	Benjamini-Hochberg p-values; statistical comparison
BWB	Buckthorn Witches' Broom phytoplasma
clr	Centered log-ratio
CZM	Count Zero Multiplicative
CPP	Chontrol Peat Paste
DMC	Dirichlet Monte Carlo
ECM	Ectomycorrhizal fungi
ERM	Extraradical mycelium
FASTQ	Text-based format for storing both a biological sequence (usually nucleotide sequence) and its corresponding quality score
LSU	Large subunit ribosomal RNA gene
ITS	Internal transcribed spacer ribosomal RNA gene
ISU	Identical sequence units
MCL	Maximum Composite Likelihood, phylogenetic tree construction method
ML	Maximum Likelihood; phylogenetic test
MPS	Multiple-Pathogen Strategy
N	Nucleic acid representing any of the four DNA bases: Thymine, Adenine, Cytosine, Guanine
NGS	Next Generation Sequencing
NJ	Neighbour Joining, phylogenetic tree construction method
nu rRNA	nuclear ribosomal RNA gene
OTU	Operational taxonomic unit
rRNA	ribosomal RNA
PCR	Polymerase Chain Reaction
SOM	Soil Organic Matter
SSU	Small subunit ribosomal RNA gene
TTLT	Thames Talbot Land Trust

USDA	United States Department of Agriculture
UTRCA	Upper Thames River Conservation Authority
VAM	Vesicular Arbuscular Mycorrhizae; see AMF
VTX	Virtual Taxon

Chapter 1: Introduction

Non-native plants are commonly introduced into North America through landscape trades such as horticulture, agriculture, and forestry (Reichard and Hamilton 1997). As of 1997, 235 intentionally introduced woody plant species have naturalized in North America (Reichard and Hamilton 1997). In some cases, these naturalized species proliferate and persist to the detriment of the environment (Mack et al. 2000). Invasive species are thought to be the second biggest cause of biodiversity loss, next to habitat loss (Heneghan et al. 2006, IUCN 2014). Invasive plants, in particular, have been implicated in disrupting forest ecosystems by altering species composition (Heneghan et al. 2006), chemically modifying soils (Barto et al. 2011), and hindering interspecies interactions (Heneghan et al. 2006).

There are more species of invasive plants in Ontario than any other Canadian province (OMNRF 2012). The Ontario Invasive Plants Council highlights 16 species of terrestrially invading plants that pose a threat to the province's natural diversity (http://www.invadingspecies.com/invaders/plants-terrestrial/). Of the 5 267 plots surveyed in southern Ontario between the period of 2005 to 2010, 11% of the top four dominant plant species were invasive (Puric-Mladenovic et al. 2012). Common buckthorn (*Rhamnus cathartica*) was the most commonly recorded (in 333 plots), followed by purple loosestrife (*Lythrum salicaria*) (85 plots), European frog-bit (*Hydrocharis morsus-ranae*) (78 plots), Tartarian honeysuckle (*Lonicera tatarica*) (66 plots), garlic mustard (*Alliaria petiolata*) (50 plots), cow vetch (*Vicia cracca*) (39 plots), and bittersweet nightshade (*Solanum dulcamara*) (33 plots). Buckthorn was considered to be an aggressive invader, attaining 9% average cover in all plots where it was identified, and reaching a maximum cover of 80% (Puric-Mladenovic et al. 2012). In London, Ontario, the title for "most common tree" is held by two invasive species—European Buckthorn by number of stems and Norway Maple by total size (UFORE 2012). Buckthorn's effects on its surrounding environment is still an understudied subject, and there is little information on the native fungi that it may have interactions with.

1.1 COMMON BUCKTHORN (*RHAMNUS CATHARTICA*)

1.1.1 Invasion History

Rhamnus cathartica L. (Common Buckthorn, European Buckthorn) was first imported to North America from its native Eurasian range during the early 1800s for its medicinal qualities, namely the cathartic properties of its fruits (see Chapter 3, Section 3.1.2) (Kurylo and Endress 2012). It was so commonly seen in New England by the early 19th century that it was assumed to be a native shrub in many plant catalogues (Kurylo and Endress 2012). Most of its spread within North America was due to human-mediated movement as an ornamental, hedge, and/or shelterbelt plant because of its growth rate, hardiness, ease of propagation, and low susceptibility to herbivory (Kurylo and Endress 2012). An 1824 issue of the *New England Farmer* mentioned Common Buckthorn's superiority as a hardy hedge species and, by 1864, Toronto nursery catalogs listed buckthorn as an available hedge plant (Kurylo and Endress 2012). By 1877, this species was present in cultivated grounds near Castleton, northeast of Toronto (Kurylo and Endress 2012) and, by the 1930s, it had been introduced (and subsequently abandoned) in western Saskatchewan as a potential shelterbelt species (Archibold et al. 1997). Now, *R. cathartica* is identified as a noxious weed in six US states and two Canadian provinces (NRCS 2013; http://www.omafra.gov.on.ca/english/crops/facts/info_buckthorn.htm).

1.1.2 Ecosystem Impacts

Buckthorn is able to outcompete and displace native understory vegetation, transforming the forest into dense monoculture thickets (Becker et al. 2013) that are not found in its native area in Europe (Knight 2006). Exotic species that form monocultures are typically rarer in their native ranges. It is believed that the growth of an invasive in non-native environments is promoted by altering its interaction with native plants. In some cases, invasive plants owe their success to the production of biochemicals that are novel in the invaded range (Vandenkoornhuyse et al. 2003). A study on buckthorn's soil ecosystem impacts was done by Stinson et al. (2006), who observed a reduction of native diversity through the alteration of interspecies interactions or ecosystem properties. Buckthorn is adaptable to a wide range of soil moisture and light levels; it has an extended range of

optimal photoperiods, high seed production, effective seed dispersal through frugivorous birds, high seedling germination, low seedling mortality rates, and produces allelopathic compounds (Au and Tuchscherer 2014). These and other advantages allow buckthorn to compete vigorously for limiting resources in native forested communities, with removal being costly and difficult once established.

1.1.3 Changes in Soil Nutrient Availability

Buckthorn has high foliar nitrogen (Heneghan et al. 2007), a common limiting nutrient in many soil habitats and its leaves remain green after they are shed (Becker et al. 2013). Litter high in nitrogen typically has more simple sugars that make it attractive to microbial decomposers as well as detritivores (ranging from protozoans to earthworms) (Stinson et al. 2006).

Buckthorn thickets are associated with elevated soil nitrogen, carbon, pH, and water content. Invaded areas show twice the percentage of soil nitrogen (mean, 0.54%) than open areas (native species woodlots) (mean, 0.27%) and 80% more carbon (mean 6.83%) than open areas (mean, 3.81%), which was hypothesized to occur due to the rapid incorporation of nitrogen-rich buckthorn litter into the soil (Heneghan et al. 2006). Soil pH was significantly higher in invaded areas as was gravimetric water content —40% higher in buckthorn areas (Heneghan et al. 2006). Site-specific differences were seen in extractable nitrogen and nitrogen mineralization (ammonification, nitrification and total nitrogen mineralization) rates, alongside a slightly lower overall carbon:nitrogen ratio (Heneghan et al. 2006). Since this was not a manipulative study, distinguishing the cause and effect of buckthorn invasion in regards to changes in soil nutrient levels was not possible, but other studies demonstrate the ability of invasive plants to alter soil chemical properties (nitrogen and carbon) (Ehrenfeld et al. 2001; Vitousek and Walker 1989; Wall et al. 2002; Witkowski 1991).

1.1.4 Competitive Growth Advantages

Some invasive species are able to utilize resources when native plants are inactive, or are able to use the available resources more efficiently (Zhou et al. 2004). Comparing the

ecophysiological responses of two invasive shrubs, *R. cathartica* and *Lonicera x bella* (Showy fly honeysuckle), to two native shrubs, *Cornus racemosa* (gray dogwood) and *Prunus serotina* (black cherry), Zhou et al. (2004) demonstrated an invasive plant's ability to take advantage of high light situations. Of the four species, buckthorn showed the greatest response to increased light availability, with greater trunk diameter increments in open wetland systems than closed canopies (Gourley 1985; Zhou et al. 2004). *Rhamnus cathartica* is in leaf an average of 58 days longer than gray dogwood and black cherry (Catling and Mitrow 2012), during which it gains most of its annual carbon (Zhou et al. 2004). Early emerging buckthorn saplings shade the ground layer with the potential to inhibit photosynthesis in smaller native herbs and seedlings during critical life stages. Buckthorn saplings are light tolerant but capable of growing in shaded conditions until canopy openings trigger a rapid growth response (Catling and Mitrow 2012) .

1.1.5 Reproductive Success

Buckthorn takes at least 9–20 years to reach reproductive age (Gourley 1985). It is a dioecious plant, with female trees being noted for their prolific seed production, which can be from 2 to 6 times higher in open fields than in closed canopy environments (Catling and Mitrow 2012). The seeds set between late July to early August and are generally untouched by most birds in both naturalized and native ranges (Catling and Mitrow 2012). Often, berries remain on the parent plant until late winter or early spring, leading to large seed banks below the trees, some reported to contain up to 5000 seeds/m^2 (Becker et al. 2013). After the removal of the parent tree due to natural or restoration events, seeds in the seed banks may germinate in canopy openings for up to 2–6 years (Becker et al. 2013; Converse 1984; Delanoy and Archibold 2007; Pergams and Norton 2006), with seed viability increasing after digestion and/or flesh removal (Catling and Mitrow 2012). High germination success of 85–95% have been seen under the parent tree (Catling and Mitrow 2012).

1.1.6 Interspecies Interactions

Multiple invasive species originating from the same native ranges typically maintain their associations in invaded ranges, and may lead to a compounded decline in ecosystem health

because of advantageous co-evolutionary interactions. These synergistic interactions are termed ‘invasional meltdowns’ (Simberloff and Von Holle 1999). Meltdowns are best viewed at the community level, where there is an increased impact and/or rate of establishment of the invasive species (Simberloff and Von Holle 1999).

Buckthorn thickets are characterized by a conspicuous reduction in the litter layer in comparison to native forests (Heimpel et al. 2010), with soil exposure occurring within the first few weeks of spring (Stinson et al. 2006). Buckthorn leaf litter has ideal chemical and physical properties, including high nitrogen and calcium, low tannins, and comparatively softer leaves that are easier for consumption by decomposers (Heimpel et al. 2010). This makes the leaf litter layer under buckthorn-dominant canopies an ideal habitat for the invasive European earthworm (dew worm), *Lumbricus terrestris* (Hale et al. 2005). It is hypothesized that native earthworm populations did not survive the Pleistocene glacial events, and that most of the northern current-day populations originate from invasive European species (Heimpel et al. 2010; Stinson et al. 2006). The detrimental impacts of invasive earthworms on woodland habitats has been confirmed (Catling and Mitrow 2012; Groffman et al. 2004; Gundale 2002). In one instance, a negative correlation was observed between the earthworm, *Lumbricus rubellus*, and an endangered fern, *Botrychium mormo*, due to the loss of mycorrhizae in the litter layer stemming from increased earthworm activity (Gundale 2002). Different European earthworm species predominate in disturbed habitats, depending on the type of disturbance. The invasive dew worm dominates woodland and fencerow soil faunal communities, whereas other earthworm species prefer agricultural and adjacent woodlot sites (Hale et al. 2005)

A litter decomposition study using buckthorn, sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), and white oak (*Quercus alba*) leaves was done in locations characterized by low, medium, and high abundances of dew worms (Stinson et al. 2006). Earthworms have observable distribution heterogeneity within heterogeneous forest systems (Groffman et al. 2004; Stinson et al. 2006). Earthworms displayed preference for buckthorn and sugar maple litter bags, which resulted in the accelerated decomposition rate of both leaf types, whereas the decomposition of red and white oak litter was slower, indicating lower immediate litter quality (Heneghan et al. 2007). Despite the heterogeneous

distribution of earthworm populations, there is a substantial mass loss of *R. cathartica* litter in bags that allowed earthworm access; 50% of the litter decomposed in less than 3 months, demonstrating preferential decomposition despite it being in areas of low earthworm numbers (Stinson et al. 2006).

Lumbricus terrestris prefers the shadier, cooler understories that are found in buckthorn monoculture micro-environments. The bare soil resulting from increased earthworm activity is ideally suited for the germination and survival of buckthorn seedlings (Knight et al. 2007; Stinson et al. 2006). Lower germination success are seen with buckthorn seedlings that fall in more competitive native understory environments because of increased herbaceous plant density and larger litter O-horizons (typically 10–15 cm thick) (Frelich et al. 2006). The types of mycorrhizae (see section 1.2) that typically associate with buckthorn roots are not negatively affected by *L. terrestris* in comparison to those that are associated with native tree species (e.g., sugar maples) (Catling and Mitrow 2012).

The associations between *R. cathartica* and other invasive species go beyond the changes in soil organic matter (SOM) and disturbances in leaf litter dynamics (Heimpel et al. 2010). To begin, buckthorn creates favourable conditions for invasive European earthworm growth and reproduction, which in turn assists with the spread of the introduced Asian flatworm (*Bipalium adventitium*), a specialist earthworm predator. Buckthorn is also the primary overwintering host for oat crown rust (*Puccinia coronata*), which is a problematic plant pathogen for cultivated oats (*Avena sativa*) and other cereal grains (Liu and Hambleton 2013). *Rhamnus cathartica* is also the obligate overwintering host for the invasive soybean aphid (*Aphis glycines*). Buckthorn growth, alongside the increased planting of soybean crop—the aphid's summer host—has led to the aphid becoming the most important soybean pest in North America since 2000 (Ragsdale et al. 2004). The soybean aphid is also linked to three other invasive predatory insects, the lady beetle (*Harmonia axyridis*), the ground beetle (*Agonum muelleri*), and the Asian parasitoid of the soybean aphid (*Aphelinus certus*) (Heimpel et al. 2010).

Finally, the primary dispersal of buckthorn seeds is done by a number of bird species including the European starling (*Sturnus vulgaris*) (Heimpel et al. 2010). Rhamnaceae seeds in native European ranges are typically dispersed by birds (Godwin 1943); however, European starlings are not one of the main dispersal agents in Germany or the United Kingdom (Heimpel et al. 2010). Up to 8.3% of the stomach contents of European starlings caught in New York state consisted of common buckthorn berries (Lindsey 1939). The purgative effects of buckthorn fruit on starlings isn't yet known, so there may only be a partial link between the European starling and the spread of buckthorn in North America (Heimpel et al. 2010).

1.2 Arbuscular Mycorrhizal Fungi (Glomeromycota)

1.2.1 AMF and Plant Symbiosis

Arbuscular mycorrhizal fungi (AMF) represent a phylum of fungi, the Glomeromycota (Schüßler et al. 2001), in which four orders have been described: the Glomerales, the Diversisporales, the Archaeosporales and the Paraglomerales (Krüger et al. 2009). These fungi are obligate biotrophs and form associations with approximately 67% of surveyed land plants (Brundrett 2009) based on a 400 million-year-old reciprocal exchange system (Taylor et al. 1995). AMF provides limiting nutrients (primarily phosphorus) in exchange for carbon assimilates (van der Heijden and Horton 2009, Smith and Read 2008) by creating arbuscules, tree-like nutrient exchange structures surrounded by the host membrane, within root cortical cells (Bever et al. 2001; Chandramohan et al. 2002). Members of the suborder *Glomineae* also forms vesicles (storage organs) within plant cortical cells (Bever et al. 2001). Buckthorn associates with AMF in both native and invaded ranges (Knight 2006), but details on the functional and taxonomic types of the associations are unknown. Other major mycorrhizal groups include ectomycorrhizal (ECM) and ericoid mycorrhizal fungi (Smith et al. 1997), but these are not the focus of this study since they do not associate with buckthorn (Au and Tuchscherer 2014; Knight 2006).

The arbuscule, surrounded by the host membrane, is a structure with high surface area where the fungus primarily provides phosphates in exchange for plant-derived carbohydrates (Bever et al. 2001). Since AMF are obligate biotrophs, anywhere from 4%

to 17% of the host's fixed carbohydrates may be taken (Dong et al. 2005). Some nitrogen and trace elements (Cu and Zn) can also be absorbed by the extraradical mycelium (ERM) of AMF (Smith and Read 2008). Nitrogen sources taken up by the ERM include NH_4^+ (Bennett and Wallsgrove 1994; Hawkins et al. 2000), NO_3^- (Bennett and Wallsgrove 1994; Hawkins et al. 2000; Walker et al. 2003) and organic nitrogen sources (Hawkins et al. 2000; Leigh et al. 2009; Mithöfer and Boland 2012).

A reciprocal reward system has been observed where an influx of carbon from the plant to the fungal partner results in increased P uptake and transfer to the host (Ellison and Barreto 2004; Hasan and Wapshere 1973). Similarly, inorganic and organic nitrogen uptake and transport in the ERM are enhanced in response to experimental addition of sucrose (carbohydrates) in the root compartment (Fellbaum et al. 2012). This demonstrates the ability of AMF to benefit the plant by providing the most limiting nutrient (P or nitrogen) as a response to increases in carbon supply (Fellbaum et al. 2012).

1.2.2 Methods of Dispersal

Arbuscular mycorrhizal fungi, like other mycorrhizae, propagate using infective hyphae, hyphal fragments, or asexual spores (Bever et al. 2001). The colonization of a root, leading to the formation of vesicles and arbuscules within the cortical cells, may also lead to the extension of the hyphal network from one root to another or to different hosts (Bever et al. 2001). Spores are formed within the root cortex or the soil, having the potential to colonize another host with an optional period of dormancy in between (Bever et al. 2001).

Mycorrhizal fungi colonize and connect roots of similar or different plant species, constructing a mycelial network for resource distribution regardless of plant size, identity, age, or forest dominance (van der Heijden and Horton 2009). Apart from nutrient acquisition, arbuscular mycorrhizae influence plant growth, improve plant resistance to stressors such as drought, prevent nutrient leaching, facilitate bacterial dispersion, and bind soil particles (van der Heijden and Horton 2009).

1.2.3 Host Specificity and AMF Diversity

Mycorrhizal diversity may not directly reflect aboveground plant diversity. In some cases, species-poor coniferous forests may have hundreds of ECM fungi (Trappe 1997), but species-rich tropical or temperate forests may only boast one to two dozen AMF species (Allen et al. 1995).

Aboveground plant community structure affects fungal community composition (Bever et al. 2001), and vice versa. There are two explanations for the maintenance of AMF diversity within a forest community: (1) all of the species have similar ecological niches within the plant roots, or (2) fungal species are ecologically distinct and occupying different niches (Bever et al. 2001). The first hypothesis holds true if diversity is maintained by random drift processes, and has invariably (possibly incorrectly) been substantiated by the observation of single fungal isolates colonizing multiple plant hosts, and single plant species hosting multiple fungal species (Bever et al. 2001). However, multiple fungi have been shown to differ in their effects on plant hosts (Nemec 1979; Powell et al. 1982). The second hypothesis assumes that individual fungi are more competitive in their respective roles, which means that multiple niches within a habitat maintain a diverse community (Bever et al. 2001). Individual species play different roles in plant communities (phosphorus facilitation, pathogen protection, etc.), so a full complement of fungi would improve the plant community's productivity (Newsham et al. 1995).

The question of whether AMF display narrow or wide ranges of host specificity has been explored with trap cultures in soils from various ecosystems, including tallgrass prairies, sand dunes, California grasslands, chalk grasslands, and agricultural fields (Bever et al. 2001). In all systems, fungi that were "trapped" by various host plants sporulated differently depending on the plant species (Bever et al. 1996). Bever et al. (2001) showed dominance of the mycorrhiza *Acaulospora colossica* when grown with only field garlic (*Allium vineale*), but the same mycorrhiza was only a minor component of a community containing planted *Plantago lanceolata*. *Scutellospora calospora* displayed the reverse relationship. A similar distribution of mycorrhizae relating to host specificity was also

observed *in vivo* (Bever et al. 1996; Schultz 1996). Differences in AMF temporal abundance were also observed; some were active in the fall and winter months, resulting in sporulation in late spring, and others were active in the spring and summer months, and sporulated at the end of summer (Dumbrell et al. 2011; Santos-González et al. 2007; Schultz et al. 1999).

Plants can exude chemical signals that attract mycorrhizal fungi when they lack nutrients, or can reduce root colonization and mycorrhizal phosphorus uptake during high nutrient availability (van der Heijden and Horton 2009). Not all plants equally benefit from mycorrhizae (van der Heijden and Horton 2009, Barto et al. 2011), and multiple AMF species may simultaneously associate with a single plant, each with its own cost-benefit relationship (van der Heijden and Horton 2009). Some woody species, such as maple (*Acer saccharum*, *A. rubrum*) and ash (*Fraxinus americana*) (Barto et al. 2011), and understory herbs (van der Heijden and Horton 2009) are thought to be AMF dependent, especially during seedling emergence and establishment (Barto et al. 2011). Perennials can be colonized by AMF in as little as 3 to 6 days after seedling emergence (van der Heijden and Horton 2009).

Within the complex dynamics of plant-fungal relationships, there is the potential to develop positive and negative feedback growth loops. In a positive feedback dynamic, the fungus that promotes the highest growth rate of the host will in turn have a higher relative growth rate on the host, if it is the preferred associated species (Bever et al. 2001). Positive feedback may lead to a local loss of mycorrhizal diversity and contribute to the small-scale heterogeneous spatial structuring of forest populations, but also promote the stability of large scale diversity, where each host supports the growth of a different mycorrhiza (Bever et al. 1997). Alternatively, local and large scale plant and fungal diversity may be maintained if the fungus promoting the growth of one host has a higher growth rate on another host species. This is a negative feedback dynamic, where there is a reduction in the benefit a plant species receives from its fungal partner over time (Bever et al. 2001).

1.2.4 Habitat Disturbance

Fungi have a strong effect on forest plant succession (Gange et al. 1990; Janos 1980). Nonmycotrophic plants dominate in environments where disturbance reduces the density of infective fungal parts (spores and hyphae) (Medve 1984). With the eventual invasion of fungi, plants that are facultative or obligate fungal symbionts should have a higher competitive advantage (Janos 1980). Plant restoration benefits from the inoculation of these fungi into the soil (Aerts and Honnay 2011; Korb et al. 2003). Evidence for fungal successional dynamics has also been observed (Johnson et al. 1991; Kernaghan 2005), indicating that the presence or absence of fungi may also influence later stages of plant succession.

1.3 Research Objectives

Several studies on the mechanisms of invasion and the ecological impacts of invasive species in North America view sugar maple communities as an integral part of native temperate forest ecosystems (Barto et al. 2011; Stinson et al. 2006). In this study, sugar maple (*Acer saccharum* Marsh.) stands were surveyed to observe the changes in native soil AMF communities in the midst of buckthorn (*Rhamnus cathartica* L.) invasion because maples associate with and rely on AMF during all life stages (Barto et al. 2011).

This project explores two aspects of the biology of the invasive European buckthorn in Southern Ontario. It will document 1) the aboveground fungi that are associated with buckthorn, and 2) buckthorn's belowground impacts on arbuscular mycorrhizal fungi (AMF) associated with sugar maple trees and seedlings through allelochemical exudates and leachates.

There is limited information on buckthorn-associated fungi in Canada; many of the documented observations originate in Europe and the USA. Preliminary surveys in London, Ontario, and the surrounding area identified multiple undocumented species on buckthorn, which necessitated a more thorough analysis.

Co-evolutionary resistance to buckthorn allelochemicals developed by European AMF communities may not yet have occurred in invaded North American ranges. It is

hypothesized that the addition of buckthorn allelochemicals to naïve sugar maple seedlings will result in a change in the native root-associated AMF communities, benefitting the growth of tolerant mycorrhizae over those that are not. Differences in allelochemical tolerance will be observed through changes in community composition and abundance between treated and untreated samples.

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Chapter 2: Fungi associated with *Rhamnus cathartica* in Southwestern Ontario

2.1 Introduction and Objectives

Common buckthorn (*Rhamnus cathartica*) thrives in a variety of habitats due to its high tolerance to a wide range of light and soil conditions (Heneghan et al. 2006). It is often found in woodlots and open fields that are subject to disturbances, growing into dense single-species stands that effectively choke out native understory plants (Catling and Mitrow 2012; Knight et al. 2007). Buckthorn seeds are mainly distributed with the help of birds and animals that consume the fruits. Seeds have high germination success and seedlings are difficult to eradicate once established (Catling and Mitrow 2012). This becomes problematic when buckthorn grows in or near diverse native communities as it is a better competitor for light and resources (Klionsky et al. 2011). The reduction of native plant biodiversity is a monumental problem because many organisms have evolved a dependence on specific plant hosts to survive. Ignoring the spread of invasive plants in small areas may lead to a decline in the health of an entire ecosystem, and so the development of cost-effective buckthorn removal strategies is crucial.

2.1.1 Chemical and Mechanical Buckthorn Removal

The estimated annual cost of damages and invasive species management in Canada in the forest sector is $20 billion, and $2.2 billion for invasive plants in the agricultural sector (Environment Canada 2012). In London, Ontario, buckthorn accounts for about 19.5% of the urban forest tree population and is the most prevalent tree in five of the seven land use types in the city (UFORE 2012). It has the highest occurrence in disturbed habitats, including medium/high density residential areas, natural areas/open spaces, and industrial areas (UFORE 2012).

Eradication of buckthorn from invaded plant communities can be difficult for a number of reasons. Established buckthorn populations can have densities of several thousand stems per hectare, cut stumps will readily resprout unless chemically treated, seedbank densities and germination rates can be high, with seed viability remaining for 2–6 years after the adults are removed, and there may be a constant supply of seeds brought

in by birds feeding in untreated areas (Heimpel et al. 2010). Buckthorn sapling removal from invaded areas and maintenance of uninvaded areas requires constant monitoring (Converse 1984; Larkin et al. 2014), which may be expensive and/or unfeasible depending on the level of degradation of a site.

In 2004, the City of London began experiments in woodlots and municipal parks to determine the best management practices for buckthorn. Mechanical techniques spanning a three-year removal program showed promise: the first year would remove all seed-bearing stems, the second year would remove any stems above the knee, and the third would remove any stems above the ankle (Bergsma and De Young 2012). There are multiple options for mechanical control of buckthorn, as outlined by the Upper Thames River Conservation Authority (UTRCA 2007), which include cutting/mowing, girdling, pulling/excavation, burning, underplanting, and restoring water levels. Common buckthorn vigorously resprouts from the buds at the base of the stem after cutting. Revisiting and cutting buckthorn every year in early June and late August for three successive years may be effective enough to weaken the root systems (Converse 1984). Girdling buckthorn plants involves cutting into the phloem (inner bark) of the plant but leaving the xylem (sapwood) intact. This allows the roots to send nutrients up to the aboveground structures, but does not allow the delivery of photosynthates to the roots, and may take anywhere from one to two years for the plant to die (UTRCA 2007). The removal of seedlings and small plants can be done when the soil is moist using hand pulling or excavation with a grubbing hoe, although this technique may activate dormant seeds within the seedbank. Burning buckthorn and the restoration of water levels back to historic conditions, specifically in wetlands, has been shown to control buckthorn (UTRCA 2007) but are not techniques that are currently used in London. Common management programs within London run by the UTRCA typically rely on basal bark sprays, manual pulling, and foliar control, with consideration to the size of the treatment area, stem density, sensitivity of the habitat in the management area and adjacent land, and availability of resources for any given project (Pers. comm. Brandon Williamson, UTRCA, June 2016). Following any invasive removal, underplanting the disturbed soil with native woody species is required to encourage the natural rehabilitation of the area. However, it was seen that underplanting with sugar maple seedlings in oak woods of Morton Arboretum Illinois had poor success under buckthorn

canopies (Converse 1984), indicating that the choice of rehabilitative species is important and there may be legacy effects within the soil.

Chemical applications of glyphosate (Roundup®) or triclopyr (Garlon®) on cut stumps are a common strategy for buckthorn removal (Converse 1984; Reinartz 1997), but also require multiple applications over a period of years to prevent germinating seeds from re-establishing. Unfortunately, vigorous re-sprouting from a cut buckthorn stem is common (Pers. comm. Alastair Biscaia, Credit Valley Conservation, September 2016), and stump applications of Garlon RTU or glyphosate may be ineffective or unfeasible, especially in wet conditions (Dornbos Jr. and Pruim 2012) or due to the ineffective mode of delivery (spray bottles). Within London, Ontario, chemical control is limited to late fall or early winter, while buckthorn is still growing, to reduce harm done to dormant native plants. Stump and basal bark chemical applications employ Garlon RTU (pre-mixed at 23% solution in mineral spirits) and foliar applications use RoundUp Weathermax mixed in water (Pers. comm. Brandon Williamson, UTRCA, June 2016). The Thames Talbot Land Trust (TTLT) employ glyphosate (Roundup®) on cut buckthorn stumps when temperatures are above ~10 °C, and Garlon RTU on cut stumps or painted onto the base of the tree (up to ~15 cm in diameter) during colder weather (pers. comm. Daria Koscinski, TTLT, February 2017). Both RoundUp Weathermax and Garlon RTU have proven effective at controlling adult and seedling buckthorns, and have the advantage of reduced labour and physical disturbance to the soil and groundcover compared to cutting or pulling methods. However, chemical control may not always be feasible due to pesticide spray drift affecting the surrounding environment, especially near water, or harming human health (Bales and Krick 2012).

2.1.2 Biocontrol agents

There is growing interest in developing biocontrol agents for buckthorn and other invasive plants. Traditional methods involve the testing and release of co-evolved host pathogens from its native Eurasian range, or the application of natural pathogens to target the invasive species early in the growing season to kill it or reduce its competitive ability (Templeton 1979). Arthropod species including internal feeders and sap suckers (those that feed from

the interior or the exterior of the plant, respectively) were prioritized for biological control of buckthorn, but none were monospecific at the genus or species level, targeting native plants in the genus *Rhamnus* as well (Gassmann and Tosevski 2014). The discovery of '*Candidatus Phytoplasma rhamni*', an obligate bacterial pathogen that lives in the phloem tissue of buckthorn and causes Buckthorn Witches' Broom (BWB) phytoplasma in Germany, sparked interest for use as a biocontrol. However, high transmission risks of BWB to the native *Rhamnus alnifolia* in North America (Gassmann and Tosevski 2014) as well as the occurrence of the phytoplasma in 25% of surveyed *R. cathartica* in Europe without BWB symptoms means it is a weak and unreliable pathogen-host relationship for effective use as a biocontrol (Jovic et al. 2011).

The use of fungal pathogens as a biocontrol (mycoherbicides) is a viable solution for buckthorn management. This technology has already been studied for invasive management programs, with the first agent, the rust *Puccinia chrondrillina*, being released in Australia in 1972 to control *Chrondilla juncea* (skeleton weed) (Hasan and Wapshere 1973). Since then, over 25 introductions have been made, with a large number having a major impact on invasive alien weed populations (Evans 2002), but the technology is still in its early stages. Pathogenic opportunistic fungi that are present in invaded ranges can be cultured and formulated as a product to be applied in the appropriate season. Commercial mycoherbicides have already been introduced in North America: *Colletotrichum gloeosporioides* is used for the control of northern jointvetch (*Aeschynomene virginica*), and fresh preparations of *Phytophthora palmivora* are used against stranglervine (*Morrenia odorata*) (Ellison and Barreto 2004). The natural fungal plant pathogen *Chondrostereum purpureum* sold under the Chontrol Peat Paste (CPP) label is intended for use on broad-leaved plants as a biocontrol. Applications to buckthorn during the early spring on girdled trees resulted in a 90% mortality (Au and Tuchscherer 2014), although caution may be required in some settings since *Chondrostereum* is known to cause silver leaf disease of commercial fruit trees (Agrios 2005). Seasonal limitations on the use of this product resulted in lower success in late summer and late fall periods due to temperatures beyond the optimal 15–25 °C range, as well as low efficacy on cut stump applications (Au and Tuchscherer 2014). In Manitoba, CPP paste applied onto girdled stems resulted in 70-90% of stems showing no regrowth in the following spring, whereas paste application on cut

stumps higher regrowth (Nature Manitoba 2014). It was suspected that the initial available colonisable area available to the fungus increased its efficacy the next growing season (Nature Manitoba 2014) Similar opportunistic pathogenic fungi may be used in a spore mixture and applied to buckthorn to help control regrowth after the use of mechanical controls.

One solution may be the use of a "Multiple-Pathogen Strategy" (MPS) to create a mixture consisting of at least three targeted pathogenic fungi that may increase the effectiveness of the product as well as the mortality of plants (Chandramohan 1999). This will allow for compensation by other species if one pathogen fails, reduce the chance of resistance development in the target weed, and favour the potential synergism between pathogens to enhance efficacy (Chandramohan 1999). The simultaneous control of northern jointvetch (*Aeschynomene virginica*) and winged waterprimrose (*Jussiaea decurrens*) was achieved through the addition of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* and *C. gloeosporioides* f. sp. *jussiae* (Boyette et al 1979). A pre-injection of *Alternaria macrospora* allowed for the control of spurred anoda (*Anoda cristata*) by *Fusarium lacteritium* (Crawley and Walker 1983). In 2002, three grass pathogens (*Drechslera gigantea, Exserohilum longirostratum, and E. rostratum*) were tested individually and as a mixture for the control of 7 grasses in a mixed plot, as well as a separate field trial of guineagrass (*Megathyrsus maximus*). The three-pathogen mixture was just as effective as single isolates in controlling both trials, with no guineagrass regrowth for ~10 weeks (Chandramohan et al. 2002). Spore suspensions on the same three-pathogen mixture from Chandramohan et al. (2002) was further tested on green foxtail (*Setaria viridis*), which resulted in a noticeable damage on seedlings one day after treatment and substantial seedling death after one week (Casela 2010). Constraints on the type of pathogen strains used to develop bioherbicides include finding those that are virulent, destructive, and have high host specificity (Chandramohan 1999). Without a comprehensive, up-to-date list of fungi that naturally occur on the target species, the development of successful bioherbicides would be difficult.

2.1.3 Fungi on Buckthorn

Of all known insect and fungal species associated with buckthorn, only a few occur within its naturalized range. A total of 30 fungal species have been reported on *R. cathartica*, of which just five species: *Cucurbitaria rhamni* (Barr 1990) and four members of the *Puccinia coronata* complex (Conners 1967; Ginns 1986; Jin and Steffenson 1999; Liu and Hambleton 2013), have been documented in Canada (Table 2.1). A few additional fungi, such as *Cercospora rhamni*, *Nectria cinnabarina*, *Phyllosticta rhamni*, *Pyrenopeziza morthieri* [as *Pezicula morthieri*], and *Schizophyllum commune*, have been noted on other species of *Rhamnus* in Canada (Conners 1967; Ginns 1986).

2.1.4 Aims and Objectives

A thorough survey of fungi on buckthorn has yet to be done for Southern Ontario populations. Many of the documented cases outside of Europe are found in the USA, thereby increasing the likelihood of similar observations being made in Canada. The objective of this chapter is to survey and identify fungi growing on buckthorn (*Rhamnus cathartica*) in open- and closed- canopied environments in the London and surrounding area.

Table 2.1. Fungi associated with dead or living common buckthorn (*Rhamnus cathartica*); summarizing records from the Systematic Botany and Mycology Laboratory (SBML) Database (Farr and Rossman), Diseases and Pests of Ornamental Plants (Pirone 1978), and Agriculture Canada (Conners 1967; Ginns 1986; Liu and Hambleton 2013)

Fungus	Location(s)
Asteromella vogelii	Europe
Berkleasmium dudkae	Europe
Biscogniauxia simplicior	Europe
Cercospora rhamni [= *Passalora rhamni*]	Europe, USA (New Jersey, New York, Wisconsin)
Cladosporium aecidiicola	Europe
Coniothyrium dumeei	Europe
Cucurbitaria rhamni	Canada (Ontario) and Europe
Diaporthe fibrosa	Europe
Dothiorella sp.	USA (North Dakota)
Erysiphe friesii [= *Microsphaera friesii*]	Europe and Asia
Eutypa lata	Europe
Eutypella extensa	Europe
Fomitiporia punctata [= *Phellinus punctatus*]	Europe, USA (North Dakota)
Leucostoma persoonii [as *Cytospora leucostoma*]	Europe
Lophiostoma rugulosum	Europe
Lophiostoma viridarium [as *L. desmazieri*]	Europe
[*Oidium* sp. - anamorphic *Erysiphe*]	Europe
Peniophora violaceolivida	Europe
Phellinus rhamni	Europe
Phyllactinina alnicola [= *Microsphaera alni*]	USA (Wisconsin)
Phyllactinia guttata [= *Phyllactinia suffulta*]	Europe
Phyllosticta cathartici	Europe
Phyllosticta rhamni	Europe and USA (Wisconsin)
Phyllosticta rhamnicola	Europe
Phyllosticta sp.	USA (Wisconsin)
Phymatotrichopsis omnivora	USA
Phytophthora ramorum	USA (California)
Puccinia coronata [= *P. lolii* and *P. rhamni*, and includes material previously named *Aecidium rhamni*, *Dicaeoma rhamni*, *P. coronifera* and *P. aecidii-cathartici*; *P. coronata* var. *avenae* and *P. coronata* var. *coronata*]	Europe, Canada, and USA
Puccinia coronati-hordei [= *P. coronata* var. *hordei*]	Canada (Manitoba, Saskatchewan) and USA (North Dakota, South Dakota)
Puccinia coronati-brevispora	Canada, Europe
Puccinia coronati-agrostis	Canada, Europe

Septoria rhamni-catharticae	Europe
Sphaeropsis rhamni	USA (Oklahoma)
Tubercularia sp. [anamorphic *Nectria*]	USA (North Dakota)

2.2 Materials and Methods

2.2.1 Sampling Regions

Macrofungi were sampled in multiple locations within or around the city of London, Ontario (Table 2.2, Figure 2.1). At least 100 buckthorns from both open habitats and within closed canopy forest were sampled within each location in early spring, summer, fall, and early winter. A random wandering survey design was used. Fungal fruiting bodies, including a section of the substrate bark, branch, or leaf, were collected in individual paper bags, air dried at low heat (~35 °C) with forced air in a commercial food dehydrator, and stored in the lab.

2.2.2 Fungi Identification

Samples were rehydrated with a drop of 95% ethanol followed by deionized water for 1 minute, thinly sectioned and mounted on slides. Mountants for microscopy included KOH (2% aqueous) and Melzer's reagent (1.5 g potassium iodide, 20 mL distilled H_2O, 0.5 g iodine, 20 g chloral hydrate), the latter to stain for amyloidity (Kirk et al. 2008). Morphological features were recorded and photographed. Identification keys were used to identify the sample to genus and species level. Vouchers are labeled with collector's name and date in the UWO Herbarium.

Table 2.2. Sampling locations, coordinates, and seasons visited for all fungi on common buckthorn (*Rhamnus cathartica*) surveys.

Location	Co-ordinates (UTM, Zone 17T)	Year/season visited
Five Points Forest - Driedger Tract, near Putnam, ON	505949mE 4761949mN	2014: fall
AFAR Trail[1] west of Western University campus, London ON	477085mE 4761818mN	2013: fall; 2015 spring
Kains Woods ESA, London, ON	471261mE 4758802mN	2014: spring; fall
Kilally Meadows ESA, London, ON	482984mE 4765179mN	2014: fall
Komoka Provincial Park, London, ON	467893mE 4754927mN	2014: spring, summer, fall; 2015: spring
Medway Valley Heritage Forest ESA, London, ON	476649mE 4761462mN	2013: fall, winter; 2014: spring, summer, fall; 2015: spring
rare Charitable Research Reserve, Cambridge, ON	552191mE 4803346mN	2014: spring, summer, fall; 2015 summer
Sifton Bog ESA, London, ON	473676mE 4757663mN	2013: fall; 2014: spring, fall,
Warbler Woods ESA, London, ON	471258mE 4756433mN	2014: summer, 2015: spring
Westminster Ponds ESA, London, ON	481962mE 4755073mN	2014: fall; 2015 summer

[1] Trail joins with Medway Valley Heritage Forest ESA

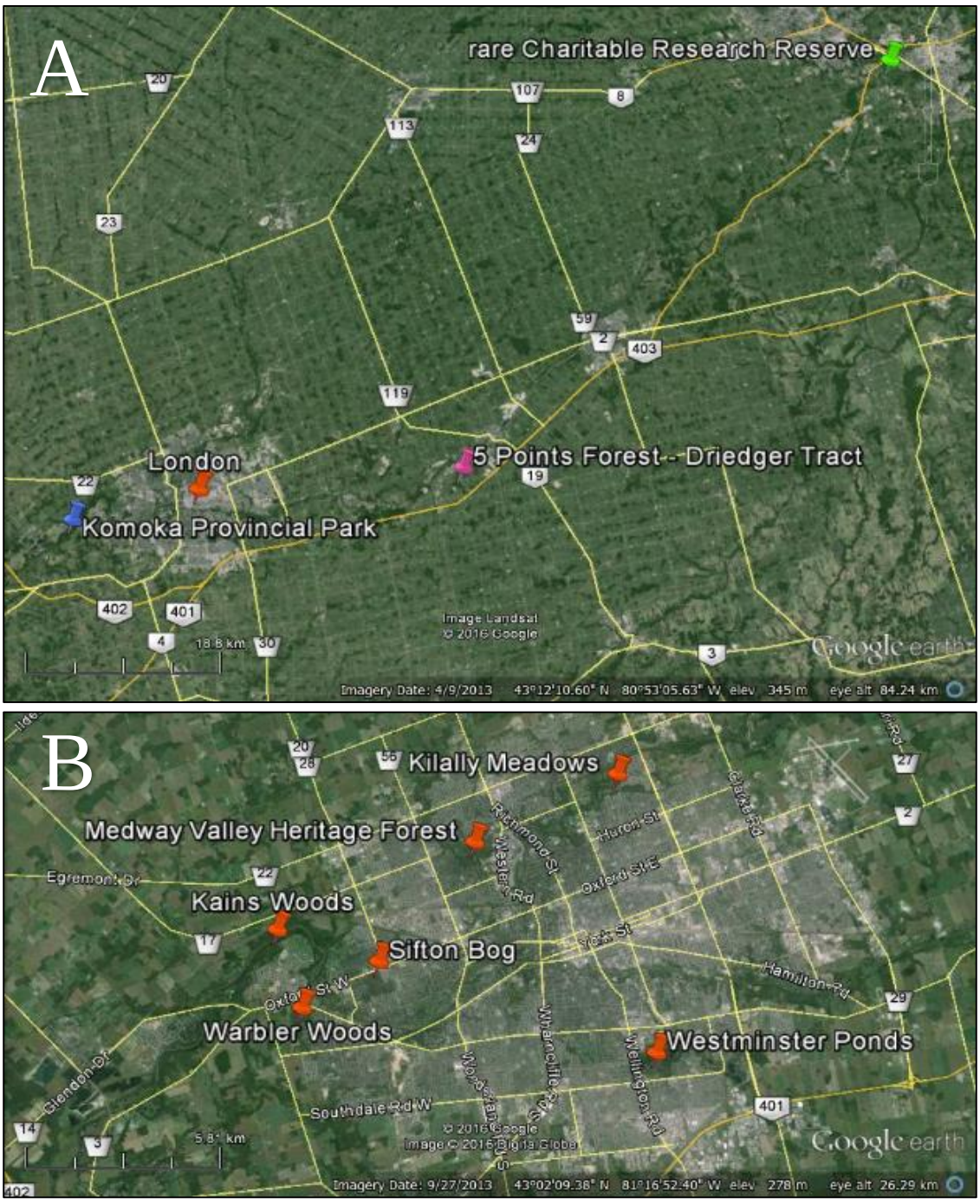

Figure 2.1. Survey locations for fungi on common buckthorn (*Rhamnus cathartica*) within **A)** Komoka Provincial Park (London ON), *rare* Charitable Research Reserve (Cambridge, ON), and Five Points Forest – Driedger Tract (Putnam, ON) (17T 509350mN and 4783358mW. Google Earth**.** April 9, 2013); **B)** Environmentally Significant Areas (ESA) in London, ON (17T 478815mN and 4870148mW. Google Earth**.** September 27, 2013)

2.3 RESULTS

The survey of macrofungi growing on common buckthorn (*Rhamnus cathartica*) resulted in the addition of 23 additional species from a total of 45 observations to the known list (Table 2.1). Two species (*Puccinia coronata* and *Cucurbitaria rhamni*) had been previously identified in Canada and the USA (Farr and Rossman, Barr 1990, Conners 1967; Ginns 1986, Liu and Hambleton 2013), bringing the total number of fungi known on buckthorn to 52 species, with 28 found in Canada (Table 2.3).

A cluster of the honey mushroom (*Armillaria mellea* s.l.) root pathogen was seen (Figure 2.2 A) at the base of a fallen buckthorn in Sifton Bog ESA (Sifton Bog), a year after characteristic rhizomorphs were seen underneath the peeled bark of another dead buckthorn. The teleomorph stage of the coral spot fungus group (*Nectria cinnabarina*) (Figure 2.2 B & C), a weak twig and branch pathogen, had not been seen on buckthorn in North America prior to this survey, but its anamorph *Tubercularia* sp. had been seen in North Dakota (Farr and Rossman). During the survey period, the teleomorphic coral spot was seen in nearly all sampling locations in the fall. The canker fungi *Hypoxylon fuscum* and *H. perforatum* were found in Medway Valley Heritage ESA (Medway Valley), and in Sifton Bog (Figure 2.2 D & E) on dying buckthorn branches without bark. A weak opportunistic branch pathogen, *Cylindrobasidium evolvens*, was found in multiple locations (Figure 2.2 F).

Although *Schizophyllum commune* (split gill) has a ubiquitous distribution on dead wood as a white rot fungus (Schmidt and Liese 1980), it was found only once in two locations, Sifton Bog and Westminster Ponds ESAs (Figure 2.2 G). Other primary decomposers included *Antrodia malicola*, *Datronia mollis*, *Irpex lacteus*, *Polyporus alveolaris*, *Plicaturopsis crispa*, *Steccherinum ochraceum*, and *Phlebia radiata* (Figure 2.2 H-N). Weaker primary decomposers and secondary decomposers included *Crepidotus calolepis*, *Crepidotus caspari*, *Daldinia concentrica*, *Hyphoderma* cf. *mutatum*, *Lachnum virgineum*, *Merismodes fasciculata*, *Morrisographium persicae*, *Mycena meliigena*, *Peniophora incarnata*, and *Peniophora cinerea* (Figure 2.2 O-X).

Table 2.3. Survey results of fungi found on dead or living common buckthorn (*Rhamnus cathartica*) within Medway Valley ESA (MV), Sifton Bog (SB), Westminster Ponds (WP), AFAR trail (AFAR) on Western's campus, Five Points Forest (FPP), *rare* Charitable Research Reserve. Detection methods include visual (V) or visual and microscopy (VM).

	ESA						
Fungus	**MV**	**SB**	**WP**	**AFAR**	**FPP**	***rare***	**Detection**
Hypoxylon perforatum	*						VM
Antrodia malicola group		*					VM
Crepidotus calolepis		*					VM
Crepidotus caspari		*					VM
Hypoxylon fuscum		*					VM
Hyphoderma cf. *mutatum*		*					VM
Lachnum virgineum		*					VM
Mycena meliigena		*					V
Phlebia radiata		*					VM
Plicatura crispa		*					VM
Morrisographium persicae				*			VM
Daldinia concentrica					*		V
Datronia mollis					*		V
Steccherinum ochraceum					*		VM
Armillaria mellea group	*	*					V
Merismodes fasciculata	*	*					VM
Peniophora cinerea	*	*					VM
Polyporus alveolaris	*			*			V
Schizophyllum commune		*	*				V
Irpex lacteus		*				*	VM
Peniophora incarnata	*	*			*		VM
Cylindrobasidium evolvens		*	*		*		VM
Nectria cinnabarina[2]	*	*	*	*		*	VM
Puccinia coronata[1]	*	*	*	*	*	*	V

[1] Previously identified on buckthorn (*R. cathartica*) in Table 2.1

[2] including *Tubercularia* anamorphs

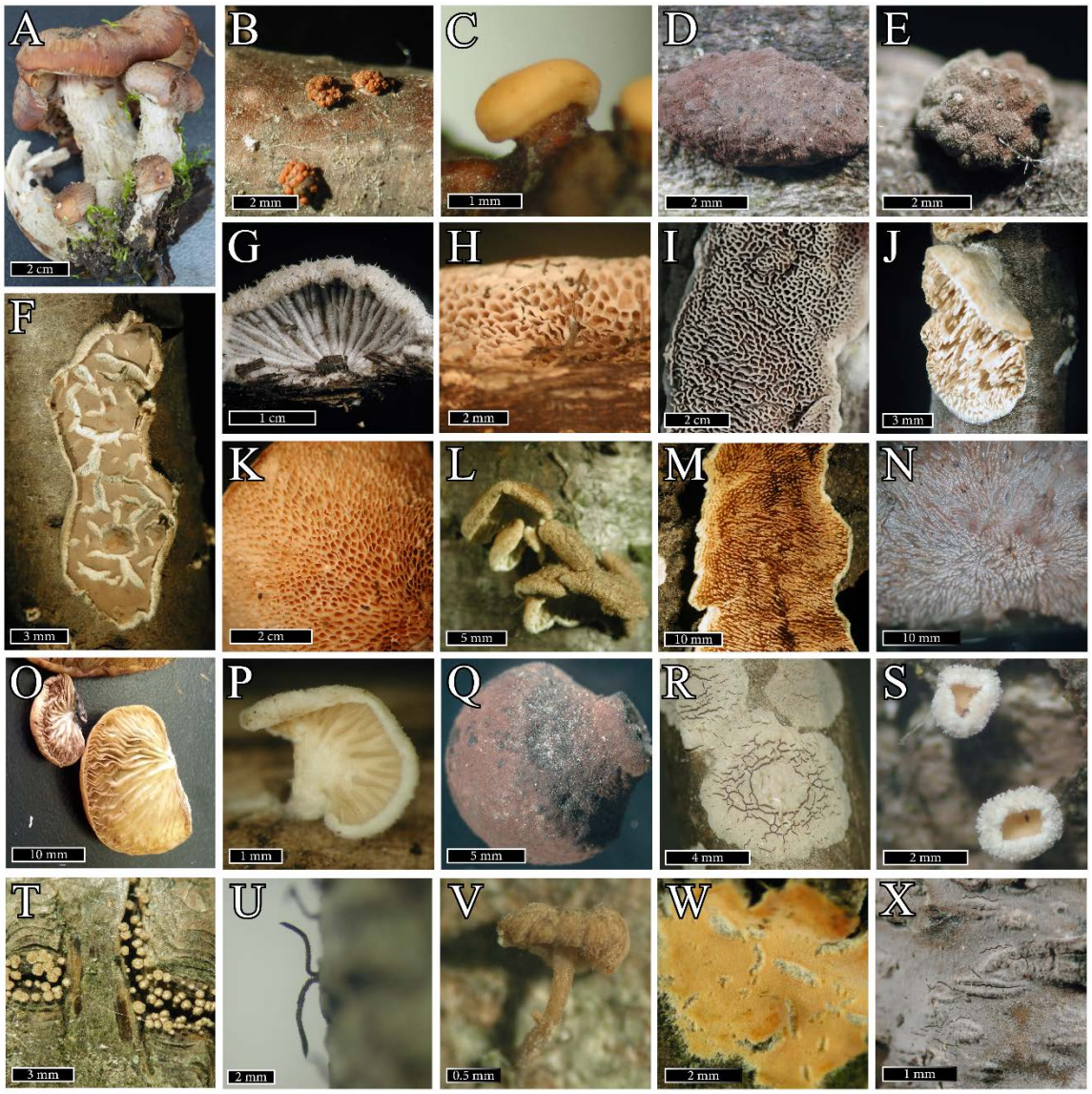

Figure 2.2. Fruiting bodies of all fungi found on buckthorn (*Rhamnus cathartica*). A) *Armillaria mellea* (Vahl) P. Kumm. s.l. (honey mushroom); **B)** *Nectria cinnabarina* (Tode) Fr. group (coral spot) and its **C)** *Tubercularia* anamorph (asexual stage); **D)** *Hypoxylon fuscum* (Pers.) Fr.; **E)** *Hypoxylon perforatum* (Schwein.) Fr.; **F)** *Cylindrobasidium evolvens* (Fr.) Jülich; **G)** *Schizophyllum commune* Fr. (split gill); **H)** *Antrodia malicola* (Berk. & M.A. Curtis) Donk group; **I)** *Datronia mollis* (Sommerf.) Donk; **J)** *Irpex lacteus* (Fr.) Fr.; **K)** *Polyporus alveolaris* (DC.) Bondartsev & Singer (=*Neofavolus alveolaris*); **L)** *Plicatura crispa* (Pers.) Rea; **M)** *Steccherinum ochraceum* (Pers.) Gray; **N)** *Phlebia radiata* Fr.; **O)** *Crepidotus calolepis* (Fr.) P. Karst; **P)** *Crepidotus caspari* Velen.*;* **Q)** *Daldinia concentrica* (Bolton) Ces. & De Not. (coal fungus, King Alfred's cake); **R)** *Hyphoderma*

cf. *mutatum* (Peck) Donk; **S)** *Lachnum virgineum* (Batsch) P. Karst.; **T)** *Merismodes fasciculata* (Schwein.) Donk; **U)** *Morrisographium persicae* (Schwein.) Illman & G.P. White; **V)** *Mycena meliigena* (Berk. & Cooke) Sacc.; **W)** *Peniophora incarnata* (Pers.) P. Karst.; and **X)** *Peniophora cinerea* (Pers.) Cooke

Many of the saprobic fungi were found in Sifton Bog because of its high buckthorn stem count as well as the ongoing management of the invasive in the area by the UTRCA. This left weakened or dying trees susceptible to the invasion of opportunistic fungi as well as a large number recently dead trees available for the natural succession of fungal decomposers. Similarly, the entranceway to Komoka Provincial Park and Five Points Forest, having many buckthorn brush piles due to management programs, yielded many more fungi than other survey areas such as *rare* CRR, and other ESAs. In total, 3 observations were made along the AFAR trail connecting to Medway Valley, 7 in Five Points Forest, 2 in Killaly, 2 in Medway Valley, 3 in *rare* CRR, 22 in Sifton Bog, 3 in Warbler Woods, and 3 in Westminster Ponds (Appendix I).

Fungi were rarely seen on open-field buckthorn, despite surveying an equal number of open-field and closed-canopy trees in each location. The only exception was a large, ~10+ year-old buckthorn growing by the parking lot entrance to Kilally Woods with multiple fungi growing on dead branches underneath its full canopy. Thirty-three of the 45 identified fungi (77.8%) were seen on closed-canopied buckthorn, either on the tree itself, a fallen branch, or in a human-mediated buckthorn brush pile. One observation (*I. lacteus*; 2.2% of observations) was made at the forest edge, three fungi (*P. incarnata*, and two *H.* cf. *mutatum*; 6.7% of observations) were seen in an open-field buckthorn, and six fungi (*D. concentrica*, *P. incarnata*, *S. ochraceum*, *D. mollis*, and two *C. evolvens*; 13.3% of observations) were seen in an open-field brush pile of buckthorn branches after a buckthorn management crew had passed through Five Points Forest. *Puccinia coronata* (not included in the total recorded count of 45) was found ubiquitously on buckthorn regardless of its location within or outside forests in the late summer to fall seasons.

2.4 Discussion

Gaining thorough knowledge of an invasive species' range of natural enemies is important in determining its impact in its invaded regions. Fungi growing on the invasive common buckthorn (*Rhamnus cathartica*) have been recorded in its native range in Eurasia, as well as its invaded range in the central and northern United States, but sightings in Canada are limited. In Ontario, two fungi (*Puccinia coronata* and *Cucurbitaria rhamni*) had been identified on buckthorn. However, because of the overlap in biomes across central and northern USA into Canada, it is expected that fungi found associated with the invasive in the United States would also be found in the southern area of this province. A wandering survey of open- and closed- canopied buckthorn trees that spanned all four seasons across 20 months yielded 23 species that can be added to the list of buckthorn-associated fungi.

Armillaria, the causal agent of *Armillaria* root disease, is a facultative necrotroph—colonizing living roots, killing root tissue, and feeding off dead tissue for nutrients. After plant death, *Armillaria* survives as a white-rotter on the infected root system (Redfern and Filip 1991). For this reason, the genus has been well studied in forest communities to determine its trigger for pathogenicity. It is one of the most important genera of fungal root pathogens worldwide, affecting not only tree species, but agroeconomic crops in many climates (Baumgartner et al. 2011). *Armillaria*'s rootlike rhizomorphs can be observed under the bark on root and trunk systems of dead, diseased or healthy host plants (McDonald et al. 1987). Its pathogenicity between isolates can range from very high to obligately saprophytic, where pathogenic severity tends to increase as management intensifies (McDonald et al. 1987). Its mycelium often survives in residual debris, after the clearing of infected forest stands or fruit/nut crop, until the next crop (Redfern and Filip 1991). In Queensland, Australia, *Armillaria* was found in nearly all stumps after clear-cutting an introduced pine forest (McDonald et al. 1987), and chemical and mechanical killing has been linked to increased *Armillaria* activity in hardwood forests (Pronos and Patton 1977; Swift 1972). All three *Armillaria* samples in this study were collected in Sifton Bog, the most intensively managed ESA for buckthorn, where plenty of recently cut or chemically weakened trees and seedlings remained to decompose. Interestingly, parasitism of *Armillaria* by *Entoloma abortivum* resulting in misshapen fruiting bodies

called carpophoroids (Czederpiltz et al. 2001) was also observed in this study (Appendix I).

The coral spot fungus, *Nectria cinnabarina*, and its "*Tubercularia*" anamorph were seen in nearly all sampling areas on dead buckthorn twigs and fallen branches. The *Nectria cinnabarina* group (Rossman 1983) consists of at least 20 morphologically indistinguishable varieties of *N. cinnabarina* (Hirooka et al. 2011) as well as several species of *Tubercularia* anamorphs. It is a common saprobe species, occurring on a range of hardwood trees and woody shrubs in temperate areas (Hirooka et al. 2011). Rarer occasions of facultative pathogenicity on apple and other hardwoods are known as "coral spot", where the fungus typically infects compromised wood, but can later spread (Sinclair and Lyon 2005). *Nectria* cankers were reported on *Acer, Aesculus, Prunus, Robinia, Spiraea, Tilia* and *Ulmus* in 1883 by Mayr (Hirooka et al. 2011), as well as other hardwood shrubs and trees around the world (Sinclair and Lyon 2005).

Hypoxylon fuscum and *H. perforatum* canker fungi are endophytes that develop into wood saprotrophs (Granito et al. 2015). In beech forests, fruiting bodies of *Hypoxylon fragiforme* (Pers.) J. Kickx develop during tree water stress (Chapela and Boddy 1988) and the tree is therefore more exposed to fungal attack in drier conditions (Granito et al. 2015). Water stress may not be the cause for *Hypoxylon* infection on buckthorn since the invasive is known to tolerate a wide range of environmental conditions, and so, the cause of its growth on buckthorn is likely a result of other sources of stress.

The white rot *Cylindrobasidium evolvens* is a pioneer saprobe and weak branch pathogen that colonizes recently dead coniferous and deciduous wood, especially fresh cut surfaces (Vasiliauskas and Stenlid 1998) of corticated branches and trunks (Eriksson and Ryvarden 1976). A comparison of saprobic fungi among *Cylindrobasidium torrendii, Fistulina hepatica, A. mellea,* and *S. commune* shows that *C. torrendii* and *S. commune* are intermediates between white and brown rot fungi, degrading all wood components but leaving the central lamella intact, similar to soft rot (Floudas et al. 2015).

Fungi previously reported on buckthorn in North America but not found in this study included: *Cercospora rhamni [=Passalora rhamni*], *Cucurbitaria rhamni,*

Fomitiporia punctata [=*Phellinus punctatus*], *Phyllosticta rhamni*, *Phymatotrichopsis omnivora*, *Phytophthora ramorum*, and *Sphaeropsis rhamni* (Table 2.1). *Cercospora rhamni* is a leaf spot described on buckthorn in Wisconsin, New Jersey, and New York in the 1960 U.S.D.A. Agriculture Handbook (USDA 1960). Similarly, leaf spot fungi such as *Phyllosticta rhamni*, reported in Wisconsin (Greene 1945; USDA 1960), *P. ramorum*, cause of Sudden Oak Death in California (Ivors et al. 2006), and *Sphaeropsis rhamni* in Oklahoma (Preston 1945) were not sampled in this study. In Wisconsin, USA, *Phyllactina alnicola*, the cause of a powdery mildew of buckthorn leaves, has the ability to impair photosynthesis, stunt growth, and increase senescence of its host plant (Pirone 1978). However, instances of severe buckthorn infection by *P. alnicola* have not been documented. *Cucurbitaria rhamni* has been previously reported in Ontario (Barr 1990) as well as Europe, but was not adequately verified. Samples having similar morphological features, globose black pyrenomycete fruiting body clusters erupting from basal stromatic tissue, were collected, although repeated attempts at culturing or morphological visualization of the characteristic small ovoid ascospores were not successful (http://fungi.myspecies.info/all-fungi/cucurbitaria-rhamni). Similarly, *F. punctata* (synonymous with *P. punctatus*) was not positively identified in this study, although samples with brown resupinate sporocarps were seen in Five Points Forest, which warrants further investigation. Leaf diseases and microfungi were not the focus of this study as identification would have required culturing and sequencing, and leaf spots rarely become serious enough to cause harm (Pirone 1978). However, adequate sampling material of unnamed microfungi can be found in the UWO Herbarium for eventual sequencing studies. The wandering surveys did not include every buckthorn in the area, but great effort was made to find trees with obvious signs of fungal growth, so leaf diseases may have been missed.

Sequencing of all samples within identified species groups, such as *A. mellea*, *N. cinnabarina*, and *A. malicola* would help determine if particular varieties are more commonly associated with buckthorn. Further steps would involve looking at other regions of Canada and the USA, to include both urban and disturbed pockets in rural areas, and to identify a relatively robust fungus or combination of fungi that are able to take advantage of a weakened buckthorn and aid in its eventual eradication from the rehabilitation area.

Extending lists of associated organisms capable of using problematic invasive plants as hosts is key to the ongoing management and removal of the invasives. The success of biocontrol agents can be difficult to predict *in vivo*. Although the application of the causal agent of silverleaf disease, *Chondrostereum purpureum*, in the Chontrol Peat Paste mycoherbicide used on cut buckthorn stumps has met with some success, its efficacy can vary (Au and Tuchscherer 2014). In Manitoba, CPP use is encouraged in sensitive habitats or during seasons when other chemical methods are not as effective (Nature Manitoba 2014). Buckthorn is a vigorous plant, and for this reason the development of an effective and cheap adjunct that can be used in conjunction with the current 'standard' treatment would be beneficial. The use of fungal pathogens to formulate mycoherbicides in a single or multiple-pathogen strategy may become a viable method of control, once we have a complete picture of the buckthorn mycobiota.

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Chapter 3: Common buckthorn (*Rhamnus cathartica*) allelopathy and arbuscular mycorrhizal fungi

3.1 Introduction and Objectives

3.1.1 Chemical Defense Mechanisms

Plants, as a rich source of nutrients for many organisms, have developed a range of structural, chemical, and protein-based defenses (Freeman and Beattie 2008). It has been estimated that plants are able to synthesize more than 200 000 specialized metabolites (Pichersky and Lewinsohn 2011), some of which are secondary metabolites that are toxic, anti-digestive, or unpalatable and help defend against bacteria, fungi, protists, insects, and vertebrates (Mithöfer and Boland 2012). Secondary metabolites also include attractants that allow for, or enhance, the communication between plants and symbiotic insects, epiphytes, and soil microorganisms (e.g., nitrogen-fixing bacteria and mycorrhizal fungi) (Santi et al. 2013; Schmitz and Harrison 2014), as well as allelopathic compounds that result in the inhibition of germination or growth of other plants (or organisms). The release of allelopathic compounds has been implicated in the invasional success of multiple non-native plants in North America, such as garlic mustard (*Alliaria petiolata*) (Barto et al. 2011; Cantor et al. 2011), spotted knapweed (*Centaurea maculosa*) (Bais et al. 2003; Thelen et al. 2005), and common buckthorn (*Rhamnus cathartica*) (Knight 2006; Seltzner and Eddy 2003), among others (Callaway and Aschehoug 2000; Orr et al. 2005). The rationale for considering allelopathy as a mechanism for invasional success hinges on the observation that invasive plants often establish monocultures where diverse native communities once were, and that allelopathy may be more effective in invaded ranges than in originating ones. This is known as the novel weapons hypothesis (Burke and Chan 2010; Callaway and Ridenour 2004; Hierro and Callaway 2003; Thorpe et al. 2009). An example can be seen with the Canada goldenrod (*Solidago canadensis*) species complex, a vital component in diverse grassland and prairie communities in North America, but considered an invasive, rapidly-spreading weed in China (Dong et al. 2005). Conversely, the Eurasian invasive garlic mustard causes significant shifts in mycorrhizal (see 3.1.4) and bacterial

community composition and structure in North America because of allelopathic compounds released into the soil (Thorpe et al. 2009).

3.1.2 Allelochemicals in Common Buckthorn

The purgative effects of common buckthorn have been recorded in English herbal literature dating from 1633 (Kurylo and Endress 2012). Common buckthorn had a long history of pharmaceutical applications, but had fallen out of use by the early 19th century because its effects were “more offensive, and operate more severely” (Coxe 1806, cited in Kurylo 2012) than fruits of other medicinal trees listed in The American Dispensatory from 1806 (Kurylo and Endress 2012).

Despite buckthorn’s decline in medicinal popularity, the plant’s cathartic properties enabled its wide dispersal and low susceptibility to herbivory. This may be partly attributed to the presence of anthraquinones—a chemical class of secondary metabolites—found in all parts of the plant. Over 170 naturally occurring compounds are considered anthraquinones, of which more than half are produced by fungi (e.g., *Penicillium* and *Aspergillus*, mushrooms, and lichens), and others in flowering plants and some insects (Wink 2010). Several plant families, including the Rhamnaceae, the Rubiaceae, and the Fabaceae are rich in anthraquinones (van den Berg et al. 1988).

The most common anthraquinones produced by *Rhamnus* spp. are emodin, rhein, chrysophanol, aloe-emodin, madagascin, and physcion (Genovese et al. 2010; Newman 1966). The compound madagascin was confirmed in *R. cathartica* fruits (Epifano et al. 2012) as well as a newly discovered anthraquinone derivative: 1,8-dihydroxy-2-[(z)-4-methylpenta-1,3-dien-1-yl]anthraquinone). Other known lesser-known compounds include: 2-acetyl-3,8-dihydroxy-6-methoxy-anthraquinone and glucofrangulin (both anthraquinones), dendrochrysanene (a phenanthrene derivative), β-sorigenin and geshoidin (lactones), pruniflorone H (xanthone), rumejaposide I (oxanthone), and kaempferol and quercetin (flavonols) from various parts of a *R. cathartica* plant (Hamed et al. 2014).

As a secondary metabolite, emodin is not essential to the survival and reproduction of plants. The compound was first described over 75 years ago as frangula-emodin (Kurylo

and Endress 2012), but its biological properties have only recently been elucidated. Emodin has been identified in at least 17 plant families (28 genera and 94 species) with a worldwide distribution in tropical, subtropical and temperate regions (Mummey and Rillig 2006). Some of the better known sources include the plant families Fabaceae (*Cassia*), Polygonaceae (*Polygonum, Rheum,* and *Rumex*) and Rhamnaceae (*Rhamnus* in the north temperate zones and *Ventilago* in Australasia). Emodin and other anthraquinones are stored in plants as inactive glycosides (Newman 1966). The most common emodin-related glycosides are emodin-8-glucose, frangulin, and glucofrangulin (Newman 1966). The distribution of emodin among plant organs is ubiquitous, with it being found in the stem, bark, root, and foliage, as well as reproductive organs (flower, fruit, seeds, and pods) (Mummey and Rillig 2006). Secondary metabolites with an adaptive function are found in unequal concentrations in plant organs (Mummey and Rillig 2006). Light intensity and season temporally affect the levels of emodin in *Rhamnus* and other plants. In *R. frangula* bark, three peaks have been observed in April, July-August, and November (Newman 1966), and *R. purshiana* showed a significantly increased emodin content when exposed to a daily photoperiod of 12 h (van den Berg et al. 1988). This may relate to possible functions of emodin in photoprotection from UV radiation as well as the inhibition of superoxide radicals (Newman 1966). In *Rheum undulatum,* anthraquinone content (where 50% was emodin) is highest in spring, having a continuous decrease during the summer (Paneitz and Westendorf 1999). This suggests the occurrence of a tradeoff between defense and development, where the potential for herbivory is highest in the spring and other metabolic activities takes precedence in the summer (growing, flowering, and fruiting) (Mummey and Rillig 2006).

Emodin and its derivatives have purgative effects (Newman 1966). In mammals, emodin glycosides are not absorbed until they reach the large intestine, where bacteria metabolize them into aglycones. In turn, aglycones damage epithelial cells, inhibit Cl^- channels across colon cells (Rauwald 1998), and affect the immune system as well as vasomotor and other metabolic processes (Mummey and Rillig 2006). Emodin extracted from *R. alnifolia* leaves and mixed into an artificial diet was an effective feeding deterrent for the larvae of species such as gypsy moths (*Lymantria dispar*) (Trial and Dimond 1979). Emodin may be responsible for the lower number of recorded phytophagous insects found

on *R. cathartica* in Canada than in Europe, where they are native (Malicky et al. 1970). Emodin can be toxic to some birds and mammals; starlings (*Sturnus vulgaris*) and redwing blackbirds (*Agelaius phoeniceus*) have a LD_{50} of >100 mg kg^{-1}, whereas white footed mice (*Peromyscus leucopus*) avoid emodin-containing foods (Schafer et al. 1983). Emodin-containing plants such as *R. alaternus* allow for fruit dispersal by birds while maintaining low seed predation by invertebrates and microorganisms (Knight et al. 2007). Any unripe, fleshy fruits are well protected against seed predation due to the presence of anthraquinones, as seen in Old World *Rhamnus alaternus* and *R. palestina*, and New World *R. cathartica*, where most bird species do not consume unripe fruits (Newman 1966). The process of fruit ripening is thought to break down secondary metabolites. In *R. alaternus*, emodin deceases during ripening but does not fully disappear (Tsahar et al. 2002). Avian frugivores that act as primary seed dispersers have been seen consuming fruit of *R. cathartica*. The seeds are protected during digestive passage by a moisture-sensitive envelope that splits and ejects the seed after exposure to dry air, allowing germination to be independent of the gut characteristics of dispersers (Izhaki and Safriel 1990). Izhaki (2002) suggested that emodin within the flesh of the fruit may deter germination based on the observation that the removal of fruit pulp (by hand or through digestive passage) is required for the germination of *R. cathartica* and *R. alaternus* seeds.

Growth inhibition of the roots and shoots of sunflower (*Helianthus annus*, LD_{50} = 45 mg L^{-1}) and popcorn (*Zea mays* var. *everta*, LD_{50} = 65 mg L^{-1}) was observed with emodin concentrations ranging from 10 to 100 mg L^{-1} (Hasan 1998). Ninety-eight percent of the sunflowers in the control group germinated, but with exposure to 50 and 100 mg L^{-1} of emodin, percent germination dropped to 76% and 55%, respectively (Hasan 1998). Lettuce seedlings (*Lactuca sativa*) were inhibited by 1.85×10^{-4} M (50 ppm) emodin, with concentrations greater than 3.7×10^{-4} M (100 ppm) inhibiting root and hypocotyl (leaf sheath) growth (Inoue et al. 1992). Klionsky et al. (2011) suspected that growth and germination of herbaceous woodland seedlings (*Eurybia macrophylla*, *Thalictrum dasycarpum*, *Symphyotrichum lateriflorum*, and *Geranium maculatum*) were hindered by surrounding *R. cathartica*. It is also hypothesized that emodin leached from fallen fruits and leaves into the soil and slowed the growth of competing plants (Izhaki 2002). Seltzner and Eddy (2003) assessed the inhibition of alfalfa germination by emodin derived from

buckthorn roots, bark, fruits, and leaves. Full strength (100%) drupe extract had the highest percentage germination inhibition, with 1 alfalfa seed germinating from 2000, and 256 seeds germinating at 50% extract concentration (both significantly less than in the control). Leaf extracts (100%) had the second-highest percentage of germination inhibition, with 1167 of 2000 seeds germinating ($p<0.05$). Neither root nor bark extracts significantly affected germination, even at full concentration.

Allelochemicals have been shown to influence nutrient availability within the soil by indirectly affecting soil nutrients and rates of nutrient cycling through the influence of microorganisms (Gerdemann and Nicolson 1963). The addition of emodin was shown to indirectly decrease Mn^{2+} and PO_4^{3-} availability and increase Na^+ and K^+ availability by influencing soil microbes and their subsequent nutrient uptakes (Inderjit and Nishimura 1999). Information about antimicrobial influences within the soil is less clear. Emodin may have a role in protecting plants from disease *in vivo* (Liu and Wang 2003). Addition of *Aloe vera* anthraquinones caused the inhibition of nucleic acid synthesis in *Bacillus subtilis* (Schultz et al. 1999). *In vitro* exposure to emodin (concentrations of 10-200 μg mL^{-1} inhibited nine soil microbial species (*Arthrobacter globiformis*, *Chlorella pyrenoidosa*, *Bacillus megaterium*, four *Rhizobium* spp., and *Azotobacter chroococcum*) (Clapp et al. 1995). Emodin isolated from *Cassia nodosa* inhibited the growth of all pathogenic microorganisms tested, namely the bacteria *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and the fungi *Aspergillus flavus*, *A. niger*, *Gibberella fujikuroi* [as *Fusarium moniliforme*] and *Macrophomina phaseolina* [as *Rhizoctonia bataticola*]), with MICs ranging from 1×10^{-3} to 1×10^{-5} mg mL^{-1} and maximum activity against *F. moniliforme* (Brundrett et al. 1999). Highly effective inhibition of spore germination of 17 tested fungal species was observed with emodin isolated from *Rhamnus triquetra* bark (Fogel and Hunt 1979). Maximum inhibition (100%) was seen with *Aspergillus awamori* [as *A. luchuensis*], *Botrytis cinerea*, *Cladosporium cladosporioides*, *Helminthosporium* sp., and *Trichothecium* sp. at 2000 μg mL^{-1}, but growth inhibition was also observed at lower concentrations (Fogel and Hunt 1979). Fewer than 50% spore inhibition was achieved at emodin concentrations of 500 μg mL^{-1}, with a maximum inhibition seen with the pathogenic basidiomycetous fungus *Heterobasidion annosum* (Lugo and Cabello 2002). Hempel et al. (2007) subjected 11 isolated compounds as well

as the crude extract from *R. cathartica* to an antimicrobial activity test with *E. coli* (G -ve), *S. aureus* (G +ve), *Candida albicans* (yeast), and *A. niger*. All isolated compounds inhibited *S. aureus*, whereas there was no inhibition zone in any *A. niger* plates (Hempel et al. 2007). Compound 1 (a newly identified anthraquinone), emodin, and rumejaposide (a glucosyl anthrone) had significant antibacterial effects on the bacteria and yeasts (Hempel et al. 2007).

A greenhouse study of seed germination and seedlings examined the effects of macerated root extracts of *R. cathartica* (European invasive) and *Fallopia japonica* (Japanese knotweed; Asian invasive) on arbuscular mycorrhizal fungal (AMF; see 3.1.3) associated with *Ulmus alata*, *U. parvifolia* and *U. minor* (Pinzone 2016). Tree seedlings resisted the allelopathic effects from co-evolved plant species, but *R. cathartica* had the most effect on *U. parvifolia* from East Asia, whereas *F. japonica* affected the European *U. minor*. Only buckthorn treatments showed indirect effects on *Ulmus* spp., witnessed by the reduction in the abundance of arbuscules, and the rare occurrence of vesicles. This study, unlike many others, assessed growth inhibition using crude *R. cathartica* extracts and not isolated compounds, making it a more accurate representation of *in vivo* conditions. Emodin is a highly reactive anthraquinone, but it does not act alone in the environment.

3.1.3 Arbuscular Mycorrhizae

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that form a large, spreading hyphal network in the soil and have multiple points of invasion within roots of various host plants (Smith and Read 2008). The mycelium is multinucleate and coenocytic (with no septa separating cells), facilitating its primary function of nutrient transfer (Zolan and Pukkila 1986). Hyphal filaments penetrate root cortical cells, forming a trunk with a highly branched, terminal tree-like structure, an arbuscule, where bi-directional nutrient exchange takes place (Smith and Read 2008). Vesicles (storage organs) may or may not form inter- or intra- cellularly. Some vesicles thicken to form intraradical or extraradical spores (Smith and Read 2008).

3.1.4 Invasives Altering Soil Communities

Arbuscular mycorrhizae influence growth and mediate interactions between plants, with the potential to enhance competitiveness of invasive plants (Marler et al. 1999; Stampe and Daehler 2003; Walling and Zabinski 2004), and reduce it in others (Stampe and Daehler 2003). In certain cases, plant communities can be altered by invasive plants when sensitive species of AMF are replaced with more resistant species (Brundrett 2009). As observed with some invasive species in North America, allelopathic chemicals may inhibit the growth of surrounding plants directly (Bainard et al. 2009; Bais et al. 2003; Dorning and Cipollini 2006; Lawrence et al. 1991; Ridenour and Callaway 2001) or by limiting AMF growth (Callaway et al. 2008; Seltzner and Eddy 2003; Stinson et al. 2006).

The invasive garlic mustard (*Alliaria petiolata*) disrupts native mycorrhizal communities. Garlic mustard is non-mycorrhizal and produces a host of secondary metabolites that change soil microbial communities. Garlic mustard-induced community shifts show no change in AMF richness but undergo the replacement of sensitive species with more resistant ones, and a suppression of AMF colonization (Barto et al. 2011). Stinson et al. (2006) confirmed garlic mustard's antifungal effects, where its extracts added to potted native sugar maples (*Acer saccharum*) were just as effective as the living plant at reducing AMF colonization and spore germination. Similar reductions in mycorrhizal diversity have been reported with other invasive species such as *Centaurea maculosa* (Spotted knapweed) (Mummey and Rillig 2006), *Solidago canadensis* (Canada goldenrod) (Zhang et al. 2007), and *Tamarisk* spp. (Meinhardt and Gehring 2012).

Buckthorn forms mycorrhizal connections in its native and invaded ranges (Knight 2006), but not much is known about the associations in invaded habitats. In its invaded range, the connections consist of coiled and straight hyphae, arbuscules, and oval-shaped vesicles within aniline blue-stained buckthorn roots; although, the taxonomic classifications of these fungi are still unknown. No molecular work has been done on the types of AMF communities of buckthorn in its invaded range, or on the changes that native woodland AMF communities may undergo during and after buckthorn invasion.

3.1.5 Past Methods of Describing AMF Communities

Early researchers used classical methods of quantifying mycorrhizae by clearing and staining roots to determine the proportion that were mycorrhizal (Chandramohan et al. 2002). One popular method was the (grid) line intersect technique developed by Newman (1966), which was modified and standardized by Powell et al. (1982) to include arbuscules, vesicles, and root length containing hyphae in order to estimate the degree of mycorrhizal colonization on roots. This technique involves counting the number and type of mycorrhizal structures within stained, sectioned roots that intersect lines of a grid (or hair-line of the eyepiece); the number of intersections with AMF divided by the total number of root–grid intersections gave the percent colonization (Newman 1966, Sun and Tang 2012).

Quantifying mycorrhizal infection within a root does not identify species since roots were rarely seen with identifiable spores. Identification of mycorrhizae was accomplished through wet-sieving for spores in soil (direct estimates) or 'trapping' fungi in pots with host plants (trap cultures) followed by wet-sieving (indirect estimates). Current wet-sieving and decanting techniques for spore and hypha isolation, developed by Gerdemann and Nicolson (1963), use sodium pyrophosphate (NaPyrP) to help break soil colloids (Fogel and Hunt 1979; Pacioni 1992), mechanical agitators to help thoroughly mix the slurry (Pacioni 1992), and centrifugation in sucrose to separate the spores (based on Jenkins 1964).

Fungal surveys based on spores in soil may not detect species that sporulate in host root systems and not the soil (Liu and Wang 2003), or those that have seasonal variation in spore development (Hempel et al. 2007; Lugo and Cabello 2002). Other detection methods were developed, including trap culturing on mycorrhizal host plants to promote the growth and sporulation over a period of months within a greenhouse (Bever et al. 2001). Choosing appropriate host species affected spore densities in pots. Some hosts such as white clover (*Trifolium repens*) were better than others (*Z. mays*, *Nicotiana tabacum*, and *Potentilla anserina*) for the quantification of fungal diversity in soils (Liu and Wang 2003). However, certain AMF, such as *Acaulospora colossica,* would grow only when host and greenhouse

conditions were typical of North Carolinian winter and spring months (Schultz et al. 1999). Invariably, differences in methodology (greenhouse conditions, host species, treatment of soil prior to trapping, season of field soil sampling, etc.) would lead to the proliferation of different species that were more suited to those conditions (Bever et al. 2001; Öpik et al. 2014), and a considerable effort would be necessary to determine effective host plants and conditions for each AM fungus.

Problems occur with trap cultures when opportunistic species, those that are suited to disturbance, proliferate (e.g., *Glomus* sp.), which results in the misrepresentation of their abundance in natural conditions (Brundrett et al. 1999; Öpik et al. 2014). Numerous abiotic and biotic factors affect sporulation spatially and temporally (Hempel et al. 2007). Sporulation may be necessary for some taxa (e.g., Gigasporaceae) to complete their life cycles, whereas others (e.g., Glomeraceae) rely on infective hyphae and the extension of hyphae between colonized roots (Smith and Read 2008). Fungi also display differences in biomass allocation between the roots and rhizospheres of the same plant; *Scutellospora* shows a preference for growth in the rhizosphere whereas *Glomus* shows increased allocation within the roots (Clapp et al. 1995). This leads to differences in composition within and around the plant, as well as differences in sporulation patterns among species (Öpik et al. 2014). Trap cultures are invaluable when certain treatment conditions cannot or should not be carried out in the field, but any inferences drawn from the data should be done carefully.

3.1.6 AMF Taxonomy

The development of AMF taxonomy was originally developed on the basis of discrete spore subcellular structures obtained by wet sieving potted or field soil. A comprehensive manual of spore identification of AMF was developed by Schenck and Perez (1987). By 2001, AMF were classified into 7 genera on the basis of spore wall characteristics and ontogeny, and approximately 145 described species were accepted (Bever et al. 2001). However, identification of spores directly from field soil was not always reliable due to morphological differences between intraradical and extraradical spores of the same species (Stockinger et al. 2009). The species of a genus have a limited number of differences in

morphology and hyphal structure, viable spores are ephemeral, direct examination may not reveal all species present, and soil conditions may alter the appearance spores, making it difficult to differentiate species (Bever et al. 2001; Stockinger et al. 2010). Currently, any morphology-based identification of Glomeromycota requires microscopy expertise and adequate literature on the topic (Öpik et al. 2014).

3.1.6.1 DNA-based Techniques

The use of DNA as a tool to catalogue biodiversity and generate phylogenies provides a more objective and reliable alternative to morphological identification of AMF. Extraction of genomic DNA for any molecular analysis involves disruption of cells or tissues, denaturation of nucleoprotein complexes, and removal of contaminants (RNA, proteins, carbohydrates, lipids, etc.), while maintaining quality and integrity of the final product by inactivation of nucleases (DNase) (Tan and Yiap 2009). Advancement in DNA extraction technology resulted in solution-based or column-based protocols that could accept tissue (e.g., ground cultured mycelium or sectioned mushroom) or soil as a raw DNA source (Tan and Yiap 2009). DNA extraction followed by PCR amplification of target DNA gene regions became a rapid, simple, and reliable procedure to collect data on single or multiple taxa within samples (Hudson 2008). However, picking the most appropriate gene region for sequencing can itself be a challenge.

3.1.6.3 Sequencing Platforms

Parallel advancements in sequencing technology provided the basis for molecular and/or taxonomic analyses, starting with classical cloning and Sanger sequencing methods in the early 1990s (Sanger 1977; Swerdlow et al. 1990), leading up to 1996 where the full genetic code of the first eukaryotic and fungal organism, baker's yeast (*Saccharomyces cerevisiae*), was sequenced (Goffeau et al. 1996). Multiple different DNA sequences of mixed microbial communities were separated by cloning—each clone taking up only one copy of PCR-amplified DNA—and then the PCR inserts in individual clones were sequenced (Bianciotto et al. 2011). However, classical cloning and Sanger sequencing techniques can be costly and time-consuming for multiple samples containing complex communities, as they usually sample a small fraction of each community (Horn et al. 2014). Sanger

sequencing (using an ABI 3730xl) can yield 400~900 bp × 96 samples per run with 1.9~84 kb of data per run, 454-pyrosequencing using the Roche FLX platform (ceased in 2013) could sequence up to 700 bp with 1 M reads per run (Liu et al. 2012b), and the MiSeq (Illumina) Next Generation Sequencing (NGS) platform can sequence 250 bp paired-end reads with 15~20 M reads per run (http://genecore3.genecore.embl.de/genecore3/illumina.cfm). With the advance of high throughput NGS technology such as 454 and Illumina, AMF taxonomic studies began to look in greater depth at species-level distributions in environmental samples (Öpik et al. 2014; Shendure and Ji 2008). PCR-based NGS sequencing technologies produce millions of short sequence reads, varying from tens of base pairs to ~800 bp (Luo et al. 2012) using amplicons from PCR reactions. Even with technology advancements, these lengths may not be sufficient for confident species-level identification (Öpik et al. 2014). Ideal AMF identification requires a larger 1 500 bp fragment spanning the SSU-ITS-LSU region of the rRNA gene (see 3.1.6.2 Gene Markers) (Krüger et al. 2009b; Öpik et al. 2014). Studies outlining limitations and advantages of various NGS platforms have already established that the Roche 454 platform, previously one of the more popular NGS technologies, has high homopolymer error rates (Quince et al. 2009) and 11% to 35% of the sequences are the result of artificial replicates (Gomez-Alvarez et al. 2009). The single nucleotide detection method in Illumina, another popular NGS platform alternative, avoids this issue but has base calling biases (phasing and fading) (Erlich et al. 2008). Many error estimates and sequence bias studies have been based on simple DNA samples (Quince et al. 2009) that have low relevance to complex community samples (Luo et al. 2012). The analysis of a complex freshwater planktonic community was done on the Roche 454 and Illumina platforms by Luo et al. (2012), and both were considered reliable for quantitatively assessing genetic diversity within the community. However, Illumina yielded longer, more accurate contigs despite the shorter read length compared to Roche 454, and Roche 454 retrieved 14% fewer complete genes than Illumina due to A-T rich homopolymer regions. Monetarily, the Illumina dataset was one fourth of the cost of the Roche 454 data and was considered to be more appropriate for short-read metagenomic studies, whereas Roche 454 is more appropriate for repetitive sequences, palindromes, or for metagenomic analyses based on longer, unassembled fragment lengths (Luo et al. 2012). In terms of read depth, an

important factor to consider when sequencing large communities (Caporaso et al. 2011), the Illumina dataset contained 23.82 million reads, whereas Roche 454 contained 1.28 million reads (Luo et al. 2012).

Within the Illumina NGS platform, PCR amplicons of desired gene regions are attached to short complementary nucleotide adaptor regions to allow binding onto a flow cell microchip. Platform-specific chemistry converts dsDNA into single-stranded fragments, then copying single fragments to create clusters, and cyclically attaching fluorescently labeled nucleotides that are imaged using LASER excitation. Compilation of the images allows for the simultaneous recording of sequence information from each amplicon cluster as they are being built in first the forward and then the reverse direction (http://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf). Output data files are analyzed to remove PCR and sequencing errors, cluster sequences, and provide a formatted table of sequences and sample groups for use in any molecular or phylogenetic downstream analyses (Gloor et al. 2010). Unfortunately, inadequate reference sequence data are still a constraint for taxonomic studies using NGS platforms, so the continued sequencing of known reference strains or specimens from culture collections or herbaria is necessary to build suitable databases (Lumini et al. 2010; Stockinger et al. 2009).

3.1.6.2 Gene Markers

The locus used to quantify fungal diversity must be a short sequence that is universally present in target lineages and is a compromise between the possibility of designing universal primers for PCR amplification and having sufficient sequence variation to distinguish species (Vialle et al. 2009). The marker used by the Consortium for the Barcode of Life for most eukaryotes is the mitochondrial gene encoding the cytochrome *c* oxidase subunit (*CO1* or *COX1*) (http://www.barcodeoflife.org/content/about/what-dna-barcoding). This functions well for some fungal genera (*Penicillium*) but poorly for other Ascomycota—*Fusarium* have multiple *CO1* copies—as well as species in the orders Neocallimastigales and the pathogenic Microsporidia since they completely lack mitochondria (Bullerwell and Lang 2005). Mycologists have converged to the nuclear

ribosomal RNA gene region (nu rRNA gene region or nu rDNA) as the most informative region of study because of the ease of its extraction from total genomic material as well as the level of taxonomic detail present in multiple 500- to 800- bp sections of the gene. The nu rRNA gene region consists of the small subunit (SSU or 18S), 5.8S, and large subunit, (LSU or 28S) rRNA genes as a transcribed unit of RNA polymerase I. Two internal transcribed spaces (ITS1 and ITS2) are spliced out after the transcription of the ribosome gene. These two regions, including the 5.8S gene, are referred to as the ITS region, which is the official barcode for fungi due to its hypervariability and ability to delineate to species and subspecies levels (Schoch et al. 2012). Unfortunately, related species in many fungal groups (e.g., *Penicillium*) lack distinguishing variation in their ITS region (Skouboe et al. 1999), and the sequence information is "saturated" over broader evolutionary comparisons, precluding the use of ITS data in phylogenetic analyses, for instance, to place unknown sequences from soil into families or orders (Liu et al. 2012a). For arbuscular mycorrhiza, the same hypervariability presents problems because of heterogeneity in repeat ITS copies within a single isolate, their asexual lifecycle, and the possibility of clonal diversity complicating AMF species boundaries (Öpik et al. 2014). Nucleotide variation between and within Glomeromycota species in the ITS and the LSU rRNA gene is such that no single fragment is able to distinguish among all species (Stockinger et al. 2010). Krüger et al. (2009b) analyzed the species-level resolving power of multiple sections of the nu rRNA gene; the largest fragment, 1 500 bp spanning the 3' end of SSU, ITS, and 5' end of LSU gene regions provided the ability to resolve to species level with confidence for AMF sequences. Other fragments, including 800 bp of the nu LSU rRNA gene, three 400 bp fragments throughout the ITS2, LSU-D1, and LSU-D2 were not sufficient on their own. Other markers such as the mitochondrial LSU rRNA gene and intergenic region have been applied to describe intraspecific species variation (de la Providencia et al. 2013). Multilocus analyses are preferred for studies of evolutionary relationships because no single locus is best suited to answer all the questions (Robert et al 2011). This invariably led to phylogenetic analyses that looked at gene relatedness instead of species relatedness (Öpik et al. 2014). However, single-gene analyses are still a practical tool for metagenomic studies because multiple samples containing multiple taxa can be analyzed through a single

NGS run, and high-throughput sequencing technology cannot yet sequence large fragments.

Even though SSU variation is insufficient to identify species of later-diverging fungi (within the Ascomycota and Basidiomycota, for example), early diverging lineages such as the Glomeromycota show better species-resolution with SSU and LSU regions (Schoch et al. 2012, Öpik et al. 2014). A simplified comparison of ITS, LSU, and ITS + LSU sequences between 42 species (606 sequences) of Glomeromycota showed high levels of intraspecific variation (Schoch et al. 2012). A consideration for the use of the SSU over the LSU for AMF studies is the low number of sequences deposited in GenBank for the LSU region for arbuscular mycorrhizal fungi (Lumini et al. 2010). Dunthorn et al. (2012) compared two popular hyper-variable regions of the SSU, the variable regions 4 and 9 (V4 and V9), for microbial eukaryotes (ciliates). Both regions were attractive options, but in ciliates the genetic distances within and among species in the same genus were more similar when using just the V4, or whole SSU, than comparing the V9 and SSU. Many projects that use NGS sequencing to describe the AMF community composition employ the V4 region of the nuclear SSU rRNA gene (Lumini et al. 2010; Öpik and Davison 2016). Multiple primer pairs are available to amplify the SSU of AMF preferentially (Figure 3.1). The first primer pair NS31-AM1 (Helgason et al. 1998; Simon et al. 1992) used to detect AMF communities using 454-pryosequencing, failed to pick up some occurrences of the basal families Ambisporaceae, Archaeosporaceae, and Paraglomeraceae (Daniell et al. 2001). Lee et al. (2008) improved upon this primer set and created AML1 and AML2 which showed better coverage and recovery of taxa. AML2 and NS31 have been used in Roche 454 studies to sequence the V4 and part of the V5 region of the SSU (Van Geel et al. 2014). Another primer set, AMV4.5N-F and AMDG-R (Sato et al. 2005), that strictly covered the V4 region in a study by (Lumini et al. 2010), was shown to retrieve a broader spectrum of AMF sequences in higher proportion than the NS31/AM1 set (Van Geel et al. 2014). The comparison of results using different primer pairs in NGS studies is difficult. Van Geel et al. (2014) critically evaluated six different primer pairs (4 from the SSU, 2 from the LSU) in silico as well as with surface-washed roots from apple orchards. The highest nucleotide diversity was found in the V4 region between primers AMV4.5NF-AMDGR, with AMDG-R and AML2 having the highest in silico AMF specificity.

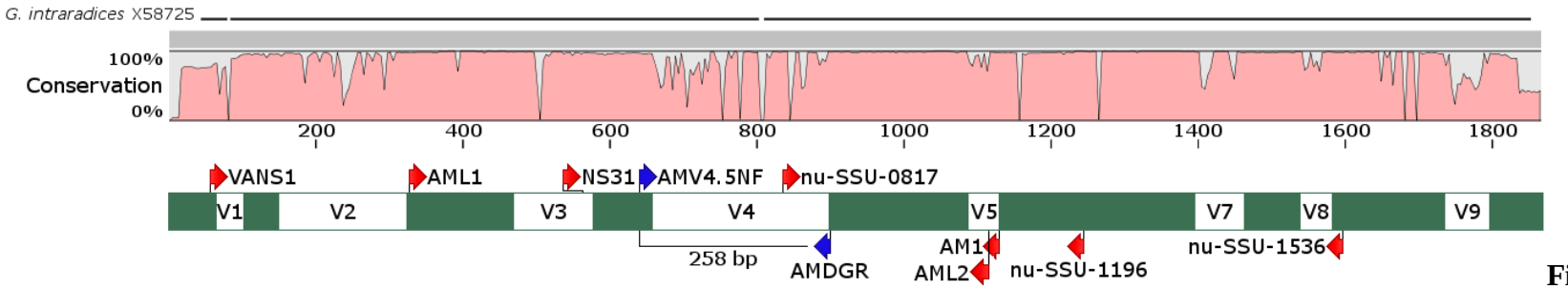


Figure 3.1. Primers amplifying arbuscular mycorrhizal fungi (AMF), phylum Glomeromycota, in the nuclear ribosomal RNA gene region small subunit (SSU). Percent conservation line plot made using reference sequences found in (Krüger et al. 2012) (http://www.arbuscular-mycorrhiza.net/amphylo_downloads.html, version 2) including *Glomus intraradices* X58725 as a positional reference, aligned and visualized using CLC Sequence Viewer (http://www.clcbio.com/). The primers used for this analysis, AMV4.5F-AMDGR (Sato et al. 2005), span 258 bp of the *G. intraradices* gene. Primers AML1, AML2 (Lee et al. 2008), NS31, VANS1 (Simon et al. 1992), AMV4.5F, AMDGR, AM1 (Helgason et al. 1998), nu-SSU-0817-5′, nu-SSU-1196-3′ and nu-SSU-1536-3′ (Borneman and Hartin 2000) have been used in high-throughput sequencing AMF studies.

Primers AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 were all powerful enough to characterize the community, but AMV4.5NF-AMDGR favoured Glomeraceae sequences over the Ambisporaceae, Claroideoglomeraceae, and Paraglomeraceae (Van Geel et al. 2014). Despite this drawback, the AMV4.5NF-AMDGR primer set is the only one yielding an amplicon short enough (300 bp) to be used in Illumina NGS sequencing platform while still covering the largest variable region (V4) in the SSU.

3.1.7 Aims and Objectives

The aim of this chapter is to determine whether there are changes in abundance and diversity of AMF communities associated with the native sugar maple (*Acer saccharum*) tree due to the presence of buckthorn (*Rhamnus cathartica*). Arbuscular mycorrhizalfungi have been shown in other studies to be responsive to invasives, allelochemicals, or disturbance, and mycorrhizal communities are expected to show a shift from sensitive to more resistant generalists during or after buckthorn invasion.

Objective 1

A comparison of soil and roots of sugar maples (*Acer saccharum*) within buckthorn (*Rhamnus cathartica*) invaded or uninvaded stands will assess the differences in AMF communities in established forests.

Objective 2

A manipulative common-garden experiment will assess the effects of different sources of buckthorn allelochemicals (roots, leaves and fruit) on AMF communities in sugar maple from soils previously unexposed to buckthorn.

3.2 Materials and Methods

3.2.1 Soil Sampling

Objective 1

Sampling sites included plots within Komoka Provincial Park (Komoka, Ontario; Figure 3.2 A), and *rare* Charitable Research Reserve (referred to as *rare* CRR) (Cambridge, Ontario; Figure 3.2 B and C). Latitude and longitude coordinates of sampling sites using the universal transverse Mercator system (UTM) are provided in the figure captions. Uninvaded sites were defined as mature sugar maple stands at least 30 m away from nearest mature fruiting buckthorn and at least 10 m away from any garlic mustard plants, another allelochemically active invasive species. Invaded sites were classified as mature sugar maple stands with at least one mature, fruiting buckthorn tree (approximately 9–20 years old) growing underneath the drip line (canopy). Uninvaded and invaded plots were at least 10 m apart.

Feeder roots and associated soil were collected from mature maples in uninvaded and invaded stands in the summer on June 12, 2014 (at *rare* CRR) and July 14, 2014 (at Komoka), and in the fall on October 23, 2014 (at *rare* CRR) and October 28, 2014 (at Komoka). Within each sampling area, three maple trees were sampled with three root samples per tree. Root excavation followed a main root from the trunk to a point where a 15 × 15 × 20 cm pit was dug and soil was collected in a plastic sampling bag. A total of 32 samples was collected from 18 uninvaded and 18 invaded sites.

Objective 2

Sugar maple seedlings (15–20 cm in height) and potting soil were collected on May 22nd and 23rd, 2014—to avoid the disturbance of spring ephemerals—from a woodlot at Shady Maples Farm, Ilderton, Ontario (Figure 3.2 D). The plots were dominated by sugar maple trees (>50%), with no history of buckthorn influence, and at least 30 m away from the nearest mature, fruiting buckthorn. Extra potting soil was taken from each site. Buckthorn seedlings were taken from the edge of a buckthorn-invaded woodlot at the Environmental

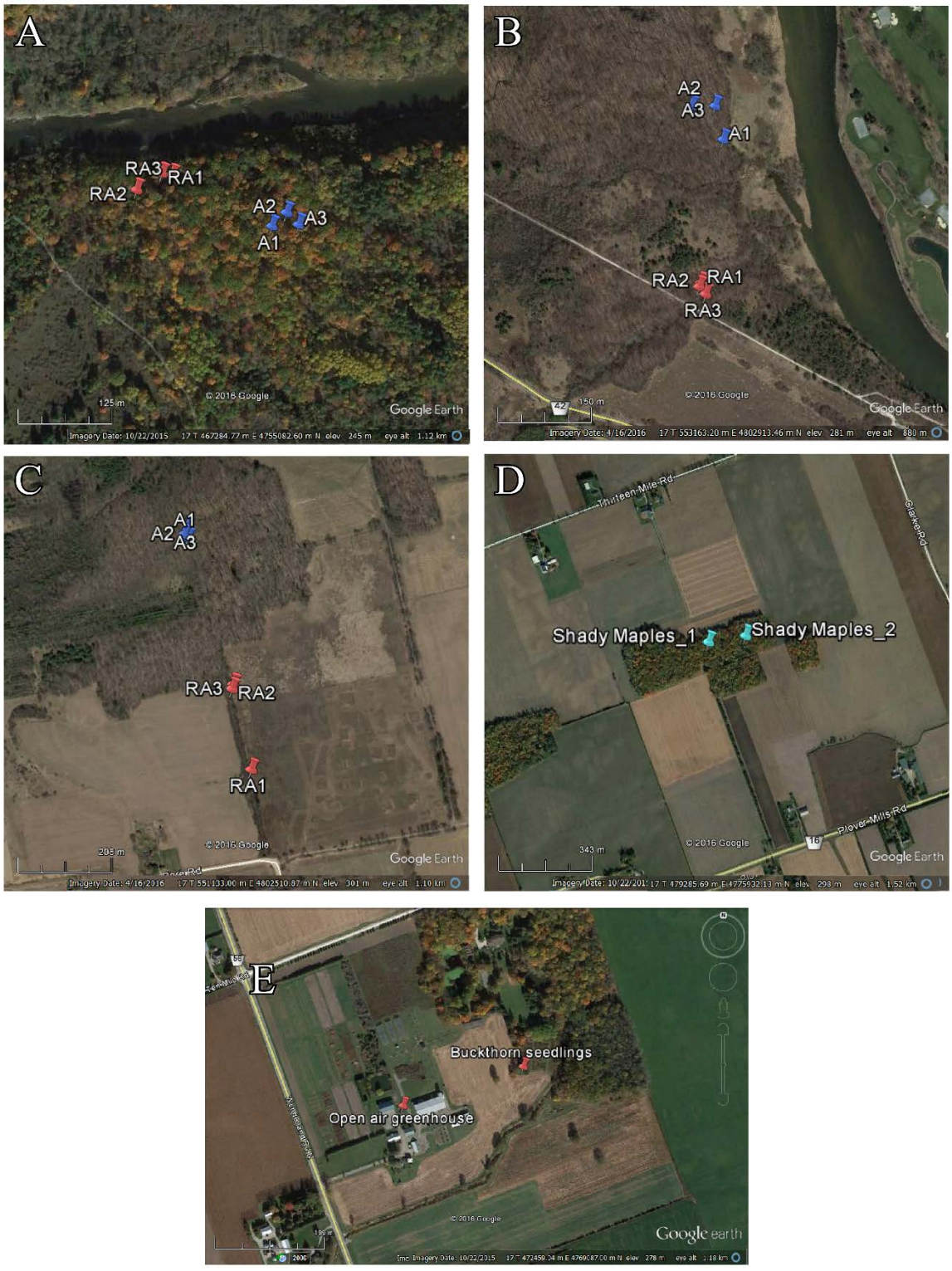


Figure 3.2. Sampling locations for Objective 1 and 2 in **A)** Komoka Provincial Park, Komoka, ON (17T 467284mE and 4755082mN. Google Earth. September 10, 2015.); **B)** Cliffs and Alvars forest at *rare* Charitable Research Reserve, Cambridge, ON (17T 553163

mE and 4802913mN. Google Earth. April 16, 2016); **C)** Grand Alee and Indian Woods forest at *rare* Charitable Research Reserve, Cambridge, ON (17T 551133mE and 4802510mN. Google Earth. April 16, 2016.) and **D)** Uninvaded sugar maple (*Acer saccharum*) seedlings at Shady Maple Farm, Ilderton, ON (17T 478949mE and 4775237mN. Google Earth. September 22, 2015). **E)** Open-air greenhouse location and seedling sampling location for buckthorn (*Rhamnus cathartica*) seedlings at the Environmental Sciences Western Field Station, Middlesex Centre, ON (17T 472643mE and 4769095mN. Google Earth. September 22, 2015). Pins labeled "A" indicate sugar maple trees in uninvaded plots, those labeled "RA" indicate sugar maple trees with mature fruiting buckthorn in invaded plots.

Sciences Western Field Station on May 28, 2014 (Middlesex Centre, ON; Figure 3.2E). A total of 72 sugar maple seedlings (including associated soil) and 48 buckthorn seedlings (excluding associated soil) were collected. Shady Maple Farm was revisited on November 4th, 2014, at the end of the treatment period, to collect six field control seedlings from each site.

3.2.2 Seedling Treatments

Seedlings (sugar maples alone, or sugar maples with buckthorns; see Fig. 3.4) were immediately planted in 15 cm diameter pre-cleaned pots and kept in a partially shaded enclosure at the Environmental Sciences Western Field Station (Figure 3.2E). Watering regimes included natural precipitation as well as early morning watering to prevent drought and seedling death. Seedlings were left to grow from May until the end of October, 2014. Weeds were actively removed from the pots, taking care to reduce soil disturbance, and no fertilizer was added.

Buckthorn amendments were applied in twice during the testing season since there are two major periods of increased buckthorn leachate in soils, when berries are picked off and are excreted by frugivores in mid-fall, and when leaves fall in early winter (personal observation). Due to growing and timing constraints, amendment additions mirroring natural timelines were not possible, so treatments occurred once in June and again in

September, 2014. Leaves were picked in June of 2014 and berries from the previous year's growing season were picked in March 2014, both from buckthorn trees growing on Western's campus (477001 m E and 4761149 m N; Figure 3.3). The leaves were dried at 60 °C for 24 hours, and the berries were freeze-dried at 64 mtorr for 6 hours. The berries were coarsely mulched with a cold mortar and pestle. Each pot received 12 g or approximately 60 fruits (equivalent to 202 fruits/m^2; Seltzner and Eddy 2003) and/or an equal weight of leaves. Treatment 4 (fruit + leaves) was the combined mulched weight of treatments 2 and 3 (Figure 3.4).

The treatment period ended October 30th, 2014. Maple seedlings were carefully removed from the pots, and soil was gently washed away to keep the fine roots intact. The entire root mass was clipped, placed in sterile 50 mL tubes, and frozen at –20 °C. Clippers and buckets were cleaned between each treatment to reduce the chance of cross-contamination.

3.2.3 Soil DNA Extraction

Collected soils from Objective 1 were washed with 1M sodium pyrophosphate (NaPyrP) (Anachemia) to help break up soil particles. Up to 20 g wwt of soil was washed with 200 mL of NaPyrP in clean glass jars. Samples were left to sit for 5 min, hand-shaken for 1 min to break up soil colloids, then strained through a coarse (No. 16, 1.18 mm), medium (No. 60, 250 µm), and fine (No. 270, 53 µm) sieve (VWR Scientific, West Chester, PA). Roots were separated by hand from the coarse and medium sieve. Organic matter, including mycelia and spores, were pipetted out from the fine sieve using 1 mL broad tips into 50 mL Falcon tubes together with the root material. Sieves were washed with soapy water, rinsed for 1–2 min with dH_2O, and cleaned in 70% ethanol between samples. Sample tubes were centrifuged for 3 min at 2 000 rpm, the supernatant was removed and the pellet was frozen until lyophilization. For Objective 2, frozen fine sugar maple roots were washed with NaPyrP as described above.

Figure 3.3. Collection locations for buckthorn (*Rhamnus cathartica*) fruits and leaves (collected in March and June, 2014, respectively) (17T 477001mE and 4761149mN. Google Earth. September 22, 2015).

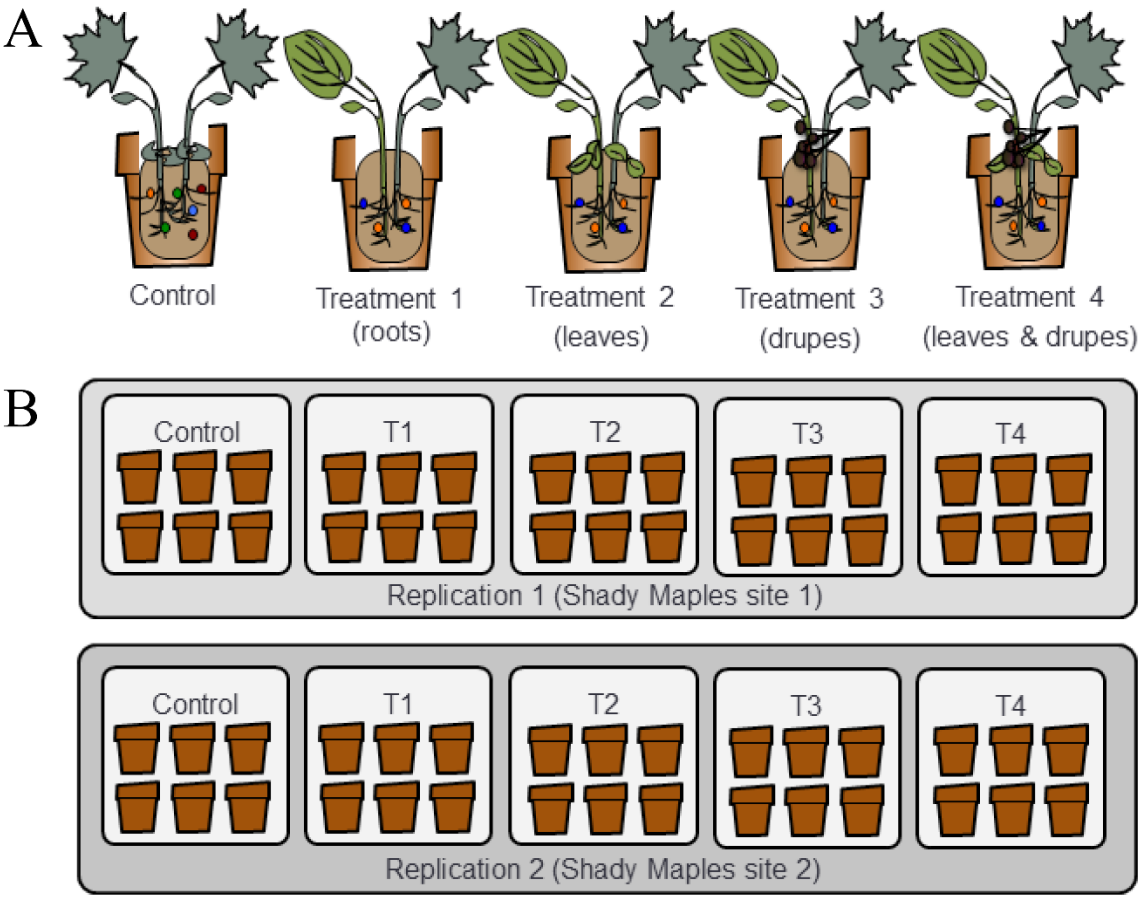


Figure 3.4. Experimental design of the potted greenhouse experiment, Objective 2. A) Open-air greenhouse design using two replications of four buckthorn (*Rhamnus cathartica*) allelochemical treatments: (1) roots only (no leaves or fruits); (2) leaves; (3) fruits; (4) leaves and fruits. Control pots had two *Acer saccharum* seedlings to control for effects of root disturbances and competition; all treatment pots had one *R. cathartica* seedling and one *A. saccharum* seedling. **B)** Complete replicated design.

All samples were freeze-dried for 24 h at 52–64 mtorr (Virtis Bench Top 3.5 Freeze Dryer). Care was taken to prevent cross-contamination by plugging the open end of each tube with a paper towel. Samples were ground in liquid nitrogen, with acid-washed mortars and pestles, to break up soil particles, and release spore or cell contents. After grinding, samples were kept frozen at –20 °C in sealed 50 mL Falcon tubes until DNA extraction.

DNA isolation from roots and soils was done using the ZR Soil Microbe DNA MicroPrep (Zymo Research Corporation). Up to 0.25 g of ground sample was added to the ZR Bashingbead™ Lysis tube with 750 µL Lysis Solution, and processed in a FastPrep FT120 for 45 s at speed setting 4. Isolated DNA was immediately quantified using a nanodrop (Nanodrop 2000, Thermo Scientific) to determine DNA concentration. If DNA concentrations were below 15 ng/µL, additional extractions were performed and combined. Extracts were stored at –20 °C until PCR amplification.

3.2.4 Soil and Root PCR Amplification

The nuclear ribosomal small subunit was targeted to provide taxonomic identification and abundances of buckthorn AMF and soil/root-associated fungi. For sequencing using the Illumina MiSeq (MiSeq) platform, the primer set AMV4.5F-AMDGR (Sato et al. 2005) was chosen to amplify species within the Glomeromycota. The 5’ end of the forward and reverse primers were modified to include the forward or reverse Illumina adapter, a 4 bp linker (NNNN), and an 8 bp barcode sequence that allowed recognition of products from different samples following Illumina sequencing (Gloor et al. 2010) (Appendix II).

PCR reactions were set up on ice to minimize primer dimerization. PCRs were carried out in 25 µL reactions with variable DNA loading volumes (4-8 µL), 1.25 µL of 5 µM each of forward and reverse primers (0.5 nmol per reaction), 0.5 µL loading dye, and 12.5 µL Accustart II PCR ToughMix mastermix. PCRs using the ToughMix mastermix and AMF primers used the following thermal profile: 94°C for 1 min, 29 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 18 s. No final extension step was used.

DNAs from potted sugar maple roots (Objective 2) were individually amplified within PCR reactions and each treatment within a replication was pooled into one tube to be sequenced under one barcode pair. For example, the 6 individual PCRs from all 6 Control seedlings in replication 1 were PCR amplified using one set of barcoded primers. Performing multiple PCRs was considered unnecessary as increasing the number of pooled replicates had not shown an observable increase in sequencing depth, especially when using the MiSeq platform (Hale et al. 2005).

PCR products were visualized using gel electrophoresis in a 1% Agarose-A gel to verify PCR success before pooling replicates. Pooled replicates were lyophilized and reconstituted with 30 µL mH_2O and stored at –80 °C before submission for sequencing at the London Regional Genomics Centre at Robarts Research Institute (London, Ontario).

3.2.5 Bioinformatic Analysis

The Illumina MiSeq platform was used to sequence the DNA samples. Raw FASTQ data were processed using a custom MiSeq data processing pipeline (https://github.com/ggloor/ miseq_bin/tree/master) using the AMV4.5F-AMDGR primer set (labelled as V4AMF in the online documentation). PANDAseq (https://github.com/neufeld/pandaseq) (Masella et al. 2012) was used to overlap forward and reverse reads with a minimum overlap distance of 30 nt. Sequences containing ambiguous basecalls (N) were removed, as well as sequences with mismatches to the primer sequence due to sequencing errors. Both barcode and primer sequences were trimmed prior to clustering. The pipeline groups individual reads into identical sequence units (ISUs) and checks for chimeras using the UCHIME de-novo algorithm (Edgar et al. 2011). It then groups ISUs into operational taxonomic units (OTUs) at 97% identity around

the most abundant centroid sequence using the UCLUST algorithm in the USEARCH v7.0.1090 program (Edgar 2010). Although the distance cut-off is considered arbitrary and controversial, it has been used in other sequencing studies and allows for comparisons between them (Lumini et al. 2010; Santos-González et al. 2007). A preliminary taxonomic assignment was given to each sequence by the built-in Mothur v1.34.0 (Schloss et al. 2009) classification program based on the Silva 16S rRNA gene reference dataset (Pruesse et al. 2007).

3.2.6 Statistical Analyses and Data Visualization

Samples were combined for differential abundance analysis using ALDEx2 in R 3.3.1 (R Core Team 2016), ALDEx2 package (Fernandes et al. 2013), zCompositions package (Palarea-Albaladejo and Martín-Fernández 2015), and CoDASeq Microbiome Tutorial by Dr. Greg Gloor and Jean Macklaim (https://github.com/ggloor/CoDa_microbiome_tutorial/wiki). No OTUs were removed from the analysis. Cluster dendrograms using the Aitchison distance metric and the Ward D2 clustering method were created to visualize community composition in all samples (see documentation for the hclust command in the ‘stats’ package for other options). ALDEx2, a univariate comparison tool, incorporates the Bayesian estimate of taxon abundance into a compositional framework. ALDEx2 estimates the distribution of taxon abundance by sampling from 1000 Dirichlet Monte Carlo (DMC) replicates—the distribution of posterior probabilities of observing each taxon. Data were transformed by the centered log-ratio (clr) transformation and used to conduct a univariate statistical test between observed and posterior probabilities, and distributions of P and Benjamini-Hochberg (BH) adjusted P values were given. ALDEx2 is designed to identify significant taxa between treatment groups despite the large variation in metagenomics datasets.

A phi analysis combines the correlation of direction of variance and the correlation of amount of variance into one number (the phi value) to measure the strength of association between OTUs (Lovell et al. 2015). A constant ratio between OTUs—those that respond similarly between environments or treatments—is said to have high association, which corresponds to a low phi value. This can be compared to the expected

value of phi calculated from the 1000 DMC replicates in the ALDEx analysis after the centered zero value (CZM) replacements. Phi cutoffs of 0.2 and 0.3 (Objective 1) and 0.2 and 0.15 (Objective 2) were used for this dataset to help simplify the output, although typical metagenomic studies use phi cutoffs between 0.2 to 0.3. The compositional biplot highlights all positively correlated taxa clustered by colour and the highly significant taxa in the ALDEx analysis are shown as grey dots (Aitchison and Greenacre 2002). All phi-metric analyses were done using R and code provided in the CoDa Microbiome Tutorial by Greg Gloor and Jean Macklaim (https://github.com/ggloor/CoDa_microbiome_tutorial/wiki/Part-3%3A-OTU-Correlations-with-Phi) and based on Lovell et al. (2015).

3.3 Results

The MiSeq run had a total of 704 293 raw reads that clustered into 31 008 ISUs with 1 055 (3.4%) rejected as possible chimeras. The remaining ISUs clustered into 1 279 OTUs (529 348 reads) at 97% identity. Of those, 143 OTUs that were present at an abundance less than 0.01% in any sample and 278 OTUs with a sum of less than 5 reads across all samples were discarded. This left 858 OTUs (528 076 reads, 75.0% of the raw read count) for taxonomic analysis.

The BLAST query function in the MaarjAM online database (http://maarjam.botany.ut.ee/; Öpik et al. 2010) was used to identify possible virtual taxon (VTX) numbers for all OTU sequences. This was done on top of the preliminary Mothur assignments because the SILVA database did not have adequate Glomeromycota reference sequences (e.g., many *Glomus* and *Claroideoglomus* remained unclassified, and some *Paraglomus* were misclassified as Basidiomycota). All potential Glomeromycota reads were pooled and aligned using command line Muscle v3.8.31 (Edgar 2004) and made into Neighbour Joining (NJ) and Maximum Likelihood (ML) trees using MEGA v7.0.18 (Kumar et al. 2016). The dataset also included sequences of the VTX Type (listed in the MaarjAM database), MaarjAM top matches for each OTU, SSU AMF reference sequences

from (Krüger et al. 2012), with *Mortierella hyalina* JQ040259.1 as an outgroup (Appendix III).

Eighty-six OTUs had high matches to *Paraglomus* VTX00308 (*P*308) sequences within the MaarjAM database. Of these, all but two had initially been assigned to Basidiomycota using the SILVA reference dataset within Mothur; one remained unclassified and the other was assigned to Glomeromycota. Neighbour Joining and ML trees were created to determine a more parsimonious phylogenetic placement of all potential *Paraglomus* OTUs, using the top MaarjAM VTX sequence hits, VTX Type sequence, and named BLAST sequences (Edgar 2010) with the closest distance tree matches (Koski and Golding 2001) (Appendix IV). Eight of the 86 OTUs clustered consistently among the *P*308 and *P. laccatum* VTX281 (*Plac*281) type sequences in both NJ and ML trees, with 78 OTUs excluded since they clustered within the Basidiomycota.

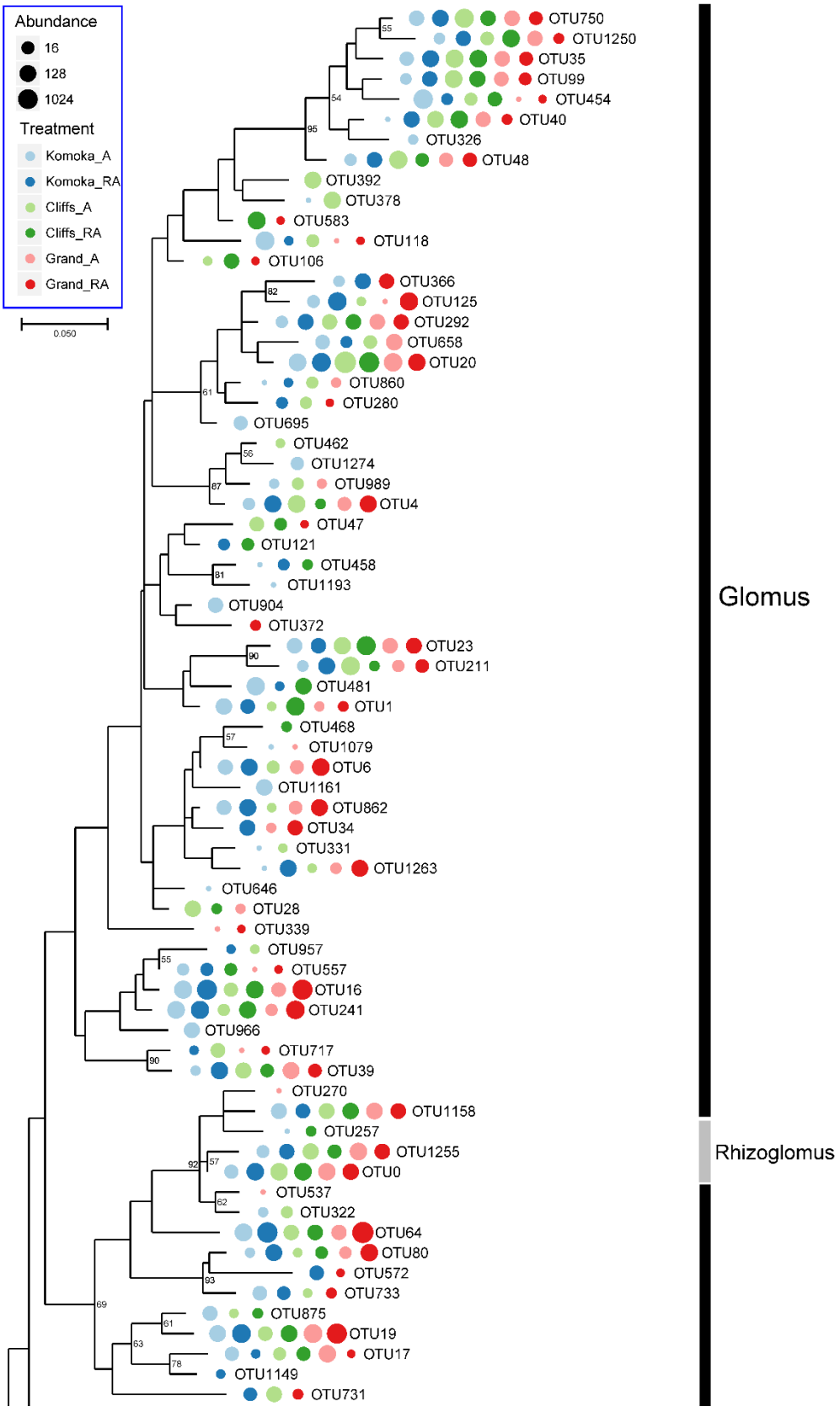
The remainder of the dataset included 449 Fungal OTUs (454 233 reads, 86.0% of final reads) as well as one Florideophycidae OTU, one Monosigidae (Choanomoda) OTU, two Ichthyosporea OTUs, and three Ochrophyta OTUs (229 total reads, 0.0434% of final reads), and 402 unclassified OTUs (73 460 reads, 13.9% of final reads). Within the Fungi, 132 OTUs belonged to the Glomeromycota (335 666 reads, 73.9% of final reads), 1 Blastocladiomycota OTU (61 reads, 0.0134% fungal reads), 122 Chytridiomycota OTUs (8.04% fungal reads), 17 Ascomycota OTUs (1 475 reads, 0.325% fungal reads), 134 Basidiomycota OTUs (69 984 reads, 15.4% fungal reads), 10 Zygomycota OTUs (1 527 reads, 0.336% fungal reads), and 33 unclassified Fungi OTUs (8 868 reads, 1.95% fungal reads). Of the Glomeromycota reads, there were 8 genera, including *Acaulospora* (2 OTUs; 1 VTX), *Claroideoglomus* (14 OTUs, 10 VTX), *Diversispora* (3 OTUs, 3 VTX), *Funneliformis* (1 OTU, 1 VTX), *Glomus* (99 OTUs, 40 VTX), *Paraglomus* (8 OTUs, 2 VTX), *Rhizoglomus* (3 OTUs, 2 VTX), and *Septoglomus* (2 OTUs, 1 VTX).

3.3.1 Objective 1

Dataset 1 (Objective 1) had 101 Glomeromycota OTUs with 14 688 reads (2.78% of total reads), which comprised 55 unique VTX: 1 *Acaulospora lacunosa*, 7 *Claroideoglomus* spp., 1 *Claroideoglomus lamellosum*, 3 *Diversispora* spp., 1 *Funneliformis mosseae*, 36

Glomus spp., 1 *Glomus macrocarpum*, 1 *Paraglomus* spp., 1 *Paraglomus laccatum*, 1 *Rhizoglomus fasciculatus*, 1 *Rhizoglomus vesiculiferus*, and 1 *Septoglomus constrictum* (Appendix V).

No OTUs were found with significant BH-adjusted p-values (< 0.05) or with moderate to large effect sizes between June and October data, so reads were merged between the sampling seasons. Average read counts within each site were relativized to 10 000 reads per sample and then visualized on a ML tree as OTUs (with 100 bootstrap replicates) (Figure 3.5). Some OTUs within the *Glomus-Rhizoglomus* clades (322, 326, 392, 378, 695, 891, 904, 966, 989, 1161, and 1274) have higher reads in A (uninvaded) plots, whereas others (OTUs 121, 372, 458, 468, 572, and 583) show higher reads in RA (invaded) plots. OTU164 (*Scon*64) was present in all samples, with a lower read count found in buckthorn-invaded than pristine plots, and the second OTU108 (*Scon*64) was amplified in all uninvaded plots but was present in the highest amount in the Cliffs buckthorn invaded plot. OTU617 (*Fmos*67) had a higher read count in the invaded Komoka soils, but was not amplified in any of the pristine sugar maple soils except Komoka. The *Claroideoglomus branch* had five OTUs (32, 60, 154, 158, and 252) that did not show site- or invasion- specific patterns. OTUs 480 and 980 were amplified only in pristine Komoka soils, OTU432 was amplified in Grand Alee in invaded buckthorn soils, and OTU320 seemed to have site-specific amplification in Komoka soils. OTU551 was present in both Komoka and Grand Alee, but had higher read counts in both invaded plots. *Paraglomus laccatum* OTU161 was seen only in Komoka soils, with a higher relative abundance in pristine sugar maple stands, whereas unknown *Paraglomus* spp. OTUs 986 and 718 were amplified in Komoka invaded and uninvaded soils and smaller amounts within uninvaded Grand Alee soils. Among *Diversispora* spp. phylotypes, OTUs 96 and 307 were seen in all soils, but OTU177 was present in all uninvaded plots and only in invaded Cliffs soils. Finally, the two *Acaulospora lacunosa* OTUs 482 and 113 were only picked up in Grand Alee sugar maple stands.


Abundance
16
128
1024
Treatment
Komoka_A
Komoka_RA
Cliffs_A
Cliffs_RA
Grand_A
Grand_RA
0.050
OTU750
OTU1250
OTU35
OTU99
OTU454
OTU40
OTU326
OTU48
OTU392
OTU378
OTU583
OTU118
OTU106
OTU366
OTU125
OTU292
OTU658
OTU20
OTU860
OTU280
OTU695
OTU462
OTU1274
OTU989
OTU4
OTU47
OTU121
OTU458
OTU1193
OTU904
OTU372
OTU23
OTU211
OTU481
OTU1
OTU468
OTU1079
OTU6
OTU1161
OTU862
OTU34
OTU331
OTU1263
OTU646
OTU28
OTU339
OTU957
OTU557
OTU16
OTU241
OTU966
OTU717
OTU39
OTU270
OTU1158
OTU257
OTU1255
OTU0
OTU537
OTU322
OTU64
OTU80
OTU572
OTU733
OTU875
OTU19
OTU17
OTU1149
OTU731
Glomus
Rhizoglomus

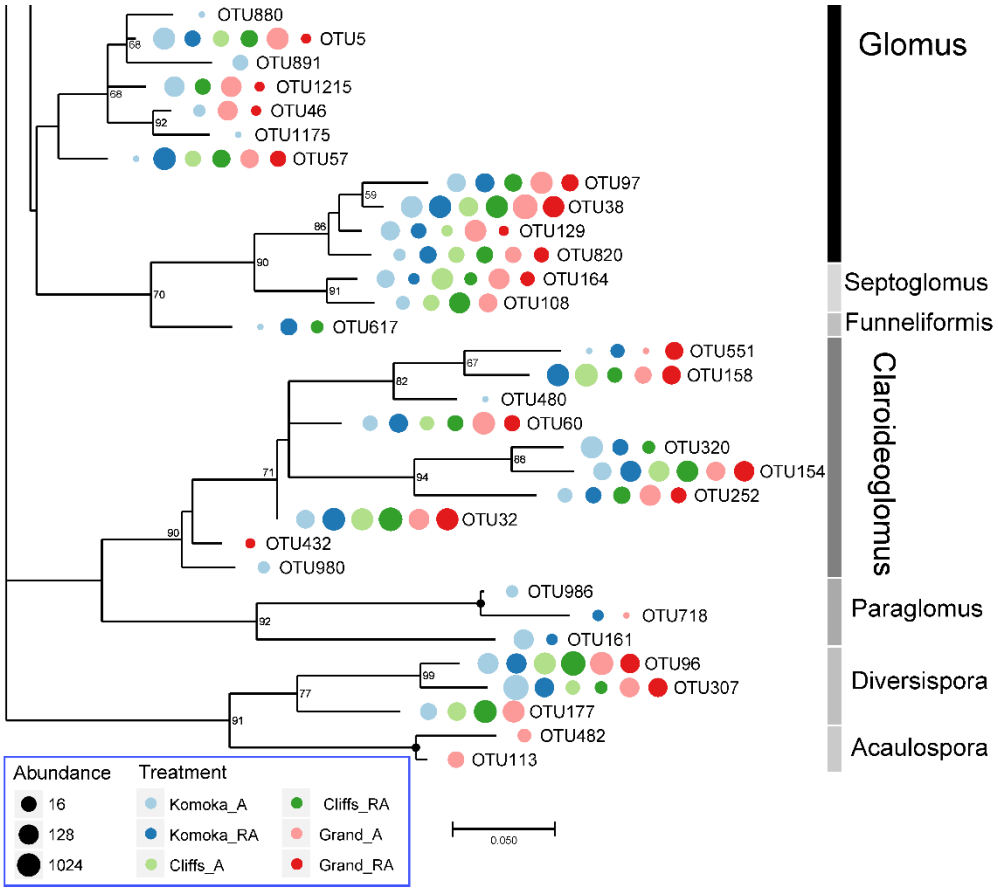


Figure 3.5. Molecular phylogenetic analysis by Maximum Likelihood method. Maximum Likelihood tree based on the Tamura-Nei model (Tamura and Nei 1993) of all OTUs found in invaded and uninvaded field samples (Objective 1). The tree with the highest log likelihood is shown. The percentage of trees in which associated taxa clustered together >50% of the time is shown next to the branches (bootstrap values of 1000 replicates) (Felsenstein 1985). Values that are ≥ 70% are highlighted in bold. Initial trees were obtained using the Neighbour-Joining and BioNJ algorithms from a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Branch lengths are proportional to the number of substitutions per site. The analysis involved 101 nucleotide sequences, and all positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. A total of 213 positions were used in the final dataset. Raw OTU reads were relativized to 10 000 reads per sample between invaded and uninvaded buckthorn sites (n=2) and are represented on a log2 scale next to each terminal node, and are colour coded by treatment type. Evolutionary analysis was conducted in MEGA7

(Kumar et al. 2016), abundance and treatment information was done through the phyloseq R package (McMurdie and Holmes 2013). Thirty-three OTUs were found in all 6 sites, 10 in 5, and 24 were found only in sites without buckthorn, including both OTUs of *Acaulospora* spp., one of *Paraglomus* sp., two of *Claroideoglomus* spp., and 20 of *Glomus* spp.

A coloured biplot function mapping VTX against sampling locations (n=12) with June and October sampling times is shown in Figure 3.6. Principal Components axes 1 and 2 explain 21.6% and 19.4% of the variation, respectively, with PC3 dropping to approximately 14% explained variance. Clustering of June samples can be seen in the center of the plot, having little bearing on components 1 and 2, whereas larger diversity is visible with October samples. Invaded October plots within Grand Alee and Komoka (OG_RA and OK_RA) are more similar to one another's OTU composition than invaded October Cliffs (OC_RA) plots, which are characterized by eight different *Glomus* and one *Claroideoglomus* OTUs. Uninvaded October Komoka plots (OK_A) had the largest difference between all the plots, characterized by nine *Glomus* OTUs, one *Diversispora* OTU (*D*62), one *Claroideoglomus* OTU (*C*279), and one *Paraglomus laccatum* OTU (*Plac*261). The presence of *D*356 has the highest influence on the October uninvaded Cliffs (OC_A) sample, driving it away from the central cluster.

Similar patterns of seasonal variability can be seen within the cluster dendrogram when considering all VTX in each sample (Figure 3.7). Both OK_AR/OG_AR and OK_A/OG_A samples clustered together, whereas both October Cliffs uninvaded and invaded (OCA and OC_AR) sites fell together within the June sampling point cluster. Both JC_AR and JC_A clustered within the same branch indicating similar VTX communities and a stronger plot effect, whereas the grouping of JK_AR and JG_AR shows more of a treatment (buckthorn invasion) effect. OC_A has a unique community of Glomeromycota, with high proportions of *G*199 and *G*88 comprising nearly 60% of the read count.

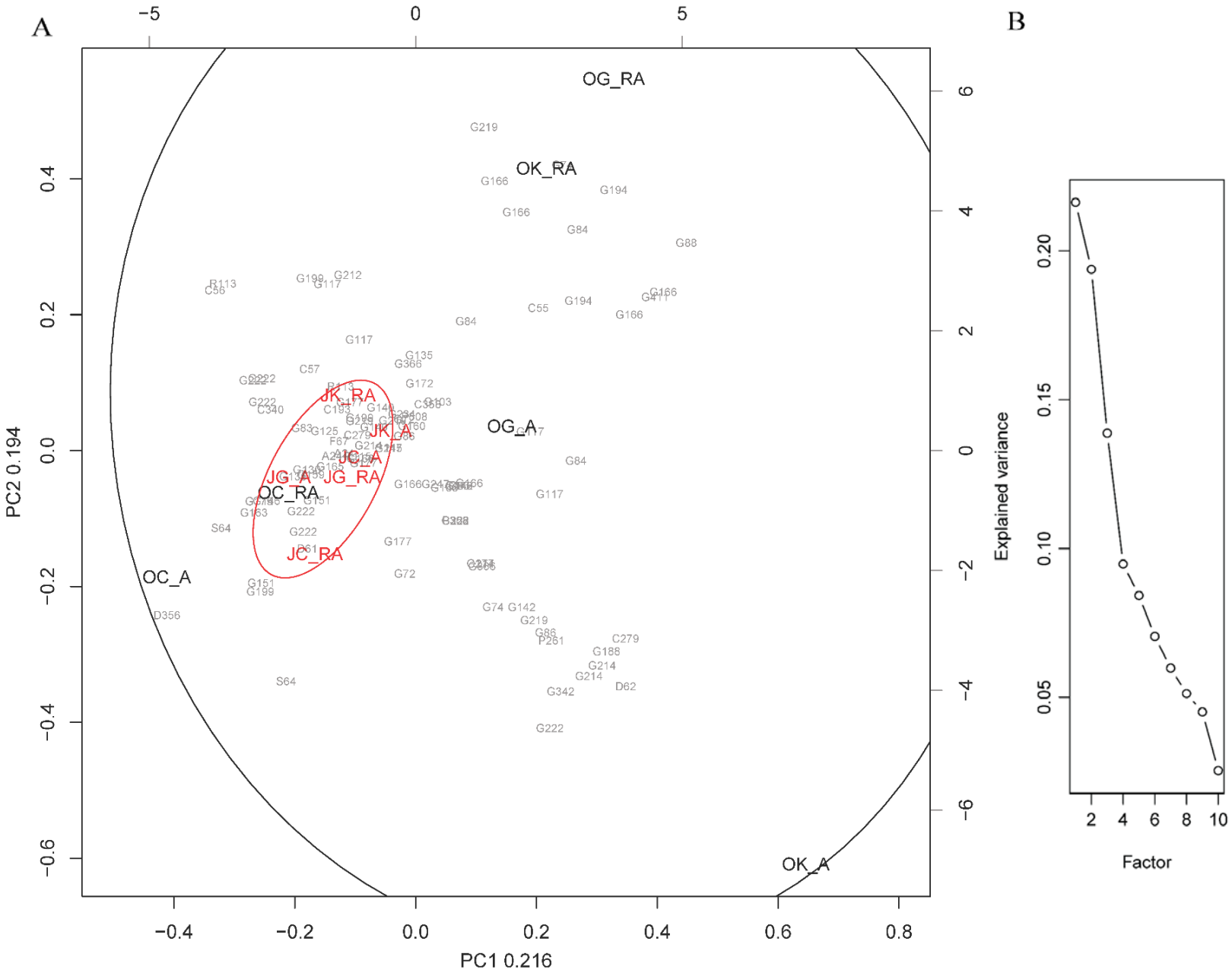

A
B
OG_RA
OK_RA
OG_A
OC_RA
OC_A
OK_A
JK_RA
JK_A
JG_A
JG_RA
JC_RA
PC1 0.216
PC2 0.194
Explained variance
Factor

Figure 3.6. Covariance biplot (A) and scree plot of eigenvalues (B) based on virtual taxa found in buckthorn (*Rhamnus cathartica*) invaded (RA) and uninvaded (A) sugar maple (*Acer saccharum*) soils (Objective 1), taken from plots within Komoka Provincial Park (K), London, ON, Grand Alee-Indian Woods (G) and Cliffs and Alvars (C), in rare Charitable Research Reserve, Cambridge, ON. Samples taken from each location are marked with the time of year they were taken, June (J) in red or October (O) in black. 95% confidence ellipses indicate lower degree of arbuscular mycorrhizal fungi (AMF) variation in June and higher AMF variation in October. Virtual taxa are shortened to the first letter of the genus, followed by the last three digits of the taxon number, as referred to in the MaarjAM database (Öpik et al. 2010). Genera found in this study are *Acaulospora* – A, *Claroideoglomus* – C, *Diversispora* – D, *Funneliformis* – F, *Glomus* – G, *Paraglomus* – P, *Rhizoglomus* – R, *Septoglomus* – S. The scree plot histogram shows eigenvalues (% explained variance) for the covariance biplot in other Principal Component axes.

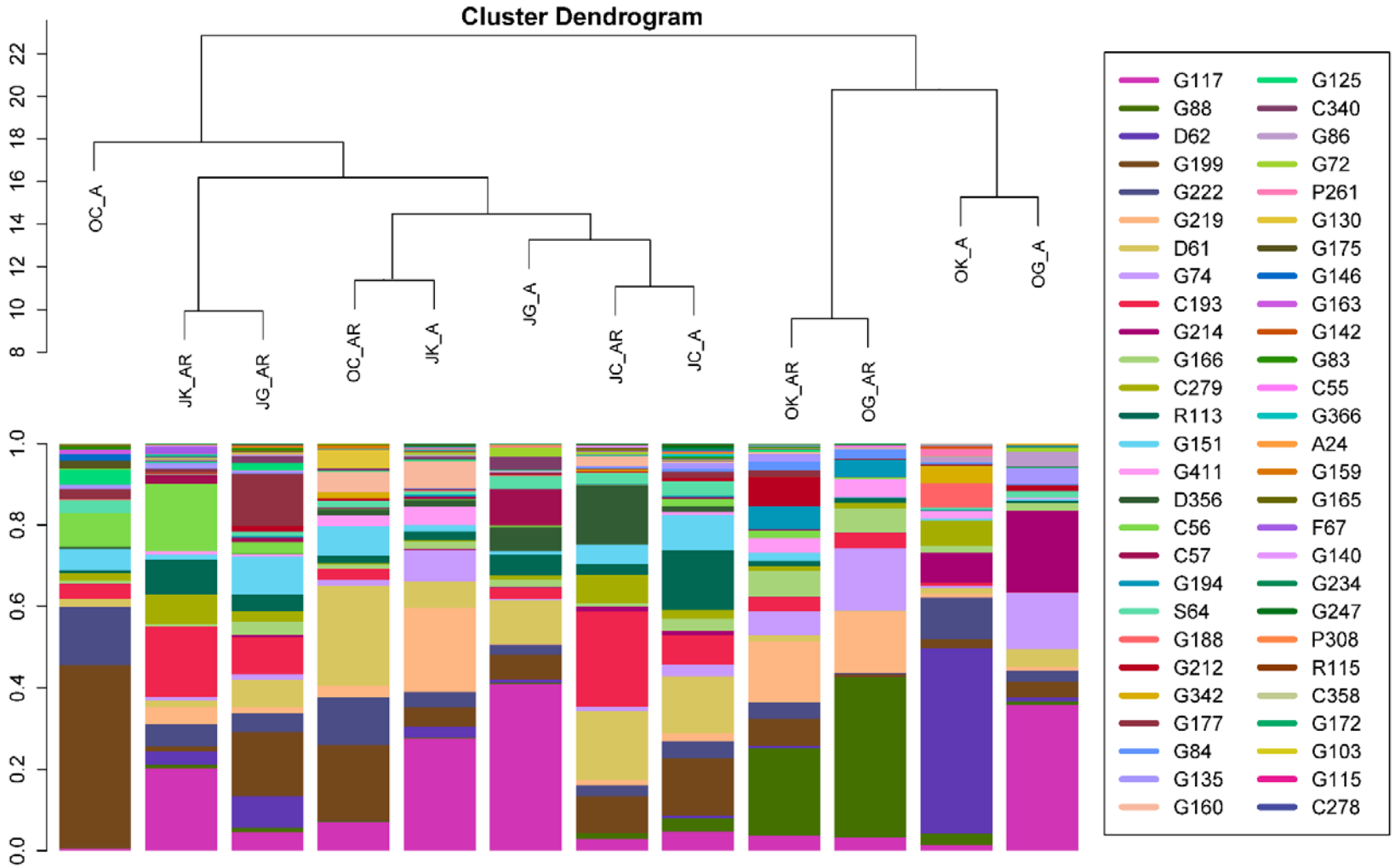


Figure 3.7. Cluster dendrogram and abundance barplot of all unique Virtual Taxa found in buckthorn (*Rhamnus cathartica*) invaded and uninvaded sugar maple (*Acer saccharum*) plots (Objective 1). October (O) and June (J) sampling times for each sampling location (Cliffs & Alvars – C, Grand Alee-Indian Woods – G, Komoka Provincial Park – K) is shown with buckthorn invaded (RA) or uninvaded (A) sites. Virtual Taxa are represented by the first letter of the genus (*Acaulospora*

– A, *Claroideoglomus* – C, *Diversispora* – D, *Funneliformis* – F, *Glomus* – G, *Paraglomus* – P, *Rhizoglomus* – R, *Septoglomus* – S) followed by the last three digits of the VTX number as found in the MaarjAM database (Öpik et al. 2010). A small seasonal effect is seen with the October Komoka and Grand Alee sites clustering together, whereas October Cliffs was clustered within the June sample branches. No definite pattern in VTX abundance is seen within the June samples. The top four abundant VTX among all samples were G117, *G*88 and *G*199 (all *Glomus* spp.), and *Diversispora* sp. VTX62. Figure generated using R 3.3.1 and the CoDASeq microbiome tutorial (https://github.com/ggloor/ CoDa_microbiome_tutorial/wiki). Inner branches are scaled; terminal branches are not scaled.

Differences between the October pristine samples was seen in the presence of *D*64 in Komoka, *G*117 in Grand Alee, and *G*119 in Cliffs & Alvars. On the other hand, June uninvaded samples had a prevalence of *G*117 in both Grand Alee and Komoka sites and an equal proportion of *G*88, *D*61, and *Rfas*113 in Cliffs & Alvars.

OTUs with differential abundances (those with moderate to large effect sizes due to larger between-group than within-group differences) in buckthorn uninvaded (A) versus invaded (RA) sites were highlighted with the ALDEx2 package in R. All OTUs and samples were kept in the dataset since OTU subsampling measures (e.g., retaining those with reads > 0.1% of the total dataset read count) did not substantially increase PC variance contained in axes 1 and 2. Two OTUs 129 and 164 belonging to *G*117 and *Scon*64 were considered moderately influential, with effect sizes within the range of -0.8 and -1 (Figure 3.8; Appendix VI). *Glomus* sp. VTX117 was present in five of the six pristine sites (147 total read count) and in two of the invaded sites (4 total read count), and *S. constrictum* VTX64 was present in all pristine sites (141 total read count) and in three of the invaded sites (5 total read count). No significant taxa were identified with the BH-adjusted p-values in relation to invaded or uninvaded soils.

Positively associated OTUs (those with low phi values) were shown within eight clusters (represented by their assigned genus and VTX number) at phi $\leq$ 0.3 (Figure 3.9). Six of the eight clusters contained OTUs within the same genus, whereas the other two contained a *Glomus*-*Claroideoglomus* pairing and a *Glomus*-*Glomus*-*Diversispora* pairing. A positive correlation among these clusters indicates similar increases or decreases in abundances across samples. A stronger positive correlation was seen with phi $\leq$ 0.2 clusters containing *C*279-*C*193, and *G*74-*G*219 (Figure 3.9).

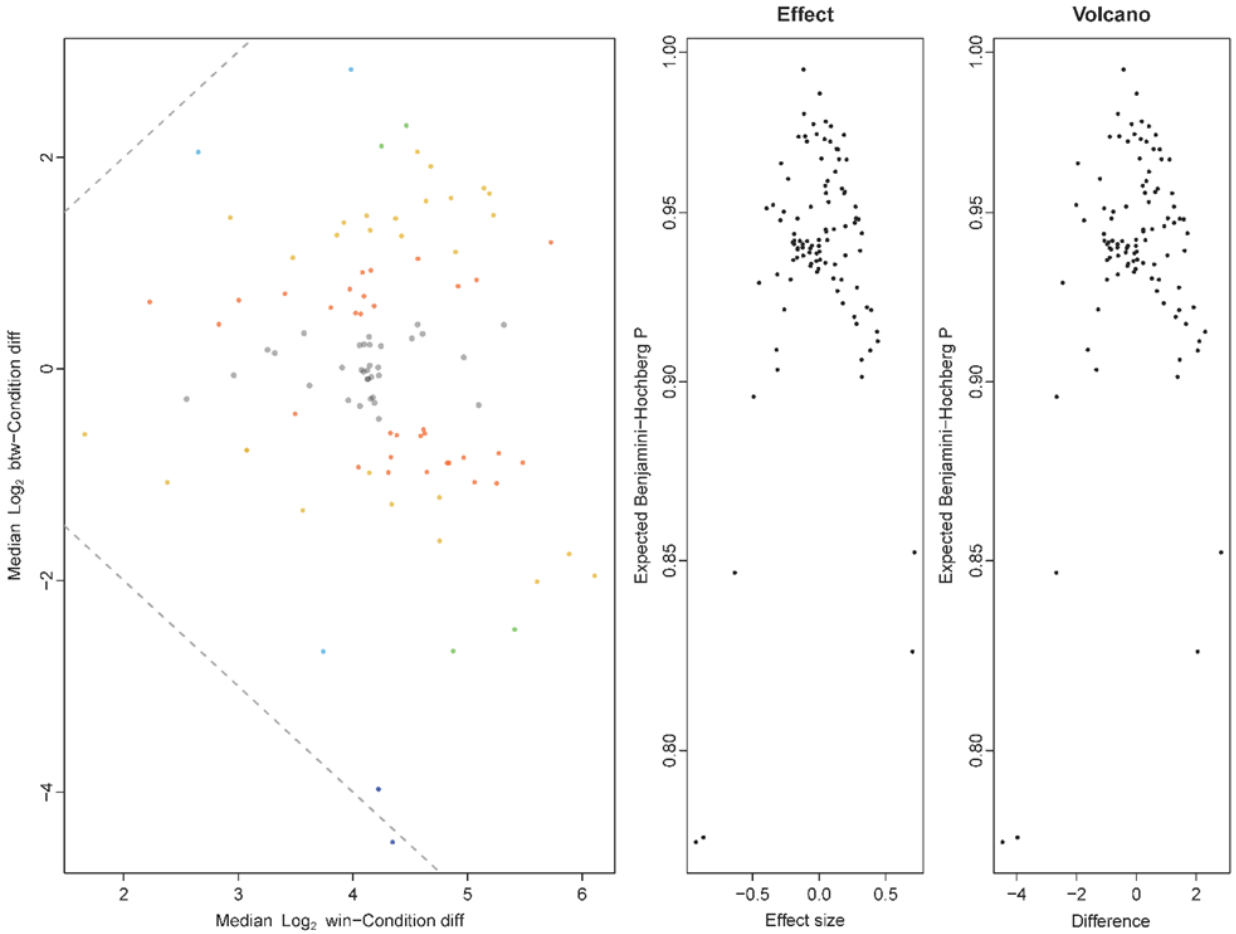


Figure 3.8. Analysis of OTUs in invaded versus uninvaded plots with differential variations (Objective 1); A) Scatterplot of the within- to between-condition differences in OTU variation among sample types. Dark blue dots represent samples with moderate effect sizes between -0.8 and -1 (those with larger between-group variation in comparison to within-group variation), and black dashed lines represent the line of equivalence for the within- and between- group values. Taxa that are more abundant than the mean in pristine (A) samples have negative y values, taxa that are more abundant than the mean in invaded (RA) samples have positive y values; **B)** Plot of effect size vs the BH adjusted P value; **C)** Volcano plot for reference. Figures generated using the ALDEx2 package (Fernandes et al. 2013) in R 3.3.1 (R Core Team 2016) using the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_ microbiome_tutorial/wiki).

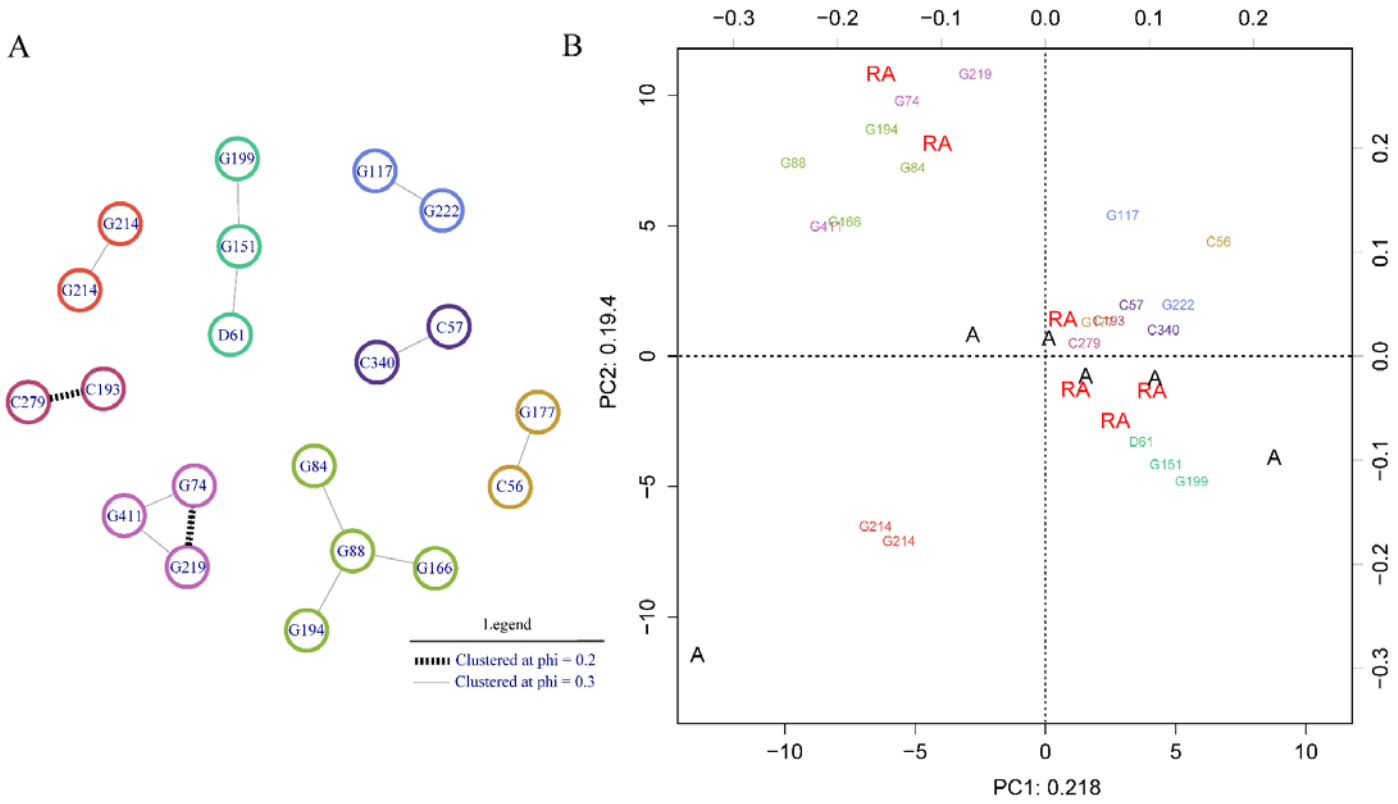


Figure 3.9. Positively correlated OTUs shown in an A) ordinal diagram (phi ≤ 0.3), and B) covariance biplot (Objective 1) based on buckthorn (*Rhamnus cathartica*) invaded and uninvaded sugar maple *(Acer saccharum*) soils. All positively correlated OTUs are represented by Virtual Taxa (VTX) designation. First letter genus abbreviations (C – *Claroideoglomus*, D – *Diversispora*, G – *Glomus*) are followed by the last three digits of its VTX numeric identifier. Stronger positive correlations (at phi ≤ 0.2) between taxa are indicated using heavy dashed lines. Coloured clusters from the ordinal diagram (A) are shown in the covariance biplot (B). Figures generated using R 3.3.1 and the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_microbiome_tutorial/wiki).

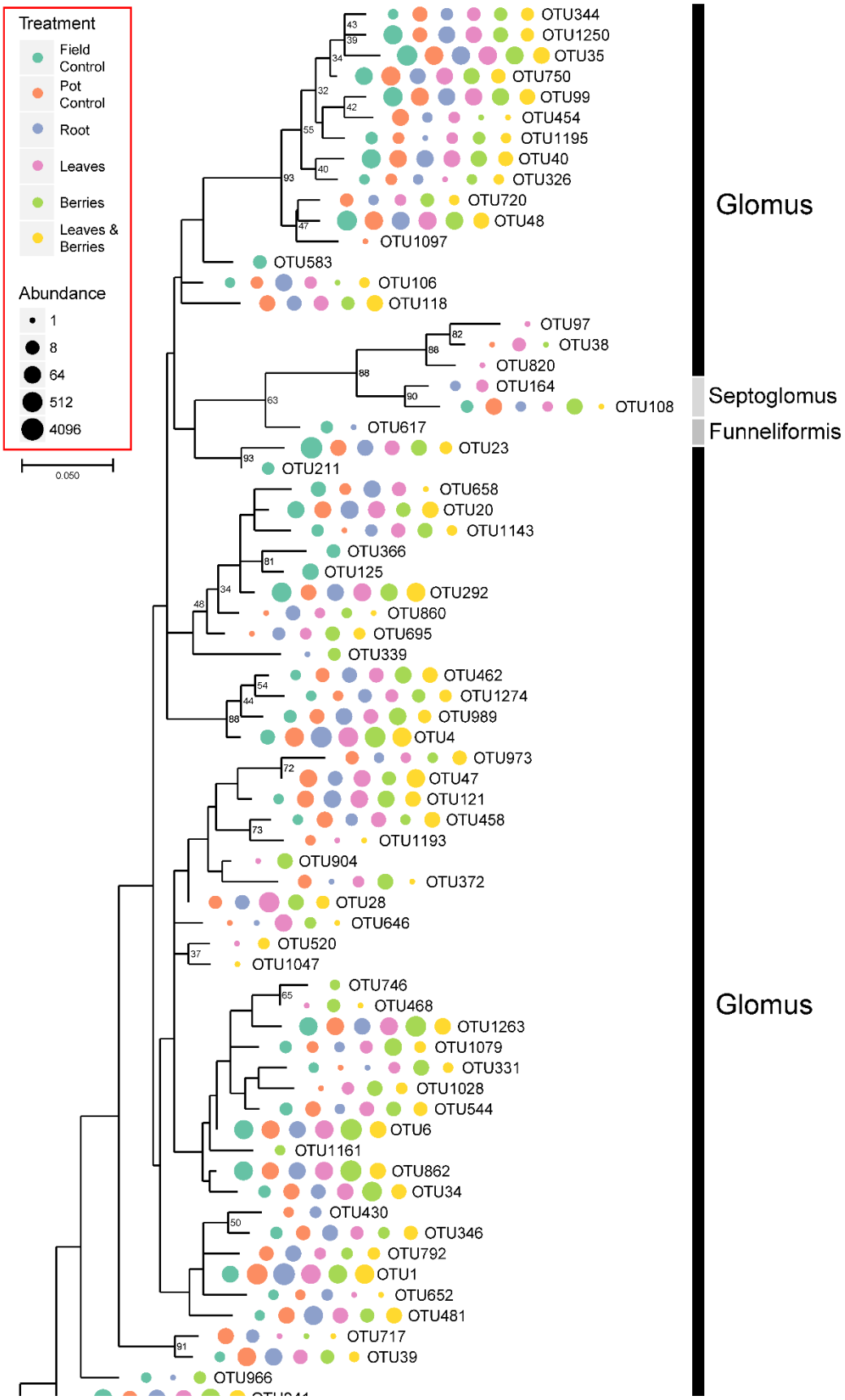
3.3.2 Objective 2

Dataset 2 (Objective 2) had 126 Glomeromycota OTUs with 165 805 reads (31.1% of total reads), which comprised 55 unique VTX: 1 *Acaulospora lacunosa,* 8 *Claroideoglomus* sp., 1 *Claroideoglomus lamellosum,* 2 *Diversispora* sp., 1 *Funneliformis mosseae,* 36 *Glomus* sp., 1 *Glomus macrocarpum,* 1 *Paraglomus* sp., 1 *Paraglomus laccatum,* 1 *Rhizoglomus fasciculatus,* 1 *Rhizoglomus vesiculiferus,* and 1 *Septoglomus constrictum* (Appendix VII).

Average read counts between replications were relativized to 10 000 reads within each treatment and placed into a ML tree (with 1000 bootstrap replications) as OTUs (Figure 3.10). *Glomus* spp. sequences clustered into three groups, separated by *Septoglomus* spp., *Funneliformis* spp., and *Rhizoglomus* spp. clades. *Glomus* spp. OTUs 97, 38, 820, 617, and 23 clustered with the *Septoglomus*/*Funneliformis* branch with high bootstrap confidence (>80), indicating that these sequences may be phylogenetically closer to the last two genera than to *Glomus* spp.

All zero values within the read count table were replaced with the count zero multiplicative (CZM) method and converted to proportions before creating a cluster dendrogram visualizing all unique VTX between samples (Figure 3.11). Both field control (CF) samples clustered on a separate branch from potted control (CP) samples, indicating the presence of a potting effect on the dataset. Control replications are sister groups, and Root 1 (R1) replicate clustered alongside the Control branch, showing similar VTX composition but higher *G*177 proportions and a subsequent reduction in *Rfas*113. Root 2 (R2) replicate clustered with Leaves & Berries 1 (LB1), Leaves 1 (L1), and Berries 1 (B1), whereas a slight replication effect was seen with the branches ending with Berries 2 (B2) replicate, Leaves 2 (L2) and Leaves & Berries 2 (LB2) terminal nodes.

Both control replications of the field (CF) and potted (CP) samples were combined (n=4) and both replications of all emodin treatments, Roots (R), Leaves (L), Berries (B) and Leaves/Berries (LB) were combined (n=8) since a minimum of 3 replicates are required for the ALDEx2 package.


Treatment
Field Control
Pot Control
Root
Leaves
Berries
Leaves & Berries
Abundance
1
8
64
512
4096
0.050
OTU344
OTU1250
OTU35
OTU750
OTU99
OTU454
OTU1195
OTU40
OTU326
OTU720
OTU48
OTU1097
OTU583
OTU106
OTU118
OTU97
OTU38
OTU820
OTU164
OTU108
OTU617
OTU23
OTU211
OTU658
OTU20
OTU1143
OTU366
OTU125
OTU292
OTU860
OTU695
OTU339
OTU462
OTU1274
OTU989
OTU4
OTU973
OTU47
OTU121
OTU458
OTU1193
OTU904
OTU372
OTU28
OTU646
OTU520
OTU1047
OTU746
OTU468
OTU1263
OTU1079
OTU331
OTU1028
OTU544
OTU6
OTU1161
OTU862
OTU34
OTU430
OTU346
OTU792
OTU1
OTU652
OTU481
OTU717
OTU39
OTU966
Glomus
Septoglomus
Funneliformis
Glomus

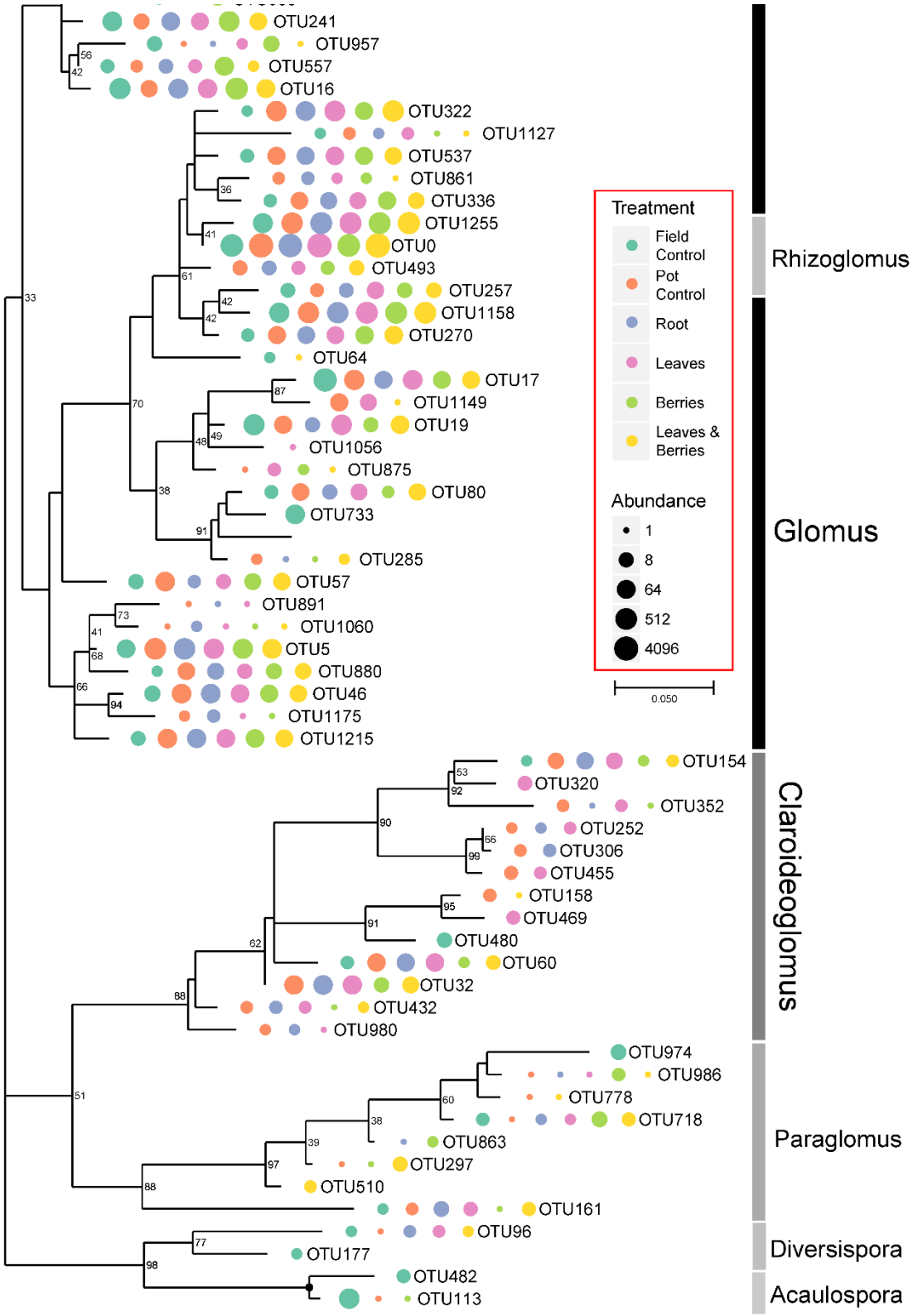

OTU241
OTU957
OTU557
OTU16
OTU322
OTU1127
OTU537
OTU861
OTU336
OTU1255
OTU0
OTU493
OTU257
OTU1158
OTU270
OTU64
OTU17
OTU1149
OTU19
OTU1056
OTU875
OTU80
OTU733
OTU285
OTU57
OTU891
OTU1060
OTU5
OTU880
OTU46
OTU1175
OTU1215
OTU154
OTU320
OTU352
OTU252
OTU306
OTU455
OTU158
OTU469
OTU480
OTU60
OTU32
OTU432
OTU980
OTU974
OTU986
OTU778
OTU718
OTU863
OTU297
OTU510
OTU161
OTU96
OTU177
OTU482
OTU113
Treatment
Field Control
Pot Control
Root
Leaves
Berries
Leaves & Berries
Abundance
1
8
64
512
4096
0.050
Rhizoglomus
Glomus
Claroideoglomus
Paraglomus
Diversispora
Acaulospora

Figure 3.10. Molecular phylogenetic analysis by Maximum Likelihood method of all OTUs found in potted samples (Objective 2). Maximum Likelihood tree based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood is shown. The percentage of trees in which associated taxa clustered together >30% of the time is shown next to the branches (bootstrap values of 1000 replicates) (Felsenstein 1985). Values that are ≥70% are highlighted in bold. Initial trees were obtained using the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Branch lengths are measures in the number of substitutions per site. The analysis involved 245 nucleotide sequences, and all positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. A total of 211 positions were used in the final dataset. Raw OTU reads were relativized to 10 000 reads per sample and averaged between replications (n=2). Data are represented on a log2 scale next to each terminal node, and are colour coded by treatment type. Evolutionary analysis was conducted in MEGA7 (Kumar et al. 2016), abundance and treatment information was done through the phyloseq R package (McMurdie and Holmes 2013). Fifty-eight OTUs were found in all six treatments, 4 were only seen in the Field control, and 11 OTUs were found only in Treatment samples, including two of *Paraglomus* spp., two of *Claroideoglomus* spp., and 7 of *Glomus* spp

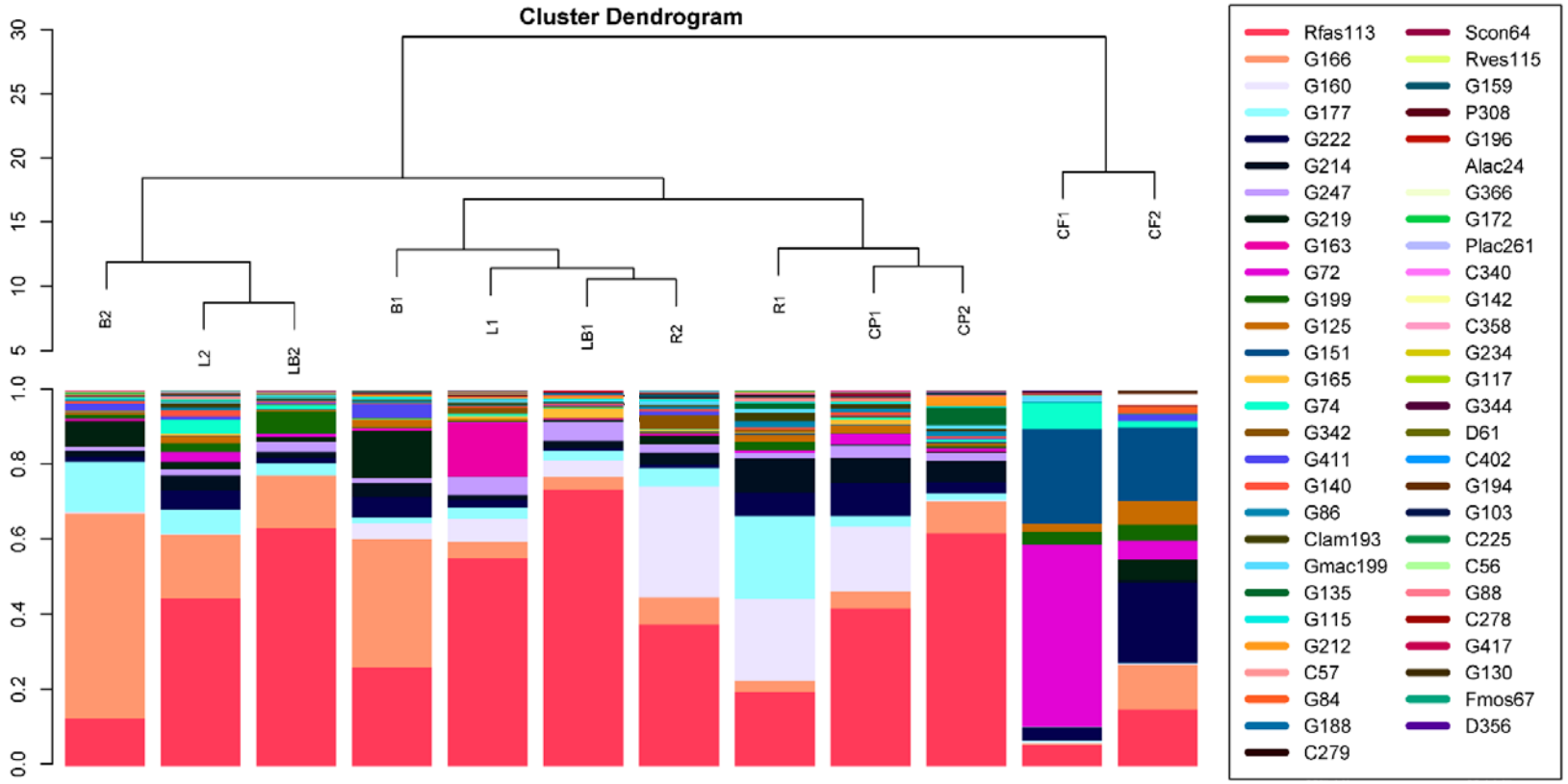


Figure 3.11. Cluster dendrogram and abundance barplot of all unique Virtual Taxa (VTX) found in potted Control (*Acer saccharum*) and Treatment (*Acer saccharum, Rhamnus cathartica,* plus allelochemicals) samples (Objective 2). VTX found in Potted Control (CP) and Field Control (CF) are clustered alongside buckthorn (*R. cathartica*) allelochemical treatment pots (R – Root, L – Leaves, B – Berries, LB – Leaves and Berries). All VTX are listed with the genera's first letter and first three letters of the species, if known (A – *Acaulospora lacunosa*, C – *Claroideoglomus*, Clam – *C. lamellosum*, D – *Diversispora*, Fmos – *Funneliformis mosseae*,

G – *Glomus*, Gmac – *G. macrocarpum*, P – *Paraglomus*, Plac – *P. laccatum*, Rfas – *Rhizoglomus fasciculatus*, Rves – *Rhizoglomus vesiculiferus*, and Scon – *Septoglomus constrictum*), and the last three digits of its VTX identifier (Öpik et al. 2010). Clustering patterns show a potting effect with the CF samples. The top three abundant VTX in the potted samples (*Rfas*113, *G*166, and *G*160) are not found in high abundance in the CF samples, whereas *G*72, *G*151, and *G*222, make up the largest proportion. Legend lists taxa in order of decreasing overall abundance starting from the top left. Figures generated using R 3.3.1 and the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_microbiome_tutorial/wiki).

All OTUs and samples were kept in the dataset since OTU subsampling measures (e.g., retaining those with reads > 0.1% of the total dataset read count did not substantially increase PC variance contained in axes 1 and 2). Five OTUs (4, 462, 17, 23 and 48) had large effect sizes (> 1, or < -1) between groups, and 6 OTUs (99, 40, 35, 1255, 695, and 1274) had moderate significant effect sizes (between 0.8 and 1, or -0.8 and -1) (Figure 3.12, summarized in Appendix VIII). All of the OTUs with moderate or large effect sizes between Control and Treatment plots were *Glomus* spp. sequences. Both OTUs of *G*177 had low reads in the control samples and significantly more in the treatment samples (positive y-axis values). In contrast, *G*151, *G*125, and *G*72 were higher in control than the four treatments (negative y-axis values). No OTUs were found to have significant BH-adjusted p-values (<0.05).

A cluster dendrogram was created using taxa with large and moderate effect sizes (Figure 3.13). Both CF samples were strikingly different than all the potted samples, with a much higher proportion of OTU23 (*G*151), and CF2 having the highest proportion of OTU17 (*G*72) than all other samples. Within the potted samples, OTU4 steadily increased in proportion, with potted controls having the lowest (disregarding B1 & B2 samples), followed by Leaves, Leaves/Berries, and Roots. The B1 replication clustered with the two CP1 and CP2 replications, whereas the B2 replication was most similar to both R1 and R2. Disturbance of the soil caused by the potting procedure appears to have encouraged growth of OTU4 (*G*177), which was present in both field controls in very low counts in the raw data (13 reads between both sites). OTUs 695, 1274, and 462 are considered absent from field samples (2 reads between both sites) but show an increase after disturbance. The *G*177 VTX, represented by OTUs 4, 462, and 1274 all increased after potting and buckthorn disturbance.

Positively associated OTUs (phi ≤ 0.15) are clustered by colour in Figure 3.14A. Taxa with large effect sizes highlighted in Figure 3.12 are shown with grey centers in the ordination plot (Figure 3.14A), and as grey dots on the covariance biplot (Figure 3.14B). The potting effect is visible along PC1 (27.1% explained variance), with both CF treatments clustering away from potted samples.

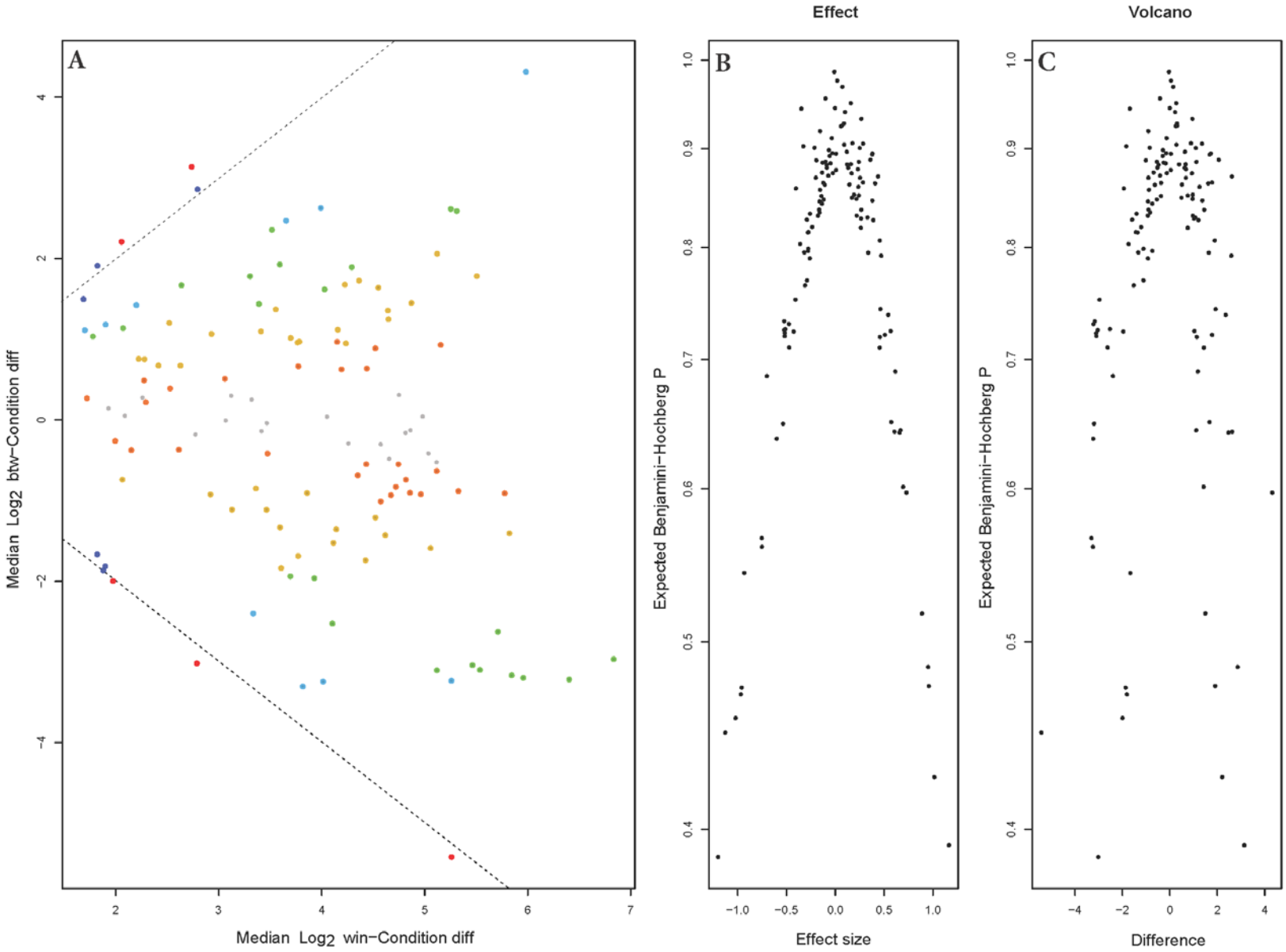

A
Median Log2 btw−Condition diff
Median Log2 win−Condition diff
Effect
B
Expected Benjamini−Hochberg P
Effect size
Volcano
C
Expected Benjamini−Hochberg P
Difference

Figure 3.12. Analysis of OTUs in control versus treatment samples with differential variations (Objective 2); **A)** Scatterplot of the within- to between- condition differences in OTU variation among samples. Red dots represent those with large effect sizes between > 1 or < -1 (those with larger between-group variation in comparison to within-group variation), dark blue dots represent samples with moderate effect sizes (between 0.8 and 1, or -0.8 and -1) and black dashed lines represent the line of equivalence for the within- and between- group values. Taxa that are more abundant than the mean in Control (Field Control and Potted Control) samples have negative y values, taxa that are more abundant than the mean in Treatment (Roots, Leaves, Berries, and Leaves & Berries) samples have positive y values; **B)** Plot of effect size vs the BH adjusted P value; C) Volcano reference plot. Figures generated using the ALDEx2 package (Fernandes et al. 2013) in R 3.3.1 (R Core Team 2016) through the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_microbiome_tutorial/wiki).

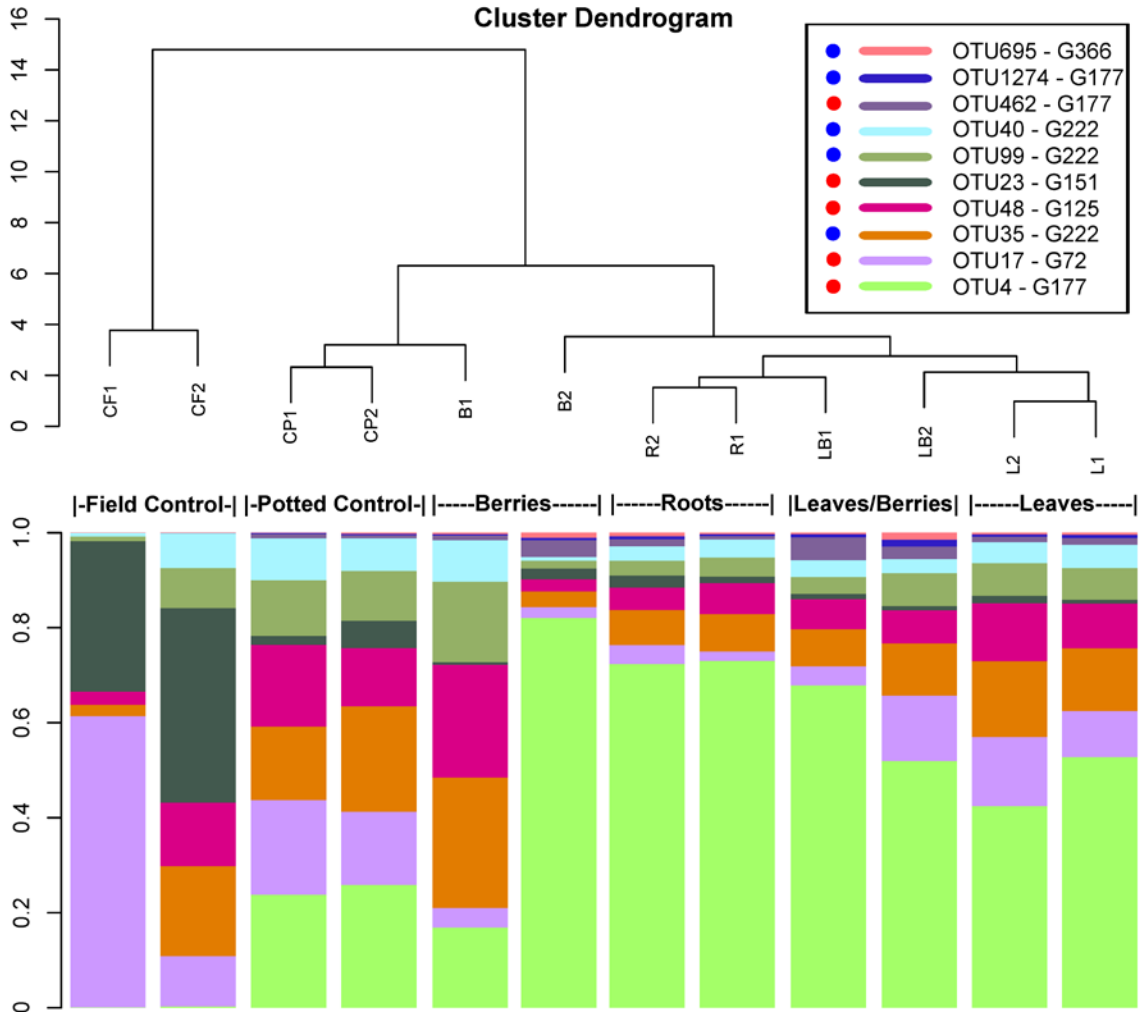


Figure 3.13. Cluster dendrogram and abundance barplot of taxa with large (red dot) and moderate (blue dot) effect sizes (Objective 2). A potting effect is shown as the biggest difference between Field Control (CF) and potted samples (CP – Potted Control, R – Roots, L – Leaves, B – Berries, LB – Leaves & Berries), with the appearance of 4 taxa (OTUs 695, 1274, 462, and 4) after potting, and the reduction in proportion of OTUs 23 and 17 after potting. Treatment effects (except for B1 and B2) include a large increase in the proportion of OTU4 and subsequent decrease in abundance of all other OTUs. B1 shows no treatment effect and clusters with both Controls, B2 shows an extreme increase in OTU4. Figures generated using R 3.3.1 (R Core Team 2016) and the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_microbiome_ tutorial/wiki).

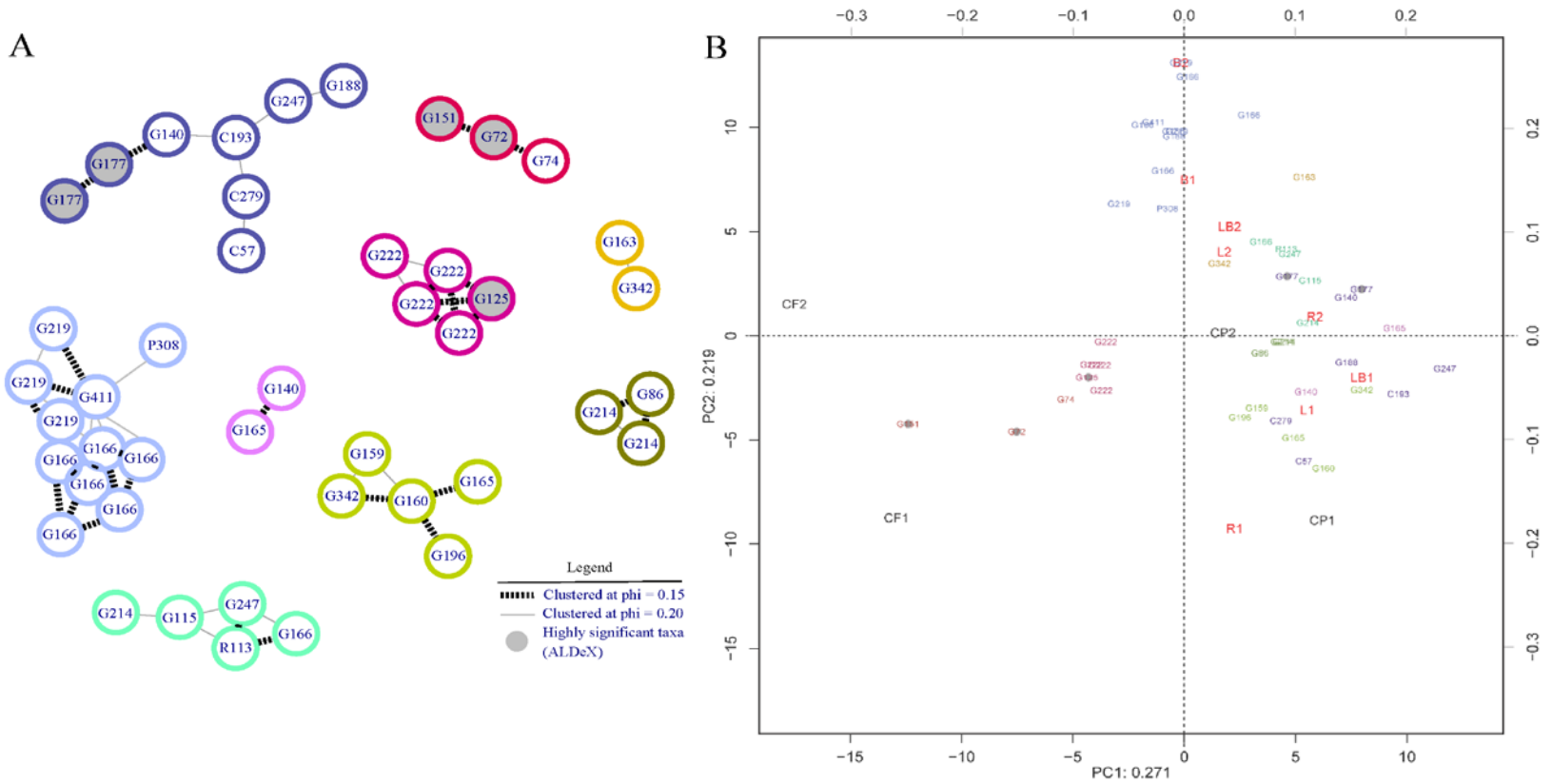


Figure 3.14. Positively correlated OTUs shown in an A) ordinal diagram (phi ≤ 0.2) and B) covariance biplot based on Control and Treatment potted samples (Objective 2). Ordinal diagram visualizing positively correlated VTX at both phi ≤ 0.2 (grey line) and 0.15 (dashed line). OTUs with large effect sizes are shown in grey. Coloured clusters from the ordinal diagram (A) are shown in the covariance biplot (B). Biplot PC1 explained 27.1% of the variation between potting effects, with Field Control (CF) samples clustering separately from Potted Control (CP) and all other treatments: Roots (R), Leaves (L), Berries (B) and Leaves & Berries (LB). PC2

explained 21.9% of the variance due to sampling location (disregarding TB1 as an outlier), with potted replicates 1 and 2 clustering above and below the PC2 axis. First letter VTX abbreviations (C – *Claroideoglomus*, G – *Glomus,* P – *Paraglomus,* R – *Rhizoglomus*) are followed by the last three digits of its VTX numeric identifier. Numbers following sample names indicate sampling location (1 or 2). Figures generated using R 3.3.1 (R Core Team 2016) and the CoDASeq microbiome tutorial (https://github.com/ggloor/CoDa_microbiome_ tutorial/wiki).

Treating B1 as a potential outlier, sampling location variance (21.9%) can be explained by PC2 where there is separation between sampling locations 1 and 2 above and below the dashed line. The associated scree plot (Figure 3.8B) of eigenvalues and PC components confirms that most of the variation can be explained by PC1 and PC2. The ordination diagram (Figure 3.14A) shows a clearer view of OTUs and AMF genera (C – *Claroideoglomus*, G – *Glomus*, P – *Paraglomus*, and R – *Rhizoglomus*) that are positively correlated with both phi cutoffs (0.15 and 0.1). VTX are expected to respond similarly to treatment effects and would typically fall into clusters amongst themselves (as seen in the two clusters comprised mainly of *G*166 or *G*222). Six of the 9 clusters (at phi $\leq$ 0.2) all contain the same genus (*Glomus* spp.), and 3 contain different genera (*Glomus*-*Claroideoglomus*, *Glomus*-*Paraglomus*, and *Glomus*-*Rhizoglomus*). Clustering at phi $\leq$ 0.15 (dark dashed lines) all involve OTUs within the same genus, except for the *Glomus*-*Rhizoglomus* group, and all involve at least one different VTX.

A covariance biplot of OTUs found in the manipulative experiment, after the outlying B1 sample was removed, shows the separation of CF replications 1 and 2 from all potted replications along PC1 (29.1% explained variance), and clear site-specific clustering of samples and OTUs along PC2 (20.4% explained variance) (Figure 3.15A). The scree plot of eigenvalues shows a drop in explained variance in PC3 to approximately 11% (Figure 3.15B). Despite the Shady Maples sites being within the same forest, approximately 400 m from one another (Figure 3.2D), there are observable differences in AMF communities. The 95% confidence ellipses (calculated using standard error) exclude the more variable CF replications within each site, as well as the B2 replicate.

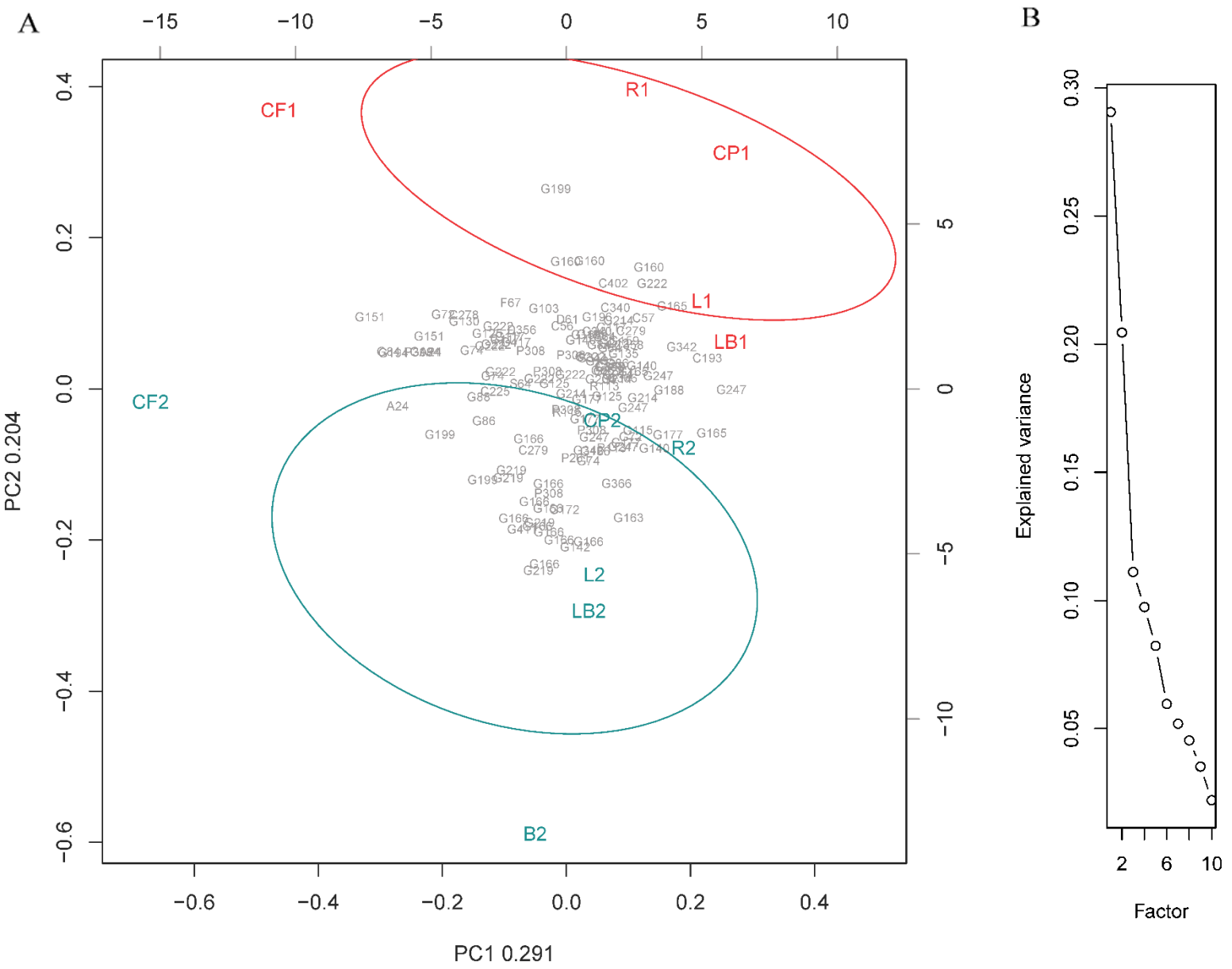

A
B
CF1
R1
CP1
L1
LB1
CF2
CP2
R2
L2
LB2
B2
PC1 0.291
PC2 0.204
Explained variance
Factor

Figure 3.15. Covariance biplot (A) and scree plot of eigenvalues (B) based on virtual taxa found in potted Control (*Acer saccharum*) and Treatment (*Acer saccharum, Rhamnus cathartica,* plus allelochemicals) samples (Objective 2). VTX found in each replication (1 or 2) in Potted Control (CP), Field Control (CF), and buckthorn allelochemical treatment pots (R – Root, L – Leaves, B – Berries, LB – Leaves and Berries), with B1 removed as an outlier. Virtual taxa are shortened to the first letter of the genus, followed by the last three digits of the taxon number, as referred to in the MaarjAM database (A – *Acaulospora lacunosa*, C – *Claroideoglomus*, Clam – *C. lamellosum*, D – *Diversispora*, Fmos – *Funneliformis mosseae*, G – *Glomus*, Gmac – *G. macrocarpum*, P – *Paraglomus*, Plac – *P. laccatum*, Rfas – *Rhizoglomus fasciculatus*, Rves – *Rhizoglomus vesiculiferus*, and Scon – *Septoglomus constrictum*) (Öpik et al. 2010). 95% confidence ellipses (red for replication 1, cyan for replication 2) indicate site specific differences in VTX abundances. The scree plot histogram shows eigenvalues (% explained variance) for the covariance biplot in other Principal Component axes.

3.4 Discussion

The spread of invasive plants into native communities has been associated with changes in soil AMF community composition through disturbance or allelochemicals (Barto et al. 2011; Meinhardt and Gehring 2012; Mummey and Rillig 2006; Stinson et al. 2006; Zhang et al. 2007).

The first objective was to determine whether local invasion of buckthorn (*Rhamnus cathartica*) into pristine sugar maple (*Acer saccharum*) forests would affect fungal communities, most likely due to the release of multiple secondary metabolites through roots, decomposing berries, and litter. An increase in disturbance—and allelochemical—tolerant AMF and a decrease in sensitive or rare species in sugar maple forests was hypothesized because of similar occurrences documented with other invasive plants (Epifano et al. 2012; Genovese et al. 2010; Hempel et al. 2007). However, data revealed that AM fungal communities in sugar maple forests varied by site and sampling time as much or more than in their response to buckthorn invasion.

The second objective was to find out whether any short-term AMF community changes would occur in pots containing sugar maple seedlings exposed to buckthorn root exudates and leachates from leaves and berries. Maple seedlings and naïve soil were collected, and the maples were planted alongside root-washed buckthorn seedlings and allowed to grow over the summer from May to October. The seedlings were given time to establish for one and a half months before buckthorn allelochemical additions using mulched leaves, coarsely ground berries, or a combination of both. Sequencing of mycorrhizal DNA from root-washed sugar maple seedlings (as well as undisturbed field controls) revealed a strong potting/greenhouse effect as well as a weaker treatment effect between samples, indicating the potential for buckthorn influence on native seedlings. Potting effects include the compaction of soil near the bottom of the pot, as well as the removal of water-soluble compounds from drainage holes over time.

Despite the difficulty in generalizing the overall changes in community composition and abundance, changes in local AMF dynamics can still provide insight into

buckthorn's invasional success. It was possible to put names onto previously unknown AMF that may be associated with, and affected by, buckthorn during invasion. Considering every sample taken from buckthorn-invaded or -uninvaded soils, only 24 OTUs were seen solely in uninvaded sites, including sequences belonging to species of *Acaulospora*, *Paraglomus*, *Claroideoglomus*, and *Glomus*. Despite high variance among samples, a prevailing pattern in seasonal AMF dynamics became apparent when comparing June and October sample dates. Here, similar mycorrhizal communities consisting of *Claroideoglomus* spp. and *Glomus* spp. VTX were observed between some summer (June/July) sample dates, and different taxa dominated in the fall for each area (species from *Diversispora*, *Rhizoglomus*, and *Glomus*). Seasonal shifts in AMF dominance were seen in the same location despite the presence or absence of buckthorn, i.e., between pristine Komoka/Cliffs & Alvars, and invaded Grand Alee/Komoka plots.

Significant differences were not evident between paired sites when all three sampling locations and both temporal replications were considered, which necessitated a closer look at paired sites to identify site-specific changes during buckthorn invasion. No AMF VTX differences between invaded and pristine soils were seen in Cliffs & Alvars in June, an unexpected finding given that the invaded plot had more mature buckthorn stems than invaded Komoka or Grand Alee sites. This may indicate influences of soil composition on mycorrhizae in the area, that buckthorn did not substantially affect mycorrhizal communities, or that the 'pristine' Cliffs & Alvars site may already have disrupted AMF communities due to the advancing garlic mustard front along the periphery. In general, October samples from Komoka and Grand Alee were considered similar in AMF composition when invaded and uninvaded sites were compared, and only the June invaded sites were similar across the two locations. Here, comparable edaphic factors within Komoka and Grand Alee may be driving the parallel trajectory of AMF community development in these separate locations.

Low explained variance in the first two principal components in this study may be attributed to AMF community variance among sampling locations due to soil properties such as pH, soil fertility and texture (Jansa et al. 2014), soil carbon, gravimetric water, site-specific changes in extractable nitrogen and nitrogen mineralization (Barto et al. 2011;

Heneghan et al. 2006), and the presence of other mycorrhizal native plants (Davison et al. 2012; Helgason et al. 2014). The visible differences in dominant AMF VTX across all sampled pristine locations demonstrates spatial structuring, something that has been observed in scales as small as < 1 m (Mummey and Rillig 2008), as well as the functional redundancy of these organisms (Gosling et al. 2016). This suggests that generalizing AMF community patterns is difficult without proper representative sampling (a known limitation in this study) as well as appropriate metadata, a recommended component for future investigations involving buckthorn and AMF.

Positive correlations in VTX abundances were seen using the compositional association analyses, where six of the eight OTU clusters demonstrated coordinated genus-level fluctuations across all samples; of these, four contained only *Glomus* spp. sequences. This may be the result of 1) the preferential amplification of Glomeraceae DNA (genera in this study include: *Glomus* spp., *Rhizophagus* spp., and *Septoglomus* spp.) known to occur with the AMV4.5NF-AMDGR primer pair (Van Geel et al. 2014), 2) the functional redundancy and functional synergy of mycorrhizae within a niche (Doherty 2009), or 3) the incomplete separation of sequences into OTU clusters (pers. comm. Gregory B. Gloor, 2016) (Gloor et al. 2016; Lovell et al. 2015). In the third case, the 3% OTU cutoff for the V4 SSU region may be too high for adequate AMF species delineation using the current primers, resulting in pairs or groups of sequences having proportional changes in abundance because they originally stemmed from a single organism. In this study, OTUs belonging to *G*166 and *G*222 grouped tightly within each respective cluster, and may indicate that current species delineation within these *Glomus* spp. VTX may not correlate with the differences within their genetic sequences, specifically the V4 region of the SSU. This alludes to the limitations of using short-read sequence studies for this DNA region, where the nucleotide differences between species are not congruent with OTU clustering. Phi clusters containing different genera may indicate functionally redundant organisms within the cluster in sugar maple roots, increasing or decreasing in abundance due to the same factors (treatment or other environmental influences). In this study, clusters containing sequences belonging to *Rhizoglomus*-*Glomus*, *Paraglomus*-*Glomus*, and *Claroideoglomus*-*Glomus* were observed. The last two groups were seen only at phi ≤ 0.2,

and the *Rhizoglomus-Glomus* cluster had a stronger positive correlation, possibly due to its close phylogenetic relatedness to one another or their role within a niche.

The manipulative garden experiment allowed for the direct addition of buckthorn allelochemicals to naïve sugar maple seedlings and associated soil arbuscular mycorrhizae (Objective 2). Pooling control samples and comparing them to the four allelochemical treatments highlighted two OTUs both belonging to *G*177 that were recorded in higher abundance in treatment samples, and three OTUs belonging to *Glomus* spp. (*G*151, *G*125 and *G*72) that were higher in controls. *G*151 and *G*72 were highest in field control samples, and dropped in reads in the potted control replications, indicating that they are sensitive to potting disturbances. Certain disturbance-tolerant Glomeraceae such as *G. intraradices* and *F. mosseae* [≡*G. mosseae*] produce large amounts of spores and are found in disturbed sites (Jansa et al. 2003; Öpik et al. 2006). The hyphal networks of the Glomeraceae, as opposed to the family Gigasporaceae, are better integrated within soils because they have more hyphal fusions, are faster root colonizers, are able to allocate a larger fraction of fungal biomass into the host root, and form lipid-storing vesicles (Maherali and Klironomos 2007; van der Heijden and Scheublin 2007). Similarly, *G*177, having very low counts in the field control, which increased 100-fold in the potted control, seemed to be better adapted to disturbance (both potting and buckthorn treatment effects). *Glomus* spp. (*G*166 and *G*130) and closely related *Rhizoglomus fasciculatus* (*Rfas*113) also responded favorably to potting disturbances, whereas field control maples maintained higher associations with *Glomus* spp. *G*72, *G*22 and *G*151—presumed to be species that are better suited to undisturbed habitats. Interestingly, both *G*151 and *G*72 abundances were positively correlated despite their distant phylogenetic relatedness within the ML phylogenetic analysis (Appendix III). The likelihood of these OTUs belonging to a single, improperly clustered species is low, and may instead indicate functional dependency or redundancy between separate mycorrhiza within the same ecosystem.

Taxa with large and moderate effect sizes with higher between treatment variation than within treatment variation showed relatively even proportions between six OTUs in the potted controls, with five decreasing in evenness in response to buckthorn leaves, leaves and berries, and roots, as the proportion of OTU4 (*G*177) increased. It was expected that

the leaves and berries treatment would have a greater effect on AMF abundance as it had the combined weight of crushed leaves and ground berries than in the separate leaves only and berries only treatments, but this was not the case in this experiment.

The buckthorn berries were picked in March and it is possible that freeze-thaw temperature cycles of winter as well as the natural reduction in allelochemical concentrations, due to a defense and development tradeoff, in mature fruit made them less potent (Mummey and Rillig 2006; Newman 1966; Paneitz and Westendorf 1999). Other comparative allelochemical studies used fresh buckthorn extracts from berries collected in the midsummer (Epifano et al. 2012) or fall (Seltzner and Eddy 2003). The extreme AMF community shift seen in the B2 replication may be due to the emergence of buckthorn seedlings from the added berries, later in the growing season. Buckthorn seedlings were not removed alongside other weeds, as germination is part of the natural progression of fallen fruit. It is unknown whether the majority of germinated seedlings were found in the B2 replication, since notes on which pots contained the newly germinated seedlings were not made at the time of root harvest. There may be a stronger than anticipated root effect in the potted soils. Root exudates may have been actively produced by buckthorn seedlings growing alongside sugar maples, resulting in an extreme shift to *G*177 (OTU4) dominated mycorrhizal communities. Buckthorn's below-ground influences have been observed on three of four tested native forbs after the removal of buckthorn canopy cover in the field, demonstrating that its inhibitory effects within the soil are at least as large as its shading effects (Klionsky et al. 2011). The below-ground root effect diminished in L and LB treatments amended with leaves and/or berries, which warrants further investigation. Starting seedlings from seed in native forest soil and growing for multiple season would have removed potting and greenhouse disturbance variation to better resemble natural conditions. In this way, a buckthorn seedling may be planted beside each maple seedling, and litter bags containing buckthorn leaves and berries may be added to the soil surface to begin the treatment. In this case, care must be taken to fully remove the buckthorn and monitor for any germination of seedlings after the treatment period to prevent the reduction of site quality after the experiment.

The AMV4.5F-AMDGR primers have been shown preferentially to amplify AMF sequences from the Claroideoglomeraceae, Gigasporaceae, and Glomeraceae families, while underrepresenting the Ambisporaceae, Diversisporaceae, and Paraglomeraceae in a primer evaluation using five orchard soil samples (Van Geel et al. 2014). In this study, sequences belonging to the Gigasporaceae were not identified in either experiment, and the highest amplification was seen with Glomeraceae (nearly 98% of all reads), with lower amplification of Claroideoglomeraceae, Paraglomeraceae, Diversisporaceae and Acaulosporaceae. The high proportion of Glomeraceae found in this study can also be partly explained by their natural occurrence within sugar maple forests. Spore analyses on AM populations in three sugar maple forests showed the presence of 8 Glomeraceae spp. (*Glomus hoi, G. macrocarpum, G. aggregatum, G. microaggregatum, Funneliformis mosseae* [≡*G. mosseae*], *Funneliformis geosporum* [≡*G. geosporum*], *Rhizoglomus clarum* [≡*G. clarum*], and *Sclerocystis rubiforme* [≡*G. rubiforme*]), as well as unknown *Glomus* spp., *Acaulospora* spp., and AMF spp. (Moutoglis and Widden 1996). Sequences obtained from the V4 region in this study identified *G. macrocarpum* and *A. lacunosa*, with 76 *Glomus* spp. OTUs, as well as the renamed *Funneliformis mosseae* [≡*G. mosseae*] (Schüßler and Walker 2010), *Rhizoglomus fasciculatus* [≡*G. fasciculatus*] (Schüßler and Walker 2010), *R. vesiculiferus* [≡*Glomus vesiculiferum*] (Redecker et al. 2013; Sieverding et al. 2015), and *Septoglomus constrictum* [≡*Glomus constrictum*] (Oehl et al. 2011).

The transplanting procedure from forest to open-air greenhouse had an immediate effect on sugar maple seedlings, where visible stress, namely yellowing and browning along the leaf edges of the maple seedlings, was seen in the leaves within the first week after planting. Buckthorn plants, despite the root-washing procedure to remove associated soil, showed no visible signs of stress after replanting into pots. The sensitivity of the sugar maple to potting disturbance, as well as the introduction of an invasive plant into its root zone, may greatly reduce the AMF community's resistance to the effects of invasion, as seen in the dominance of *Glomus* spp. after potting. Abrupt disturbances are not common in forests unless through human activity, and sugar maple forest communities involving not just seedlings but their mature counterparts, as well as many other native trees, shrubs, and herbs, have larger sources of soil variation, and with that, better resistance to invasion than a single seedling within a pot. As a result, more field studies on the effects of

buckthorn invasion in forests are needed. Although different VTX were highlighted as associated with buckthorn invasion in both experiments, they were all Glomeraceae, a family with phylogenetic traits that are better suited to disturbed environments (de la Providencia et al. 2005; Hart and Reader 2002; Jansa et al. 2003; Morton and Benny 1990; Öpik et al. 2006; van der Heijden and Scheublin 2007). Seasonal variation had a stronger influence over AMF community changes than the presence or absence of buckthorn, and so this particular invasive may not directly influence mycorrhizal communities as seen with other allelochemically active plants (garlic mustard) (Barto et al. 2011; Cantor et al. 2011; Stinson et al. 2006). The allelochemicals of *R. cathartica* may have a stronger influence on surrounding plants and seedling germination inhibition instead of directly affecting the mycorrhizae (Klionsky et al. 2011; Seltzner and Eddy 2003). Disrupting the root functions of nearby native plants and subsequently affecting their health could lead to a natural shift in AMF communities, where established mycorrhizae associated with undisturbed trees (*S. constrictum, Glomus* spp.) are replaced with disturbance-loving AMF (specifically *Glomus* VTX117 and VTX177) that may not functionally support native trees and forest communities as well as their replaced counterparts. However, mycorrhizal species undergo temporal changes in dormancy naturally throughout the year, and these fluctuations may not indicate an overall loss of diversity or changes in community health. Comparing sequence information from other studies may help to determine whether increases in disturbance-loving AMF negatively affect plant communities.

Subsequent studies may require increasing the number of paired sample locations to reduce the influence of spatial heterogeneity and seasonal variation. Difficulty in finding appropriate paired sites within a sampling region was and will continue to be a limitation for this type of study (see 3.2.1 Soil Sampling). The difficulty lies in finding buckthorn-invaded sites without garlic mustard nearby, which is made unlikely by the affinity of both invasive species for disturbance and their prolific spread once established. Nevertheless, assessing sequence data from a larger number of samples alongside other metadata (soil pH, carbon, nitrogen, phosphates, herb and tree inventories, emodin, and other allelochemical concentrations) may help to tease apart the effects of edaphic factors from those that more directly arise from buckthorn influence. Soil samples collected from buckthorn monoculture sites without the influence of any native trees would yield

information about the final composition of AMF communities after invasion, when all native plants have died. An attempt to sequence soil from buckthorn monocultures was made in this study from five different ESA locations but the extracted DNA from these sites consistently failed to amplify so that sequencing was not possible, possibly due to soil variables acting upon PCR success. Assessment of AMF in multiple buckthorn-dominated stands will assist in determining whether all sites converge to similar mycorrhizal communities or whether mycorrhizae in buckthorn monocultures are more influenced by stochastic processes, resulting in different soil communities across sites. Field observations of buckthorn invasion into plant communities that are dependent on ectomycorrhizal (ECM) or ericoid mycorrhizal fungi would supplement observations made by Pinzone (2016), where seed germination, root infection, and seedling growth of ECM-associated *Betula* species were severely reduced. Comparing sequence information from soils taken in the native Eurasian range of common buckthorn would help determine whether there are any major differences between North American and Eurasian AMF communities. Parallel soil chemistry studies showing the concentration and residence times of buckthorn allelochemicals and other effects of buckthorn on soil fertility and structure are also necessary. With more information, it may be possible to determine the impact of *Rhamnus cathartica* invasion in native forest communities, whether allelochemicals directly influence arbuscular mycorrhizae or directly alter plant communities during invasion. Any information will help with the ever-growing problem of buckthorn management and forest rehabilitation plans across the continent.

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Chapter 4: General Discussion

The primary objective of this thesis was to determine buckthorn's (*Rhamnus cathartica*) interactions and associations with Ontario's native fungal communities. Specifically, I tried to determine what fungi colonize and degrade open-field and closed-canopy buckthorn, the correlations of species within communities of arbuscular mycorrhizal fungi (AMF) in sugar maple (*Acer saccharum*) forests during invasion, and how different sources of buckthorn allelochemicals affect AMF associated with sugar maple seedlings during a growing season. By using a wandering survey of fungi on buckthorn, by sequencing soil fungi from buckthorn-invaded and -uninvaded forests, and from a greenhouse experiment in which maple seedlings were grown with and without added buckthorns, their leaves and fruit, the nature of buckthorn's interaction with fungi in this region was illuminated.

4.1 Context and Significance of This Study

Prior to this survey, 30 fungal species had been documented on buckthorn across the globe, of which just five species had been reported in Canada and ten in the United States (Conners 1967; Farr and Rossman; Ginns 1986; Liu and Hambleton 2013). After a 20-month survey of buckthorn in and around London, Ontario, I have added 23 fungi to Canadian records, including the root rotter *Armillaria mellea* s.l., the canker fungi *Hypoxylon fuscum* and *H. perforatum*, the weak branch pathogens *Nectria cinnabarina* s.l. and *Cylindrobasidium evolvens*, as well as multiple primary and secondary decomposers (Chapter 2, Figure 2.2). Fungi were rarely found on healthy buckthorn trees in open-field environments, whereas sites undergoing management practices (chemical spraying and mechanical removal) had the highest occurrences of fungal colonization on dead or dying buckthorn matter. This suggests that active physical or chemical management will be necessary to control open-grown buckthorns. In their study surveying forest stands in southern Italy, Granito et al. (2015) confirmed that some fungi were preferentially found in actively managed plots, namely those with consistent sources of disturbance that resulted in increased coarse woody debris. *Armillaria mellea*, *C. evolvens*, *H. fuscum*, and *P. crispa* were all found in managed plots, whereas *D. mollis*, and *S. commune* were seen in old-growth plots and/or mature plots. In my study, managed plots consistently harboured more

fungi than unmanaged plots, and certain species were found only in managed plots, such as *A. malicola, A. mellea, C. calolepis, C. caspari, H. fuscum, L. virgineum, M. meliigena,* and *P. crispa* in Sifton Bog ESA during ongoing buckthorn management, and *D. concentrica, D. mollis, S. ochraceum* in managed Five Points Forest. Management and the subsequent accumulation of coarse woody debris increases the chance encounters for necrotroph/parasitic and saprobic fungi. *Armillaria* is known to occur more frequently in highly managed zones (McDonald et al. 1987), where it may survive in residual debris after clearing (Redfern and Filip 1991) by chemical or mechanical means (Pronos and Patton 1977; Swift 1972). *Plicatura crispa* was seen on a dying open-canopy buckthorn outside of the survey period in Westminster Ponds ESA (personal observation), which confirms that these fungi can occasionally be found on buckthorn beyond actively human-mediated zones. The higher occurrence of opportunistic and pioneer saprobes can be expected in any region that is undergoing management. Any increase in fungal activity will assist in the degradation of cleared wood, but might also lead to higher infection pressures on native plants growing in the area. Because of this, after successful invasive species management, planting seeds or saplings of native species with associated mycorrhizae may be required to stabilize the soil communities and promote rehabilitation (Cuenca et al. 1997; Medina and Azcón 2010; Mendes Filho et al. 2010).

The second research objective was to compare differences in AMF communities pristine sugar maple soils and those being invaded by buckthorn. Twenty-four OTUs belonging to *Acaulospora* sp., *Paraglomus* spp., *Claroideoglomus* spp. and *Glomus* spp. were seen only in pristine sites and a moderate increase in relative abundance of *Glomus* VTX177 and *Septoglomus constrictum* VTX64 was seen in disturbed sites. Trends in AMF activities in response to seasonal changes is not a ubiquitous phenomenon, having been observed in studies from different ecosystems from deserts (Panwar and Tarafdar 2006) to dunes (Stürmer and Bellei 1994), temperate grasslands (Escudero and Mendoza 2005), as well as greenhouse soils (Liu et al. 2013), but not in a mature mixed forest in Estonia (Davison et al. 2012). Genus-level patterns in seasonal variation were observed in this study with higher relative abundances of *Claroideoglomus* spp. and *Glomus* spp. in summer, contrasted with higher relative abundances of *Diversispora* spp., *R. fasciculatus* and other *Glomus* spp. in the fall, depending on the location and despite buckthorn

encroachment. This partly agrees with a study of AMF spore abundance in a grassland in Inner Mongolia, where *Funneliformis caledonium* was significantly higher in spring, while *Claroideoglomus etunicatum, R. fasciculatus* and *Glomus warcupii* were abundant in the fall (Sun et al. 2013). *Glomus* remains a phylogenetically diverse, poorly-defined genus (Schwarzott et al. 2001) and contains species that are active at different times of the year. In soils collected from Grasslands National Park, Saskatchewan, *Glomus viscosum, G. mosseae*, and *G. hoi* were better suited to moist July soils, and were replaced by three unknown *Glomus* species during the warm and dry conditions of late August (Yang et al. 2010). Sequences belonging to *Rhizoglomus* spp., *Glomus* spp., and *Septoglomus constrictum* were positively correlated with treatments, an expected result as *Septoglomus*, and more recently *Rhizoglomus*, were delineated from *Glomus* (Oehl et al. 2011; Redecker et al. 2013; Sieverding et al. 2015). Functional similarity among closely related species in arbuscular mycorrhizae has been seen when growing *Plantago lanceolata* with Glomeraceae and Gigasporaceae mycorrhizae (Maherali and Klironomos 2007; van der Heijden and Scheublin 2007). The complementary nature of Glomeraceae and Gigasporaceae (higher fungal mass allocation outside vs. within the root, respectively) led to increases in biomass of *P. lanceolata*, whereas the plant grown with only one or the other did not change in biomass (Maherali and Klironomos 2007).

The second research objective also included a common-garden experiment to determine whether the addition of buckthorn leachates and root exudates to naïve potted sugar maple seedlings and associated AMF would change the proportions of mycorrhizal communities in comparison to field seedlings and potted controls. *Glomus* VTX177 responded favourably to disturbance (potting, buckthorn allelochemicals, or both) and was recorded in higher relative abundance in all treatment samples and potted controls than in the field. In contrast, *G*151, *G*125, *G*222, and *G*72 were consistently more proportionally abundant in potted controls across both replicates, with *G*151 and *G*72 demonstrating sensitivity to potting disturbance and being found mostly in field control sugar maple roots. Disturbances due to potting soil for greenhouse studies has been shown to cause shifts in mycorrhizal communities (Hazard et al. 2013; Sýkorová et al. 2007). A comparison of AMF ITS sequences from greenhouse pots using bait plants and field soil showed similar patterns: *Glomus mosseae* was never detected in the field samples but increased to 25% in

bait plants (grown in field sites) and 50% in greenhouse compartments, whereas *Glomus badium* was never found in greenhouse soils, only in small amounts in association with bait plants, and highest in undisturbed field soils (Sýkorová et al. 2007). However, *G. mosseae* disappeared from the greenhouse soils over time, possibly due to fungal successional dynamics, characterizing it as an early-stage colonizer. The greenhouse study described in this thesis spanned 5 months in comparison to the 20-month study by Sýkorová et al. (2007), and so changes in relative abundances beyond the single growing season could not have been observed. Other AMF phylotypes tended to occur in cultivated or natural environments: *Funneliformis constrictum*, and sister groups *Claroideoglomus luteum* and *C. etunicatum* showed growth preferences of early successional colonizers. *Rhizophagus intraradices*, similar to both sequencing studies in Chapter 2, was the most frequently detected in all systems and had growth patterns of a generalist mycorrhiza (Sýkorová et al. 2007). As *G*177 increased in proportion across treatments, a decrease in other *Glomus* VTX were observed. Explaining the source of the shift in AMF abundance is difficult as multiple possibilities may have occurred: 1) *G*177 had a competitive advantage over other mycorrhizae during buckthorn disturbance, resulting in increased allocation of sugars from the roots of the sugar maple, 2) the other 9 *Glomus* species were more negatively affected by disturbance/buckthorn, leaving behind vacant niche space for *G*177 to expand into, or 3) the use of relative abundances in sequencing studies to monitor changes in taxa may result in data that incorrectly implicate a decrease in one taxon or taxa because of an observed proportional increase of another. Because only sugar maple roots were analysed, an increase in *G*177 after potting and treatment disturbance indicates that this specific OTU may have been more beneficial to the native plant during times of stress.

4.2 Study Limitations and Future Directions

Buckthorn surveys in other regions of Canada, especially in moist and northern areas, may add to the list of associated fungi, and sequencing specimens within species complexes (*Armillaria mellea*, *Nectria cinnabarina*, and *Antrodia malicola*) would improve on the identification of these collections.

Another exotic invasive plant spreading across Ontario is *Vincetoxicum rossicum* (dog-strangling vine), which is highly dependent upon mycorrhizal connections in both invaded and native Eurasian ranges. A greenhouse soil experiment compared paired sites with no record of invasion or decades of invasion over the course of a single growing season of 29 weeks (Day et al. 2015). The authors found that fungal composition in greenhouse soils did not mirror those of invaded field soils after the study period, most likely due to the generalist nature of dog-strangling vine. Less is known about buckthorn's dependency on AMF in native ranges, and obtaining sequence data for AMF communities in buckthorn monoculture soils would clarify the structure of communities after long periods of invasion, specifically whether stochastic processes and low host specificity lead to different community compositions across sampling sites or whether specific AMF are better suited to buckthorn invasion. Similarly, it would be interesting to sequence AMF directly from buckthorn roots to see if they yield the same or different taxa associating with buckthorn in each invasion scenario. If *G*177 was also found in high abundances in roots of both maple and buckthorn, there is a possibility for lateral nutrient transfer between plants, and further studies may be able to determine in which direction it may occur. Environmental metadata, including soil pH, C, N, P, plant inventories, and allelochemical quantification would have helped to explain some of variation present in the datasets (Hazard et al. 2013; Yang et al. 2012). Furthermore, additional replications in the greenhouse experiment would have allowed for the separate statistical comparison of field and potted controls, as both were pooled in this study to allow for adequate statistical power. Future studies focusing on the invasion of buckthorn into forests dominated by ectomycorrhizal or ericoid mycorrhizal associations would improve our understanding of its invasional biology (Pinzone 2016). It would be interesting to determine whether buckthorn requires AMF to proliferate in these soils and successfully invade, or whether it utilizes other means to compete with native communities such as alteration of soil nutrient cycling, high light competition, increased relative growth rate after canopy openings, and reduced herbivory due to allelochemicals (Catling and Mitrow 2012).

The advancement of sequencing technology as well as reference databases for AMF will be an integral part of building upon the data presented. Many of the sequences obtained from this study are from unnamed species belonging to *Glomus*, a genus that contains

"species of uncertain position" (Redecker et al. 2013). This impediment will have to be addressed in multiple ways, including the re-evaluation of phylogenetic relationships among members within the Glomeromycota as more reference specimens are discovered and sequenced, the improvement of DNA amplification and sequencing technologies to reduce error rates, improvements in the reproducibility of direct amplification from soil, increased sequencing length to capture larger and more diagnostic fragments of the genome, and continually refining bioinformatics analytical methods to cluster and compare sequence data. The cryptic nature of mycorrhizae and the difficulties culturing AMF makes studying these organisms challenging, as non-congruencies of morphological and molecular characters are observed (Redecker et al. 2013), and sequencing of genetically heterogeneous multinucleate organisms leads to problems equating read count numbers to biomass and abundance (Corradi et al. 2007; Schlaeppi et al. 2016). Increasing the ability of sequencing technology to compare longer sections of the mycorrhizal genome (e.g., ~1550 bp of rDNA) will allow for the better identification and phylogenetic placement of OTUs (Krüger et al. 2009). This has been accomplished through the advent of a new single molecule real time (SMRT) methodology that was able to sequence a *ca*. 1.5 kbp fragment spanning the SSU-ITS-LSU region (Schlaeppi et al. 2016). The ability to increase the phylogenetic resolution in metagenomic studies will continue to improve as SMRT sequencing, its successor the 'Sequel' system, and others become more broadly available (Schlaeppi et al. 2016).

4.3 Integrating Management And Restoration Plans

By learning about the ways in which buckthorn changes the environment after a large-scale invasion, it will be possible to mitigate the loss of resources and time during the rehabilitation process. The potential to formulate stump sprays or foliar applications of parasitic fungal spores for buckthorn management hinges on the discovery of associated fungi, and from there identifying isolates that are effective in killing weakened trees. Similarly, any legacy effects that remain in soils after buckthorn removal may have to be reversed through the addition of mycorrhizal inoculum to the root zone of native seedlings prior to restoration (Cuenca et al. 1997; Rowe et al. 2007). This may help decrease seedling mortality and also provide the foundation to engineer heterogeneous environments of fungi

that contribute to the maintenance of plant diversity well into the future. Continuing research on the biology of buckthorn invasion and building upon the current standard of chemical and mechanical removal would provide tremendous benefit to the many organizations across North America tasked to eradicate this noxious weed.

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Appendices

Appendix I. Sample collection and identification information (Chapter 2). Green/underlined names are samples that were identified through microscopy, the remainder were visually identified based on morphological characteristics**.**

ID Number	Date collected	Location	UTM Coordinates (Zone 17T)	Latin Name & Authority	Description	Location Notes	ID Notes
NMW 130609/01	6/9/2014	*rare* Research Reserve	552191.45mE 4803346.61mN	*Rhamnus cathartica voucher (male)*	Male flowering *Rhamnus cathartica* voucher	Open field	Det: NMW
NMW 130925/01	9/25/2013	AFAR Trail	477085.79mE 4761818.65mN	*Nectria cinnabarina* (Tode) Fr.	Salmon pink, raised fruiting bodies Smooth surface on fine twigs	Canopied	Det: RGT
NMW 131023/01a	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Armillaria mellea* (Vahl) P. Kumm. group rhizomorphs	Rhizomorphs of fruiting body found on dead trunk of *R. cathartica* across the trail from another *R. cathartica* tree with aborted fruiting body	Canopied	Det: RGT and NMW

					(NMW-0001b-2013)		
NMW 131023/01b	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Armillaria mellea* (Vahl) P. Kumm. group aborted fruiting bodies	Dark, cream colour with light patches found at base of *R. cathartica* (dead, sprayed) Moist, rubbery before drying, no visible gills, misshapen	Canopied	Honey mushroom aborted fruiting body
NMW 131023/04	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Mycena meliigena* (Berk. & Cooke) Sacc.	Small, beige fruiting body, dried out on trunk of *R. cathartica* 1.5mm height, 1 mm width	Canopied	RGT Visual ID
NMW 131023/05	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Crepidotus calolepis* (Fr.) P. Karst	Bracket gilled fungi, yellow-brown	Canopied	Det: NMW and RGT
NMW 131023/06	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Nectria cinnabarina* (Tode) Fr.	Salmon pink, raised fruiting bodies Smooth surface on fine twigs	Canopied	Visual ID based on NMW 131023/06

NMW 131023/08	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Peniophora cinerea* (Pers.) Cooke	Gray-pink, powdery, amorphous	Canopied	ID: RGT
NMW 131023/09	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Irpex lacteus* (Fr.) Fr.	Old fruiting body, yellow, elongated pores/toothy, not resupinate white when young, almost toothy, resupinate Ranges from 1 mm to 10 mm wide, amorphic shape	Canopied	Det: NMW
NMW 131023/11	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Merismodes fasciculata* (Schwein.) Donk	Beige fruiting bodies, clusters between cracks of bark	Canopied	Det: RGT
NMW 131023/12	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Hyphoderma* cf. *mutatum* (Peck) Donk	White powder, creamy raised center, dry and cracked Spore shape is wrong, but looks similar under dissecting microscope	Open field	Det: RGT

NMW 131023/13	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Cylindrobasidium evolvens* (Fr.) Jülich	Gray purple central body, resupinate Fluffy white fringe *R. cathartica* brush pile	Canopied	Clumped non-amyloid basidiospores Leptocystidia (smooth, fusiform)
NMW 131023/15	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Nectria cinnabarina* (Tode) Fr.	Salmon pink, raised fruiting bodies Smooth surface on fine twigs	Canopied	Visual ID based on NMW 131023/06
NMW 131023/16	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Plicatura crispa* (Fr.) Rea	Bracket fungi, brown to light-brown underside, gilled surface of branch, 5 mm long, 2-3 mm tall	Canopied	Det: NMW and RGT
NMW 131023/17	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Peniophora incarnata* (Pers.) P. Karst.	Orange-pink-brown amorphic crust	Canopied	Visual ID based on NMW 141127/09
NMW 131023/19	10/23/2013	Sifton Bog ESA	476988.10mE 4761930.75mN	*Lachnum virgineum* (Batsch) P. Karst.	Small white cups, 1 mm diameter	Canopied	Det: NMW and RGT
NMW 140527/01	5/27/2014	Precious Blood Field behind	476804.76mE 4761257.87mN	*Rhamnus cathartica* L.	Buckthorn voucher for June 2014 samples	Open field	Det: NMW

		Brescia College					
NMW 140527/02	5/27/2014	Precious Blood Field behind Brescia College	476773.57mE 4761208.31mN	*Puccinia coronata* Corda	Voucher of *P. coronata* on *R. cathartica*	Open field and canopied	Det: NMW and RGT
NMW 140527/03	5/27/2014	Medway Valley ESA	476649.92mE 4761462.29mN	*Hypoxylon perforatum* (Schwein.) Fr.	Brown mounds in mature, dying *R. cathartica* tree near the entrance to Medway Valley interior trails	Canopied	Det: NMW
NMW 140605/01	6/5/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Irpex lacteus* (Fr.) Fr.	See description for NMW 131023/09	Forest edge	Visual ID based on NMW 131023/06
NMW 140605/03	6/5/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Peniophora cinerea* (Pers.) Cooke	Purple/gray crust	Canopied	Visual ID on NMW 131023-08
NMW 140605/04	6/5/2014	Sifton Bog ESA	473600.19mE 4757301.29mN	*Irpex lacteus* (Fr.) Fr.	See description for NMW 131023/09	Canopied	Visual ID based on NMW 131023/06
NMW 140620/01	6/20/2014	Warbler Woods ESA	471258.72mE 4756433.11mN	*Hyphoderma* cf. *mutatum* (Peck) Donk	White crust, cracked, dried, yellow to white with central	Canopied	Visual ID based on NMW 131023/12

					mound, amorphic		
NMW 140620/02	6/20/2014	Warbler Woods ESA	471258.72mE 4756433.11mN	*Merismodes fasciculata* (Schwein.) Donk	Beige fruiting bodies, clusters between cracks of bark	Canopied	Visual ID based on NMW 131032/11
NMW 140620/03	6/20/2014	Warbler Woods ESA	471258.72mE 4756433.11mN	*Hyphoderma* cf. *mutatum* (Peck) Donk	White crust, cracked, dried, yellow to white with central mound, amorphic	Canopied	Visual ID based on NMW 131023/12
NMW 141023/01	10/23/2014	*rare* Research Reserve	551291.02mE 4802734.24mN	*Tubercularia* sp. J.C. Carter	Salmon pink, raised fruiting bodies Smooth surface on fine twigs	Canopied	Visual ID based on NMW 131023/06
NMW 141023/03	10/23/2014	*rare* Research Reserve	551291.02mE 4802733.98mN	*Irpex lacteus* (Fr.) Fr.	See description for NMW 131023/09	Canopied	Visual ID based on NMW 141023/03
NMW 141023/06	10/23/2014	*rare* Research Reserve	551291.02mE 4802733.98mN	*Hyphoderma* cf. *mutatum* (Peck) Donk	White crust, cracked, dried, yellow to white with central mound, amorphic	Canopied	Visual ID based on NMW 131023/12

NMW 141028/01	10/28/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Schizophyllum commune* Fr.	*R. cathartica* banch on ground	Canopied brush pile	Det: NMW
NMW 141028/03	10/28/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Hypoxylon fuscum* (Pers.) Fr.	Brown mounds on large dying buckthorn underneath peeled bark	Canopied	Det: NMW
NMW 141028/04	10/28/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Antrodia malicola* (Berk. & M.A. Curtis) Donk	Bracket polypore on roots of dead *R. cathartica* (same tree as NMW 141028/05) Brown rot	Canopied	Det: NMW and RGT
NMW 141028/05	10/28/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Armillaria mellea* (Vahl) P. Kumm. group	Fruiting body; *Armillaria mellea* group	Canopied	Det: NMW
NMW 141028/07	10/28/2014	Sifton Bog ESA	473718.92mE 4757959.11mN	*Crepidotus caspari* Velen.	White bracket, beige gills, small	Canopied	Final taxonomy determined by spore shape and size
NMW 141127/01	11/27/2014	Killaly Meadows	482984.61mE 4765179.20mN	*Peniophora incarnata* (Pers.) P. Karst.	Bright-pale orange crust on undersde of branch	On open field buckthorn by parking lot	Visual ID based on NMW 141127/09

NMW 141127/02	11/27/2014	Killaly Meadows	482984.61mE 4765179.20mN	*Hyphoderma* cf. *mutatum* (Peck) Donk	White crust	On open field buckthorn by parking lot	Visual ID based on NMW 131023/12
NMW 141127/05	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.90mN	*Cylindrobasidium evolvens* (Fr.) Jülich	Crust, purple-gray	Canopied	RGT Visual ID
NMW 141127/06	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.95mN	*Daldinia concentrica* (Bolton) Ces. & De Not.	In *R. cathartica* brush pile	Open field brush pile	Det: RGT in field
NMW 141127/09	11/27/2014	Five Points Driedger	505337.91mE 4761948.95mN	*Peniophora incarnata* (Pers.) P. Karst.	Bright-pale orange crust on undersde of branch	Open field brush pile	Det: NMW and RGT
NMW 141127/11	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.95mN	*Steccherinum ochraceum* (Pers.) Gray	*R. cathartica* brush pile	Open field brush pile	Visual IDs on NMW 131023-13
NMW 141127/12	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.95mN	*Cylindrobasidium evolvens* (Fr.) Jülich	See description for NMW 141130/02	Open field brush pile	RGT Visual ID
NMW 141127/13	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.95mN	*Datronia mollis* (Sommerf.) Donk	Polypore, white	Open field brush pile	Det: RGT
NMW 141127/14	11/27/2014	Five Points Driedger Tract	505337.91mE 4761948.95mN	*Cylindrobasidium evolvens* (Fr.) Jülich	See description for NMW 141130/02	Open field brush pile	Det: NMW and RGT

NMW 141130/01	11/30/2014	Westminster ponds ESA	481962.44mE 4755073.24mN	*Phlebia radiata* Fr.	Resupinate grey-purple jelly-like fungus on *R. cathartica* branch pile	Canopied brush pile	Visual IDs on NMW 131023-13
NMW 141130/02	11/30/2014	Westminster ponds ESA	481962.44mE 4755073.24mN	*Cylindrobasidium evolvens* (Fr.) Jülich	See description for NMW 141130/02	Canopied	Visual ID based on NMW 131023/06
NMW 141130/03	11/30/2014	Westminster ponds ESA	481962.44mE 4755073.24mN	*Nectria cinnabarina* (Tode) Fr.	Coral spot fungus	Canopied	Visual ID on NMW 131023-08
NMW 141130/04	11/30/2014	Westminster ponds ESA	481962.44mE 4755073.24mN	*Schizophyllum commune Fr.*	*R. cathartica* brush pile		
RGT 141226/06	12/26/2014	Medway Valley ESA	476667.63mE 4761876.21mN	*Peniophora cinerea* (Pers.) Cooke	Gray-pink, powdery, amorphous	Canopied	RGT Visual ID
NMW 150309/01	3/18/2015	AFAR Trail	476988.10mE 4761930.75mN	*Polyporus alveolaris* (DC.) Bondartsev & Singer	AKA *Neofavolus alveolaris*	Canopied	Det: NMW and RGT
NMW 150309/04	3/18/2015	AFAR Trail	476988.10mE 4761930.75mN	*Morrisographium persicae* (Schwein.) Illman & G.P. White	Spear like projections from bark, black	Canopied	Det: NMW

Appendix II. Primer and tag information for Illumina MiSeq sequencing using AMF-specific primers spanning the V4 SSU (Chapter 3, Objective 1).

Primer label	Primer name_label	Illumina adaptor	Linker	Barcode	Primer sequence (Sato et al. 2005)	Total length
Forward						
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8F	AMV4.5N_8F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT	nnnn	aagatggt	AAG CTC GTA GTT GAA TTT CG	65
9F	AMV4.5N_9F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT	nnnn	agttaacc	AAG CTC GTA GTT GAA TTT CG	65
10F	AMV4.5N_10F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT	nnnn	cttcctgg	AAG CTC GTA GTT GAA TTT CG	65
11F	AMV4.5N_11F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT	nnnn	gcttgatg	AAG CTC GTA GTT GAA TTT CG	65
12F	AMV4.5N_12F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT	nnnn	tgacttca	AAG CTC GTA GTT GAA TTT CG	65
Reverse						
5R	AMDG_5R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	agttaacc	CCC AAC TAT CCC TAT TAA TCA T	71
6R	AMDG_6R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	ccaaggcc	CCC AAC TAT CCC TAT TAA TCA T	71
7R	AMDG_7R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	ataacgaa	CCC AAC TAT CCC TAT TAA TCA T	71
8R	AMDG_8R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	tgacttca	CCC AAC TAT CCC TAT TAA TCA T	71
9R	AMDG_9R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	agccacac	CCC AAC TAT CCC TAT TAA TCA T	71
10R	AMDG_10R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	caggctta	CCC AAC TAT CCC TAT TAA TCA T	71

11R	AMDG_11R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	cttcctgg	CCC AAC TAT CCC TAT TAA TCA T	71
12R	AMDG_12R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	gactaatc	CCC AAC TAT CCC TAT TAA TCA T	71
13R	AMDG_13R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	gcttgatg	CCC AAC TAT CCC TAT TAA TCA T	71
14R	AMDG_14R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	tggcggct	CCC AAC TAT CCC TAT TAA TCA T	71
15R	AMDG_15R	CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T	nnnn	gtaagcgc	CCC AAC TAT CCC TAT TAA TCA T	71
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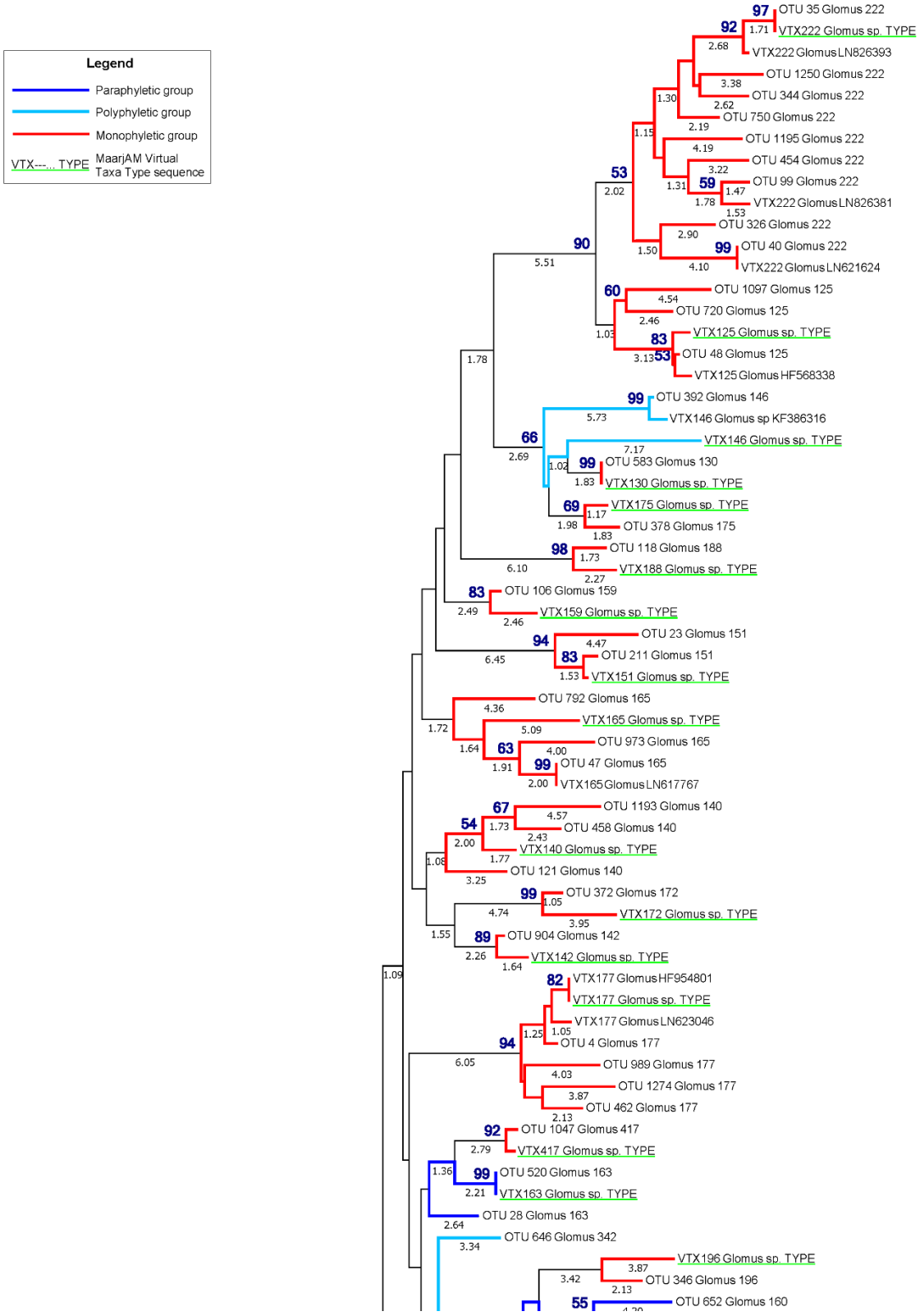
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Monophyletic group
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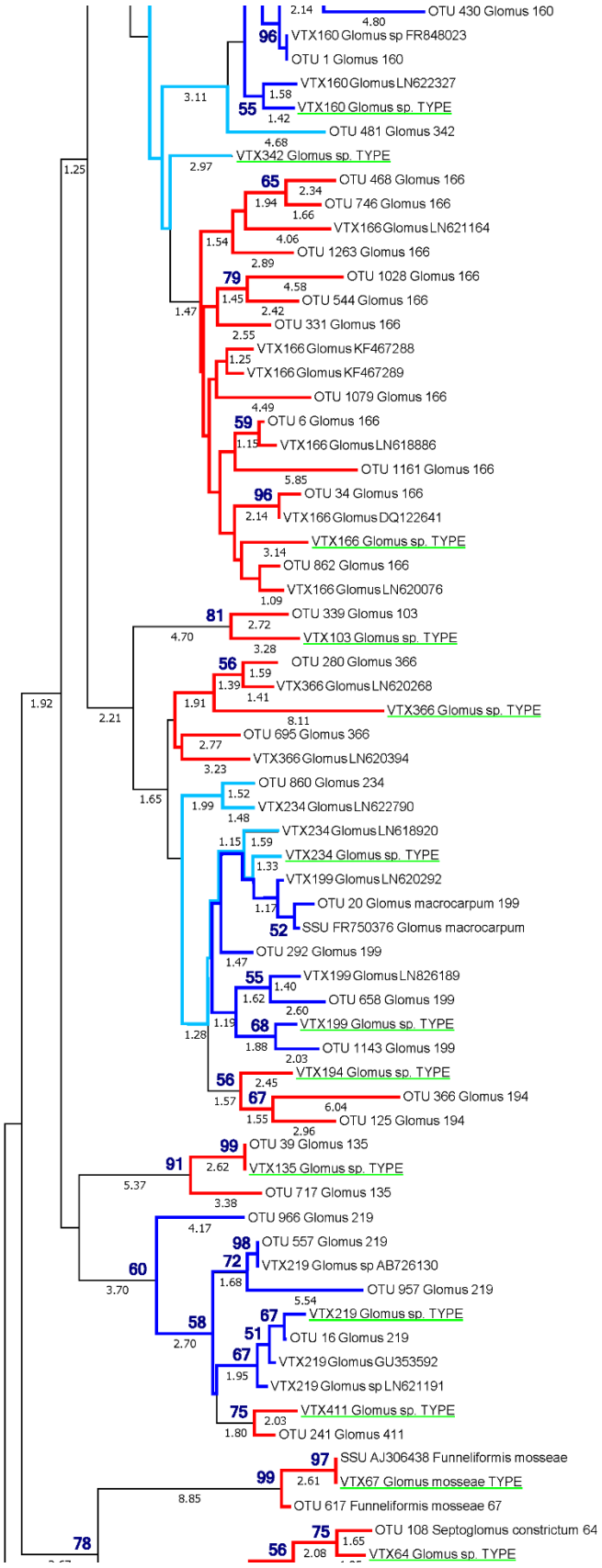
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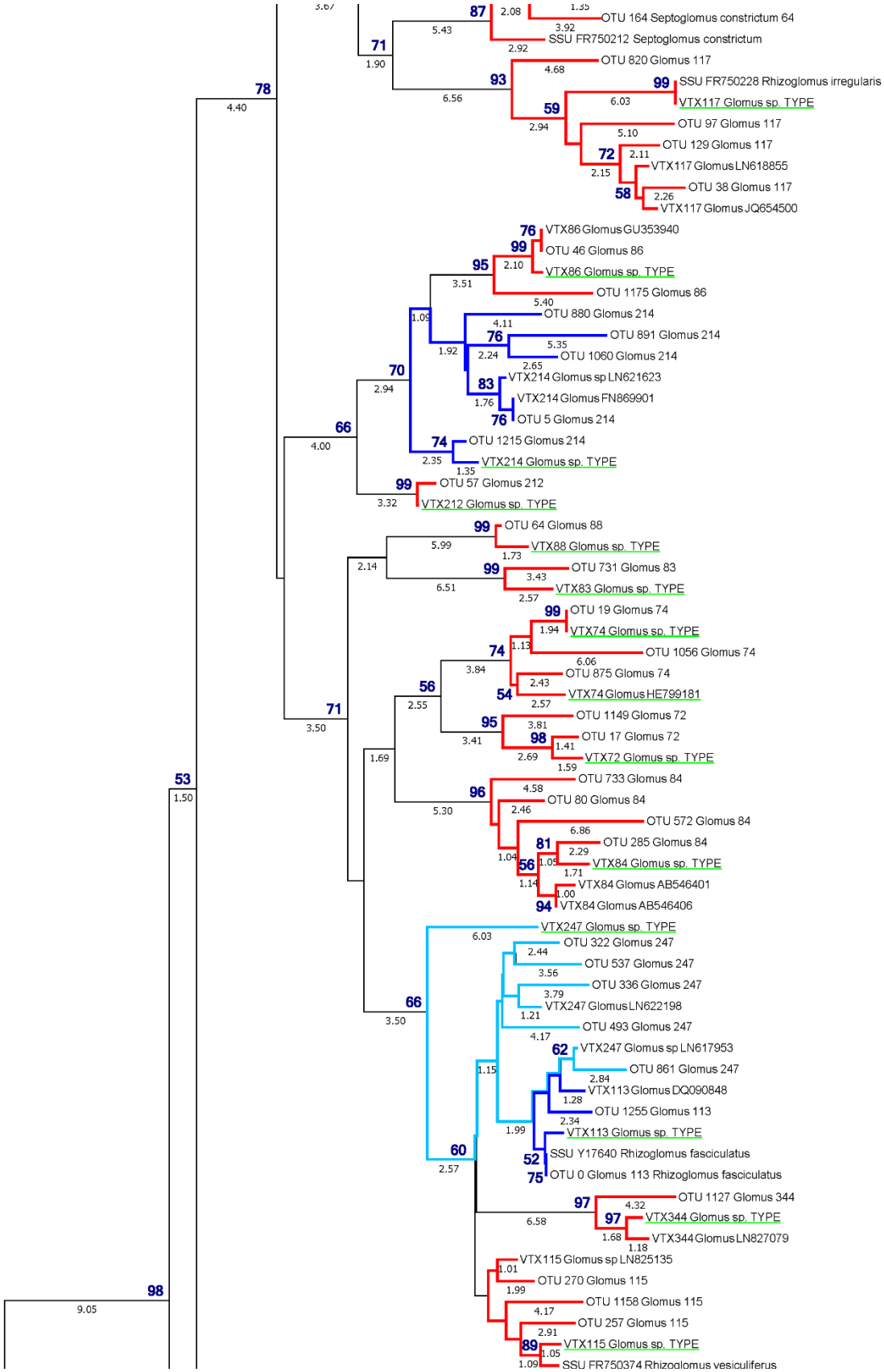
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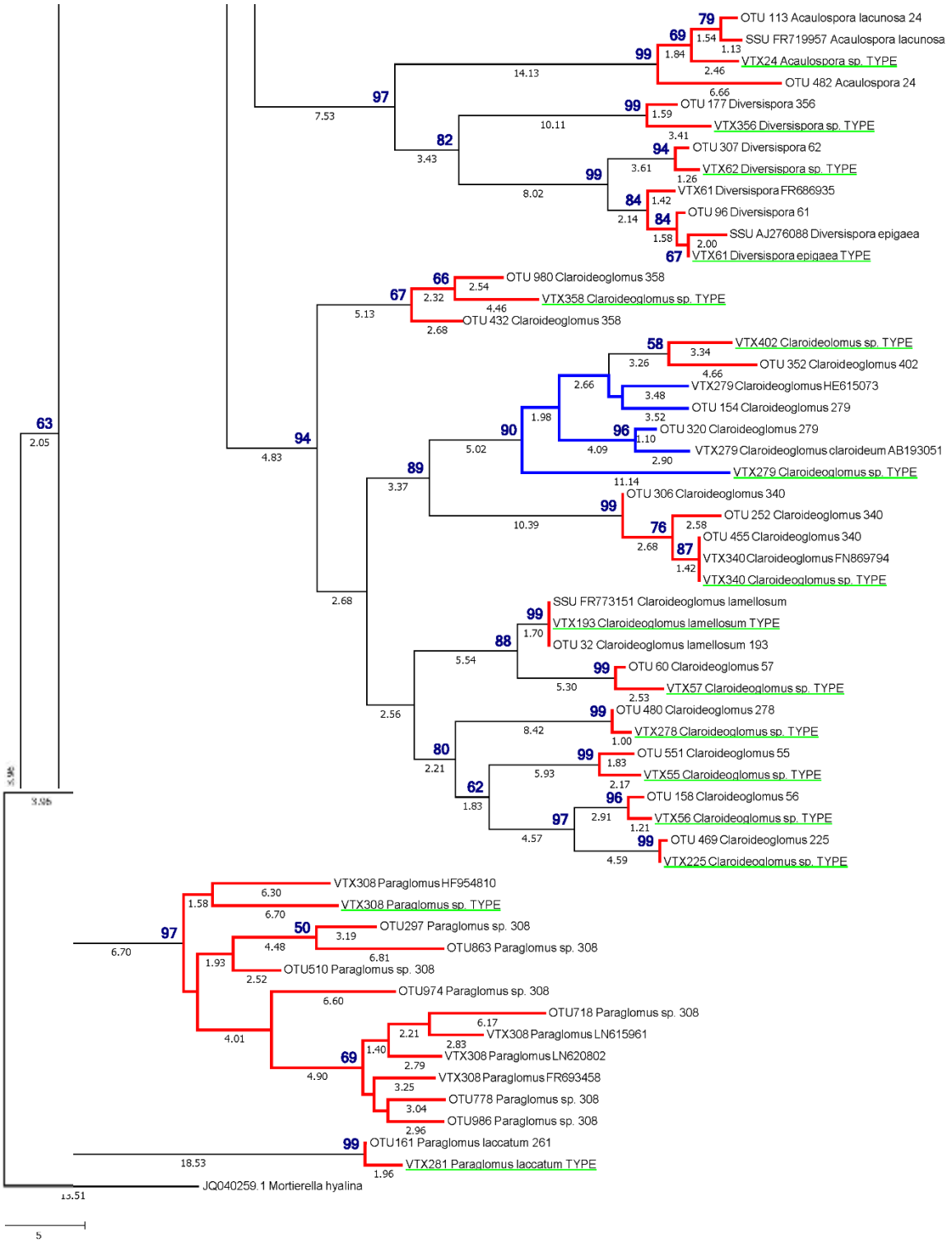
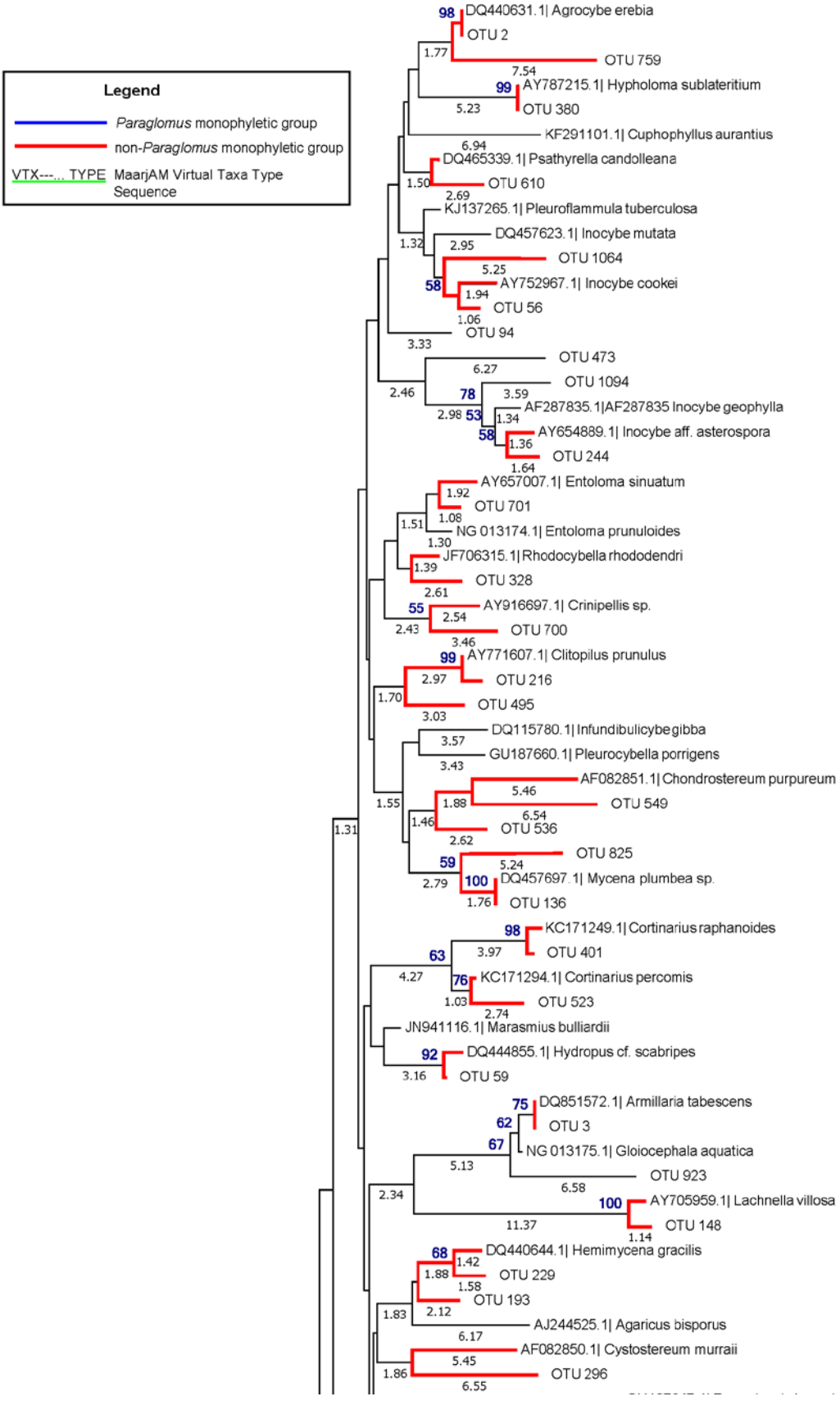


Appendix III. Molecular phylogenetic analysis by Maximum Likelihood methods of unknown OTUs and reference sequences from NCBI, Krüger et al. (2012), and MaarjAM databases (Öpik et al. 2010) and using *Mortierella hyalina* JQ40259.1 as an

outgroup. Initial trees were obtained using the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maxiumum Composite Likelihood (MCL) approach (bootstrap values of 1000 replicates) (Felsensetein 1985). Values that are > 50% are highlighted in bold. Branch lengths are measures in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Terminal groups connected by navy blue lines are paraphyletic, those connected by a light blue line are polyphyletic, and those connected by a red line are monophyletic. MaarjAM Virtual Taxa type sequences are underlined in green. OTUs are listed with the OTU number, followed by genus and VTX of closest MaarjAM BLAST match. Evolutionary analysis was conducted in MEGA7 (Kumar et al. 2016).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DQ465339.1| Psathyrella candolleana
OTU 610
KJ137265.1| Pleuroflammula tuberculosa
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OTU 56
OTU 94
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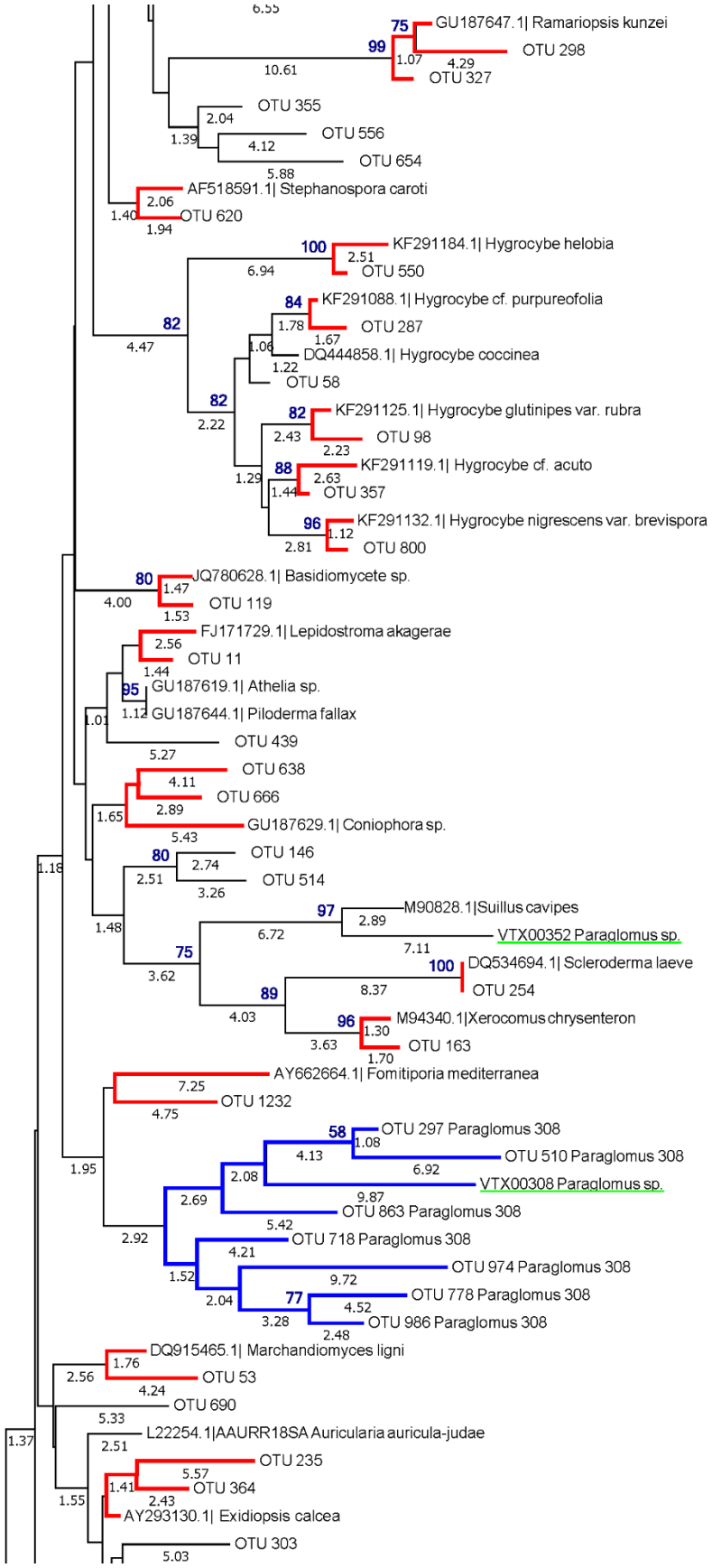
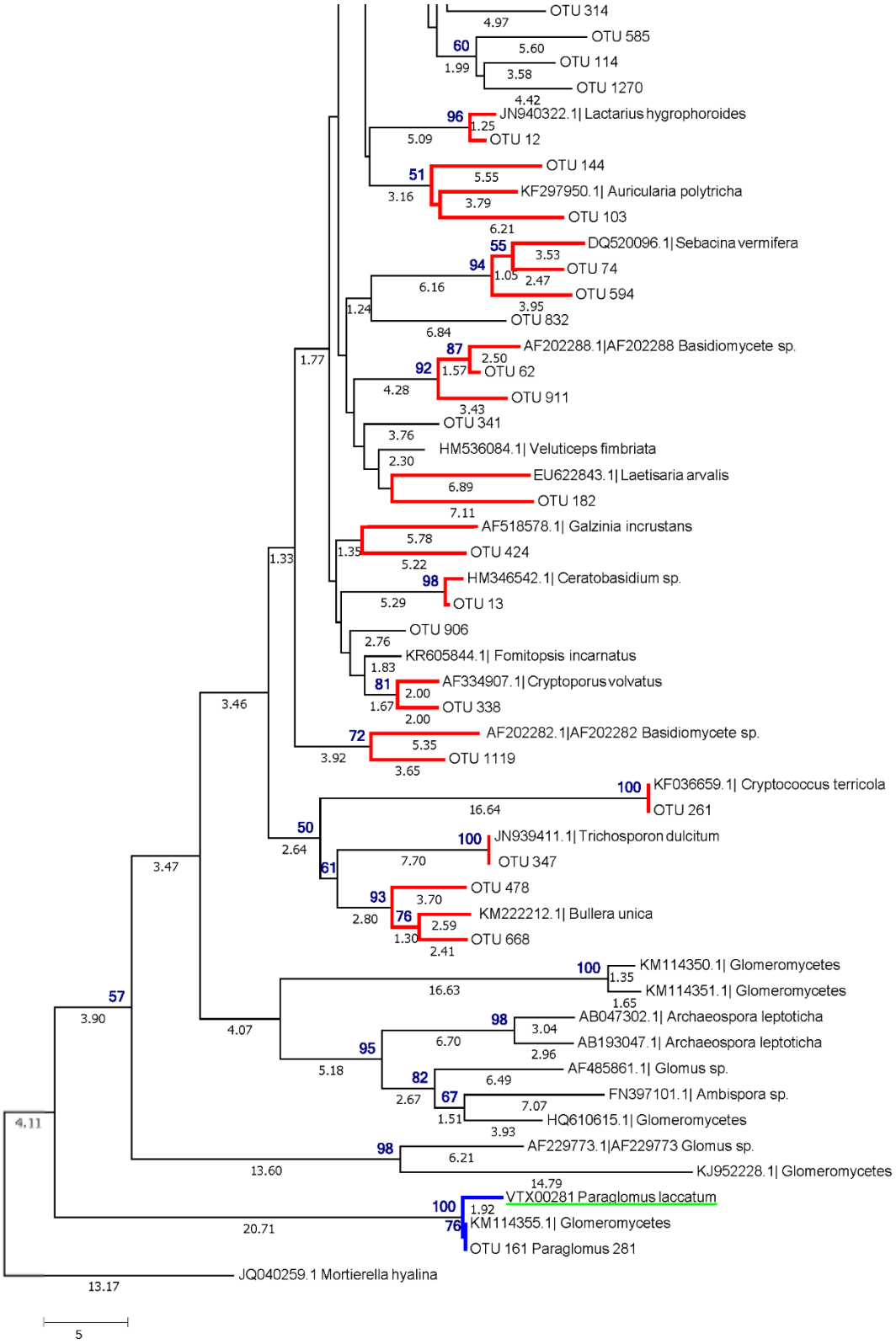
AY657007.1| Entoloma sinuatum
OTU 701
NG 013174.1| Entoloma prunuloides
JF706315.1| Rhodocybella rhododendri
OTU 328
AY916697.1| Crinipellis sp.
OTU 700
AY771607.1| Clitopilus prunulus
OTU 216
OTU 495
DQ115780.1| Infundibulicybe gibba
GU187660.1| Pleurocybella porrigens
AF082851.1| Chondrostereum purpureum
OTU 549
OTU 536
OTU 825
DQ457697.1| Mycena plumbea sp.
OTU 136
KC171249.1| Cortinarius raphanoides
OTU 401
KC171294.1| Cortinarius percomis
OTU 523
JN941116.1| Marasmius bulliardii
DQ444855.1| Hydropus cf. scabripes
OTU 59
DQ851572.1| Armillaria tabescens
OTU 3
NG 013175.1| Gloiocephala aquatica
OTU 923
AY705959.1| Lachnella villosa
OTU 148
DQ440644.1| Hemimycena gracilis
OTU 229
OTU 193
AJ244525.1| Agaricus bisporus
AF082850.1| Cystostereum murraii
OTU 296


6.55
75
GU187647.1| Ramariopsis kunzei
99
OTU 298
1.07
4.29
10.61
OTU 327
OTU 355
2.04
1.39
OTU 556
4.12
OTU 654
5.88
AF518591.1| Stephanospora caroti
2.06
1.40
OTU 620
1.94
100
KF291184.1| Hygrocybe helobia
2.51
6.94
OTU 550
84
KF291088.1| Hygrocybe cf. purpureofolia
82
1.78
OTU 287
4.47
1.67
1.06
DQ444858.1| Hygrocybe coccinea
1.22
OTU 58
82
2.22
82
KF291125.1| Hygrocybe glutinipes var. rubra
2.43
OTU 98
2.23
88
KF291119.1| Hygrocybe cf. acuto
2.63
1.29
1.44
OTU 357
96
KF291132.1| Hygrocybe nigrescens var. brevispora
1.12
2.81
OTU 800
80
JQ780628.1| Basidiomycete sp.
1.47
4.00
OTU 119
1.53
FJ171729.1| Lepidostroma akagerae
2.56
OTU 11
1.44
95
GU187619.1| Athelia sp.
1.12
GU187644.1| Piloderma fallax
1.01
OTU 439
5.27
OTU 638
4.11
OTU 666
2.89
1.65
GU187629.1| Coniophora sp.
5.43
1.18
80
OTU 146
2.74
2.51
OTU 514
3.26
97
M90828.1|Suillus cavipes
2.89
1.48
6.72
VTX00352 Paraglomus sp.
7.11
75
3.62
100
DQ534694.1| Scleroderma laeve
8.37
OTU 254
89
4.03
96
M94340.1|Xerocomus chrysenteron
1.30
3.63
OTU 163
1.70
AY662664.1| Fomitiporia mediterranea
7.25
OTU 1232
4.75
58
OTU 297 Paraglomus 308
1.08
4.13
OTU 510 Paraglomus 308
6.92
1.95
2.08
VTX00308 Paraglomus sp.
9.87
2.69
OTU 863 Paraglomus 308
5.42
2.92
OTU 718 Paraglomus 308
4.21
OTU 974 Paraglomus 308
1.52
9.72
2.04
77
OTU 778 Paraglomus 308
4.52
3.28
OTU 986 Paraglomus 308
2.48
DQ915465.1| Marchandiomyces ligni
1.76
2.56
OTU 53
4.24
OTU 690
5.33
1.37
L22254.1|AAURR18SA Auricularia auricula-judae
2.51
OTU 235
5.57
1.41
OTU 364
2.43
1.55
AY293130.1| Exidiopsis calcea
OTU 303
5.03



Appendix IV. Molecular phylogenetic analysis by Maximum Likelihood methods of unknown "*Paraglomus* sp." OTUs and closest BLAST matches from NCBI and

MaarjAM databases (Öpik et al. 2010), with *Mortierella hyalina* JQ40259.1 as an outgroup. Initial trees were obtained using the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maxiumum Composite Likelihood (MCL) approach (bootstrap values of 1000 replicates) (Felsensetein 1985). Values that are > 50% are highlighted in bold. Branch lengths are measures in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Terminal groups connected by navy blue lines belong to a *Paraglomus* monophyletic groups, those connected by a red line belong to a non-*Paraglomus* monophyletic groups. MaarjAM Virtual Taxa type sequences are underlined in green. OTUs are listed with the OTU number, NCBI closest BLAST matches are listed by accession number followed by identity of the sequence, MaarjAM Virtual Taxa reference sequences are underlined in green. Evolutionary analysis was conducted in MEGA7 (Kumar et al. 2016).

Appendix V. Table of OTU read numbers found in buckthorn (*Rhamnus cathartica*) invaded and uninvaded sugar maple (*Acer saccharum*) soils.

Key to seasons: O – October; J – June

Key to sites: C – Cliffs & Alvars, *rare* Charitable Research Reserve; G – Grand Alee & Indian Woods, *rare* Charitable Research Reserve; K – Komoka Provincial Park, London, Ontario

Key to site type: A – buckthorn uninvaded; RA – buckthorn invaded

OTU	OC RA	OK RA	JK RA	OG RA	JG RA	JC RA	OK A	OC A	JK A	OG A	JC A	JG A	Phylogenetic VTX Assignment
113	0	0	0	0	0	0	0	0	0	0	0	8	*Acaulospora lacunosa* 24
482	0	0	0	0	0	0	0	0	0	0	0	3	*Acaulospora lacunosa* 24
480	0	0	0	0	0	0	1	0	0	0	0	0	*Claroideoglomus* 278
154	2	15	25	36	7	43	15	30	2	0	6	23	*Claroideoglomus* 279
320	0	4	0	0	0	1	164	0	0	0	0	0	*Claroideoglomus* 279
252	5	3	1	0	5	0	0	0	4	0	0	72	*Claroideoglomus* 340
432	0	0	0	1	0	0	0	0	0	0	0	0	*Claroideoglomus* 358
980	0	0	0	0	0	0	2	0	0	0	0	0	*Claroideoglomus* 358
551	0	2	0	13	0	0	1	0	0	0	0	1	*Claroideoglomus* 55
158	1	27	56	9	8	1	0	131	0	0	5	9	*Claroideoglomus* 56
60	2	6	7	3	3	1	1	1	4	0	1	199	*Claroideoglomus* 57
32	23	55	58	99	25	150	21	58	0	2	20	61	*Claroideoglomus lamellosum* 193
177	12	0	0	0	0	93	1	7	9	0	4	130	*Diversispora* 356
96	205	22	5	8	19	109	40	32	33	44	39	241	*Diversispora* 61
307	1	8	11	0	22	0	1320	0	13	12	2	21	*Diversispora* 62

617	0	0	6	0	0	1	0	0	1	0	0	0	*Funneliformis mosseae* 67
339	0	0	0	1	0	0	0	0	0	1	0	0	*Glomus* 103
270	0	0	0	0	0	0	0	0	0	0	0	1	*Glomus* 115
38	55	48	57	72	11	14	3	8	124	279	8	714	*Glomus* 117
97	4	6	9	9	0	2	22	0	1	40	0	113	*Glomus* 117
129	0	3	0	1	0	0	15	0	14	35	1	82	*Glomus* 117
820	1	3	3	2	2	3	0	0	2	5	4	2	*Glomus* 117
48	2	10	1	3	5	1	2	58	2	2	0	7	*Glomus* 125
583	37	0	0	0	1	0	0	0	0	0	0	0	*Glomus* 130
39	0	31	3	5	1	3	2	13	0	37	2	5	*Glomus* 135
717	0	0	1	0	1	0	0	5	0	1	2	0	*Glomus* 135
121	0	1	1	0	0	2	0	0	0	0	0	0	*Glomus* 140
458	0	2	0	0	0	1	0	0	1	0	0	0	*Glomus* 140
1193	0	0	0	0	0	0	1	0	0	0	0	0	*Glomus* 140
904	0	0	0	0	0	0	18	0	0	0	0	0	*Glomus* 142
392	0	0	0	0	0	0	0	26	0	0	0	0	*Glomus* 146
23	60	6	4	1	20	30	11	15	8	2	17	17	*Glomus* 151
211	1	26	0	0	6	0	3	70	0	1	7	3	*Glomus* 151
106	7	0	0	0	1	1	0	0	0	0	1	0	*Glomus* 159
1	41	7	0	2	0	16	2	0	34	1	1	1	*Glomus* 160
28	0	0	0	0	0	1	0	18	0	0	0	2	*Glomus* 163
47	0	0	0	1	0	2	0	7	0	0	0	0	*Glomus* 165
6	0	25	1	59	2	0	16	0	0	7	3	1	*Glomus* 166
34	0	14	0	13	0	0	0	0	0	1	0	1	*Glomus* 166
331	0	0	0	0	0	0	1	0	0	0	1	0	*Glomus* 166
468	0	0	0	0	0	1	0	0	0	0	0	0	*Glomus* 166
862	0	29	0	36	0	0	12	1	2	3	0	4	*Glomus* 166
1079	0	0	0	0	0	0	1	0	0	1	0	0	*Glomus* 166
1158	9	6	1	12	4	5	18	9	6	5	4	32	*Glomus* 166

1263	0	25	0	33	3	0	0	1	1	2	0	1	*Glomus* 166
372	0	0	0	2	0	0	0	0	0	0	0	0	*Glomus* 172
378	0	0	0	0	0	0	0	32	1	0	0	0	*Glomus* 175
4	0	26	3	4	36	1	3	36	1	1	3	7	*Glomus* 177
462	0	0	0	0	0	0	0	1	0	0	0	0	*Glomus* 177
989	0	0	0	0	0	0	2	1	0	0	1	2	*Glomus* 177
1274	0	0	0	0	0	0	6	0	0	0	0	0	*Glomus* 177
118	0	1	0	1	0	0	177	3	1	0	0	1	*Glomus* 188
125	0	76	0	94	1	0	3	0	2	0	1	1	*Glomus* 194
366	0	14	0	13	0	0	3	0	0	0	0	0	*Glomus* 194
292	7	13	1	12	1	1	0	7	4	4	3	15	*Glomus* 199
658	0	1	1	0	0	0	0	4	12	32	0	1	*Glomus* 199
57	5	114	1	2	4	2	0	2	1	13	2	7	*Glomus* 212
5	0	4	0	0	1	5	149	1	0	140	3	2	*Glomus* 214
880	0	0	0	0	0	0	0	0	1	0	0	0	*Glomus* 214
891	0	0	0	0	0	0	6	0	0	0	0	0	*Glomus* 214
1215	0	0	0	0	1	3	57	0	0	61	0	1	*Glomus* 214
16	24	237	12	382	3	6	2	1	101	7	4	4	*Glomus* 219
557	0	1	2	0	1	2	0	0	4	1	0	0	*Glomus* 219
957	0	1	0	0	0	0	0	0	0	0	1	0	*Glomus* 219
966	0	0	0	0	0	0	25	0	0	0	0	0	*Glomus* 219
35	27	21	5	4	3	3	1	50	9	9	7	9	*Glomus* 222
40	27	16	0	1	1	3	0	22	1	7	0	8	*Glomus* 222
99	6	7	3	1	3	6	3	43	0	4	0	8	*Glomus* 222
326	0	0	0	0	0	0	2	0	0	0	0	0	*Glomus* 222
454	4	2	0	0	1	1	276	3	0	0	0	1	*Glomus* 222
750	7	17	4	1	5	1	11	110	7	2	3	18	*Glomus* 222
1250	27	2	7	2	0	3	0	2	3	5	2	12	*Glomus* 222
860	0	1	0	0	0	0	0	0	1	2	2	0	*Glomus* 234
322	0	0	0	0	0	0	1	0	1	0	2	0	*Glomus* 247

537	0	0	0	0	0	0	0	0	0	0	0	1	*Glomus* 247
481	12	1	0	0	0	3	120	0	0	0	0	0	*Glomus* 342
646	0	0	0	0	0	0	1	0	0	0	0	0	*Glomus* 342
280	0	1	1	1	0	0	0	0	0	0	2	0	*Glomus* 366
695	0	0	0	0	0	0	7	0	2	0	0	0	*Glomus* 366
241	22	58	3	114	2	0	53	0	23	3	2	1	*Glomus* 411
17	0	0	1	1	0	4	7	3	2	8	0	50	*Glomus* 72
1149	0	1	0	0	0	0	0	0	0	0	0	0	*Glomus* 72
19	11	95	4	388	4	6	4	0	38	138	7	6	*Glomus* 74
875	0	0	0	0	0	1	14	0	1	0	1	0	*Glomus* 74
731	0	4	0	0	2	0	0	11	0	0	1	0	*Glomus* 83
80	0	23	1	56	0	2	2	0	0	3	1	1	*Glomus* 84
572	0	7	0	1	0	0	0	0	0	0	0	0	*Glomus* 84
733	0	4	0	2	0	0	12	0	0	0	1	0	*Glomus* 84
46	0	0	0	0	1	0	0	0	2	39	0	0	*Glomus* 86
1161	0	0	0	0	0	0	34	0	0	0	0	0	*Glomus* 86
1175	0	0	0	0	0	0	1	0	0	0	0	0	*Glomus* 86
64	0	344	3	1003	3	9	81	2	1	8	9	8	*Glomus* 88
20	150	91	2	5	43	57	67	714	8	3	36	114	*Glomus macrocarpum* 199
161	0	0	1	0	0	0	48	0	0	0	0	0	*Paraglomus laccatum* 261
718	0	1	0	0	0	0	0	0	0	1	0	0	*Paraglomus* sp. 308
986	0	0	0	0	0	0	2	0	0	0	0	0	*Paraglomus* sp. 308
0	13	15	24	18	8	17	0	8	9	6	28	51	*Rhizoglomus fasciculatus* 113
1255	3	5	5	13	4	1	3	4	2	0	13	64	*Rhizoglomus fasciculatus* 113
257	0	0	0	0	0	1	0	0	1	0	0	0	*Rhizoglomus vesiculiferus* 115

108	15	0	0	0	0	17	0	4	3	15	0	6	*Septoglomus constrictum* 64
164	0	0	1	0	3	1	14	49	1	1	10	66	*Septoglomus constrictum* 64

Appendix VI. Variance analysis of OTUs found in buckthorn (*Rhamnus cathartica*) invaded and uninvaded soils. Taxa listed below had moderate effect sizes between buckthorn invaded (RA) and pristine (A) sugar maple (*Acer saccharum*) plots. Key to table headings: rab.all – median clr value for all samples in the feature; rab.win.A – median clr value for the A group of samples; rab.win.RA - median clr value for the RA group of samples; dif.btw – median difference in clr values between A and RA groups; dif.win – median of the largest difference in clr values within A and RA groups; effect - median effect size: diff.btw /max(dif.win) for all instances; overlap - proportion of effect size that overlaps 0 (i.e., no effect)

OTU	rab.all	rab.win. A	rab.win. RA	diff.btw	diff.win	effect	overlap	Taxa
129	0.553687	3.737143	-0.66007	-4.38511	4.221231	-0.91193	0.167278	G117
164	1.124166	3.526518	-0.51474	-3.91217	4.289638	-0.83722	0.15928	S64

Appendix VII. Table of OTUs found in potted greenhouse experiment involving sugar maple *(Acer saccharum*) seedlings exposed to buckthorn (*Rhamnus cathartica*) allelochemicals.

Key to samples: B – Berries; CF – Field Control; CP – Potted Control; LB – Leaves & Berries; R – Roots; L – Leaves

Key to sites: 1 – Shady Maples site 1, Ilderton, Ontario; 2 – Shady Maples site 2, Ilderton, Ontario

OTU	B2	CF1	CP1	B1	LB1	LB2	R2	CF2	L2	L1	R1	CP2	Phylogenetic VTX Assignment
113	6	0	1	0	0	0	0	226	0	1	1	2	*Acaulospora lacunosa* 24
482	0	0	0	0	0	0	0	6	0	0	0	0	*Acaulospora lacunosa* 24
469	0	0	0	0	0	0	0	0	34	0	0	0	*Claroideoglomus* 225
480	1	10	1	0	0	0	0	0	0	0	0	0	*Claroideoglomus* 278
154	4	1	43	6	10	25	20	0	61	61	104	37	*Claroideoglomus* 279
320	1	0	0	0	0	1	0	0	44	0	0	0	*Claroideoglomus* 279
252	0	0	4	1	0	1	1	0	12	10	9	7	*Claroideoglomus* 340
306	1	0	15	0	1	0	3	0	0	0	18	2	*Claroideoglomus* 340
455	1	0	26	0	3	0	2	0	14	6	0	1	*Claroideoglomus* 340
432	2	0	10	2	6	11	5	0	10	10	12	4	*Claroideoglomus* 358
980	0	0	7	1	1	2	3	0	6	3	6	4	*Claroideoglomus* 358
352	1	0	16	2	5	0	0	0	0	19	7	1	*Claroideoglomus* 402
158	0	0	19	0	9	0	0	0	2	0	0	4	*Claroideoglomus* 56
60	8	3	175	2	44	26	24	0	249	64	184	82	*Claroideoglomus* 57
32	16	0	311	28	105	98	109	0	313	241	403	149	*Claroideoglomus lamellosum* 193
177	0	1	0	0	1	0	0	0	0	2	0	0	*Diversispora* 356
96	0	0	0	0	19	0	6	1	2	17	6	3	*Diversispora* 61
617	0	3	0	0	1	0	1	0	1	0	2	0	*Funneliformis mosseae* 67
339	0	0	0	24	5	0	0	0	0	2	5	0	*Glomus* 103

270	135	1	109	92	370	184	101	2	116	79	49	81	*Glomus* 115
38	1	0	3	4	1	0	0	0	0	47	2	0	*Glomus* 117
97	0	0	0	0	0	0	0	0	0	8	0	0	*Glomus* 117
820	0	0	0	0	0	0	0	0	0	4	0	0	*Glomus* 117
48	121	78	592	273	140	133	77	474	519	171	387	161	*Glomus* 125
720	12	0	27	21	9	8	2	0	15	7	8	6	*Glomus* 125
1097	1	0	6	0	0	2	0	0	3	0	1	0	*Glomus* 125
583	0	7	0	0	0	0	0	0	1	0	0	2	*Glomus* 130
39	16	0	28	15	18	6	19	1	36	28	277	827	*Glomus* 135
717	1	0	1	4	4	2	1	0	7	2	30	146	*Glomus* 135
121	182	1	121	28	61	125	185	0	461	55	140	131	*Glomus* 140
458	5	1	132	3	219	16	18	0	27	66	1	2	*Glomus* 140
1193	0	0	9	1	8	2	0	0	5	3	1	2	*Glomus* 140
904	90	0	0	1	1	4	1	0	5	3	0	0	*Glomus* 142
23	104	895	65	6	25	17	42	1452	68	15	82	75	*Glomus* 151
211	0	1	0	1	0	0	0	3	0	0	0	0	*Glomus* 151
106	3	0	13	2	15	2	271	1	2	22	19	8	*Glomus* 159
1	176	21	5428	605	2851	54	8091	30	112	2048	4469	81	*Glomus* 160
430	0	0	11	0	5	0	6	0	0	3	10	0	*Glomus* 160
652	0	0	8	2	10	0	9	1	0	5	6	0	*Glomus* 160
28	73	0	0	29	40	24	54	0	23	4788	0	29	*Glomus* 163
520	0	0	1	1	29	0	0	0	0	5	0	0	*Glomus* 163
47	23	0	413	13	1483	43	56	0	103	187	1	7	*Glomus* 165
792	7	0	57	6	41	0	96	0	1	17	33	0	*Glomus* 165
973	7	0	32	1	103	2	6	0	3	12	2	1	*Glomus* 165
6	6115	0	26	1165	183	232	137	279	757	140	65	526	*Glomus* 166
34	1479	0	9	285	71	76	38	4	210	29	18	126	*Glomus* 166
331	85	0	0	21	9	7	4	1	13	4	2	6	*Glomus* 166
468	26	0	0	1	0	9	1	0	5	1	1	2	*Glomus* 166
544	48	0	6	7	29	56	6	6	67	8	4	84	*Glomus* 166

746	9	0	0	1	0	1	0	0	0	0	1	1	*Glomus* 166
862	4792	3	21	1241	176	132	156	244	664	107	102	255	*Glomus* 166
1028	65	0	0	13	16	15	2	0	22	3	0	3	*Glomus* 166
1079	282	0	2	83	17	13	8	3	28	5	2	14	*Glomus* 166
1158	1238	8	1312	1483	1519	4021	1523	189	2656	1088	369	388	*Glomus* 166
1263	3365	1	31	594	171	188	94	154	493	104	72	363	*Glomus* 166
372	111	0	29	0	0	10	3	0	18	2	0	5	*Glomus* 172
4	3825	3	818	194	1503	988	1179	10	1802	963	4363	339	*Glomus* 177
462	163	0	33	12	106	50	24	1	48	26	45	9	*Glomus* 177
989	291	2	51	21	42	14	77	3	35	44	122	14	*Glomus* 177
1274	23	0	6	3	15	27	10	1	22	12	25	3	*Glomus* 177
118	26	0	99	4	291	34	27	0	55	25	37	30	*Glomus* 188
125	0	0	0	0	0	0	0	39	0	0	0	0	*Glomus* 194
366	0	0	0	0	0	0	0	8	0	0	0	0	*Glomus* 194
346	5	1	56	10	81	9	77	3	9	26	44	3	*Glomus* 196
292	275	104	13	23	51	2045	36	321	613	47	209	94	*Glomus* 199
658	0	17	14	0	11	4	0	0	10	44	268	2	*Glomus* 199
1143	58	2	0	7	0	22	3	1	63	1	14	5	*Glomus* 199
57	12	9	74	87	289	18	12	0	14	35	5	486	*Glomus* 212
5	369	4	1633	361	841	350	666	59	781	368	1589	944	*Glomus* 214
880	32	0	137	30	108	44	60	1	41	18	35	22	*Glomus* 214
891	0	0	6	0	1	0	2	0	2	3	4	1	*Glomus* 214
1060	2	0	4	1	3	7	3	0	7	0	7	1	*Glomus* 214
1215	112	4	342	148	297	59	145	4	352	42	263	191	*Glomus* 214
16	1875	0	75	1682	260	415	587	381	568	31	6	42	*Glomus* 219
557	257	0	7	127	13	9	35	6	18	15	0	7	*Glomus* 219
957	44	0	4	19	6	4	6	9	5	5	0	3	*Glomus* 219
966	14	0	0	3	1	0	3	1	0	0	0	0	*Glomus* 219
35	151	67	532	315	173	209	120	671	677	241	472	291	*Glomus* 222
40	39	20	302	100	79	56	50	260	188	89	227	90	*Glomus* 222

99	76	28	403	195	78	132	50	300	289	122	240	138	*Glomus* 222
326	1	0	12	6	13	7	2	1	4	4	6	5	*Glomus* 222
344	5	0	64	24	24	27	7	1	27	18	11	8	*Glomus* 222
454	1	0	221	2	7	1	1	0	2	20	9	6	*Glomus* 222
750	46	0	1168	92	47	61	10	89	164	109	107	45	*Glomus* 222
1195	7	2	9	10	18	6	2	1	17	3	3	5	*Glomus* 222
1250	39	2	34	27	37	28	34	269	120	87	194	26	*Glomus* 222
860	5	0	2	2	4	9	10	0	6	9	54	3	*Glomus* 234
322	132	2	721	39	3115	795	333	0	317	1331	216	323	*Glomus* 247
336	116	0	116	81	69	95	87	3	82	44	24	38	*Glomus* 247
493	17	0	24	6	64	26	21	0	19	11	11	15	*Glomus* 247
537	125	5	172	43	244	34	159	0	49	273	36	46	*Glomus* 247
861	7	0	11	2	5	4	19	0	9	5	0	5	*Glomus* 247
481	17	0	158	26	232	18	1013	1	19	116	114	18	*Glomus* 342
646	9	0	0	7	4	4	5	0	2	450	0	7	*Glomus* 342
1127	1	0	8	2	6	4	3	1	5	16	5	9	*Glomus* 344
280	1	0	1	1	15	18	6	0	2	9	10	2	*Glomus* 366
695	50	0	1	3	6	28	12	0	14	8	14	3	*Glomus* 366
241	641	1	48	529	141	175	257	133	192	16	3	22	*Glomus* 411
1047	0	0	0	0	10	0	0	0	0	0	0	0	*Glomus* 417
17	109	1732	685	48	89	263	65	376	617	177	119	202	*Glomus* 72
1149	2	0	216	0	1	9	1	0	129	1	0	1	*Glomus* 72
19	20	254	104	16	190	374	14	131	1110	223	22	109	*Glomus* 74
875	6	0	5	1	5	10	1	0	24	3	0	0	*Glomus* 74
1056	0	0	0	0	0	0	0	0	6	0	0	0	*Glomus* 74
80	14	0	90	1	244	14	7	6	11	116	37	71	*Glomus* 84
285	4	0	5	0	16	0	1	0	0	2	4	6	*Glomus* 84
572	0	0	2	0	0	1	0	0	0	1	0	0	*Glomus* 84
733	0	0	0	0	0	0	0	126	0	0	1	0	*Glomus* 84
46	97	3	345	89	198	78	169	12	247	76	353	242	*Glomus* 86

1161	9	0	0	0	0	0	0	0	0	0	0	0	*Glomus* 86
1175	4	0	9	2	4	1	7	0	2	2	10	3	*Glomus* 86
64	2	1	0	0	0	13	0	0	0	0	1	0	*Glomus* 88
20	41	58	42	5	207	140	299	2	99	238	253	196	*Glomus macrocarpum* 199
161	2	1	1	2	3	45	52	0	34	4	0	17	*Paraglomus laccatum* 261
297	2	0	1	1	78	3	2	0	2	0	0	2	*Paraglomus* sp. 308
510	0	0	0	0	33	0	0	0	0	1	0	0	*Paraglomus* sp. 308
718	35	0	2	16	25	28	9	4	6	5	1	5	*Paraglomus* sp. 308
778	0	0	0	0	6	3	0	0	0	0	2	4	*Paraglomus* sp. 308
863	0	0	0	7	0	0	3	0	0	1	0	0	*Paraglomus* sp. 308
974	0	0	0	0	0	0	0	11	0	0	0	0	*Paraglomus* sp. 308
986	9	0	4	10	6	3	3	0	0	5	0	0	*Paraglomus* sp. 308
0	2798	195	11720	2160	45759	16588	7797	983	10107	17691	3542	12127	*Rhizoglomus fasciculatus* 113
1255	1309	9	1492	1604	3110	5126	2548	145	3062	1230	516	607	*Rhizoglomus fasciculatus* 113
257	18	1	22	30	37	68	25	7	51	117	7	5	*Rhizoglomus vesiculiferus* 115
108	128	3	182	1	2	4	0	1	9	1	8	14	*Septoglomus constrictum* 64
164	0	0	1	0	2	0	7	0	3	24	2	0	*Septoglomus constrictum* 64

Appendix VIII. Variance analysis of OTUs found in the potted greenhouse experiment. Taxa listed below had large effect sizes between sugar maple (*Acer saccharum*) Control and Field Control pots (C) and those with buckthorn (*Rhamnus cathartica*) allelochemical treatments (T)

Key to table headings: rab.all – median centered log ratio (clr) value for all samples in the feature; rab.win.C – median clr value for the C group of samples; rab.win.T - median clr value for the T group of samples; dif.btw – median difference in clr values between A and RA groups; dif.win – median of the largest difference in clr values within A and RA groups; effect - median effect size: diff.btw /max(dif.win) for all instances; overlap - proportion of effect size that overlaps 0 (i.e., no effect)

OTU	rab.all	rab.win.C	rab.win.T	diff.btw	diff.win	effect	overlap	taxa
462	2.130989	0.661496	2.724349	2.207336	2.056791	1.014875	0.0925	G177
23	3.077592	7.228731	2.28584	-5.42048	5.26009	-1.12797	0.110945	G151
48	5.472669	6.672472	4.476688	-1.99961	1.972655	-1.02201	0.121	G125
17	5.002389	7.388068	4.213059	-3.01941	2.787897	-1.2014	0.054	G72
4	7.073497	5.195408	7.528629	3.137322	2.735607	1.164232	0.062	G177

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