Belowground Fungal Community Change Associated with Ecosystem Development

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ABSTRACT (Academic)

Numerous studies have looked at biotic succession at the aboveground level; however, there are no studies describing fungal community change associated with long-term ecosystem development. To understand ecosystem development, the organisms responsible for shaping and driving these systems and their relationships with the vegetation and soil factors, it is critical to provide insight into aboveground and belowground linkages to ultimately include this new information into ecosystem theory. I hypothesized that fungal communities would change with pedogenesis, that these changes would correlate with vegetation community change, and that they should show change of composition and diversity as the seasons change. Chapter 1 discusses the main topics related to this dissertation. Chapter 2 includes a publication draft that describes a study of sand-dune soil samples from northern Michigan that were analyzed to pinpoint the structural change in the fungal community during the development of the ecosystem. The samples were analyzed by pyrosequencing the soil DNA, targeting the internal transcribed spacer region. Chapter 3 contains a coauthored published paper that describes plant invasion of fields in Virginia to determine how they impact soil bacterial and fungal communities. The bacterial and fungal communities that were invaded by 3 different plant species exhibited similar changes, regardless of plant species, suggesting that some functional traits of invasives may have similar impacts on belowground communities. Chapter 4 remarks the conclusions of this research.

Belowground Fungal Community Associated with Ecosystem Dynamics

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ABSTRACT (General Audience)

Ecosystems, including the soils underneath, are the environments that surround us perform a large number of critical human-relevant functions (playing roles in production of food, filtration of water for drinking, sequestration of carbon and nitrogen to build soil organic matter, and buffer against flooding). Yet, how these systems naturally develop over time are still in need of detailed study. One particular area of interest and need is the study of belowground fungal communities. It is not commonly known, but plants and ecosystems are highly dependent on the underground web of fungal hyphae that transform nutrients and provide water to plants. A first step in gaining this understanding utilized a natural ecosystem development gradient known as a chronosequence. It was expected that fungal communities would change as soil and ecosystem development progressed and that they would mimic changes in soil and vegetative properties. Discerning if these linkages occur is the first step to assessing how they work together to create ecosystems and their valuable environmental services. Chapter 1 provides a discussion of the main topics in this dissertation. Chapter 2 is at the heart of the dissertation via a study of fungal communities in a developmental soil ecosystem in northern Michigan in addition, in Chapter 3, I include a coauthored published paper that describes plant invasion of fields in Virginia. Chapter 4 remarks on the major conclusions of this Master thesis, supporting the role that vegetation and fungal community change in soil are associated with one another.

DEDICATION

To my son Matthew and my niece Daniela because they are the future…

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Finally, **this thesis would not have been possible without the love of my husband Robert, my parents Fernando and Lexia; and my sister Marcela—for their endless love, support, and encouragement**.

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ATTRIBUTION

All the manuscripts listed in this document, i.e., chapters 2, 3 and 4 have co-authors. Contribution of all co-authors is explained as follow:

- **Rosana P. Pineda, M.S.:** I earned a Bachelor Degree in Agronomy and a Master of Science in Biotechnology in the Universidad Nacional de Colombia with a grade average of 3.49 and 3.79 respectively. I participated in all topics related with this research: processing of soils, DNA extraction from soils, PCR assays planning and performance, sample preparation for sequencing, data analysis and interpretation from the bioinformatics process. I authored the first draft of the documents in Chapters 1, 2, and 4 except Chapter 3. In this Chapter, I contributed in many topics related with this publication; I processed the soils, extracted DNA from soils, planned and performed the PCRs assays, prepared samples for sequencing. I also in collaboration with Richard Rodrigues prepared the tables in this publication. I also along with the other authors analyzed of the data coming from the bioinformatics process and interpreted results.
- **Mark A. Williams, Ph.D.:** Professor, Horticulture Department at Virginia Tech. He has participated in research proposal preparation, experimental methods and reviewing all manuscripts. He is the principal investigator of the NSF funded project entitled: Microbial community change during vegetative succession and soil-ecosystem development who directed all the aspects of the research.
- **Richard R. Rodrigues, Ph.D.:** Postdoctoral researcher, Department of Pharmaceutical Sciences at Oregon State University. He has participated with the bioinformatics processing used in the study and reviewing the manuscripts. He is the first author in the manuscript in Chapter 3.
- **William B. Whitman, Ph.D.:** Professor, Department of Microbiology at University of Georgia. He is co-principal investigator in the NSF funded project: Microbial community change during vegetative succession and soil-ecosystem development.
- **Kamlesh Jangid, Ph.D.:** Scientist at National Centre for Cell Science. He is co-principal investigator in the NSF funded project entitled: Microbial community change during vegetative succession and soil-ecosystem development.

- **Jacob N. Barney, Ph.D.:** Associate professor in the Department of plant pathology, physiology and weed science at Virginia Tech. He has contributed analyzing the data and reviewing the manuscript in Chapter 3.
- **Erik Nilsen, Ph.D.:** Professor, Department of Biological Sciences at Virginia Tech. He has contributed analyzing the data and reviewing the manuscript in Chapter 3.
- **John Barrett, Ph.D.:** Associate professor, Department of Biological Sciences at Virginia Tech. He has contributed analyzing the data and reviewing the manuscript in Chapter 3.

CHAPTER 1

Rosana P Pineda

LITERATURE REVIEW

Introduction

Microbial communities are strongly connected to ecosystem processes. Their fundamental role in belowground processes, especially nutrient cycling and plant-microbe interactions have been widely studied (Bardgett & Wardle, 2010; Van Der Heijden et al., 2008; Jangid et al., 2011; Wardle et al., 2004) but their integration into ecological theory (e.g. succession, niche theory) is still in the early stages of scientific discovery. It has been known for many decades, for example, that a huge diversity of fungal taxa undertake a primary role in the decomposition of organic matter and crucial to the release of nutrients of nitrogen and phosphorus into forms available for uptake by plants. In this way, fungi support the growth of plants and the development of soils and their associated ecosystems. Members of the fungal community can also have a more direct role in plant growth and productivity, through their mutualistic and antagonistic interactions with belowground root systems. Fungal communities and their activities in soils are thus considered a major keystone group fundamental to ecosystems and their development. Determining *in situ* changes in fungal communities using molecular techniques provide a new means to understanding their role in ecosystem processes.

Despite great strides in linking plant and microbial communities to understand ecosystems, many details of the dynamics and interaction between aboveground and

belowground communities remain unclear (Van Der Heijden et al., 2008). For example, it has been hypothesized and shown that bacterial community change is consistent with a simple two step model of negative plant-microbial feedbacks during early steps, and positive feedbacks latter in ecosystem development. However, the application of this simple model to multiple ecosystems, and to fungal communities remain largely untested. Researching patterns of succession to include belowground microbial communities in addition to aboveground plant communities will support integration of microbial communities into ecological theory and ultimately models of ecosystem processes (Fierer et al., 2010; Tarlera et al., 2008).

The foundations of ecosystem succession, underpinned by plant community change and ultimately pedogenesis, will benefit from the addition of fungal community dynamics. Comprising from 35 to 76% of the soil microbial biomass (Joergensen & Wichern, 2008) and integral to plant growth and development, fungi are fundamental determinants of plant-ecosystem diversity and function (Talbot et al., 2014). The research in this dissertation will determine how soil fungal communities change in response to ecosystem development and invasion of non-native plants in to native plant dominated ecosystems. It will determine whether there are predictable patterns of fungal community change related to pedogenesis, plant succession, and plant invasion. The second chapter (#2) will describe soil fungal community change during soil pedogenesis, plant succession, and ecosystem development in Northern lower Michigan (Wilderness Park; WP) with soil depositional ages ranging from 105 to 4010y; and chapter 3, focuses on the role that plant invasion can have on belowground fungal and bacterial communities over shorter time periods (< 10 y).

Succession and ecosystem development

Succession is the shift in species composition and its associated substrate over time. Succession theory was described over 100 years ago by Cowles (Cowles, 1899); who recognized that species change was related to time since vegetative establishment on stabilized sedimentary parent material. This was first observed on aggrading sand dunes near the southern tip of Lake Michigan by Clements (Clements, 1916), furthermore, recognized that plant succession is a complex process associated with multiple factors (Clements, 1928). In contrast Gleason, focused on individual and population plant performance as the driving force for succession (Gleason, 1927). Still today, there are many different opinions about the underlying mechanisms that can best be used describe vegetative and ecosystem succession. The process of pedogenesis and associated soil phosphorus and nitrogen dynamics have played an increasingly important role as a descriptor of vegetative and ecosystem succession (Aber et al., 1990; Walker & Syers, 1976).

Primary succession and progression

The process of ecosystem development on barren surfaces where most vestiges of biological activity have been removed is defined as primary succession. For the current study at WP, the shift from the Pleistocene to the Holocene resulted in periods of glacial melting and lake development. The lake was underlain by sandy and stable crystalline minerals that with time tended to accumulate through the shoreline (Davis Jr, 2013). During periods of drought, sedimentary materials are blown as aeolian deposits to create new

dunes, and remain in place as the shoreline of the lake gradually recedes. (Davidson-Arnott, 2010) to form the chronosequence of interest.

Primary ecological succession is associated with disturbances that expose or result in the accumulation of primary parent materials, such as sediments and sands. Driven by both allogenic and autogenic factors, biotic turnover (Kimmins, 1997) occurs over time scales measured in years, decades, thousands, and millions of years. Vegetative change, for example, often occurs fast in the first decades and hundreds of years but then slows during the progressive stage of development. Plant species diversity and composition tends to increase concomitantly (Molles, 2005). Pedogenesis is also a fundamental component of succession, and in particular, the accrual of N and C can occur quickly on the same temporal scales as vegetation. The process of weathering and phosphorus loss, in contrast, tend to occur over longer time periods, with mineralogical changes in the soil driven by temperature, rainfall, the type of parent material, and biological activity (especially plant roots and soil microbes). Replacement and change in plant abundance occurs and is related to altered soil nutrient pools, and plant traits, such as the way in which plants compete for available resources (Connell & Slatyer, 1977). Facilitation by plant species through mutualisms with nitrogen fixing bacteria, for example, drive the accumulation of soil N during early ecosystem development. The accrual of N benefits the colonization and growth of many plant species, which is thought to be factor driving the replacement of early colonizers by faster growing plant species (Perry et al., 2008). These processes are well described among developing ecosystems undergoing progression, however, important questions about the feedbacks that reverberate between the above- and belowground communities are still not well understood. Belowground fungal communities can support

or deter plant establishment and control soil nutrient cycles, and therefore may play a fundamental role in the process of ecosystem succession and development.

Retrogression

Retrogression, or the so-called decline phase of ecosystem development occurs in the absence of major ecosystem disturbance, and results from weathering induced losses of nutrients, especially phosphorus e.g. (Crews et al., 1995; Parfitt et al., 2005). This decline coincides with reductions in plant production and standing biomass. Nutrient losses of phosphorus occur through leaching and erosion, as well as chemical transformations from inorganic into less bioavailable organic forms during soil development

Ecosystem retrogression can have profound effects on plant biomass and species composition whereby productivity is lowered. Retrogression is reversed through a large rejuvenating disturbance (e.g. landslide) that resets the system; this differs from age-related declines in forest productivity that is driven by shorter-term depression of nutrient availability and plant ecophysiological process rates that occur during succession (Peltzer et al., 2010). Reductions in ecosystem productivity and standing plant biomass, declines in the availability of nutrients, and shifts in both aboveground and belowground are thus characteristics of retrogression process.

The retrogressive stage of ecosystem development has thus often been defined by its contrast to early soil and ecosystem development when nitrogen accrual occurs quickly through biological nitrogen fixation (Menge & Hedin, 2009). Nitrogen availability thus tends to limit plant communities on very young soils, while phosphorus is limiting on mature soils (Laliberté et al., 2012; Peltzer et al., 2010; Vitousek & Farrington, 1997).

Between the progressive and retrogressive phases, there can be relatively long periods of relatively high nutrient availability, however, at all stages the role that microbes play in competition with plants and the turnover of nutrients are well-known (Bardgett & Wardle, 2010; Schulz et al., 2006). These interactions between plants, microbes, and between plants and microbes during pedogenesis and ecosystem development set the stage for feedbacks that can help define the communities of an ecosystem. It is not expected that retrogression is yet an important component of the WSP development ecosystem, however, overs tens of thousands of years the system may begin to undergo this process.

Chronosequences

Chronosequences are used, as in this thesis, as surrogates or proxies for primary succession and pedogenesis (Harden, 1982). They are a space for time substitution, whereby similar parent materials are deposited at different periods of time in the past: tens, hundreds, thousands, millions of years. (Walker et al., 2010). Chronosequences are a useful proxy to study long-term ecosystem development and how the feedback between biological communities and pedogenic processes drive ecosystem development (Peltzer et al., 2010). Chronosequences are found in a variety of landscapes as sand dunes, glacial moraines, river and marine terraces. The chronosequence parent material at WSP is a largely sandy substrate.

Soil chronosequences are recognized as a valuable tool to investigate the rate and direction of soil development, registering pedogenic changes over time-scales ranging from years to millions of years (Huggett, 1998). Also, it is a way to follow the succession patterns or changes in species composition and ecosystem state occurring over decades to

hundreds of years in response to disturbance (Aber & Melillo, 2001). Huggett in his review concluded that the soil chronosequence are powerful tool for pedological research and to test pedological theories (Huggett, 1998). The critical assumption of chronosequences is that each site has traced same history for both biotic and abiotic factors, being age the only factor of change in the sequence (Johnson & Miyanishi, 2008). Technically, while there is no chronosequence that can perfectly meet this critical assumption, there are numerous examples that appear to come close, and thus provide information about ecosystem development that would otherwise be difficult or impossible to test. The WSP chronosequence are viable representations of the process of ecosystem development, and formed from parent material that appear to have be relatively uniform over the last 4500 years (Lichter, 1998b).

Since the mid and late 1900's Walker and collaborators (Walker & Syers, 1976) made important contributions to the knowledge about the dynamics of phosphorus during long-term ecosystem development and established the important role played by soils during vegetative succession. Their studies in chronosequences (mainly in New Zealand) laid the bases for the understanding of soil pedogenesis and nutrient dynamics (P, C, S and organic matter dynamics) as drivers shaping aboveground communities (plant vegetation succession). The long-term P dynamics model proposed by Walker and his colleges about how phosphorus become less available to plants over thousands of years, losing off the ecosystem by processes like occlusion, precipitation, and run off had important implications to the better understanding of the ecosystem development and how this affect other ecosystems players.

Lichter who in the late 1900's made important contributions to the understanding of the organic matter dynamics in the horizon formation and weathering and mineral depletion processes along the Lake Michigan Dunes chronosequences (Lichter, 1995, 1997, 1998b). His research described the changing aboveground and belowground properties during succession (e.g. plant composition, soil nutrients) (Lichter, 1998a). This work was of course built upon the research of others reaching back to the 19th century, whereby it was recognized that ecosystems change in predictable patterns, but with a major focus on the aboveground process of plant succession (Cowles, 1899).

The sand dunes at Wilderness Park (WP) described in this thesis, form a chronosequence formed adjacent to Lake Michigan (Lichter, 1995). The formation of dune-capped beach ridges occurred during drought episodes, the co-occurrence of falling and low lake levels, and the aeolian deposition of dried lake sediments into dunes (Lichter, 1995). ^{14}C dating of macrofossils from dune plants were used to develop a chronology of dune-ridge formation (Lichter, 1997). This chronosequences comprises seventy-two dunes ridges that have been formed over the past 4500 years and are considered geomorphically stable and thus can provide gradational changes in vegetation and soil properties suitable for assessing pedogenesis and succession along the chronosequence (Lichter, 1998b). The vegetation succession surveys at WP have been reported by Lichter and Williams (Lichter, 1998a; Williams et al., 2013) where the first dunes were dominated by grasses, then shrubs, and then during latter succession mixed forest and especially pine replaced grass and shrub species. In this thesis, 9 dune ridges spanning the entire age range of the chronosequence were chosen for detailed study.

Previous studies carried out by Lichter on soil properties in Michigan chronosequences showed that percentage of moisture of the upper mineral soil increased with increasing dune age. Soil C and N also increased up to ~500 years, but then remained at steady-state thereafter (Lichter, 1998b). Soil organic matter content and soil cations were measured by Williams showed that the levels of soil Ca, Mg, soil organic matter and total soil organic C (but not mineralizable C) decreased from younger to older soils; they observed patterns of change as declining concentrations of mineral nutrients and soil organic matter during pedogenesis. Overall, patterns of pedogenesis and ecosystem change are consistent with ecosystem development, however, it is notable that the ecosystem has low soil P (~7ug g-1) (Williams et al., 2013). Clear patterns of succession have also been documented at WP (Lichter, 1998b).

Fungi as an ecosystem driver

The fungal kingdom is diverse both functionally and phylogenetically; playing major roles as soil decomposers and determinants of nutrient cycling in ecosystems. Special types of mycorrhizal fungi can be associated with plant roots providing mineral nutrition to the host plant (Carroll & Wicklow, 1992). Hundreds to thousands of fungal species inhabit a gram of soil, however many factors such as plant, nutrient and physical characteristics of the soils are known to affect the species composition (Pelczar et al., 2010). Fungi, through their activity in soil, assert large control over the fluxes of ecosystem C and N (Dighton, 2003) and earth's biogeochemical cycles (Falkowski et al., 2008; Gadd, 2008). However, gaps remain in the role of fungal community changes during pedogenesis, vegetative succession, and ecosystem development.

Fungal relative to bacterial biomass tends to increase as primary succession proceeds, however the functional significance of the shifts between fungal and bacteria dominance is not well understood (Bardgett & Wardle, 2010). The fungal: bacterial ratio is usually substantially higher in natural/remnant compared to managed soils, and the contribution made by fungi in terms of biomass to nutrient cycling may also be greater (Jangid et al., 2008). Fungi play many roles through decomposition of labile and recalcitrant organic matter, transport of nutrients from mineral to litter layers and through structuring vegetation through symbiotic and pathogenic relationships (Klein & Paschke, 2004). Microbial biomass is positively related with plant biomass, (Wardle, 1992), however the compositional changes that are related to shifts in vegetation or vegetation productivity with ecosystem development are not widely described.

Fungal and bacterial dominance tend to change in association with soil pH, with the former relatively more abundant with low soil pH, and the latter at higher pH (Rousk et al., 2010), though these results are not always consistent (Baath & Anderson, 2003). These observations have often been explained as the result of direct impact of soil acidity on microbial community structure, however, pH change is associated with the overall process of pedogenesis, and thus is associated with a complex dynamic in the chemical and biological properties of the soil habitat. Other studies have suggested that shifts in microbial community structure are related to soil N availability or to effects of N supply on plant belowground C allocation (Högberg et al., 2007). Indeed, studies show that multiple soil properties might play a role in determining microbial community structure (Jangid et al., 2013; Jangid et al., 2011; Michel & Williams, 2011; Mitchell et al., 2010; Mitchell et al., 2012; Williams et al., 2013).

Plant Invasions

Positive and negative feedbacks are thought to be important drivers of succession, but also of plant invasion (Allison & Vitousek, 2004; Hayward et al., 2015). Invasive plants are a major driver of ecosystem change, and have been shown to be specifically associated with changes in nutrient cycling following replacement of native vegetation (Allison & Vitousek, 2004; Liao et al., 2008; Vilà et al., 2011). In this since they are disruptor to the processes of natural succession endemic to an ecosystem as the major drivers of nutrient cycling, changes in soil microbial communities have been implicated as reinforcing plant invasion (Reinhart & Callaway, 2006). Surprisingly, there have been few studies that have attempted to make explicit links between plant invasion and microbial community changes using next generation DNA-based methods (Coats & Rumpho, 2014). Knowing the details of community change will help move the state of science from describing invasion related broad microbial shifts to more specific description of specific microbial groups and functions that are impacted by invasion.

Knowing whether microbial community changes during invasion support plant invaders or are simply an artifact of plant species change is difficult to discern. It is nevertheless, a puzzle, worth determining because it would offer a mechanistic description that could aid in the management of plant invasion. If, for example, plant invaders support the growth of a specific group of nitrifying bacteria not supported by native vegetation, bacterial inoculum or management of the system to reduce or offset the effect of the invader specific plant growth promoter could be instituted. The ability to alter nutrient cycling, for example has been shown to be associated with the invasion of several exotic grasses

(Hawkes et al., 2005), and thus knowing the mechanisms driving this change could provide needed descriptions to help manage or deter plant invasion. Knowing the ecology of native species, likewise, can be used to the advantage of land managers to support their growth. Mycorrhizae of native plants (*Pinaceae*) are well known to support and sustain their growth (Nuñez et al., 2009). In contrast, certain types of mycorrhizae have been implicated in causing the opposite effect, and supporting plant invasion (Hayward et al., 2015). Though it is becoming clearer that soil microbial communities are important in plant invasion, there are many questions that remain about the role they play in native and invasive impacted ecosystems. Furthermore, discerning whether or not there are microbial related invasive traits that are shared across many different invasive plant species would help to unify ideas about the invasive species connection to belowground microbial communities.

In addition to nutrient cycling dynamics as a hypothesized driver supporting a positive feedback for plant invasion, another common hypothesis that helps to explain invasion is related to pathogen release. In this scenario, invasive plants outcompete native and non-invasive plants because, unlike the former, the latter are more susceptible to pathogen attack (Klironomos, 2002; Klironomos, 2003; Maron et al., 2014). This could provide a profound advantage to invasive plants compared to that of natives. Describing microbial communities using DNA based methods can provide clues to the potential for pathogen based effects during invasion.

Some of the most convincing studies implicating the importance of microbial communities in plant invasion have involved the reciprocal transplant of native and invasive plants between home and foreign soil (Blank & Sforza, 2007; Rout & Callaway, 2012). These and other studies have supported the direct role that soil microbial

communities have on plant invasion (Castro-Diez et al., 2014). However, results are mixed and appear to be context specific (Tamura & Tharayil, 2014). Discerning the functional significance and whether invasive traits are common among different plant species (e.g. supporting a similar shift in soil community composition) are needed to understand and begin the process of rationally managing plant invaders.

Tools for studying soil-plant-associated belowground microbial communities

During the twentieth century the fields of ecology, evolutionary biology, and molecular genetics have increasingly converged, and brought new tools and perspectives that help to identify microorganisms in the soil and form the base of the soil ecosystem functioning (Feder & Mitchell-Olds, 2003). The DNA coding for the ribosomal RNA genes or their spacer regions has proven extremely useful for detection of fungi in complex environmental samples. Some of the characteristics enabling this region to be a powerful marker are: it is essential to protein synthesis and therefore ubiquitous to all microorganisms, it is structurally and functionally conserved, rRNAs are readily isolated and identified, and exhibit regions that are both relatively variable and conserved. These properties essentially reflect evolutionary changes in microorganisms which can then be used to describe phylogeny and taxonomy of the organisms. Identification of organisms from a growing body of fully sequenced cultured isolates and type strains is increasingly useful for discerning potential functioning of microbial communities

Eukaryotic ribosomes are formed by rRNAs and ribosomal proteins. The rRNA genes are the DNA sequences that direct formation of precursor molecule, which is

processed to yield the mature RNA constituents of the ribosome (Sollner-Webb & Mougey, 1991) There are four rRNA genes in Eukaryotes, 28S, 18S, 5.8S and 5S. The three first genes belong to a single transcription unit; however, the last one is transcribed from a separate gene (Cooper, 2000). The nuclear 18S rRNA gene is called the Small-subunit rRNA (SSU) and the nuclear 28S rRNA gene is referred as Large-subunit rRNA (LSU). The ribosomal cistron rDNA contains the Internal transcribed spacer 1 (ITS1), the 5.8S gene and the ITS2 region (Torres-Machorro et al., 2010). The great success in the use of these RNA gene markers support their use for describing the change in microbial phylogeny during succession and plant invasion (Olsen et al., 1986).

The identification and microbial diversity described in soils (e.g. Buée et al., 2009; Lauber et al., 2009; McGuire et al., 2013; Roesch et al., 2007) have helped to show that soil properties and land management are important drivers of community change. A gap remains to be filled regarding the drivers shaping the soil microbial communities during the non-managed process of ecosystem development and plant succession (Schmidt et al., 2014).

The knowledge of processes influencing soil fungal diversity and composition as they occur through processes, such as ecosystem development or plant invasion can help to determine potential strategies associated with biodiversity conservation, microbial community management, bioremediation, and agroecosystem management (Zhou et al., 2002). Microbial community change is related to the development of the soil ecosystem and associated changes in vegetation (Tarlera et al., 2008). This thesis seeks to further describe the processes and organisms that drive functioning in both native plant systems, and the large impacts driven by invasion dominated ecosystems. One of the main

challenges in soil microbial ecology is to better understand and predict the processes that drive soil microbial diversity and the ways in which this diversity feedback to impact ecosystems and associated plant communities (Maron et al., 2011).

Research Objective

The main objective in this study was to discern the patterns of fungal community change in soil and its relationship with vegetative and soil properties in native and invasive plant dominated ecosystems. **It was hypothesized that changes in plant communities, whether by invasion or through succession would be associated with changes in belowground fungal communities.**

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CHAPTER 2

Soil fungal communities along the Michigan sand-dune soil chronosequence and their relationship with ecosystem properties

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Abstract

Belowground fungal communities are a critical part of ecosystem function but their role during the natural process of ecosystem development remain understudied. Only a few studies have described fungal communities change during long-term (greater than 1,000 years) of ecosystem development and its relationship with vegetation and pedogenesis. I hypothesized that fungal community structure and richness would change with ecosystem development and be associated with pedogenesis, and vegetative succession. I also expected fungal communities to change with season, though these changes would be much smaller than those related to longer time scales of ecosystem development. The objective of this study was to establish whether plant succession and pedogenic related soil properties were correlated with fungal community change. To meet this objective samples of sand-dune soils from northern Michigan, composed of 9 age classes ranging from 105 to 4,010 years following deposition, were sampled from the incipient A-horizon (~0 to 10 cm depth). The samples were analyzed by pyrosequencing the soil DNA, targeting the internal transcribed spacer (ITS) region. Bray-Curtis ordination indicated two primary patterns related to axis 1 and axis 2. This 27% in each axis would explain the change in community structure dominated by the phyla Ascomycota and Basidiomycota. Accounting for 49% and 15% respectively of the community, fungal change was greatest early (105 to 460 years) during ecosystem development. Community structure stabilized during later stages (845 to 4010 years). In support of the main hypothesis, fungal communities changed with soil indicators of pedogenesis and plant community succession. The observed patterns of change associated with long-term ecosystem development support the idea that the

characteristics of fungal community structure and vegetation may be linked through plant-microbial-soil feedbacks.

Introduction

Microorganisms are strongly connected to ecosystem processes, performing many key belowground functions from nutrient cycling to developing symbiotic relationships with plants that affect, and in turn, are effected by pedogenesis and ecosystem development (Van Der Heijden et al., 2008). Yet, the connections between belowground communities, plant succession, and ecosystem development over hundreds to thousands of years still need further investigation. Considerable research over the last decades suggest that early pedogenesis and ecosystem development are periods of continuous change. Yet, there is still debate about the factors that drive fungal community change during ecosystem development. Ultimately, it is the goal of the research to understand how functional relationships of fungi relate to nutrient cycling, plant community composition, and biogeochemical processes (Michel and Williams, 2011; Mitchell et al., 2012; Mitchell et al., 2010). Understanding the fungal-ecosystem link has the potential to inform ecosystem and global conservation and restoration efforts, and the ability of ecosystems to function as environmental filters.

Fungi are microorganisms estimated to have originated over 600 million years ago (Berbee and Taylor, 2010). Since their emergence, they have been key players in soil formation, rock dissolution, decomposition of organic residues, and nutrient mineralization (Dighton, 2003). Fungal-plant mutualisms and more generally plant – microbial feedbacks are major contributors to global biogeochemical cycles (Van Der Heijden et al., 2008;

Humphreys et al., 2010), but the relationship of fungal community structural changes relevant at longer and climate relevent temporal scales are still vaguely described (Fierer, 2008). Do changes in fungal communities, for example, during ecosystem development determine ecosystem vegetation structure, and if so feedback to positvely reinforce vegetation, or negatively cause vegetation turnover?

Vegetation may also affect the soil fungal community both directly and indirectly. Plant-mycorrhizal mutualisms are a means of direct interaction. Indirect effects of soil communities on vegetation may occur through their effect on the quantity and quality of organic matter inputs. This dynamic between plant root and microbes have been studied, and clearly have consequences for both plant and fungal growth (Bever et al., 1997; Anacker et al., 2014). Over longer time scales feedback mechanisms are altered to influence the type of fungi and plants that ultimately survive and dominate ecosystems. It is this natural process of primary succession where a considerable amount is known about vegetation but not belowground microbial communities. Predicting the factors that shape microbial communities and the plant-microbial feedback system that, along with changes in pedogenesis are thought to be the most likely drivers of ecosystem development and thus ecosystem function (Ohtonen et al., 1999; Mitchell et al., 2010).

This aim of the study was to describe change in soil fungal communities along a series of developmental sand-dune soils that form a chronosequence. The chronosequence of focus borders northern Lake Michigan that have formed a series of dunes ranging in depositional age from 105 to 4,000 years. Soil fungal community change along this series of developing soils was studied through the use of 454 pyrosequencing of fungal specific

ITS genes. It was hypothesized that soil fungal communities would follow a pattern of change related to shifts in plant succession and pedogenesis.

Materials and Methods

Study Site

The sand-dune chronosequence bordering Lake Michigan at Wilderness State Park in the peninsula of Michigan were selected as the sample site for this study. The site consists of an extensive strandplain of about 108 arcuate dune ridges (that is, dune-capped beach ridges) with depositional ages from present day to about 4,500 years. The dune ridges are about 2.5 km long, 10 to 30 m wide, and generally between 3 and 5 m in height (Lichter, 1998b, Lichter, 2000). Age represents the estimated time since deposition of the parent material

Soil Sampling

Five replicates of soil samples for study of the soil fungal communities were collected at 10-m intervals across transects along each dune's crest. Each replicate consisted of five to six subsamples collected from 0 to 15 cm, using 5-cm diameter stainless steel cores. Subsamples were homogenized, packed in sterile Whirlpak® bags, and frozen until use. Similarly, soil samples from the beach were collected to assess the community composition of the sand that would become, in part, the eolian deposits of the dune soils. All soil samples were collected in two seasons during 2008, in summer (August) and winter (December). Vegetation was surveyed in summer, with the dominant overstory vegetation

observed shown in Table 1. Further details of the soils can be found in Williams et al, (2013).

Table 1. Observed dominant overstory vegetation during summer season.

Dominant cover for each age*	Red Pine	White Pine	Spruce	Fir	Oak	Juniper	Little Bluestem	>30% Bare surface
105							X	X
155						X	X	X
210						X		
450	X		X	X	X			
845	X		X	X				
1,410	X		X	X				
2,385	X	X						
3,220	X	X						
4,010	X							

*Dominant (>10%) cover for each site age based on relative biomass. Understory of forest systems were diverse but with low vegetative biomass

DNA Extraction, PCR Amplification, and Pyrosequencing

For the small subunit SSU and ITS analyses, 0.5 g of freeze-dried soils were weighed, and DNA was extracted from each soil sample using a PowerSoil® DNA Isolation Kit (MoBio), according to the manufacturer's protocol. DNA quality was checked on a 0.8% (w/v) agarose gel. DNA concentrations were determined by spectrophotometry quantification using the Nanodrop 2000 (Thermo Scientific™). An aliquot of the total DNA was diluted to a final concentration of 5 ng/μL and stored in a -20°C freezer for PCR assays in order to check the amplification performance of the samples.

The confirmation of DNA amplification was performed with the small subunit rRNA using the following primers: nu-ssu-0817F and nu-ssu-1196R; nu-ssu-0817F and nu-ssu-1536R (Borneman and Hartin, 2000); and ITS1f and ITS4 (Gardes and Bruns, 1993; White et al., 1990; White et al., 1991) (Table 2). The 50 μL PCR reaction mixture contained

2 μL of each primer (20 μM), 25 ng of template DNA, 1 μL of BSA (20 mg/mL), and 25 μl of the enzyme ImmoMix™ Red (Bioline). The following PCR conditions were used: a denaturation step at 95°C for 7 minutes, 35 cycles at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, an extension step at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

As described above, DNA of each sample was prepped for submission to the Next Generation Sequencing Service Provider (Molecular Research DNA Laboratory). The ultimate region of interest was amplified using PCR primers ITS1f and ITS4 (Gardes and Bruns, 1993) (Table 2). The PCR reaction for these primers was performed using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). The PCR conditions used were as follows: a denaturation step at 94°C for 3 minutes, 28 cycles at 94°C for 30 seconds, an annealing step at 53°C for 40 seconds, an extension step at 72°C for 60 seconds, and a final extension at 72°C for 5 minutes.

Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Beckman Coulter). Samples were sequenced by MR DNA®, utilizing the Roche 454 FLX titanium sequencer and using reagents per manufacturer's guidelines. Bioinformatic analysis included the removal of barcodes and primers the resulting sequence data. Sequences were denoised, operational taxonomic units (OTUs) generated, and singletons and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity).

Table 2. Primers sequence used for DNA amplification and their target region.

Primer name	Sequence (5' – 3')	Target region
nu-ssu-0817F	TTAGCATGGAATAATRRAATAGGA	V4 (partial) and V5 variable region
nu-ssu-1196R	TCTGGACCTGGTGAGTTTCC	
nu-ssu-0817F	TTAGCATGGAATAATRRAATAGGA	V4 (partial), V5, V7, and V8 (partial) variable regions
nu-ssu-1536R	ATTGCAATGCYCTATCCCCA	
ITS1f	CTTGGTCATTTAGAGGAAGTAA	Targeting both ITS1 and ITS2 introns
ITS4	TCCTCCGCTTATTGATATGC	

Diversity Analyses

The OTU table provided by MR DNA was filtered to remove non-fungal OTUs and converted to biom format. A cutoff of 250 sequences per sample was used for following diversity analyses in QIIME v1.8 (Caporaso et al., 2010). The alpha diversity and taxonomic summaries were studied with respect to season and age of soil. For the Chao1 observed species (Chao, 1984), Shannon and Simpson indices were used to calculate alpha diversity. The rarefaction plots were generated for Chao1 and observed species metrics with respect to soil age and season. Permutational multivariate analysis of variance (PERMANOVA), including adonis, analysis of similarities (ANOSIM), and multi-response permutation procedures (MRPP), were used to compare the samples with respect to years (age of soil), and season. OTU were also analyzed using Bray Curtis ordination using a bray-curtis distance measure. OTU were transformed by using the general relativization procedure in PCORD version 6.0 (MjM software design) to assess change in fungal community structure with site age. One-way analysis of variance (ANOVA) and Student's t-test and Tukey-Kramer HSD using JMP®, Version 11 (SAS Institute Inc., Cary, NC, 1989–2007) were used to compare richness and diversity indices associated with

age and season. SigmaPlot version 11.0 (Systat Software, San Jose, CA) was used to make some of graphical output from PCORD.

Results

The overstory vegetation was dominated, roughly in order of successional stage, by grasses (*Schizachyrium scoparum*, juniper (*Juniperus communis*), oak (*Quercus rubra*), fir (*Abies balsamea*), spruce (*Picea glauca*), white pine (*Pinus strobus*), and red pine (*Pinus resinosa*). Though these species dominated the overstory in our sampling, a more comprehensive description of ~100 plant species (cover >0.1%) were reported by Lichter (1998a). Some other common species with greater than 10% cover in his survey included *Ammophilia breviligulate*, *Arctostaphylos uva-usi*, and *Prunus virginiana*. The reason for the differences between the two surveys can be explained by the much greater sampling resolution by Lichter, of eleven dune ridges younger than 845y, which described a high rate of species turnover across these different age ridges. As an example, plant species at 450y were completely different from those at 105y. Plant species at 845y were similarly, different from those at 210y. The data described in this thesis also indicate considerable change ($p<0.001$) in plant species with ridge age, each describing relative snapshot of this larger plant diversity. Vegetation was dominated early by grasses (105 to 155y), juniper-oak shrub (210y), spruce-fir (450 to 1410y), and then Pine (2385 to 4010). For simplicity, these changes were described in even broader terms in Figure 1.

Fungal Community Structure and Ecosystem Development

When grouped at the 97% similarity level, there were 3,412 OTUs observed. Bray-Curtis ordination was used to provide a description of the variation in the fungal

community structure using the most abundant OTUs (based on 150,075 sequences per age) across the chronosequence of summer and winter samples. This ordination indicated two axes, each describing 27% of the variation in the data (Fig 1). The spread of the ordinates in axis 1 show a pattern that matches the chronology of soil age, which is less clear but notable along Axis 2. Overall, the results show a pattern which indicate a relationship between soil fungal community structure and ecosystem development (age, $p<0.01$). However, the relationship was not linear, but instead fungal communities changed the most early and tended to stabilize during latter ecosystem development.

There was a significant linear correlation ($r^2=0.39$; $p<0.005$) with change in vegetation and fungal community structure (Beta diversity). This relationship reflects both the early shifts and the later tendency for relatively stable fungal and vegetative communities. It is notable, however, that spruce and fir disappeared from the oldest canopy, but fungal communities showed no similar evidence of significant change during these several thousand years of soil and ecosystem development. Despite this, the linkage between fungal and vegetative community change, as noted, was apparent. Change in fungal communities were thus tested and found to be also linked to shifts in broad plant functional groups (grasses, shrubs, pines, $p<0.001$).

The fungal community structure of the beach sand was found to be different from that of the chronosequence, however, the differences were not as large as expected. Differences, based on Bray-Curtis distances between the young and the old dunes were no larger than those of the young soils and those of the beach sand. Though possible, it does not seem likely that the communities in the developing soils of the dune ridges were derived from those of the sometimes water immersed beach sand. The fact that there was some

resemblance between the beach and the ridge soils may be the result of the relatively low resolution of the ITS gene region for partitioning fungi into distinct phylogenetic groups.

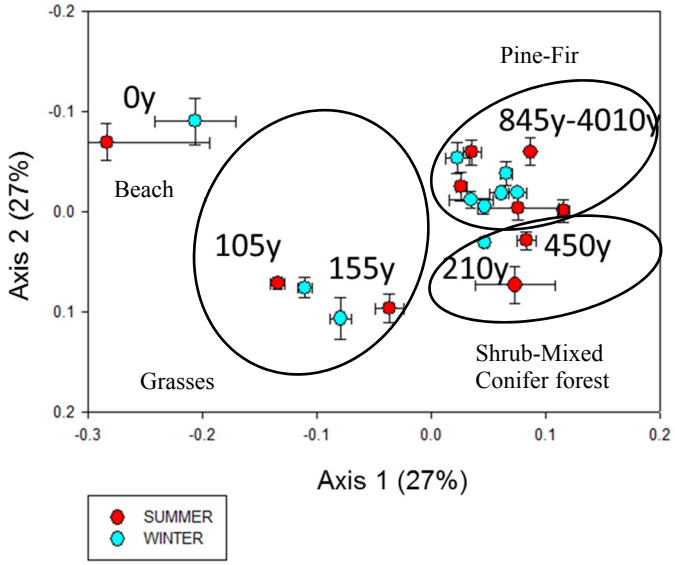

Figure 1. Bray-Curtis ordination plot showing the relationship between soil ecosystem development and fungal community composition. The 269 most abundant OTUs were used for the ordination. Percentages on each axis denote the amount of variability associated with each axis.

Phylum-level change in community composition during ecosystem development

Phylum level changes in community structure were significantly different with age ($p<0.01$), however, there were no clear patterns of change that stood out or showed a relationship with vegetative succession. The relatively rare phyla of the community, Glomeromycota, averaged less than 1% of the community rRNA sequences, and were no more dominant in the early stages of succession than in later stages. Ascomycota, the phylum with the greatest number of OTU, averaged approximately 50% of the representative phyla, but also did not change significantly with soil and ecosystem development. The results for the other phyla that were represented by a larger pool of OUT

than Glomeromycota, but smaller than Ascomycota were also not significantly different with age. A full 98.2% of the ITS sequences were matched with fungi, and so the veracity of the results appears to be strong. Thus, at the phylum level, the effects of ecosystem development, pedogenesis, and vegetative succession do not appear to impact fungal community structure.

Other phyla observed in our surveys included Basidiomycota (15%), other Dikarya (16%) (Fig 2, 3). Chytridiomycota, like Glomeromycota, were detected in pyrosequencing but were rare (averaging 0.5 to 1% of sequences). Surprisingly, though the phylum Glomeromycetes was present in all samples, it was detected in greater abundance in the sandy beach samples and 2,385-year soil compared to other soil ages. Sequences belonging to Ascomycota correspond mainly to the subphyla Pezizomycotina and Saccharomycotina.

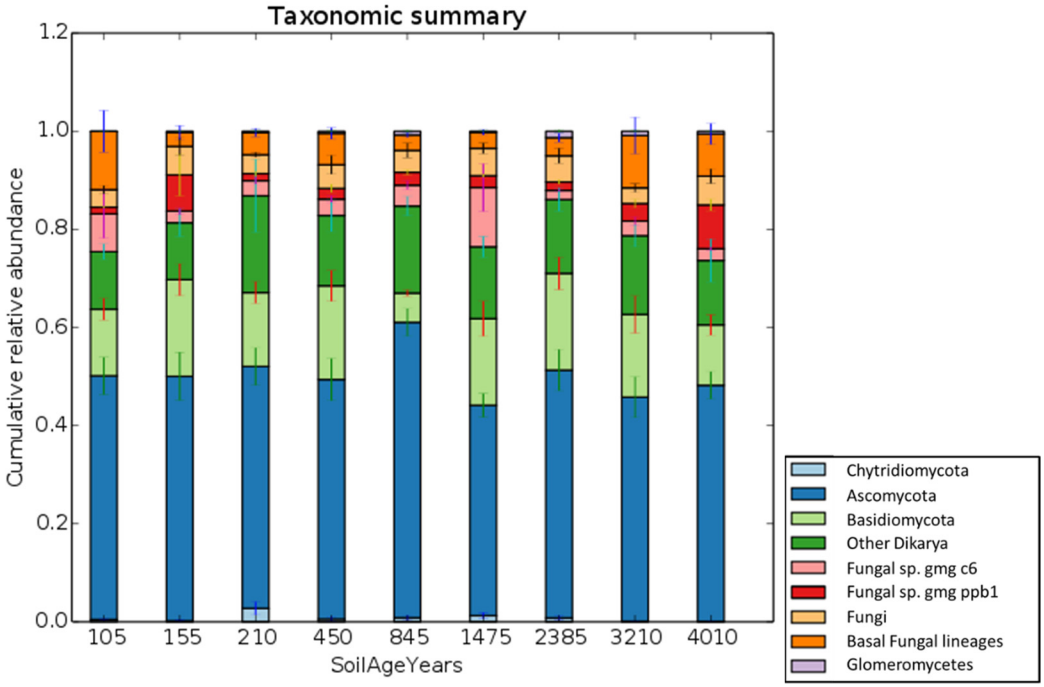

Figure 2. Relative abundance of sequences aligning to different soils in the Michigan chronosequence.

OTU-level changes across the ecosystem gradient

As part of the community change described by multivariate Bray-Curtis ordination (Fig 1), the OTU used in that analysis were described as vectors of the multidimensional space. Several OTU were identified that could significantly explain the variation along Axis 1, derived from the transition from beach sand to developing soils (Table 3 ad Table 4). Taxa associated with the shift from beach sand to developing soils were most closely related to *Sclerotinia, Mycosphaerella, Helotiales sp,* and *Ceratobasidium*.

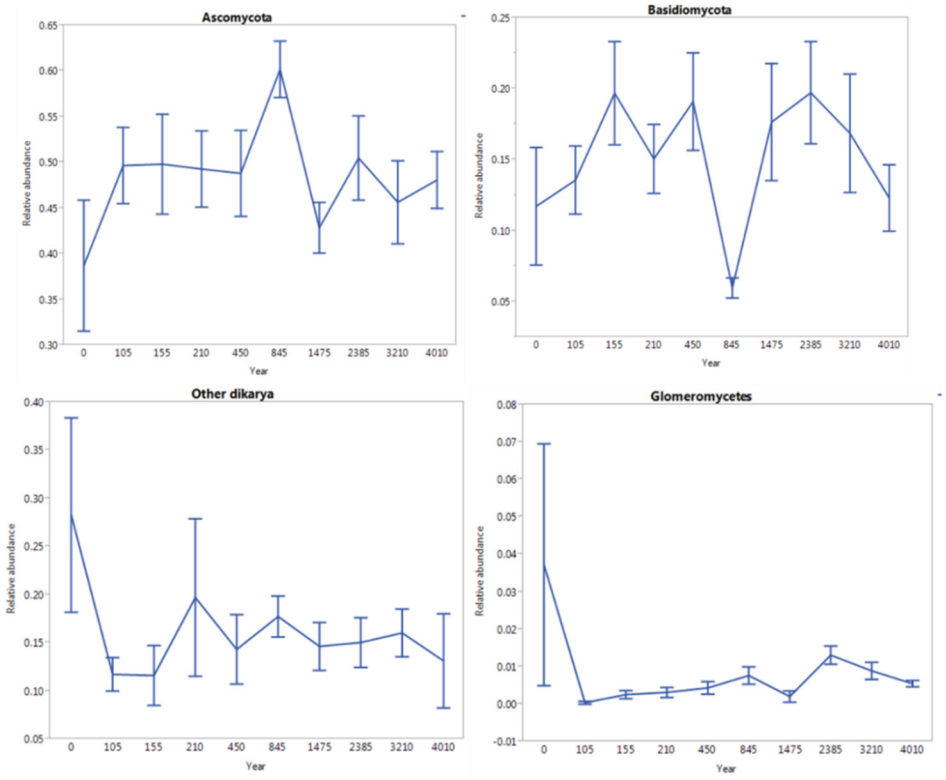

Figure 3. Relationship between relative abundance of three individual phyla and other dikarya across the ecosystem development in the Michigan chronosequence. Each point in the graph is the average of the percentage abundance of each phylum at each stage of development (summer and winter).

Table 3. OTU with r=0.5 or greater for Axis 1 of the Bray-Curtis ordination

OTU	r value in Axis 1	Taxonomy (Phyla, subphyla, family, genera, species)
2	0.592	Ascomycota; Pezizomycotina; *Sclerotinia trifoliorum*
175	0.560	Ascomycota; Pezizomycotina; Arthopyreniaceae
79	0.558	*Helotiales sp.**
169	0.530	Ascomycota; Pezizomycotina; *Mycosphaerella milleri*
94	0.508	Ascomycota; Pezizomycotina; *Sclerotinia trifoliorum*
235	0.499	Basidiomycota; Agaricomycotina; *Ceratobasidium sp*

*Initially, this OTU was classified as fungal_sp._gmg_c6, but additional blast search provided a classification of *Helotiales sp*. (max score of 905).

Using the same analysis, but with a focus only on the samples from the youngest to the oldest soils undergoing pedogenesis also were described by increasing relative abundance of taxa closely related to *Sclerotinia sp*., but differed in that *Chaetomium* and *Oidodendron* also increased with ecosystem development. Other taxa closely related to Chytridiomycota and Arthopyreniaceae also shifted along Axis1, and thus associated with changes during early ecosystem development.

Table 4. OTU with r=0.5 or greater for Axis 1 of the Bray-Curtis ordination without inclusion of the beach sand in the Bray-Curtis analysis.

OTU ID	r value	Taxonomy (Phyla, subphyla, family, *genera, species*)
196	0.691	Ascomycota; Pezizomycotina; *Sclerotinia trifoliorum*
132	0.654	Ascomycota; Subphylum; Pezizomycotina; *Chaetomium globosum*
85	0.597	*Oidiodendron**
26	0.584	Chytridiomycota
228	0.569	Ascomycota; Pezizomycotina; Arthopyreniaceae
89	0.544	Ascomycota; Pezizomycotina; *Sclerotinia trifoliorum*

*Initially, this OTU was classified as Dikarya, but an additional blast search provided a classification of *Oidiodendron*. (max score of 894).

Fungal Diversity

Shannon, Simpson, and Chao1 indices were used to estimate fungal diversity and richness. The Shannon index corrected for the number of observed species. Table 5 shows the diversity, evenness, and estimated richness of OTUs for each soil. Values for richness ranged between 172 and 335. Shannon diversity indices ranged between 5.40 and 6.22 and Simpson from 0.93 to 0.97. There was no statistically significant difference with soil age, there may be a clustering of richness in groups. For example, the two youngest soils (105 and 155 years) have a very similar richness that is lower than that for the older soils. These younger soils are dominated by grasses and bare ground. Though only an observation without statistical veracity, it is also interesting that richness is greatest at two main shifts in vegetative succession at 210 and 2,385-year soils (Table 5; Fig 4). It should also be noted that changes in soil properties correlate with fungal community change (e.g. Ca; $r=0.41$; $p<0.01$), however soil property changes are more continuous relative to the patterns of fungal change. Though these soil property changes are likely to influence community structure, the change associated with vegetation more closely mimics those of the fungal community change (Table 6). Moreover, fungal community diversity correlated ($r=0.60$; $p=0.008$) with vegetative diversity, supporting the hypothesis of the link between the two (Fig 5, 6). Overall, it is concluded that the association between fungal community richness and diversity with that of vegetative structure are supportive of the overall hypothesis of this study.

Table 5. Estimates of richness (Mean, SE[a]) of the OTUs (Chao1 index) and diversity indices (Shannon and Simpson) calculated from sequences of ITS rRNA of fungi from the Michigan chronosequence soils.

Age of Soil	Estimated Chao1 Richness of the OTU	Shannon Diversity Index	Simpson Diversity Index	Evenness Index
105	173 (8.7)	5.73 (0.23)	0.94 (0.02)	0.80 (0.03)
155	172 (31.8)	5.40 (0.39)	0.94 (0.02)	0.77 (0.03)
210	335 (55.4)	6.22 (0.63)	0.93 (0.05)	0.78 (0.06)
450	267 (29.1)	5.97 (0.25)	0.95 (0.01)	0.79 (0.03)
845	251 (38.8)	6.08 (0.15)	0.97 (0.003)	0.82 (0.02)
1,475	212 (47.8)	5.95 (0.27)	0.96 (0.01)	0.83 (0.03)
2,385	331 (89.5)	5.99 (0.36)	0.95 (0.01)	0.79 (0.03)
3,210	264 (13.1)	6.08 (0.21)	0.96 (0.01)	0.79 (0.03)
4,010	280 (33.9)	6.06 (0.37)	0.96 (0.01)	0.79 (0.04)

[a] The standard error (SE) of the mean is given in parenthesis.
[b] Calculations based on the Operational Taxonomic Units (OTU) formed at an evolutionary distance of <0.03.

Fungal richness (Chao1) was greatest in the 210-year soil and 2,385-year soil, with the lowest richness values during initial soil development and at 1475y. Richness did not

change as mature soils aged. (>1475y; Fig 2). The fungal richness mimics shifts in vegetation type, from primarily grasses (105 to 155 year), to tree shrubs (210 year), and finally to mixed forest and pine forest (450 to 4,010 years) (Lichter, 1998a; Williams et al., 2013). There appears to be a change in richness when going from the two youngest soils to the next oldest soils, as new vegetation results in greater plant cover. There was relatively high variation in the richness and diversity (differences of up to 2X) between fungal communities across the chronosequence that were related to both changes in vegetative diversity and periods of changing ecosystem vegetative structure. The results suggest that vegetation may play a strong role in the alpha and beta diversity of soil fungal communities.

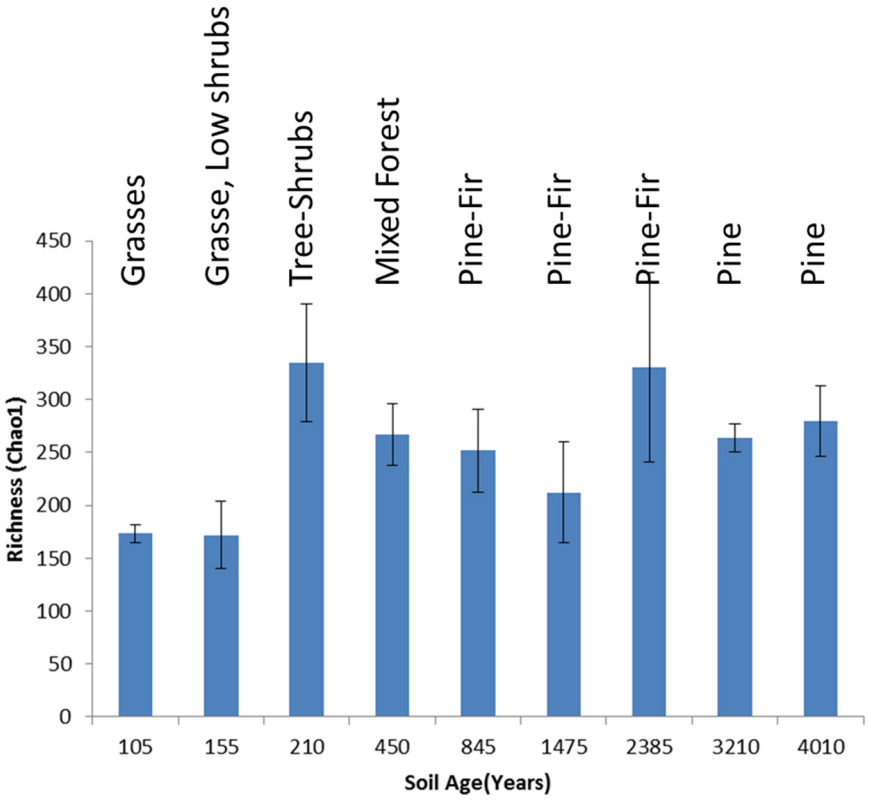

Figure 4. Richness of OTUs based on a total number of 150,075 sequences across the chronosequence. Dominant vegetation is shown above each bar.

Table 6. Mehlich-3 extractable soil cations and selected soil properties from the mineral soil across the chronosequence.

Age (years)	Ca	K	Mg	pH
		(µg/g)		
105	1289 a	18 a	115 a	7.6 a
155	744 b	18 a	121 b	7.1 b
210	685 b	19 a	100 a	5.8 c
450	120 c	20 a	23 c	3.8 d
845	110 c	26 a	10 c	3.7 d
1475	115 c	23 a	10 c	3.6 d
2385	126 c	25 a	11 c	3.6 d
3210	111 c	24 a	10 c	3.7 d
4010	101 c	24 a	11 c	3.5 d
r-value	0.84	0.65	0.85	0.82

[a]Soil properties with significant log-linear correlation to soil age ($p<0.05$). P (~ 4 µg/g) did not show a significant correlation with the soil age. Means within columns followed by the same letter are not significantly different ($P<0.05$).

[b] Results are averages from summer and winter samples.

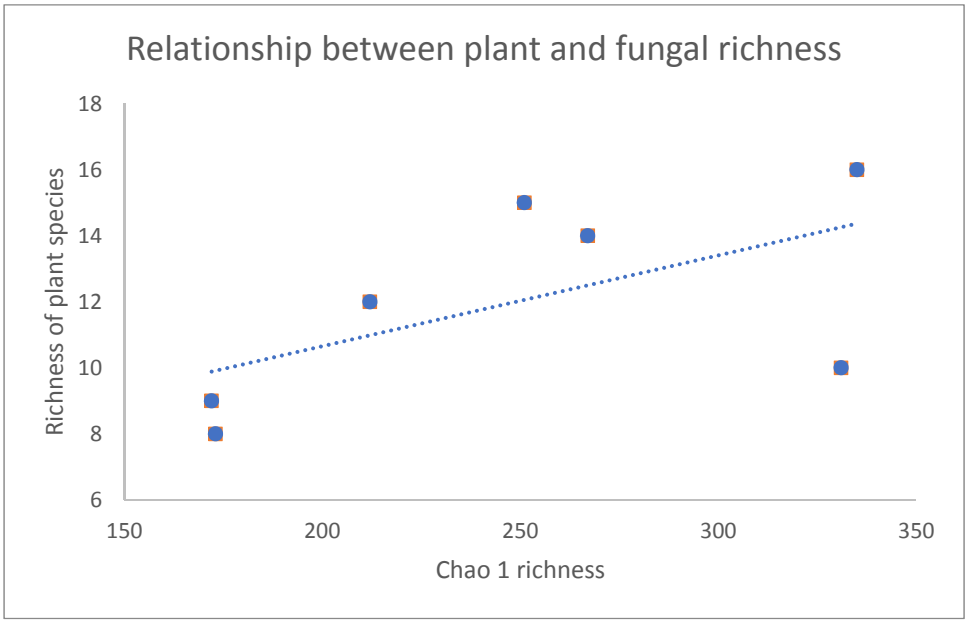

Figure 5. Richness of OTUs based on a Chao 1 estimator. Data for the dominant vegetation with greater than 0.5% cover were used to determine plant richness ($r=0.60$; $p=0.008$). Note the data only reflect up to 2385y, as per the data available from Lichter, 1998a)

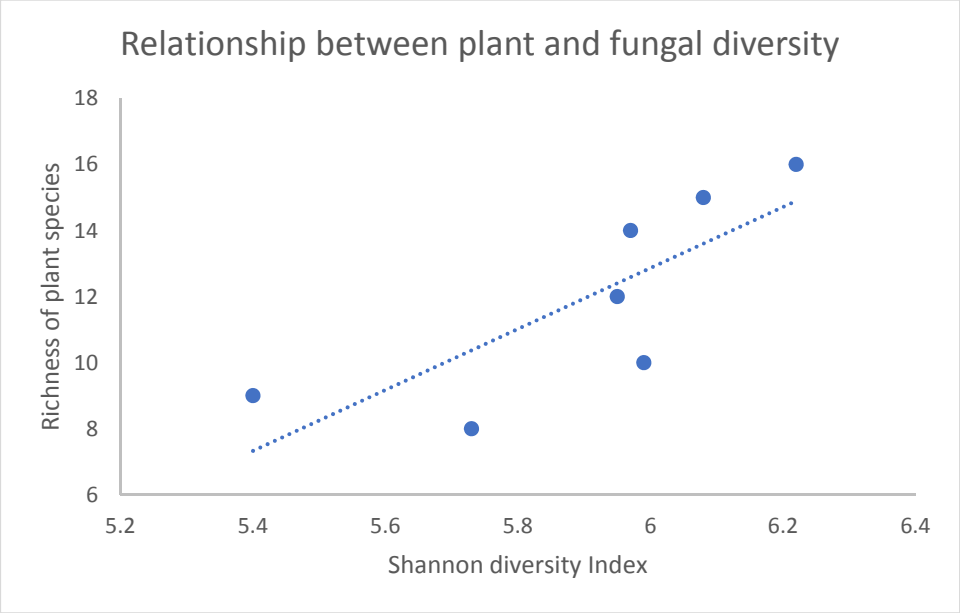

Figure 6. Richness of OTUs based on the Shannon diversity index. Data for the dominant vegetation with greater than 0.5% cover were used for plant richness (r=0.60; p=0.002). Note the data only reflect from 105 up to 2385y, per the data available from Lichter, (1998a).

Discussion

Previous work has shown changes in microbial communities (bacterial and fungal) during early ecosystem succession (between 0 to 150 years) in retreating glaciers (Blaalid et al., 2012; Brown and Jumpponen, 2014; Ohtonen et al., 1999; Welc et al., 2012). In the present study, shifts in community structure were also greatest early (105 to 845y), and support the hypothesis that fungal community change is associated with developmental ecosystem processes such as vegetative succession. Fungal community structure, however, was relatively stable between 845 and 4010 years. Though the overall degree of vegetative change was much lower during this time, there were some shifts in plant dominance from fir, spruce, and pine dominated to a primarily pine dominated ecosystem. It is notable that

despite changes in plant genera, these latter plants are all from the *Pinaceae* family, a group of trees shown to undergo lower nucleotide substitutions, compared to angiosperms, by 15 fold (Buschiazzo et al., 2012). This lower rate of nucleotide change may translate into smaller differences in functional interactions among the *Pinaceae* with soil fungal communities than those that occur between many other genera of plants. There is a need for further research into the role of plants in determining soil fungal communities and what plant factors, if any, are a major driver of soil fungal community composition and structure. Microbial related factors driving shifts in plant functional types (grasses, shrubs, pines) or changes in soil properties associated with pedogenesis are hypotheses that need further consideration.

Vegetation and Soil Property shifts associated with Fungal Community change

In some respects, the change in fungal communities during ecosystem development that ultimately reach a stable climax are reminiscent of the theory of vegetation succession (Clements, 1916). However, this stability is not intended to imply it occurs perpetually, but rather, that at some point during development that ecosystem change slows, resulting in relatively slow change in ecosystem properties. Once soil pedogenesis results in substantial change in soil nutrient reservoirs, however, the system would shift into retrogression, with concomitant change in plant and soil microbial communities (Jangid et al 2013). Indeed, proxies of pedogenic processes, such as the loss of calcium and magnesium from the soil during early ecosystem development, coincide with changing bacterial communities. Soil pH, for example, has typically been associated with bacterial community change (Fierer et al., 2010). The relationship between fungal community change and pH are weaker than that

found for bacterial communities (Rousk et al., 2010; Williams et al 2013). There is nevertheless evidence indicating that soil properties play a role in both stabilizing and supporting fungal community dynamics during ecosystem development. There were, however, also fungal community changes that occurred despite stable values of pH and extractable soil nutrients. Hence, though both soil properties and vegetative change help to explain shifting communities during ecosystem development, other factors appear to be at play in determining soil fungal community structure.

Though fungal colonization and community change are linked and often hypothesized to be driven by vegetation, there has also been consideration that fungal community establishment and stability feedback to determine aboveground plant communities (Zobel & Öpik, 2014; García de León et al., 2016). Arbuscular mycorrhizal fungi, for example, have been shown to play a strong role in structuring plant communities and favoring species under phosphorus-limiting conditions where the hyphal growth is stimulated by root exudates (Bardgett and Wardle, 2010; Grime et al., 1987). Once niches are filled and established, fungal communities (or plant communities) may be resistant to change. The role of plant-microbial feedbacks together driving habitat and biological changes that determine both belowground and aboveground communities have theoretical support (Wardle et al., 2004; Horn et al., 2017), particularly over the short term of many years, however more research into the role that fungal and plant communities shift in response and drive one another over longer time periods need further empirical testing.

Consistent with the idea that plant-microbial feedbacks shift from positive to negative during ecosystem development, OTU associated with the oftentimes pathogenic fungi *Mycosphaerella* and *Sclerontinia* (Bolton et al., 2006) increase with ecosystem

development. The occurrence of these fungi could reduce the abundance or occurrence of certain plant taxa, (Wardle et al., 2004; Williams et al.,2013). *Chaetomium globosum* can play multiple roles in soil, but a number of reports indicate it can act as a biocontrol agent against attack by pathogenic fungi (Hug et al., 2015; Park et al., 2015; Aggarwal et al., 2014). The Ascomycete *Helotiales*, like many of the above fungi are an ecologically diverse group of soil saprobes, plant pathogens, mutualistic ericoid and ectomycorrhizal (ECM) fungi, and dark septate endophytes. These broad roles of these organisms make it difficult to come to firm conclusions about the functional relevance of changing fungal community structure on ecosystem development. The relative increase of these oftentimes pathogenic microbes during ecosystem development, however, may play a role in the direction of vegetative succession, as previously shown and described (Kardol et al., 2006; Kardol et al. 2007; Jangid et al., 2013).

Seasonal Effects associated with Soil Fungal Community Change

The influence of seasonal changes on soil fungal communities during ecosystem development has not been explored extensively. Environmental variables are expected to be different, with more belowground carbon flow to roots leading to more microbial activity during the summer, as opposed to the winter (Kaiser et al., 2010). However, our research, unexpectedly, did not detect that fungal communities were different between seasons. One other study that we are aware used DNA based ITS markers to assess change in arbuscular mycorrhizal fungi with season, and like our study showed no detectable variation with season. The relative lack or low variation in fungal communities between seasons (Davison et al., 2012; Wang et al., 2012); suggests that once established, and not

impacted by disturbance, microbial communities can be relatively stable, as previously shown for bacterial communities (Williams et al., 2013; Jangid et al., 2013). If the relative abundance of DNA marker sequences is a good indicator of living fungal communities, this has broad implications for ecology. Moreover, this relative stability is unique from that of soil directly associated with the rhizosphere where dynamics are often shown to occur over time (Shi et al., 2015; Bencherif et al., 2016).

In the research of this thesis, it may have been difficult to statistically detect differences by season across a diverse chronosequence of soils. Indeed, *a posteriori* testing of each age separately indicates that 7 of the 9 were statistically different by season. It is not the point of this analysis to reverse course and change the results based on expected outcomes. The latter, after the fact analysis is a tool for testing a future hypothesis, but does raise the question of the importance of small relative differences with season for community function. Indeed, many changes in soil fungal communities, when validated statistically, sometimes (Voříšková et al., 2014) but not always (Morrison et al., 2016), involve a small minority of the fungal community taxa. Thus, the importance of small changes in fungal communities for ecosystem function, when they occur, need to be described in terms of not only statistical differences, but of ecological relevance. Based on current data, it was not shown, but would be hypothesized that season has a small but important functional change between growing seasons, likely driven by changes in the annual cycle brought about by photosynthesis.

The findings that transitions between winter and summer result in no or small change in fungal communities are primarily surprising in the context of ecosystem carbon flow and nutrient cycling. These changes are described by relatively large dynamics of

microbial biomass and activity over the annual cycle in temperate ecosystems (Williams, 2007). While there is no ecological rule that states a 30% change in microbial biomass would equate to similar dynamics in community structure, the dynamics of a complex, diverse fungal community were expected to reflect these dynamics in energy and carbon between seasons. Yet, in the context that fungal communities were found to be relatively stable in older soils with depositional ages ranging over several thousand years, it is less surprising that effects were not detected over the relatively short time periods between stages of the annual cycle. The high degree of consistency and stability in community structure associated with the plant-microbial components of the ecosystems remains to be more fully understood. It is clear that fungal communities can be highly dynamic, as indicated by change in the first few hundred years of ecosystem development, however, the relatively high degree of stability in later ecosystem development need further investigation. Clearly, the scale of temporal resolution would be expected to play a strong role in the measurement of microbial community structure. Hence, further investigations into the importance of these different scales of change and their relevance to ecosystem function are warranted.

Stability and Dynamics associated with Soil Fungal Community Structure

It has been postulated that DNA extracted from soils are not necessarily reflective of the dominant and most active members of the community. If DNA is stabilized in soil then much of the community structure would reflect fungal community history (legacy) rather than or as much as a single snapshot of fungal community structure. Fungal communities, when assayed using molecular techniques that are thought to be more

indicative of an active community, sometimes do have more dynamic shifts between soils or because of soil disturbance (Carini et al., 2016, Barnard et al., 2013, Freedman and Zak, 2015). Yet, these results are not easily rectified against the stability of fungal community structure (based on DNA) as soils aged over many thousands of years. If soils do accumulate DNA, and if the DNA can survive many hundreds of years, this would also suggest that as new DNA is deposited into soils over these long-time spans of thousands of years, that new community development would alter the relic DNA pool, and thus the observed changes in fungal communities. Hence, by this logic, there was nevertheless a change toward relatively stable communities during later ecosystem development. Relic DNA in soil organic matter could potentially explain part but not all of the observations of stable fungal community structure during latter ecosystem development.

Looking to the ecology of plant communities might offer some insights into the observations of fungal communities. The tallgrass prairie of Kansas where remnant vestiges of the native ecosystem can be observed, there are both indications of dynamics and stability (Jangid et al., 2008). Elements of organism and community dynamics and stability (Young et al., 2005) across landscapes are commonly documented and observed against the backdrop of major fluxes of energy, carbon and other nutrients related to plant species productivity. Interactions between predators and prey, moreover, and grazers with vegetation ensure that energy, and carbon are relatively dynamic. The communities that cycles these nutrients also undergo relative cycles of boom and bust, however, whole regional scale sized ecosystems can also exist in relatively stable organism-habitat states for many hundreds if not thousands of years (Scheffer et al., 2001). Scale of physical or temporal focus has a strong impact on the understanding of this relationship, but the

concept nevertheless has merit. The application of these ideas to microbes, which survive and proliferate at physically and temporally very different scales than the macro-world, however, need further scrutiny.

Potential for change among Mycorrhizal Fungal Communities

It is a common view that the majority of land-plant species form mutualism with fungi, such as Arbuscular mycorrhizal fungi (AMF), which support nutrient acquisition, growth, and reproduction (Heckman et al., 2001). Arbuscular mycorrhizal fungi, however, were not widely observed across the developmental ecosystem gradient, even among the grasses. It should be noted that the focus of the experiment and selection of primers were not specifically directed toward of AMF or other mycorrhizal fungi (Řezáčová et al., 2016). If plant-microbial feedbacks were important for determining fungal community structure, the lack of AMF is somewhat surprising, however. While fungi such as Gigaspora were shown to have closely related taxa in the soils that were surveyed, they also did not explain differences across the chronosequence, despite there often successful mutualisms with pine species (Dunstan et al., 1998). The Pezizomycotina subphylum, which belongs to Ascomycota, includes numerous species that form ectomycorrhizae (Spatafora et al., 2006). The orders Pezizales, in particular, include some of the largest numbers of EcM fungal lineages (Tedersoo et al., 2010). Ecto and endo-mycorrhizal plant-fungal interactions occur across numerous phyla and families (Trocha et al., 2012), but the detail needed to confirm the presence of these taxa were not possible using the current methodology. The occurrence of these mycorrhizal fungi would be, however, consistent

with the observations herein, where soil fungal community change was observed to be coupled with a change in vegetation, especially during early ecosystem development.

Phosphorus levels tend to be relatively low in these soils (<7 ug g^{-1} soil) and the availability of the nutrient might be a major limiting factor for plant growth. This latter point would tend to suggest that mycorrhizae would be necessary for the growth of vegetation across the chronosequence, however, if levels of phosphorus are greater in subsoil this could preclude the problem of P acquisition. In this regard it is important to note that many mycorrhizal fungi may be more prevalent in the O-layer above the mineral surface and thus not observed using our methods.

Mycorrhizal fungi, and particularly AMF are considered to have been some of the first fungi to inhabit land (Buschiazzo et al., 2012). Their early interaction with newly developing types of vegetation during the last ~500 million years have been used to explain the broad distribution of mycorrhizae across fungal phyla and their symbiosis with so many different types of plant species (Wilkinson, 2001). The widespread nature and diversity of fungi that can form mycorrhizae with plants make it difficult to discern if the presence or absence of a particular taxa are indicative of this plant-fungal interaction. It has been documented using mycorrhizal specific primers that plant host identity is associated with mycorrhizal fungal community composition during ecosystem development (Martinez-Garcia et al., 2015). So, though it is not possible to come to firm conclusions about the role of mycorrhizae during ecosystem development at WP, further investigation of the fungi, using more specific molecular DNA markers could help to discern whether mycorrhizal fungi are ubiquitous and unchanging or a major part of community change during ecosystem development.

Overall, the general pattern of fungal community change is consistent with expected changes in plant communities and soil properties during ecosystem development. While it is not known how well our surveys may present as pathogens, saprophytes or symbiotic fungi, the degree of belowground community change is consistent with the generally fast pace of vegetation turnover in the first several hundred years, relative to the more stable plant community structure that occurs during the last several thousand years of ecosystem development that were measured.

Richness and alpha diversity of Soil Fungal Communities

The factors that influence the diversity (alpha diversity) and richness of fungal or other microbial communities in soil have been widely (Broeckling et al., 2008) studied, but have come to only a few firm conclusions. Ecosystem type and to a lesser extent, pH, have both been shown to be associated with changes in bacterial diversity, with the latter suggesting that lower soil pH results in lower diversity compared to neutral pH. Indeed, pH had been described as a major driver of both bacterial richness and diversity (beta-diversity) and structure (alpha-diversity), while having small to no effect on fungal communities (Rousk et al., 2010). The research at WSP, like many other studies also suggest that pH is not a primary driver of soil fungal community alpha diversity.

Fungal community diversity has been linked to latitude whereby tropical systems were shown to have greater diversity than temperature and boreal systems, however, this was not true for all groups of fungi (Tedersoo et al., 2014). Due to limitations in measuring a multitude of functions simultaneously, the functional importance of diversity, per se, in soil are not well described (Zak et al., 2003). There are also questions about whether 1000

or 2000 taxa of bacteria or fungi in a soil relate to biogeochemically different outcomes, especially if there is considerable functional redundancy. The results in thesis, as described below, have relevance toward this question.

Over a decade ago it was postulated that soil microbial diversity would be related to surface area, pore size distribution, and particle size (Zhou et al., 2002). The idea was relatively simple, suggesting that microbes such as bacteria could exist and remain safe from predation in soils with numerous small pores. The data in our experiment would tend to support this idea whereby pedogenesis increase isolated pore spaces and therefore fungal richness and diversity, but the link is still weak because there are no direct measures of surface area in the soils at Wilderness Park. Regardless of age, however, a comparison of plant and soil fungal diversity present stronger support for a linkage between plant and fungal taxa diversity across the developing ecosystem.

Though fungal richness mimics shifts in vegetation and thus may be indicative of both direct and indirect plant-microbial feedbacks that influence soil fungal and plant communities during soil-ecosystem development, the highest richness values may be associated with periods of ecosystem or vegetative transition, in which plant diversity, root exudates, and litter quality allow for a greater number of species types to simultaneously, though temporarily, co-exist. This idea that transitions or disturbances have influence microbial diversity compared to that of stable semi-native ecosystems (Schnoor et al, 2011; Jangid et al, 2008) has been reported and thus deserves further investigation; including their effects on nutrient cycles (Broeckling et al., 2008).

Plant diversity was shown to be related to beta diversity across a number of different grasslands (Prober et al., 2015), and other similar links between plant and fungal

diversity have been observed when using small numbers of plant and fungal taxa (Van der Heijden, 1998), but at field relevant scales the linkages between plant and fungal diversity need further testing to understand this relationship. Indeed, because plant-microbial feedbacks can be both positive and negative from the pot to the ecosystem scale, untangling the relationship will require a full range of many different types of research studies. Results from WSP indicate that there was lower fungal richness early when plant richness was also low, and that when fungal diversity was highest also corresponded with greater plant diversity (>450y; Lichter, 1998a). The results from the research in this thesis thus support the idea of a linkage between fungal and plant diversity at ecosystem relevant scales. The functional relevance of these changes is not known, but could be seen to be related to differences in direct plant-fungal interactions, and the utilization of a greater diversity of metabolic substrates.

Conclusion

The pattern shown by the soil fungal community herein correlated with plant succession during thousands of years of ecosystem development. Dynamics during early and stability during latter ecosystem development give clues of a possible intimate relationship between the fungal community and plant succession during ecosystem development. Changes in the soil fungal community between summer and winter were not large, if at all, which suggests fungal resilience to short-term environmental changes. The patterns of fungal community change and diversity associated with succession support the idea that changes during ecosystem development are not only related to the dynamics and stability of plant communities, but may also support the idea of biotic plant-soil feedbacks.

Overall, the results indicate fungal community alpha and beta diversity are shaped at the ecosystem level but also potentially related to individual soil properties such as Ca, pH, and Mg.

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CHAPTER 3

Plant Invasions Associated with Change in Root-Zone Microbial Community Structure and Diversity

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RESEARCH ARTICLE

Plant Invasions Associated with Change in Root-Zone Microbial Community Structure and Diversity

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Abstract

The importance of plant-microbe associations for the invasion of plant species have not been often tested under field conditions. The research sought to determine patterns of change in microbial communities associated with the establishment of invasive plants with different taxonomic and phenetic traits. Three independent locations in Virginia, USA were selected. One site was invaded by a grass (*Microstegium vimineum*), another by a shrub (*Rhamnus davurica*), and the third by a tree (*Ailanthus altissima*). The native vegetation from these sites was used as reference. 16S rRNA and ITS regions were sequenced to study root-zone bacterial and fungal communities, respectively, in invaded and non-invaded samples and analyzed using Quantitative Insights Into Microbial Ecology (QIIME). Though root-zone microbial community structure initially differed across locations, plant invasion shifted communities in similar ways. Indicator species analysis revealed that Operational Taxonomic Units (OTUs) closely related to *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Ascomycota* increased in abundance due to plant invasions. The Hyphomonadaceae family in the Rhodobacterales order and ammonia-oxidizing *Nitrospirae* phylum showed greater relative abundance in the invaded root-zone soils. Hyphomicrobiaceae, another bacterial family within the phyla *Proteobacteria* increased as a result of plant invasion, but the effect associated most strongly with root-zones of *M. vimineum* and *R. davurica*. Functional analysis using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) showed bacteria responsible for nitrogen cycling in soil increased in relative abundance in association with plant invasion. In agreement with phylogenetic and functional analyses, greater turnover of ammonium and nitrate was associated with plant invasion. Overall, bacterial and fungal communities changed congruently across plant invaders, and support the hypothesis that nitrogen cycling bacteria and functions are important factors in plant invasions. Whether the changes in microbial communities are driven by direct plant microbial interactions or a result of plant-driven changes in soil properties remains to be determined.

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Introduction

Invasive plants are implicated in altering plant community dynamics, disturbance regimes, net primary productivity, and nutrient cycles [1–3], which threaten ecosystem functioning and stability. The soil microbial community plays a central role in ecosystem functioning, including serving as plant symbionts, mediating plant nutrient acquisition, nutrient cycles, and soil formation [4]. These belowground communities have been implicated in invasive species success, but only a few studies have assessed how belowground microbial taxa change with plant invasions into ecosystems [5].

Important feedbacks between plants and the soil biotic community have begun to shed new light on plant rarity and invasiveness. High density of native species, such as *Rhododendron maximum*, reduced soil nutrient availability and mycorrhizae abundance associated with surrounding plants [6–9]. *Alliaria petiolata* in contrast, an invasive plant, reduced arbuscular mycorrhizal fungi (AMF) colonization of native trees and overall tree growth [10]. It was thought that the reduction in AMF occurred as a result of the plant releasing glucosinolate containing root exudates [5]. Relatively uncommon native plants were also shown to be more negatively affected by pathogens while invaders, in contrast, showed evidence of more positive plant-microbial feedbacks [11, 12]. These results have been further corroborated using reciprocal transplant studies of plant-soil-microbial feedbacks associated with invaded and native ranges of *Triadica sebifera* [13] and *Pinus contorta* [14]. Still, other effects related to soil nutrient cycling indicated that a mixture of the exotic grasses *Avena barbata* and *Bromus hordeaceous* had elevated levels of nitrate, ammonia oxidizers, microbial N, and gross nitrification rates compared to the native grass *Nasella sp*. [15]. Overall, these results show that microbial communities and their processes are altered due to the invasion of exotic plants, and provide evidence that invader and plants native to an ecosystem have underlying differences in their interactions with belowground microbial communities. Meta-analysis have concluded, specifically, that nitrogen turnover is greatly altered and often greater following exotic plant invasion of ecosystems dominated by native plants [16, 17].

Most of the microbial studies conducted have either been based on greenhouse plantings or field establishment of plants rather than observing changes that occur due to natural invasion in the landscape. There are also few studies that have measured microbial communities in the root-zones of native and invaded soil-ecosystems to determine the structure and composition of microbial communities and whether these field observations corroborate the multitude of different litter-based and experimental observations [18]. A recent meta-analysis suggested the importance of invader-ecosystem interactions and the lack of studies across taxonomic groups and habitats [19]. Meta-analyses help to unify ideas and hypotheses, but can mask the relationship between invasive plant species and their influence on soil nutrient pools and microbial dynamics, which are thought to be quite species specific [20, 21]. Studies that are inclusive of multiple invasive plants and their effects on root-zone microbial community structure and function can thus help to inform whether belowground changes are specific, or broadly associated with plant invasion.

Our overall objective was to understand the effects of plant invasions on soil microbial community structure and its potential linkages to plant-ecosystem function. Specifically, we had two main questions: (1) Do invading species with different taxonomy and phenetic traits have similar or unique effects on microbial communities in root-zone soils?; and (2) are changes in root-zone communities consistent with changes associated with microbial function and soil processes?

Materials and Methods

Species and site descriptions

Study sites were selected that met the following criteria: (1) each site must have invaded and non-invaded (reference) areas, the latter of which represents the site pre-invasion; and (2) one invasive species dominates its strata in the invaded plot—no more than 10% cover of other invasive species are located in the invaded plot. Based on these criteria, three sites were selected in the Ridge and Valley Province of the central Appalachian Mountains in Virginia, USA (Table 1). One site (M) was invaded by a C_4 subcanopy grass (*Microstegium vimineum* [Trin] A. Camus; Japanese Stiltgrass) (Mv), another (R) was invaded by a shrub (*Rhamnus davurica* ssp *davurica* Pall.; Dahurian Buckthorn) (Rd), and the third (A) was invaded by a tree (*Ailanthus altissima* (Mill.) Swingle.; Tree of Heaven) (Aa). All three populations were chosen at locations where a nearby non-invaded reference site was available that was similar in plant community composition, slope, and aspect as the invasion. The native vegetation from these non-invaded sites was used as reference (MvR, AaR, RdR). In all cases it was concluded that the reference site was capable of being invaded, and did not have overarching preexisting difference from the invaded site (Table 1). The term "invasion" is used to differentiate between invaded and non-invaded effects. Two sites were in use for another research grant funded by the USDA Joint Venture program (11-1480-01, 2011–2015). David Carr at the Blandy Experimental Farm provided permission to sample soils in the *Rhamnus* and reference sites. William McShea provided permission to sample soils at the Smithsonian Conservation Biology Institute forest site in *Ailanthus* and reference locations. We obtained permission from Eastern Divide District to sample soils at the Jefferson National Forest site in *Microstegium* and reference locations. The lands were public and no protected species were sampled.

Microstegium vimineum is a shade-tolerant C4 annual grass common to much of the Eastern US where it has been implicated in reducing tree recruitment (e.g., [20]), decreasing microarthropod diversity [24], and changing soil chemistry and soil microbial communities [25].

Table 1. Details of Sampling Locations.

Location	Invasive Species	Soil Type	Native Species
A: Smithsonian Conservation Biology Institute, Front Royal at an elevation of 378m. (Latitude = 38.88553N, Longitude = -78.13844W)	*Ailanthus altissima* (**Aa**)	Montalto loam. Taxonomic class: Fine, mixed, semiactive, mesic Ultic Hapludalfs.	**AaR**: Red oak species (*Quercus species*), tulip poplar (*Liriodendron tulipifera*), and common hackberry (*Celtis occidentalis*). The understory had an abundance of spice bush (*Lindera benzoin*) and infrequent dunal pawpaw (*Asimina triloba*) and bush honeysuckle *(Lonicera maackii*).
M: Jefferson National Forest, Montgomery County at an elevation of 2280m. (Latitude = 37.28108N, Longitude = -80.47523W)	*Microste-gium vimineum* (**Mv**)	Berks-Weikert composition on slopes from 15 to 25 percent [22]. Taxonomic class: Loamy-skeletal, mixed, active, mesic Typic Dystrudepts.	**MvR**: The forest canopy is primarily red maple (*Acer rubrum*), white oak (*Quercus alba*), and red oak (*Quercus rubra*). The understory community composition is typical of Appalachian forests of Virginia with total site richness of 78 species [23].
R: Blandy Experimental Farm, Boyce at an elevation of 183m. (Latitude = 39.05923N; Longitude = -78.05428W)	*Rhamnus davurica* (**Rd**)	Timberville silt loam. Taxonomic class: Fine, mixed, active, mesic Typic Hapludults Poplimento-Rock outcrop complex. Taxonomic class: Fine, mixed, subactive, mesic Ultic Hapludalfs.	**RdR**: Perennial grasses (e.g., *Panicum virgatum*) and infrequent annual and perennial herbaceous weeds

The following experimental groups were studied: (i) location (A, M, and R); (ii) invasion status (Invasive plants (I) and Native plants (N)); and (iii) interaction of location and invasion status (Aa, AaR, Mv, MvR, Rd, and RdR).

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This *M*. *vimineum* invasion is located near an old homestead upslope from the site, but the exact date of establishment is unknown. The reference site was selected across an ephemeral stream likely acting as a barrier to dispersal to the *M*. *vimineum* population.

Rhamnus davurica ssp. *Davurica* is a deciduous short-lived shrub native to China, North Korea, Mongolia, eastern Siberia and Japan. It was commonly planted in the Northwestern US plains for windbreaks in the 1930's. Both *R*. *davurica* and *Rhamnus cathartica* L. (Common Buckthorn) were incorporated into the Virginia Arboretum in 1939, but only *R*. *davurica* has invaded into the Blandy Experimental Farm in Boyce, Virginia, USA. The site invaded by *R*. *davurica* has been unmanaged for over 3 decades and has not for the Blandy Experimental Farm. The *R*. *davurica* invasion into the grassland is well documented at this farm and has occurred over a 25-year period.

Ailanthus altissima is a common urban, roadside, and natural area invasive tree capable of growing in a variety of non-managed and disturbed systems worldwide; spreading both sexually and clonally [20, 25, 26]. This fast growing tree has putative allelopathic effects [23], though the ecological impacts of *A*. *altissima* are largely unknown [27]. The *A*. *altissima* invasion occurred at this site over the last 40 years following a clear cut on one side of a logging road. The other side of the road was not logged and is an non-invaded reference area. While logging removed overstory vegetation, the impacts on soil were relatively small.

Soil sampling and analyses

Soil sampling locations were selected by a stratified random technique. A 50 m transect was established along one edge of each plot (same for both invaded and non-invaded plots). The transect was divided into five replicate 10 m reaches. A random number generator was used to pick a meter mark within each 10 m reach for establishing a perpendicular transect. Once the position of the transect was identified, the random number generator was used to select a distance along the perpendicular transect for the soil sample. At this location, a coin was flipped to choose the right or left side of the perpendicular transect to sample. The soil sample was taken 1 m away from the perpendicular transect. If the final location was occupied by a rock or tree, the closest location where a soil sample could be taken was used. Soils were sampled at each location using a standard 7-cm soil corer (Model # 402.25, AMS Inc., American Falls, ID, USA).

At each sample location, the litter and humus layers were removed. The soil corer was washed with 95% alcohol before sampling and between each soil sample. The soil sampler was then driven in to a depth of 10 cm using a professional slide hammer (Model 57780, AMS Inc., American Falls, ID, USA). Leaf litter, roots, and large debris were removed from each sample (100 cm^3) and the soil samples were placed in a sterile zip-top bag and refrigerated in a cooler until the samples could be stored at -5°C in the lab at Virginia Tech. This resulted in ten randomly selected soil samples at each site, five of which were from the invaded and five from the adjacent non-invaded reference. Each soil sample was sieved through an alcohol washed #20 soil sieve (Model H-3903, M & L Testing equipment, Calgary, Alberta, Canada), and individually mixed and homogenized. All precautions against contamination were taken. Subsamples of the sieved soil were analyzed for several nutrient cations and anions, extractable nitrogen, and microbial diversity. The subsamples for nutrient cation analysis were extracted with 1*M* KCL and analyzed using ICP. Soil parameters measured were: pH, cation exchange capacity, and concentrations of P, K, Ca, Mg, Zn, Mg, Cu, Fe, and B.

A separate subsample was incubated for seven days at field moisture water potential. Directly before and following the seven days of incubation, samples were extracted with 1M KCl to determine extractable inorganic nitrogen content. Based on water content and particle size analysis, it was estimated that water potential for all soil samples ranged between -100 to

-500 KPa. Sampling in May ensured that each sample was near saturation and similarly moist. Total nitrate and ammonium ions (μg g^{-1}) were measured with a Lachet autoanalyzer (Quikchem 8500 Series 2) and turnover (T_1-T_0) x (100/ T_0) was calculated following a one week incubation of soil (25°C). Wilcoxon (rank-sums) test with a normal approximation to the two-sample test was performed in JMP® Pro, Version 11 (SAS Institute Inc., Cary, NC, 1989–2007) to check whether the turnover was different between invaded and non-invaded samples. Microbial community structure and diversity were determined on another subsample of soil DNA (see below).

Univariate statistical analysis on soil nutrients

A two-way analysis of variance was used to determine significant effects of location, invasion status (invaded or non-invaded), and their interaction on soil nutrition. Means were separated using Tukey HSD at alpha = 0.05. All ANOVAs were performed with JMP statistical software (SAS Institute Inc., Cary, North Carolina).

DNA extraction and amplification

For both the 16S rRNA gene analyses and the ITS analyses, 0.5 g of freeze-dried homogenized soil was weighed and DNA was extracted from each soil sample using PowerSoil® DNA Isolation Kit (MoBio) according to the manufacture's protocol. DNA quality was checked on a 0.8% (w/v) agarose gel. DNA concentrations were determined by fluorometric quantification using the Qubit® 2.0 platform with Qubit dsDNA HS Assay Kit (Life Technologies). DNA was diluted to 50 ng μL^{-1} and stored in a -20°C freezer. It was used for the PCR-based protocol described in [28], using the PCR bacteria/archaeal primers 515F/806R targeting the V4 region of the 16S rRNA. ITS1FI2/ ITS2R were used to amplify the spacer ITS1 of the internal transcribed spacer (ITS) rDNA region [29, 30]. The reverse amplification primer also contained a twelve base barcode sequence. Both PCR primers contain sequencer adapter regions. The enzyme used in the PCR reaction was KAPA2G Robust (5 U/μL) from Kapa Biosystem. For 16S rRNA assay the 25 μL reaction mixture contained 0.5 μL of dNTPs (10 mM), 0.5 μL of each primer (10 μM), 50 ng of the DNA template, 1 μL of DMSO (100%), 0.2 μL of the enzyme (5U/μL) and 5 μL of Buffer GC (Kapa Biosystem). For the ITS assay, the PCR reaction final volume was 25 μL, containing 0.5 μL of dNTPs (10 mM), 0.625 μL of each primer (10uM), 50 ng of the DNA template, 1.25 μL of DMSO (100%), 0.2 μL of the enzyme (5 U/μL) and 5 μL of Buffer A (Kapa Biosystem). The PCR conditions used were as follows: for the 16S assay, there was a denaturation step at 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, an annealing step at 60°C for 60 seconds, an extension step at 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. For the ITS assay, there was a denaturation step at 95°C for 15 seconds, 35 cycles of 95°C for 30 seconds, an annealing step at 55°C for 30 seconds, an extension step at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The specificity of the PCR was further evaluated by running on a 1.2% (w/v) agarose gel. The concentration of the DNA was obtained by Fluorometric Quantitation (Qubit® 2.0 Life Technologies) before sending samples to sequencing. From the bacterial experiments, two out of the 30 samples did not show 16S rRNA gene amplification. Hence, 28 samples were sent for 16S rRNA gene sequencing, whereas all 30 samples were sent for ITS rDNA sequencing. Sequencing on the Illumina MiSeq platform was conducted by the Virginia Bioinformatics Institute core facility.

Sequence data analyses

In the bacterial data, an 'Rd' sample (F8) was removed from further analysis due to contamination on the sequencing plate. The paired end reads were stitched using *Pandaseq* [31]. For the

fungal data, only read-2s with a quality threshold of 30 were used for further analyses. The bacterial and fungal sequencing data were analyzed using *QIIME* [32]. Briefly, reads were clustered into OTUs based on 97% sequence similarity using *uclust* [33] and *usearch61* [33], for bacteria and fungi respectively, using an open reference OTU-picking strategy. The representative sequence of an OTU was used to assign it a taxonomy, using *uclust* against the Greengenes reference database version 13_8 [34, 35] for bacteria, and *RDP classifier* [36] against the UNITE reference database version 12_11 [37] for fungi.

Comparison and statistics on groups

A sampling depth threshold of 80,000 and 3,200 sequences per sample, for bacteria and fungi respectively, was used for the diversity and taxonomic summary analyses. The beta diversity was calculated using weighted and unweighted Unifrac [38] (for bacteria), and Bray-Curtis [39](for fungi) distance metrics. To identify group differences, the distances were used for Principle Coordinate Analysis [40] and visualized in 3D-plots using EMPeror [41]. The chao1 [42] and observed species metrics were used to plot alpha rarefaction curves. The alpha diversity was calculated using PD whole tree (for bacteria only), chao1, observed species, and Shannon and Simpson indices for bacteria and fungi. The bar graphs with standard error bars were used to visualize microbial taxonomic summaries of the interaction between location and invasion at different levels and generated using custom python scripts. Multivariate data analysis methods of adonis [43] and Analysis of Similarity (ANOSIM) [44] were used to identify whether groups were significantly different. Indicator species analysis (ISA) [45] in PC-ORD Version 6 [46] was used to identify taxa that were significantly (indicator value > 70 and p-value < 0.01) associated with invasion when blocked by geographic sites/location. A seed of 16 and 18 with 5000 runs was used for the bacteria and fungi, respectively.

Functional analyses

The actual abundance (counts) of the OTUs belonging to the significant genera from ISA was used for functional analyses using *PICRUSt* [47]. OTUs not part of the closed reference OTU picking were filtered out. Using default parameters, the filtered OTU table was normalized by the 16S rRNA copy number abundance to identify true abundance followed by metagenome functional prediction for each sample. The metagenomes were collapsed into KEGG pathways. Using STAMP [48], two-sided Welch's t-test [49] with Benjamini-Hochberg [50] and Storey [51] multiple testing corrections were performed to identify KEGG pathways that were significantly different (q-value < 0.05) between invaded and non-invaded samples.

Results

Soil nutrients change associated with invasion

Many soil parameters, particularly pH, P, K, Mg, Zn, and B varied among locations (Table 2). Four soil parameters varied between invaded and non-invaded plots across locations (Table 2). Interestingly, 7 of the 11 soil parameters varied between invaded and non-invaded plots among species, including pH, P, and CEC (Table 2).

In most cases, nutrient parameters were higher in the invaded patch compared to the non-invaded patch (Table 2). For example, *Microstegium vimineum* increased pH, K, and Ca, *Rhamnus davurica* increased K and Mn, while *Ailanthus altissima* lowered pH, Ca, Mn, Fe, and B (Table 2).

Concentrations of nitrate in soil ranged from 1.5 to 18.3 and ammonium from 9 to 29 $\mu g\ g^{-1}$ soil. Following one week of incubation (22°C), the concentrations increased, on average, ranging

Table 2. Mean Values (St. Dev.) and Two-Way Analysis of Variance on Soil Nutrition Parameters from Three Sites in Central Appalachian Mountains with Invaded and Non-Invaded Locations.

Location	M		R		A		Location	Invasion Status	Location x invasion status
Invader	*Microstegium vimineum*		*Rhamnus davurica*		*Ailanthus altissima*				
Invasion status	Invaded	Non-invaded	Invaded	Non-invaded	Invaded	Non-invaded			
pH	5.36** (0.27)	4.9 (0.15)	6.69* (0.2)	6.66 (0.12)	6.29* (0.12)	6.67 (0.31)	**<0.001**	0.608	**0.001**
P	2.4 (0.5)	2.2 (0.5)	11.8 (6.8)	4.4 (1.1)	2.6 (0.8)	2.0 (0.00)	**<0.001**	**0.015**	**0.016**
K	106.8** (28.3)	52.4 (5.9)	104.0* (23.8)	72.4 (18.5)	150.4 (35.8)	126.4 (37.6)	**0.002**	**0.002**	0.442
Ca	553.2** (208.1)	156.2 (26.3)	1151.6 (175.9)	1123.6 (117.4)	1174.0* (285.4)	1634.8 (265.6)	**<0.001**	0.872	**0.000**
Mg	65.0 (11.2)	32.8 (2.6)	97.0 (7.3)	88.4 (9.2)	164.0 (46.8)	208.4 (25.7)	**<0.001**	0.887	**0.003**
Zn	2.18 (0.37)	1.88 (0.29)	1.36 (0.31)	1.28 (0.25)	4.72 (1.18)	5.12 (1.11)	**<0.001**	0.980	0.538
Mn	12.62*** (1.12)	15.4 (7.61)	11.44*** (5.7)	7.48 (0.64)	14.96** (2.58)	32.3 (7.74)	0.478	**0.004**	**<0.001**
Cu	1.4 (0.22)	1.48 (0.50)	0.60 (0.23)	0.82 (0.18)	1.24 (0.55)	0.78 (0.19)	**0.003**	0.677	0.091
Fe	18.5 (4.93)	22.4 (5.37)	16.4 (18.1)	18.5 (4.93)	3.98* (1.08)	2.46 (0.67)	**0.027**	0.118	0.185
B	0.3** (0.1)	0.2 (0.0)	0.5 (0.1)	0.5 (0.1)	0.8** (0.2)	1.4 (0.3)	**0.000**	**0.017**	**<0.001**
CEC	6.4 (0.42)	6.1 (1.16)	6.9 (0.86)	6.5 (0.62)	8.14 (1.60)	10.26 (1.50)	0.052	0.222	**0.034**

Parameter = soil nutrition trait; Location = the three locations where each species was sampled; Invasion Status = invaded and non-invaded plots. Bolded values indicate significant ($p \leq 0.05$) effects. All nutrient units are μg element g^{-1} soil. The statistical test (Tukey HSD means separation) is between invaded and non-invaded within site.

* = $p \leq 0.05$

** = $p \leq 0.01$

*** = $p \leq 0.001$

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from non-detectable to 24 for nitrate and 33 to 51 μg g^{-1} ammonium. Wilcoxon (rank-sums) test with a normal approximation to the two-sample test showed that turnover of nitrate during the one week incubation was observed to be significantly greater in association with invasion (p-value = 0.014), averaging 137 and 61 percent per week of incubation in invasive and non-invasive factors, respectively (Table 3). On the other hand, turnover of ammonium during the one week incubation was observed to be greater, but not significant, in association with invasion, averaging 154 and 123 percent per week of incubation in invasive and non-invasive factors, respectively. These results suggest that invasion increased the rate of N cycling and availability of nitrogen for plant uptake from soil. The results also agree with the phylogenetic and

Table 3. Turnover (Percentage) of Inorganic Nitrogen (Mean, SE[a]) in Non-Invaded and Invaded Locations at Three Sites in Central Appalachian Mountains.

Location	M		R		A			
Invader	*Microstegium vimineum*		*Rhamnus davurica*		*Ailanthus altissima*		*All plant species*	
Invasion status	Invaded	Non-invaded	Invaded	Non-invaded	Invaded	Non-invaded	Invaded	Non-invaded
NO_3	42 (8)	-20 (20)	236 (106)	196 (70)	108 (24)	33 (5)	137 (45)	61 (31)
NH_4	247 (41)	347 (45)	6 (19)	-61 (20)	209 (48)	83 (17)	154 (35)	123 (48)

[a] The standard error (SE) of the mean is in given in parenthesis.

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functional analyses which showed greater N cycling genes, and greater relative abundance of nitrifying and putative nitrogen-fixing bacteria in the invasive compared to non-invasive soil.

Alpha diversity of microbial communities associated with invasion

Bacteria. A total of ~17.8 million high quality 16S rRNA gene sequence reads were obtained from the invaded and non-invaded plots. The sequences from 27 samples possessed a 254-bp average length and will be submitted to the NCBI Sequence Read Archive according to MIMS standard. There were a total of 210,007 distinct OTUs (observations) across samples with a total of 4,444,765 sequences (counts) that were assigned to these OTUs. The observation refers to the number of distinct OTUs; whereas the count refers to the abundance of bacteria belonging to these OTUs in samples. The mean and median counts per sample were 164,621 and 158,958, respectively. A sampling depth threshold of 80,000 counts per sample removed one sample from further analyses. The average Good's coverage for the bacterial data across 26 samples was 96.1%.

Chao1, observed species, Shannon, Simpson, and PD whole tree metrics were used to calculate alpha diversity (species diversity within the community). A non-parametric test with the default 999 Monte Carlo permutations with an FDR correction showed significant differences ($\alpha<0.05$) between locations and between location x invasion for alpha diversity but not between invaded and non-invaded samples (Shannon and Simpson metrics were not used) (Data not shown). However, the rarefaction curves, which are sample size independent, showed trends that non-invaded samples have lower alpha diversity (S1 Fig). Without the sampling depth threshold on the 26 samples, a one-tail Mann-Whitney test showed that the alpha diversity of invasive samples was significantly greater ($\alpha<0.05$) than that in non-invaded samples for all five diversity metrics (Table 4). Kruskal Wallis test with a Chi-Square approximation of one-way test in JMP® Pro, Version 11 (SAS Institute Inc., Cary, NC, 1989–2007) suggested that the diversity metrics (except Simpson index) were significantly different

Table 4. Alpha Diversity Metrics for Invasion, Location, and Location x Invasion in Bacteria.

	Chao1	Observed Species	Shannon	Simpson	PD Whole Tree
Invasion status					
I (n = 11)	24,563	15,024	10.83	0.998	604
N (n = 15)	20,566	12,328	10.54	0.997	512
p-value (one-tail)	**0.012**	**0.004**	**0.006**	**0.007**	**0.007**
Locations					
A	25,687	15,326	10.82	0.997	616
M	17,512	11,000	10.43	0.998	448
R	23,460	13,987	10.75	0.997	591
p-value (two-tail)	**0.001**	**0.002**	**0.003**	0.817	**0.002**
Location x Invasion status					
Aa	27,684	16,806	10.98	0.998	666
AaR	23,691	13,845	10.65	0.997	566
Mv	19,507	12,108	10.61	0.998	494
MvR	15,915	10,114	10.28	0.997	410
Rd	26,875	16,398	10.87	0.998	668
RdR	22,093	13,023	10.70	0.997	559
p-value (two-tail)	**0.003**	**0.002**	**0.002**	0.103	**0.002**

Bolded values indicate significant ($\alpha<0.05$) effects.

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Table 5. Alpha Diversity Metrics for Invasion, Location, and Location x Invasion in Fungi.

	Chao1	Observed species	Shannon	Simpson
Invasion status				
I (n = 15)	814	537	6.30	0.947
N (n = 15)	728	483	5.87	0.935
p-value (one-tail)	**0.039**	**0.023**	0.076	0.221
Locations				
A	863	600	6.60	0.962
M	800	512	6.29	0.959
R	650	420	5.36	0.902
p-value (two-tail)	**0.015**	**0.022**	**0.006**	**0.006**
Location x invasion status				
Aa	844	570	6.65	0.965
AaR	883	629	6.55	0.959
Mv	935	604	6.56	0.961
MvR	666	421	6.02	0.958
Rd	664	438	5.68	0.916
RdR	636	401	5.03	0.889
p-value (two-tail)	**0.011**	**0.014**	**0.020**	**0.030**

Bolded values indicate significant ($\alpha<0.05$) effects.

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($\alpha<0.05$) between samples as per location and interaction of location and invasion status. Since the sample size variation can affect the diversity metrics, the sampling depth threshold was utilized for further analyses by taking a random subsample of 80,000.

Fungi. The read 1s were not used for the analysis due to the poor quality of sequences. A total of 204,835 high quality read 2s of the ITS gene sequence were obtained from the invaded and non-invaded plots. The sequences from 30 samples possessed a 230-bp average length and will be submitted to the NCBI Sequence Read Archive according to the MIMS standard. There were a total of 4,419 distinct OTUs (observations) across samples with a total of 182,009 sequences (counts) that were assigned to these OTUs. The mean and median counts per sample were 6,067 and 4,927 respectively. A sampling depth threshold of 3,200 counts per sample did not remove any sample from further analyses. The average Good's coverage for the fungal data across 30 samples was 95.5%.

Chao1, observed species, Shannon, and Simpson metrics were used to calculate alpha diversity. A non-parametric test with the default 999 Monte Carlo permutations with FDR correction showed significant differences ($\alpha<0.05$) between locations, invasion status, and their interaction (location x invasion status) for alpha diversity (Shannon and Simpson metrics were not used) (Data not shown). Similarly to the bacterial data, the rarefaction curves showed trends that non-invaded samples have lower alpha diversity (S2 Fig). Without the sampling depth threshold, a one-tail Mann-Whitney test showed that the alpha diversity of invasive samples is significantly higher ($\alpha<0.05$) than that in non-invaded samples for chao1 and observed species metrics (Table 5). Kruskal Wallis test with a Chi-Square approximation of one-way test in JMP® Pro, Version 11 (SAS Institute Inc., Cary, NC, 1989–2007) suggested that the diversity metrics were significantly different ($\alpha<0.05$) between samples as per locations and interaction of locations and invasion status. Since the sample size variation can affect the diversity metrics, the sampling depth threshold was utilized for further analyses by taking a random subsample of 3,200.

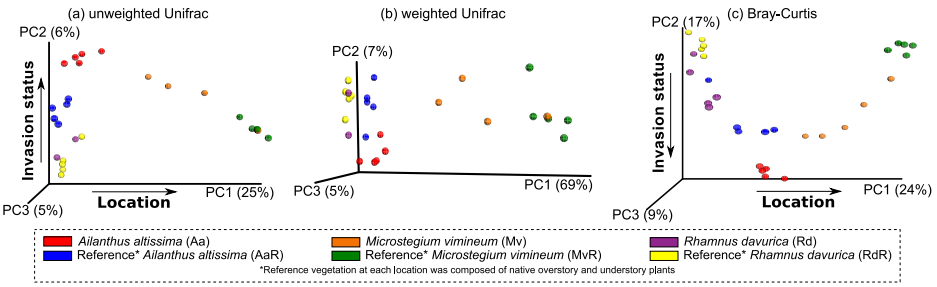

Fig 1. PCoA plot describing (a) un-weighted and (b) weighted Unifrac for bacteria and (c) Bray-Curtis distances for fungi in the invaded and non-invaded sites. Each circle indicates a sample. Multivariate data analysis methods of adonis and ANOSIM were used to identify whether groups were significantly different.

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Beta diversity of microbial communities associated with invasion

Bacteria. Multivariate data analyses using adonis, ANOSIM, and MRPP on weighted and unweighted Unifrac distances showed significant differences ($\alpha<0.01$) in the beta diversity of the location and the interaction of location and invasion status.

Fungi. The beta diversity of location, invasion status, and their interaction were significantly different ($\alpha<0.01$) as shown by adonis, ANOSIM, and MRPP on Bray-Curtis distances, with an exception of ANOSIM indicating a p-value of 0.014 for invasion.

The PCoA analysis of the weighted and unweighted Unifrac (for bacteria), and Bray-Curtis (for fungi) distances showed that the samples clustered as per the location and invasion (Fig 1), with location explaining the maximum variation (PC1). For the unweighted Unifrac and Bray-Curtis distances, invasion status (across all locations) consistently accounted for the second most variation (6% for bacteria and 17% for fungi on PC2). There was a lot of variation associated with the Rd samples as shown in Axis 2 of Fig 1. Overall, these results indicated the effects of invasion and location x invasion status. There were, thus clear patterns of change in soil microbial communities following the invasion of each species across geographically separated ecosystems.

Taxonomic summary and identification of microbial communities associated with invasion. Taxonomic summaries showed that *Acidobacteria* (~30%) and *Proteobacteria* (~22%), and *Ascomycot*a (~47%) and *Zygomycota* (~13%) were the most dominant phyla of bacteria and fungi, respectively (Fig 2). A major proportion of taxa could not be assigned (~34%) to

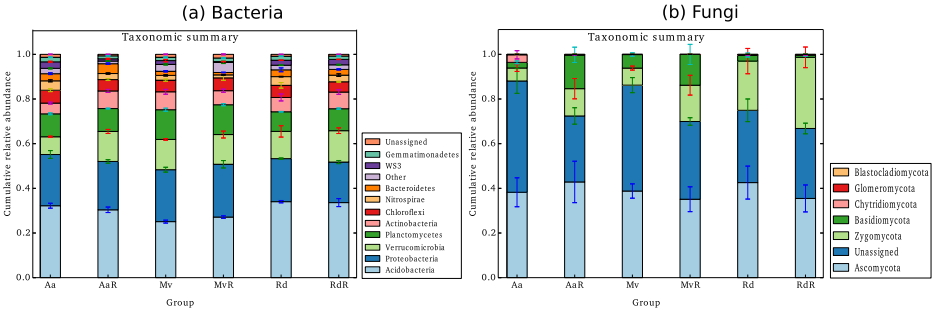

Fig 2. Taxonomic summary of the relative abundance of (a) bacterial and (b) fungal phyla in the invaded and non-invaded sites. The taxa are arranged as per total relative abundance across all samples, with the most abundant phyla at the bottom and the least abundant phyla at the top of the y-axis. Similarly, the phylum names in the legend are arranged from the least abundant at the top to the most abundant at the bottom.

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Table 6. Genera with a Greater Relative Abundance Associated with Invasion and Determined to have a Significant Effect Based on Indicator Species Analysis (IV > 70 and p-value < 0.01).

Bacteria						
Phylum	**Class**	**Order**	**Family**	**Genus**	**I (%)**	**N (%)**
Acidobacteria	-	-	-	-	0.30	0.17
Acidobacteria	Holophagae	Holophagales	Holophagaceae	Geothrix	0.01	0.00
Acidobacteria	iii1-8	SJA-36	-	-	0.03	0.01
Acidobacteria	RB25	-	-	-	0.25	0.12
Acidobacteria	S035	-	-	-	0.08	0.05
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	0.02	0.01
Actinobacteria	Actinobacteria	Actinomycetales	Williamsiaceae	Williamsia	0.01	0.00
Chloroflexi	TK10	-	-	-	0.02	0.01
Gemmatimonadetes	Gemmatimonadetes	-	-	-	0.03	0.01
Nitrospirae	Nitrospira	Nitrospirales	-	-	0.02	0.00
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	0.52	0.10
OD1	SM2F11	-	-	-	0.01	0.00
OP3	koll11	-	-	-	0.01	0.00
OP3	PBS-25	-	-	-	0.01	0.00
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	-	0.21	0.07
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	0.06	0.02
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	-	0.01	0.00
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	0.03	0.00
Proteobacteria	Deltaproteobacteria	NB1-j	MND4	-	0.17	0.05
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter	0.05	0.02
WS2	SHA-109	-	-	-	0.06	0.03
*Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	Actinomadura	0.00	0.01
Fungi						
Phylum	**Class**	**Order**	**Family**	**Genus**	**I (%)**	**N (%)**
Ascomycota	-	-	-	-	1.34	0.34
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Cladosporium	0.05	0.01
Ascomycota	Leotiomycetes	-	-	-	0.37	0.11
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	-	6.52	2.10
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cylindrocarpon	0.95	0.45
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	0.83	0.14
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Neonectria	0.15	0.02
Ascomycota	Sordariomycetes	Incertae sedis	Plectosphaerellaceae	Plectosphaerella	0.24	0.03
Ascomycota	Sordariomycetes	Sordariales	-	-	1.07	0.53

The hyphen (-) indicates that no taxonomic information was available for that OTU at that level. The bacterial OTU indicated with asterisk (*) was the only OTU associated with non-invaded samples in the ISA. The last two columns indicate the percentage of relative abundance of taxa in the invaded and non-invaded samples, respectively.

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known taxa for the fungal data; however, they were a very minor portion for bacteria. The genus level taxonomic summaries were used for indicator species analysis (ISA) to identify taxa that were more abundantly associated with invaded or non-invaded samples (Table 6). Overall, the results suggested numerous types of taxa associated with invasion, whereas only one taxa was associated with non-invasion.

Bacteria. After removing OTUs assigned to archeal and unassigned taxa, OTUs with a total relative abundance of less than 0.1% across all samples were removed. The remaining 416

taxa were re-relativized and used for ISA blocked using soil/geographic locations. Out of 22 OTUs (Table 6) that showed significantly different abundance in invaded and non-invaded samples, 21 OTUs were associated with invasion. OTUs within *Proteobacteria* (6 OTUs), *Acidobacteria* (5 OTUs), and *Actinobacteria* (3 OTUs) had greater sequence abundance due to invasion as revealed by ISA and blocked across soil/geographic locations. Bacterial taxa responsible for nitrogen cycling in soil were increased in abundance in association with plant invasion. Taxa belonging to the ammonia-oxidizer *Nitrospirae* (phylum) and Nitrospira (class) were among the bacteria each with 1.5 times greater abundance in the invaded (3.5% compared to 2.4% in non-invaded) root-zone soils. Nitrifying bacteria appear to be a major result and perhaps driver of invasive plant species change in ecosystems.

The nitrogen-fixing bacterial community was also an important potential indicator of change noted in plant invasions. Several bacterial groups which are known to contain taxa involved in nitrogen-fixation were shown to increase as a result of plant invasion in our data. Rhodobacterales are commonly identified as nitrogen-fixing bacteria [52], and found to collectively contribute to (2.7 times) greater abundance in the invaded root-zone soils in our data (0.22% compared to 0.08% in non-invaded) and previous literature [53]. Hyphomicrobiaceae, another bacterial family within the phyla *Proteobacteria* were also greater as a result of plant invasion, but the effect was most strongly associated with the root-zones of *M. vimineum* (1.4 times abundant, 3.7% compared to 2.6% in non-invaded) and *R. davurica* (1.2 times abundant, 1.7% compared to 1.4% in non-invaded). Though nitrogen-fixation symbiosis are not widely considered key traits among the invasive plant species in this research study, the greater relative abundance of these putative diazotrophic taxa support the idea that these traits may be important associations for many plant invader types.

Fungi. After removing OTUs assigned to unassigned taxa, OTUs with a total relative abundance of less than 0.1% across all samples were removed. The remaining 226 taxa were re-relativized and used for ISA blocked across soil/geographic locations. All of the 9 OTUs (Table 6) that showed significantly different abundance in invaded and non-invaded samples were associated with invasive samples. OTUs within Ascomycota (9 OTUs) had a greater sequence abundance due to invasion as revealed by ISA blocked across soil/geographic locations. Taxa belonging to the Sordariomycetes were among the fungi with 1.2 times greater abundance in the invaded (21.3% compared to 17.5% in non-invaded) root-zone soils.

Predicting microbial functions in non-invaded and invasive samples

Currently, PICRUSt can only be used for functional analysis of bacterial taxa. To the best of our knowledge, we could not find a program for functional analysis of fungi, analogous to PICRUSt for bacteria. The fungal data resources AFTOL (http://aftol.org/) and FunSecKB [54] provide relevant but incomplete data for our purpose.

The actual counts from the OTU table were obtained for the bacterial species belonging to the genera that were significant from the ISA. OTUs not part of the closed reference OTU picking method were filtered out from the 3,385 OTUs belonging to the 22 significant genera and the remaining 365 OTUs (~11%) were used for functional analyses using PICRUSt. The 16S rRNA copy number normalized abundance was used to predict metagenome and collapse into KEGG pathways. Two-sided Welch's t-test with multiple testing corrections in STAMP was performed to identify KEGG pathways at different levels that are significantly different (q-value < 0.05) between invaded and non-invaded samples. At Level 2 of KEGG, BH and Storey corrections found 9 and 27 pathways, respectively, to be significantly different between root-zone bacteria of invaded and non-invaded samples (S1 and S2 Tables). At Level 3 of KEGG, BH correction did not detect pathways to be significantly different between invaded and non-

invaded root-zone bacteria. However, for the same level, Storey FDR detected 60 pathways to be different (S3 Fig). The significant processes were descending sorted as per the average of mean relative frequency (%) in non-invaded and invaded samples. The top 20 abundant processes were categorized as belonging to non-invaded (N) or invaded (I) samples depending on the difference of mean relative frequency (%) (S3 Table).

As expected from the taxonomies of bacteria from the ISA, nitrogen metabolism was also observed to be higher in the root-zone bacterial communities of invasive plants as compared to that of the non-invaded plants (S3 Table). The increase in nitrogen metabolism by invasive plants and the associated benefits to invasion are well known [55–57].

Discussion

Plant invasion theory has developed a broad number of hypotheses to explain the success of invasive plants [58]. Despite their likely importance, however, there is a dearth of research into aboveground-belowground linkages across landscape scales that have determined the effects of plant invasion on soil or root-zone microbial communities [59, 60]. Here we show that at three independent locations, three invasive plants are associated with uniform shifts in belowground root-zone soil microbial communities. This is important, further, because each of the invasive plants has a distinct phylogeny and life form. Our results are broadly relevant because belowground interactions between soil microbes and plants provide an important linkage to support plant invasions.

Bacterial community shifts due to plant invasion

Compared to adjacent non-invaded patches, fungal and bacterial communities were described by consistent ordinal shifts associated with invasion. Nitrospira sp. and *Nitrospirae* were among the bacteria with greater abundance in the invaded soils. Overall *Nitrospirae* was very abundant, and greater in the invaded (3.5% compared to 2.4% in non-invaded) root-zone soils. Previous studies have shown that *Nitrospirae*, which are most often found to be chemolithic autotrophs, and include taxa that are drivers of nitrification, tend to account for 0.2 to 0.7% of OTUs in grasslands, agricultural systems, and forests [61, 62]. However, 2% or more have been observed in remnant deciduous forests [63], which is consistent with the forests described herein. Furthermore, our results corroborate that plant invasions are associated with major changes in the nitrogen cycle [18, 60] by showing greater rates of root-zone soil N turnover due to invasion.

Importantly, the results of our experiments support a major mechanism of plant invasion success, and link microbial phylogeny with functional measurements of nitrogen turnover. The greater rates of nitrogen turnover and estimates of metagenome composition and function using PICRUSt are in agreement that N cycling processes are important components of invader success. Nitrogen-fixing bacterial communities are also an important indicator of change previously documented in plant invasions [64]. The link between nitrogen-fixation and bacterial phylogeny, however, is not as strong as that with nitrification. Several bacterial groups which are known to contain taxa well known for nitrogen-fixation were observed to increase in our study as a result of plant invasion. Nitrogen-fixers can be free-living, and their abundance in soil tends to be low ($2.4x10^5$ copies g^{-1}); however, associative diazotrophs are generally more common ($1.3x10^7$ copies g^{-1}) in the root-zones of numerous types of plants if carbon is available to drive the energetically expensive process of N_2 reduction to ammonium [65]. Since these bacteria are closely linked to plant roots, their greater abundance, and the confirmation that nitrogen fixation genomes are available to support greater nitrogen fixation (PICRUSt) associated with invaded soils, are in support of the argument that the result is not due to *a priori* soil habitat

differences, but rather the impact of the root-zones of plant invaders. If greater N-fixation is the result of increasing abundance of diazotrophs, then greater supplies of N could help to foster greater nitrogen availability for plants and nitrifiers alike. These types of interactions have the potential to act as a positive feedback to support the habitat needs of the invader. Negative consequences of increased nitrogen-fixation and nitrification could also come from the leaching of nitrate to groundwater and gaseous losses through denitrification (N_2O).

Connections between plant traits and root-zone associated microbial communities have been considered [19, 60]. Less work, however, has been conducted to determine how root-zone soil microbes directly benefit and support the longer-term spread of invasive plants [66]. Though the work presented here does not directly address the long-term nature of invasion, they are representative of fairly mature invasions (>5y) and the potential consequences of changing microbial communities and alterations in ecosystem nutrient cycles.

The field results presented help to fill a major gap in understanding plant invaders and mechanisms of invasion success. The evidence provided in the research reported here are consistent with the idea that plant invaders shape belowground communities, and positively feedback to support the success of the plant invader. In addition, the research has shown that plant invaders are associated with change in soil properties which might be driven by the plant invader and facilitated by positive feedbacks resulting from microbial community processes. Alterations in nutrient cycling have previously been described as potential drivers that feedback to support plant invasion. Often these results are tied to changes in plant tissue chemistry and the decomposition [21, 25] but less attention has been paid to the potential effects that plant roots might have more directly on soil nutrient bioavailability. Plant root systems have the capacity to alter soil pH and therefore chemical equilibria and pH sensitive biological processes. Nitrification, for example, has been described as limited by pH below 5.5–6.0 [67]. Chemical equilibrium of soil nutrients, such as phosphorus, potassium, and iron, furthermore, are strongly impacted by soil pH. The significant changes in bioavailable soil nutrient pools suggest further attention is needed to understand their role in sustaining plant invasions.

Fungal community shifts due to plant invasion

It was expected that invaded soils would tend to be less diverse and support greater dominance if invasive plants stimulated the activity of specific microbes that feedback to support invader growth. Invasion, however, was associated with greater diversity and richness of fungi (and bacteria). The importance and contribution of this microbial diversity to the success of the invaders is an open question, however, and despite attempts to link microbial diversity to function, diversity in soils is large and generally difficult to interpret. It is clear, though, that certain microbial types were associated with greater abundance in invaded soil and have the potential to feedback and support the growth and reproduction of invaders. The large changes in microbial diversity, though not straightforward to interpret, require further research and consideration of how it impacts plant invader success.

Unlike the structural and functional linkages that were made associated with bacterial community change and plant invasion, fungal communities in the current study were not as clearly demarcated phylogenetically nor linked with specific processes. There were, however, very similar directional shifts in fungal community structure that help to support the findings observed for bacterial communities. Indeed, shifts in fungal community structure accounted for up to 17% of the variation in the PCoA plot (Fig 1). Fungi play critical ecosystem roles as saprotrophs, mutualists, and pathogens and though pinpointing the exact nature of the effects are not possible in the current study, the patterns of community change support the idea that plant invaders drive and are driven by a positive plant-microbial feedback model that fuel their success.

The Ascomycota showed greater abundances associated with invasion, and as the compositionally largest phylum of fungi with 64,000 species and a range of traits that include saprobe, pathogens, and mutualists, the effects of the change are likely to be functionally important [68, 69]. It is important to recognize that fungi, like bacteria, can have multiple ecological roles; for example, many mycorrhiza are also saprotrophs. Using their methodology to sort orders into an ecological context, however, Sordariales were overwhelmingly characterized as Saprobes, and the Hypocreales and Capnodiales form a mix of saprobes, plant associates and plant pathogens. So although the primary ecological changes that were observed using these methodologies are still broad, they show the potential that phylogeny has for predicting fungal ecology and the effects of plant invasion.

It is notable that a considerable amount of study has been given to the pathogenic roles played by many of the fungal taxa in our surveys. Dothideomycetes and Nectriaceae, for example, are found to play multiple antagonistic roles to plants and plant growth. It cannot be known, however, if these fungi actually play this type of role or are perhaps recruited to support plant invasion through antagonization of non-invaded plant species [70]. Whether serving as a loose plant affiliate or a plant-microbial interaction, there would be opportunity for invasive plants to disrupt plant communities if invaders themselves were less prone to the antagonistic effects of the pathogens. Research is needed to understand the nature of the changes in fungal community structure and their consequences for plant invader success.

Conclusion

It is well known that invasive species have direct and indirect effects on the surrounding non-invaded plant community, especially through root exudates: *Centaurea* spp. [71]; *Ailanthus altissima* [72]; and *Artemisia vulgaris* [73]. Our study offers insights into microbial communities and plant invasions by showing a link between invasion and belowground community change. Functional predictions based on the phylogeny of bacteria agreed with field measurements of N turnover rates and suggest that changes in N cycling bacteria, which include nitrifiers and diazotrophs, may be a significant cog in the success of invasive plant encroachment and success into non-invaded/remnant ecosystems. If these results are further confirmed, management scenarios may soon be utilized to change the soil properties and outcome of plant-driven changes in microbial communities to help favor non-invaded plants and restore native ecosystem functions.

Supporting Information

S1 Fig. Rarefaction plots of bacterial alpha diversity for invaded and non-invaded samples using (a) chao1, (b) observed species, and (c) PD whole tree.
(EPS)

S2 Fig. Rarefaction plots of fungal alpha diversity for invaded and non-invaded samples using (a) chao1 and (b) observed species.
(EPS)

S3 Fig. KEGG pathways (level 3) predicted by PICRUSt that were significantly different between root-zone bacteria of invaded and non-invaded samples using two-sided Welch's t-test with Storey FDR for multiple testing corrections.
(EPS)

S1 Table. KEGG pathways (level 2) predicted by PICRUSt that were significantly different between root-zone bacteria of invaded and non-invaded samples using two-sided Welch's t-test with Benjamini Hochberg FDR for multiple testing corrections. I and N

indicate pathway was abundant in root-zone bacteria of invaded and non-invaded samples, respectively.
(DOCX)

S2 Table. KEGG pathways (level 2) predicted by PICRUSt that were significantly different between root-zone bacteria of invaded and non-invaded samples using two-sided Welch's t-test with Storey FDR for multiple testing corrections. I and N indicate pathway was abundant in root-zone bacteria of invaded and non-invaded samples, respectively.
(DOCX)

S3 Table. Top 20 abundant and significant ($\alpha < 0.05$) level 3 KEGG processes by Storey FDR. First, the significant processes were descending sorted as per the average of mean relative frequency (%) in native and invasive samples. The top 20 abundant processes were categorized as belonging to native (N) or invasive (I) samples depending on the difference of mean rel. freq. (%). Finally, in each category, the processes were descending sorted as per the difference in mean rel. freq. (%) between I and N.
(DOCX)

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Author Contributions

Conceived and designed the experiments: RRR RPP JNB ETN JEB MAW. Performed the experiments: RRR RPP. Analyzed the data: RRR RPP MAW ETN. Contributed reagents/materials/analysis tools: RRR RPP JNB ETN JEB MAW. Wrote the paper: RRR MAW JNB RPP ETN.

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CHAPTER 4

Rosana P. Pineda and Mark A. Williams

CONCLUSION

Conclusion

Two different sets of experiments assessing the relationship between vegetation and fungal communities were undertaken in this thesis. There were many differences in the ecosystem type and soils in the study. The soils associated with the chronosequence at Wilderness State Park (WP) in Michigan were derived from the same or similar sandy parent material that was younger and less weathered than those of the invasive plant experiment in Virginia. Though all soils are perpetually altered and receive continual inputs of material and nutrients (e.g. atmospheric deposition), the primary parent material of the soils in the study sites from Virginia ranged from relatively young Inceptisols with deep, well drained soils that formed from the residuum of shale and sandstone to more highly weathered mixed alluvial and colluvial Alfisols.

The scales of change that were assessed were also much different between the two studies. At WP, changes in fungal communities were assessed over hundreds to thousands of years following deposition and aging of parent material. In Virginia, community change was assessed in weathered soils that were invaded or uninvaded by plants within the last 10 years. Fungal community change was shown, as hypothesized, to be associated with differences in soil properties and the arrival of new plant species. Unfortunately, because the two studies utilized different primers and sequencing technologies it is difficult to make

direct comparisons regarding fungal community change between studies. Taken together, however, plant species were a consistent factor associated with fungal community change even when soil properties were not largely altered (e.g. Rhamnus-affected versus reference soil).

In addition to change, fungal communities were stable (unchanged) when aboveground vegetation tended to stabilize during later ecosystem development at WSP. Not surprisingly, soil properties measured on site also did not change during this period of relative fungal community stability. Hence, the continued working hypothesis derived from the research in this thesis is that both soils and plants can drive fungal community change. Though other researchers have come to similar conclusions, the research herein is unique in that it describes change over multiple temporal scales.

Each study also had its own unique aspects. In the case of plant invasion, the results were some of the first to directly test, in the field, whether different functional types of invasive plants alter, in similar or different ways, soil fungal communities. Very different functional groups of plant species were shown to alter the structure and function of microbial communities in similar ways (multivariate ordination shifts). This suggests that the trait of invasiveness may be associated with specific types of plant-soil-microbial feedbacks that support invasion success.

At WP, studies showing plant change associated with pedogenesis during primary succession over hundreds to thousands of years are not yet widely published (Lichter, 1998). The results do agree with studies that were undertaken over periods of time that were much shorter or longer and through focus on special functional groups such as arbuscular mycorrhizal fungi (Martínez-García et al 2015). Together these results show

that vegetation change over a broad range of scales from years to hundreds and thousands of years are observable and may represent important ecological shifts.

The degree of change is also important to consider, and though not directly tested, some clues to the relative changes between the two studies can be inferred. Generally, changes due to invasion involved significant changes of less than 0.5% for an OTU, with 9 fungal taxa acting as good indicators of change. Yet, one community member closely related to the fungal family *Nectriaceae*, changed from representing 2.1 to 6.2% of taxa as a result of invasion. During ecosystem development, one of the more dominant members of the community, *Sclerotina* changed from 15 to 35% of taxa between early and late stages of pedogenesis and ecosystem development. Though the change in communities, not surprisingly, may be judged to be larger over the longer time scales of the chronosequence, changes due to plant invasion show how quickly plants can impact soil fungal communities.

Many fungi are classified as monoecotypic, meaning they have a primary, but not always, lifestyle strategy that would classify them as saprotrophic, pathogenic, and/or mutualists (Rodriguez and Redman, 1997). A large majority have been described as saprophytic. However, some researchers suggest that fungi are often better classified as having a multiphasic lifestyle (Rodriguez et al., 2009). This makes the description of fungal community function a difficult task. It also shows a huge diversity of metabolisms and potential interactions that would be expected in complex ecosystems and during ecosystem change. It is notable in this regard that dominant fungi, such as *Sclerotina*, have numerous members that are considered pathogenic to plants. Though still highly speculative, the increasing relative contributions of these two taxa due to invasion and ecosystem

development, respectively, may help to explain hypothesis related to plant-microbial feedbacks which control ecosystem vegetation and processes.

In plant invasion ecology, the enemy release and accumulation of pathogens hypotheses are consistent with the findings described in this thesis. In essence, invasive plants, unlike in their native range, are not impacted by the pathogens of the new environment (Gundale et al., 2014), and furthermore may increase the abundance of pathogens (invasive meltdown) in the invaded system (Jeschke et al., 2012). During ecosystem development, it has been hypothesized that increasing levels of pathogens feedback and select for specific and stable plant communities during the latter stages of ecosystem and soil development. Both of these hypotheses need further study using both controlled greenhouse and observational field based studies.

It is important to note the key role that soil fungi play as ecosystem decomposers and drivers of nutrient cycling. Obtaining their nutrients by releasing enzymes, saprophytic fungi are decomposers that derive and recycle nutrients from their surrounding environment. However, with the possibility of facultative trophic forms, fungi could have biphasic lifestyles. For example, ectomycorrhizal fungi are capable of obtaining carbon and nutrients both biotrophically and saprotrophically (Koide et al., 2008). Saprotrophs are a functional group that participate in numerous ecosystem services, such as soil formation, rock dissolution, particle binding and soil fertility by the decomposition of organic residues, nutrient mineralization, and soil stability (Dighton, 2003). These fungi obtain carbon from litter, invading dead cells of tissues such as stems, roots, and leaves; also, it is thought that these fungi may transfer nutrients (such as phosphorus or nitrogen) to plant roots based on studies of the net movement of phosphorus or nitrogen into litter (Koide et

al., 2008). It is thus not surprising that many of the fungi were identified as taxa resembling saprotrophs.

Both Ascomycota and Basidiomycota fungi could have members with mycorrhizal and saprotrophic capacity, which are thought to have evolved repeatedly from saprotrophic ancestral fungi (Egger, 2006). Ectomycorrhizal and saprotrophic decomposer fungi play key roles in nutrient supply and litter decomposition, respectively, which make them both very common in most forest soils. Both of these fungi groups participate in the nutrient cycles in soils. Through their mycelia they may sequester and release large quantities of nutrients (Johnson et al., 2002). Despite these possibilities, the data collected in this study cannot confirm the role of these fungi, however, now that these fungal communities have been identified, whether as a result of invasion or ecosystem development, their specific roles can be further described.

Though widely hypothesized, it is not known whether soil fungal succession is linked with plant succession. Tight linkages and feedbacks between plants and fungi may result in relatively quick concurrent shifts in both communities. Species turnover could be a factor influencing the fungal succession trajectory. Soil legacies, however, have been shown to have persistent effects, and it is thus possible that changes in belowground communities may lag behind those of the aboveground vegetation (Grove et al., 2012). Yet, data from the invasion study show that community turnover and change can in some regards also be relatively large over periods of less than a decade. Results from this thesis, nevertheless, support the idea that plant communities and plant-microbial feedbacks play an important role in determining soil fungal communities.

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