MICROBIAL COMMUNITIES AS INDICATORS OF ECOSYSTEM FUNCTIONS AT TWO SITES IN GREAT SMOKY MOUNTAINS NATIONAL PARK

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By: Elin Richard Swank
Thesis Director: Dr. Sean O'Connell
Thesis Committee: Dr. Anjana Sharma, Dr. Beverly Collins, Dr. Greg Adkison

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ABSTRACT

MICROBIAL COMMUNITIES AS INDICATORS OF ECOSYSTEM FUNCTIONS AT TWO SITES IN GREAT SMOKY MOUNTAINS NATIONAL PARK

Elin Richard Swank, M.S. Biology

Western Carolina University (June 2023)

Director: Dr. Sean O'Connell

Differences in soil nutrient cycling, including nitrogen cycling and bioavailability have been linked to the inhabiting microbial and plant community interactions (Powell et al., 2015; Levy-Booth et al., 2014). Understanding the community relationships and their effects may help us describe the role of microbial communities in influencing plant communities and ecosystem functions (Phillips et al., 2013; Isobe et al., 2011; Hacquard and Schadt, 2015). Data quality (accurate, informative etc.) and quantity is needed to describe causal relationships among microbial communities, plant communities, and their distributions in an ecosystem (Mushinski et al., 2017). To address the need for and demonstrate the utility of, functional and process focused data, microbial community analysis was conducted focusing on known bacterial nitrogen cyclers and mycorrhizal fungi. Samples were cored from the mineral soil within the mycorrhizosphere surrounding a Northern Red Oak (*Quercus rubra)* at both the Purchase Knob (35.59173°N, 83.05997°W) and Cataloochee (35.58760°N, 83.08064°W) All Taxa Biodiversity Inventory (ATBI) plots (n=12 from each site). Samples were pooled and homogenized by sub-site location around the oak tree at each site (up slope, side slope, down slope; n=3 from each site). DNA

extraction and next generation sequencing of the bacterial 16S rRNA and fungal internal transcribed spacer (ITS) rRNA regions were performed by Azenta, Inc. (South Plainfield, NJ). Important bacterial genera found include the metabolically diverse *Burkholderia*, as well as known participants in soil nitrogen cycling *Nitrospira*, *Bradyrhizobium*, *Rhodoplanes*, and *Pedomicrobium*. Ectomycorrhizal (ECM) fungi genera that are important plant symbionts and transporters of nutrients within soil (Allen et al., 2003; Churchland and Grayston, 2014) were found, including *Sebacina*, *Russula*, *Elaphomyces* and the potentially ECM *Pseudotricholoma*. Principal components analysis (PCA) of the distributions of sequences showed a clear difference between the Cataloochee and Purchase Knob site samples with greater variability in sample community composition at Cataloochee for both bacteria and fungi. Key and prevalent groups identified are known to be involved in nitrogen cycling, plant associations, and nutrient transport suggesting that their relationships play some role in biogeochemical and nutrient cycling. Future analyses are needed to describe the pathways between microbial and plant, taxa and communities, and the emergent properties relating them to nutrient cycling and ecosystem functions. Supplementary files include the files 16S_otu_taxa_table.csv (bacteria dataset), ITS_otu_taxa_table.csv (fungi dataset), MIDI_Functional_Genes.csv (functional gene dataset), Figures_Tables.pdf (data tables and visualizations) and Figures_Tables.Rmd (R markdown script used to create Figures_Tables.pdf). The raw sequence files for next generation sequencing of bacterial 16S rRNA and fungal ITS regions are available through the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/bioproject/978337 with accession numbers SAMN35549206-SAMN35549229.

INTRODUCTION

It is known that plants, microbes (including fungi), and soil abiotic factors act on one another dynamically, but the spatial patterns and mechanisms are not always well understood (Berg and Smalla, 2009; Horz et al., 2004; Mushinski et al., 2017; Norman and Barrett, 2014). Because it is logistically difficult, time consuming, and expensive to probe and analyze soil microbial communities, there is a shortage of research in this area regarding spatial distribution and functional gene assemblages within soils or across landscapes (Levy-Booth et al., 2014; Mushinski et al., 2017). The inability to culture many soil bacteria in laboratories likely leads to reservations when deciding on research to be funded and this contributes to a lack of microbial species resolution regarding what species are present and their function in the soil environment (Horz et al., 2004; Mushinski et al., 2017). To establish causal relationships there is a need for data quantity and quality regarding soil microbial communities and their spatial distribution in ecosystems (de Vries et al., 2012; Mushinski et al., 2017; Baldrian, 2019). Linking the diversity of microbial communities to ecosystem functions and process rates requires a focus on functional genes relevant to biogeochemical processes (Van Der Heijden et al., 2008; McGuire and Treseder, 2010; Baldrian, 2019). Spatial information of these communities allows us to know where they are in soil environments, and when combined with measures of abundance and functional genes, can indicate the ecological importance of what they do *in situ*. Ammonia oxidation is a critical process in the global nitrogen cycle regulating plant available forms of nitrogen and nitrogen losses from the system (Norman and Barrett, 2014). Mycorrhizal fungi regulate nutrient exchanges in forested soil environments and account for a substantial amount of

the total soil microbial biomass (Allen et al., 2003; Churchland and Grayston, 2014). Oak species are both economically and ecologically important in their environmental range (Conrad et al., 2020). The purpose of this research was to investigate the microbial communities within the mycorrhizosphere of Northern Red Oak (*Q. rubra*) in southeastern U.S. hardwood forests of the Appalachian Mountains. We hoped to uncover relationships among microbial community members and between microbial community members and the host tree that may inform us as to which biological entity exerts the most influence on the distributions and abundances of the microbial community members.

BACKGROUND

Nitrogen Cycling and Climate Change

Climate change is significantly altering Earth's biogeochemical cycles, which is affecting ecosystem functions in poorly understood ways. The nitrogen (N) cycle (Figure 1) is composed of several chemical transformations of compounds containing N that are crucial for ecosystem production. Nitrogen cycling is composed of three main steps where initial atmospheric dinitrogen (N_2) gas is deposited in soil as ammonium (NH_4^+) through the process of nitrogen fixation. Subsequent transformation of NH_4^+ via nitrification into nitrite (NO_2^-) and nitrate (NO_3^-) allows for the third chemical transformation. Nitrite and nitrate in the soil are ultimately reduced biochemically through use in anaerobic microbial respiration making way for the final transformation of N species. Denitrification is the final process in which nitrous oxide (N_2O) and nitric oxide (NO) are biochemically reduced to N_2 and all three gases can be released into the atmosphere ultimately denitrifying the soil (Levy-Booth et al., 2014).

Nitrogen cycling is predominantly mediated by microbes in the soil, with individual processes largely performed by specialized taxonomic groups (Isobe et al., 2011). Soil nitrogen cycling is displayed in a simplified form in Figure 1. Soil nitrification begins with bacterial and archaeal oxidation of ammonia into nitrate using the ammonia monooxygenase (AMO) enzyme, encoded partially by *amoA* genes (Horz et al., 2004; Levy-Booth et al., 2014). The AMO enzyme catalyzes the rate limiting step of ammonia oxidation, which itself is the rate limiting step in nitrification, a process that is essential for increasing N bioavailability in N limited ecosystems (Horz et al., 2004; Isobe et al., 2011; Levy-Booth et al., 2014; Norman and Barrett,

2014). NH_4^+ and NO_3^- are the main forms by which living organisms obtain N from soil (Isobe et al., 2011; Levy-Booth et al., 2014). Nitrification and denitrification dictate N availability and loss in terrestrial systems where N limitations are common (Levy-Booth et al., 2014; Mushinski et al., 2017). Nitrification losses occur when NO_3^- leaches through the soil solution, reducing groundwater and stream quality, causing soil acidification and habitat eutrophication in receiving systems (Isobe et al., 2011; Laverman et al., 2001; Levy-Booth et al., 2014). Denitrification losses occur through emission of N_2O and NO gases into the atmosphere, contributing to global climate change by trapping heat, where N_2O has shown to be many times more potent than carbon dioxide (CO_2) over a 100-year period, and has also been linked to ozone depletion (Horz et al., 2004; Isobe et al., 2011; Levy-Booth et al., 2014).

Increased N compound leaching and emission from forested systems, predominantly through NO and N_2O dissolution into water is largely attributed to increasing N deposition in the system which is caused by industrial processes and fossil fuel combustion (Aber et al., 1991; Aber et al., 1998; Galloway et al. 2003; Isobe et al., 2011). Increased N deposition likely alters soil microbiomes through changes in microbial community structure and function, as well as N cycling (Horz et al., 2004; Isobe et al., 2011). Nitrogen addition rate and duration have been shown to affect soil respiration, increasing negative effects through changes in soil pH and carbon (C) to nitrogen (C:N) ratio (Yang et al., 2022). Microbial communities may be a mitigating factor regarding climate change, shifting of ecosystems and functions, and may be utilized for forest conservation management purposes if better understood.

Oak Significance

It is likely that plants alter microbiomes in the soil they inhabit, and it is thought that the microbial communities within soil affect the health and fitness of the plant (Hacquard and

Schadt, 2015; Pinho et al., 2020). Rhizosphere and bulk soil have been found to harbor different microbial communities due to differences in root exudates and rhizodeposition (Pinho et al., 2020). This effect has been seen in several tree species (oak, beech, spruce, and poplar) where various root exudates select for microorganisms that affect host growth, health, and productivity (Pinho et al., 2020). Bacterial endophytes have been found to prime plant immune responses, aid in pathogen resistance, strengthen mycorrhizal formation, transform, and mobilize nutrients, and alter root branching and allocation patterns through varying mechanisms like producing plant hormone precursors (Hacquard and Schadt, 2015). The oak genus, *Quercus*, is regarded as a highly ecologically and economically valuable contributor to eastern U.S. forested ecosystems (Conrad et al., 2020; Radcliffe et al., 2021). Some are considered keystone species because of their contributions to wildlife food supplies through acorn production, and leaf hosting of highly abundant and diverse insect taxa (Greenberg et al., 2014; Radcliffe et al., 2021). In this regard oak species diversity may also mitigate food supply shortages through differences in "red" vs "white" subgenus acorn production cycles, especially when acorn production losses occur (Radcliffe et al., 2021). Predicted water stress is likely altering habitat selection of oak species of the southeastern U.S., forcing migration to higher elevations (Speer et al., 2009). The oak decline phenomenon, which consists of a complex suite of abiotic and biotic factors whose relationships are little understood, is affecting many oak-dominated forests (Conrad et al., 2020; Radcliffe et al., 2021). Oak replacement by more shade-tolerant mesophytic tree species, and oak regeneration failure have also been observed in several study areas (Radcliffe et al., 2021). The relationships between soil microbes and plant cohorts may be intrinsic to these phenomena and represent a gap in scientific understanding that could benefit from increased attention.

Mycorrhizal Fungi

Understanding plant-microbe interactions can elucidate the effects of microbiomes on plant lifestyles and lifecycles (de Vries et al., 2012; Hacquard and Schadt, 2015). These interactions have also been linked to differences in nutrient cycling and availability (Phillips et al., 2013; Isobe et al., 2011). Recent studies have indicated the importance of soil microbiomes in plant health, especially within the rhizosphere (Berg and Smalla, 2009; Hacquard and Schadt, 2015; Pinho et al., 2020). Mycorrhizal fungi are ubiquitous, contribute to plant growth and nutrient cycling, and have shown to organize around plant functional type (Allen et al., 2003; Phillips et al., 2013; Churchland and Grayston, 2014; Terrer et al., 2016; Averill et al., 2018).

Arbuscular and ectomycorrhizal (AM and ECM respectively) fungi are likely associated with most tree species in temperate forests and reflect differences in biogeochemical processes between them (Phillips et al., 2013; Averill et al., 2018). AM associated plants tend to have faster decomposing litter and require AM to scavenge soil for nutrients, unlike ECM associates that can extract nutrients from organic matter and whose litter tends to decompose more slowly (Phillips et al., 2013; Read and Perez-Moreno, 2003). Phillips et al. (2013) found that ECM associated trees were well correlated to the ratio of organic N to inorganic N in the upper surface soil and increasing percentages of AM associated trees were correlated to increased N losses in a system. Nutrient cycling and bacterial communities have been linked to processes involving mycorrhizae; this is especially so in soil around the mycorrhizal root system, termed the mycorrhizosphere (Allen et al., 2003; Phillips et al., 2013; Churchland and Grayston, 2014; Terrer et al., 2016; Averill et al., 2018).

Microbial Communities and Ecosystem Functions

Microbial community data are generally regarded as noisy (containing complex

information from unaccounted variables), sparse (many observations are zero), and compositional (community composition is derived from interactions of the individuals) (Allison and Martiny, 2008; Busato et al., 2023; Weiss et al., 2016). These factors make microbial community correlation analysis difficult and lead to loss of aspect specificity in some interactions, especially when more than two aspects of a community are involved (Busato et al., 2023; Weiss et al., 2016). Although there has been little progress in relating microbial communities to in situ ecosystem biogeochemical functioning, this is being remedied with advanced molecular techniques and modeling (Allison and Martiny, 2008; Graham et al., 2016; Isobe et al., 2011; Powell et al., 2015). Microbial gene abundance has been correlated with changes in process rates, and soil characteristics when using quantitative analysis to analyze functional gene sequences (Levy-Booth et al., 2014). Also, statistical models have shown to be more accurate and predictive of ecosystem function and soil characteristics when incorporating microbial functional genes (Graham et al., 2016; Powell et al., 2015). The spatial patterns of functional communities in soil likely play an important role in determining where specific functions take place and may indicate a spatially specific ability to mitigate changing environmental conditions (de Vries et al., 2012). Ubiquity and complexity of microorganisms in the soil may preclude the direct application of studies regarding them into issues of resource or conservation management. Finer details may need to be resolved for specific issues and environments, especially when scaling up to the ecosystem level. But these qualities also may present a background on which issues in these areas become apparent or understood more clearly, such as understanding the mycorrhizae plant complex's influence on the distribution of biogeochemically functional microbes within an ecosystem. Knowing how these complex species associations interact in their environment may give us a way to isolate functional species

within an ecosystem to a specific niche, allowing us to derive a spatially explicit map of ecosystem functions.

METHODS

Samples were taken at Cataloochee and Purchase Knob ATBI (All Taxa Biodiversity Inventory) in Great Smoky Mountains National Park on May 12, 2022, with the help of Paul Super and Sean O'Connell. At each site a mature Northern Red Oak tree was chosen as the sample area from which mineral soil samples would be taken by coring. Four replicates each were gathered from up slope, cross slope, and down slope of the main stem for a total of 24 samples: 12 from each site. After removing leaf litter and humus to expose bare soil, samples were taken by using a soil corer to a depth of approximately 12-18 inches. Approximately 75 grams were collected from the lower portion of the corer into WhirlPak® bags and flash frozen on dry ice in a cooler. Samples were stored in a -80°C freezer on Western Carolina University's campus.

DNA was extracted using the Qiagen DNeasy® Powersoil® Pro Kit. Following the quick start protocol (May 2019) I substituted step 2 with 30 seconds at 2,500 rpm in a bead beater, then 1 minute on ice, followed by another 30 second bead beating. This was done because the vortex adapter referenced in step 2 has been implicated in the extreme shearing of DNA. DNA extractions were completed June 27, 2022, and stored at -20°C. Agarose gel electrophoresis was performed to test DNA quantity and quality, and polymerase chain reaction (PCR) was performed to amplify bacterial and fungal DNA.

Initial PCR primers used included 341F (forward) with a GC clamp and 907R (reverse) for bacterial 16S rRNA region (Laverman et al., 2001; Zhalnina et al., 2014; Ribbons et al., 2016; Pinho et al., 2020), EF4 (forward) and ITS4R (reverse) for the first reaction of a nested

PCR method for fungi, ITS1F (forward) with a GC clamp and ITS2R (reverse) for the second reaction (Selosse et al., 2002; Nguyen et al., 2013; Ribbons et al., 2016; Pinho et al., 2020). To investigate the presence of the *amoA* sub-unit among bacteria and *amoA* gene among archaea in our samples, primers used included *amoA*1F (forward) and *amoA*2R (reverse) for the bacterial *amoA* sub-unit, and amo111F (forward) and amo643R (reverse) for the archaeal *amoA* gene (Dillow 2009). All PCR products were stored at 4°C and tested by agarose gel electrophoresis for quality and quantity.

Originally, I had planned to use denaturing gradient gel electrophoresis (DGGE) to separate PCR products for community diversity analysis and use resultant bands to obtain DNA sequences identifying the bacterial and fungal species present. Test runs of DGGE were performed using the BioRad D-Code™ Universal Mutation Detection System. While testing the DGGE process it was found that the chemicals and equipment used could not produce the results required for this project. This irreproducibility led to discarding DGGE in favor of other data collection methods.

The remaining soil samples (which had been preserved at -20ºC due to a malfunction in the ultralow freezer) were combined into 6 total samples, 3 from each site (C = Cataloochee, P = Purchase Knob), 1 each from up slope (C1, P1), side slope (C2, P2), and down slope (C3, P3). Approximately 1g of soil from each sample was sent to GENEWIZ of Azenta Life Sciences (South Plainfield, NJ) for next generation sequencing and initial statistical analysis.

Approximately 50 grams of soil from each of the 6 samples were sent to Microbial Insights (Knoxville, TN) for QuantArray® sequencing and quantification of functional genes. Total bacteria (EBAC) and total archaea (ARC) were amplified and quantified using qPCR (quantitative polymerase chain reaction) along with the functional genes for sulfate reducing

bacteria (APS), sulfate reducing archaea (SRA), iron reducing archaea (IRA), iron reducing bacteria - other (IRB), iron reducing *Geobacter* (IRG), iron reducing *Shewanella* (IRS), iron oxidizing bacteria (FeOB), manganese oxidizing bacteria (MnOB), sulfur oxidizing bacteria (SOB), ammonia oxidizing bacteria (AMO), ammonia oxidizing archaea (AOA), nitrite oxidizing bacteria (NOR), anaerobic ammonia oxidizers (AMXNIRK), anaerobic ammonia oxidizers (AMXNIRS), nitrogen fixing bacteria (NIF), denitrifying bacteria (nirK), denitrifying bacteria (nirS), denitrifying archaea (ANIRK), denitrifying archaea (ANIRS), methanogens (MGN), fermenters (FER), acetogens (AGN), and acetylene degraders (AHY).

Next generation sequencing data and QuantArray® functional gene data analyses were done in R studio (R Core Team, 2023). Azenta had normalized sequence data in their report by dividing each sample read total by the lowest sample total giving the correction values displayed in Table 1 and Table 2. The normalized values were grouped by taxonomic level and classification for display. Unclassified operational taxonomic units (OTUs) were grouped into unclassified sets including non-bacterial or non-fungal OTUs. OTUs that accounted for less than one percent of sample total reads in all samples were grouped into minor contributors including non-bacterial or non-fungal OTUs. Non-bacterial or non-fungal OTUs that were classified at the taxonomic level displayed and accounted for more than one percent of reads in at least one sample were grouped with minor contributors. Functional gene data was summed across samples for each gene at each site (Cataloochee, Purchase Knob) for display.

Alpha diversity measures and Good's coverage were calculated for taxonomic data including bacteria and fungi. Quantitative species estimates included Chao1 and ACE and the abundance and diversity estimates included Shannon's H and Simpson's diversity measures and Pielou's measure of evenness. To find highly correlated taxa two-sided Pearson correlation

(confidence interval = .99) analyses of both phyla and genera were performed using Bonferroni multiple hypothesis test correction on p-values (alpha = .001) (Busato et al., 2023; Weiss et al., 2016). To assess differences in community functional capacities functional gene data were subjected to two-sided t-tests (confidence interval = .99) of site means with Bonferroni multiple hypothesis test correction on p-values (alpha = .001). Principal components analysis (PCA), including functional gene data as supplementary quantitative variables, was performed on OTU count data from each site, one each for bacteria, fungi, and a combined dataset.

RESULTS

Agarose Gels

Figures 2 through 6 display the agarose gel electrophoresis and DGGE test results of genomic DNA and targeted PCR products. Figure 2 shows a good yield of genomic DNA from all samples with minimal shearing present. PCR protocol on the bacterial 16S rRNA region was successful for all samples tested (Figure 3). A single band was observed for the bacterial *amoA* PCR (Figure 4) in the Cataloochee down slope sample lane (center) (the positive control did not show amplification of the target sequence). Figure 5 shows bands in all sample lanes and a faint band in the positive control, indicating that all samples contained some archaea with the *amo* gene however, the bands were surrounded by sheared DNA. The results of the DGGE test for 16S rRNA PCR products showed few resolved bands and inadequate signal strength for further analysis. Repeated trials of DGGE with fresh reagents, altered gel running voltages and times, and the use of a gel stacking technique did not yield adequate results.

Next Generation Sequencing Metadata

Table 1 and Table 2 show meta data for bacteria and fungi including raw read counts and correction factors used to reverse normalization of next generation sequencing data of the 16S rRNA and ITS genes provided by Azenta. The meta data shows that sample C1 and C2 had the lowest total reads for fungi and bacteria, respectively. There were many unclassified bacteria at the kingdom level and some individuals were classified in the domain Archaea (Table 1). **Error! Reference source not found.** also shows that some fungi were unclassified to kingdom and some individuals were classified into kingdom Plantae, the most being in sample C3.

Sample Diversity Measures

Alpha diversity measures for taxonomic data including bacteria and fungi are shown in Table 3 along with a count of species observed and the Good's coverage calculation for each sample. The Good's coverage value reflects the probability that the sequence library included the sequences found in the sample, and higher values indicate lower probability that sequences were not covered in the library. The Good's coverage value for each sample suggests that there is a low probability that the sequences found were not covered in the library used to match sequences. Chao1 and ACE are abundance-based species richness estimates, estimating the actual number of species present within each community sample. Both ACE and Chao1 indices for all sites estimate similar numbers of species, and the standard error ranges overlap one another lending increased confidence to these estimates.

Pielou's evenness is a calculation of species abundance and diversity indicating the relative abundance of each species in the sample. Pielou's evenness approaches one when all species observed occur in similar abundances and approaches zero when species abundances vary wildly from one another. The Pielou's evenness values are .50 and greater suggesting moderate community evenness in all samples, with increased evenness in the Purchase Knob samples (.65), and the highest community evenness seen in sample C3 (.74) (Table 3). Shannon H and Simpson's indices are indicators of diversity within samples utilizing species richness and evenness measurements. The calculation of Shannon H weights species richness higher than evenness, and Simpson's calculation weights evenness higher than richness. Higher values for Shannon's H indices and Simpson's values approaching 1 indicate larger amounts of diversity within the sample. Simpson's diversity shows values close to one in all samples indicating high diversity and Shannon's H values are large for each sample also indicating high diversity.

Taxon Correlations

Two-sided Pearson correlation analysis (with Bonferroni correction) of the combined fungal and bacterial data set showed no significant correlations between individual phyla, but many significant correlations between individual genera (Table 5; 253 out of 14,706 distinct possible paired interactions between 172 genera). Two-sided Pearson correlation detects only the presence of linear relationships (positive or negative) between two variables, in this case the OTU at the classification level of phyla and genera. This result is difficult to interpret without broader contextual information and only describes a single and relatively simple interactive capability between these microbial community members. Microbes likely engage in multiple non-linear relationships with other community members and this type of analysis is the first step in regarding the whole of these interactions.

Sample Community Phyla and Genera

In Figures 7 through 10 bacterial and fungal phyla and genera are shown as proportions of normalized sample total sequence reads. Figure 7 shows the bacterial phyla Proteobacteria and Acidobacteria co-dominate in all samples along with a large proportion of unclassified OTUs in sample C2. Chloroflexi, AD3 and Actinobacteria account for a smaller total proportion with Nitrospirae, Gemmatimonadetes, Verrucomicrobia, WPS-2, and GAL15 accounting for the smallest total proportions in each sample. Figure 8 displays bacterial genera showing the largest proportion by a significant amount to be unclassified, with *Rhodoplanes*, *Bradyrhizobium*, *Pedomicrobium*, and *Burkholderia* making up smaller proportions.

In Figure 9 Basidiomycota and Ascomycota co-dominate samples C3, P1, P2, and P3, with Basidiomycota and Mucoromycota co-dominating in sample C2. Sample C1 is dominated by Mucoromycota with smaller proportions of Basidiomycota and Ascomycota.

Mortierellomycota contribute small proportions to samples C2, C3, P1, P2, and P3, and Glomeromycota contribute small proportions to samples C3, P2 and P3. Figure 10 shows fungal genera were found to be the most diverse of all taxonomic classifications regarded in this study. Unclassified genera had the highest proportion of reads in sample C3, *Mucor* dominate in C1, and *Russula* dominate in P3. *Sebacina* showed highest sample proportions in Purchase Knob (P) samples while the largest proportion of *Pseudotricholoma* was in sample C2. *Saitozyma* were present in all samples but show larger proportions from Purchase Knob samples. *Leohumicola* were also present in similar proportions in all samples showing larger proportions in P2 and P3. *Elaphomyces* proportions were highest in C1 and P1, and *Geminibasidium* show highest proportions in C3, P1, and P2. The largest proportion of *Sagenomella* was found in P2, with smaller proportions in C3, P1, and P3. Similarly, the largest proportion of *Lactarius* was found in P1 with smaller proportions in C3, P2, and P3. *Scleroderma* showed a large proportion in sample P2; *Mortierella* was highest in C3 with smaller proportions in Purchase Knob samples; *Astraeus* was most predominant in P1 with small proportions in P2 and P3; *Penicillium* was present in all samples with highest proportions in C3, P2 and P3; the largest proportion of *Trichoglossum* was found in C2; C3 contained the largest proportion of *Cenococcum* as well as *Clavulinopsis* and *Trichoderma*; the largest proportions of *Inocybe* were found in P1 and P2; P2 and P3 contained the largest proportions of *Pachyphloeus*; C3 contained the largest proportion of *Hydnobolites* as well as *Tuber*.

Site Functional Genes

Figure 11 shows the functional genes that were sequenced and quantified in each sample summed into totals by site. For sulfur oxidizing bacteria (SOB), nitrite oxidizing bacteria (NOR), methanogens (MGN), iron reducing *Geobacter* (IRG), total bacteria (EBAC), total

archaea (ARC), sulfate reducing bacteria (APS), and ammonia oxidizing bacteria (AMO) genes, similar amounts of cells per gram of soil were found at each site. Ammonia oxidizing archaea (AOA), anaerobic ammonia oxidizers (AMXNIRS) and denitrifying bacteria (nirS) genes were only found at Purchase Knob, and nitrogen fixing bacteria (NIF), iron reducing bacteria- other (IRB) and iron oxidizing bacteria (FeOB) were found in amounts that varied by at least one power of ten between sites. Sulfate reducing archaea (SRA), denitrifying bacteria (nirK), manganese oxidizing bacteria (MnOB), iron reducing *Shewanella* (IRS), iron reducing archaea (IRA), fermenters (FER), denitrifying archaea (ANIRS and ANIRK), anaerobic ammonia oxidizers (AMXNIRK), acetylene degraders (AHY), and acetogens (AGN) were not found in quantifiable amounts at either site.

Community Diversity and Functional Gene PCA

Figures 12 through 14 display the results of PCA using OTU abundances in the bacterial, fungal, and combined data sets respectively, and include the functional gene data. Figure 12 shows bacterial communities from Cataloochee samples differing largely from one another across dimension 1, with the side slope (C2) differing widely from the up slope (C1), and down slope (C3) along dimension 2 with a predominant negative relationship. The Purchase Knob sample bacterial communities showed similar negative relationships along dimension one, and differed most along dimension two, with side slope (P2), and down slope (P3) sites showing positive relationships, and up slope (P1) showing a negative relationship. AOA, IRB, ARC, nirS, MGN, AMXNIRS, NOR, EBAC and SOB (not shown) cluster closest to P2 and P3, while FeOB, AMO, and APS cluster nearest to P1 and C2. IRG and NIF are nearest to C1, and C3 is the farthest from all quantified genes.

Figure 13 shows that C3 is largely different from all other fungal communities displaying

the only positive relationship with dimension one and a slight positive relationship with dimension 2. C1 and C2 show similar negative relationships with dimension one with C1 exhibiting a more negative relationship with dimension two than C2. The Purchase Knob samples' fungal communities share a similar negative relationship with dimension one, while P2 shows the most positive relationship with dimension 2. AOA, IRB, MGN, AMXNIRS, nirS, FeOB, ARC and EBAC (not shown) cluster nearest to all Purchase Knob samples, with NOR, AMO, APS and SOB nearest to C2, and NIF nearest to C1. C3 is farthest from all quantified genes with IRG being the closest.

In Figure 14 sample C3 is positively correlated with both dimensions, most positively with dimension one. C1 is positively correlated to dimension one and much more negatively with dimension two. C2 is negatively correlated with both dimensions but more negatively with dimension 2. Sites P1 and P3 are negatively correlated with dimension 1, while P1 is slightly positively correlated with dimension two, P3 is slightly negatively correlated with dimension two. Site P2 is largely positively correlated with dimension two and negatively correlated with dimension one. AOA, IRB and MGN cluster nearest to P2; ARC, nirS, AMXNIRS, and EBAC cluster close to P1 and P3; AMO, APS, NOR and SOB are nearest to C2, with NIF closest to C1, and IRG closest to C3 which is farthest from all quantified genes.

DISCUSSION

Microbial Data and Ecosystem Functions

Due to the noise, sparsity, and compositionality inherent in microbial data, and our current lack of ability to resolve many taxonomic individuals with real confidence, data was grouped at the phyla and genera taxonomic levels for Pearson correlation analysis. Grouping data at higher taxonomic classifications likely reduces perceived interaction complexity between OTUs, but is commonly used to avoid other computational discrepancies, such as artificial indicators of interactions (Busato et al., 2023; Weiss et al., 2016). Typically, noise is used to describe information detected in analysis but not pertinent to the study in one sense or another (can come from instruments, experimental design, etc.), sparsity refers to the many zeros encountered in microbial community data, and compositionality refers to the idea that the data gathered are part of much broader and more complex whole.

We use community members as indicators of ecosystem functions which combined with diversity measures can help indicate ecological relevance and potential sustainability. We have attempted to uncover relationships between microbial community members and the host tree through diversity and correlation analysis of the host mycorrhizosphere community. The types of data and analysis used in this study help us to answer the questions of "who" among mycorrhizosphere community members (microbial, plant, etc.) exerts the predominant influence on the community structure, and what functions are attributed to communities under that specific influence. In this way, we can investigate ecosystem functions at finer spacial scales by linking biological community structures with the entities influencing that structure and the entities

creating ecosystem functions.

Bacteria

Summary descriptions of bacterial genera are displayed in Table 4. Within the Gram-negative phylum Proteobacteria there are several classes of organisms, such as Alphaproteobacteria, which includes both the *Pedomicrobium*, *Rhodoplanes* and *Bradyrhizobium* (Garrity et al., 2015). Many *Pedomicrobium* strains have been shown to reduce nitrate, with some strains able to reduce nitrite and ammonium (Hirsch, 2015). *Rhodoplanes* are a group of facultative *photoorganotrophs* that can perform nitrogen fixation and gain nitrogen from nitrate and urea (Hiraishi and Imhoff, 2021). Many species of *Rhodoplanes* participate in denitrification by utilizing nitrate as a terminal electron acceptor for metabolic processes (Hiraishi and Imhoff, 2021). The genus *Bradyrhizobium* contains chemoorganotrophs, utilizing nitrates and amino acids as nitrogen sources (Kuykendall, 2015). Species within *Bradyrhizobium* are characterized by the ability to participate in nitrogen fixation, predominantly through symbiotic relationships with plants from the family Leguminosae but some can fix nitrogen in a free-living state (Kuykendall, 2015). Within recent years the *Rhodoplanes* and *Bradyrhizobium* have been phylogenetically linked much closer than previously thought, with studies calling into question their placement in separate families (Hördt et al., 2020). Another functionally important class within Proteobacteria is the Betaproteobacteria, which contains the genus *Burkholderia* that has constituents known to occupy varied ecological niches through the utilization of an array of metabolic capabilities (Garrity et al., 2015; Vandamme and Eberl, 2018). *Burkholderia* participate in a wide variety of metabolic processes exemplified by *Burkholderia cepacia* which was shown to utilize any of 100 organic compounds (Vandamme and Eberl, 2018).

Genus *DA101* of the phylum Verrucomicrobia has been found in large abundances in

soils, with highest abundances recorded in grassland soils (Brewer et al., 2016). Brewer et al. (2016) suggests that *DA101* prefer soils that do not overlap in range of forest soils dominated by nonsymbiotic *Bradyrhizobium*. The functional capacity of these organisms may be important for both terrestrial and freshwater ecosystems due to their participation in C and N cycling and their occupation of many and varied ecosystems. Of the phyla found, individuals from the Gram-negative Acidobacteria and Proteobacteria phyla can be found in many environments typically making up large proportions of the sequenced microbial community (Thrash and Coates, 2015a; Garrity et al., 2015). Individual species from these phyla display varied physiological traits, including some species that are known to participate in ecosystem functions like carbon, manganese (Mn), iron (Fe) and N cycling (Thrash and Coates, 2015a; Garrity et al., 2015).

Of the six genera within Acidobacteria, *Geothrix* is the only known to contain species that utilize nitrate (NO_3^-) as an electron acceptor during metabolism (Thrash and Coates, 2015b). This reaction is likely part of the larger nitrogen cycle, chemically transforming soil nitrate into nitrite (NO_2^-). Within Proteobacteria, the genus *Rhizobium* are known plant symbionts, and some species participate in the leguminous form of nitrogen fixation within plant roots (Kuykendall, 2015). While neither *Geothrix* nor *Rhizobium* were found explicitly within this study there is a possibility that they were not able to be classified to genus during next generation sequencing.

Fungi

Summary descriptions of fungal genera are displayed in Table 4. Basidiomycota, commonly known as club fungi is an ecologically diverse group that includes plant pathogens, decomposers (particularly wood and litter), and many ECM species (Kjøller and Rosendahl, 2014). Ascomycota, commonly known as sac fungi is also an ecologically diverse group that was recently found to be most dominant in many soils, exhibiting high frequencies of genes

conferring stress-tolerance and competitive strategies (Egidi et al., 2019). The phylum Mucoromycota include both AM and ECM species, chitinolytic and plant saprotrophs, as well as mycoparasites, plant pathogens and opportunistic animal pathogens (Chang et al., 2022). Mortierellomycota is closely phylogenetically related to Mucoromycota having only recently been accepted as a distinct phylum (Tedersoo et al., 2018). Glomeromycota are predominantly AM plant symbionts and potentially heterokaryotic (containing different nuclei) (Tisserant et al., 2013).

Mucor fungi belonging to phylum Mucoromycota, are primarily ubiquitous saprobic soil dwellers found on decaying organic matter, but some opportunistic plant, animal, and fungi pathogens are known, including human pathogens (Morin-Sardin et al., 2017). No mutualistic relationships to plants have been discovered but some species may form mycorrhizal symbioses (Morin-Sardin et al., 2017). Some species have been used in food and pharmaceutical industries as fermenters, metabolite producers and for biotransformations of pharmaceutical compounds, they are however also involved in spoilage and contamination in these industries as well (Morin-Sardin et al., 2017). *Russula* is a large diverse genus belonging to phylum Basidiomycota, having a large geographic distribution and broad ecological niche utilization; they form ECM relationships with a diverse array of plants (Wang et al., 2015). Many *Russula* species are edible and, in some areas, contribute to local economies, providing nutritional sources of food (Wang et al., 2015). *Sebacina* is a common and widely distributed species rich group containing ericoid, orchid and ECM root symbionts, as well as saprotrophs and root endophytes (Tedersoo et al., 2014). The most common taxa have been found to exhibit little or no host specificity and little is known of their ecology due to difficulty of isolation into pure cultures (Tedersoo et al., 2014).

Pseudotricholoma species have been found in soil, woodlands and grasslands in the

northern hemisphere and the genus likely contains biotrophic and potentially ECM species (Sánchez-García et al., 2014). *Saitozyma* includes some common yeast species that were isolated across all altitudes in a study by Moreira et al. (2020) suggesting a wide distribution and are typically found in acid soils with high organic and moisture content. Hambleton et al. (2005) found *Leohumicola* in Canadian soils to be closely related to unidentified fungi from studies regarding genetic diversity of endophytes in ECM and ericoid mycorrhizal roots from Italy, Norway, and Australia. *Elaphomyces* known as "deer truffles" are important ECM fungi in temperate and subarctic forests where some species provide food sources for mammals (Paz et al., 2017). They have been shown to dominate ECM communities in poor, acidic soils, and exhibit drought tolerance (Paz et al., 2017). Nguyen et al. (2013) isolated *Geminibasidium* species from forest, grassland, and blueberry field soils that had been historically burned. These species were related to environmental sequences found globally, one of which was reported to be from ECM root tips by Nguyen et al. (2013). *Sagenomella* grows under alkaline conditions and in a study by Tayyab et al. (2022) *Sagenomella* sequences were significantly negatively correlated to total N, organic matter, available phosphorous and soil water content (Feng et al., 2023). In high latitudes of the northern hemisphere *Lactarius* is a diverse and abundant group of ECM species of fungi displaying increased diversity in boreal forest types compared to arctic tundra (Geml et al., 2009). Geml et al. (2009) found that vegetation played a major role in the composition of *Lactarius* species assemblages in Alaska with phylogroups displaying preference to either lowland or upland forest.

Scleroderma was shown to increase phosphorous mobilization in the rhizosphere of *Castanea henryi* (European Chestnut) and significantly increased seedling dry biomass through ECM colonization (Chen et al., 2023). Typically exhibiting a garlic-like odor, *Mortierella* are

diverse soil saprobes that are used in dietary supplement production and biofuel industries (Uehling et al., 2017). In *Mortierella* isolates recovered from *Populus* roots and soils, Uehling et al. (2017) found an endosymbiotic bacterium *Mycoavidus cysteinexigens* that exhibits vertical transmission. *Mortierella* isolates showed increased growth and produced more aerial hyphae when the endosymbiotic bacteria were removed (Uehling et al., 2017). *Astraeus* is an ECM fungal group that exhibits associations with *Pinus*, *Pseudotsuga*, *Alnus*, *Eucalyptus*, and *Castanea* (Fangfuk et al., 2010). *Penicillium* are found in terrestrial and aquatic habitats, known to be important decomposers they may also be important nutrient cyclers and pollutant degraders in the intertidal zone (Park et al., 2019). *Trichoglossum* sometimes known as "hairy earth tongues", have a wide geographic range, are predominantly saprobic in soils, and have been found in associations with mosses (Dasgupta et al., 2022). A highly abundant and ubiquitous species of *Cenococcum, C. geophilium* is an ECM fungus tolerant of a range of environmental factors that is especially adapted to water stress; however, molecular evidence suggests that it could be multiple cryptic species (Fernandez and Koide, 2013). *Clavulinopsis fusiformis* has been known to grow in grassland and woodland soils with grasses, mosses, and under hardwoods, conifers, and shrubs (Keleş and Kaya, 2021). *Trichoderma* are typically thought to be ubiquitous free-living soil inhabitants although some species have been reported in human infections and increasing evidence suggests this group to be opportunistic plant symbionts (Samuels, 2006). *Inocybe* is generally considered ECM but may form orchid and arbutoid mycorrhizal associations.

Microbial Communities and Ecosystem Functions

While we are not able to define distinct relationships between mycorrhizosphere community members from this study, we can however describe a communal functional dynamic.

Many groups of fungi found in the study likely help the host plant manage fluctuations in nutrient supply due to their mycorrhizal capacity. Mycorrhizal fungi are known for transporting and partitioning nutrients and water and can explore larger areas of soil more efficiently than their host plant (Allen et al., 2003). This likely means that nutrients made available by biological processes like the ones conferred by the functional genes found in this study can be provided to the broader system through mycorrhizal connections. Conversely this may also mean that mycorrhizal fungi can prevent access to water and nutrients in times of stress, potentially parasitizing or decomposing the host or other community members as suggested by the presence of opportunistically pathogenic species. Because mycorrhizae can facilitate nutrient exchange and uptake by plants, they also play a role in C dynamics by increasing access to limiting nutrients allowing more C to be stored in tissues. This likely also works conversely under stressful conditions which may alter the trophic dynamic by increasing decomposition and respiration, releasing more C from decaying tissues and into the atmosphere.

Nitrogen chemical species flow via genes from nitrogen fixation (NIF) to ammonia oxidation (AOA, AMO), anaerobic ammonia oxidation (AMXNIRK, AMXNIRS), and nitrite oxidation (NOR), and then on to the denitrification (nirK, nirS, ANIRK, ANIRS) as shown in Figure 1 and represented for this study in Figure 11. Most N entering ecosystems happens through biological means and N typically limits ecosystem productivity (Levy-Booth et al., 2014). Outside of total archaea and bacteria, nitrogen fixation was the most abundant gene group found in this study suggesting the importance of this process for the system. An abundance of N fixation and nitrification genes may indicate a larger available N pool providing substrates for denitrification and related processes. Cataloochee samples had a higher abundance of N fixation genes which may indicate highly active N extractive processes that increase

demand. The presence and abundance of archaeal and anaerobic ammonia oxidation genes in Purchase Knob samples suggests environmental stress and anaerobic conditions during the sampling period. High abundance of denitrifying bacteria in Purchase Knob samples may be due to an increased abundance of oxidized chemical N species. Purchase Knob samples also showed a relatively high abundance of iron oxidizing bacteria compared with Cataloochee samples which may be due to increased iron availability or chemical conditions suitable to the oxidation of iron.

Functional genes found at both sites indicate similar functional capabilities and capacities of microbial communities, apart from nirS, AMXNIRS and AOA which were only found in Purchase Knob samples. The similar abundance of APS, IRB, IRG, SOB, AMO, NOR and MGN genes suggest that the associated processes may be locally ubiquitous. Sulfate and iron reduction can be used to remove electrons during chemoheterotrophic or chemolithotrophic metabolism. Sulfur oxidation is a chemolithotrophic metabolic strategy and evidence is suggesting that sulfur and iron cycling may be strongly linked to one another possibly occurring simultaneously with sulfur fueling iron reduction (Hansel et al., 2015). Ammonia oxidation and nitrite oxidation are also chemolithotrophic metabolic strategies contributing to the transportation of N through soil by increased mobility of chemical species formed (Zhalnina et al., 2014). Methanogenic bacteria contribute to the release of methane (CH_4) and CO_2 into the atmosphere through the degradation of organic compounds during metabolism (Garcia, 1990), and this group may have major implications for carbon sequestration under climate change.

Environmental Factors and Geography

The geographic location of both sites is very close when considering whole ecosystem processes, but it is also worth noting that the relative positions of each site likely confer different suites of environmental factors and disturbance. The Cataloochee site was roughly 4600-4700

feet in elevation on a south facing slope and the Purchase Knob site at roughly 4300 feet on somewhat of a northern slope. The aspect, incline and rainfall dynamics of a site can determine whether nutrients and soil are collected or dispersed. What may have had the largest impact on differences seen (although none statistically significant) between Cataloochee and Purchase Knob site samples is their slope position. The Cataloochee site gets the most direct sun because it is facing predominantly south, and the Purchase Knob site is mostly protected from direct sun by surrounding inclines. The surrounding inclines at the Purchase Knob site may also contribute to high nutrient low oxygen conditions due to large surface area drainage of soil water towards that location.

Small scale geographic differences may have profound effects on the functional ability of landscapes with some processes being strongly correlated to specific areas. This would make management and planning difficult for ecosystem sustainability suggesting that some areas deemed important enough due to their functional contributions be specifically targeted for conservation or remediation. Although this may be possible it brings up important questions about the interconnectedness of processes within geographically defined systems. The sheer number of microbial and plant relationships, and their complex nature currently prevent us from defining them explicitly but the patterns they exhibit are observable and provide some understanding as to how they collate into a larger functional system.

Host Microbial Communities and Relationships

The connection between microbial community members and the mycorrhizosphere host may be predicated on a balance of supply and demand. Many groups of fungi and some bacteria found in this study are known to have the potential to infect plants and other organisms opportunistically. Acute oak decline is known to be caused by abiotic and biotic factors, and oak

species may acidify soil to promote nutrient uptake and availability, combat unfavorable environmental conditions and withstand disease (Pinho et al., 2020). The effects of oak induced acidification on the microbial community in the rhizosphere of the oak are little understood, and it may prevent or preempt decline (Pinho et al., 2020). The drastic alteration of mycorrhizosphere environmental factors may lead to increased virility of pathogens, loss of functional capability, and reduced nutrient transport. Establishing clear connections between mycorrhizosphere hosts and microbial communities is a necessary step towards understanding their physiological relationships and how they affect ecosystem functions on small scales.

Data Utility and Importance

Multiple data types including environmental, chemical, and taxonomic are likely required to answer questions regarding ecosystem functions. Although this study did not directly link functional genes to a taxonomic level, the results suggest that we can have some confidence when linking environmental functions to microbial community members in soil. We see phyla and genera known to contain individuals with abilities to perform ecosystem functions can be linked *in situ* to the genes which produce those environmental effects. The use of quantified functional genes to improve the ability of environmental models to reflect the fluctuations of microbially driven processes has already been undertaken and led to improvements in model predictions (Graham et al., 2016; Powell et al., 2015). More ecological data pertaining to microbial community dynamics is required to link whole community functions to ecosystem functions and dynamics.

Future Goals and Objectives

Although many bacteria are unculturable in lab settings and their functional capacities remain unknown, every organism participates in some aspect of ecosystem functions through

direct or indirect means. Microbial ecology harbors a wealth of information, has implications in all environments, and is a field of study where more research is required to extrapolate into reliable resources for land management and conservation. To conserve an ecosystem as a functioning entity the actors in the processes creating the function must also be conserved. Under climate change the robustness of landscapes and ecosystems may be mediated by soil communities with the potential to provide benefits including sustainability to landscape level systems and local communities that rely on them. These soil communities also may provide an indication of future landscape changes and be useful as a tool for decision making as they reflect landscape level processes relevant to management goals and strategies. By describing the community relationships of the predominant actors in soils we better understand why and how they occur in the spatial orientation we observe. The implications of these relationships may help link ecosystem nutrient exchanges to the underlying plant and microbial community complexes that perform transformation and transportation of nutrients. We may be able to describe a path to developing a spatially explicit method to describe the arrangement of soil microbial communities, and through this describe the spatial qualities of ecosystem functions. Better understanding of how and why the plant, mycorrhizae, and bacteria influence one another allows for more informative modelling of biogeochemical cycles, feedback mechanisms, and responses to changing environmental conditions. Characterizing microbial communities and their contributions to ecosystem form and function through DNA sequencing in concert with biotic and abiotic environmental measures is the first step in the process of understanding these connections. Ongoing bacterial community surveys may be an effective way of collecting fine scale spatial data while incorporating a community engagement role that allows partners to learn about local landscapes and how they have effects on a global scale.

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