University of Arkansas, Fayetteville
ScholarWorks@UARK

Theses and Dissertations

12-2017

Fungi of Forests: Examining the Diversity of Root-associated Fungi and Their Responses to Acid Deposition

Donald Jay Nelsen
University of Arkansas, Fayetteville

Follow this and additional works at: http://scholarworks.uark.edu/etd

Part of the Environmental Indicators and Impact Assessment Commons, Environmental Microbiology and Microbial Ecology Commons, Forest Biology Commons, Fungi Commons, and the Terrestrial and Aquatic Ecology Commons

Recommended Citation
Nelsen, Donald Jay, "Fungi of Forests: Examining the Diversity of Root-associated Fungi and Their Responses to Acid Deposition" (2017). *Theses and Dissertations*. 2543.
http://scholarworks.uark.edu/etd/2543

This Dissertation is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu, ccmiddle@uark.edu.

Fungi of Forests: Examining the Diversity of Root-associated Fungi and Their Responses to Acid Deposition

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biology

by

Donald Nelsen
Minnesota State University, Mankato
Bachelor of Science in Biology, 2010
Louisiana State University
Master of Science in Plant Health, 2013

December 2017
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

____
Dr. Steven Lee Stephenson
Dissertation Director

____
Dr. Fred Spiegel
Committee Member

____
Dr. Burt H. Bluhm
Committee Member

____
Dr. Ravi Damodar Barabote
Committee Member

Abstract

Global importance of forests is difficult to overestimate, given their role in oxygen production, ecological roles in nutrient cycling and supporting numerous living species, and economic value for industry and as recreational zones. Fitness of the forest-forming trees strongly depends on microbial communities associated with tree roots. In particular, fungi impact tree fitness: mycorrhizal species provide water and nutrients for the trees in exchange for C, endophytic fungi play key roles in host defense against pathogenic organisms, and saprotrophic fungi decompose dead organic matter and facilitate nutrient cycling. In addition, pathogenic fungal species strongly affect forest fitness. Despite their importance, fungal communities associated with forest trees are largely unknown because the typical morphological assay takes into consideration a scarce portion of fungal diversity: species that produce visible fruiting bodies at relatively frequent intervals. A more accurate assessment of fungal diversity in forests has become possible with the development of next-generation sequencing, where fungal species are being identified based on the presence of their DNA in the sample. In this work, DNA-metabarcoding was utilized to assess the diversity of fungi associated with roots of forest-forming trees within the families Fagaceae and Betulaceae (Europe), and Fagaceae, and Juglandaceae (North America). The data obtained provided unprecedented insight into hidden richness of root-associated fungi, which approached 1756 OTUs (a proxy for species) in the European dataset, and 2769 – in the North American dataset. Variation in fungal community composition was largely explained by geographical location (ca. 30%). However, the effect of host specificity (ca. 9-15% of variation) was significant as well. DNA-based data revealed strong positive and negative patters in fungal co-occurrence (e.g., a positive relationship was observed between *Cenococcum geophilum* and species of Russulaceae), which could indicate interactions between fungal species. In addition to

diversity assays, fungal responses to acid precipitation were quantified, and revealed strong declines in fungal richness and abundance, including ectomycorrhizal species. I conclude that compositional shifts in root-associated fungal communities could be particularly suitable for monitoring of forest ecosystems, given an optimal response time in fungi (not too slow as in wooded plants and not as high as in bacteria).

Acknowledgements

Only a few times in life, I think, do we pause and take a moment to appreciate our accomplishments and the people who helped to make them possible. For me, this document, and the work it represents, is one of the most valued accomplishments of my life. There are many to whom I owe a debt of gratitude and thanks for their help along the way, and I'd like to take the time to recognize them here.

First, I would like to extend my sincere gratitude for the guidance and mentorship of my academic mentors during my undergraduate studies at Minnesota State University, Mankato, for without them I would have been unable to find my way through the trials of undergraduate and graduate education. My thanks go out to Dr. John Krenz, who taught me as an undergraduate what research entails by guiding me in my first research project. I appreciate very much the willingness of Dr. Alison Mahoney to mentor me in my desire to pursue independent studies in mycology as an undergraduate. Thanks go out to Dr. Brad Cook for teaching me that a university education is about so much more than grades. I am also thankful for the continued friendship and occasional advice proffered by Dr. Christopher Ruhland.

I would also like to thank the professors who guided me through my masters research at Louisiana State University: Dr. Cathy Aime, Dr. Meredith Blackwell, Dr. Chris Clark, and Dr. Marc Cohn. Their contribution to my development as a scientist and student made it possible to continue on to this point.

All of which brings me to my current studies and the people who have been very important and influential in the development of this work. First and foremost I need to thank my advisor, Dr. Steve Stephenson. A long time ago, before I had any experience in mycology, Dr. Stephenson took a chance on me and gave me the opportunity to research fungal biodiversity abroad. It is

because of his mentorship, advice, and willingness to trust in my abilities that I have been able to progress to the point of conducting independent research in pursuit of a PhD. I owe a debt of gratitude to my committee members as well, without which none of this would have been possible. Thank you to Dr. Burt Bluhm for believing in my desire to do good research, and for the logistical support that made it possible for me to actually do it. Thank you to Dr. Fred Spiegel for providing lively critical debate and friendly conversation about my research goals and the academic lifestyle, as well as the opportunity to assist with the mycology course. Thank you also to Dr. Ravi Barabote, for excellent advice about much of the molecular and bioinformatic components of my research project.

A very big thank you to my lab mates: Isadora Cuelho, Geoff Zahn, Laura Walker, Karen Willard, Sittie Macabago, Brandy Garrett-Kluthe, Eric Hearth, Rajaa Al-Anbagi, and Autumn Lynn Coffey Olsen. Being surrounded by your good company throughout graduate school was a valuable thing during some very stressful times. The same can be said of my fellow graduate students in the department. A thank you to the following for being a laborer, an ear, a shoulder, or sharing a coffee or beer: Anant Deshwal, Brian Becker, Auriel Fournier, Brad Austin, Hal Halvorson, Laura Berryman-Williams, Melissa Welch, and so many others I'm not sure I can list them all!

Finally, I would like to extend my deepest gratitude to the family and friends who have stood by me through what must seem to them to be a never-ending pursuit of all things complicated. To my mom, who believed in me all the way to the end, thank you and I miss you every day. To my dad, who loves talking about my progress and is always excited to hear what I'm up to, thank you. To my sisters Teri and Jodi, without the support of you and your families I would have been unable to make it this far, thank you so much! Lastly, I would like to acknowledge the kind,

generous, helpful, thoughtful, and loving person who helped me keep my eyes on the goal and achieve this milestone – my wife, Tatiana Semenova-Nelsen. Thank you for being an island of calm in heavy seas.

Dedication

This book is dedicated in memory of my mother, Denise Audrey Hoeft, who died shortly before I was able to begin this work. The world was made lesser at her loss, and this work was made possible by her loving understanding of my need to explore the unknown. It is in that spirit that I dedicate this book to her, and those who seek to know that which they do not.

Table of Contents

I. Introduction ... 1
- A. Forests and Fungi ... 1
- B. Next Generation Sequencing ... 3
- C. Acid Deposition & Ecosystems ... 6
- D. Objectives ... 10
  - Root-associated Fungal Communities in Two European Deciduous Forests ... 10
  - Root-associated Fungal Communities in Deciduous Forests of Eastern North America ... 12
  - Effects of Acid Deposition on Root-associated Fungal Communities ... 14
- E. References ... 16

II. Diversity, distribution and specificity of fungi associated with root material of four tree species in two European forests ... 20
- A. Abstract ... 20
- B. Introduction ... 22
- C. Methods ... 24
  - Study sites and root sampling ... 24
  - DNA extraction, PCR and sequencing ... 25
  - Bioinformatics ... 26
  - Statistical analyses ... 27
- D. Results ... 29
  - Diversity of root-associated fungi ... 29
  - Factors affecting communities of root-associated fungi ... 33
  - Host specificity of fungi ... 35
  - Fungal co-occurrence patterns ... 37
- E. Discussion ... 40
- F. Conclusion ... 44
- G. References ... 45
- H. Supplemental Materials ... 51

III. Root-associated fungal communities of selected trees in Eastern North American temperate deciduous broadleaf forests ... 61
- A. Abstract ... 61
- B. Introduction ... 63

C. Methods ... 66
Study sites and root-tip sampling ... 66
DNA extraction and sequencing ... 68
Bioinformatics and statistical analyses ... 69
D. Results ... 71
Diversity and distribution of root-associated fungi ... 71
Community structure is driven by location and host-association ... 77
Fungal co-occurrence patterns ... 80
E. Discussion ... 81
F. Conclusions ... 85
G. References ... 86
H. Supplementary ... 91
IV. Compositional, taxonomic and functional responses of fungi associated with three species of Fagaceae to experimental acid deposition in a North American deciduous forest ... 97
A. Abstract ... 97
B. Introduction ... 99
C. Methods ... 102
Site selection ... 102
Root tip and soil sampling ... 102
DNA extraction and sequencing ... 103
Bioinformatics analyses ... 104
Statistical analyses ... 105
D. Results ... 107
Experimental acid deposition alters fungal community composition ... 107
Changes in abundance of fungi in response to experimental acid deposition ... 111
Indicator species analyses of treatment/control conditions and host taxa ... 113
E. Discussion ... 114
F. Conclusions ... 118
G. References ... 119
H. Supplemental Materials ... 126
V. Discussion and Conclusions ... 130
A. NGS and fungal diversity ... 131

B. Fungal host-specificity 133
C. Regional variation in fungal communities 135
D. Fungal responses to acid deposition 136
E. Responses of fungal taxonomic and ecological groups to acid deposition 136
F. Fungal-fungal interactions 138
G. Future research 139

List of Tables

I. Introduction – no tables

II. Diversity, distribution and specificity of fungi associated with root material of four tree species in two European forests

Table S1. Molecular identification tag (MID) assignment for samples collected from Netherlands and German sites……………………………………………..............51

Table S2. Ecological function of OTUs on four European tree species……………………53

Table S3. Vector identification for NMDS ordination of fungal OTUs on trees in Germany and the Netherlands………………………………………………………………...54

Table S4. Identification of vectors and their r values for separate ordinations of trees sampled in Germany and the Netherlands……………………………………….55

Table S5. Indicator Species Analysis of fungal OTUs occurring on host trees in Germany and the Netherlands………………………………………………………………...56

Table S6. Topsoil physical and chemical properties for each sample site. Data retrieved from the Harmonized World Soil Database Viewer (V 1.21)…………………...58

III. Root-associated fungal communities of selected trees in Eastern North American temperate deciduous broadleaf forests

Table S1. Richness of OTUs in fungal genera on host trees in temperate deciduous forests located in the central Appalachian Mountains and the Ozark Mountains……….93

IV. Compositional, taxonomic and functional responses of fungi associated with three species of Fagaceae to experimental acid deposition in a North American deciduous forest

Table 1. The richness response of ecological functional groups and fungal taxa to experimental acid deposition as revealed using NMDS in PC-ORD…………..109

Table 2. Abundance shifts in ecological functional groups and fungal taxa in response to experimental acid deposition as revealed through Comprehensive Meta-Analysis……………………………………………………………………...112

Table S1. Indicator species analyses of host and treatment variables in PC-ORD software……………………………………………………………………...128

V. Discussion and Conclusions – no tables

List of Figures

I. Introduction

Figure 1. The cycle of anthropogenic acid deposition from industrial source air pollution to wet and dry deposition 6

Figure 2. Location of field sampling sites in the Netherlands (N) and Germany (G)....11

Figure 3. Locations of field sites in North America include four sites in AR (Pea Ridge National Military Park, Ozark National Forest, Lake Wilson Park, and Lost Valley Park), one site in WV, and one site in VA....13

Figure 4. Map of the watershed boundaries in the Fernow Experimental Forest near the town of Parsons in West Virginia....15

II. Diversity, distribution and specificity of fungi associated with root material of four tree species in two European forests

Figure 1. Community richness and diversity of fungal OTUs associated with model tree species in sample sites....30

Figure 2. Diversity of root-associated fungi....31

Figure 3. Richness of OTUs in the top 29 fungal genera occurring on four model tree species in two European forests....32

Figure 4. Two-dimensional NMDS ordination of fungal communities associated with trees at sample sites in Netherlands and Germany....34

Figure 5. Non-metric Multidimensional Scale ordination of sampled trees by fungal OTUs present....35

Figure 6. Four-way Venn diagram of OTU distribution across four host species in two families....36

Figure 7. Species co-occurrence matrix of 86 fungal species found across 40 sampled trees....39

Figure S1. Values of Good's coverage on the four tree species sampled at both sites....51

Figure S2. Rarefaction curves for ITS2 DNA sequence reads from 40 sampled trees in Germany and the Netherlands....52

Figure S3. Co-occurrence matrix of ECM fungi....59

Figure S4. Co-occurrence matrix of 35 saprotrophic fungi showing positive and negative interaction patterns of occurrence greater than that expected by chance...............60

III. Root-associated fungal communities of selected trees in Eastern North American temperate deciduous broadleaf forests

Figure 1. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Carya ovata*..73

Figure 2. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Fagus grandifolia*................................74

Figure 3. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Quercus rubra*....................................75

Figure 4. Proportions of OTUs belonging to different ecological functional groups specific to Carya ovata (a), Fagus grandifolia (b), Quercus rubra (c), or occurring on all three tree species (d) across the study sites of temperate deciduous forests of eastern North America...76

Figure 5. Nonmetric multidimensional scaling ordination of root-associated fungal OTUs on host trees...78

Figure 6. Venn diagram of OTUs occurring on the roots of Quercus rubra, Carya ovata, and Fagus grandifolia in temperate deciduous forests of the central Appalachian Mountains and the Ozark Mountains..79

Figure 7. Co-occurrence analyses of root-associated fungal assemblages on three host tree taxa in two temperate deciduous forests of eastern North America (Ozark Mountains and central Appalachian Mountains)..80

Figure S1. Mean values of Good's Coverage estimator for each sample at each location.....91

Figure S2. Mean richness, diversity, and evenness of fungal OTUs on host trees at different sampling sites in temperate deciduous forests of eastern North America............92

Figure S3. Co-occurrence analyses of root-associated fungi on Carya ovata, Fagus grandifolia, and Quercus rubra based on different geographic locations: a) communities occurring on roots in the central Appalachian Mountains, b) the Ozark Mountains, and c) both localities together..96

IV. Compositional, taxonomic and functional responses of fungi associated with three species of Fagaceae to experimental acid deposition in a North American deciduous forest

Figure 1. Nonmetric Multi-dimensional Scaling ordinations of root-associated fungal communities under experimental acidification conditions and control conditions....................108

Figure S1. Statistical analyses of sequencing effort, diversity metrics, and fungal biomass..126

V. Discussion and Conclusions – no figures

I. Introduction

A. Forests and Fungi

Globally, forests account for approximately 4 billion hectares (31%) of terrestrial land cover (Keenan et al., 2015) and contribute vital habitat for wildlife, resources for human industrial and leisure activities, food, shelter, and performance of other necessary ecosystem services such as carbon sequestration. These forests include those located in polar/boreal, temperate, subtropical, and tropical regions of the world. Among these classifications, subtropical and tropical forests have been in decline, polar/boreal forests showed no significant change between 1990 and 2015, and temperate forests were found to be increasing in extent (FAO, 2015). Temperate regions in the northern and southern hemispheres have a variety of forest types: coniferous, mixed coniferous/deciduous, and deciduous forests are broad categories of temperate forests found in these regions around the globe (Archibold, 2012). In temperate deciduous forests of the northern hemisphere, canopy-level members of these forest communities include species in the families Betulaceae, Fagaceae, and Juglandaceae among a number of other families. Eastern North American deciduous forests are populated by large numbers of oak species (*Quercus*) and American beech (*Fagus grandifolia* Ehrh.), both members of the Fagaceae (Braun, 1950). Another common group of forest trees in the Central Appalachian Mountains and Ozark Mountains of Eastern North America are species of hickory (*Carya*) of the family Juglandaceae (Pell and Bukenhofer, 1999). Across the Atlantic Ocean, on the mainland of the European continent, temperate deciduous forests consist largely of species related to those found in their North American counterparts. These forests often contain the canopy-forming species European beech (*Fagus sylvatica* L.) and European oak (*Quercus robur* L.) in the Fagaceae, as well as members of the Betulaceae, including silver birch (*Betula pendula* Roth) and the European

hornbeam (*Carpinus betulus* L.) (Svenning, 2002). These dominant tree-forming plant families form an important part of diverse temperate deciduous forest canopies, a diversity that is linked to significant increases in ecosystem services (Gamfelt et al., 2013).

The importance of this diversity and habitat availability is not limited to above-ground forest communities. Below the leaf litter, in the rhizosphere, there occurs a rich diversity of microbial communities. One group of these belowground inhabitants consists of the root-associated fungi, consisting of taxa that make their home on and in the roots of host plants. Lifestyles of these fungi vary in functionality and include pathogens, commensals, symbionts, and saprotrophs. Pathogenic fungi have a negative impact on their host organisms while commensal fungi such as root and foliar endophytes have no apparent cost to their host plants. Saprotrophic fungi are essential as decomposers, providing carbon cycling for the ecosystem they inhabit (Hanson et al., 2008). Among the approximately 390,000 terrestrial vascular plants (Kew, 2016), an estimated 86% are associated with symbiotic mycorrhizal fungi in their root systems (Brundrett, 2009), including the dominant canopy-members of temperate deciduous forests in the northern hemisphere.

Mycorrhizal associations provide increased access to soil water and nutrients for host plants in exchange for photosynthates. These associations include arbuscular, ectomycorrhizal, ericoid, and orchidaceous lifestyles. Arbuscular mycorrhizae exchange nutrients with host plants across the cell membrane of root cells that have been colonized by hyphae forming branched structures called arbuscules (Parniske, 2008), this is the most common mycorrhizal association in vascular plants (Brundrett, 2009). Ectomycorrhizae form associations with plant roots by exchanging nutrients in the intercellular spaces of roots where they form a hyphal network called a Hartig net (Blasius et al., 1986), which anchors the outer sheath of hyphal cells to the plant root. Ericoid

mycorrhizae, so named due to their close association with members of the family Ericaceae, develop coils of intracellular hyphal cells in the epidermis of plant roots that, like arbuscular mycorrhizae, do not penetrate the plant cell membrane but provide a location for nutrient exchange (Read, 1996). Mycorrhizal associations involving orchids include a wide variety of fungi with different nutritional modes that are found in and on orchid roots, providing essential nutrition for the germination of orchid seeds (via mycoheterotrophy of hyphal cells) as well as increased access to nutrients in the nearby environment of the orchid (Rasmussen, 2002). Identification and characterization of fungi associated with roots has changed over time as a wider variety of techniques have become available. Morphological assays involving the examination of colonized roots, isolation and culturing of fungi on media, and identification of species from fruiting bodies collected on and around suspected host plants represent the majority of efforts to describe root-associated fungal communities. In recent decades, the continuing development of molecular tools has allowed for the increased identification of fungal taxa using DNA sequence information in place of traditional morphological assessments, opening up the black box of uncultured/unknown fungi in environmental samples.

B. Next Generation Sequencing

Sanger sequencing (chain termination sequencing) of the Internal Transcribed Spacer (ITS) region of the fungal ribosomal RNA gene (Scooch et al., 2012) has made possible the DNA-based identification of fungi. This particular locus provides accurate identification for most fungal taxa, provided there are adequate correctly-identified and curated reference sequences in available DNA databases, but Sanger sequencing is still limited by the need for isolated DNA from a single organism for sequencing. Most recently it has become possible to assess fungal

communities in environmental samples containing multiple species using Next Generation Sequencing (NGS) techniques.

As DNA sequencing technology has changed, so too has our capacity to ask questions about the communities of organisms we can sample. NGS technologies offer an opportunity to examine communities of fungi that are present in an environmental sample without necessarily being able to isolate and grow them on media or to observe their morphology for traditional taxonomic identification (Shokralla et al., 2012). This has led to an increase in our understanding of the diversity of fungi in aerial (Núñez et al., 2017), aquatic (Zinger et al., 2012), and soil samples (Buée et al., 2009). Several platforms are available for high-throughput sequencing of environmental samples. Notable examples of NGS platforms include Roche 454 (pyrosequencing), Illumina MiSeq (sequencing by synthesis), and Ion Torrent Personal Genome Machine (PGM) (semiconductor sequencing). Liu et al. (2012) conducted a comparative analysis of these and other NGS technologies in addition to new Third Generation sequencing technologies. A cost to output quality assessment for these platforms shows a variety of trade-offs between selected technologies with no clear winner in terms of both cost effectiveness and quality of data (Liu et al., 2012). Roche 454 offers read lengths up to 700 bp and high accuracy while being more expensive than other platforms. Illumina MiSeq provides paired-end sequencing of reads which provides read lengths up to 600 bp (300 x 2) depending on the kit purchased for library preparation. Ion Torrent PGM provides high quality reads up to 400 bp in length, and there is some additional flexibility in library preparation costs for sequencing when compared to the proprietary consumables required for the Illumina platform.

Biodiversity studies have benefitted greatly from the use of NGS sequencing by allowing the rapid assessment of organismal diversity from a variety of environmental samples. NGS

technologies are a set of useful tools providing data on communities of organisms inhabiting a variety of plants (Sun and Guo, 2012; Kemler et al., 2013) and animals (Yoon et al., 2015), soils (Tedersoo et al., 2014), and waterways (Tan et al., 2015). These NGS tools provide information useful for a variety of land, animal, and forest management challenges, including potential biological controls of plant pathogens by endophytic fungi (Berg et al., 2008), and the evaluation of the health and genetic diversity of animal populations (Waits and Paetkau, 2005). Community analysis of NGS data has also recently been used to assess the impact of more pressing anthropogenic factors such as climate change (Geml et al., 2016) and environmental pollution (Yergeau et al., 2012) on microorganisms.

C. Acid Deposition & Ecosystems

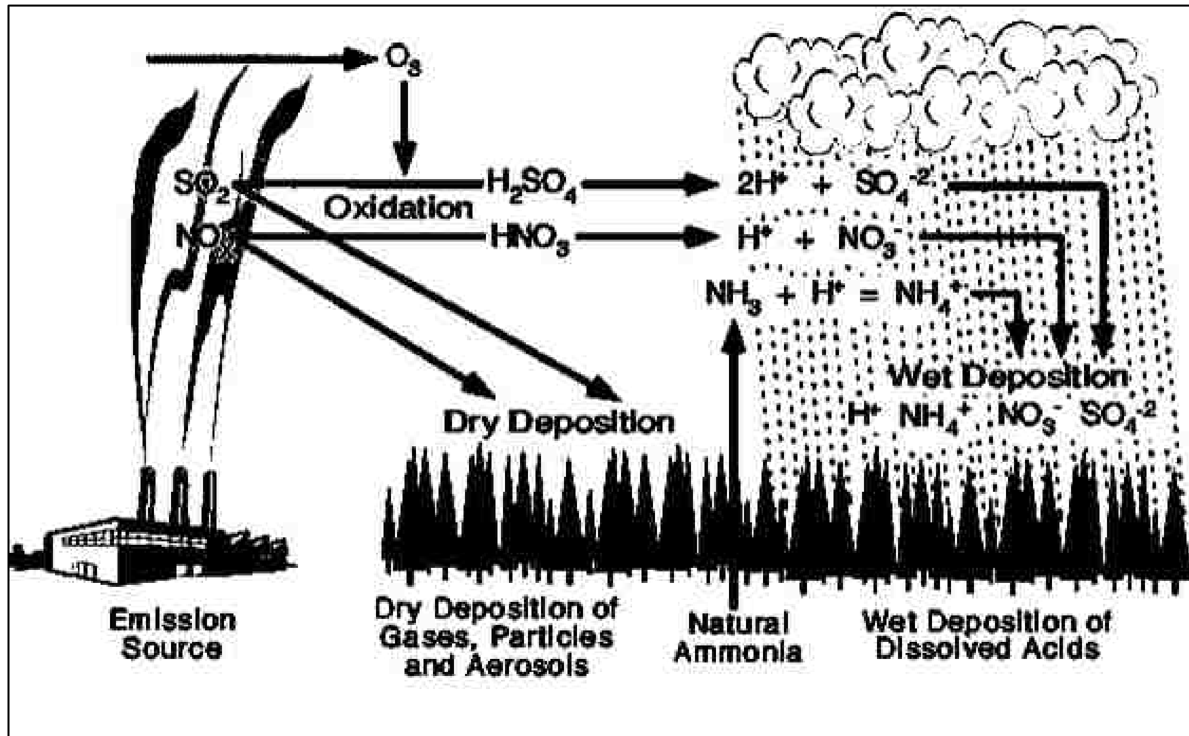


Figure 2. The cycle of anthropogenic acid deposition from industrial source air pollution to wet and dry deposition (Source: *https://environmental-chemistry.wikispaces.com/Acid+Deposition*).

Environmental pollution is a concern for its effect not only on the wellbeing of humans, but also due to the impact on ecosystems and the species of wildlife that inhabit them. One area of concern in human caused environmental pollution is acid deposition (Figure 1). This can take the form of either dry or wet deposition depending on whether the pollution consists of particulates spread on the wind and fog or acidic compounds falling to the surface in precipitation, thus linking air pollution to terrestrial and aquatic ecosystems. The first reports of acid deposition in North America come from data collected in an experimental forest in New Hampshire in the 1960s (Likens et al., 1972). The composition of wet and dry acid deposition consists of gasses and particulates containing nitrogen oxides (NO_x), ammonia (NH_3), and sulfur dioxide (SO_2) (Driscoll et al., 2001). In the United States, controls on emissions of SO_2 began in the 1970s with amendments to the Clean Air Act, and continued in 1990 with passage of portions of the Acid Deposition Control Program which called for further reduction in SO_2 emissions and the control of NO_x emissions from utility services (Driscoll et al., 2001). This has had a positive impact on acidification, but nitrogen deposition remains an active area of interest for those concerned about the effects of acid rain (Dentener et al., 2006). Acidity in precipitation can be measured using the pH scale, which is the negative logarithm of the concentration of hydrogen ions in a solution. The scale ranges from 0 to 14, with 7 being neutral and lower numbers corresponding to logarithmically higher concentrations of hydrogen ions. On the acidic side of the scale, a pH measurement of 6 contains 1 μeq/l of H^+ ions, a pH measurement of 5 contains 10 μeq/l, a measurement of 4 contains 100 μeq/l and so on (Likens et al., 1979). In wet deposition, or acid rain as it is otherwise known, acidic compounds are present in precipitation and lower the pH of the terrestrial and aquatic systems where they are deposited, thus causing a host of cascading effects (Likens and Bormann, 1974).

Various ecosystems have differential responses to acid rain. In freshwater aquatic ecosystems, acid rain has been linked to the reduction or disappearance of fish populations (Haines and Baker, 1986), increases in N concentrations and disruption of nutrients for animal and plant communities leading to eutrophication (Lepori and Keck, 2012), along with richness reduction (Baldigo et al., 2009) and shifts in communities of aquatic invertebrates based on acidity (Lepori et al., 2003). In recent years, pollution abatement strategies have led to reduction in aquatic ecosystem acidification and an improvement in the chemical quality of surface waters in North America and Europe (Garmo et al., 2014). Terrestrial ecosystems have also been negatively impacted by wet acid deposition. Grasslands have experienced loss of species (Clark and Tilman, 2008), changes in community composition (Duprè et al., 2010), and reduction of diversity (Stevens et al., 2010) due in large part to nitrogen deposition and acidification caused by nitrogen deposition. Recent efforts in Europe to reduce environmental pollution of nitrogen compounds from emissions or fertilizers have led to evidence of grassland biodiversity recovery (Storkey et al., 2015). Forest responses to acid deposition are complex and vary depending on the plant species involved and the chemistry of the underlying soil and bedrock. In the Hubbard Brook Experimental Forest of New Hampshire, a mixture of deciduous and coniferous trees has been found to respond to acidification with reduction in overall forest biomass, likely due to changes in available soil nutrients (Likens et al., 1996). In the Fernow Experimental Forest of West Virginia, all measured growth metrics of Japanese larch have shown reduced growth in response to ammonium sulfate treatment, while some deciduous species experienced early growth increases and later slowed growth due to acidification treatment over a 14-year period (DeWalle et al., 2006). In Maine, 10 years of ammonium sulfate treatment showed an increase in growth of sugar maple, but no growth response by red spruce (Elvir et al., 2003). Experimental

addition of ammonium nitrate over a 9-year period to a pine forest and deciduous broadleaf forest in Massachusetts also resulted in decreased growth in pines and some increased growth in the hardwood deciduous broadleaf trees (Magill et al., 2000). These growth trends are presumed to be related to changes in soil chemistry and exchangeable cations in forest soils caused by artificial acidification, and these changes appear to result in differential responses by deciduous and coniferous species (Magill et al., 2000; Elvir et al., 2003; De Walle et al., 2006). Aquatic and terrestrial ecosystems respond in a variety of ways to wet deposition, and among terrestrial ecosystems the visible above-ground changes appear to be related to changes in the soil.

Soil chemistry and soil microbial communities change and shift in response to artificial acidification. Acid rain causes increased leeching of cations out of soils, which is mediated at least partially in locales where soil formation from underlying bedrock replaces lost cations (Johnson et al., 1990). In addition to leeching of Ca^{++}, Mn^{++}, and other important ions in the soil (Adams et al., 2007), nitrogen saturation and Al^{3+} increases in soil are suspected of causing changes in aboveground communities (De Walle et al., 2006) and soil microbial communities (Adams and Angradi 1996; Pennanen et al., 1998; DeForest et al., 2004). Our understanding of acidification effects on soil microbes is growing but remains limited. Shifting microbial communities in forest soils could play a role in the responses of plants to acidification, but little is known about the impact of acid rain on root-associated fungal community structure and function. A comprehensive understanding of the effect of acidification on soil microbial communities in general—and root-associated fungi in particular—would be of great benefit to our understanding of the effects of acidification on forest ecosystems as a whole.

D. Objectives

Root-associated Fungal Communities in Two European Deciduous Forests

In this first research chapter, the communities of fungi associated with the roots of members of the families Fagaceae and Betulaceae (*Fagus sylvatica* and *Quercus robur*, *Betula pendula* and *Carpinus betulus,* respectively) in a temperate deciduous forest located in Wassenaar, South Holland, Netherlands and another sampling site near Baden-Württemberg, Southwest Germany (Figure 2) are described. In addition to describing community richness and diversity, the distribution of taxa on the four selected host species, the level of specificity in the relationship of fungus to each host, and the potential fungal-fungal interactions at the root using statistical analysis to determine if fungal presence/absence patterns are related to those of other fungi are discussed.

Figure 3. Location of field sampling sites in the Netherlands (N) and Germany (G). Image source (By Alexrk2 - Own workData from/Données issues de http://naturalearthdata.com/, Scale: 1:10 Mio, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=9701652)

Root-associated Fungal Communities in Deciduous Forests of Eastern North America

This chapter details the communities of fungi associated with roots of members of the families Fagaceae (*Fagus grandifolia* and *Quercus rubra* L.) and Juglandaceae (*Carya ovata* [Mill.] K. Koch) from roots of host trees sampled at sites in Arkansas, Virginia, and West Virginia (Figure 3). Fungal community taxonomy, species richness, distribution across different host species and locales, and the level of host specificity are described. In addition, this chapter describes the root-associated fungal community found on *Carya ovata*, which represents, to the best of my knowledge, the first attempt to characterize of the root-associated communities of this host tree species.

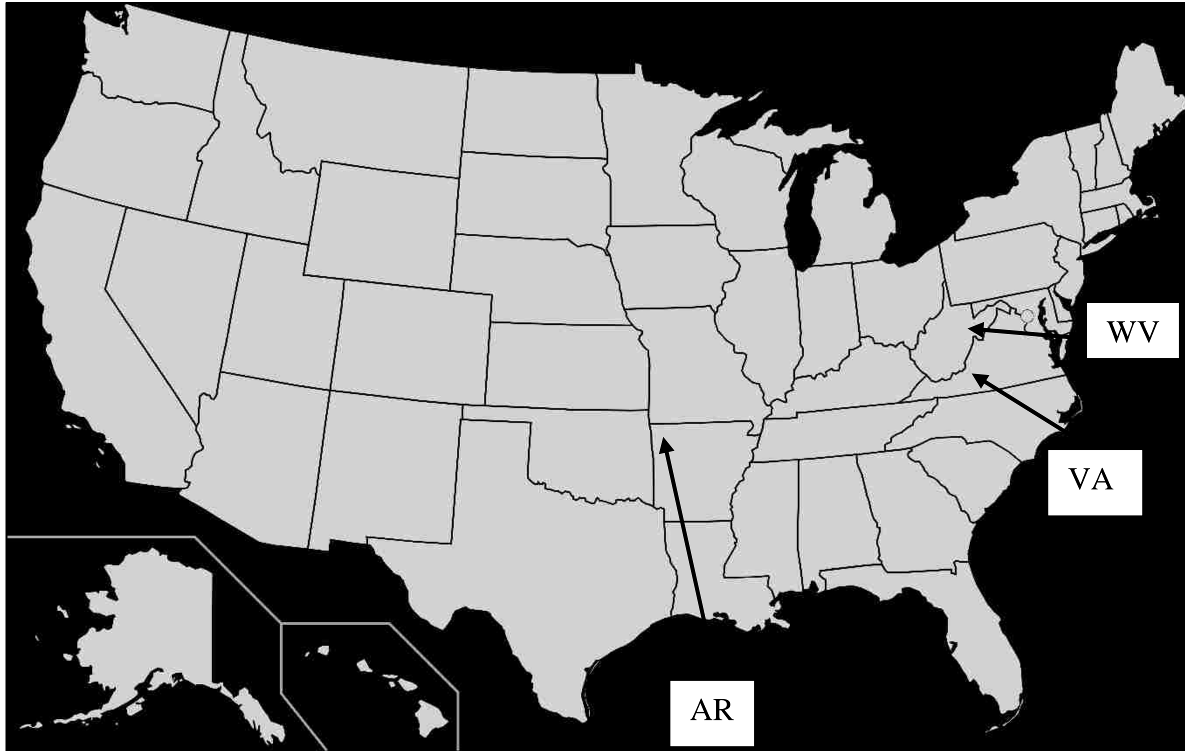


Figure 4. Locations of field sites in North America include four sites in AR (Pea Ridge National Military Park, Ozark National Forest, Lake Wilson Park, and Lost Valley Park), one site in WV, and one site in VA. Image adapted from original source: By Theshibboleth - own work, based on Electoral map.svg, inspired by BlankMap-World.png, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=941237

Effects of Acid Deposition on Root-associated Fungal Communities

The final research chapter in this dissertation examines the effect of artificial acid deposition treatments in the Fernow Experimental Forest near Parsons, West Virginia (Figure 4) on communities of fungi associated with the roots of *Fagus grandifolia, Quercus alba* L.*,* and *Q. rubra*. In this chapter, the impact of anthropogenic activities resulting in acidification of forested areas is quantified with respect to root-associated fungal communities. The influence of a treatment with ammonium sulfate fertilizer on community composition (abundance and richness), taxonomy, and functional guilds across different host tree species are assessed.

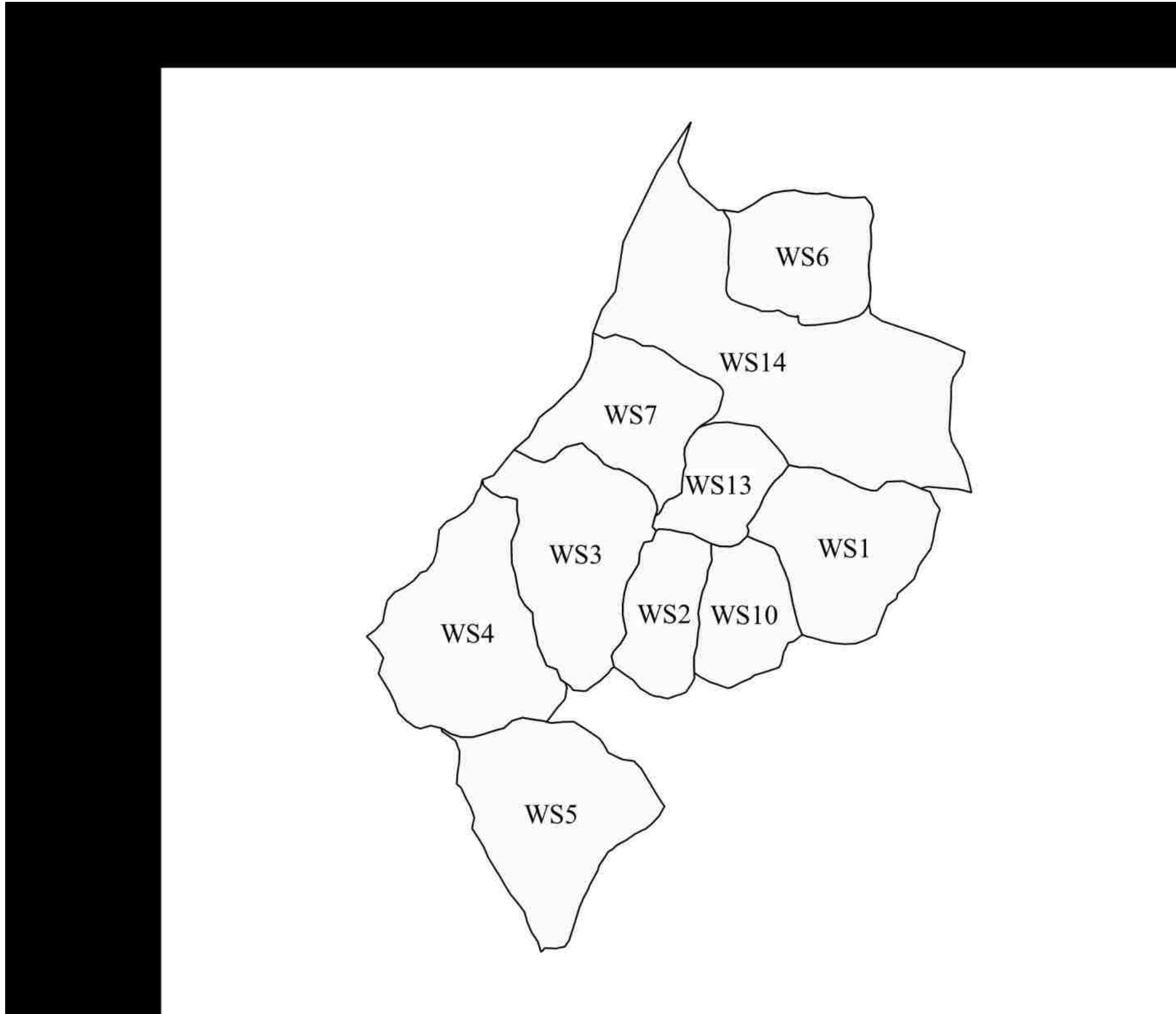


Figure 5. Map of the watershed boundaries in the Fernow Experimental Forest near the town of Parsons in West Virginia. WS3 is treated with ammonium sulfate fertilizer tri-annually. Adjacent watersheds contain comparable vegetation but are not treated.

E. References

Adams, M. B., and Angradi, T. R. (1996). Decomposition and nutrient dynamics of hardwood leaf litter in the Fernow whole-watershed acidification experiment. *Forest Ecology and Management* 83(1), 61-69.

Adams, M. B., Kochenderfer, J. N., and Edwards, P. J. (2007). The Fernow watershed acidification study: ecosystem acidification, nitrogen saturation and base cation leaching. *Water, Air, and Soil Pollution: Focus* 7(1-3), 267-273.

Archibold, O. W. (2012). Ecology of World Vegetation. Springer Science and Business Media.

Baldigo, B. P., Lawrence, G. B., Bode, R. W., Simonin, H. A., Roy, K. M., and Smith, A. J. (2009). Impacts of acidification on macroinvertebrate communities in streams of the western Adirondack Mountains, New York, USA. *Ecological Indicators* 9(2), 226-239.

Berg, G., Müller, H., Zachow, C., Opelt, K., Scherwinski, K., Tilcher, R., Ulrich, A., Hallmann, J., Grosch, R., and Sessitsch, A. (2008). Endophytes: structural and functional diversity and biotechnological applications in control of plant pathogens. *Ecological Genetics* 6(2).

Blasius, D., Feil, W., Kottke, I., and Oberwinkler, F. (1986). Hartig net structure and formation in fully ensheathed ectomycorrhizas. *Nordic Journal of Botany* 6(6), 837-842.

Braun, E. L. (1950). Deciduous Forests of Eastern North America. Blakiston, Philadelphia.

Brundrett, M. C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant and Soil* 320(1-2), 37-77.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R. H., Uroz, S., and Martin, F. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist* 184(2), 449-456.

Clark, C. M., and Tilman, D. (2008). Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature* 451(7179), 712.

DeForest, J. L., Zak, D. R., Pregitzer, K. S., and Burton, A. J. (2004). Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal* 68(1), 132-138.

Dentener, F., Drevet, J., Lamarque, J. F., Bey, I., Eickhout, B., Fiore, A. M., Hauglustaine, D., Horowitz, L.W., Krol, M., Kulshrestha, U.C. and Lawrence, M. (2006). Nitrogen and sulfur deposition on regional and global scales: a multimodel evaluation. *Global Biogeochemical Cycles* 20(4).

DeWalle, D. R., Kochenderfer, J. N., Adams, M. B., Miller, G. W., Gilliam, F. S., Wood, F., Odenwald-Clemens, S. S., and Sharpe, W. E. (2006). Vegetation and acidification. In *The Fernow Watershed Acidification Study* (pp. 137-188). Springer Netherlands.

Driscoll, C. T., Lawrence, G. B., Bulger, A. J., Butler, T. J., Cronan, C. S., Eagar, C., Lambert, K. F., Likens, G. E., Stoddard, J. L., and Weathers, K. C. (2001). Acidic Deposition in the Northeastern United States: Sources and Inputs, Ecosystem Effects, and Management Strategies:

The effects of acidic deposition in the northeastern United States include the acidification of soil and water, which stresses terrestrial and aquatic biota. *AIBS Bulletin* 51(3), 180-198.

Duprè, C., Stevens, C. J., Ranke, T., Bleeker, A., Peppler-Lisbach, C., Gowing, D. J., Dise, N. B., Dorland, E. D. U., Bobbink, R., and Diekmann, M. (2010). Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. *Global Change Biology* 16(1), 344-357.

FAO Global Forest Resources Assessment 2015. FAO Forestry Paper No. 1 UN Food and Agriculture Organization, Rome (2015)

Gamfeldt, L., Snäll, T., Bagchi, R., Jonsson, M., Gustafsson, L., Kjellander, P., Ruiz-Jaen, M.C., Fröberg, M., Stendahl, J., Philipson, C.D., and Mikusiński, G. (2013). Higher levels of multiple ecosystem services are found in forests with more tree species. *Nature Communications* 4, 1340.

Garmo, Ø. A., Skjelkvåle, B. L., de Wit, H. A., Colombo, L., Curtis, C., Fölster, J., Hoffmann, A., Hruška, J., Høgåsen, T., Jeffries, D.S., and Keller, W. B. (2014). Trends in surface water chemistry in acidified areas in Europe and North America from 1990 to 2008. *Water, Air, and Soil Pollution* 225(3), 1880.

Geml, J., Semenova, T. A., Morgado, L. N., and Welker, J. M. (2016). Changes in composition and abundance of functional groups of arctic fungi in response to long-term summer warming. *Biology Letters* 12(11), 20160503.

Haines, T. A., and Baker, J. P. (1986). Evidence of fish population responses to acidification in the eastern United States. *Water, Air, and Soil Pollution* 31(3), 605-629.

Hanson, C. A., Allison, S. D., Bradford, M. A., Wallenstein, M. D., and Treseder, K. K. (2008). Fungal taxa target different carbon sources in forest soil. *Ecosystems* 11(7), 1157-1167.

Johnson, D. W., Cresser, M. S., Nilsson, S. I., Turner, J., Ulrich, B., Binkley, D., and Cole, D. W. (1990). Soil changes in forest ecosystems: evidence for and probable causes. *Proceedings of the Royal Society of Edinburgh, Section B: Biological Sciences* 97, 81-116.

Keenan, R. J., Reams, G. A., Achard, F., de Freitas, J. V., Grainger, A., and Lindquist, E. (2015). Dynamics of global forest area: results from the FAO Global Forest Resources Assessment 2015. *Forest Ecology and Management* 352, 9-20.

Kemler, M., Garnas, J., Wingfield, M. J., Gryzenhout, M., Pillay, K. A., and Slippers, B. (2013). Ion Torrent PGM as tool for fungal community analysis: a case study of endophytes in Eucalyptus grandis reveals high taxonomic diversity. *PLoS One* 8(12), e81718.

Kew, R. B. G. (2016). The state of the world's plants report–2016. Royal Botanic Gardens, Kew.

Lepori, F., and Keck, F. (2012). Effects of atmospheric nitrogen deposition on remote freshwater ecosystems. *Ambio* 41(3), 235-246.

Lepori, F., Barbieri, A., and Ormerod, S. J. (2003). Effects of episodic acidification on macroinvertebrate assemblages in Swiss Alpine streams. *Freshwater Biology* 48(10), 1873-1885.

Likens, G. E. and Bormann, F. H. (1974). Acid rain: a serious regional environmental problem. *Science* 184, 1176-1179.

Likens, G. E., Bormann, F. H., and Johnson, N. M. (1972). Acid rain. *Environment: Science and Policy for Sustainable Development* 14(2), 33-40.

Likens, G. E., Driscoll, C. T., and Buso, D. C. (1996). Long-term effects of acid rain: response and recovery of a forest ecosystem. *Science* 272(5259), 244.

Likens, G. E., Wright, R. F., Galloway, J. N., and Butler, T. J. (1979). Acid rain. *Scientific American*, 241(4), 43-51.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., and Law, M. (2012). Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology* 251364-251364.

Magill, A. H., Aber, J. D., Berntson, G. M., McDowell, W. H., Nadelhoffer, K. J., Melillo, J. M., and Steudler, P. (2000). Long-term nitrogen additions and nitrogen saturation in two temperate forests. *Ecosystems* 3(3), 238-253.

Núñez, A., de Paz, G. A., Ferencova, Z., Rastrojo, A., Guantes, R., García, A. M., Alcamí, A., Gutiérrez-Bustillo, A.M., and Moreno, D. A. (2017). Validation of the Hirst-type spore trap for simultaneous monitoring of prokaryotic and eukaryotic biodiversities in urban air samples by next-generation sequencing. *Applied and Environmental Microbiology* 83(13), e00472-17.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* 6(10), 763-775.

Pell, W. F., and Bukenhofer, G. A. (1999). Ozark-Ouachita highlands assessment: terrestrial vegetation and wildlife (Vol. 5). US Dept. of Agriculture, Forest Service, Southern Research Station. Asheville, North Carolina.

Pennanen, T., Fritze, H., Vanhala, P., Kiikkilä, O., Neuvonen, S., and Bååth, E. (1998). Structure of a microbial community in soil after prolonged addition of low levels of simulated acid rain. *Applied and Environmental Microbiology* 64(6), 2173-2180.

Rasmussen, H. N. (2002). Recent developments in the study of orchid mycorrhiza. *Plant and Soil* 244(1), 149-163.

Read, D. J. (1996). The structure and function of the ericoid mycorrhizal root. *Annals of Botany* 77(4), 365-374.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., Crous, P.W., and Miller, A. N. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences* 109(16), 6241-6246.

Shokralla, S., Spall, J. L., Gibson, J. F., and Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology* 21(8), 1794-1805.

Stevens, C. J., Duprè, C., Dorland, E., Gaudnik, C., Gowing, D. J., Bleeker, A., Diekmann, M., Alard, D., Bobbink, R., Fowler, D., and Corcket, E. (2010). Nitrogen deposition threatens species richness of grasslands across Europe. *Environmental Pollution* 158(9), 2940-2945.

Storkey, J., Macdonald, A. J., Poulton, P. R., Scott, T., Köhler, I. H., Schnyder, H., Goulding, K.W.T., and Crawley, M. J. (2015). Grassland biodiversity bounces back from long-term nitrogen addition. *Nature* 528(7582), 401.

Sun, X., and Guo, L. D. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. *Mycology* 3(1), 65-76.

Svenning, J. C. (2002). A review of natural vegetation openness in north-western Europe. *Biological Conservation* 104(2), 133-148.

Tan, B., Ng, C., Nshimyimana, J. P., Loh, L. L., Gin, K. Y. H., and Thompson, J. R. (2015). Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. *Frontiers in Microbiology* 6.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., and Smith, M. E. (2014). Global diversity and geography of soil fungi. *Science* 346(6213), 1256688.

Waits, L. P., and Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management* 69(4), 1419-1433.

Yergeau, E., Lawrence, J. R., Sanschagrin, S., Waiser, M. J., Korber, D. R., and Greer, C. W. (2012). Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities. *Applied and Environmental Microbiology* 78(21), 7626-7637.

Yoon, S. S., Kim, E. K., and Lee, W. J. (2015). Functional genomic and metagenomic approaches to understanding gut microbiota–animal mutualism. *Current Opinion in Microbiology* 24, 38-46.

Zinger, L., Gobet, A., and Pommier, T. (2012). Two decades of describing the unseen majority of aquatic microbial diversity. *Molecular Ecology* 21(8), 1878-1896.

II. Diversity, distribution and specificity of fungi associated with root material of four tree species in two European forests

Donald J. Nelsen*[1,2], Tatiana A. Semenova-Nelsen[2], József Geml[2,3], Sandeep Sharma[4], Burt H. Bluhm[4], and Steven L. Stephenson[1].

1. Department of Biological Sciences, 850 W. Dickson Street SCEN 601, Fayetteville, AR 72701, USA

2. Biodiversity Dynamics Research Group, Naturalis Biodiversity Center, Leiden, the Netherlands

3. Faculty of Science, Leiden University, Leiden, the Netherlands

4. Department of Plant Pathology, University of Arkansas, Plant Sciences Building room 217, 495 Campus Drive, Fayetteville, AR 72701, USA

*Corresponding author: djnelsen@uark.edu

A. Abstract

Temperate mixed deciduous forests are economically and ecologically important ecosystems. Ectomycorrhizal fungi and other root-associated fungi play an important role in the maintenance of these ecosystems. Knowledge of the diversity and distribution of fungi associated with specific tree species in these forests is incomplete. The focus of this research was to assess diversity, distribution, host specificity, and species co-occurrence patterns of root-associated fungi to better understand the ecology of underground microbial communities of the dominant tree species *Fagus sylvatica, Quercus robur, Betula pendula,* and *Carpinus betulus* in two European forests. DNA from root-tips of host trees was used to identify fungi using ITS meta-barcoding on the Ion Torrent NGS platform. This resulted in 1756 OTUs across 40 sampled trees. Mean richness was lowest for *Q. robur,* and highest for *C. betulus.* Root-associated fungi were found to represent more than 50 taxonomic orders. The distribution of root-associated fungi was largely driven by locality, with host family and host genus also playing a significant role.

OTUs were distributed across different hosts unequally, with 27.3% of all OTUs found only on *C. betulus*. Fifty-six point five percent of all fungal OTUs occurred on only one host tree species, while 63.4% were restricted to host families, suggesting some level of host specificity. Fungal co-occurrence analyses of all identified OTUs, saprotrophic OTUs, and ectomycorrhizal OTUs showed that within and among ecological functional groups, there are positive and negative fungal-fungal interactions.

Keywords: meta-barcoding, fungal diversity, co-occurrence, host specificity

Acknowledgements

The authors would like to acknowledge the following sources of funding support: this work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1008895; research was also funded in part by the Slime Mold Project at the University of Arkansas. A special thank you to the Naturalis Biodiversity Center's Martin Fellowship for providing the funding necessary to complete sampling in Europe and for facilitating a collaborative relationship between labs in the USA and the Netherlands. In addition, the authors would like to thank Nicolas Pastor for assistance with sample collecting in the Netherlands, and Luis N. Morgado for lending technical expertise and critical assessment of raw sequence analysis.

Author contributions: Donald J. Nelsen developed this research project, collected and processed samples, analyzed the data, and wrote the paper. Tatiana A. Semenova-Nelsen collected and processed samples, assisted with data analysis and writing the paper. József Geml helped develop the research, collect samples, and provided laboratory resources. Sandeep Sharma sequenced the samples. Burt H. Bluhm provided laboratory consumables and sequencing resources. Steven L. Stephenson helped develop research and provided laboratory resources.

B. Introduction

Temperate forests make up one of the most important biomes on Earth, covering an area of more than 570 million hectares (FAO & JRC 2012). In the European Union, approximately 42% of land coverage consists of forests and woodlands, and these areas are continuously increasing due to on-going forestation programs (*www.ec.europa.eu*). The importance of forests is difficult to overestimate, given their key roles in oxygen production and air filtering, as habitats for numerous species, their importance for watershed protection, prevention of soil erosion, and as a source of wood for various industries. Forests are of particular importance for the global carbon (C) budget; according to FAO estimations, in 2005 C content in European forests approached nearly 180 Gt, including 110 Gt stored in forest soils (ftp.fao.org). Due to their importance as a C sink, forests play key roles in mitigating climate change (Pan et al., 2011).

Fungi are also an important and diverse component of forest ecosystems. Saprotrophic fungi regulate forest C cycling through decomposition, and some species are the only organisms able to decompose lignin (de Boer et al., 2005). Mycorrhizal fungi mediate belowground allocation of C (Clemmensen et al., 2013) by accessing C compounds from roots of the forest trees in exchange for soil-derived nutrients. Many woody plants in temperate forests form obligate symbioses with ectomycorrhizal (ECM) fungi that are essential for the development of a large proportion of woody plants. Across Western Europe, dominant tree species of mixed deciduous forests such as *Fagus sylvatica* L., *Quercus robur* L., *Betula pendula* Roth, and *Carpinus betulus* L. are involved in biotic interactions with ECM fungi.

Although the importance of ECM fungi for establishment of forests is well known, our knowledge of their diversity remains limited. Particularly little is known of the factors that affect diversity and distribution of ECM fungi. It is generally assumed that ECM assemblages reflect

soil nutrient availability and water content, soil texture and forest type, as well as factors that impact the above-mentioned parameters, such as geographic zone or forest management activities (Wubet et al., 2012). However, there is no clear understanding regarding the relative impact of these factors. For example, the impact of host specificity has been estimated from being almost negligible (Horton & Bruns 1998; Kennedy et al., 2003; Tedersoo et al., 2008; Richard et al., 2009; Kennedy et al., 2012) to strongly important (Smith et al., 2009). Given the high variation in biology of ECM fungi, it is difficult to provide any generalizations regarding the extent of host specificity; however, there is evidence for particular ECM genera to grow in symbiosis with representatives of specific taxonomic families or genera of trees (Molina et al., 1992; Newton and Haigh 1998; Massicotte et al., 1999). Assessments of ECM fungal diversity across various habitats, including different forest types or edaphic gradients, became possible with the recent advancement of next-generation sequencing techniques. In addition, deep sequencing data provided a potential for unraveling fungal-fungal interactions using species co-occurrence data to reveal possible examples of cooperation and competition in fungi (Ovaskainen et al., 2013).

DNA-based studies have dramatically increased our knowledge of fungal diversity and species richness in the dominant ECM genera. In European mixed forests, however, the majority of studies have been carried out on fungal communities isolated from soil. To our knowledge, no studies have focused on root material gathered directly from the ECM root-tips of trees in European forests, and this may limit our ability to build conclusions with regards to ECM diversity, host-association, and community structure. In the present study we collected ECM colonized root-tips of four tree species growing in two mixed deciduous forests, the first located in the Netherlands and the second in Germany. Our objectives were to address the following

research questions: (1) What is the diversity of fungi inhabiting roots of *Fagus sylvatica, Quercus robur, Betula pendula,* and *Carpinus betulus* in temperate European mixed deciduous forests? (2) What is the extent of host-specificity in fungal community compositions; if apparent, does the host-specificity occur in spatially disparate forests? (3) Are there any patterns in fungal species co-occurrence indicative of fungal-fungal interactions (cooperation or competition)?

C. Methods

Study sites and root sampling

Root-tip samples were taken from host tree taxa at collecting sites in the Netherlands and Germany in June and July 2015, respectively. The Netherlands sampling site consisted of a mixed deciduous forest area in Wassenaar, South Holland (Lat. 52° 7'44.70"N, Lon. 4°23'19.63"E) dominated by *Fagus sylvatica* L., *Quercus robur* L., and *Betula pendula* Roth. The sampling site in the Netherlands was ca. 314,453 m^2 in total extent. The German sampling site (Lat. 48°54'44.77"N, Lon. 8°28'43.53"E) consisted of a mixed deciduous forest area in Baden-Württemberg, Southwest Germany. Prominent deciduous tree species at this site included the same taxa represented in the Netherlands sample site. The sampling site in Germany was approximately 809,698 m^2. The linear distance between sample sites was 460.5 km.

We assessed fungal communities associated with roots of four tree species belonging to two taxonomic families. These species were: *Fagus sylvatica* and *Quercus robur* in the Fagaceae, and *Betula pendula* and *Carpinus betulus* L. in the Betulaceae. Five replicates of each host tree were sampled at each site, resulting in a total of 20 trees sampled per site. Trees were opportunistically sampled on both sites at a distance of at least 10 m from one another to avoid sampling the same genet repeatedly and minimize same-community resampling (Dahlberg and

Stenlid 1990, 1994; Bonello et al., 1998; Gherbi et al., 1999; Zhou et al., 1999, 2001; Fiore-Donno and Martin 2001; Kretzer et al., 2004).

The upper soil and litter layers were removed prior to root sampling, and roots were visually inspected to verify that they were emerging from the tree of interest. Sampling was carried out at 90° increments around the base of each sampled tree, and collected roots were combined and placed in 15 ml screw-top tubes containing 7 ml of 2% CTAB solution. Sample tubes were kept frozen in -20°C until processing for DNA extraction in the laboratory within two weeks of collection. Root samples were washed with distilled water to remove soil and organic particulates. The material was then examined under a dissecting scope and root-tips were carefully collected from each sample with heat sterilized forceps. Collected root-tips were placed in 2 ml microcentrifuge tubes and lyophilized. Root material was then lysed with sterile 3 mm glass beads in a Tissue Lyser (QIAGEN™), and thoroughly mixed prior to DNA extraction.

DNA extraction, PCR and sequencing

For each of the samples, two independent DNA extractions were carried out using a total of ca. 50 mg of homogenized root tissue. DNA was extracted using a Macherey-Nagel NucleoSpin Plant II DNA extraction kit, following the manufacturer's protocol for PL1. Extracted DNA was eluted twice in 30 µl of PE buffer solution. Prior to PCR, replicate DNA extractions for each sample were pooled together. Subsequent 40 µl PCR reactions were carried out as in Geml et al. (2014) and contained 1 µl of DNA template, 4 µl of 10X buffer, 1.5 µl of dNTP solution (2.5 mM), 1.5 µl each of forward and reverse primers (10 mM), 0.5 µl BSA (10 mg/ml), 4 µl $MgCl_2$ (50 mM), 0.4 µl BIOTAQ polymerase (5 U/µl), and 25.6 µl of MQ water. Primers used were fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) labeled with sample-specific Multiplex Identification DNA (MID) tags (Table S1) to amplify ca. 250-450 bp of the ITS2

region (Toju et al., 2012). PCR reactions were carried out under the following conditions: one cycle of 95°C for 5 min, followed by 25 cycles of 95°C for 20 sec, 54°C for 30 sec, and 72°C for 1.5 min, ending in a terminal elongation at 72°C for 7 min. Three replicate PCR reactions and a negative control consisting of MQ water instead of template were carried out for each sample. PCR products were verified via agarose gel electrophoresis, and the resultant products were pooled for each sample.

Pooled PCR products were assessed for DNA concentration and amplicon size distribution using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). Short fragments and reagents were removed using 0.9X Ampure® beads (Beckman-Coulter, Beverly, MA, USA). Equimolar concentrations of PCR product were achieved by dilution with MQ water, and all products were pooled for subsequent sequencing. Emulsion PCR of 250 µl of the pooled samples was carried out in accordance with the protocol for the Ion PGM™ 200Xpress™ Template Kit. The resultant library was sequenced using the Ion 318™ Chip on an Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Guilford, CT, USA), at the University of Arkansas.

Bioinformatics

Ion Torrent sequencing resulted in 9,396,405 total raw sequence reads, with a modal read length of 276 bp and a median length of 199 bp. Raw sequencing data were cleaned-up using the online platform Galaxy (https://main.g2.bx.psu.edu/root), in which the sequences were sorted according to samples, and adapters (identification tags) were removed. Poor-quality ends of the sequences were trimmed off based on 0.02 error probability limit in Geneious Pro 5.6.1 (BioMatters, New Zealand). Subsequently, sequences were filtered using USEARCH v.8.0 (Edgar, 2010) based on the following settings: all sequences were truncated to the length of 200 bp and sequences with

expected error > 1 were discarded. The resulting high-quality sequences were grouped into 4,637 operational taxonomic units (OTUs) by UPARSE algorithm in USEARCH at 97% sequence similarity, as in other fungal metabarcoding studies (e.g., Bjorbækmo et al., 2010; Geml et al., 2010; Bellemain et al., 2013; Tedersoo et al., 2014). Simultaneously, 4,658 putative chimeric sequences were excluded. We assigned sequences to taxonomic groups based on pairwise similarity searches against the curated UNITE fungal ITS sequence database containing identified fungal sequences with assignments to Species Hypothesis groups (Kõljalg et al., 2013). After discarding global singletons and OTUs that did not have at least 80% similarity (across at least 100 bp) to any fungal sequence in UNITE, the final dataset contained 1,756 OTUs. Representative sequences of fungal OTUs were submitted to GenBank with the accession numbers KY413820-KY415572.

Statistical analyses

We quantified the depth of sequencing coverage by rarefaction curve and coverage estimators. Rarefaction analysis, Good's coverage, Shannon's (H) and Simpson's diversity indexes, OTU richness (S) and evenness (H/ln S) was carried out and/or calculated using the "rarefy" function in the Vegan package (Oksanen et al., 2012) in R software for statistical computing (R Core team 2013). The distribution of fungal OTUs among the four host tree species was visualized using Venny 2.1 (Oliveros 2015) to determine the extent of OTUs associated with each host species. We also assessed the number of OTUs for specific ecological groups found on/in the root tissue of each host tree, using the dataset of Tedersoo et al. (2014) for ecological guild assignment. Criteria for ecological guild assignment consisted of taxonomic identification to the species level where possible or to genus where ecological role was consistent for that taxon level. The difference in fungal community compositions of tested host trees was visualized using Non-

metric Multidimensional Scaling (NMS) in PC-ORD v. 5.32 (McCune and Grace 2002) using two different approaches. Initially, we compared the data for both the German and the Netherlands sampling sites, and then analyzed fungal community compositions separately for German and Netherlands sample sites. The latter allowed us to visualize the extent of host fidelity in the communities of fungi associated with different tree species in each site. The primary matrix consisted of sampled trees by OTU presence-absence data (i.e., fungal community composition). Following recommendations of other fungal metabarcoding studies (e.g., Lindahl et al., 2013; Morgado et al., 2016), presence was set as ≥3 sequences on a per sample basis. The secondary matrix consisted of sampled trees by number of OTUs belonging to specific taxa (richness of fungal taxa). The dataset was subjected to 500 iterations per run using the Sørensen similarity (Bray-Curtis index) and a random starting number. To quantify the extent of host specificity of fungal community compositions, we carried out a permutation-based nonparametric MANOVA (Anderson 2001), also in PC-ORD. This software was also used to identify fungal species characteristic for a particular tree using indicator species analysis (Dufrêne and Legendre 1997). Fungal species co-occurrence was analyzed in R using the co-occur package for 86 OTUs identified to species. OTUs were chosen based on 97% or greater sequence identity to known sequences in the UNITE database and occurrence on at least three sampled trees. Where OTUs were defined as conspecific, they were collapsed if the species hypothesis was identical and retained if the species hypothesis was different. This analysis aimed at revealing possible examples of positive and negative fungal-fungal interactions (Ovaskainen et al., 2010; Ottosson et al., 2014).

D. Results

Diversity of root-associated fungi

The following values were obtained for Good's coverage: *Fagus sylvatica* (99.7±0.1%), *Quercus robur* (99.7±0.1%), *Betula pendula* (99.7±0.1%), and *Carpinus betulus* (99.7±0.2%). These values indicated equally deep OTU recovery across the treatments (Fig S1). Rarefaction analysis for all samples approached a plateau, suggesting that almost all the fungal species in the sampled plots were sequenced (Fig S2).

DNA-metabarcoding of fungal communities revealed a high diversity of fungi associated with roots of four model tree species. Mean fungal OTU richness obtained per host tree was lowest in *Fagus silvatica* (136±45 OTUs) and highest in *Carpinus betulus* (216±67OTUs), although richness of fungi associated with model trees was not significantly different among trees within each locality (Fig 1). We observed fungal OTUs belonging to more than 50 taxonomic orders (Fig 2); however, of these, more than 35 orders were characterized by very low OTU richness (fewer than 10 OTUs). On the ordinal level, diversity ranking was dominated by species in the Agaricales for all model trees; however, subsequent orders with highest richness varied depending on the tree species. For example, the Helotiales and Mortierellales had high richness in fungal communities associated with *Betula pendula*, while in *C. betulus* we observed high OTU richness in the Hypocreales and Chaetothyriales; in *Querqus robur*, the Russulales followed the Agaricales in the diversity ranking. Similar variation was observed on the level of taxonomic genera (Fig 3); for example, in *B. pendula*, the highest OTU richness was found in the ascomycete dark septate endophytic (DSE) genus *Meliniomyces* (16 OTUs) and another DSE ascomycete, *Cladophialophora,* was also present (3 OTUs), the basidiomycete ECM genus *Lactarius* (8 OTUs) and the zygomycete *Umbelopsis* (4 OTUs). In *Q. robur*, we observed high

richness in the ECM genera *Lactarius* (11 OTUs), *Russula* (9 OTUs) and *Cortinarius* (9 OTUs). Interestingly, we observed a number of OTUs in the genus *Mycena* which is generally considered saprotrophic -- 4 OTUs in the roots of *B. pendula* and 4 OTUs associated with *Q. robur*.

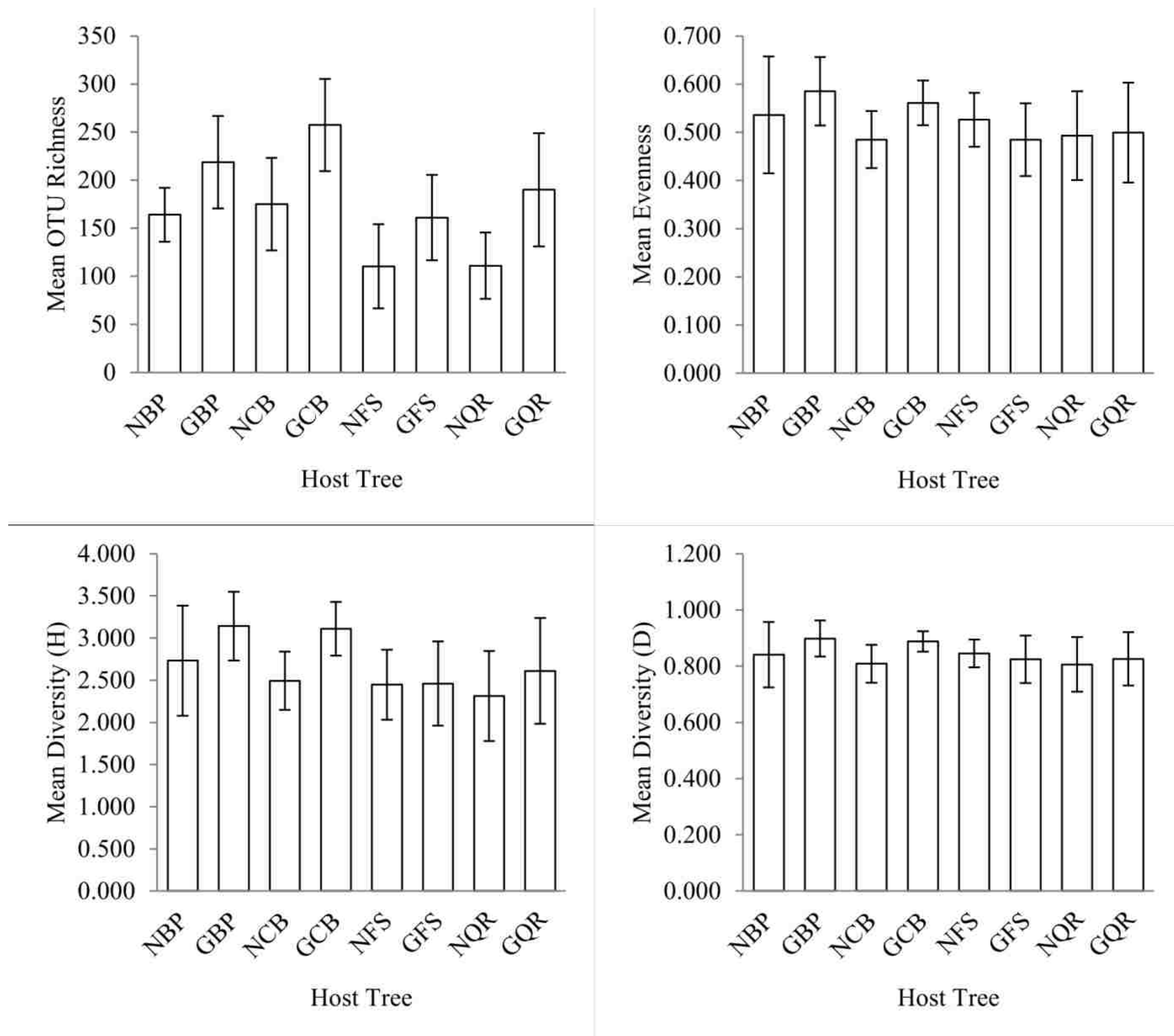


Figure 1. Community richness and diversity of fungal OTUs associated with model tree species in sample sites. No significant differences in mean richness or diversity were found on conspecific trees between sites.

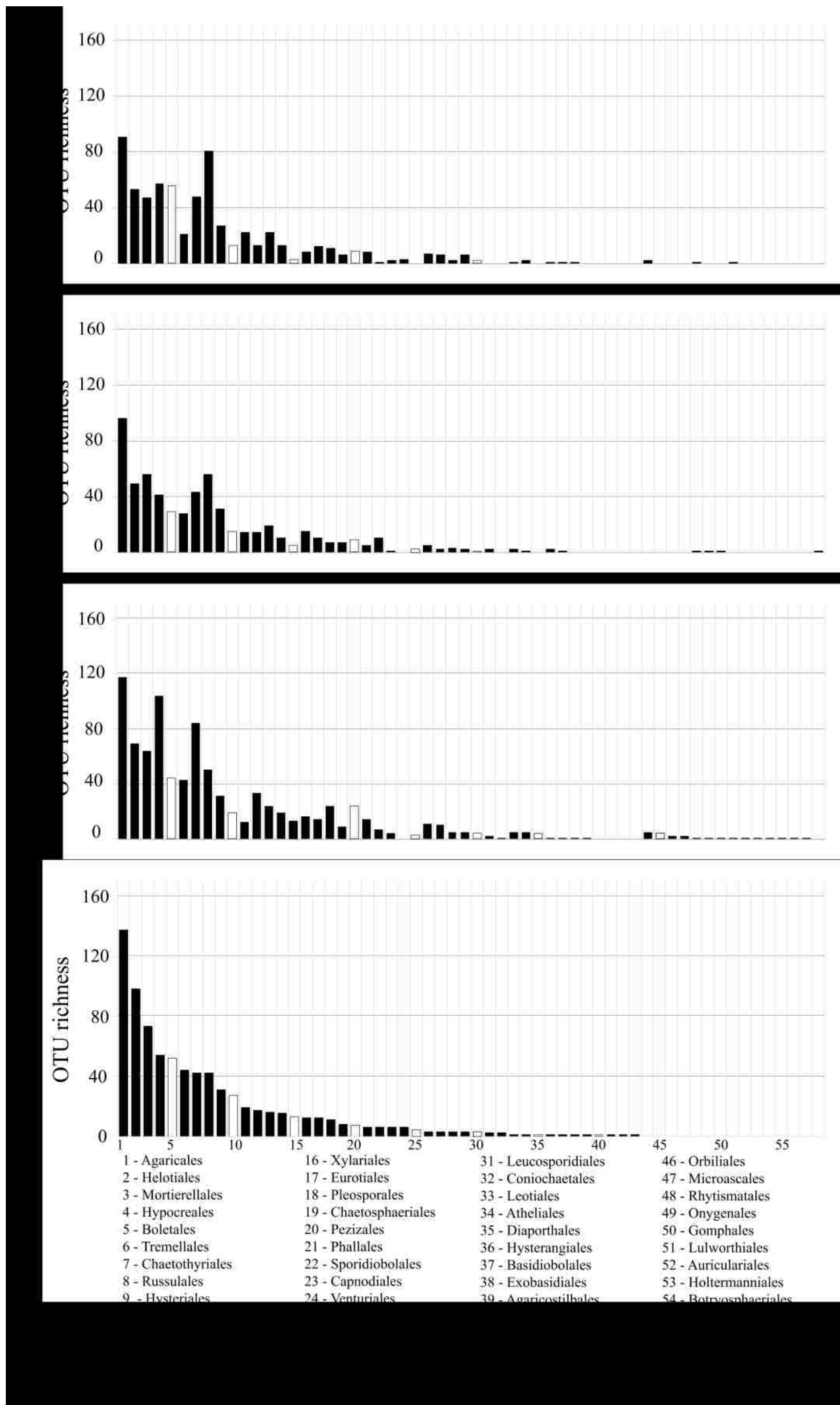


Figure 2. Diversity of root-associated fungi. Numbers of OTUs in fungal orders found on root material from four tree species in European mixed deciduous forests are indicated in each instance.

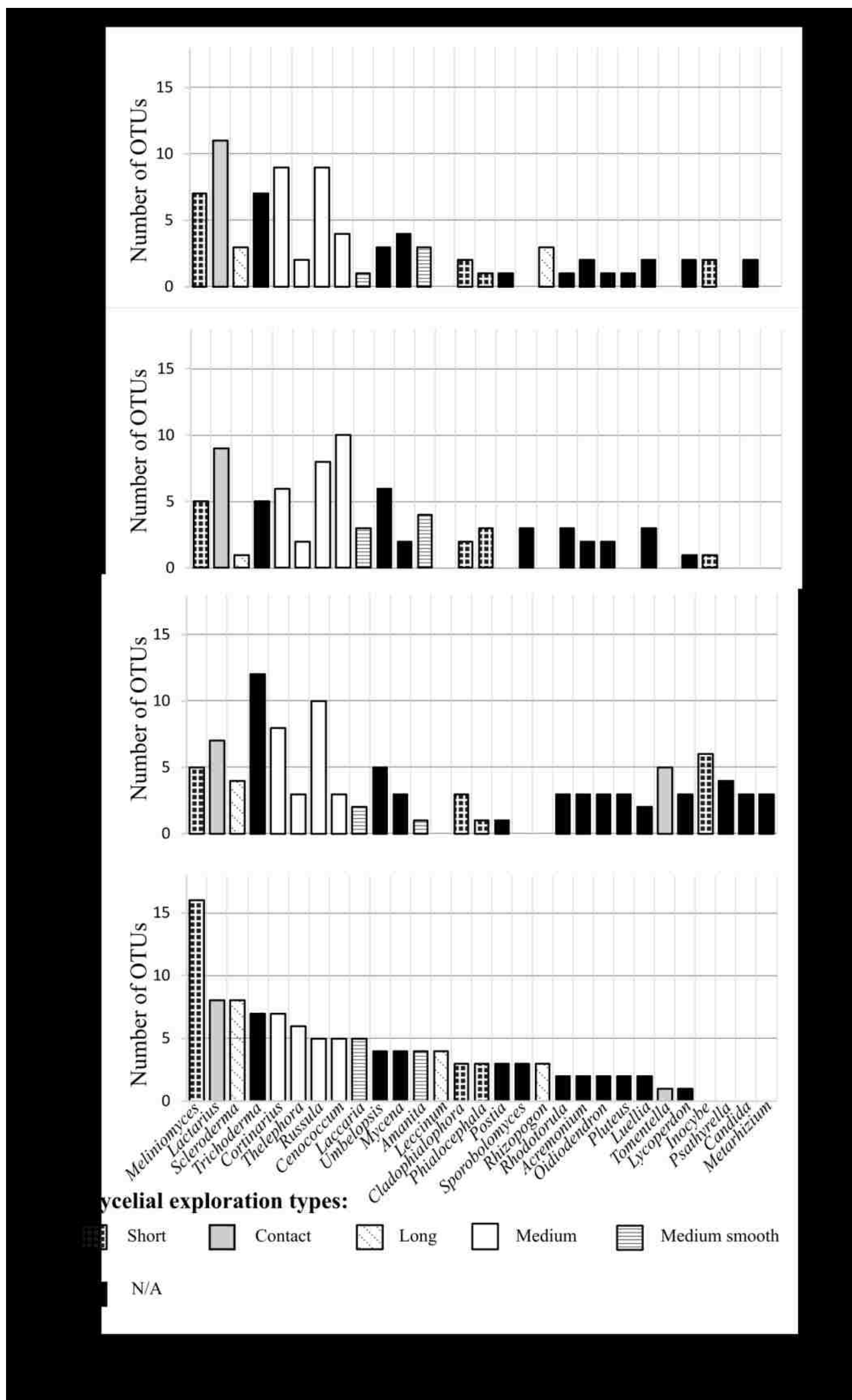


Figure 3. Richness of OTUs in the top 29 fungal genera occurring on four model tree species in two European forests. Genera with three or more OTUs on one or more tree species are included. The ECM genus *Leccinum* was found only on *B. pendula* among the sample sites, while the saprotrophic *Psathyrella* and entomopathogenic *Metarhizium* appeared in *Carpinus betulus* samples but no others. Mycelial exploration types as defined in Agerer (2001).

Factors affecting communities of root-associated fungi

Variation in community composition of root-associated fungi of the four model trees was depicted on a NMDS plot (Fig 4). Ordination analysis resulted in a 3-dimensional solution with a final stress of 12.08, final instability of 0, and axis 1: r^2=0.550, axis 2: r^2=0.188, axis 3: r^2=0.122. Ordination analysis revealed the strong effect of forest location (Germany versus Netherlands) on the communities of root-associated fungi. According to MANOVA, forest location explained 21.38% of the variation in fungal community compositions (F=6.44, P<0.0002). However, as revealed by MANOVA, both host tree family and host tree species had significant effects on fungal community composition: tree family explained 4.8% of the variation in fungal communities (F=2.01; P=0.007), and 5.52 % of this variation was explained by the tree species (F=1.58; P=0.002).

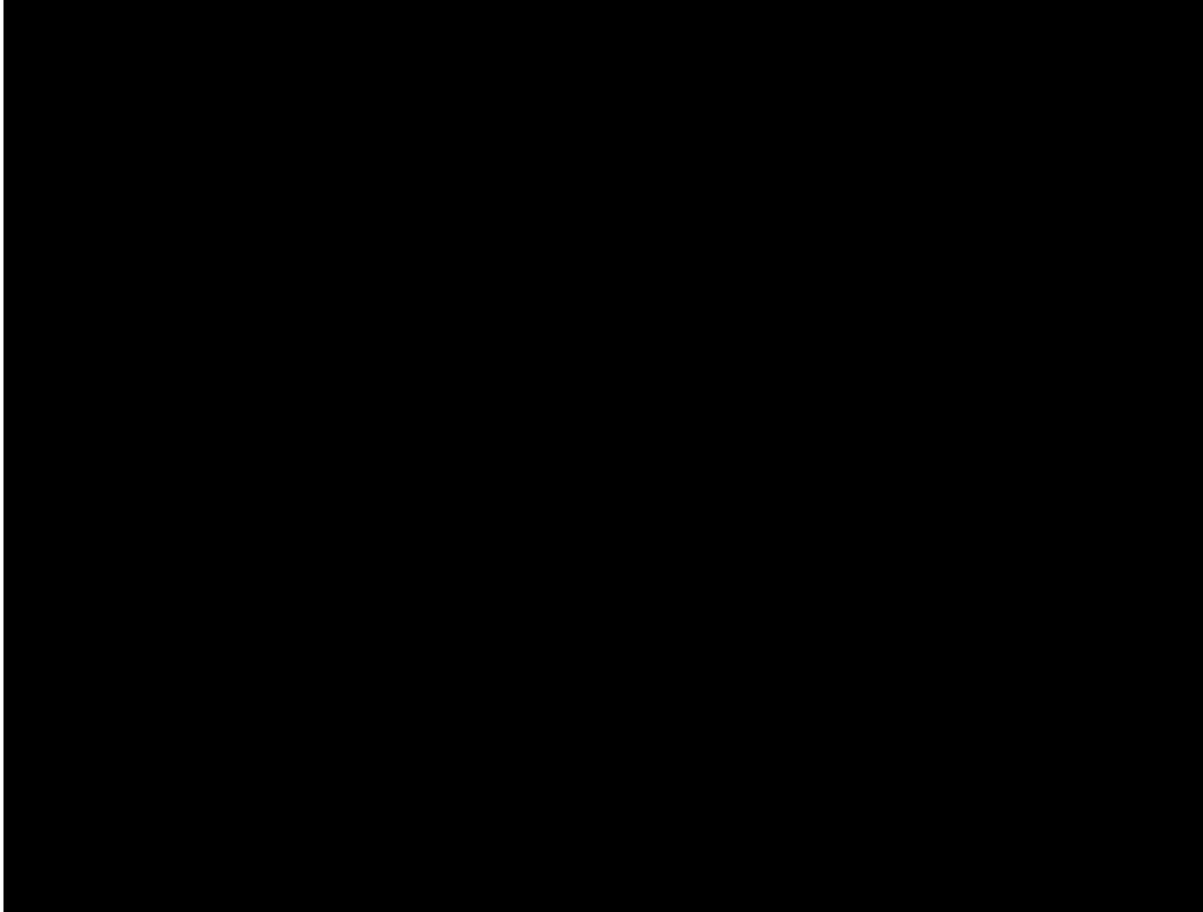


Figure 4. Two-dimensional NMDS ordination of fungal communities associated with trees at sample sites in Netherlands and Germany. Communities of root-associated fungi appear distinctly separate by sample site.

A second set of NMS ordinations conducted for each site independently, revealed the significant ($r \geq 0.5$) distribution of fungal taxa among host species (Fig 5, Table S4). Significant fungal taxa in Germany included OTUs in 22 orders and 48 genera, while in the Netherlands the significant fungal taxa were in 10 orders and 9 genera. Permutation-based nonparametric MANOVA for Germany showed that host tree species were responsible for ca. 15% of the variation in fungal assemblages, while in Germany host trees were responsible for ca. 9.13% of variation. The number of significant taxa in Germany were much larger than that found in the Netherlands.

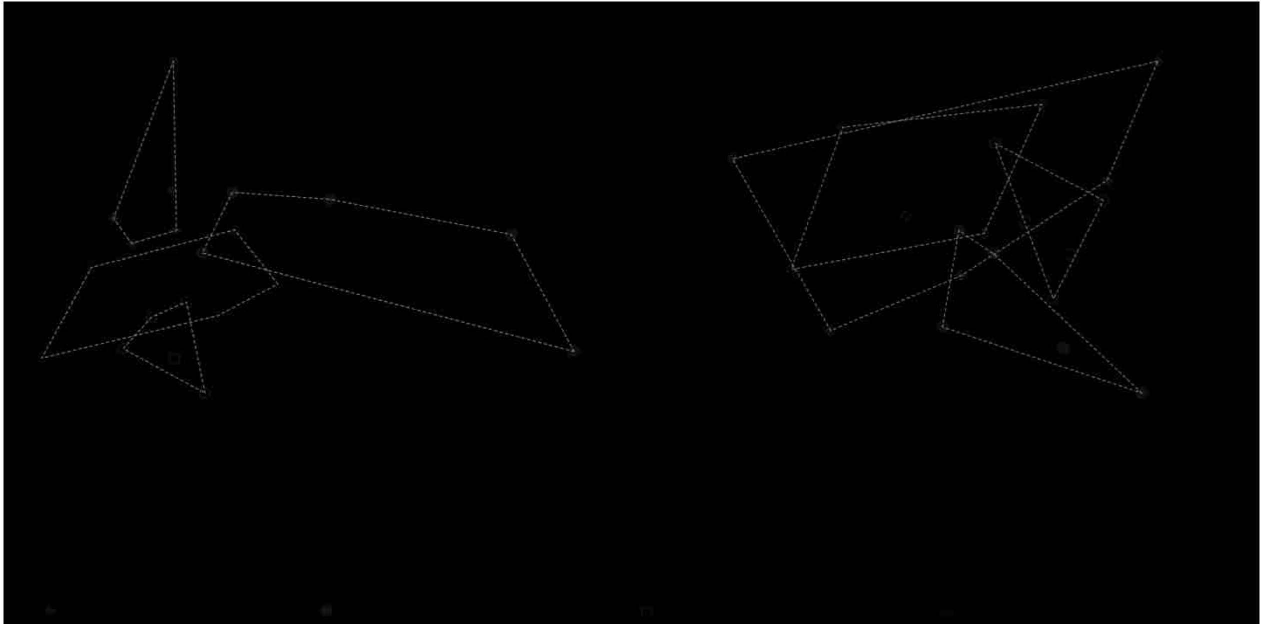


Figure 5. Non-metric Multidimensional Scale ordination of sampled trees by fungal OTUs present. Vectors represent the distribution of fungal taxa (orders and genera) in ordination space. Fungal taxa in Germany are more strongly correlated with *Carpinus betulus* than fungal taxa in the Netherlands.

Host specificity of fungi

Fungal OTUs associated with the roots of the four host tree species were assessed for the possibility of host-specific associations using a number of analyses. Venn diagram of the OTU distribution across the four tree species (Fig 6) showed that ca. 15% of the OTUs were present in all of the host trees. Proportions of the OTUs specific for the host trees varied largely, from a few percent in *Quercus robur* and *Fagus sylvatica,* to almost 30% of the OTUs specific for *Carpinus betulus* (Fig 6).

.

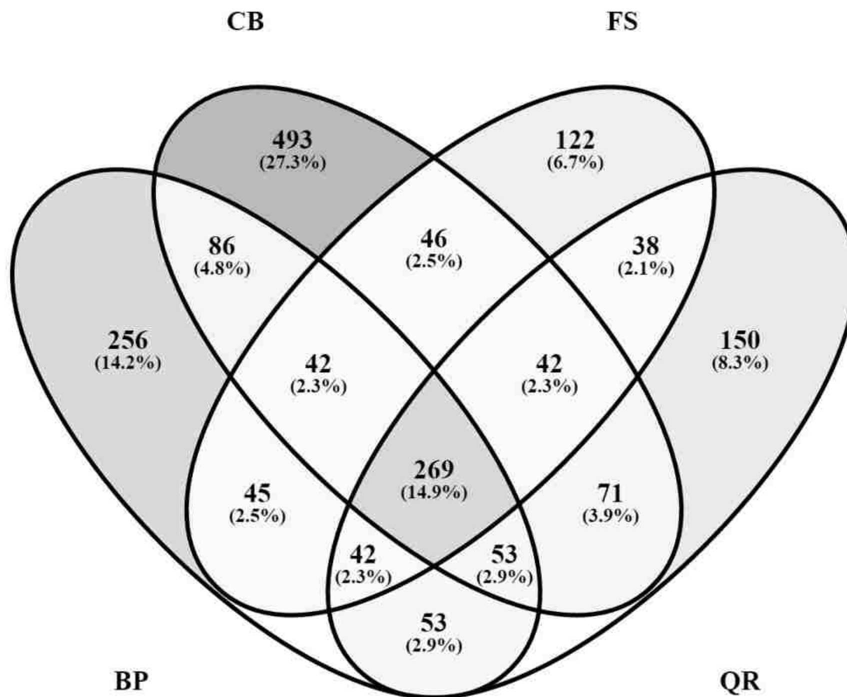


Figure 6. Four-way Venn diagram of OTU distribution across four host species in two families. Approximately half (46.3%) of all fungal OTUs were restricted to Betulaceae, while 17.1% occurred only on Fagaceae.

Indicator species analysis was conducted to assess the potential for fungal OTUs to be occurring on hosts with a frequency greater than that expected by chance (Table S2). Indicator species analysis revealed 27 OTUs characteristic for *Betula pendula*, among them a few were identified to species of DSE - *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox, and *Meliniomyces variabilis* Hambl. & Sigler , and a yeast frequently isolated from nutrient rich soils - *Cryptococcus podzolicus* Babeva & Reshetova (Mestre and Fontenla 2014). Among 19 indicators revealed for *C. betulus*, we observed *Clathrus archeri* (Berk.) Dring – a species that is

generally considered saprotrophic, and the plant parasite *Hyphodontia radula* (Pers.) Langer & Vesterh. Two OTUs indicated for *Q. robur* included ectomycorrhizal *Russula nigricans* Fr., and among 5 OTUs characteristic for *F. sylvatica*, we observed *Lopadostoma fagi* Jaklitsch, J. Fourn. & Voglmayr, which occurs frequently on the bark of the trees. The complete list of the indicator OTUs and their taxonomic affinities is presented in Table S5.

Fungal co-occurrence patterns

Eighty-six fungal OTUs identified to the species level were selected for pairwise species co-occurrence test. Of the 3655 possible species pair combinations, 1261 were omitted due to expected co-occurrence <1. The remaining 71 OTUs (2394 pair combinations) revealed 181 positive and 42 negative correlations between the fungal species (Fig 7). The maximum number of positive correlations with other fungi (23) was revealed for *Hypoxylon fragiforme* (Pers.) J. Kickx f., an ascomycete species frequently colonizing dead wood. Another species of *Hypoxylon* (*H. rubiginosum* (Pers.) Fr.) was also characterized by a high number of positive correlations (17), implying a relation between the fungal genus and co-occurrence with other fungal species. However, we did not observe any similarity in the correlations of four OTUs belonging to the genus *Trichoderma*: one of the OTUs identified as *T. pubescens* Bisset formed 3 negative co-occurrence patterns, while another OTU identified as the same species, showed 12 positive correlations with other fungi in our dataset. The species characterized by the maximum negative co-occurrence instances was *Luellia cystidiata* Hauerslev, a corticoid basidiomycete species, although another species in the same genus (*L. recondita (*H.S. Jacks.) K.H. Larss. & Hjortstam) showed 19 positive and 4 negative correlations. There was no significant difference in positive and negative correlations shown by ECM, DSE and saprotrophic ecological groups (not shown). Among the 18 ECM species with significant interactions there were 18 positive interactions and

one negative interaction. Interestingly, the negative interaction was found between the common ectomycorrhizal ascomycete *Cenococcum geophilum* Fr. and a hypogeous ascomycete, *Hydnotrya tulasnei* (Berk.) Berk. & Broome. *Scleroderma citrinum* Pers. and *S. areolatum* Ehrenb. appear to be positively associated, as well as several *Cortinarius* spp. and *Russula* spp. (Figure S3). Among 35 identified saprotrophic fungal species retained in analysis (having significant interactions) there were 93 positive interactions and 16 negative interactions (Figure S4).

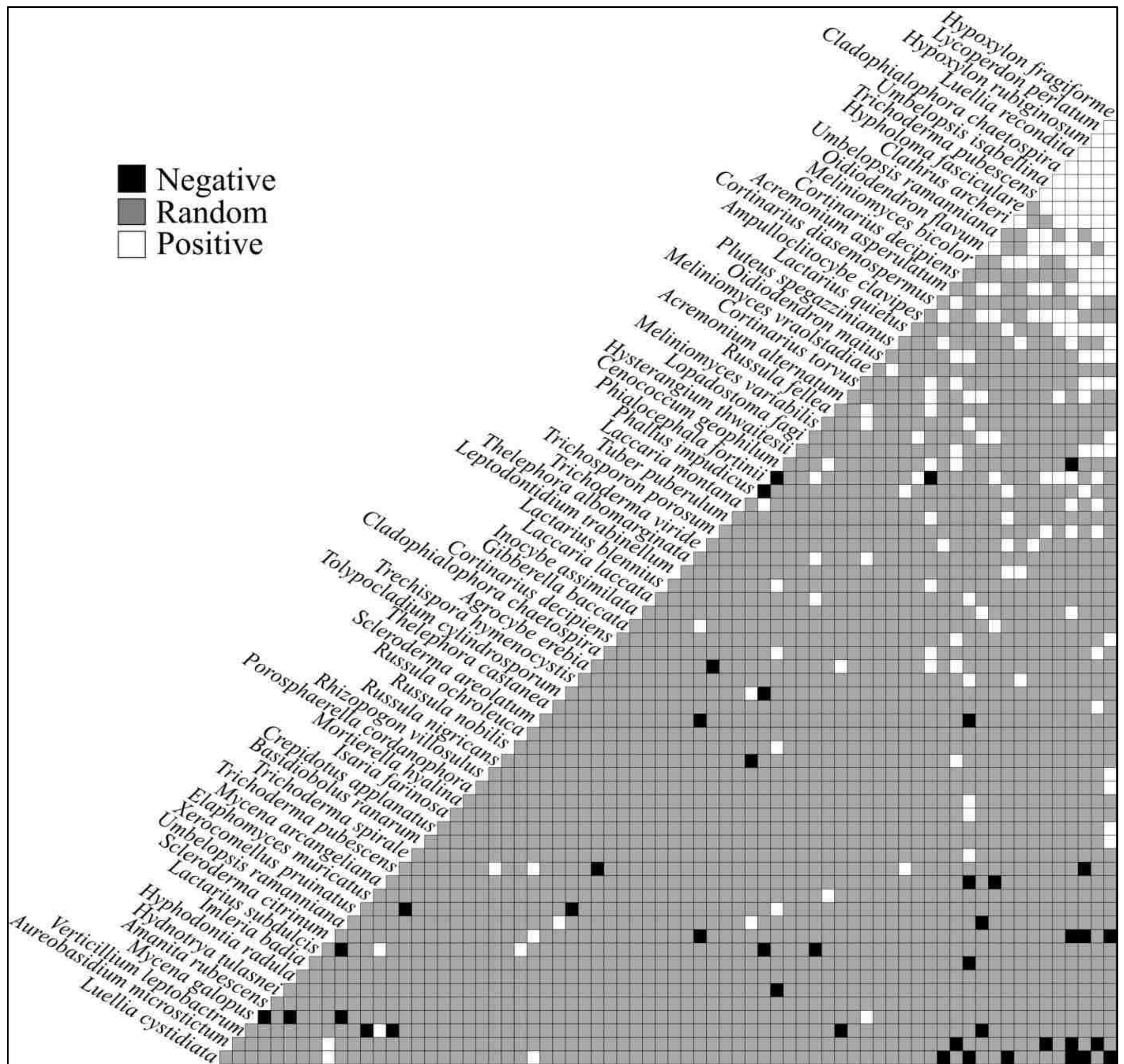


Figure 7. Species co-occurrence matrix of 86 fungal species found across 40 sampled trees. Out of 3655 species pair combinations, 1261 pairs (34.5 %) were removed from the analysis because expected co-occurrence was < 1. The remaining 71 species (2394 pairs) were included in the analysis.

E. Discussion

Temperate mixed deciduous forests are characterized by a high diversity of below-ground fungal communities, as revealed by DNA-metabarcoding of soil samples (Tedersoo et al., 2014). Determining the extent of diversity on specific hosts in mixed forests directly from root tissue would further clarify the relationships between fungal taxa and associated trees. The purpose of this study was to examine that diversity on four tree species in European mixed deciduous forests, assess patterns in the distribution of root associated fungi based on locality and host fidelity, and determine patterns in fungal species co-occurrence suggestive of fungal-fungal interactions.

Our data showed a major effect of geographical position on root-associated fungal communities, as communities associated with trees in the Netherlands were clearly separated from the ones in Germany. This effect, however, cannot be entirely attributed to geographic distance itself, and rather represents a combined effect of various factors, including such things as distance decay (community interactions decrease as geographic distance increases), soil chemistry and texture, and climate variables. For example, soil pH across the tested Netherlands and German sites differed by one unit, and even such a moderate variation has been shown to strongly affect root-associated fungal communities (e.g., Toljander et al., 2006; Martinova et al., 2015; Barnes et al., 2016). Variation in soil texture (more clay at the Netherlands site and more loam at the German site) could also account for variation in community compositions of root-associated fungi (Goldmann et al., 2016). Higher organic carbon content in German soils most likely contributed to observed difference in Netherlands and German communities, as well as explained higher species richness of the majority fungal taxa in German communities. In comparison to soil parameters, the effect of geographic position on root-associated fungi was shown to be relatively

subtle, because the habitat provided by the host tree for its symbiotic fungi buffers the effect of the surrounding environment (McCormack and Guo 2014). An example of this buffer effect was described recently: in German beech-dominated forests geographic location accounts for approximately 8% of the variation in root-associated fungal communities (Goldmann et al., 2016).

Fungal community compositions were different in the two forests, and segregating the data of German and Netherlands communities for NMDS analysis revealed a significant effect of the host tree species on fungal community composition. Host specificity explained approximately 9% of fungal community variation in the Netherlands communities and ca. 15% in German communities, and this effect was observed in fungi on host trees at both genus and family level (Betulaceae vs Fagaceae). In that respect, our results were in agreement with previous research showing strong effect of host-tree species on its associated fungal communities (Massicotte et al., 1999; Wehner et al., 2014) but disagreed with other research where this effect was not confirmed (Dean et al., 2015; Roy-Bolduc et al., 2016). Host specificity in root-associated fungi is often attributed to the niche-effect, (i.e., the ability of a particular tree and fungal species to live in a specific habitat that leads to the formation of distinct communities). For example, high levels of host-specificity in fungi associated with *Alnus* sp. were shown to result from adaptations to high soil acidity and nitrate conditions (Tedersoo et al., 2009; Huggins et al., 2014). On the other hand, host specificity in root-associated fungi may result from fungal adaptations to living with a particular host tree (e.g., fungal communities may be shaped by tree root exudates) (Broeckling et al., 2007). Our results suggest that both host traits and the surrounding environment had an impact on root-associated fungal communities in our study and contributed to the observed level of host-specificity. We observed stronger host specificity in the

German forest compared to the Netherlands forest, possibly due to a variation in levels of forest disturbance. Our Netherlands sampling site was situated closer to the city, suggesting possible effects of urbanization such as altered pH, soil salinity and concentrations of heavy metals that influence species turnover, richness and community composition in root-associated fungi (Newbound et al., 2010). Forest disturbance of the Netherlands site could also explain observed higher OTU richness of fungal communities associated with trees in Germany, even though this trend was not significant due to high standard deviations obtained for the richness estimators.

Fungal communities associated with the roots of model tress were characterized by relatively high OTU richness (100-200 OTUs per tree replicate), comparable with similar data reported in other studies (e.g., Lang et al., 2011; Lankau and Keymer 2016). However, we did not observe lower richness in communities associated with *Carpinus betulus* compared to *Fagus sylvatica* in German mixed deciduous forest as reported previously by Lang et al. (2011) and explained by limited carbohydrate allocation to the *C. betulus* root system leading to lower diversity of ectomycorrhizal species. On the contrary, in our dataset *C. betulus* had the highest richness of associated fungi, and had significantly higher (nearly double) richness compared to *F. sylvatica* in the German site. Nearly one third of the OTUs in our dataset were associated solely with *C. betulus*, including species of ectomycorrhizal fungi in the genera *Inocybe*, *Tomentella* and *Russula*. This could be indicative of host specificity or host preference. However, it is possible that richness in tree-associated communities is not specific for particular tree taxa but may be locally controlled by other factors unrelated to tree taxonomy, such as the surrounding plant community (Kennedy et al., 2003). Among the traits specific for the tree species, the level of shade-tolerance seemed to correlate with the richness of root-associated fungal species with the long-distance type of mycelium. In fungal communities associated with shade-intolerant *Betula*

pendula we observed 16 taxa (OTUs) with long-distance mycelium (e.g.; *Scleroderma, Leccinum, Rhizopogon*) (Agerer 2001), and 6 species were found in intermediate shade-tolerant *Quercus robur*. The tree species with higher shade-tolerance, *F. sylvatica* and *C. betulus*, were mostly associated with fungi having medium, short-distance, and contact mycelium. Because fungal species with long-distance mycelium may transfer water and nutrients over greater distances, they could be particularly well-suited for trees growing in less shaded, and therefore drier, areas.

In addition to environmental filtering and host-specificity, our results show that root-associated fungal communities could be influenced by fungal-fungal species interactions. The known examples involve competition between arbuscular mycorrhizal fungi and endophytes (Wearn et al., 2012), negative interactions between specific fungal genets (Lilleskov et al., 2004), e.g., competition between *Tomentella* and *Amanita*, *Meliniomyces* and *Russula* (Burke et al., 2009) or *Russula* and other ectomycorrhizal species (Koide et al., 2005; Sun et al., 2015). Analysis of co-occurrence between ectomycorrhizal species revealed a number of positive relationships between species in a number of genera, including *Russula*, *Lactarius*, and *Cortinarius*. Various species of *Cortinarius* appear to have a positive relationship to one another, suggesting cooperation between members of this genus on host trees. Surprisingly, our species co-occurrence analyses revealed mostly positive correlations between different fungal species, except for *Trichoderma,* which may be mycopathogenic and therefore expected to negatively correlate with a number of other fungal species (Hermosa et al., 2012). On the other hand, our dataset involved fungi present inside of the plant roots, and observing mainly positive co-occurrence patterns for these fungi agreed with the results of research by Pan and May (2009) who also observed largely positive interactions between endophytic fungi infecting roots of maize. Such co-occurrence

patterns resulted from higher vulnerability of the host plant to establishing symbiosis with multiple species after the initial colonization by the pioneer endophytic strain (Pan and May 2009). Thus, the positive correlations observed in our study possibly indicate a cooperative relationship between fungi with endophytic roles and ectomycorrhizal fungi.

F. Conclusion

The diversity of fungi associated with the roots of trees in European temperate deciduous forests conform to current estimates. The degree to which these fungi are distributed based on host-specificity or host preference is significant. Further research to assess whether or not species found on only one host taxon (whether at the genus or family level) is exhibiting host specificity, host preference, or responding to environmental factors is to be encouraged. Fungal co-occurrence data revealed previously unknown positive correlations between taxa. Correlations that exist between fungi of different ecological function may be a consequence of particular conditions in the rhizosphere favorable for these fungi, indicative of a cooperative arrangement between noncompetitive species or merely a function of microhabitat fragmentation within the rhizosphere. Within ectomycorrhizal fungi, some are thought to be exclusive, specifically *Russula* spp., and this suggests that our assumptions of the relationships between fungi of this ecological group may need to be revised to include the potential for cooperation. Further experiments need to be done to determine the extent of these relationships, preferably in a controlled environment.

G. References

Agerer, R. (2001). Exploration types of ectomycorrhizae. *Mycorrhiza* 11(2), 107-114.

Barnes, C. J., Maldonado, C., Frøslev, T. G., Antonelli, A., and Rønsted, N. (2016). Unexpectedly High Beta-Diversity of Root-Associated Fungal Communities in the Bolivian Andes. *Frontiers in Microbiology* 7: 1377.

Bellemain, E., Davey, M. L., Kauserud, H., Epp, L. S., Boessenkool, S., Coissac, E., Geml, J., Edwards, M., Willerslev, E., Gussarova, G., and Taberlet, P. (2013). Fungal palaeodiversity revealed using high-throughput metabarcoding of ancient DNA from arctic permafrost. *Environmental Microbiology* 15(4), 1176-1189.

Bjorbækmo, M. F. M., Carlsen, T., Brysting, A., Vrålstad, T., Høiland, K., Ugland, K. I., Geml, J., Schumacher, T., and Kauserud, H. (2010). High diversity of root associated fungi in both alpine and arctic *Dryas octopetala*. *BMC Plant Biology* 10(1), 1.

Bonello, P., Bruns, T. D., and Gardes, M. (1998). Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. *New Phytologist* 138(3), 533-542.

Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K., and Vivanco, J. M. (2008). Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology* 74(3), 738-744.

Burke DJ, Lopez-Gutierrez JC, Smemo KA, Chan CR. (2009). Vegetation and Soil Environment Influence the Spatial Distribution of Root-Associated Fungi in a Mature Beech-Maple Forest. *Applied and Environmental Microbiology* 75(24), 7639–7648

Clemmensen, K. E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R. D., Wardle, D. A., and Lindahl, B. D. (2013). Roots and associated fungi drive long-term carbon sequestration in boreal forest. *Science* 339(6127), 1615-1618.

Dahlberg, A., and Stenlid, J. (1990). Population structure and dynamics in Suillus bovinus as indicated by spatial distribution of fungal clones. *New Phytologist* 115(3), 487-493.

Dahlberg, A., and Stenlid, J. A. N. (1994). Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. *New Phytologist* 128(2), 225-234.

de Boer, W., Folman, L. B., Summerbell, R. C., and Boddy, L. (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews* 29(4), 795-811.

Dean, S. L., Warnock, D. D., Litvak, M. E., Porras-Alfaro, A., and Sinsabaugh, R. (2015). Root-associated fungal community response to drought-associated changes in vegetation community. *Mycologia* 107(6), 1089-1104.

Dufrene, M., and Legendre, P. (1997). Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecological Monographs* 67(3), 345-366.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19), 2460-2461.

Fao, J. (2012). Global forest land-use change 1990–2005. FAO forestry paper, 169.
Fiore-Donno, A. M., and Martin, F. (2001). Populations of ectomycorrhizal *Laccaria amethystina* and *Xerocomus* spp. show contrasting colonization patterns in a mixed forest. *New Phytologist* 152(3), 533-542.

Geml, J., Kauff, F., Brochmann, C., and Taylor, D. L. (2010). Surviving climate changes: high genetic diversity and transoceanic gene flow in two arctic–alpine lichens, *Flavocetraria cucullata* and *F. nivalis* (Parmeliaceae, Ascomycota). *Journal of Biogeography* 37(8), 1529-1542.

Geml, J., Pastor, N., Fernandez, L., Pacheco, S., Semenova, T. A., Becerra, A. G., Wicaksono, C. Y., and Nouhra, E. R. (2014). Large-scale fungal diversity assessment in the Andean Yungas forests reveals strong community turnover among forest types along an altitudinal gradient. *Molecular Ecology* 23(10), 2452-2472.

Gherbi, H., Delaruelle, C., Selosse, M. A., and Martin, F. (1999). High genetic diversity in a population of the ectomycorrhizal basidiomycete *Laccaria amethystina* in a 150-year-old beech forest. *Molecular Ecology* 8(12), 2003-2013.

Goldmann, K., Schröter, K., Pena, R., Schöning, I., Schrumpf, M., Buscot, F., Polle, A., and Wubet, T. (2016). Divergent habitat filtering of root and soil fungal communities in temperate beech forests. *Scientific Reports* 6.

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158(1), 17-25.

Horton, T. R., and Bruns, T. D. (1998). Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). *New Phytologist* 139(2), 331-339.

Huggins JA, Talbot J, Gardes M, Kennedy PG (2014). Unlocking environmental keys to host specificity: differential tolerance of acidity and nitrate by Alnus-associated ectomycorrhizal fungi. *Fungal Ecology* 1-10.

Ihrmark, K., Bödeker, I. T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K. E., and Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region–evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology* 82(3), 666-677.

Kennedy, P. G., Izzo, A. D., and Bruns, T. D. (2003). There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. *Journal of Ecology* 91(6), 1071-1080.

Kennedy, P.G., Smith, D.P., Horton, T.R., Molina, R.J., 2012. *Arbutus menziesii* (Ericaceae) facilitates regeneration dynamics in mixed evergreen forests by promoting mycorrhizal fungal diversity and host connectivity. *American Journal of Botany* 99(10), 1691e1701.

Koide RT, Xu B, Sharda J, Lekberg Y, Ostiguy N. 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* 165:305–316.

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., and Douglas, B. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology* 22(21), 5271-5277.

Kretzer, A. M., Dunham, S., Molina, R., and Spatafora, J. W. (2004). Microsatellite markers reveal the below ground distribution of genets in two species of *Rhizopogon* forming tuberculate ectomycorrhizas on Douglas fir. *New Phytologist* 161(1), 313-320.

Lang, C., Seven, J., and Polle, A. (2011). Host preferences and differential contributions of deciduous tree species shape mycorrhizal species richness in a mixed Central European forest. *Mycorrhiza* 21(4), 297-308.

Lankau, R. A., and Keymer, D. P. (2016). Ectomycorrhizal fungal richness declines toward the host species' range edge. *Molecular Ecology* 25(13), 3224-3241.

Lilleskov, E. A., Bruns, T. D., Horton, T. R., Taylor, D. L., and Grogan, P. (2004). Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities. *FEMS Microbiology Ecology* 49(2), 319-332.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J., and Kauserud, H. (2013). Fungal community analysis by high-throughput sequencing of amplified markers–a user's guide. *New Phytologist* 199(1), 288-299.

Martinová, V., Van Geel, M., Lievens, B., and Honnay, O. (2016). Strong differences in *Quercus robur*-associated ectomycorrhizal fungal communities along a forest-city soil sealing gradient. *Fungal Ecology* 20, 88-96.

Massicotte, H. B., Molina, R., Tackaberry, L. E., Smith, J. E., and Amaranthus, M. P. (1999). Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Canadian Journal of Botany* 77(8), 1053-1076.

McCormack, M. L., and Guo, D. (2014). Impacts of environmental factors on fine root lifespan. *Frontiers in Plant Science* 5.

McCune, B., Grace, J. B., and Urban, D. L. (2002). Analysis of ecological communities (Vol. 28). Gleneden Beach, OR: MjM software design.

Molina, R., Massicotte, H., and Trappe, J. M. (1992). Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. *Mycorrhizal functioning: an integrative plant-fungal process* 357, e423.

Morgado, L. N., Semenova, T. A., Welker, J. M., Walker, M. D., Smets, E., and Geml, J. (2016). Long-term increase in snow depth leads to compositional changes in arctic ectomycorrhizal fungal communities. *Global Change Biology* 22(9), 3080-3096.

Newbound, M., Mccarthy, M. A., and Lebel, T. (2010). Fungi and the urban environment: A review. *Landscape and Urban Planning* 96(3), 138-145.

Newton, A. C., and Haigh, J. M. (1998). Diversity of ectomycorrhizal fungi in Britain: a test of the species–area relationship, and the role of host specificity. *New Phytologist* 138(4), 619-627.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Wagner, H., and Oksanen, M. J. (2013). Package 'vegan'. *Community ecology package, version* 2(9).

Oliveros, J. (2015). An interactive tool for comparing lists with venn diagrams. Available at: http. bioinfogp.cnb.csic.es/tools/venny/index. html.

Ovaskainen, O., Hottola, J., and Siitonen, J. (2010). Modeling species co-occurrence by multivariate logistic regression generates new hypotheses on fungal interactions. *Ecology* 91(9), 2514-2521.

Ovaskainen, O., Schigel, D., Ali-Kovero, H., Auvinen, P., Paulin, L., Nordén, B., and Nordén, J. (2013). Combining high-throughput sequencing with fruit body surveys reveals contrasting life-history strategies in fungi. *The ISME Journal* 7(9), 1696-1709.

Pan, J. J., and May, G. (2009). Fungal-fungal associations affect the assembly of endophyte communities in maize (Zea mays). *Microbial Ecology* 58(3), 668-678.

Pan, Y., Birdsey, R. A., Fang, J., Houghton, R., Kauppi, P. E., Kurz, W. A., Phillips, O. L., Shvidenko, A., Lewis, S. L., Canadell, J. G., and Ciais, P. (2011). A large and persistent carbon sink in the world's forests. *Science* 333(6045), 988-993.

Richard, F., Selosse, M. A., and Gardes, M. (2009). Facilitated establishment of *Quercus ilex* in shrub-dominated communities within a Mediterranean ecosystem: do mycorrhizal partners matter? *FEMS Microbiology Ecology* 68(1), 14-24.

Roy-Bolduc, A., Laliberté, E., and Hijri, M. (2016). High richness of ectomycorrhizal fungi and low host specificity in a coastal sand dune ecosystem revealed by network analysis. *Ecology and Evolution* 6(1), 349-362.

Smith, M. E., Douhan, G. W., Fremier, A. K., and Rizzo, D. M. (2009). Are true multihost fungi the exception or the rule? Dominant ectomycorrhizal fungi on *Pinus sabiniana* differ from those on co-occurring *Quercus* species. *New Phytologist* 182(2), 295-299.

Sun, H., Santalahti, M., Pumpanen, J., Köster, K., Berninger, F., Raffaello, T., Jumpponen, A., Asiegbu, F. O., and Heinonsaloa, J. (2015). Fungal community shifts in structure and function across a boreal forest fire chronosequence. *Applied and Environmental Microbiology* 81(22), 7869-7880.

Team, R. C. (2013). R: A language and environment for statistical computing.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L. V., Vasco-Palacios, A. M., Thu, P. Q., Suija, A., and Smith, M. E. (2014). Global diversity and geography of soil fungi. *Science* 346(6213), 1256688.

Tedersoo, L., Suvi, T., Jairus, T., and Kõljalg, U. (2008). Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of *Picea abies* and *Betula pendula*. *Environmental Microbiology* 10(5), 1189-1201.

Tedersoo, L., Suvi, T., Jairus, T., Ostonen, I., and Põlme, S. (2009). Revisiting ectomycorrhizal fungi of the genus *Alnus*: differential host specificity, diversity and determinants of the fungal community. *New Phytologist* 182(3), 727-735.

Toju, H., Tanabe, A. S., Yamamoto, S., and Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PloS One* 7(7), e40863.

Toljander, J. F., Eberhardt, U., Toljander, Y. K., Paul, L. R., and Taylor, A. F. (2006). Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. *New Phytologist* 170(4), 873-884.

Wearn, J. A., Sutton, B. C., Morley, N. J., and Gange, A. C. (2012). Species and organ specificity of fungal endophytes in herbaceous grassland plants. *Journal of Ecology* 100(5), 1085-1092.

Wehner, J., Powell, J. R., Muller, L. A. H., Caruso, T., Veresoglou, S. D., Hempel, S., and Rillig, M. C. (2014). Determinants of root-associated fungal communities within Asteraceae in a semi-arid grassland. *Journal of Ecology* 102(2), 425–436

White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18(1), 315-322.

Wubet, T., Christ, S., Schöning, I., Boch, S., Gawlich, M., Schnabel, B., Fischer, M., and Buscot, F. (2012). Differences in soil fungal communities between European beech (*Fagus sylvatica* L.) dominated forests are related to soil and understory vegetation. *PloS One* 7(10), e47500.

Zhou, Z., Miwa, M., and Hogetsu, T. (1999). Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR*). New Phytologist* 144(1), 55-63.

Zhou, Z., Miwa, M., Matsuda, Y., and Hogetsu, T. (2001). Spatial distribution of the subterranean mycelia and ectomycorrhizae of *Suillus grevillei* genets. *Journal of Plant Research* 114(2), 179-185.

H. Supplemental Materials

Table S1. Molecular identification tag (MID) assignment for samples collected from Netherlands and Germany sites.

Sample #	MID tag#	MID seq	Sample #	MID tag#	MID seq
NBP3	IonXpress_001	CTAAGGTAAC	GFS1	IonXpress_026	TTACAACCTC
NBP1	IonXpress_003	AAGAGGATTC	GFS2	IonXpress_027	AACCATCCGC
NBP2	IonXpress_004	TACCAAGATC	GFS3	IonXpress_028	ATCCGGAATC
NBP4	IonXpress_005	CAGAAGGAAC	GFS4	IonXpress_029	TCGACCACTC
NBP5	IonXpress_006	CTGCAAGTTC	GFS5	IonXpress_030	CGAGGTTATC
NCB1	IonXpress_011	TCCTCGAATC	GQR1	IonXpress_031	TCCAAGCTGC
NCB2	IonXpress_012	TAGGTGGTTC	GQR2	IonXpress_032	TCTTACACAC
NCB3	IonXpress_013	TCTAACGGAC	GQR3	IonXpress_033	TTCTCATTGAAC
NCB4	IonXpress_014	TTGGAGTGTC	GQR4	IonXpress_034	TCGCATCGTTC
NCB5	IonXpress_015	TCTAGAGGTC	GQR5	IonXpress_035	TAAGCCATTGTC
NFS1	IonXpress_016	TCTGGATGAC	GCB1	IonXpress_095	CGGACAGATC
NFS2	IonXpress_017	TCTATTCGTC	GCB2	IonXpress_037	CTTGAGAATGTC
NFS3	IonXpress_018	AGGCAATTGC	GCB3	IonXpress_038	TGGAGGACGGAC
NFS4	IonXpress_019	TTAGTCGGAC	GCB4	IonXpress_039	TAACAATCGGC
NFS5	IonXpress_020	CAGATCCATC	GCB5	IonXpress_040	CTGACATAATC
NQR1	IonXpress_021	TCGCAATTAC	GBP1	IonXpress_044	TTGGAGGCCAGC
NQR2	IonXpress_022	TTCGAGACGC	GBP2	IonXpress_045	TGGAGCTTCCTC
NQR3	IonXpress_023	TGCCACGAAC	GBP3	IonXpress_046	TCAGTCCGAAC
NQR4	IonXpress_024	AACCTCATTC	GBP4	IonXpress_047	TAAGGCAACCAC
NQR5	IonXpress_025	CCTGAGATAC	GBP5	IonXpress_096	TTAAGCGGTC

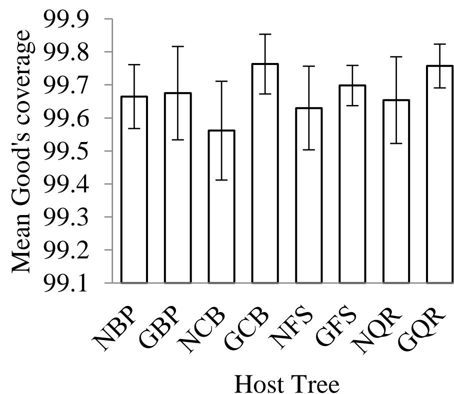


Figure S1. Values of Good's coverage on the four tree species sampled at both sites. No significant difference in depth of OTU recovery was found between species.

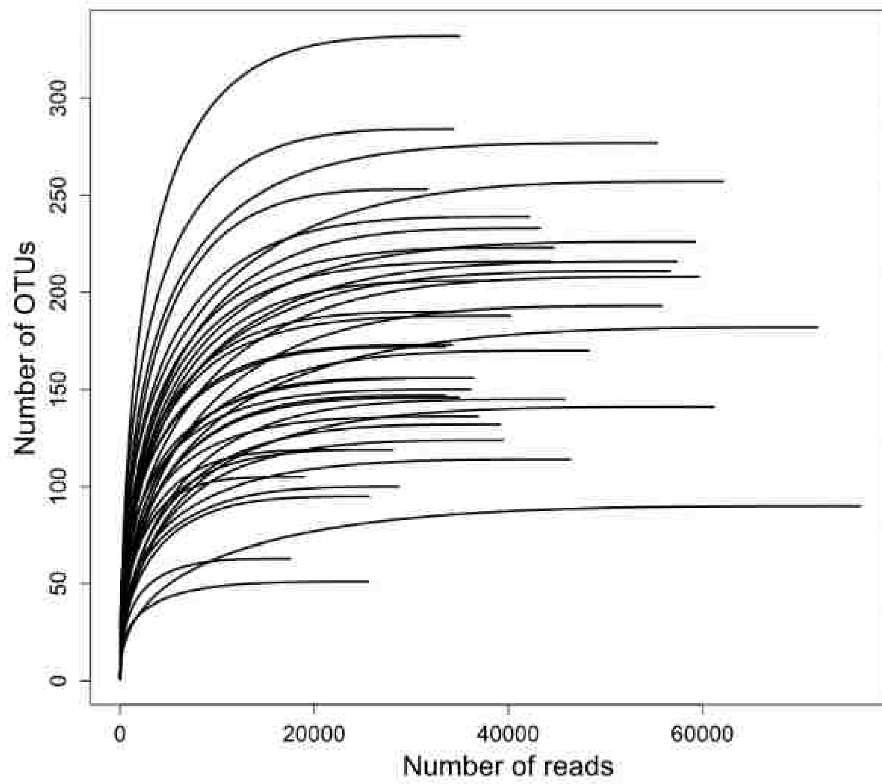


Figure S2. Rarefaction curves for ITS2 DNA sequence reads from 40 sampled trees in Germany and the Netherlands. All sampled trees approached a plateau, suggesting a thorough sampling of fungal OTUs from each tree.

Table S2. Ecological function of OTUs on four European tree species. Functional group assignment was based on OTUs identified to genus and cross referenced with Tedersoo et al. 2014. Large numbers of ECM taxa and saprotrophic taxa were found in association with colonized root-tips.

Ecological function	**BP**	**CB**	**FS**	**QR**
Animal parasite	22	28	13	17
Animal pathogen	2	4	0	0
Dark septate endophyte	1	0	2	1
Ectomycorrhizal	169	171	141	181
Endophyte	8	8	8	5
Lichenized	1	1	0	0
Mycoparasite	2	6	0	2
Plant parasite	4	4	2	0
Plant pathogen	17	24	13	15
Root associated	0	1	0	0
Saprotroph	212	282	164	185

Table S3. Vector identification for NMDS ordination of fungal OTUs on trees in Germany and the Netherlands.

Fungal taxa on the first axis	**r value**	**Fungal taxa on the second axis**	**r value**
Fimetariella	0.578	Pleosporales	0.633
Ectomycorrhizal	-0.509	Pezizales	0.626
Pezizales	-0.512	*Tricladium*	0.589
Paecilomyces	-0.516	Capnodiales	0.581
Hypholoma	-0.519	Onygenales	0.561
Cladophialophora	-0.541	*Cyphellophora*	0.548
Thelephorales	-0.547	*Mycenella*	0.548
Sebacinales	-0.551	*Peziza*	0.548
Inocybe	-0.552	*Trichophaea*	0.548
Trichosporon	-0.567	Hysteriales	0.515
Polyporales	-0.576	*Humaria*	0.504
Clathrus	-0.595	*Pestalotiopsis*	0.5
Eurotiales	-0.602	*Ampulloclitocybe*	-0.518
Trichosporonales	-0.606		
Lycoperdon	-0.613	**Fungal taxa on the third axis**	**r value**
Hypoxylon	-0.677	*Cryptosporiopsis*	0.577
Xylariales	-0.696		
Chaetothyriales	-0.726		
Agaricales	-0.735		
Saprotrophic	-0.812		

Table S4. Identification of vectors and their r values for separate ordinations of trees sampled in Germany and the Netherlands.

Germany

Fungal Taxa on the first axis	**r value**
Agaricales	0.5
Agaricostilbales	0.652
Agyriales	0.538
Archaeorhizomycetales	0.652
Chaetothyriales	0.759
Diaporthales	0.652
Dothideales	0.652
Eurotiales	0.593
Geminibasidiales	0.695
Hypocreales	0.71
Lulworthiales	0.652
Microascales	0.652
Onygenales	0.652
Pezizales	0.854
Phallales	0.519
Pleosporales	0.828
Saccharomycetales	0.79
Thelephorales	0.67
Tubeufiales	0.652
Abortiporus	0.652
Acremonium	0.589
Acrostalagmus	0.652
Aureobasidium	0.652
Bensingtonia	0.652
Bradymyces	0.652
Candida	0.853
Clathrus	0.623
Clitopilus	0.652
Clonostachys	0.652
Coniella	0.652
Conocybe	0.652
Dictyochaeta	0.538
Exophiala	0.652
Fibulobasidium	0.538
Gamsia	0.652
Geopora	0.538
Gliomastix	0.652

***Fungal Taxa on the first axis* cont.**	**r value**
Glomerella	0.652
Humaria	0.538
Hypocrea	0.652
Inocybe	0.719
Isaria	0.623
Leptodontidium	0.589
Melanogaster	0.652
Metarhizium	0.609
Minimelanolocus	0.652
Oidiodendron	0.563
Otidea	0.538
Paralepista	0.652
Penicillium	0.652
Peziza	0.864
Psathyrella	0.801
Pseudaegerita	0.538
Rutstroemia	0.557
Sarea	0.538
Schizothecium	0.652
Schwanniomyces	0.652
Scutellinia	0.652
Talaromyces	0.652
Tomentella	0.789
Trichothecium	0.652
Tubeufia	0.652
Wardomyces	0.652

Fungal Taxa on the second axis	**r value**
Helotiales	-0.592
Trechisporales	-0.586
Venturiales	-0.517
Annulohypoxylon	0.616
Lasiosphaeris	0.616
Lopadostoma	0.557
Mycena	-0.532
Rickenella	-0.561
Trechispora	-0.667

Netherlands

Fungal Taxa on the first axis	**r value**
Agaricales	0.784
Chaetothyriales	0.76
Hysteriales	0.747
Mortierellales	0.746
Polyporales	0.598
Agrocybe	0.502
Cenococcum	0.615
Cladophialophora	0.641
Cortinarius	0.533
Tolypocladium	0.518

Fungal Taxa on the second axis	**r value**
Helotiales	-0.66
Hypocreales	-0.585
Tremellales	-0.582
Elaphomyces	0.635
Trichosporon	-0.677

Fungal Taxa on the third axis	**r value**
Capnodiales	-0.679
Pezizales	0.514
Phialocephala	-0.551
Trechispora	-0.571

Table S5. Indicator Species Analysis of fungal OTUs occurring on host trees in Germany and the Netherlands.

Host species	Location	Fungal OTU taxonomy	Indicator Value	*P*-value	Accession No.	SH No.
Betula pendula	Ger	Agaricomycetes sp.	80	0.0034	FN610890	SH205313.07FU
	Ger	Ascomycota sp.	80	0.003	KJ826832	
	Ger	Ascomycota sp.	71.4	0.0036	KT581723	
	Neth	Capnodiales sp.	55.6	0.0332	JF449633	SH206778.07FU
	Neth	*Cryptococcus podzolicus*	60	0.0364	AJ581036	SH181879.07FU
	Neth	Eurotiomycetes sp.	45.7	0.03	EU292229	SH213261.07FU
	Ger	Fungi sp.	83.3	0.0012	KJ827957	
	Ger	Fungi sp.	80	0.0034	UDB007496	SH176401.07FU
	Ger	Fungi sp.	62.5	0.0124	KJ827388	
	Ger	Fungi sp.	60	0.036	KT195623	
	Ger	Fungi sp.	60	0.0416	KC588678	
	Ger	Fungi sp.	60	0.0416	KP889691	
	Neth	Fungi sp.	64	0.02	GU174309	SH205736.07FU
	Neth	Fungi sp.	60	0.0324	KT219809	
	Ger	Helotiaceae sp.	71.4	0.003	AY219881	
	Neth	Helotiales sp.	64	0.0194	KC876248	SH181081.07FU
	Neth	Helotiales sp.	60	0.0376	GU997932	SH214273.07FU
	Ger	Herpotrichiellaceae sp.	60	0.0302	HE605254	SH199199.07FU
	Ger	*Meliniomyces variabilis*	80	0.0034	AY394902	SH181078.07FU
	Neth	*Meliniomyces variabilis*	60	0.041	AY394902	SH181078.07FU
	Ger	Mycenaceae sp.	71.4	0.0034	HQ625481	SH220731.07FU
	Ger	*Phialocephala fortinii*	100	0.0006	UDB020374	SH204986.07FU
	Ger	Sordariomycetes sp.	60	0.0312	AY704744	
	Neth	Trichocomaceae sp.	60	0.041	KM242318	
	Ger	Umbelopsidaceae sp.	60	0.0304	AB846975	SH018786.07FU
	Ger	Venturiaceae sp.	71.4	0.0032	GU446639	SH219611.07FU
	Ger	Vibrisseaceae sp.	60	0.0312	FN565305	SH204986.07FU
Carpinus betulus	Ger	Basidiomycota sp.	60	0.0354	GQ223476	SH015646.07FU
	Neth	Chaetothyriales sp.	80	0.0046	FM999597	SH458421.07FU
	Ger	*Clathrus archeri*	55.6	0.03	KJ702369	SH205136.07FU
	Ger	Fungi sp.	80	0.003	FM999518	SH214279.07FU

Table S5 (Cont.)

Host species	Location	Fungal OTU taxonomy	Indicator Value	*P*-value	Accession No.	SH No.
	Ger	Fungi sp.	60	0.0302	LC033786	
	Neth	Fungi sp.	60	0.0378	KP897187	
	Ger	Helotiaceae sp.	62.5	0.01	JX042982	SH026685.07FU
	Ger	Herpotrichiellaceae sp.	71.4	0.0026	UDB005252	SH179624.07FU
	Ger	Herpotrichiellaceae sp.	60	0.0302	KC965591	SH183634.07FU
	Ger	Herpotrichiellaceae sp.	60	0.0354	JN890098	SH026648.07FU
	Ger	Herpotrichiellaceae sp.	55.6	0.0144	GU083280	SH213273.07FU
	Neth	*Hyphodontia radula*	60	0.0356	GQ411525	SH221533.07FU
	Ger	Leotiomycetes sp.	55.6	0.0194	KF617787	SH021489.07FU
	Neth	Leotiomycetes sp.	62.5	0.0158	FM999518	SH214279.07FU
	Neth	Microbotryomycetes sp.	80	0.0028	EF434040	SH193764.07FU
	Ger	Mortierellaceae sp.	60	0.0302	FJ197928	SH011035.07FU
	Neth	Mortierellaceae sp.	62.5	0.0152	FN397392	SH180120.07FU
	Neth	Myxotrichaceae sp.	80	0.0036	HM136622	SH217025.07FU
	Ger	Tremellomycetes sp.	45.7	0.0324	GU055604	SH212824.07FU
Quercus robur	Ger	*Russula nigricans*	80	0.004	UDB000011	SH219259.07FU
	Neth	Russulaceae sp.	60	0.0334	FJ946940	SH218427.07FU
Fagus sylvatica	Ger	Eurotiomycetes sp.	71.4	0.0026	EU292229	SH213261.07FU
	Ger	Eurotiomycetes sp.	55.6	0.018	EU292229	SH213261.07FU
	Ger	Fungi sp.	64	0.0172	UDB005682	SH186212.07FU
	Ger	Helotiales sp.	55.6	0.0294	JF519310	SH192278.07FU
	Ger	*Lopadostoma fagi*	64	0.0196	KC774576	SH195181.07FU

Table S6. Topsoil physical and chemical properties for each sample site. Data retrieved from the Harmonized World Soil Database Viewer (V 1.21).

Topsoil parameter	Netherlands site	Germany site
Sand Fraction (%)	85	42
Silt Fraction (%)	8	38
Clay Fraction (%)	7	20
USDA Texture Classification	loamy sand	loam
Reference Bulk Density (kg/dm3)	1.65	1.41
Bulk Density (kg/dm3)	1.3	1.3
Gravel Content (%)	15	10
Organic Carbon (% weight)	0.49	1.45
pH (H2O)	7.9	5.1
CEC (clay) (cmol/kg)	111	32
CEC (soil) (cmol/kg)	5	12
Base Saturation (%)	100	38
TEB (cmol/kg)	4.2	4.3
Calcium Carbonate (% weight)	6	0
Gypsum (% weight)	0	0
Sodicity (ESP) (%)	4	2
Salinity (ECe) (dS/m)	0.2	0.1

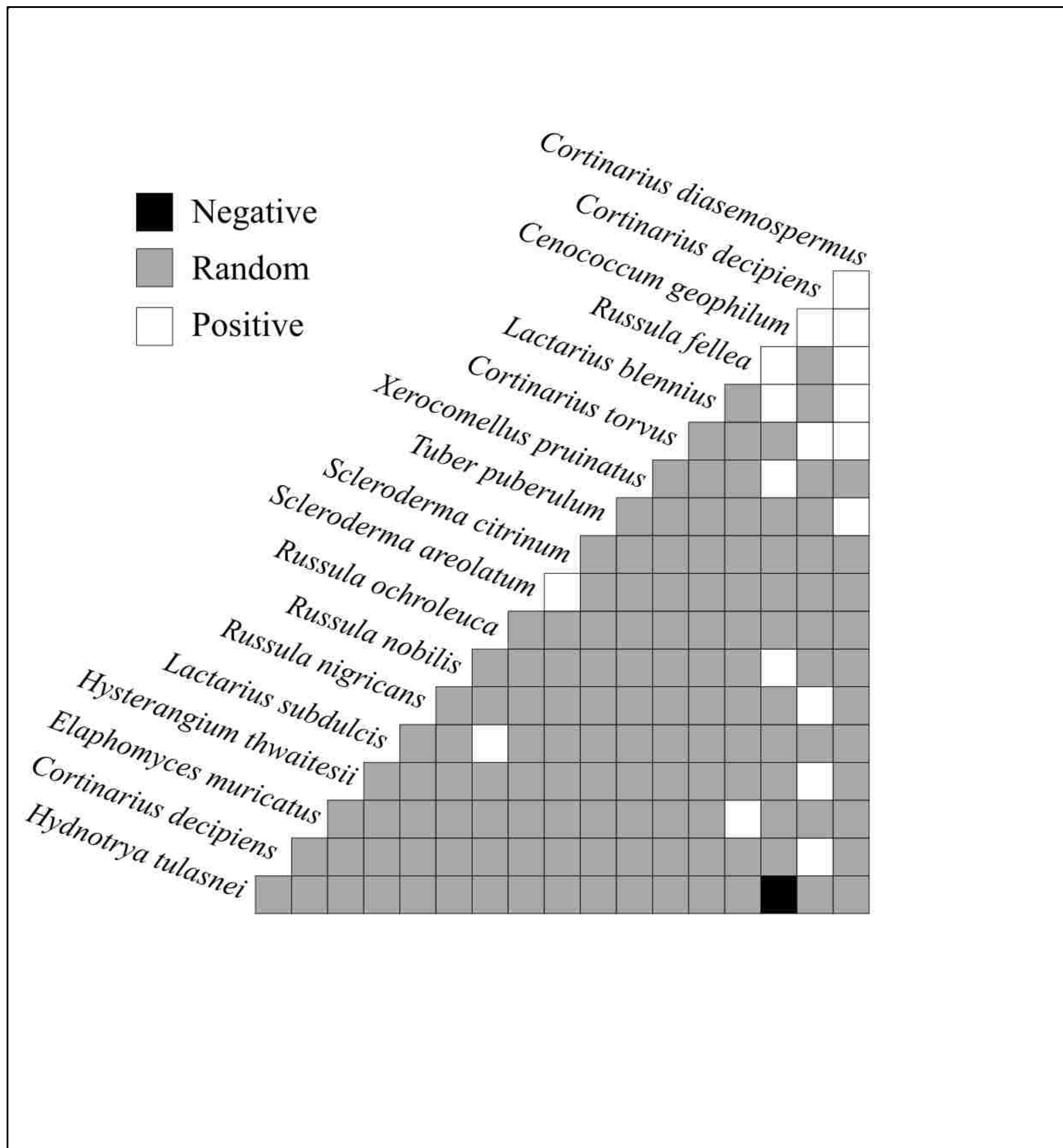


Figure S3**.** Co-occurrence matrix of ECM fungi. Fungal-fungal interactions among 30 ECM OTUs identified to the species level resulted in 18 positive, 1 negative, and 252 random associations. Of the 30 initial species, 18 were retained in the analysis with the other 12 showing neither positive nor negative associations.

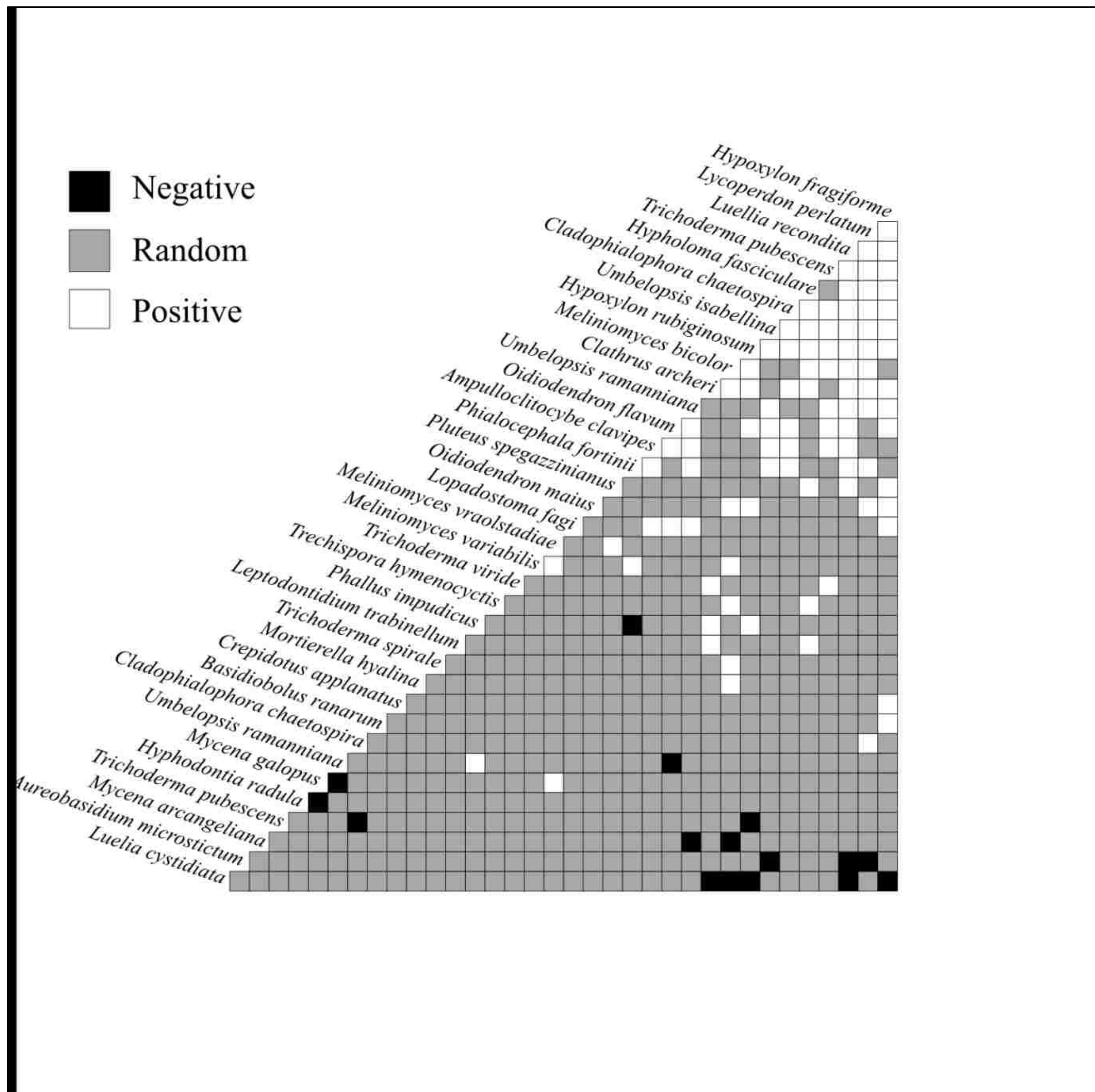


Figure S4. Co-occurrence matrix of 35 saprotrophic fungi showing positive and negative interaction patterns of occurrence greater than that expected by chance. There were a total of 16 negative and 93 positive fungal-fungal co-occurrence greater than that expected by chance. Random interactions constituted the remaining 486.

III. Root-associated fungal communities of selected trees in Eastern North American temperate deciduous broadleaf forests

Donald J. Nelsen[1*], Tatiana Semenova-Nelsen[2], Sandeep Sharma[3], Burt H. Bluhm[3], Steven L. Stephenson[1]

1. University of Arkansas, Department of Biological Sciences, 850 W. Dickson Street SCEN 601, Fayetteville, AR 72701, USA

2. University of Kansas, Kansas Biological Survey, Higuchi Hall 2101 Constant Ave, Lawrence, KS 66047, USA

3. University of Arkansas, Department of Plant Pathology, Plant Sciences Building room 217, 495 Campus Drive, Fayetteville, AR 72701, USA

*Corresponding author: donald.nelsen@gmail.com

A. Abstract

The eastern half of the United States is dominated by areas of temperate deciduous broadleaf forests that are important, both ecologically and economically. These forests support a vast diversity of thousands of species and provide billions of dollars in revenue. Among the dominant trees, American beech (*Fagus grandifolia*), northern red oak (*Quercus rubra*), and shagbark hickory (*Carya ovata*), form tight associations with below-ground microbial communities that play key roles in forest health, provide a defense against pathogens, and regulate host tree fitness. The diversity of these communities, however, remains largely unknown. This study used ITS2 rDNA to assess root-associated fungal communities of *F. grandifolia*, *Q. rubra*, and *C. ovata* in forests of the central Appalachian Mountains and the Ozark Mountains of northwestern Arkansas, USA. DNA-metabarcoding revealed a high diversity of fungi associated with the host trees in both geographic localities. Variation in community compositions was explained by geographic location by ca. 30%, while the effect of host specificity was ca. 10%. Fungal species

occurring on all the trees (“generalists”) to a large extent were ectomycorrhizal, while host-specific (“local”) communities were dominated by saprotrophs. Sequencing data provided insight in fungal-fungal interactions reflected in co-occurrence patterns of numerous ectomycorrhizal and saprotrophic species, with the most positive interactions revealed for *Cenococcum geophillum*. Because root-associated fungal communities are highly diverse, geographically distinct, and shift in response to available host taxa, we highlight the importance of DNA-based assessments to provide a more thorough understanding of these communities globally, including North American forest ecosystems.

Keywords: co-occurrence, host-specificity, fungal communities, Ion Torrent, ectomycorrhizae

Acknowledgements

The authors would like to acknowledge the following sources of funding support: this work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1008895; research was also funded in part by the Slime Mold Project at the University of Arkansas, and the Arkansas Native Plant Society. Thanks to the staff at the Fernow Experimental Forest and Mountain Lake Biological Station for providing lodging, historical data on forest composition, and directions. We extend our thanks also to Zachary Young for assistance in field collections.

Author contributions: Donald J. Nelsen developed this research project, collected and processed samples, analyzed the data, and wrote the paper. Tatiana A. Semenova-Nelsen collected and processed samples, assisted with data analysis and writing the paper. Sandeep Sharma sequenced the samples. Burt H. Bluhm provided laboratory consumables and sequencing resources. Steven L. Stephenson helped develop research and provided laboratory resources.

B. Introduction

Diverse areas of forests extend from southern tropical regions to the subarctic in North America. According to modern classifications, a wide range of forest types can be found in North America, including boreal, subalpine, montane and temperate rain forests, as well as coastal redwoods, evergreen and deciduous coniferous forests, mixed coniferous/deciduous forests, and the temperate deciduous broadleaf forests that make up much of the forested land of the eastern United States (Dyer, 2006). Forests are of vital importance, both ecologically (e.g., providing habitats for numerous living organisms) and economically (e.g., for wood production or as recreational zones) (USBC, 2006; Thomas et al., 2017). For instance, plant biodiversity of North American temperate deciduous forests was estimated as ca. 2000-3000 species (Kier et al., 2005), and species richness of soil fungi approached 2000-4000 operational taxonomic units (OTUs)–a proxy for species generally used in molecular assessments (Tedersoo et al., 2014). Globally, forests play key roles in carbon sequestration in trees (Pan et al., 2011) and soils (Lal, 2005), nutrient cycling (Prescott, 2002), and oxygen generation.

Geographically, forest types vary across the United States, with the western half characterized by Mediterranean chaparral, coastal redwood, and temperate rain forests that exist along the western coast, while montane and evergreen coniferous forests dominate further inland areas until the Great Plains (Wade et al., 2003). The eastern United States is characterized by coastal evergreen forests, mixed coniferous and deciduous forests, with some boreal forests in the northern parts of Minnesota and Maine. In general, most of the forested area of the eastern United States is dominated by assemblages of temperate deciduous broadleaf trees (Braun, 1950; Dyer, 2006), which extend from Texas to Minnesota and from Massachusetts to Nebraska (Dyer, 2006).

Dominant tree species of temperate deciduous broadleaf forests include maples (*Acer* spp., Aceraceae), birches (*Betula* spp., Betulaceae), oaks (*Quercus* spp., Fagaceae), beech (*Fagus grandifolia* Ehrh., Fagaceae), and hickory (*Carya* spp., Juglandaceae)(Braun, 1950). Globally, forests are of primary importance due to the ecological and economic value of their dominant tree species in addition to the ecological services provided by forest plants (Pearce, 2001). Timber-related industry in the United States accounted for 83.5 billion dollars of gross domestic production (GDP) in the most recent census (USBC, 2006), while the estimated revenue generated by tourism in national parks (including forested lands) surpassed 18.4 billion dollars in 2016 (Thomas et al., 2017). As pointed out by Pearce (2001), it is difficult to estimate the total value of forested lands based on incomplete data for the vast array of direct and indirect goods and services provided by forests around the world. It is, therefore, difficult to assign a value to the ecological and economic importance of the temperate deciduous broadleaf forests of eastern North America. Oak, beech, and hickory are useful tree species for industrial use as well as providing food and habitat for wildlife. Among the Fagaceae, *Quercus rubra* L. and *Fagus grandifolia* are dominant tree species in much of the temperate forest biome. The Juglandaceae are another family of important canopy-forming tree species in these forests. One species of interest in this family is *Carya ovata* (Mill.) K. Koch, which can be found in forested areas at mid-latitudes across the eastern United States. In addition to the aboveground communities that are dependent on these tree species, there is an underground diversity of microbes associated with the roots of these trees (Uroz et al., 2016).

Rhizosphere microbial communities are of great scientific interest as drivers of forest health, as a defense against pathogenic organisms, and as symbionts regulating the host fitness under stressful abiotic conditions (Vandenkoornhuyse et al., 2015). One area of primary interest with

respect to microbial communities of the rhizosphere is represented by the root-associated fungi (Walker et al., 2005; Burke et al., 2009; Comas & Eissenstat, 2009). These include taxa forming mycorrhizal associations, those serving as endophytes, and commensals or saprotrophs in the rhizosphere that are often collectively referred to as “other” fungi (Coince et al., 2014). The fitness of trees and other forest plants is greatly increased from the associations with their mutualistic fungi, both in the root and the adjacent rhizosphere (Barea et al., 2002). On the other hand, pathogenic fungi reduce the fitness of forest plants and are responsible for economic losses of forest products exceeding 7 billion dollars (Pimentel et al., 2005). Current estimates of the size of the Kingdom Fungi suggest anywhere from 1 million to 5.1 million species, with 70,000 currently described (Blackwell, 2011). Across this diversity of taxa, a wide array of different ecological functional groups is responsible for decomposition, diseases, and mutualistic lifestyles which are all part of functioning forest ecosystems. Understanding the taxa and ecological roles of root-associated assemblages of fungi is an important component to understanding forest health and function.

Quercus rubra and *Fagus grandifolia* are dominant tree species in the temperate deciduous broadleaf forests of eastern North America. Studies of root-associated fungal assemblages on these trees have focused on the description of putative ectomycorrhizal species and endophytes using a variety of morphological assays (roots and fruiting bodies) and molecular techniques (RFLP analyses and Sanger sequencing for example) (Gebhardt et al., 2007; Karpati et al., 2011). Information on the root-associated fungal communities on *Carya ovata*, another important species in the broadleaf forests of eastern North America, appears to be lacking in the scientific literature, although they have been reported as forming ectomycorrhizal associations in previous research (Comas and Eissenstat, 2009). Recent advances in sequencing technology have

provided rapid, cost effective methods for identifying communities of microorganisms using Next Generation Sequencing (NGS) platforms such as Ion Torrent. These technologies allow the description and study of root-associated communities from host tissues at a much larger scale than previously available through morphological assays, culturing, and sequencing of individual fungi.

The purpose of the research described herein was to add to our current understanding of fungal communities using ITS2 rDNA for NGS sequence-based identification of fungi from DNA extracted directly from root tissue of specific trees to describe and compare the root-associated fungal communities on *Fagus grandifolia, Quercus rubra,* and *Carya ovata* trees in geographically distant temperate deciduous broadleaf forests of eastern North America (Central Appalachian Mountains of Virginia and West Virginia and the Ozark Mountains of northwestern Arkansas) to answer the following research questions: (1) What are the communities of fungi associated with the roots of these trees in the temperate deciduous forests of eastern North America? (2) To what extent does location dictate the structure of root-associated fungal assemblages? (3) What level of host-specificity is present in fungi associated with the roots of each host species? (4) Are there patterns of occurrence among fungi on host trees that could be indicative of fungal-fungal interactions?

C. Methods

Study sites and root-tip sampling

As indicted above, two localities (the Ozarks of North Western Arkansas and the Central Appalachian Mountains) were selected for root tip sampling of *Fagus grandifolia, Quercus rubra,* and *Carya ovata* in the summer of 2014 (May-September). The Ozark Mountain sites where *Q. rubra* roots were sampled consisted of the Pea Ridge National Military Park (PR) near

Garfield, Arkansas (36°44’38” N, 94°02’58” W) and the Ozark National Forest (ONF) near Devil’s Den State Park (35°46'20.0"N 94°14'37.2"W). *Fagus grandifolia* roots were sampled at Lost Valley State Park (LV) near Kingston, Arkansas (36°00'50.8"N 93°22'55.8"W), and *C. ovata* roots were sampled near Lake Wilson Park (LW) located just outside the city of Fayetteville, Arkansas (35°59'57.8"N 94°09'37.9"W). In the Central Appalachian Mountains, *C. ovata* was sampled in the Fernow Experimental Forest (FEF) near the town of Parson in eastern central West Virginia (39°03'21.0"N 79°40'06.0"W) and near the Mountain Lake Biological Station (MLBS) in Giles County, Virginia (37°24'46.8"N 80°31'16.6"W). *Fagus grandifolia* was sampled at another site near the MLBS (37°21'58.8"N 80°32'18.9"W), and *Q. rubra* was also sampled nearby (37°21'07.1"N 80°32'08.6"W).

Host trees were sampled opportunistically at each site. A distance of at least 10 m was maintained between sampled trees to avoid sampling the same fungal genet. A total of 40 trees were sampled, including 15 *Carya ovata* (5 each from the FEF, MLBS, and LW sites), 10 *Fagus grandifolia* (5 each from the MLBS and LV sites), and 15 *Quercus rubra* (5 each from the MLBS, ONF, and PR sites). Root tips were sampled by uncovering the lateral roots of the target tree at the base of the trunk and following those roots out at 90° intervals to unearth feeder roots and colonized root tips representative of the root-associated fungal community on the root system of the target tree in the top 10 cm of soil below the litter layer. Colonized root tips were collected for each tree at each 90° interval and placed in a 15 ml screw cap tube containing 7 ml of a 2% CTAB solution so that each tube contained the pooled sample of roots for each sampled tree. Tubes were frozen in -20°C until processed for DNA extraction, PCR, and sequencing. Samples were thawed and thoroughly cleaned in distilled water to remove soil and organic particles. Using a dissecting scope and heat sterilized forceps, root tips were removed from each

sample of root material and placed in 2 ml microcentrifuge tubes, after which the root tips were lyophilized. These tubes were then submerged in liquid nitrogen and subsequently lysed and homogenized with sterile 3 mm glass beads in a Tissue Lyser (QIAGEN™) prior to DNA extraction.

DNA extraction and sequencing

DNA was extracted twice from each sample, for a total of 50 mg of homogenized tissues. DNA extractions were carried out using a Macherey-Nagel NucleoSpin Plant II DNA extraction kit using the PL1 lysis buffer protocol. Matrix-bound DNA was eluted 2 times in 30 µl of PE buffer, and pooled. PCR was carried out for each sample using the protocol established in Geml et al. (2014), 40 µl reactions were carried out with reagents at the following volumes: 1 µl of DNA template, 4 µl of 10X buffer, 1.5 µl of dNTP solution (2.5 mM), 1.5 µl each of forward and reverse primers (10 mM), 0.5 µl BSA (10 mg/ml), 4 µl MgCl2 (50 mM), 0.4 µl BIOTAQ polymerase (5 U/µl), and 25.6 µl of MQ water. The primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) were used for library prep PCR. The ITS4 primer was labeled with Multiplex Identification DNA (MID) tags specific for sample. PCR reactions amplified a 250-450 bp region of the ITS2 rDNA locus (Toju et al., 2012). PCR reactions were carried out under the following conditions established in Geml et al. (2014): 95°C for 5 min (one cycle), followed by 95°C for 20 sec, 54°C for 30 sec, and then 72°C for 1.5 min (25 cycles), followed by a terminal elongation step of 72°C for 7 min. Each sample had three replicate PCR reactions and a negative control with no template DNA added. Verification of PCR products for samples and replicates was done with agarose gel electrophoresis, and the PCR replicates were pooled for each sample.

Analysis of the size distribution and concentration of PCR products was done for each pooled sample using an Agilent D1000 ScreenTape system (Agilent Technologies, Waldbronn, Germany). Target PCR products were cleaned up using 0.9X Ampure® beads (Beckman-Coulter, Beverly, Massachusetts, USA). An equimolar pool of PCR products was then calculated from the volume and concentration of each sample for a normalized equimolar pool concentration of 15 nmol/µl. Oil emulsion PCR using 250 µl of the normalized equimolar pool was carried out in accordance with the protocol for the Ion PGM™ 200Xpress™ Template Kit. This library was then sequenced using an Ion 318™ Chip on an Ion Torrent Personal Genome Machine (Life Technologies, Guilford, Connecticut, USA). Ion Torrent sequencing resulted in 3,250,075 reads with a mean length of 228 bp.

Bioinformatics and statistical analyses

Preliminary clean-up of raw sequencing data was done using the Galaxy online platform (https://main.g2.bx.psu.edu/root) to arrange sequence reads by sample and remove MID adapter sequences. Sequences with poor-quality ends were trimmed using a 0.02 error probability limit in Geneious Pro 5.6.1 software (BioMatters, New Zealand). Sequence data were then filtered in USEARCH v.8.0 (Edgar, 2010) using the following settings: sequences were truncated to 200 bp, and those sequences with an expected error >1 were discarded. The remaining high-quality sequences were grouped into 7,730 operational taxonomic units (OTUs) using the UPARSE algorithm in USEARCH set to 97% sequence similarity as recommended in recent fungal metabarcoding studies (e.g., Bjorbækmo et al., 2010; Geml et al., 2010; Bellemain et al., 2013, Tedersoo et al., 2014; Semenova et al., 2016). A total of 7,690 putative chimeric sequences were excluded. Sequences were assigned to taxonomic groups using the UNITE fungal ITS sequence database based on pairwise similarity search. Global singletons were discarded, OTUs with less

than 80% sequence similarity to any UNITE sequences were discarded, leaving a dataset of 2,769 OTUs for subsequent analyses (Accession Numbers MG159816 - MG162584). Ecological functional groups were assigned for OTUs identified to genus (where consistent ecology existed) or species based on the dataset of Tedersoo et al. (2014), and resulted in 373 OTUs with putative functional assignment for analyses of distribution and proportions.

Depth of sequencing coverage was quantified using the Good's coverage estimator, and OTU community richness, diversity, and evenness were calculated to determine any potential differences between sampled communities. Good's coverage, Shannon-Weaver and Simpson's diversity indexes, OTU richness and Pielou's evenness was carried out and/or calculated using the Vegan package "rarefy" function (Oksanen et al., 2013) in R software for statistical computing (R Core team, 2017). To visualize the diversity of fungi occurring on roots of host trees, OTU data were assessed using the web-browser based tool Krona Plots (Ondov et al., 2011) for each host species. Krona plots were generated both for read abundance data (a limited proxy for biomass) and for richness data in order to compare the relative abundance of taxa and the diversity of taxa on each host species. Ecological functional groups of root-associated fungi were visualized in Excel to assess the distribution of functional types on different host species and those which occurred on all hosts (putative generalists). To determine the extent to which fungal OTUs were associated with each host species, Venn diagrams were generated using the online BioVenn platform (Hulsen et al., 2008) to provide comparative visualizations of area-proportional diagrams from OTU lists on each host. A presence/absence matrix was generated of OTUs on each host tree replicate, and a second matrix with categories: Geographic locality, specific site, host species, and fungal OTU taxonomies (orders and genera) was used to enable a joint-biplot analysis of subsequent ordinations. Non-Metric Multidimensional Scaling (500

iterations, Sørensen similarity/Bray-Curtis index, and a random starting number) of OTU presence/absence data was done using PC-ORD software v. 6.19 to assess the distribution of OTUs on roots in the ordination space in order to identify the extent of host specificity, site fidelity, and important fungal taxa. Additionally, plexus values (chi-squared derivatives to test a hypothesis of no difference) were calculated for each OTU pair to determine if there were instances of OTUs occurring together in ordination space more frequently than expected by chance–thus suggestive of a potential positive fungal-fungal interaction. Permutation-based nonparametric MANOVA were carried out in PC-ORD to determine the amount of variation in the dataset explained by location and host species variables and these values were recorded. Multi-Response Permutation Procedures (MRPP) were calculated for each grouping category in the secondary matrix to determine if in-group variation was greater than that expected by chance. Fungal co-occurrence patterns were assessed for 78 species-level OTUs based on location and by host in R using the R package: "cooccur" (Griffith et al., 2016) providing both positive and negative co-occurrence of fungal species greater than expected by chance in order to identify potential fungal-fungal interactions on roots of host trees.

D. Results

Diversity and distribution of root-associated fungi

Fungal communities obtained for each of the tree species had relatively high (96.7-97.7%) Good's coverage estimators, suggesting that the sequencing effort was sufficient to capture the diversity of fungi present in the samples (Figure S1). OTU richness and diversity estimators were comparable (not statistically different) between the sampled communities (Figure S2). The diversity of root-associated fungi encompassed OTUs from five fungal phyla; in addition, a high number of sequences identified no further than to Kingdom Fungi was obtained. Most of the

OTUs (73-75%) associated with the sampled trees belonged to the Ascomycota or Basidiomycota. These OTUs could be referred to 17 fungal classes, 53 orders, 120 families and 177 genera (Table S1). Visual representations of the relative abundance and OTU richness for each host tree are presented in Figures 1 (*Carya ovata*), 2 (*Fagus grandifolia*) and 3 *(Quercus rubra)*. Across all three sampled trees, proportions of the most abundant and species-rich fungal groups remained markedly similar; datasets based on sequence abundance were largely represented by the members of the Basidiomycota, whereas presence-absence (richness) data were dominated by the Ascomycota. Arbuscular mycorrhizal fungi belonging to the Glomeromycota were present but were characterized by both low richness and abundance.

Proportions of various ecological (functional) groups of fungi associated with tree roots were also similar (Figures 4a-c). However, richness of plant pathogenic fungi was higher in *Fagaceae* hosts (*Fagus grandifolia* and *Quercus rubra*) as compared to *Carya ovata*. Fungi that occurred on all host trees (generalists) were largely represented by an ectomycorrhizal ecology (ca. 64%), whereas host-specific fungi were mostly saprotrophs (50-58%). (Figure 4d).

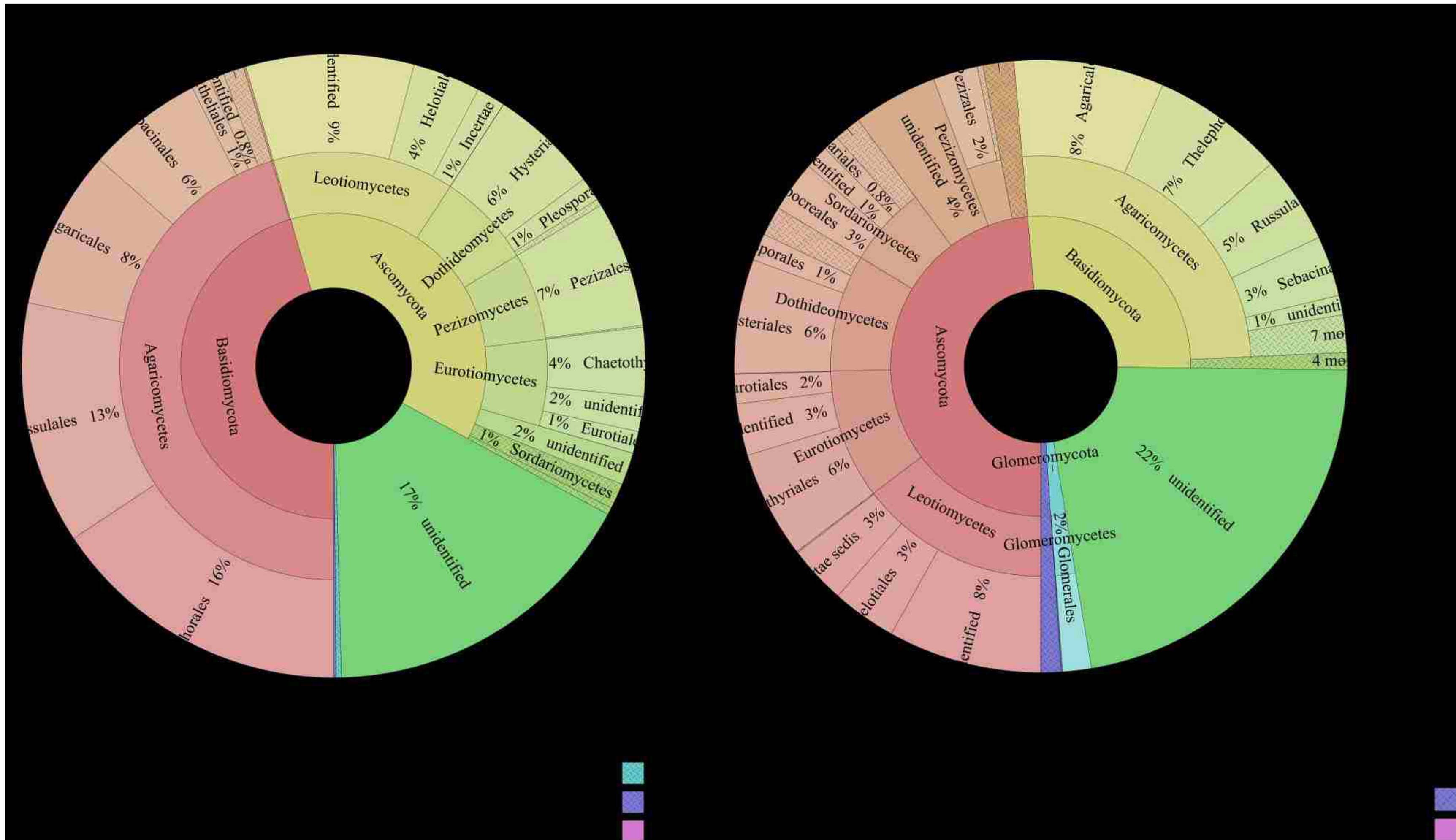


Figure 1. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Carya ovata*. The largest groups are shown in red, second largest groups in light yellow-green, and unidentified in dark green. Ascomycota comprises 49% of the richness found on *Carya*, while Basidiomycota account for 27%. In abundance, basidiomycete OTUs account for 45% of the sequence reads while ascomycetes account for 37%. A large percentage of OTUs associated with the roots of *Carya ovata* are unidentified fungal sequences.

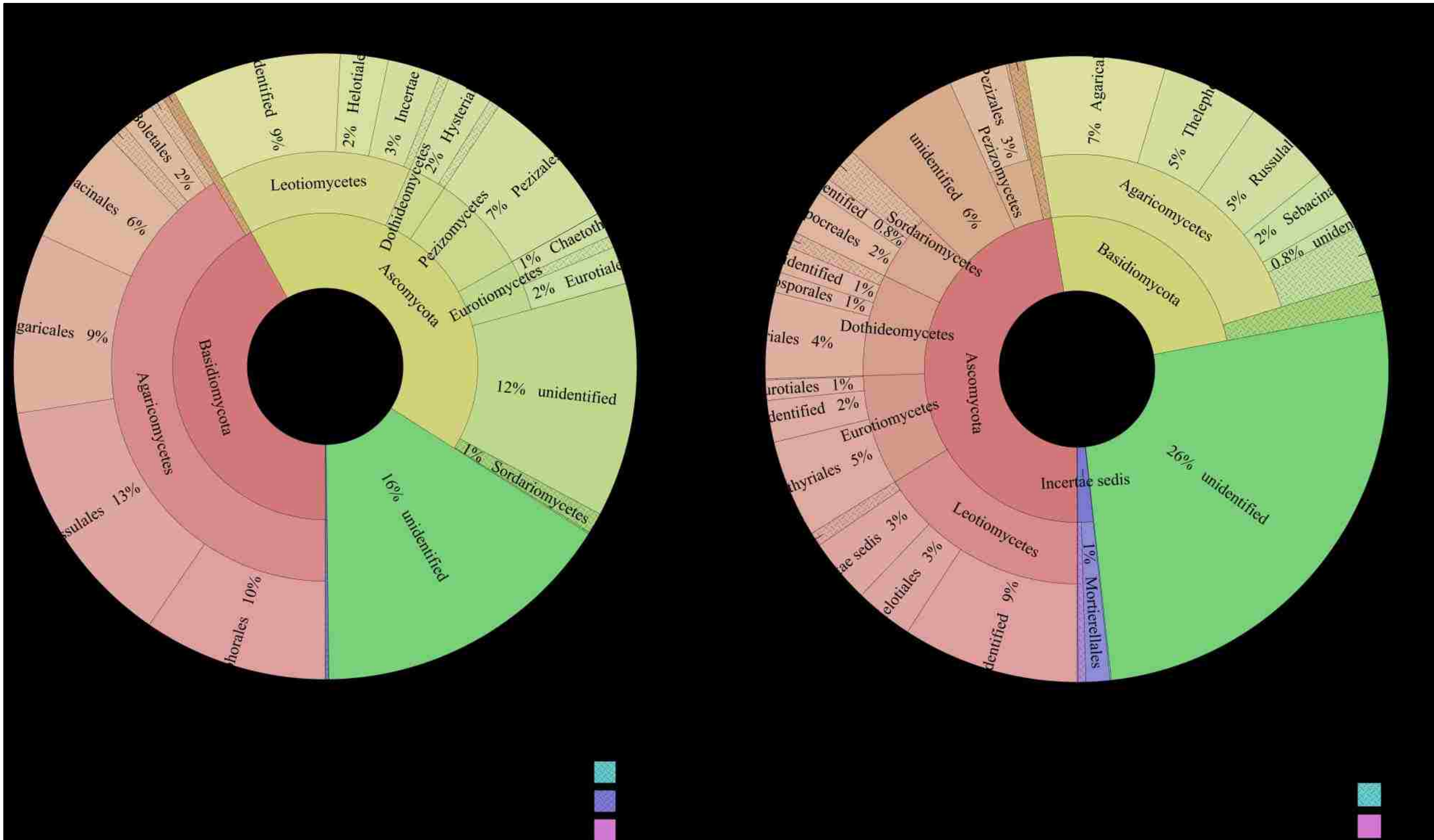
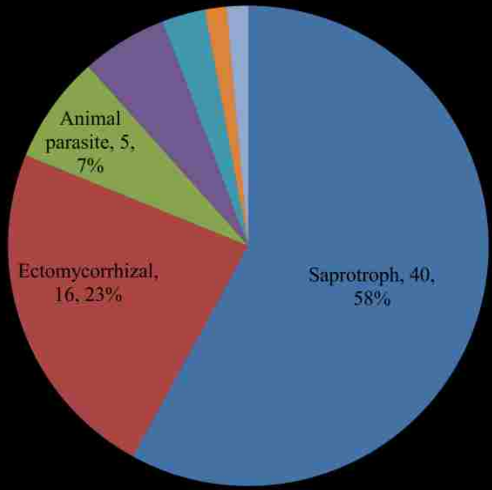
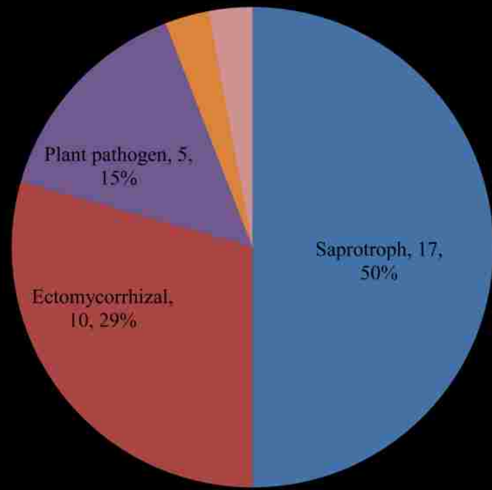
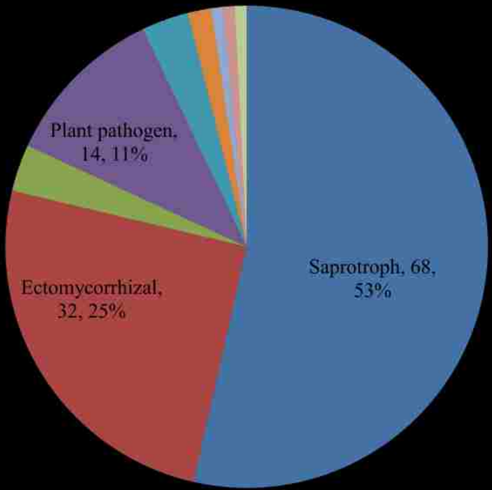


Figure 2. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Fagus grandifolia*. The largest groups are shown in red, second largest groups in light yellow-green, and unidentified in dark green. Ascomycota comprises 47% of the richness found on *Fagus grandifolia*, while Basidiomycota account for 25%. In abundance, basidiomycete and ascomycete OTUs each account for 42% of the sequence reads. More than 25% of OTUs are unidentified to phylum.

Figure 3. Krona plot diagrams of fungal OTU read abundance (a) and OTU richness (b) at the level of fungal orders on the roots of *Quercus rubra*. The largest groups are shown in red, second largest groups in light yellow-green, and unidentified in dark green. Ascomycota comprises 43% of the richness found on *Q. rubra*, while Basidiomycota account for 30%. With respect to abundance, basidiomycete OTUs account for nearly half (49%) of the sequence reads while ascomycetes account for 29% of reads. More than 25% of OTUs associated with *Q. rubra* are unidentified.


Animal
parasite, 5,
7%
Ectomycorrhizal,
16, 23%
Saprotroph, 40,
58%


Plant pathogen, 5,
15%
Saprotroph, 17,
50%
Ectomycorrhizal,
10, 29%


Plant pathogen,
14, 11%
Saprotroph, 68,
53%
Ectomycorrhizal,
32, 25%

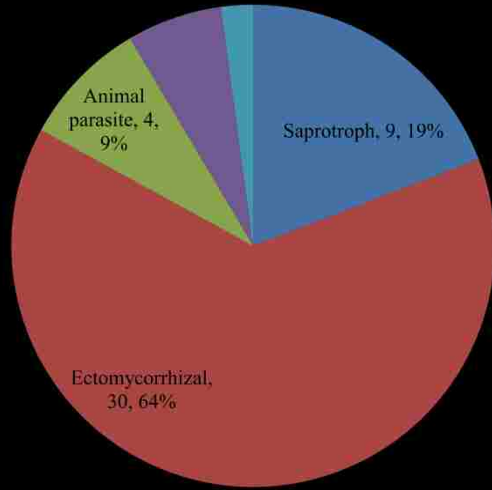

Animal
parasite, 4,
9%
Saprotroph, 9, 19%
Ectomycorrhizal,
30, 64%

Figure 4 (Previous page). Proportions of OTUs belonging to different ecological functional groups specific to *Carya ovata* (a), *Fagus grandifolia* (b), *Quercus rubra* (c), or occurring on all three tree species (d) across the study sites of temperate deciduous forests of eastern North America. Saprotrophic fungi dominate among OTUs found only on specific host trees, while ectomycorrhizal taxa dominate as generalists occurring on all sampled trees.

Community structure is driven by location and host-association

Fungal community compositions were compared by non-metric multidimensional scaling (NMDS, Figures 5a-b). An ordination plot resulted in a 2-dimentional solution with axis 1: r^2=0.560 and axis 2: r^2=0.231 and a final stress of 15.99, 0.00000 instability, and 65 iterations. Differences among groups within the dataset when grouped by location (A=0.076; P<0.00000) and host (A=0.025; P=0.00005) variables was greater than expected by chance using MRPP analysis. Variation in community composition was largely explained by location; as revealed by MANOVA statistics, location explained 29.2% of the variation in fungal OTU composition (F=3.06; P=0.0002). The effect of host specificity on community compositions was lower (10.8% by MANOVA) but still significant (P=0.0008). A joint biplot of fungal taxonomy for OTUs revealed important taxa on axes 1 and 2 (Figure 5a) and showed a pattern of host tree clustering by both location and tree species. Geographical isolation of communities in the central Appalachian Mountains and the Ozark Mountains of northwest Arkansas is demonstrated by clustering of sampled host trees by geographic locality (Figure 5b), whereas strong (measure of association >0.5) plexus lines plotted between OTUs suggest a large number of positive associations across both geographical localities.

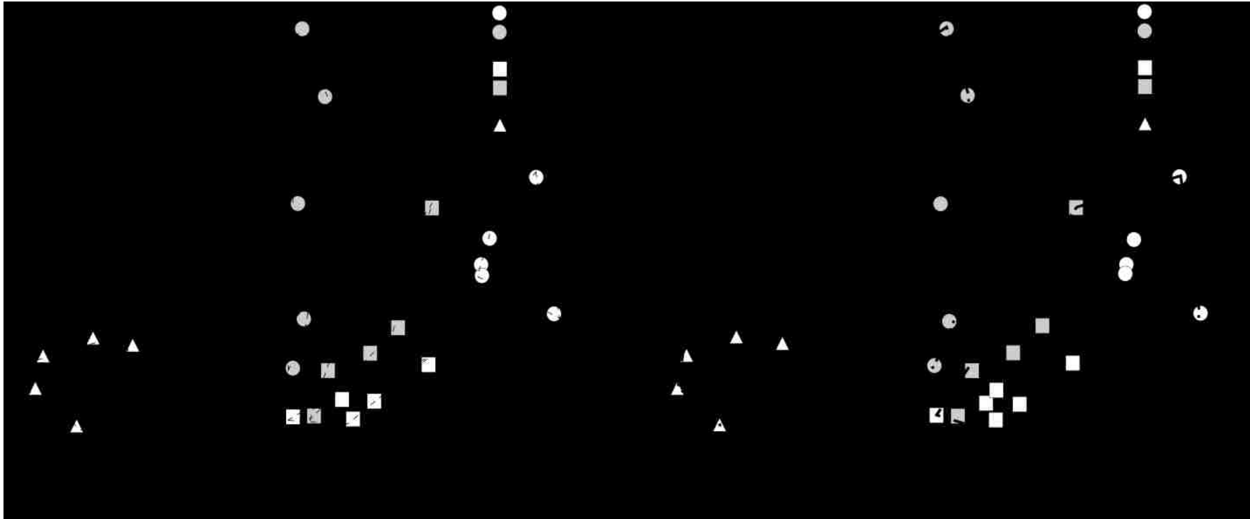


Figure 5. Nonmetric multidimensional scaling ordination of root-associated fungal OTUs on host trees. Circles represent *Carya ovata* collections at Lake Wilson (white), Fernow Experimental Forest (gray) and Mountain Lake Biological Station (ML; black). Squares represent *Quercus rubra* collectd at the Ozark National Forest (white), Pea Ridge National Military Park (gray) and ML (black). *Fagus grandifolia* collections are represented as triangles from sites at ML (white) and Lost Valley State Park (black). Fungal communities show a clear distribution influenced by location and host (a) fungal taxa with high richness associated with communities along the axis are denoted with vectors, dashed lines represent generalized fungal community limits around host tree taxa. Fungal OTUs showed a high number of positive correlations greater than that expected by chance using Yates corrected Chi-square test to generate a plexus diagram (b) in PC-ORD, the dashed/dotted centroids represent communities segregated by geographic location (Appalachian Mountains and Ozark Mountains).

In order to examine the extent of host fidelity of root-associated communities, a proportional Venn diagram was generated for all fungal OTUs occurring on each host across all sample locations (Figure 6). Individuals of *Quercus rubra* contained the largest number of OTUs in their root-associated communities (2,595) and the largest number of OTUs specific to their communities (1491). *Carya ovata* had the second largest root-associated fungal community (1,671) and number of specific fungi (722), while *Fagus grandifolia* root-associated communities were smaller in comparison (1,184 OTUs total, 414 specific). *Quercus rubra* root-associated communities shared a large number with *C. ovata* (421), *F. grandifolia* (242), and 441

OTUs occurred on all three host trees. Only 87 OTUs were shared between *F. grandifolia* and *C. ovata.*

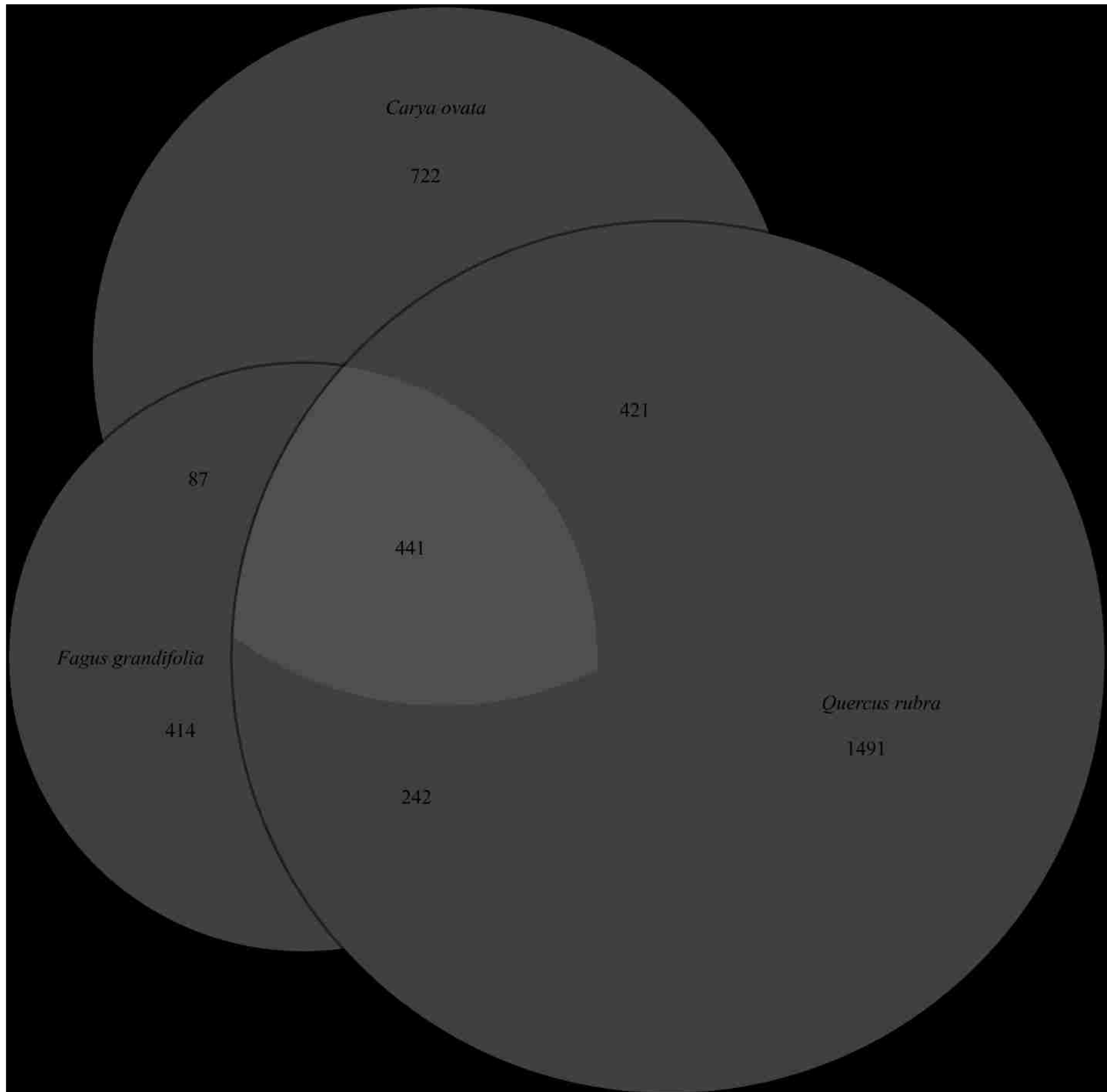


Figure 6. Venn diagram of OTUs occurring on the roots of *Quercus rubra, Carya ovata*, and *Fagus grandifolia* in temperate deciduous forests of the central Appalachian Mountains and the Ozark Mountains. 3,818 OTUs were used in analysis, with more than half (2,595) occurring on *Q. rubra* roots. 441 OTUs were generalists occurring on all three host tree taxa, while ~1,500 were specific to *Q. rubra*.

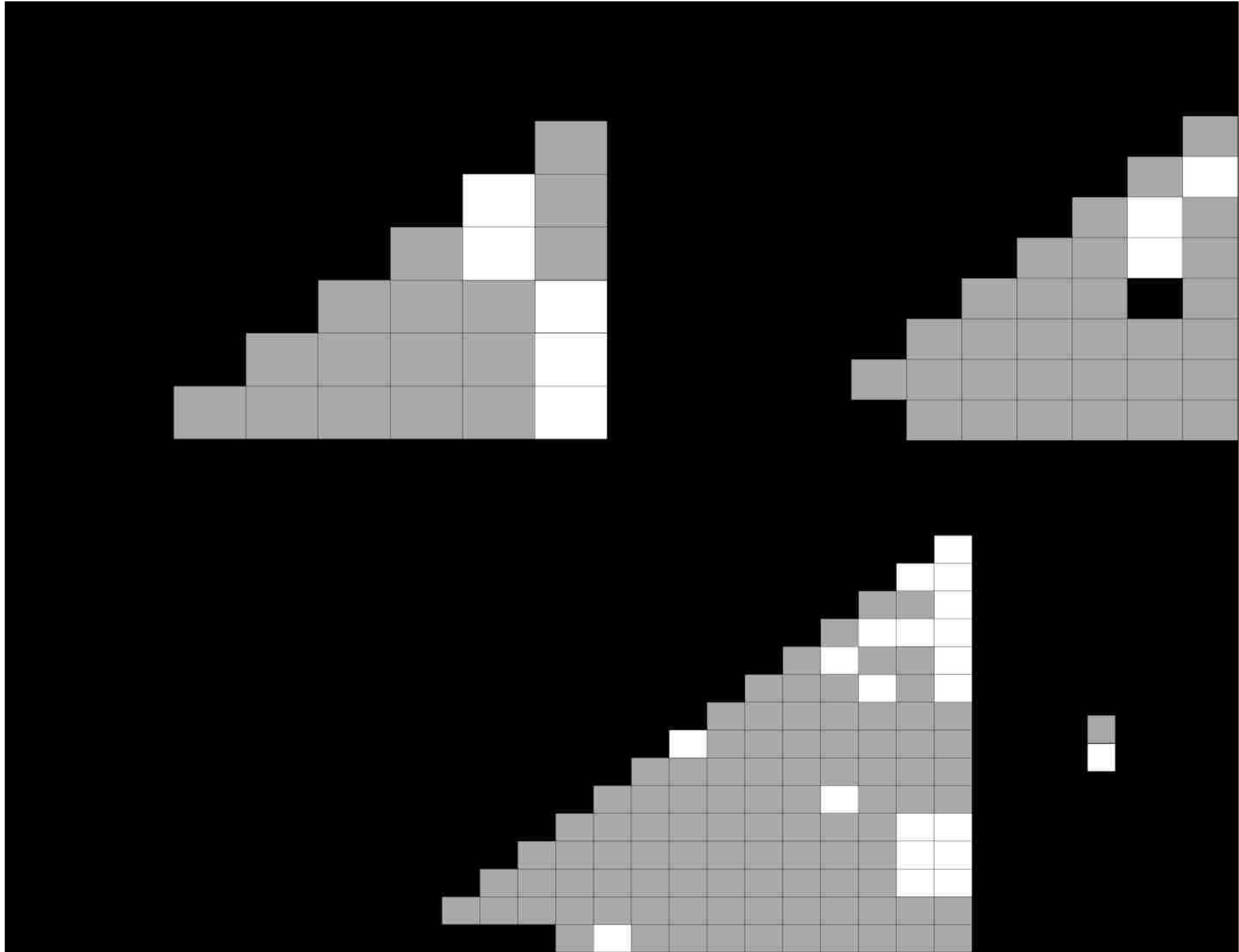


Figure 7. Co-occurrence analyses of root-associated fungal assemblages on three host tree taxa in two temperate deciduous forests of eastern North America (Ozark Mountains and central Appalachian Mountains). Host trees were: a) *Carya ovata*, b) *Fagus grandifolia,* and c) *Quercus rubra*.

Fungal co-occurrence patterns

Co-occurrence analyses revealed several positive and negative species interactions on the three host tree species across all sample locations (Figures 7a-c). On *Carya ovata* (Figure 7a) there were 63 fungal species identified across the 15 samples; of the 1,953 species pair combinations 250 pairs were retained for analysis, which resulted in 5 positive associations, 0 negative, and 245 random/non-random unclassifiable interactions. On *Fagus grandifolia* (Figure 7b), 43 fungal species, 10 samples, 135 of 903 species pairs were retained for analysis. These included 3

positive interactions, 3 negative, and 129 random/non-random unclassifiable interactions were reported. Co-occurrence analysis of 67 fungal species on *Quercus rubra* (Figure 7c) resulted in an analysis of 513 of 2,211 possible species pair combinations across the 15 sampled trees, with 20 positive, 4 negative, and 489 random/non-random unclassifiable interactions. The saprotrophic species *Mortierella elongata* Linnem. and *Oidiodendron maius* G.L. Barron were negatively associated with other saprotrophs and ectomycorrhizal fungi on *Fagus grandifolia* and *Quercus rubra* but not on *Carya ovata,* where *O. maius* appears to be positively associated with the ectomycorrhizal *Cenococcum geophilum* Fr. On *Q. rubra*, members of the ectomycorrhizal Russulaceae appear positively associated with *C. geophilum.* Co-occurrence analyses of all host trees at each locality (central Appalachian Mountains or the Ozark Mountains) and all combined were also done to assess patterns of co-occurrence in geographically distinct communities (Figure S3), and the positive interactions between *Russula* spp., *Lactarius* spp., and *C. geophilum* appear to be cosmopolitan across temperate deciduous forests of eastern North America.

E. Discussion

DNA-metabarcoding of soil samples has revealed the high diversity of soil fungal communities in forests around the globe (Tedersoo et al., 2014). By examining root material collected directly from specific host trees in the temperate deciduous forests of eastern North America we can build on existing knowledge gained by previous work and understand more fully the root-associated fungal communities found on particular tree species. The present study examined fungal communities associated with roots of *Fagus grandifolia, Quercus rubra,* and *Carya ovata* by direct sampling of tree roots in order to assess patterns in the distribution and specificity of fungal community members in different forest communities and host trees, and determine what

(if any) fungal-fungal interactions may be occurring based on patterns of co-occurrence of root-associated fungi.

Our results presented a more comprehensive assessment of root-associated communities on these three host tree species than previously available. Recent research on *Quercus rubra* and other host trees with ectomycorrhizal communities using morphotyping, RFLP analyses, baiting, and Sanger-sequencing of RFLP types resulted in fungal richness observations much more limited compared to what we found with NGS sequencing of root material (Dickie et al., 2002; Walker et al., 2005; Ishida et al., 2007; Karpati et al., 2011). Conversely, a recent study on different species of *Quercus* in California woodlands found similar numbers of ECM on roots (Morris et al., 2008), but these data were obtained via roots isolated from soil cores and not taken directly from host trees as in our study, thus likely representing ECM communities including nontarget hosts. A 21-year study of fruiting bodies collected in a mixed forest in Switzerland resulted in a maximum of 194 species found during the most diverse year (Straatsma et al., 2001), whereas in our study fungal richness was greater by more than an order of magnitude from a single sampling effort. In addition to fungi that form visible fruiting bodies and thus could be assessed morphologically, NGS data obtained in this study from root material resulted in highly diverse fungal assemblages that include many "hidden" taxa occurring on *Q. rubra, Fagus grandifolia,* and *Carya ovata* roots, which represent data not previously known to science. While morphological assessments involve largely ectomycorrhizal fungi, NGS data unraveled a diversity of fungal taxa with a variety of ecological functions, including saprotrophs, endophytes, pathogens and different types of mycorrhizae.

Root-associated fungal communities appear to be strongly influenced by their geographical location and host association. In terms of read abundance and richness of fungal taxa, all three

host tree species exhibited quite similar communities, at numbers comparable with recent soil fungal studies (Buée et al., 2009; Tedersoo et al., 2014); however, the species composition appeared to be different for each host. On *Quercus rubra*, the majority of root-associated fungi (ca. 60%) were found only in association with this host, which supports the findings of hyper-diversity on the roots of *Quercus* in previous research (Walker et al., 2005; Jumponnen et al., 2010). In this study, one third of the OTUs found on tree roots occurred on more than one host and the rest were tree-specific. This finding is in agreement with previous research showing a strong influence of host tree species on its associated fungal communities (Massicotte et al., 1999; Ishida et al., 2007; Wehner at al., 2014).

Interestingly, all the tree species sampled in this study hosted approximately same number of fungi belonging to various ecological groups. Unexpectedly, the "specificity towards a particular host" was due to fungi belonging to a saprotrophic rather than ectomycorrhizal guild. The OTUs specific to particular host trees were to a large extent saprotrophic, while "generalist species" occurring on all of the sampled trees were assigned ectomycorrhizal lifestyles. We assume that members of saprotrophic root-associated community serve as endophytes and decomposers of senescent root tip sections (Le et al., 2015) or simply exist as epiphytes in the rhizosphere, decomposing nearby organic matter in the soil horizon. On the other hand, given that some ectomycorrhizal fungi also function as saprotrophs (Phillips et al., 2014; Lindahl and Tunlid, 2015) and some saprotrophs colonize root tips as potential mycorrhizae (Vasiliauskas et al., 2007), perhaps some of the OTUs assigned a saprotrophic lifestyle in this study (as based on general knowledge) could instead have symbiotic interactions with the trees (e.g., as facultative endophytes or mycorrhizae).

Given the declining cost of next-generation sequencing, biodiversity studies such as ours are increasingly being carried out worldwide, thus providing an unprecedented insight into fungal co-occurrence patterns that could be indicative of fungal interactions. In the scope of a single study, these patterns should be discussed with caution, given that strong positive or negative co-occurrence patterns may be driven by random factors unrelated to fungal-fungal interactions, but they are still important to consider as potential ecological drivers and/or consequences of community structure and function (Ovaskainen et al., 2010). In this study, on all three tree species we observed positive interactions between *Cenococcum geophilum* and several species of saprotrophic fungi (e.g., *Cryptosporiopsis radicicola* Kowalski & C. Bartnik & *Oidiodendron maius* on *Carya ovata; C. ericae* Sigler on *Fagus grandifolia*; *Geoglossum simile* Peck*, Leotia lubrica* [Scop.] Pers.*, O. chlamydosporicum* Morrall*, Trichoderma semiorbis* [Berk.] Jaklitsch & Voglmayr & *Mortierella macrocystis* Gams on *Quercus rubra*). We also found a positive interaction with the potentially ectomycorrhizal *Sowerbyella unicisa* (Peck) Moravec and *C. geophilum* on *F. grandifolia* which has been until now considered a likely saprotroph not previously found in root-associated community studies (Hansen et al., 2013). *Cenococcum geophilum* was also found to have a positive co-occurrence with several ECM fungi, including members of the Russulaceae, previously considered to interact negatively with other mycorrhizal fungi (Koide et al., 2005; Sun et al., 2015). As a trend, it appears that *C. geophilum* is forming dynamic partnerships with both saprotrophic fungi and other mycorrhizal associates on the roots of trees in the temperate forests of eastern North America. The data reported herein can be used to develop experimental studies of the relationship between these fungi for a more definitive explanation of the observed patterns of occurrence on tree roots.

F. Conclusions

Globally, our understanding of fungal diversity is limited by the ability to accurately identify fungal taxa, most of which do not form visible fruiting bodies. Traditionally, morphological identification of fungal fruiting bodies was used to provide identifications, and the substrate upon which a particular fruiting body was found served to assign it to a certain fungal functional guild. Morphology-based description of fungal species is essential for understanding of fungal diversity; however, this approach has its limitations related to the ephemeral nature of fungal fruiting bodies in most species, including such things as the short duration of fruiting and/or infrequent production of the fruiting bodies, and their microscopic sizes. In addition, successful morphology-based assays of fungi rely heavily on skilled experts in fungal taxonomy. High-throughput sequencing technology allows more objective assessments of fungal diversity in a rapid and accurate way. Because numerous samples can be processed at the same time, a DNA-based approach could be applied on a much broader scale compared to morphological methods. In the temperate deciduous forests of North America, our DNA-based approach revealed hyper-diverse communities of fungi associated with different host trees. Communities of root-associated fungi were geographically distinct, specific to host tree species, and interacting with each other to form dynamic associations. The DNA-based assessments, as presented in this study, when applied on a broader scale, will contribute to an increased understanding of the interplay between fungal species at the host tree in addition to providing improved knowledge of fungal diversity, ecological functions, and co-occurrence patterns.

G. References

Barea, J. M., Azcón, R., and Azcón-Aguilar, C. (2002). Mycorrhizosphere interactions to improve plant fitness and soil quality. *Antonie van Leeuwenhoek* 81(1-4), 343-351.

Bellemain, E., Davey, M.L., Kauserud, H., Epp, L.S., Boessenkool, S., Coissac, E., Geml, J., Edwards, M., Willerslev, E., Gussarova, G. and Taberlet, P. (2013). Fungal palaeodiversity revealed using high-throughput metabarcoding of ancient DNA from arctic permafrost. *Environmental Microbiology* 15(4), 1176-1189.

Bjorbækmo, M.F.M., Carlsen, T., Brysting, A., Vrålstad, T., Høiland, K., Ugland, K.I., Geml, J., Schumacher, T. and Kauserud, H. (2010). High diversity of root associated fungi in both alpine and arctic Dryas octopetala. *BMC Plant Biology* 10(1), 244.

Blackwell, M. (2011). The Fungi: 1, 2, 3… 5.1 million species?. *American Journal of Botany* 98(3), 426-438.

Braun, E. L. (1950). Deciduous forests of eastern North America. Blakiston, Philadelphia.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R. H., Uroz, S., and Martin, F. (2009). 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist* 184(2), 449-456.

Burke, D. J., López-Gutiérrez, J. C., Smemo, K. A., and Chan, C. R. (2009). Vegetation and soil environment influence the spatial distribution of root-associated fungi in a mature beech-maple forest. *Applied and Environmental Microbiology* 75(24), 7639-7648.

Coince, A., Cordier, T., Lengellé, J., Defossez, E., Vacher, C., Robin, C., Buée, M., and Marçais, B. (2014). Leaf and root-associated fungal assemblages do not follow similar elevational diversity patterns. *PLoS One* 9(6), e100668.

Comas, L. H., and Eissenstat, D. M. (2009). Patterns in root trait variation among 25 co-existing North American forest species. *New Phytologist* 182(4), 919-928.

Dickie, I. A., Koide, R. T., and Steiner, K. C. (2002). Influences of established trees on mycorrhizas, nutrition, and growth of *Quercus rubra* seedlings. *Ecological Monographs* 72(4), 505-521.

Dyer, J. M. (2006). Revisiting the deciduous forests of eastern North America. *BioScience* 56(4), 341-352.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19), 2460-2461.

Gebhardt, S., Neubert, K., Wöllecke, J., Münzenberger, B., and Hüttl, R. F. (2007). Ectomycorrhiza communities of red oak (*Quercus rubra* L.) of different age in the Lusatian lignite mining district, East Germany. *Mycorrhiza* 17(4), 279-290.

Geml, J., Kauff, F., Brochmann, C., and Taylor, D. L. (2010). Surviving climate changes: high genetic diversity and transoceanic gene flow in two arctic–alpine lichens, *Flavocetraria cucullata* and *F. nivalis* (Parmeliaceae, Ascomycota). *Journal of Biogeography* 37(8), 1529-1542.

Geml, J., Pastor, N., Fernandez, L., Pacheco, S., Semenova, T. A., Becerra, A. G., Wicaksono, C. Y. and Nouhra, E. R. (2014). Large-scale fungal diversity assessment in the Andean Yungas forests reveals strong community turnover among forest types along an altitudinal gradient. *Molecular Ecology* 23(10), 2452-2472.

Griffith, D. M., Veech, J. A., and Marsh, C. J. (2016). Cooccur: probabilistic species co-occurrence analysis in R. *Journal of Statistical Software* 69(2), 1-17.

Hansen, K., Perry, B. A., Dranginis, A. W., and Pfister, D. H. (2013). A phylogeny of the highly diverse cup-fungus family Pyronemataceae (Pezizomycetes, Ascomycota) clarifies relationships and evolution of selected life history traits. *Molecular Phylogenetics and Evolution* 67(2), 311-335.

Hulsen, T., de Vlieg, J., and Alkema, W. (2008). BioVenn–a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics* 9(1), 488.

Ihrmark, K., Bödeker, I. T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K. E. and Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region–evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology* 82(3), 666-677.

Ishida, T. A., Nara, K., and Hogetsu, T. (2007). Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. *New Phytologist* 174(2), 430-440.

Jumpponen, A., Jones, K. L., David Mattox, J., and Yaege, C. (2010). Massively parallel 454-sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. *Molecular Ecology* 19(s1), 41-53.

Karpati, A. S., Handel, S. N., Dighton, J., and Horton, T. R. (2011). *Quercus rubra*-associated ectomycorrhizal fungal communities of disturbed urban sites and mature forests. *Mycorrhiza* 21(6), 537-547.

Kier, G., Mutke, J., Dinerstein, E., Ricketts, T. H., Küper, W., Kreft, H., and Barthlott, W. (2005). Global patterns of plant diversity and floristic knowledge. *Journal of Biogeography* 32(7), 1107-1116.

Koide, R. T., Xu, B., Sharda, J., Lekberg, Y., and Ostiguy, N. (2005). Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* 165(1), 305-316.

Lal, R. (2005). Forest soils and carbon sequestration. *Forest Ecology and Management* 220(1), 242-258.

Li, A., Fahey, T. J., Pawlowska, T. E., Fisk, M. C., and Burtis, J. (2015). Fine root decomposition, nutrient mobilization and fungal communities in a pine forest ecosystem. *Soil Biology and Biochemistry* 83, 76-83.

Lindahl, B. D., and Tunlid, A. (2015). Ectomycorrhizal fungi–potential organic matter decomposers, yet not saprotrophs. *New Phytologist* 205(4), 1443-1447.

Massicotte, H. B., Molina, R., Tackaberry, L. E., Smith, J. E., and Amaranthus, M. P. (1999). Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Canadian Journal of Botany* 77(8), 1053-1076.

McCune, B. and M. J. Mefford. (2011). PC-ORD. Multivariate Analysis of Ecological Data. Version 6.19 MjM Software, Gleneden Beach, Oregon, U.S.A.

Morris, M. H., Smith, M. E., Rizzo, D. M., Rejmánek, M., and Bledsoe, C. S. (2008). Contrasting ectomycorrhizal fungal communities on the roots of co-occurring oaks (*Quercus* spp.) in a California woodland. *New Phytologist* 178(1), 167-176.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H. and Oksanen, M.J. (2013). Package 'vegan'. Community ecology package, version, 2(9).

Ondov BD, Bergman NH, and Phillippy AM. (2011) Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* 12(1):385.

Ovaskainen, O., Hottola, J., and Siitonen, J. (2010). Modeling species co-occurrence by multivariate logistic regression generates new hypotheses on fungal interactions. *Ecology* 91(9), 2514-2521.

Pan, Y., Birdsey, R. A., Fang, J., Houghton, R., Kauppi, P. E., Kurz, W. A., Phillips, O.L., Shvidenko, A., Lewis, S.L., Canadell, J.G., and Ciais, P. (2011). A large and persistent carbon sink in the world's forests. *Science* 333(6045), 988-993.

Pearce, D. W. (2001). The economic value of forest ecosystems. *Ecosystem Health* 7(4), 284-296.

Phillips, L. A., Ward, V., and Jones, M. D. (2014). Ectomycorrhizal fungi contribute to soil organic matter cycling in sub-boreal forests. *The ISME Journal* 8(3), 699.

Pimentel, D., Zuniga, R., and Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52(3), 273-288.

Prescott, C. E. (2002). The influence of the forest canopy on nutrient cycling. *Tree Physiology* 22(15-16), 1193-1200.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Semenova, T. A., Morgado, L. N., Welker, J. M., Walker, M. D., Smets, E., and Geml, J. (2016). Compositional and functional shifts in arctic fungal communities in response to experimentally increased snow depth. *Soil Biology and Biochemistry* 100, 201-209.

Sun, H., Santalahti, M., Pumpanen, J., Köster, K., Berninger, F., Raffaello, T., Jumpponen, A., Asiegbu, F.O. and Heinonsalo, J. (2015). Fungal community shifts in structure and function across a boreal forest fire chronosequence. *Applied and Environmental Microbiology* 81(22), 7869-7880.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., and Smith, M. E. (2014). Global diversity and geography of soil fungi. *Science* 346(6213), 1256688.

Thomas, C. C., Huber, C., and Koontz, L. (2017). 2016 National Park visitor spending effects: economic contributions to local communities, states, and the nation (No. NPS/NRSS/EQD/NRR—2017/1421). National Park Service.

Toju, H., Tanabe, A. S., Yamamoto, S., and Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PloS One* 7(7), e40863.

Uroz, S., Buee, M., Deveau, A., Mieszkin, S., and Martin, F. (2016). Ecology of the forest microbiome: Highlights of temperate and boreal ecosystems. *Soil Biology and Biochemistry* 103, 471-488.

US Bureau of the Census. (2006). Statistical Abstract of the United States, 125th ed. Washington (DC) US Government Printing Office.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* 206(4), 1196-1206.

Vasiliauskas, R., Menkis, A., Finlay, R. D., and Stenlid, J. (2007). Wood-decay fungi in fine living roots of conifer seedlings. *New Phytologist* 174(2), 441-446.

Wade, T. G., K. H. Riitters, J. D. Wickham, and K. B. Jones. 2003. Distribution and causes of global forest fragmentation. *Conservation Ecology* 7(2).

Walker, J. F., Miller, O. K., and Horton, J. L. (2005). Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. *Molecular Ecology* 14(3), 829-838.

Wehner J., Powell J. R., Muller L. A. H., Caruso T., Veresoglou S. D., Hempel S., and Rillig M. C. (2014). Determinants of root-associated fungal communities within Asteraceae in a semi-arid grassland. *Journal of Ecology* 102(2): 425–436

White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18(1), 315-322.

H. Supplementary

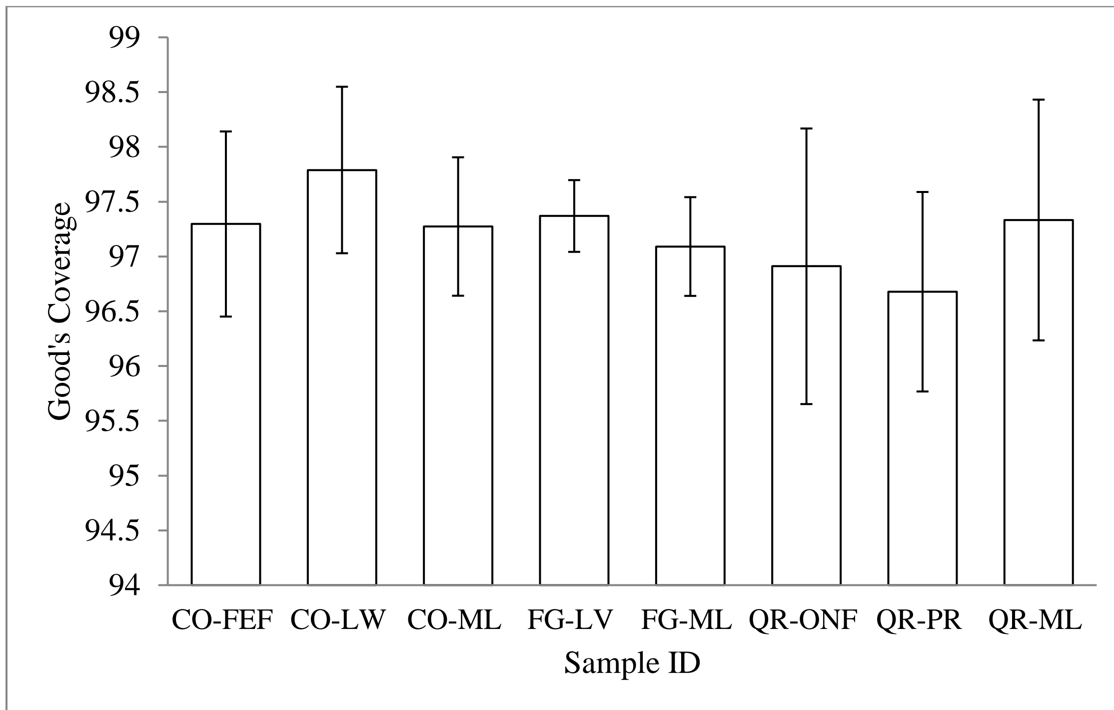


Figure S6. Mean values of Good’s Coverage estimator for each sample at each location. All values were greater than 96 and there were no significant differences between any sample mean suggesting all samples were adequately sequenced to capture the fungi present. Error bars represent ±1 standard deviation.

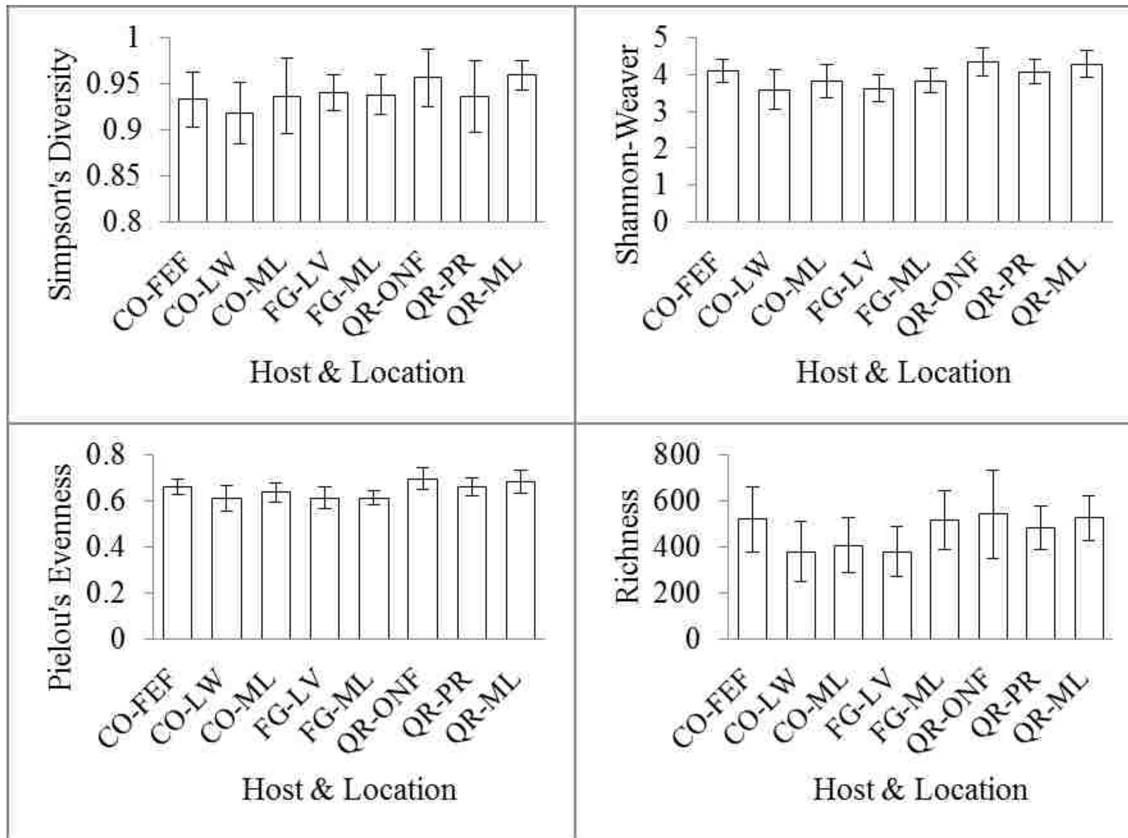


Figure S2. Mean richness, diversity, and evenness of fungal OTUs on host trees at different sampling sites in temperate deciduous forests of eastern North America. Sites include locations in the central Appalachian Mountains (Fernow Experimental Forest – FEF; Mountain Lake Biological Station – ML) and locations in the Ozark Mountains of northwestern Arkansas (Lake Wilson Park – LW; Lost Valley State Park – LV; Ozark National Forest – ONF; Pea Ridge National Military Park – PR). No significant differences exist between the samples among the different diversity indices. Error bars represent ±1 standard deviation.

Table S2. Richness of OTUs in fungal genera on host trees in temperate deciduous forests located in the central Appalachian Mountains and the Ozark Mountains. Host trees were *Carya ovata* (CO), *Fagus grandifolia* (FG), and *Quercus rubra* (QR).

Genus	CO	FG	QR	Genus	CO	FG	QR
Unidentified	2297	1312	2963	*Paraconiothyrium*	1	0	0
Cenococcum	41	26	54	*Pestalotiopsis*	1	0	0
Cryptosporiopsis	22	4	12	*Peziza*	1	0	0
Cladophialophora	17	7	9	*Phaeohelotium*	1	0	0
Oidiodendron	14	14	22	*Psathyrella*	1	0	0
Inocybe	13	9	24	*Pseudobaeospora*	1	0	0
Mortierella	10	4	12	*Pseudoclitocybe*	1	0	0
Lactarius	9	20	21	*Ramariopsis*	1	0	0
Tomentella	9	7	2	*Rhodosporidium*	1	0	0
Meliniomyces	9	4	8	*Schizophyllum*	1	0	0
Cortinarius	9	2	17	*Stachybotrys*	1	0	0
Russula	7	11	14	*Trechispora*	1	0	0
Pachyphlodes	7	2	1	*Trichoglossum*	1	0	0
Cadophora	7	1	0	*Tylopilus*	1	0	0
Pseudogymnoascus	7	0	0	*Coniochaeta*	0	3	3
Scleroderma	6	3	1	*Curreya*	0	3	0
Mycena	6	2	6	*Craterellus*	0	2	7
Phialocephala	6	1	3	*Ganoderma*	0	2	1
Metacordyceps	5	0	7	*Sporobolomyces*	0	2	0
Entoloma	4	2	0	*Cylindrium*	0	1	6
Elaphomyces	4	1	0	*Capronia*	0	1	2
Clavulinopsis	4	0	1	*Chalara*	0	1	2
Agrocybe	4	0	0	*Exobasidium*	0	1	1
Lecanicillium	3	5	7	*Phylloporus*	0	1	1
Urnula	3	4	2	*Pseudoboletus*	0	1	1
Tuber	3	4	1	*Rhodophyllus*	0	1	1
Strumella	3	3	0	*Arthroderma*	0	1	0
Hyphodontia	3	1	3	*Codinaeopsis*	0	1	0
Geoglossum	3	0	8	*Coltricia*	0	1	0
Crinipellis	3	0	3	*Discocainia*	0	1	0
Metarhizium	3	0	2	*Echinoderma*	0	1	0
Preussia	3	0	2	*Gymnomyces*	0	1	0
Flagelloscypha	3	0	1	*Hannaella*	0	1	0
Minimelanolocus	3	0	0	*Hydnocristella*	0	1	0
Monographella	3	0	0	*Naucoria*	0	1	0
Leotia	2	9	6	*Paraleptosphaeria*	0	1	0
Trichoderma	2	6	7	*Pezoloma*	0	1	0

Table S1 (Cont.)

Genus	CO	FG	QR	Genus	CO	FG	QR
Pochonia	2	3	5	*Stropharia*	0	1	0
Cryptococcus	2	1	1	*Tetrapyrgos*	0	1	0
Helicodendron	2	1	1	*Talaromyces*	0	0	6
Trichothecium	2	1	1	*Clavulina*	0	0	4
Clonostachys	2	0	5	*Cordyceps*	0	0	4
Hebeloma	2	0	2	*Hypomyces*	0	0	4
Fusarium	2	0	1	*Calcarisporium*	0	0	3
Paxillus	2	0	1	*Hypochnicium*	0	0	3
Trichosporon	2	0	1	*Penicillium*	0	0	3
Chaetomium	2	0	0	*Exophiala*	0	0	2
Crucibulum	2	0	0	*Gibberella*	0	0	2
Daedaleopsis	2	0	0	*Gymnopus*	0	0	2
Dictyochaeta	2	0	0	*Hypholoma*	0	0	2
Otidea	2	0	0	*Lactifluus*	0	0	2
Phomopsis	2	0	0	*Lauriomyces*	0	0	2
Pluteus	2	0	0	*Leucocoprinus*	0	0	2
Volutella	2	0	0	*Phacidium*	0	0	2
Sowerbyella	1	7	0	*Suillus*	0	0	2
Amanita	1	3	9	*Amphiporthe*	0	0	1
Laccaria	1	3	9	*Botryobasidium*	0	0	1
Strobilomyces	1	2	5	*Buellia*	0	0	1
Arachnopeziza	1	2	4	*Chaetosphaeria*	0	0	1
Idriella	1	2	0	*Ciliciopodium*	0	0	1
Verticillium	1	2	0	*Clitopilus*	0	0	1
Lycoperdon	1	1	3	*Coniothyrium*	0	0	1
Sebacina	1	1	3	*Cyberlindnera*	0	0	1
Tricholoma	1	1	3	*Dactylaria*	0	0	1
Ilyonectria	1	1	1	*Delicatula*	0	0	1
Rhodotorula	1	1	1	*Derxomyces*	0	0	1
Hemimycena	1	1	0	*Flavoparmelia*	0	0	1
Paraphoma	1	1	0	*Graphostroma*	0	0	1
Rectipilus	1	0	3	*Haplotrichum*	0	0	1
Genea	1	0	2	*Helicosporium*	0	0	1
Mycosphaerella	1	0	2	*Hyalopeziza*	0	0	1
Rhizophagus	1	0	2	*Hymenoscyphus*	0	0	1
Simplicillium	1	0	2	*Lecanora*	0	0	1
Gliomastix	1	0	1	*Leptodontidium*	0	0	1
Leucoagaricus	1	0	1	*Lindgomyces*	0	0	1
Mycetinis	1	0	1	*Lophiostoma*	0	0	1
Botryosphaeria	1	0	0	*Luellia*	0	0	1

Table S1 (Cont.)

Genus	CO	FG	QR	Genus	CO	FG	QR
Calvatia	1	0	0	*Myrothecium*	0	0	1
Cytospora	1	0	0	*Ochrolechia*	0	0	1
Gliophorus	1	0	0	*Paraphaeosphaeria*	0	0	1
Glomerella	1	0	0	*Passalora*	0	0	1
Gnomonia	1	0	0	*Phanerochaete*	0	0	1
Hypocrea	1	0	0	*Ramularia*	0	0	1
Hypoxylon	1	0	0	*Rhexodenticula*	0	0	1
Knufia	1	0	0	*Rhizoscyphus*	0	0	1
Lepiota	1	0	0	*Sagenomella*	0	0	1
Metapochonia	1	0	0	*Sarocladium*	0	0	1
Mycenella	1	0	0	*Umbelopsis*	0	0	1
Nectria	1	0	0	*Wickerhamomyces*	0	0	1

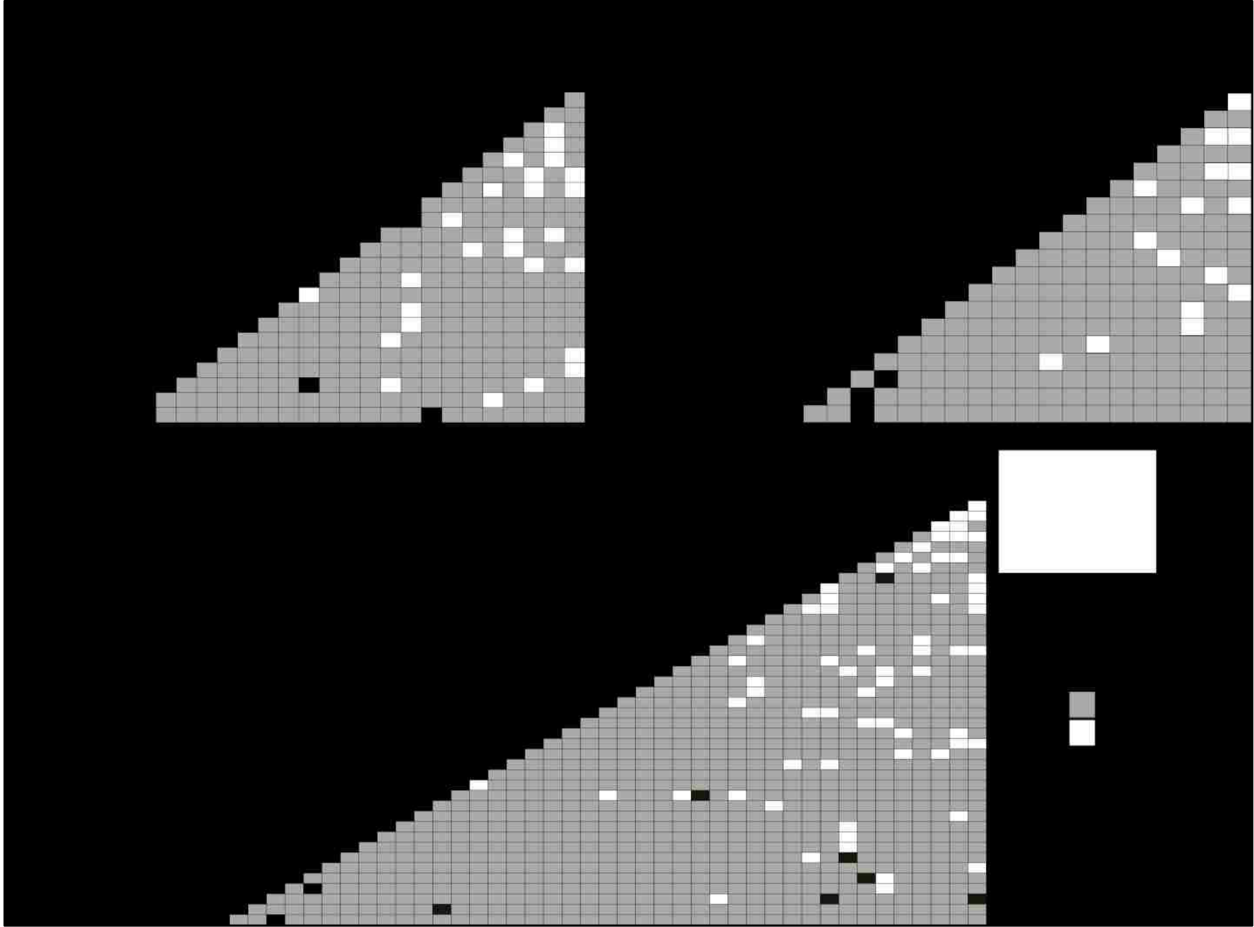


Figure S3. Co-occurrence analyses of root-associated fungi on *Carya ovata, Fagus grandifolia,* and *Quercus rubra* based on different geographic locations: a) communities occurring on roots in the central Appalachian Mountains, b) the Ozark Mountains, and c) both localities together. In the Appalachian Mountains data: there were 64 fungal species occurring, of 2,016 species pair combinations, 1391 pairs (69 %) were removed from the analysis because expected co-occurrence was < 1 and 625 pairs were analyzed resulting in 26 positive, 4 negative and 593 unclassifiable interactions. In the Ozark Mountains data: there were 53 fungal species occurring, of 1,378 species pair combinations, 966 pairs (70.1 %) were removed from the analysis because expected co-occurrence was < 1 and 412 pairs were analyzed resulting in 16 positive, 3 negative and 393 unclassifiable interactions. In the combined dataset: 77 fungal species occurred, of 2,926 species pair combinations, 2,051 pairs (70.1 %) were removed from the analysis because expected co-occurrence was < 1 and 875 pairs were analyzed resulting in 65 positive, 9 negative and 801 unclassifiable interactions.

IV. Compositional, taxonomic and functional responses of fungi associated with three species of Fagaceae to experimental acid deposition in a North American deciduous forest

Donald J. Nelsen[1*], Tatiana A. Semenova-Nelsen[2], Sandeep Sharma[3], Burt H. Bluhm[3], Steven L. Stephenson[1]

1. University of Arkansas, Department of Biological Sciences, 850 W. Dickson Street SCEN 601, Fayetteville, AR 72701, USA

2. University of Kansas, Kansas Biological Survey, Higuchi Hall 2101 Constant Ave, Lawrence, KS 66047, USA

3. University of Arkansas, Department of Plant Pathology, Plant Sciences Building room 217, 495 Campus Drive, Fayetteville, AR 72701, USA

*Corresponding author: donald.nelsen@gmail.com

A. Abstract

Atmospheric acid precipitation, generally referred to as “acid rain”, remains an important environmental problem arising from human industrial activities, crop fertilization, and cattle farming. Despite pollution abatement efforts the main cause of acid precipitation, i.e. atmospheric emissions of NOx and NH_3, continue to rise resulting in alterations to aquatic and terrestrial ecosystems worldwide. In North American deciduous forests, acid deposition is resulting in a suite of consequences such as alterations in soil variables and vegetation, including dominant canopy trees. However, very little research has been carried out so far with regards to microbial communities, including the root-associated (ectomycorrhizal and endophytic) fungi inhabiting the roots of canopy-forming trees. In this study, we quantified the responses of root-associated fungi to acid precipitation in the Fernow Experimental Forest subjected to long-term (since 1989) experimental acid deposition. We assessed fungal communities associated with roots of three forest-forming tree species belonging to the Fagaceae family - *Fagus grandifolia*,

Querqus rubra and *Q. alba*, using DNA-metabarcoding of the ITS2 rDNA region. Fungal communities were significantly different (host-specific) between the sampled tree species, and observed fungal responses varied depending on the host tree, with greater shifts found in *F. grandifolia*-associated communities. Acid deposition resulted in strong decreases in ectomycorrhizal species, likely resulting from reduced dependence of the host trees on their root-associated fungi for nitrogen acquisition, as nitrogen availability increased under the acidification treatment. Subsequently, other groups of fungi took over the vacant ecological niche, including white-rot polypores, ericoid mycorrhizae and dark septate endophytic fungi. The observed functional shifts in fungal communities imply strong alterations in ecosystem functioning with respect to nutrient cycling and decomposition.

Keywords: acidification, fungal diversity, ecology, mycology, forests

Acknowledgements

The authors would like to acknowledge the following sources of funding support: this work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1008895; research was also funded in part by the Slime Mold Project at the University of Arkansas. We appreciate the efforts of the staff at the Fernow Experimental Forest for providing lodging and background information on plot vegetation. Thank you to Dr. Benjamin A. Sikes for review and comments on the early draft of this work.

Author contributions: Donald J. Nelsen developed this research project, collected and processed samples, analyzed the data, and wrote the paper. Tatiana A. Semenova-Nelsen collected and processed samples, assisted with data analysis and writing the paper. Sandeep Sharma sequenced the samples. Burt H. Bluhm provided laboratory consumables and sequencing resources. Steven L. Stephenson helped develop research and provided laboratory resources.

B. Introduction

Human industrial activities, fuel production/consumption, and modern agricultural practices such as animal husbandry and fertilization have been dramatically increasing global nitrogen emissions in recent decades (Anderson et al., 2003; Galloway et al., 2004; Webb et al., 2005; Ye et al., 2011; Yang et al., 2017). Despite abatement policies, NOx and NH_3 emissions continue to rise and are projected to reach the level of 189 Tg N a^{-1} by the year 2050, which greatly exceeds the emission levels of the 1860s (23 Tg N a^{-1}) and 1990s (93 Tg N a^{-1}) (Galloway et al., 2004; Vet et al., 2014). Gaseous NH_3 ionizes to NH_4^+ in the atmosphere, and the latter then reacts with HCl, sulfur and nitrogen oxides to form $(NH_4)_2SO_4$, NH_3NO_3, and NH_4Cl. These and other compounds result in acid precipitation, often referred to as "acid rain" (Seinfeld and Pandis, 1998). In particular, sulfuric acid residues contribute to acid precipitation (Likens et al., 1996), resulting in excessive S and N loads in aquatic and terrestrial ecosystems worldwide (Vitousek et al., 1997; Warner et al., 2017). Due to the limited buffering capacity of soil and water, atmospheric acid deposition has been projected to cause severe disturbances in 7-17% of natural ecosystems over the next few decades (Bouwman et al., 2002).

The effect of acid rain on natural ecosystems varies geographically, based upon the level of emissions in a region from agricultural and industrial activities as well as topographical heterogeneity (Yu et al., 2016). Globally, Europe, North America, and China are experiencing increased atmospheric NH_3 due to fertilization and in the case of the United States, because of successful efforts at controlling SO_2 and NO_x emissions (Warner et al., 2017). The impact of acid deposition is also dependent on the ecosystem type, with aquatic ecosystems exhibiting rapid changes, and terrestrial ecosystem responding slower, since shifts of acidity are mediated by base cations in soils (Driscoll et al., 2001; Adams et al., 2007). In forest soils, the wet deposition of N-

based and other acidifying compounds has resulted in the leaching of exchangeable base cations by as much as 70% from the mineral soil (Heogberg et al., 2006).

Approximately 11% of global natural vegetation receives a critical load (greater than 1000 mg of N m−2 a−1), in the form of atmospheric acid deposition (Dentener et al., 2006). Increased N-accumulation has been identified as an important factor altering species composition across a number of different ecosystems (Bobbink et al., 2010). For instance, in native grasslands, acid precipitation resulted in lower species richness by as much as 23% (Stevens et al., 2004). In forested areas, acid deposition has resulted in a decrease of tree growth rate in core wood over long periods (DeWalle et al., 2006), likely related to shifts in soil nutrients in both upper (organic) and underlying (mineral) horizons (DeWalle et al., 2006; Hoegberg et al., 2006).

Despite the existing knowledge of the effects of acid deposition on water, soil, and aquatic and above-ground organisms, relatively little is known about the effect of acidification on soil microbial communities, including root-associated fungi. Few previous studies have reported decreases in total microbial biomass and effective rate of decomposition/nutrient cycling (Adams and Angradi, 1996; DeForest et al., 2004) under acid deposition treatments. Gilliam et al. (2011) report increases in fungi and declines in bacteria caused by acid deposition. However, no information has been provided so far on taxonomic diversity and shifts in functional traits of soil fungi in response to acid precipitation. Given the known effects of atmospheric acid deposition on aboveground vegetation and the well-known associations between plants and their mutualistic fungi, it seems likely to expect changes in communities of root-associated fungi in response to acidification.

The roles of root-associated fungi in forest ecosystems are difficult to overestimate, given that the vast majority of woody plants form obligate symbioses with ectomycorrhizal (ECM) and

endophytic fungi. Mycorrhizal species provide trees with nitrogen in exchange for carbon-containing compounds, and thus mediate belowground allocation of carbon (C) in forests (Clemmensen et al., 2013). In addition, root-associated fungi play key roles in host defense against pathogens (Herre et al., 2007), while also supplying essential microelements and water (Marschner and Dell, 1994; Augé, 2001; Lösch & Gansert, 2002). Saprotrophic fungi feed on the dead cells of their hosts and facilitate nutrient cycling in the ecosystem; in particular, lignin degradation is important for forest C cycling, and is being performed exclusively by fungi (de Boer et al., 2005). Fitness of the trees, however, could be largely influenced by a variety of fungal pathogens, including necrotrophic species infecting the roots (Jarosz & Davelos, 1995).

The aim of the present study was to quantify the responses of root-associated fungal communities to atmospheric acid deposition. We took advantage of the long-term (since 1989) acidification experiment being carried out on the Fernow Experimental Forest (Parsons, West Virginia), where acid precipitation has been simulated by the triannual addition of ammonium sulfate fertilizer. Ectomycorrhizal root tips from *Fagus grandifolia* Ehrh., *Quercus alba* L., and *Q. rubra* L. (Fagaceae) were sampled in an artificially acidified and adjacent, nonacidified watersheds to answer the following research questions: (1) how do root-associated fungi shift in their community composition in response to experimental acid deposition? (2) how does acidification influence richness and abundance in taxonomic and functional groups of root-associated fungi? and (3) what is the impact of host tree species on the diversity of root-associated fungi, and their responses to the acidification treatment?

C. Methods

Site selection

A study site in the Central Appalachian Mountains was selected for sampling of root tips from host tree taxa in June 2014. The Fernow Experimental Forest (FEF), located near Parsons, West Virginia (39.03° N, 79.67° W), is a mixed deciduous temperate forest with watersheds exposed to different management conditions in addition to experimental acidification (Adams et al., 2006). Watershed three (WS3) has been subject to artificial acidification annually since 1989. Three applications per year of ammonium sulfate fertilizer (spring 34kg/ha; summer 101kg/ha; fall 34kg/ha) have been applied for 25 years prior to the beginning of this study. Watersheds 4, 7, and 13 constitute adjacent unacidified watersheds that were sampled to compare root associated fungal community compositions with those of host trees in WS3.

Root tip and soil sampling

Root tips were collected opportunistically within watersheds with a distance of at least 10 m between sampled host trees. Root tips were sampled from three host tree species in the Fagaceae: *Fagus grandifolia*, *Quercus alba*, and *Q. rubra.* Each host species had 5 replicates from WS3, and 5 total from the adjacent watersheds 4, 7, and 13 (with the exception of *Q. alba,* which had only 3 trees sampled in control watersheds due to limited numbers). Root tips were collected by uncovering roots from the base of the target tree trunk at 90° intervals, unearthing lateral roots below the humic layer (0-10 cm), and following these roots to growing feeder roots and root tips. Collected root tips from the 4 quadrants of the tree were pooled as a representative sample of fungi associated with the roots of the target tree. These feeder roots and root tips were removed and stored in 15 ml screw-cap tubes containing 7 ml of 2% cetyltrimethylammonium bromide (CTAB) solution, and frozen at -20° C until processed for DNA extraction, PCR, and

sequencing. Root samples were thawed and washed with distilled water to remove soil and organic particulates. Root sections were examined with a dissecting scope and heat sterilized forceps were used to collect root tips. These collected root tips were placed in 2 ml microcentrifuge tubes and then frozen and lyophilized. Root material in these tubes was submerged in liquid nitrogen and then lysed with sterile 3 mm glass beads in a Tissue Lyser (QIAGEN™), and homogenized prior to DNA extraction.

Soil samples of approximately 100 g dry weight were collected from the top 10 centimeters of soil within 1 meter of each sampled tree. Soil samples were air dried, sifted through a 2 mm soil sieve to remove large particulates, and sent for soil chemical analyses at Brookside Laboratories (New Bremen, Ohio).

DNA extraction and sequencing

Each sample was subject to DNA extraction twice, using a total of approximately 50 mg of homogenized root tissue. DNA extractions were done using a Macherey-Nagel NucleoSpin Plant II DNA extraction kit. The extractions were done following manufacturers protocol for PL1 lysis buffer. Matrix-bound DNA went through 2 elutions in 30 µl of PE elution buffer, and replicates were pooled prior to PCR. Following the PCR protocol established in Geml et al. (2014), 40 µl reactions were carried out at these volumes: 1 µl of DNA template, 4 µl of 10X buffer, 1.5 µl of dNTP solution (2.5 mM), 1.5 µl each of forward and reverse primers (10 mM), 0.5 µl BSA (10 mg/ml), 4 µl MgCl2 (50 mM), 0.4 µl BIOTAQ polymerase (5 U/µl), and 25.6 µl of MQ water. The following primers were used: fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990). The latter was labeled with sample-specific Multiplex Identification DNA (MID) tags (Table S2). PCR reactions amplified a. 250-450 bp region of the ITS2 rDNA locus (Toju et al., 2012). As described in Geml et al. (2014), PCR reactions were carried out under the following

conditions: 95°C for 5 min (one cycle), followed by 25 cycles at 95°C for 20 sec, at 54°C for 30 sec, and then at 72°C for 1.5 min, with a final terminal elongation step at 72°C for 7 min. Each sample was amplified with three replicate PCR reactions and a negative control with no template DNA added. Verification of PCR products was done with agarose gel electrophoresis, and the PCR replicates were pooled for each sample.

Concentration and size distribution of PCR products was done for each pooled sample using an Agilent D1000 ScreenTape system (Agilent Technologies, Waldbronn, Germany). Target PCR products were separated from short fragments and PCR reagents using 0.9X Ampure® beads (Beckman-Coulter, Beverly, Massachusetts, USA). An equimolar pool of PCR products was then calculated from the volume and concentration of each sample for a normalized equimolar pool concentration of 15 nmol/µl. Oil emulsion PCR using 250 µl of the normalized equimolar pool was carried out in accordance with the protocol for the Ion PGM™ 200Xpress™ Template Kit. This generated a library which was then sequenced using an Ion 318™ Chip on an Ion Torrent Personal Genome Machine (Life Technologies, Guilford, CT, USA). Ion Torrent sequencing resulted in 2,545,132 sequence reads with a mean length of 267 bp.

Bioinformatics analyses

Preliminary clean-up of raw sequencing data was done using the Galaxy online platform (https://main.g2.bx.psu.edu/root) to sort sequence data by sample and remove adapter sequences. Sequences with poor-quality ends were trimmed using a 0.02 error probability limit using the software Geneious Pro 5.6.1 (BioMatters, New Zealand). Sequence data were then filtered using USEARCH v.8.0 (Edgar, 2010) with settings as follows: sequence lengths were truncated to 200 bp, and sequences with an expected error greater than 1 were discarded. The remaining high-quality sequences were grouped into 4,153 operational taxonomic units (OTUs) using the

UPARSE algorithm in USEARCH set to 97% sequence similarity as recommended in recent fungal metabarcoding studies (e.g. Bjorbækmo et al., 2010; Geml et al., 2010; Bellemain et al., 2013, Tedersoo et al., 2014; Semenova et al., 2016). A total of 2,970 putative chimeric sequences were excluded. Sequences were assigned to taxonomic groups using the UNITE fungal ITS sequence database based on pairwise similarity search. Global singletons were discarded, OTUs with less than 80% sequence similarity to any UNITE sequences were discarded, leaving a dataset of 1,937 OTUs for subsequent analyses (Accession Numbers MF664752 - MF666670). Ecological functional groups were assigned for OTUs based on the dataset of Tedersoo et al. (2014), and resulted in 619 OTUs with putative functional assignment for subsequent analyses.

Statistical analyses

Rarefaction curves were calculated to estimate depth of sequencing coverage. Rarefaction curves and calculations of diversity indices (Shannon's H and Simpson's D), OTU richness (S), and evenness (H/ln S) was carried out using the Vegan package "rarefy" function (Oksanen et al., 2012) in R statistical software (R Core team, 2014).

The impact of artificial acid deposition on the composition of root-associated fungal communities was estimated using PC-ORD v. 6.19 (McCune and Mefford, 2011) with presence/absence data to see if any ecological functional groups or taxa responded to the treatment. In addition, soil chemistry data were used to determine which parameters correlated with the distribution of root-associated fungal communities in treated and untreated watersheds. Non-metric multidimensional scaling (NMDS) in PC-ORD was used with the presence/absence data to assess shifts in richness of ecological functional groups or fungal taxa. OTU presence was set as 3 or more sequence reads based on similar research (Lindahl et al., 2013; Morgado et al., 2016). The main matrix consisted of presence/absence data for each OTU by sampled tree

(fungal community composition). The secondary matrix contained richness data for fungal genera, fungal orders, and OTUs identified as members of ecological functional groups based on taxonomy for each sampled tree. An additional secondary matrix containing soil chemistry parameters for each sampled tree was also generated. NMDS analyses were also conducted on a per-host basis (individual main and secondary matrices based on host) to determine ecological functional group responses to the treatment on different host species. NMDS of the main matrix was subjected to 500 iterations per run with Sorensen similarity (Bray-Curtis index) selected and a random starting number. The resulting ordination was rotated so that treatment appears on the left of the first axis and control on the right of the ordination space. Pearson's correlation coefficient $R^2 > 0.2$ can be considered indicative of correlation (McCune et al., 2002). In this study Pearson's correlation coefficient was set at $R^2 > 0.25$ to indicate strong correlations between community distribution of the main matrix and parameters of the secondary matrix. The values reported in this study ($|R| > 0.5$) represent correlations important for the characterization of fungal community response (Semenova et al., 2016) to artificial acidification treatment. The effect on OTU richness for ecological functional groups with >100 OTUs, Orders with ≥10 OTUs, and genera with ≥7 OTUs were considered significant in this study.

To test if root-associated fungi significantly shift in their community composition in response to experimental acid deposition and as a function of host-affinity, we ran permutation-based nonparametric MANOVA using both treatment and host as grouping variables (Anderson, 2001). To run this analysis, it was necessary to randomly subsample 3 of the 5 control group *Quercus alba* in order to maintain groups of equal size. PC-ORD was also used for indicator species analyses (Dufrêne and Legendre, 1997) to determine fungal OTUs characteristic for either treatment or control conditions, in addition to those OTUs characteristic for specific host taxa.

To test for changes in the abundance of OTUs between treatment and controls, we used sequence read counts as a proxy measurement and a Comprehensive Meta-Analysis software (Smith, 2014) to conduct a two-group analysis of each OTU. There is a constraint to using sequence read abundance as a proxy for fungal abundance and/or biomass due to variation in copy numbers of ITS in different species of fungi. On a per-OTU basis, however, changes in sequence read counts between samples can be considered an indication of real differences with respect to abundance and/or biomass (Amend et al., 2010). Our two-group analysis data consisted of the following data for each OTU: mean read count, standard deviation of the mean, and number of replicates for the treatment and control groups. The analysis compared the mean read count for each OTU across trees sampled in the acidified watershed, and control watersheds to calculate the size effect of the treatment within a 95% confidence interval. These analyses were conducted separately for each host tree species to examine the host-specific response of fungi to the acidification treatment. OTUs were sorted and analyzed separately based on their ecological functional group assignment, and taxonomic assignment to Order or genus.

D. Results

Experimental acid deposition alters fungal community composition

Rarefaction curves for all sampled trees reached a plateau, suggesting that root-associated fungal communities had been thoroughly sequenced for each tree (Figure S1.A.). There were no significant differences between treatment and control trees either in terms of community species richness, or estimates of community diversity and evenness (Figure S1.B.).

Fungal communities did however change significantly in response to acid deposition treatment. NMDS analysis resulted in a 3-dimentional solution with a final stress of 9.597 and final

instability < 0.00001 (Figure 1). The following correlation values were obtained: axis 1: r2 = 0.518, axis 2: r2 = 0.209, axis 3: r2 = 0.146. Significant differences between the fungal communities of acidified and control sites were revealed by both MRPP (*P* < 0.001; A = 0.026) and MANOVA (*P* < 0.001; F = 3.96). Experimental acidification treatment explained 18.56% of the variation in fungal community composition, as shown by MANOVA. Host tree species also had a significant effect on fungal assemblages (MRPP: *P* = 0.002; A = 0.018, MANOVA: *P* = 0.005; F = 1.695), however, this effect was less strong than of the acid deposition treatment; according to MANOVA, host tree species explained 8% of the variation in fungal community compositions.

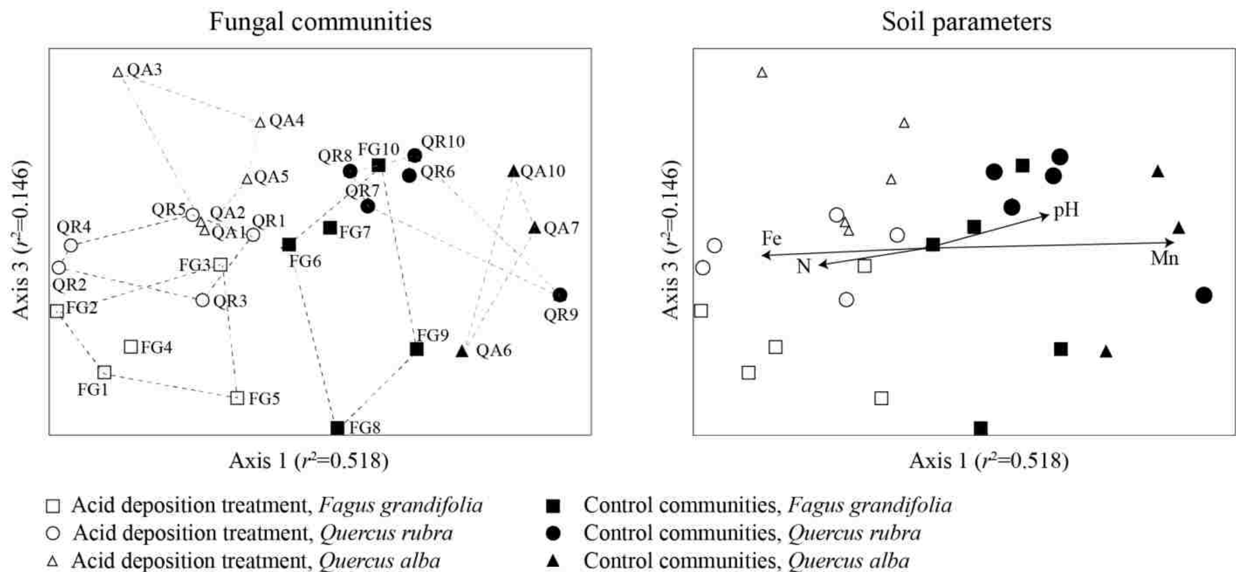


Figure 1. Nonmetric Multi-dimensional Scaling ordinations of root-associated fungal communities under experimental acidification conditions and control conditions. Fungal communities appear to be responding to treatment conditions (left) and soil parameters of N and pH (right).

The effect of acid precipitation on richness in fungal taxonomic and ecological groups is presented in Table 1. Among fungal ecological groups, we observed strong declines in richness of ectomycorrhizal fungi associated with *Fagus grandifolia* and *Quercus alba*. Among

taxonomic groups, richness declined in 11 fungal genera under acidified conditions; for example, this was the case in the ectomycorrhizal genera *Cenococcum, Laccaria, Tomentella*, *Cortinarius, Inocybe,* and *Meliniomyces* (ECM/dark-septate endophyte). Strong declines in richness were observed for *Tomentella* associated with *F. grandifolia* and *Q. alba,* and *Meliniomyces* inhabiting the roots of *F. grandifolia* and *Quercus rubra*. Among the tree species tested, root-associated communities of *Fagus grandifolia* showed the strongest response to the acid deposition treatment (i.e., richness declines were observed in the largest number of taxa) (Table 1). On the other hand, acid deposition resulted in richness increases in a few saprotrophic and ECM (*Russula* and *Lactarius*) genera on the roots of *Q. alba.* At the level of taxonomic orders, 11 taxa exhibited significant declines in richness in the acidified watershed, and 6 increased in richness in response to the treatment (Table 1). Opposite responses were observed for fungi belonging to the order Mucorales; these increased in richness on *Q. alba* roots, while declining on *F. grandifolia*.

Table 3(next page). The richness response of ecological functional groups and fungal taxa to experimental acid deposition as revealed using NMDS in PC-ORD. Correlation values are in bold for groups that correlated with ordination axis 1 at $|R| > 0.5$. Negative values indicate an increase in richness in response to artificial acidification, whereas positive values indicate a decrease in response (indicated by directional arrow).

	Fagus grandifolia			*Quercus alba*			*Quercus rubra*		
Ecological group	# of OTUs	Effect	R-value	# of OTUs	Effect	R-value	# of OTUs	Effect	R-value
Ectomycorrhizae	338	↓	**0.724**	165	↓	**0.686**	366		0.123
Saprotrophs	306		0.334	217		-0.415	386		0.000
Genus									
Arachnopeziza	<5		N/A	9	↓	**0.565**	<5		N/A
Archaeorhizomyces	22		N/A	24	↑	**-0.534**	27		N/A
Cenococcum	47	↓	**0.682**	21		N/A	50		N/A
Cladophialophora	47	↓	**0.532**	34		N/A	53		N/A
Coniochaeta	7	↑	**-0.71**	<5		N/A	9		N/A
Cortinarius	24	↓	**0.717**	10		N/A	28		N/A
Galerina	15	↑	**-0.754**	7		N/A	7		N/A
Geoglossum	8	↓	**0.624**	8		N/A	6		N/A
Inocybe	12	↓	**0.567**	14		N/A	10		N/A
Laccaria	10	↓	**0.619**	5		N/A	10		N/A
Lactarius	15		N/A	5	↑	**-0.512**	18		N/A
Meliniomyces	47	↓	**0.542**	29		N/A	66	↓	**0.618**
Mycena	7	↓	**0.759**	7		N/A	9		N/A
Oidiodendron	17	↓	**0.616**	22		N/A	18		N/A
Phialocephala	10	↑	**-0.616**	<5		N/A	10	↑	**-0.559**
Rhizoscyphus	10	↑	**-0.515**	<5		N/A	16	↑	**-0.634**
Russula	48		N/A	11	↑	**-0.614**	28		N/A
Tomentella	40	↓	**0.577**	20	↓	**0.512**	40		N/A
Umbelopsis	8		N/A	8	↑	**-0.581**	<5		N/A
Xenasmatella	11		N/A	<5		N/A	16	↑	**-0.578**
Order									
Agaricales	142	↓	**0.525**	97		N/A	166		N/A
Archaeorhizomycetales	22		N/A	24	↑	**-0.534**	27		N/A
Atheliales	22	↓	**0.565**	36		N/A	37		N/A
Boletales	28	↓	**0.716**	12		N/A	25		N/A
Cantharellales	9		0.677	<5		N/A	13		N/A
Capnodiales	18	↑	**-0.51**	5		N/A	20	↑	**-0.727**
Chaetothyriales	109		N/A	89	↑	**-0.582**	137		N/A
Eurotiales	6		-0.572	16		N/A	10		N/A
Glomerales	13		N/A	23		N/A	45	↓	**0.525**
Hysteriales	47	↓	**0.682**	21		N/A	52		N/A
Mucorales	13	↓	**0.662**	10	↑	**-0.516**	7		N/A
Pezizales	21	↓	**0.508**	22	↓	**0.888**	33		N/A
Pleosporales	21		N/A	8	↓	**0.772**	19		N/A
Polyporales	18	↑	**-0.591**	8		N/A	22		N/A
Russulales	116	↓	**0.525**	66		N/A	140		N/A
Saccharomycetales	7		N/A	8		-0.69	10		N/A
Sebacinales	21		N/A	18	↓	**0.753**	33		N/A
Sordariales	19	↑	**-0.526**	9		N/A	23		N/A
Thelephorales	104	↓	**0.59**	57	↓	**0.729**	126		N/A
Trechisporales	16		N/A	13	↑	**-0.51**	19		N/A

Changes in abundance of fungi in response to experimental acid deposition

Comprehensive meta-analysis using CMA software carried out for ecological and taxonomic groups of root-associated fungi revealed significant shifts in abundance of some groups occurred in response to experimental acid deposition (Table 2). Four ecological functional groups experienced a significant decline in abundance under treatment conditions. Among these, ECM fungi experienced a decline on *Fagus grandifolia* ($P < 0.001$) and *Quercus alba* ($P = 0.002$), saprotrophic fungi declined in abundance on *F. grandifolia* ($P < 0.001$), and declines in abundance of animal pathogens ($P = 0.029$) and yeasts ($P = 0.010$) were observed on *Q. rubra*. Among the fungal genera, we observed a decline in abundance of *Inocybe*, *Meliniomyces* and *Mortierella* among the acidified plots, while increased abundance was revealed for *Archaeorhizomyces* and *Russula* (Table 2). On the level of taxonomic orders, the Sebacinales and Thelephorales experienced significant reductions in abundance across all three host trees. Other responses were specific for a particular host tree. For example, Trechisporales increased in abundance on *Q. alba* under treatment conditions but decreased in abundance on *Q. rubra.* Members of the Atheliales also increased on *Q. alba* while decreasing on *F. grandifolia*.

Table 4. Abundance shifts in ecological functional groups and fungal taxa in response to experimental acid deposition as revealed through Comprehensive Meta-Analysis. Significant shifts (P < 0.05) in ecological and taxonomic groups are denoted in bold font, with up or down arrows indicating the direction of the effect on abundance (increasing or decreasing respectively).

	Fagus grandifolia			*Quercus alba*			*Quercus rubra*		
Ecological group	Effect	Size effect	p-value	Effect	Size effect	p-value	Effect	Size effect	p-value
Animal pathogens		-0.315	0.197		0.054	0.860	↓	**-0.632**	**0.029**
Ectomycorrhizal	↓	**-0.435**	**<0.001**	↓	**-0.218**	**0.002**		-0.042	0.416
Endomycorrhizal		0.290	0.271		-0.262	0.545		-0.043	0.844
Mycoparasites		0.125	0.667		N/A	N/A		N/A	N/A
Plant pathogens		0.136	0.373		0.095	0.677		0.054	0.698
Root endophytes		N/A	N/A		N/A	N/A		0.049	0.882
Saprotrophic	↓	**-0.248**	**<0.001**		0.068	0.301		-0.088	0.062
Yeasts		N/A	N/A		N/A	N/A	↓	**-0.627**	**0.010**
Genus									
Amanita	↓	**-0.487**	**0.035**		0.044	0.869		0.070	0.699
Archaeorhizomyces		-0.227	0.207	↑	**0.648**	**0.003**		-0.253	0.253
Cenococcum	↓	**-0.367**	**0.020**		-0.149	0.461		0.107	0.551
Cladophialophora	↓	**-0.398**	**0.005**		-0.044	0.807		-0.173	0.228
Cortinarius	↓	**-0.467**	**0.019**		-0.347	0.175		0.331	0.076
Inocybe	↓	**-0.653**	**0.001**	↓	**-0.728**	**0.001**		-0.193	0.506
Lactarius	↓	**-0.548**	**0.040**		N/A	N/A		0.462	0.059
Meliniomyces		-0.115	0.495	↓	**-0.403**	**0.030**	↓	**-0.420**	**0.002**
Mortierella		-0.393	0.138		N/A	N/A	↓	**-0.379**	**0.037**
Russula		-0.241	0.064	↑	**0.677**	**0.027**		-0.126	0.456
Tomentella	↓	**-0.573**	**0.001**		-0.325	0.140		0.049	0.766
Order									
Agaricales	↓	**-0.323**	**<0.001**		-0.170	0.051		0.056	0.411
Russulales	↓	**-0.213**	**0.012**		-0.120	0.348	↓	**-0.232**	**0.005**
Chaetothyriales		-0.148	0.095	↑	**0.242**	**0.029**		0.023	0.779
Thelephorales	↓	**-0.520**	**<0.001**	↓	**-0.448**	**<0.001**	↓	**-0.186**	**0.028**
Hypocreales	↓	**-0.315**	**0.023**		-0.239	0.158		0.025	0.858
Glomerales		0.051	0.834		0.106	0.572	↓	**-0.759**	**<0.001**
Pezizales	↓	**-0.537**	**0.001**	↓	**-0.620**	**0.001**		0.074	0.639
Atheliales	↓	**-0.559**	**0.004**	↑	**0.320**	**0.045**		0.091	0.572
Pleosporales		-0.214	0.220	↓	**-0.586**	**0.030**		-0.012	0.944
Sebacinales	↓	**-0.592**	**0.001**	↓	**-0.437**	**0.032**	↓	**-0.482**	**0.003**
Boletales	↓	**-0.345**	**0.065**		0.039	0.879		-0.034	0.865
Hysteriales	↓	**-0.367**	**0.020**		-0.149	0.461		0.010	0.955
Mortierellales		-0.193	0.372		N/A	N/A	↓	**-0.393**	**0.011**
Archaeorhizomycetales		-0.227	0.207	↑	**0.648**	**0.003**		-0.253	0.253
Tremellales		-0.312	0.173		0.185	0.579	↓	**-0.388**	**0.039**
Cantharellales	↓	**-0.777**	**0.008**		N/A	N/A		0.147	0.472
Trechisporales		-0.152	0.564	↑	**0.633**	**0.039**	↓	**-0.431**	**0.046**
Polyporales		0.174	0.511	↓	**-0.526**	**0.022**	↓	**0.609**	**0.005**

Indicator species analyses of treatment/control conditions and host taxa

Indicator species analyses resulted in 45 OTUs determined as significant ($P < 0.05$) indicators for either host species or treatment type. Across all three host tree taxa, we found 4 OTUs identified to either genus or species indicative of experimental acid deposition treatment and 33 OTUs significant as indicators of control conditions (Table S1). Three of the OTUs found to be indicative of acidification treatment were determined to be functional saprotrophs (*Cladophialophora chaetospira* [Grove] Crous & Arzanlou, *Phialocephala* sp., and *Xenasmatella* sp.) with the remaining OTU belonging to the nematophagous species *Verticillium leptobactrum* W. Gams. In the control conditions, 17 of the 33 indicator OTUs were ectomycorrhizal, dark-septate endophytes (DSE) or root-associated, 15 OTUs were saprotrophic, and a single OTU (*Circinaria* sp.), was a member of the lichenized ecological group.

Among the indicators specific for individual host tree species were ectomycorrhizal, saprotrophic, and plant pathogenic fungal taxa. Host affinity for *Fagus grandifolia* was exhibited by the ectomycorrhizal fungal species *Scleroderma citrinum* Pers. and *Russula granulata* Peck. An OTU belonging to the saprotrophic genus *Arachnopeziza* was indicated for the host *Quercus alba.* A total of 5 OTUs were indicators for the host tree species *Quercus rubra*. Among these were three ectomycorrhizal taxa (*Phylloporus rhodoxanthus* [Schwein.] Bres., *Cortinarius* sp., and *Hygrophorus* sp.), an OTU of the saprotrophic genus *Cladophialophora*, and the saprotrophic fungus *Mollisia cinerea* (Batsch) P. Karst..

E. Discussion

In this study, we assessed the responses of root-inhabiting fungi to long-term experimental acid deposition, and report strong shifts in fungal richness, abundance and community composition under artificial acidification. To our knowledge, this is the first study utilizing DNA-metabarcoding of plant root material to quantify the impacts of artificial acidification on root-associated fungal communities in temperate hardwood forests of North America. Due to contradicting trends found in previous studies with regards to changes in fungal community composition, richness, and abundance under artificial acidification, our results were difficult to compare to existing published research. For instance, studies based on phospholipid fatty acid analysis reported increases in fungal abundance (Ruess et al., 1996; Bååth et al., 1984), no change in fungal abundance (Pennanen et al., 1998), or a decline in fungal abundance (DeForest et al., 2004) under the acidification treatment in coniferous forests. On the level of individual fungal taxa, contradicting results with our study, i.e. no changes in richness/abundance under acidification treatment, were reported by Bååth et al. (1984) and DeForest et al. (2004). On the other hand, declines in richness of root-associated endophytes observed in our study were in agreement with the work of Helander et al., 1994, who reported a decline in colonization of roots by endophytic fungi in response to acid deposition. Similarly, decreases in specific ECM and AM taxa observed in our study corresponded to trends reported by Danielson and Visser (1989). We suggest that such a variety of fungal responses to acid deposition presented in previous research is heavily dependent on the ecosystems studied and the methodological approach utilized. Presumably, observed responses largely depended on initial community structure and functions in the specific ecosystem. For example, variation in the initial availability of different nutrients coupled with regular N loads could alter in different ways the temporal dynamics of

plant nutrient limitation across various ecosystems studied, thus resulting in distinct nutrient allocation shifts (and therefore, compositional shifts) in root-associated fungi (Treseder & Allen, 2002; van Diepen et al., 2010).

Our data suggested a strong impact of experimental acid deposition on soil chemistry, including alterations in soil pH and concentrations of elements, such as Ca^{++}, Mg^{++}, Fe^{++}, Mn^{++}, Cu^{++} and B^{++}, that correlated with shifts in richness and abundance of fungal taxonomic and ecological groups. Because changes in pH levels are known to affect nutrient availability (van Diepen et al., 2010), and *vice versa*, alterations in concentrations of basic and exchangeable cations shift soil pH, it was impossible to disentangle the relative effects of all contributing factors within the given experimental setup. Previous studies addressing changes in fungal community compositions associated with altered soil variables showed a key role of pH in structuring microbial assemblages (e.g., Erland & Taylor, 2002; Wubet et al., 2012; Tedersoo et al., 2014 and references therein; Goldmann et al., 2015; Zhang et al., 2016), as well as the strong impact of element concentrations on fungal richness and abundance (e.g., Oliveira et al., 2016; Xu et al., 2017). Even though we sampled fungal communities from inside of the tree roots, where the variation in pH and element concentrations are smaller compared to soils, shifts in root-associated assemblages with changing soil chemistry were expected as well (Berg & Smalla, 2009).

Perhaps the most striking result of this study was an observed strong decline in both the richness and abundance of ectomycorrhizal fungi in response to acid deposition. Similar results were obtained in other studies for specific ECM fungi; for example, a decline in commonly occurring ECM genus *Cenococcum* observed in our study corresponded to the findings of Choi et al. (2008) who found a decrease in colonization of *Larix kaempferi* (Lamb.) Carr. seedlings by *C.*

geophyllum under acidification treatment. Another study reported a decreased recovery of *Amanitaceae* sequences resulting from nitrogen deposition in coniferous forest (Weber et al., 2013), similar to what we found in our study. Given that nitrogen deposition resulted in increased tree growth at the experimental site by all measures except core wood (DeWalle et al., 2006), a decline in ectomycorrhizal fungi was unexpected, since the increases in aboveground tree biomass were assumed to correspond to increases in belowground biomass (Raich and Nadelhoffer, 1989), thus implying broader niches for root-associated fungi. Supposedly, decline in ectomycorrhizal species could indicate that trees were not strongly dependent on their mutualistic fungi for nitrogen acquisition in nitrogen-saturated soils, resulting in a functional shift in the root-associated fungal community (Wallander and Nylund, 1992; Treseder and Allen, 2002).

Nevertheless, ECM fungi declined as a functional guild, responses to acidification varied on the level of individual ECM taxa, as also observed by Cairney and Meharg (1999). For example, species of the ECM genus *Russula* increased in both richness and abundance under acid deposition, opposite to what we found for other ectomycorrhizal taxa. Because species of *Russula* tend to respond negatively to the presence of other ectomycorrhizal fungi (Koide et al., 2005; Sun et al., 2015), it remained unclear if the increase in this genus was due to the treatment itself or resulted from altered competition with other ECM fungi. Interestingly, another genus that increased in richness under the acid deposition (*Lactarius*) belonged to the *Russulaceae* family as well.

Acidification treatment resulted in increased richness and abundance in *Archaeorhizomyces*, a ubiquitous soil fungus with uncertain ecological function (Menkis et al., 2014). Because *Archaeorhizomyces* is associated with mycorrhizal fungi but does not form structures typical for

mycorrhizal fungi in the roots of the plants, it has been considered a weak parasite of mycorrhizal species (Rosling et al., 2011). Increased richness and abundance in this genus could, therefore, result from a decline in fitness of ectomycorrhizal species with subsequent proliferation of their parasites.

Even though we sampled fungal communities from the inside of the plant roots, we observed a significant portion of saprotrophic fungi among the obtained fungal OTUs. Similar results were shown in other DNA-based studies addressing fungal diversity within the plant roots, however, root-associated saprotrophs received very little attention in these studies compared to mycorrhizal species (Vandenkoornhuyse et al., 2002; Toju et al., 2013a,b). Root segments in our study were not surface-sterilized with any DNA-degrading substances, and therefore, saprotrophic species found in our dataset could potentially arise from the outside surface of the root. We, however, assume that saprotrophic fungi penetrated the plant tissues as well as mycorrhizal species, and lived inside the healthy host tissue awaiting its senescence, that would trigger the decomposition of plant material by fungi (Voříšková & Baldrian, 2013; Stone, 1987). Acidification treatment did not affect richness and abundance of saprotrophic fungi on *Quercus* hosts, however, it resulted in strong declines in saprotroph abundance on *F.grandifolia*. This trend was indirectly supported by the previous studies reporting declines in decomposition rates across experimentally acidified forests (Rechcigl and Sparks, 1985; Tamm, 1976; Wolters, 1991a,b), including beech-dominated woodlands (Wolters, 1991a). Lower decomposition rates were expected to result in accumulation of primary and secondary C-rich compounds (Wolters & Schaefer, 1994), likely causing even stronger shifts in microbial community dynamics.

F. Conclusions

The fungal responses to acidification treatment revealed in our study varied depending on the host tree species, and were stronger in beech- compared to oak- associated communities. Possibly, this difference could relate to existing variation in tree physiology, e.g., with regards to N cycling. Comparisons of soil N mineralization and nitrification rates in beech and oak rhizosphere revealed high levels of both N uptake and cycling in the *Fagus* rhizosphere, while both N mineralization and nitrification were low in soils under oak (Lovett et al., 2004). Given that fungal communities in our study were host-specific, we suggest that the variation in observed responses (i.e., the set of fungal taxonomic and functional groups that altered in response to acidification treatment) could be explained by existing variation in fungi associated with roots of different hosts. We assume that this variation could contribute to unequal fitness responses to acid deposition observed in different tree genera (Singh & Agrawal, 2006). Because fungal communities have a much faster turnover rate compared to woody plants, the state of fungal assemblages may correspond to ongoing environmental changes (including acid deposition) that will be reflected in tree traits in future years. Therefore, we highlight the importance of studies in soil microbial communities, including root-associated fungi, for predicting and managing disturbances in forest ecosystems prior to a stage when such disturbances become visible as declines in the fitness of forest trees.

G. References

Adams, M. B., & Angradi, T. R. (1996). Decomposition and nutrient dynamics of hardwood leaf litter in the Fernow whole-watershed acidification experiment. *Forest Ecology and Managemen*t, 83(1), 61-69.

Adams, M. B., DeWalle, D. R., & Hom, J. L. (eds.). (2006). *The Fernow watershed acidification study* (Vol. 11). Dordrecht, The Netherlands: Springer.

Adams, M. B., Kochenderfer, J. N., & Edwards, P. J. (2007). The Fernow watershed acidification study: ecosystem acidification, nitrogen saturation and base cation leaching. *Water, Air, & Soil Pollution: Focus*, 7(1-3), 267-273.

Aga, Y., Sasaki, H., Matsushita, N., Tange, T., & Suzuki, K. (2004). Effects of soil acidification on growth, physiological activities, and ectomycorrhizal status of *Abies firma*. *Journal of Forest Environment* (Japan).

Amend, A. S., Seifert, K. A., & Bruns, T. D. (2010). Quantifying microbial communities with 454 pyrosequencing: does read abundance count?. *Molecular Ecology*, 19(24), 5555-5565.

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26(1), 32-46.

Anderson, N., Strader, R., & Davidson, C. (2003). Airborne reduced nitrogen: ammonia emissions from agriculture and other sources. *Environment International*, 29(2-3), 277.

Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza*, 11(1), 3-42.

Bååth, E., Lundgren, B., & Söderström, B. (1984). Fungal populations in podzolic soil experimentally acidified to simulate acid rain. *Microbial Ecology*, 10(3), 197-203.

Bellemain, E., Davey, M. L., Kauserud, H., Epp, L. S., Boessenkool, S., Coissac, E., Geml, J., Edwards, M., Willerslev, E., Gussarova, G., & Taberlet, P. (2013). Fungal palaeodiversity revealed using high-throughput metabarcoding of ancient DNA from arctic permafrost. *Environmental Microbiology*, 15(4), 1176-1189.

Berg, G., & Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology*, 68(1), 1-13.

Bjorbækmo, M. F. M., Carlsen, T., Brysting, A., Vrålstad, T., Høiland, K., Ugland, K. I., Geml, J., Schumacher, T., & Kauserud, H. (2010). High diversity of root associated fungi in both alpine and arctic *Dryas octopetala*. *BMC Plant Biology*, 10(1), 244.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., & Emmett, B. (2010). Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecological Applications*, 20(1), 30-59.

Bouwman, A. F., Van Vuuren, D. P., Derwent, R. G., & Posch, M. (2002). A global analysis of acidification and eutrophication of terrestrial ecosystems. *Water, Air, & Soil Pollution*, 141(1), 349-382.

Cairney, J. W. G., & Meharg, A. A. (1999). Influences of anthropogenic pollution on mycorrhizal fungal communities. *Environmental Pollution*, 106(2), 169-182.

Choi, D. S., Jin, H. O., Chung, D. J., Sasa, K., & Koike, T. (2008). Growth and physiological activity in *Larix kaempferi* seedlings inoculated with ectomycorrhizae as affected by soil acidification. *Trees*, 22(5), 729-735.

Clemmensen, K. E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R. D., Wardle, D. A., & Lindahl, B. D. (2013). Roots and associated fungi drive long-term carbon sequestration in boreal forest. *Science*, 339(6127), 1615-1618.

Danielson, R. M., & Visser, S. (1989). Effects of forest soil acidification on ectomycorrhizal and vesicular-arbuscular mycorrhizal development. *New Phytologist*, 112(1), 41-47.

de Boer, W., Folman, L. B., Summerbell, R. C., & Boddy, L. (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews*, 29(4), 795-811.

DeForest, J. L., Zak, D. R., Pregitzer, K. S., & Burton, A. J. (2004). Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal*, 68(1), 132-138.

Dentener, F., Drevet, J., Lamarque, J. F., Bey, I., Eickhout, B., Fiore, A. M., Hauglustaine, D., Horowitz, L. W., Krol, M., Kulshrestha, U. C., & Lawrence, M. (2006). Nitrogen and sulfur deposition on regional and global scales: a multimodel evaluation. *Global Biogeochemical Cycles*, 20(4).

DeWalle, D. R., Kochenderfer, J. N., Adams, M. B., Miller, G. W., Gilliam, F. S., Wood, F., Odenwald-Clemens, S. S., & Sharpe, W. E. (2006). Vegetation and acidification. *The Fernow watershed acidification study*, 137-188. Springer Netherlands

Driscoll, C. T., Lawrence, G. B., Bulger, A. J., Butler, T. J., Cronan, C. S., Eagar, C., Lambert, K. F., Likens, G. E., Stoddard, J. L., & Weathers, K. C. (2001). Acidic Deposition in the Northeastern United States: Sources and Inputs, Ecosystem Effects, and Management Strategies: The effects of acidic deposition in the northeastern United States include the acidification of soil and water, which stresses terrestrial and aquatic biota. *AIBS Bulletin*, 51(3), 180-198.

Erland, S., & Taylor, A. F. (2002). Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. *Mycorrhizal Ecology*, 163-200. Springer Berlin Heidelberg.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., & Karl, D. M. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry*, 70(2), 153-226.

Geml, J., Laursen, G. A., Herriott, I. C., McFarland, J. M., Booth, M. G., Lennon, N., Nusbaum, C. H., & Taylor, L. D. (2010). Phylogenetic and ecological analyses of soil and sporocarp DNA

sequences reveal high diversity and strong habitat partitioning in the boreal ectomycorrhizal genus *Russula* (Russulales; Basidiomycota). *New Phytologist*, 187(2), 494-507.

Geml, J., Pastor, N., Fernandez, L., Pacheco, S., Semenova, T. A., Becerra, A. G., Wicaksono, C. Y., & Nouhra, E. R. (2014). Large-scale fungal diversity assessment in the Andean Yungas forests reveals strong community turnover among forest types along an altitudinal gradient. *Molecular Ecology*, 23(10), 2452-2472.

Gilliam, F. S., McCulley, R. L., & Nelson, J. A. (2011). Spatial variability in soil microbial communities in a nitrogen-saturated hardwood forest watershed. *Soil Science Society of America Journal*, 75(1), 280-286.

Goldmann, K., Schöning, I., Buscot, F., & Wubet, T. (2015). Forest management type influences diversity and community composition of soil fungi across temperate forest ecosystems. *Frontiers in Microbiology*, 6.

Helander, M. L., Sieber, T. N., Petrini, O., & Neuvonen, S. (1994). Endophytic fungi in Scots pine needles: spatial variation and consequences of simulated acid rain. *Canadian Journal of Botany*, 72(8), 1108-1113.

Herre, E. A., Mejía, L. C., Kyllo, D. A., Rojas, E., Maynard, Z., Butler, A., & Van Bael, S. A. (2007). Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology*, 88(3), 550-558.

Hoegberg, P., Fan, H., Quist, M., Binkley, D. A. N., & Tamm, C. O. (2006). Tree growth and soil acidification in response to 30 years of experimental nitrogen loading on boreal forest. *Global Change Biology*, 12(3), 489-499.

Ihrmark, K., Bödeker, I. T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K. E., & Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region–evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology*, 82(3), 666-677.

Jarosz, A. M., & Davelos, A. L. (1995). Effects of disease in wild plant populations and the evolution of pathogen aggressiveness. *New Phytologist*, 129(3), 371-387.

Koide, R. T., Xu, B., Sharda, J., Lekberg, Y., & Ostiguy, N. (2005). Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist*, 165(1), 305-316.

Likens, G. E., Driscoll, C. T., & Buso, D. C. (1996). Long-term effects of acid rain: response and recovery of a forest ecosystem. *Science*, 272(5259), 244.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J., & Kauserud, H. (2013). Fungal community analysis by high-throughput sequencing of amplified markers–a user's guide. *New Phytologist*, 199(1), 288-299.

Lösch, R., & Gansert, D. (2002). Organismic interactions and plant water relations. *Progress in Botany* 258-285. Springer Berlin, Heidelberg.

Lovett, G. M., Weathers, K. C., Arthur, M. A., & Schultz, J. C. (2004). Nitrogen cycling in a northern hardwood forest: do species matter?. *Biogeochemistry*, 67(3), 289-308.

Marschner, H., & Dell, B. (1994). Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil*, 159(1), 89-102.

McCune, B., Grace, J. B., & Urban, D. L. (2002). Analysis of ecological communities (Vol. 28). Gleneden Beach, OR: MjM software design.

McCune, B., Mefford, M.J. (2011). PC-ORD. Multivariate Analysis of Ecological Data. Version 6.19. MjM Software, Gleneden Beach, Oregon.

Menkis, A., Urbina, H., James, T. Y., & Rosling, A. (2014). *Archaeorhizomyces borealis* sp. nov. and a sequence-based classification of related soil fungal species. *Fungal Biology*, 118(12), 943-955.

Morgado, L. N., Semenova, T. A., Welker, J. M., Walker, M. D., Smets, E., & Geml, J. (2016). Long-term increase in snow depth leads to compositional changes in arctic ectomycorrhizal fungal communities. *Global Change Biology*, 22(9), 3080-3096.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Wagner, H., & Oksanen, M. J. (2013). Package 'vegan'. *Community ecology package, version*, 2(9).

Oliveira, E. C. A., Navarrete, A. A., Peluzio, J. M., de Oliveira Junior, W. P., Valadares, A. D. A., Tsai, S. M., & Morais, P. B. D. (2016). Structure of fungal communities in sub-irrigated agricultural soil from Cerrado floodplains. *Diversity*, 8(2), 13.

Pennanen, T., Fritze, H., Vanhala, P., Kiikkilä, O., Neuvonen, S., & Bååth, E. (1998). Structure of a microbial community in soil after prolonged addition of low levels of simulated acid rain. *Applied and Environmental Microbiology*, 64(6), 2173-2180.

R Core Team. (2014). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

Raich, J. W., & Nadelhoffer, K. J. (1989). Belowground carbon allocation in forest ecosystems: global trends. *Ecology*, 70(5), 1346-1354.

Rechcigl, J. E., & Sparks, D. L. (1985). Effect of acid rain on the soil environment: A review 1. *Communications in Soil Science & Plant Analysis*, 16(7), 653-680.

Rosling, A., Cox, F., Cruz-Martinez, K., Ihrmark, K., Grelet, G. A., Lindahl, B. D., Menkis, A., & James, T. Y. (2011). Archaeorhizomycetes: unearthing an ancient class of ubiquitous soil fungi. *Science*, *333*(6044), 876-879.

Ruess, L., Sandbach, P., Cudlín, P., Dighton, J., & Crossley, A. (1996). Acid deposition in a spruce forest soil: effects on nematodes, mycorrhizas and fungal biomass. *Pedobiologia*, 40(1), 51-66.

Seinfeld, J. H., & Pandis, S. N. (1998). From air pollution to climate change. Atmospheric Chemistry and Physics. John Wiley & Sons. Hoboken, New Jersey.

Semenova, T. A., Morgado, L. N., Welker, J. M., Walker, M. D., Smets, E., & Geml, J. (2016). Compositional and functional shifts in arctic fungal communities in response to experimentally increased snow depth. *Soil Biology and Biochemistry*, 100, 201-209.

Smith, J. (2014). Comprehensive Meta-Analysis (Version 2) [Computer software]. Englewood, NJ: Biostat. Available from http://www.comprehensive.com

Stevens, C. J., Dise, N. B., Mountford, J. O., & Gowing, D. J. (2004). Impact of nitrogen deposition on the species richness of grasslands. *Science*, 303(5665), 1876-1879.

Sun, H., Santalahti, M., Pumpanen, J., Köster, K., Berninger, F., Raffaello, T., Jumpponen, A., Asiegbu, F. O., & Heinonsaloa, J. (2015). Fungal community shifts in structure and function across a boreal forest fire chronosequence. *Applied and Environmental Microbiology*, 81(22), 7869-7880.

Tamm, C. O. (1976). Acid precipitation: biological effects in soil and on forest vegetation. *Ambio*, 235-238.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L. V., Vasco-Palacios, A. M., Thu, P. Q., Suija, A., & Smith, M. E. (2014). Global diversity and geography of soil fungi. *Science*, 346(6213), 1256688.

Toju, H., Sato, H., Yamamoto, S., Kadowaki, K., Tanabe, A. S., Yazawa, S., Nishimura, O., & Agata, K. (2013b). How are plant and fungal communities linked to each other in belowground ecosystems? A massively parallel pyrosequencing analysis of the association specificity of root-associated fungi and their host plants. *Ecology and Evolution*, 3(9), 3112-3124.

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PloS one*, 7(7), e40863.

Toju, H., Yamamoto, S., Sato, H., Tanabe, A. S., Gilbert, G. S., & Kadowaki, K. (2013a). Community composition of root-associated fungi in a *Quercus*-dominated temperate forest:"codominance" of mycorrhizal and root-endophytic fungi. *Ecology and Evolution*, 3(5), 1281-1293.

Treseder, K. K., & Allen, M. F. (2002). Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test. *New Phytologist*, 155(3), 507-515.

van Diepen, L. T., Lilleskov, E. A., Pregitzer, K. S., & Miller, R. M. (2010). Simulated nitrogen deposition causes a decline of intra-and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community structure in northern hardwood forests. *Ecosystems*, 13(5), 683-695.

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J., & Young, J. P. W. (2002). Extensive fungal diversity in plant roots. *Science*, 295(5562), 2051-2051.

Vet, R., Artz, R. S., Carou, S., Shaw, M., Ro, C. U., Aas, W., Baker, A., Bowersox, V. C., Dentener, F., Galy-Lacaux, C., & Hou, A. (2014). A global assessment of precipitation chemistry and deposition of sulfur, nitrogen, sea salt, base cations, organic acids, acidity and pH, and phosphorus. *Atmospheric Environment*, 93, 3-100.

Wallander, H., & Nylund, J. E. (1992). Effects of excess nitrogen and phosphorus starvation on the extramatrical mycelium of ectomycorrhizas of *Pinus sylvestris* L. *New Phytologist*, 120(4), 495-503.

Warner, J. X., Dickerson, R. R., Wei, Z., Strow, L. L., Wang, Y., & Liang, Q. (2017). Increased atmospheric ammonia over the world's major agricultural areas detected from space. *Geophysical Research Letters*, 44(6), 2875-2884.

Webb, J., Menzi, H., Pain, B. F., Misselbrook, T. H., Dämmgen, U., Hendriks, H., & Döhler, H. (2005). Managing ammonia emissions from livestock production in Europe. *Environmental Pollution*, 135(3), 399-406.

Weber, C. F., Vilgalys, R., & Kuske, C. R. (2013). Changes in fungal community composition in response to elevated atmospheric CO2 and nitrogen fertilization varies with soil horizon. *Frontiers in Microbiology*, 4.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), 315-322.

Wolters, V. (1991a). Effects of acid rain on leaf-litter decomposition in a beech forest on calcareous soil. *Biology and Fertility of Soils*, 11(2), 151-156.

Wolters, V. (1991b). Biological processes in two beech forest soils treated with simulated acid rain—a laboratory experiment with *Isotoma tigrina* (Insecta, Collembola). *Soil Biology and Biochemistry*, 23(4), 381-390.

Wubet, T., Christ, S., Schöning, I., Boch, S., Gawlich, M., Schnabel, B., Fischer, M., & Buscot, F. (2012). Differences in soil fungal communities between European beech (*Fagus sylvatica* L.) dominated forests are related to soil and understory vegetation. *PloS One*, 7(10), e47500.

Xu, X., Chen, C., Zhang, Z., Sun, Z., Chen, Y., Jiang, J., & Shen, Z. (2017). The influence of environmental factors on communities of arbuscular mycorrhizal fungi associated with *Chenopodium ambrosioides* revealed by MiSeq sequencing investigation. *Scientific Reports*, 7, 45134.

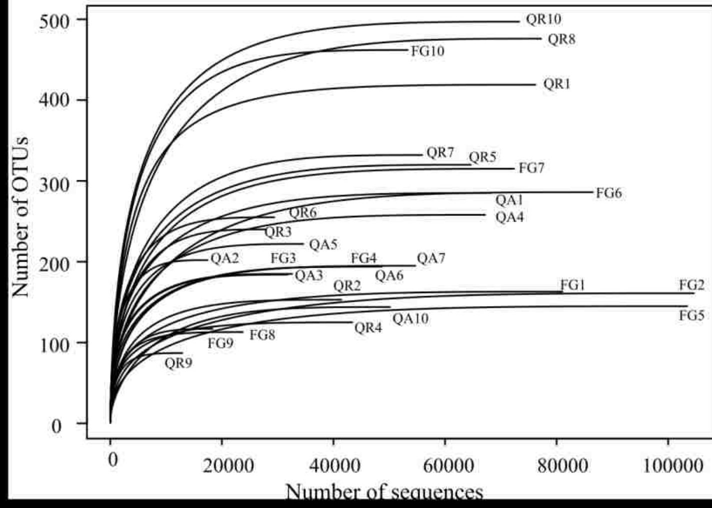
Yang, W., Jiao, Y., & Jia, Y. (2017). The status of ammonia (NH3) emissions and achievements of emissions reduction technology in farmland ecosystems. *Journal of Resources and Ecology*, 8(3), 296-303.

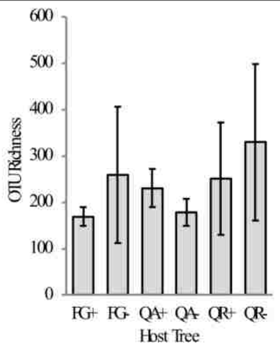
Ye, S. C., Lin, Z. C., Dai, Q. G., Jia, Y. S., Gu, H. Y., Chen, J. D., Xu, L. S., Wu, F. G., Zhang, H. C., Huo, Z. Y., & Xu, K. (2011). Effects of nitrogen application rate on ammonia volatilization and nitrogen utilization in rice growing season. *Chinese Journal of Rice Science*, 25(1), 71-78.

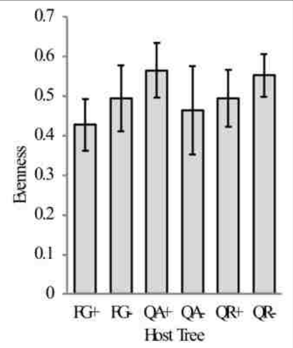
Yu, H., He, N., Wang, Q., Zhu, J., Xu, L., Zhu, Z., & Yu, G. (2016). Wet acid deposition in Chinese natural and agricultural ecosystems: Evidence from national-scale monitoring. *Journal of Geophysical Research: Atmospheres*, 121(18).

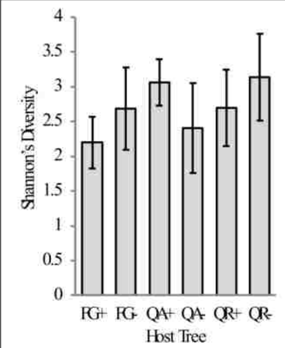
Zhang, T., Wang, N. F., Liu, H. Y., Zhang, Y. Q., & Yu, L. Y. (2016). Soil pH is a key determinant of soil fungal community composition in the Ny-Ålesund Region, Svalbard (High Arctic). *Frontiers in Microbiology*, 7.

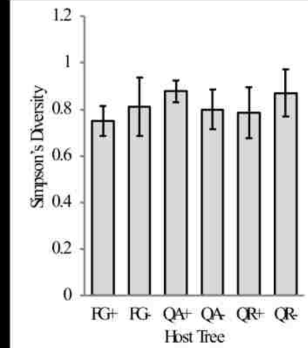
H. Supplemental Materials











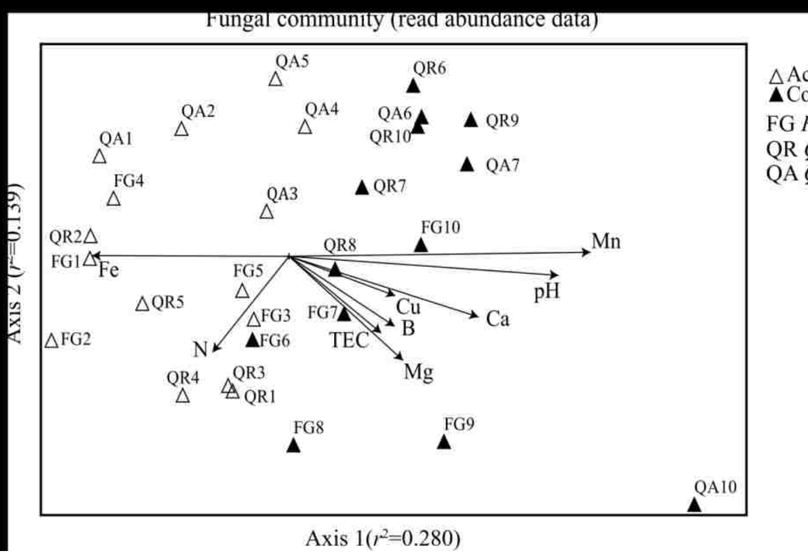


Figure S1 (previous page). Statistical analyses of sequencing effort, diversity metrics, and fungal biomass. Rarefaction curves of each sampled community (A) show plateau for all samples suggesting all community members represented in the sample were sequenced. Diversity statistics (B) show variation in richness, evenness, and diversity between treatment and control conditions on three Fagaceae species, but no significant differences. NMDS ordination of fungal community abundance data (C) is similar to presence/absence with respect to community response to artificial acidification. Abundance appears to be sensitive to more soil chemistry parameters than presence/absence.

Table S1. Indicator species analyses of host and treatment variables in PC-ORD software. Significant indicator species ($P < 0.05$) identified to the genus and species levels are included in the table. Ecological function was determined by taxonomic classification. Eight OTUs were indicators for the three host species, four OTUs were indicators for the acidification treatment, and 33 OTUs were indicators for control conditions.

OTU	**Host/Treatment**	**Fungal Taxon**	**Ecological group**	**p-value**
41	*F. grandifolia*	*Scleroderma citrinum*	Ectomycorrhizal	0.047
1825	*F. grandifolia*	*Russula granulata*	Ectomycorrhizal	0.005
61	*Q. alba*	*Arachnopeziza* sp.	Saprotrophic	0.006
196	*Q. rubra*	*Phylloporus rhodoxanthus*	Ectomycorrhizal	0.038
500	*Q. rubra*	*Cortinarius* sp.	Ectomycorrhizal	0.004
702	*Q. rubra*	*Cladophialophora* sp.	Saprotrophic	0.02
777	*Q. rubra*	*Mollisia cinerea*	Plant pathogen	0.02
991	*Q. rubra*	*Hygrophorus* sp.	Ectomycorrhizal	0.022
796	Acidification	*Cladophialophora chaetospira*	Saprotrophic	0.019
31	Acidification	*Phialocephala* sp.	Saprotrophic	0.035
312	Acidification	*Verticillium leptobactrum*	Nematophagous	0.039
129	Acidification	*Xenasmatella* sp.	Saprotrophic	0.032
13	Control	*Amanita* sp.	Ectomycorrhizal	0.043
171	Control	*Amanita* sp.	Ectomycorrhizal	0.049
143	Control	*Archaeorhizomyces* sp.	Root-associated	0.015
924	Control	*Cenococcum geophilum*	Ectomycorrhizal	0.01
69	Control	*Cenococcum geophilum*	Ectomycorrhizal	0.046
1171	Control	*Chaetosphaeria chloroconia*	Saprotrophic	0.045
419	Control	*Chaetosphaeria* sp.	Saprotrophic	0.013
14	Control	*Chaetosphaeria* sp.	Saprotrophic	0.013
499	Control	*Circinaria* sp.	Lichenized	0.044
809	Control	*Cladophialophora* sp.	Saprotrophic	0.004
244	Control	*Cladophialophora* sp.	Saprotrophic	0.013
178	Control	*Cryptosporiopsis* sp.	Saprotrophic	0.012
336	Control	*Cyphellophora* sp.	Saprotrophic	0.014
218	Control	*Dendrosporium* sp.	Saprotrophic	0.019
156	Control	*Hymenoscyphus* sp.	Saprotrophic	0.048
375	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.001
154	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.003
1518	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.013
2918	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.013
3005	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.044
1987	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.047
1549	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.047
454	Control	*Meliniomyces* sp.	Ectomycorrhizal/DSE	0.048
1063	Control	*Mortierella* sp.	Saprotrophic	0.016
1318	Control	*Mortierella* sp.	Saprotrophic	0.047
174	Control	*Mycena* sp.	Saprotrophic	0.004
115	Control	*Oidiodendron* sp.	Saprotrophic	0.003
688	Control	*Oidiodendron* sp.	Saprotrophic	0.037
380	Control	*Pezicula radicicola*	DSE	0.043
1169	Control	*Pseudaegerita* sp.	Saprotrophic	0.041
7	Control	*Russula vesca*	Ectomycorrhizal	0.043
49	Control	*Tomentella* sp.	Ectomycorrhizal	0.002
741	Control	*Tuber* sp.	Ectomycorrhizal	0.046

Table S2. IonXpress Multiplex Identification DNA sequence tags assigned to sampled roots of Fagus grandifolia (FG), Quercus alba (QA), and Q. rubra (QR).

Sample ID	IonXpress MID#	MID sequence
FG1	1	CTAAGGTAAC
FG2	2	TAAGGAGAAC
FG3	3	AAGAGGATTC
FG4	4	TACCAAGATC
FG5	5	CAGAAGGAAC
FG6	6	CTGCAAGTTC
FG7	7	TTCGTGATTC
FG8	8	TTCCGATAAC
FG9	68	TCAAGAAGTTC
FG10	69	TTCAATTGGC
QA1	32	TCTTACACAC
QA2	33	TTCTCATTGAAC
QA3	34	TCGCATCGTTC
QA4	35	TAAGCCATTGTC
QA5	36	AAGGAATCGTC
QA6	37	CTTGAGAATGTC
QA7	38	TGGAGGACGGAC
QA10	84	CTTCCATAAC
QR1	58	TCCTAGAACAC
QR2	59	TCCTTGATGTTC
QR3	60	TCTAGCTCTTC
QR4	61	TCACTCGGATC
QR5	96	TTAAGCGGTC
QR6	63	CCTTAGAGTTC
QR7	64	CTGAGTTCCGAC
QR8	65	TCCTGGCACATC
QR9	66	CCGCAATCATC
QR10	67	TTCCTACCAGTC

V. Discussion and Conclusions

This dissertation presents data on the diversity of root-associated fungi occurring on several selected host tree species in temperate deciduous broadleaf forests of western Europe and eastern North America, including the responses of root-associated fungal communities to experimental wet acid deposition (acid rain). The data contained herein represent a deeper look into the incredible diversity of fungi associated with host tree roots in this forest type than previously available to researchers. In total, nearly 15.2 million sequence reads were generated from samples collected in Europe and North America (ca. 9.4 million reads from Germany and The Netherlands, ca. 3.3 million reads from trees in eastern North American forests, and ca. 2.5 million reads at the Fernow Experimental Forest acidification study). These will be deposited in publicly available databases (e.g. DRYAD) once the manuscripts in which they are reported have been accepted for publication. These sequences represent a significant contribution to our understanding of the diversity of root-associated fungi and may serve as an informational baseline to future DNA-based monitoring efforts with respect to fungal communities in temperate deciduous forests and their response to changing abiotic factors such as those associated with climate change and environmental pollution. The raw sequencing data were clustered into fungal operational taxonomic units (OTUs) and given putative taxonomic identifications during the course of this research, and those OTU sequences were deposited in Genbank (accession numbers: KY413820-KY415572, European OTUs; MG159816 - MG162584, North American OTUs; MF664752 - MF666670, Fernow Experimental Forest acidification study OTUs). These deposited OTUs were obtained by clustering the sequence reads that passed quality filtering so that only high-quality sequence reads remained, these high-quality reads were truncated to equal lengths, and then clustered based on 97% sequence

similarity across the length of homologous nucleotides. As our understanding of the variation of the ITS2 region within and among different fungal taxonomic groups increases, it is likely that there will be changes in the methodology for clustering and identification of OTUs to increase the capability of this technology to take environmental samples and derive meaningful and accurate fungal identities from extracted DNA. Across the three data sets, an average of 16.6% of fungal OTUs were assigned species-level identification, while the majority (54.3%) of OTUs were identified to the level of order. Future taxonomic research efforts are essential to increase the number of taxonomic identities assigned to unidentified/unresolved fungal OTUs and will likely also provide information on ecological function of OTUs associated with the roots of host trees. In the future, this work could be improved by analyses using more complete data on fungal taxonomy and ecology, and the interpretations of the data in this dissertation may change in response to our increased knowledge. In this final chapter, I summarize what is known about the diversity of root-associated fungal communities in European and North American temperate deciduous broadleaf forests, their ecological roles on the roots of host trees, and the response of these communities to acid rain based on the current state of the science of fungal taxonomy, ecology, and available technology.

A. NGS and fungal diversity

Next generation sequencing technology makes possible the rapid and cost-effective identification of fungal DNA found in environmental samples and samples with multiple fungal community members present, such as the pooled root tips used to represent fungal root-associated communities in the body of work presented in this dissertation. Across the three studies presented herein, a conservative threshold of 80% sequence identity to curated sequences in the UNITE database was used to assign an OTU to the kingdom Fungi. This approach may exclude

deeply-divergent fungal lineages yet to be undescribed in the literature, but it reduces the likelihood of misidentification of OTUs belonging to nonfungal lineages being included in the analyses. Because the temperate deciduous broadleaf forest biomes of Europe and North America are among the more thoroughly studied in terms of fungal diversity (Tedersoo et al., 2014), it is expected that a sequence with 80% or greater identity to known fungi should be considered as within the fungal lineage. While some fungal sequences may have been excluded as a consequence, such a conservative approach was also expected to exclude the majority of remaining erroneous reads in the data sets. In the study of European root-associated fungi, there were ca. 1760 OTUs identified using this approach, 2769 OTUs in the North American study, and 1937 OTUs in the study of the effect of artificial acidification on root-associated communities. The mean richness of fungal OTUs per sampled host species/site was within the range of 110-257 in European forests, 381-542 OTUs in North American forests, and 169-329 in the Fernow acidification study. Within each study, there was no significant difference between mean richness on the hosts sampled, but there does appear to be a difference in richness between Europe and North American root-associated fungal communities on the selected genera (*Fagus* and *Quercus*), and a reduction of richness values on the Fernow Experimenal Forest when compared to other North American forests. This could be a function of updates to the curated database that occur at infrequent intervals as fungal taxa are more thoroughly understood. Across all three studies in this body of work, the proportion of OTUs in the Ascomycota (37-49%) was greater than for the Basidiomycota (23-30%) on all host trees. Rare fungal phyla on host tree roots included the Glomeromycota (0.03-4%), Chytridiomycota (0.1-0.2%), Zygomycota (1-2%), and Rozellomycota (0.5-0.6%). All four rare phyla occurred in the acidification study, the Glomeromycota and Chitridiomycota occurred in the European study, and only the

Glomeromycota appeared as a rare occurrence in the study of root-associated fungal diversity in North America. Within the Ascomycota across all studies, the greatest species richness occurred in the orders Helotiales, Hypocreales, Hysteriales, and Chaetothyriales. The Helotiales represented a much greater proportion of the root-associated community in the artificial acidification study (11-13%) than in either the European or North American studies (3-5%). With respect to the Basidiomycota, the greatest number of species occurred in the orders Agaricales, Russulales, Thelephorales, Boletales, Tremellales, and Sebacinales. The Thelephorales and Sebacinales made up a greater proportion of the root-associated communities on hosts in North America (4-7% and 1-3%, respectively) versus those in Europe (0.9-2% and 0.1-0.5%, respectively).

Across all data sets, the number of unidentified fungal OTUs was approximately 25 percent. This inability to resolve the identities of OTUs, in addition to the lack of knowledge with respect to individual fungal species ecological lifestyles *in situ*, makes the determination of ecological functional group assignment complex and at times uncertain. In the studies presented herein, 15-25% of OTUs were assigned an ecological function (ranging from ca. 375 – 650 OTUs) based on taxonomic assignment of taxa where that information is available in the current literature. This number was considered sufficient for analyses of ecological guilds in root-associated communities. Saprotrophic and ectomycorrhizal guilds were the most dominant in root-associated communities, followed by plant pathogenic fungi.

B. Fungal host-specificity

Current opinion on the importance of host-specificity for root-associated fungi ranges from unimportant/negligible (Dean et al., 2015; Roy-Bolduc et al., 2016) to highly important (Massicotte et al., 1999; Wehner et al., 2014) with respect to fungal community composition, and

this may vary depending on the host tree species under study. In the context of this work, host species was considered important, since it explained approximately 9-15% of the variation in fungal communities on host root systems. Among European trees, the number of OTUs specific to any one tree species ranged from 122-493. That range was dramatically larger for host tree roots in North American deciduous forests (414-1491). This trend was markedly different in terms of overall proportion of specific occurrence among root-associated communities on members of the Fagaceae as well (6-8% of fungal community specific to the Fagaceae in Europe, 10-40% in North America). This trend may not be significant, as these two studies consisted of different host taxa in Fagaceae, as well as different species in the Betulaceae and Juglandaceae between the two continents. However, it is evident that diversity and specificity on the Fagaceae in North America appears greater than the European root-associated communities on closely related species. Generalists in Europe and North America were of comparable proportions (14.9% and 11.6%, respectively). Specificity may play a role in the increased OTU richness observed in the North American data, among other factors such as different disturbance regimes and more comprehensive taxonomic sequence databases.

Analysis of host-specific and generalist root-associated fungal community members with assigned ecological functional groups revealed an interesting observation. Among host-specific communities, saprotrophic taxa appear to dominate the communities and suggest that host niche specialization is a successful strategy for fungi making a living via decomposition. Conversely, among the community members occurring on all host tree taxa, the dominant lifestyle was ectomycorrhizal. In the case of symbionts, it appears that a strategy of associating with whatever is available is preferable to specialization.

C. Regional variation in fungal communities

There appear to be distinct geographic and latitudinal differences in fungal communities from soils sampled globally (Tedersoo et al., 2014). The results of the work reported herein are consistent with that finding and describe the variation in root-associated fungal communities from geographically disparate host tree populations not from soil, but directly from root tissue. In North America, the extent of community composition explained by location was nearly 30%, while location in the European study explained more than 21% of the community composition. Currently, there are multiple explanations of the spatial variation in community structure of microorganisms within and between continents and across oceans (Green and Bohannan, 2006). On a landscape scale, communities of ectomycorrhizal fungi vary based on differences in dispersal strategy (Nara, 2009). On a larger continental or global scaling, other factors, such as spore dispersal limitations, adaptive evolution, and biogeographical histories must be considered, and it is reasonable to expect that spatially disparate places will develop unique fungal assemblages over time (Peay et al., 2010). This is likely the case for root-associated fungal communities on conspecific hosts in the different forests considered in the present work; to the extent that they are geographically isolated, exposed to different geological and biogeographical histories, and adapting to local conditions over time it makes sense that these communities will–at least to some extent–be compositionally distinct from one another across a spatial scale. Historical contingency–the order and timing of individual species arrival in a community–can cause priority effects (Fukami, 2015) that are also a likely driver of differences in fungal community composition at some spatial scale.

D. Fungal responses to acid deposition

The results of our analyses showed strong responses in fungal communities to experimental wet acid deposition via tri-annual application of ammonium sulfate fertilizer. Community composition shifted in response to the treatment, and 18.56% of the variation in fungal communities was explained by the acid deposition treatment. As a general trend, richness and read abundance of fungi declined in response to the treatment, although that was not true for all fungal taxa (see next section). Soil chemistry analysis and ordination showed that pH was an important factor for explaining community composition, as well as soil nutrients, including nitrogen, iron, and manganese. Forest trees typically show an increase in growth during the first several years of acid deposition due to increased nitrogen availability, but over long periods of chronic acid deposition some trees will slow their growth rate and even experience a reduction in annual growth of core wood in response to nutrient leeching (a consequence of acid deposition and lack of buffering capacity in some soils) (DeWalle et al., 2006). It takes years to see the effects of acid deposition on forest trees, but their root-associated fungi appear to shift strongly in response to acid deposition and perhaps could represent a method of monitoring forest health for management purposes.

E. Responses of fungal taxonomic and ecological groups to acid deposition

To determine the responses of fungal taxonomic groups and ecological functional groups to experimental acid deposition, analyses of both richness and abundance were carried out. A conservative approach was taken to minimize the chances of small sample sizes causing strong correlation coefficients. OTU richness values of taxonomic groups and ecological functional groups were set to ≥ 7 OTUs minimum (at the level of genera), ≥ 10 OTUs in fungal orders, and

≥100 OTUs for ecological functional groups. Strong Pearson's correlations were considered as (|R|>0.5) to provide a conservative estimate of groups responding to treatment.

With respect to richness responses to acid rain, ectomycorrhizal fungi and many fungal taxa experienced strong declines. Most notably for ectomycorrhizal genera, two (*Russula* and *Lactarius*) increased in richness in response to treatment. The Russulaceae have been shown to respond negatively to the presence of a community of other ectomycorrhizal fungi (Koide et al., 2005) and may be taking advantage of the reduction in other ECM fungi in the community (Sun et al., 2015). This suggests that at least the functional role of mycorrhizae on root tips is being fulfilled by some fungi under conditions of acid deposition, and this could lead to a method for monitoring forest health by comparing community richness of the Russulaceae in acidified forests with other ECM communities in non-acidified forests.

Comprehensive meta-analyses were done for sequence read counts on a per-OTU basis to assess responses in abundance to acid deposition. Copies of the ITS region in fungal genomes vary in number depending on the taxa involved, and this may not offer the best way to examine abundance shifts when comparing individual taxa (Amend et al., 2010). However, in the case of examining the same OTU across different treatments this method can be considered a useful proxy for biomass or abundance. In this way we could determine the shifts in fungal abundance of ecological functional groups and fungal taxa responding to acid rain treatment. In this analysis, ECM and saprotrophic fungi both experienced a decline in abundance/biomass. Ectomycorrhizae declined as a group, but species of *Russula* increased in abundance, perhaps taking advantage of increased availability of uncolonized root tips as other ECM taxa decreased.

Reduction in richness and abundance of ECM and saprotrophs, the two largest ecological groups of root-associated fungi, in response to acid deposition could lead to shifts in nutrient cycling as

the taxa involved in this ecosystem function may not persist under harsh conditions of acidification. There are conflicting reports in the current literature as to the responses of fungi to acidification, including increasing (Ruess et al., 1996), decreasing (DeForest et al., 2004), or no change (Pennanen et al., 1998). However, researchers have shown an increase in acetate incorporation into fungal ergosterol under acidified conditions (Rousk et al., 2009), which could indicate that those fungal taxa which remain or increase in richness and abundance under acid rain conditions will fulfill the roles of the fungi and bacteria which are extirpated. We suggest that in addition to this, local community structure (fungal species present) and functional guilds of taxa (what they do in the environment), site characteristics and soil chemistry parameters such as buffering capacity and N availability, are likely to dictate such a wide variety of measured responses to acid deposition in fungal communities. Ergosterol-based analyses such as those mentioned above would benefit from the addition of a DNA-based approach to determine taxa involved in such a variety of responses to acid deposition.

F. Fungal-fungal interactions

Analysis of fungal co-occurrence patterns was done in both European and North American data sets to identify any potential fungal-fungal interactions. This analysis identified patterns in co-occurrence that happened more often than (positive) or less often than (negative) expected by chance. Across both sets of data there appeared to be both positive and negative co-occurrence patterns between ectomycorrhizal fungi, saprotrophic fungi, and combinations of the two lifestyles. These patterns could indicate fungal partnerships between ECM and saprotrophs, and potentially within functional guilds.

Given the fact that *Cenococcum geophilum* (a likely species-complex still listed under the umbrella of a single name [Douhan et al., 2005]) was shown to decrease in both richness and

abundance in response to acid deposition, it was somewhat surprising that they are positively associated in both European and North American datasets with members of the ectomycorrhizal Russulaceae (both species of *Russula* and *Lactarius*) which show an opposite response to acid deposition. It appears that in a healthy forest rhizosphere environment, these two groups of ectomycorrhizal fungi form some sort of partnership rather than being exclusive. Based on our data alone, it is rather difficult to speculate on the nature of the relationships, both positive and negative, evidenced in the observed co-occurrence patterns. However, these data do highlight potential areas of interest for future work to determine precisely what interactions are happening between fungal taxa in the root-tip environment.

G. Future research

The research described herein provides significant improvements to our knowledge of the diversity and distribution of fungi associated with tree roots in temperate deciduous broadleaf forests of Europe and North America. The DNA-based approach to assessing community structure provides a dramatic increase in taxonomic data. However, there exists a very real need for comprehensive taxonomic work with culturable fungi to characterize and deposit sequence data for the > 25% of unknown members of kingdom Fungi, and even more unresolved species in known genera or other higher taxa that are detectable via environmental metabarcoding studies. The DNA-based approach can tell us what fungal DNA is present in a sample but not what taxa are functionally part of the root-associated community. In addition, the functional assignment of individual taxa based on taxonomy and the current understanding of ecology in closely related taxa is not direct evidence of an ecological lifestyle. An approach that uses transcriptomics of fungal isolates in experimental conditions, coupled with metabarcoding of the fungal community, would be useful to describe the functional roles of individual fungi in the

community in a direct way. Similarly, the interactions between fungi characterized as positive and negative co-occurrences in this study would benefit from future research assessing one-on-one interactions in vitro and more complex interactions in a controlled experimental multifactorial setting to uncover the mechanisms at play.

This research is apparently the first study using a DNA-based approach to identify and observe responses (shifts in richness and abundance) to acidification in individual fungal taxa and ecological functional groups. As such, it could serve as a baseline dataset to inform future researchers the extent of taxonomic and functional shifts in communities at the Fernow Experimental Forest in this snapshot of time. Similar studies in other ecosystem types and forest biomes could and perhaps should be used to capture the extent of fungal responses to acidification across multiple ecosystems. There is a potential that root-associated fungi such as those in this study are buffered by their presence on the surface–or inside–of tree roots, and thus respond differently to acidification than their soil-borne counterparts. A future study examining the differences in both community structure and function, and response to acid deposition, between soil fungi and root-associated fungi would be one way to determine the differential response of communities in these two habitat types.

In the future, the recent technological advancement allowing single-molecule sequencing without PCR amplification will presumably become an affordable technique for a more accurate assessment of fungal community richness and abundance of root-associated fungi. The fact that fungi and fungal communities have a greater rate of turnover in response to abiotic factors than trees and other forest species, may make them a good environmental indicator of forest health for locations experiencing increased acid deposition or any of numerous other ecosystem disturbances including climate change, forestry activities, or drought.

References

Amend, A. S., Seifert, K. A., and Bruns, T. D. (2010). Quantifying microbial communities with 454 pyrosequencing: does read abundance count?. *Molecular Ecology* 19(24), 5555-5565.

Dean, S. L., Warnock, D. D., Litvak, M. E., Porras-Alfaro, A., and Sinsabaugh, R. (2015). Root-associated fungal community response to drought-associated changes in vegetation community. *Mycologia* 107(6), 1089-1104.

DeForest, J. L., Zak, D. R., Pregitzer, K. S., and Burton, A. J. (2004). Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal* 68(1), 132-138.

DeWalle, D. R., Kochenderfer, J. N., Adams, M. B., Miller, G. W., Gilliam, F. S., Wood, F., Odenwald-Clemens, S. S., and Sharpe, W. E. (2006). Vegetation and acidification. The Fernow watershed acidification study, 137-188. Springer Netherlands

Douhan, G. W., and Rizzo, D. M. (2005). Phylogenetic divergence in a local population of the ectomycorrhizal fungus *Cenococcum geophilum*. *New Phytologist* 166(1), 263-271.

Fukami, T. (2015). Historical contingency in community assembly: integrating niches, species pools, and priority effects. *Annual Review of Ecology, Evolution, and Systematics* 46.

Green, J., and Bohannan, B. J. (2006). Spatial scaling of microbial biodiversity. *Trends in Ecology and Evolution*, 21(9) 501-507.

Massicotte, H. B., Molina, R., Tackaberry, L. E., Smith, J. E., and Amaranthus, M. P. (1999). Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Canadian Journal of Botany* 77(8), 1053-1076.

Nara, K. (2009). Spores of ectomycorrhizal fungi: ecological strategies for germination and dormancy. *New Phytologist* 181(2), 245-248.

Peay, K. G., Bidartondo, M. I., and Elizabeth Arnold, A. (2010). Not every fungus is everywhere: scaling to the biogeography of fungal–plant interactions across roots, shoots and ecosystems. *New Phytologist* 185(4), 878-882.

Pennanen, T., Fritze, H., Vanhala, P., Kiikkilä, O., Neuvonen, S., and Bååth, E. (1998). Structure of a microbial community in soil after prolonged addition of low levels of simulated acid rain. *Applied and Environmental Microbiology* 64(6), 2173-2180.

Rousk, J., Brookes, P. C., and Bååth, E. (2009). Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Applied and Environmental Microbiology* 75(6), 1589-1596.

Roy-Bolduc, A., Laliberté, E., and Hijri, M. (2016). High richness of ectomycorrhizal fungi and low host specificity in a coastal sand dune ecosystem revealed by network analysis. *Ecology and Evolution* 6(1), 349-362.

Ruess, L., Sandbach, P., Cudlín, P., Dighton, J., and Crossley, A. (1996). Acid deposition in a spruce forest soil: effects on nematodes, mycorrhizas and fungal biomass. *Pedobiologia* 40(1), 51-66.

Sun, H., Santalahti, M., Pumpanen, J., Köster, K., Berninger, F., Raffaello, T., Jumpponen, A., Asiegbu, F. O., and Heinonsalo, J. (2015). Fungal community shifts in structure and function across a boreal forest fire chronosequence. *Applied and Environmental Microbiology* 81(22), 7869-7880.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., Ruiz, L. V., Vasco-Palacios, A. M., Thu, P. Q., Suija, A. and Smith, M. E. (2014). Global diversity and geography of soil fungi. *Science* 346(6213), 1256688.

Wehner J., Powell J. R., Muller L. A. H., Caruso T., Veresoglou S. D., Hempel S., and Rillig M. C. (2014) Determinants of root-associated fungal communities within Asteraceae in a semi-arid grassland. *Journal of Ecology* 102(2): 425–436