INCREASED FUNGAL DIVERSITY ASSOCIATED WITH *APHAENOGASTER* SPP.: MORE EVIDENCE FOR KEYSTONE MUTUALISMS

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ABSTRACT

INCREASED FUNGAL DIVERSITY ASSOCIATED WITH *APHAENOGASTER* SPP.: MORE EVIDENCE FOR KEYSTONE MUTUALISMS

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Food webs in Southern Appalachian forest soils are complex. Because of climate uncertainty, it is imperative that we gain a baseline understanding of the nutrient fluxes associated with each trophic level of the forest ecosystem. Microbial carbon deposition and respiration are responsible for large portions of the soil carbon flux but there is little research detailing microbial interactions with soil macro-fauna. This study examines the effect of *Aphaenogaster* ants on soil microbial diversity. These ants participate in a keystone mutualism with a guild of myrmecochorous plants in Southern Appalachian forests. The data presented here demonstrate that *Aphaenogaster* interactions extend beyond the guild of herbaceous plants. Fungal diversity increases in ant nest soil relative to bulk soil ($p = 0.028$). Elucidating the ecology of *Aphaenogaster* spp. could be a key to understanding nutrition webs in the deciduous forests of eastern North America.

INTRODUCTION

Complex nutrition webs exist in the soils of Southern Appalachian forests. Although soil trophic systems are still poorly understood, a growing body of literature addresses specific interactions (Peck and Niwa, 2005; A'Bear *et al.*, 2010 and Zhang *et al.*, 2010). As more of these interactions are described in detail, an informative picture of the dynamics of nutrient cycling in temperate forest soils is emerging (Blankenship *et al.*, 2011). The next trophic interactions chosen for study should be keystone interactions where species of relative low numbers and biomass account for a significant mass of litter processed (Adl, 2003). Studies that connect data sets of already well defined interactions (Rantalainen *et al.*, 2008 and Hyodo *et al.*, 2010) will best enhance our understanding of complex soil food webs. In order to construct a model of the entire soil food web we must first have quantitative data regarding each species or functional group though it can be difficult, in situ, to determine the value of a given interaction to the overall system because coring destroys any spatial heterogeneity that may contribute to function (Adl, 2003). This study uses molecular methods to analyze the influence of *Aphaenogaster* spp. ants on the community structure of soil microbes.

Soil Nutrient Cycles

The ecology of litter decomposition and biomineralization is comprised of biotic and abiotic components operating spatially and temporally on global (Lavelle, *et al.* 1993) and local scales (Frey, 2007). In temperate forest soils, microbes are

largely responsible for the bioavailability of carbon, nitrogen, phosphorus and other essential nutrients (Dilly, 2004) but the efficacy of microbial nutrient cycling is dependent on climate. For instance, in boreal regions abiotic factors such as variation of moisture and temperature are almost solely responsible for the baseline rate of decomposition of vegetable debris (Jansson, 1985). Additions to the stable soil nutrient pool are dependent upon disturbances such as fire (Horwath, 2007). In the tropics where climate is consistent and the organic matter is relatively homogeneous, litter turnover is rapid but macroinvertebrates spatially influence microbial activity (Lavelle, *et al*. 1993). This microbial activity is reflected in turnover rates of soil organic carbon in different ecosystems (Table 1).

Not counting living vegetable biomass, the total global stock of soil carbon is calculated by Amundson (2001) to be double the atmospheric pool. Therefore, it becomes important to understand the possible controls on the flux of carbon due to soil respiration. In temperate forests, carbon inputs to soil include litter from the aerial portions of vegetation, below ground vegetation and symbionts, and rhizodeposition. While almost all of the soil carbon influx is fixed in these materials through photosynthetic pathways (Fig. 1), it is partitioned for decomposition depending on the complexity of the carbon molecular structure (Paterson, *et al.* 2008). In general, fast growing heterotrophic bacteria (r-strategists) utilize the soluble carbon-substrates such as sugars, proteins and other macromolecules while the bacteria with slower but more varied metabolic pathways (k-strategists) and saprophytic fungi acquire more carbon from the insoluble lignin and other more recalcitrant materials. Some of these complex substrates such as cellulose, lignin

metabolites and polyphenols require specialized decomposers and can remain stable in the soil as humic substances for thousands of years. In temperate forests polyphenols can account for up to 30 percent of dry weight of some plants (Horwath, 2007).

Microbes are also responsible for most of the bioavailable nitrogen in soils. Between one and five percent of the dry weight of plant cells is comprised of nitrogen (Raven, 1999) which in most soils is the rate limiting element (Robertson and Groffman, 2007). The largest pool of global nitrogen exists in the atmosphere as dinitrogen gas, but is relatively inert and unavailable to plants because of the triple covalent bond between nitrogen atoms. Fortunately, diazatrophy (nitrogen fixation) is widely distributed through the domains Bacteria and Archaea (Henson, 2004). Many diazatrophs are associated with plant roots. The nitrogen fixed by these symbionts is transferred to plants and utilized in various plant tissues as proteins, nucleic acids and hormones until senescence. Ammonia oxidizing and denitrifying prokaryotes also contribute to the flux of nitrogen in soils. However, Long, *et al.* (2012) show that the population increase of ammonia oxidizing bacteria normally associated with nitrogen fertilization diminishes with elevated CO_2. In addition to rapidly increasing CO_2 levels, anthropogenic sources of atmospheric reactive nitrogen deposition have increased ten-fold in the last one-hundred and fifty years (Galloway, 2008). This trend is another important variable for researchers to consider when modeling forest soil nutrition webs since increased availability of nitrogen is known to change plant community composition (Bobbink, 2010).

As scientists seek to understand the effects of global climate change, most studies focus on above ground biota. This is, in part, because the dynamics of below ground systems are not well described and little base-line data is available to compare to new levels of system functioning. Blankenship (2011) conducted a meta-analysis of responses of soil biota to climate change and concluded that population density and biomass of detritivores increase with rising CO_2 and temperature. Bacteria responded favorably to higher levels of CO_2 while fungal biomass was positively correlated with moisture levels but not CO_2 levels.

The nuances of microbial response to climate change will become increasingly important as we attempt to mitigate the effects of new climatic regimes on human agriculture and natural ecosystems. Early carbon cycle models attributed most soil carbon deposition to the activity of plants because they transport carbon compounds to underground tissues and form litter when above-ground tissues senesce. As we learn more about below ground processes, it becomes apparent that the mycorrhizal and bacterial symbionts also play a large role in the carbon cycle.

The Role of Mycorrhizae and Other Microbes

Three fungal phyla have co-evolved with land plants to increase the range of nutrients available to both partners. The plants receive mineral nutrients that the fungi have chemically extracted from soil organic matter and inorganic particles. In return the fungi receive photosynthate. Arbuscular mycorrhizae are all within and exclusively comprise the order Glomales. These arbuscular structures appeared in

the fossil record during the Silurian (*c.* 420 Ma) (Schussler *et al.*, 2001) and Glomales continued to co-evolve with land plants through the Devonian period. About 80% of plant species worldwide host arbuscular mycorrhizae (Smith and Read, 2008) and are rewarded with increased uptake of phosphorus (Bucher, 2007) and other nutrients. Though arbuscular mycorrhizae can be dispersed as spores or hyphae, they are obligate mutualists with plants. To obtain nutrients from the host, the fungus must infect the root through signal mediated invaginations of the epidermis. Afterwards it can spread into the root cortex and receive as much as 30% of plant photosynthate (Drigo *et al.,* 2009). This fixed carbon is then released more slowly to the wider soil community. Arbuscular mycorrhizal fungi are known to employ different metabolic strategies. Some species focus on rapid host colonization while others spend more energy on soil “exploration”. There are also differences in induction of sugar translocation from host plants (Denison and Kiers, 2011).

Because increased atmospheric CO_2 is correlated with increased levels of carbon assimilation by many plants (Elser *et al.*, 2010) we would expect to see an associated increase in arbuscular mycorrhizal activity though this is not supported by Blankenship (2011). Arbuscular mycorrhizae do not release chelating chemicals into the soil (Hanselwandter, 2008) so while they facilitate a major proportion of plant carbon deposition, they do not, in general, contribute directly to bioweathering. However, because they release protons during active transport (Ferrol, 2000) and take up nitrate and ammonium (Johansen *et al*., 1993) they do have an effect on soil pH.

Ectomycorrhizal fungi are found in the “higher” phyla Basidiomycota and Ascomycota. The fossil record contains evidence for ectomycorrhizae starting in the Eocene, but molecular clock data place the origins of this symbiosis with plants in the Jurassic (*c.* 180 Ma) and the divergence of Ascomycota from Basidiomycota no less than 400 Ma (Taylor and Berbee, 2006). About three percent of plant species worldwide host ectomycorrhizal fungi. Many are in the families Pinaceae, Fagaceae and Betulaceae (Smith and Read, 2008). Since these make up the dominant trees in temperate forests, and the associated fungi account for a significant portion of soil biomass, it becomes apparent that the ectomycorrhizae are a critical component of this ecosystem.

Ectomycorrhizal fungi participate in a less invasive relationship with their plant partners than the Glomales. Ectomycorrhizal hyphae, in most cases, penetrate only into the first layer of exterior epidermal cells. These develop around the cortex of new root tips in a reticulated structure called a Hartig net that is integrated into the epidermal cells. Then a thin exterior mantle of fungal fibers encloses the root tip, essentially becoming the plant’s biochemical interface with the soil (Massicotte, 1998). Ectomycorrhizal symbiosis is non-specific with most fungal species enjoying a broad range of hosts and competition between fungi for “root-space” has been observed (Kennedy, 2010).

The ectomycorrhizal phyla contain a variety of species capable of weathering soil organic matter. These mycorrhizae also exude low molecular weight organic acids and siderophores which cause metal leaching from mineral grains and free up essential nutrients for delivery to plant partners (van Hees *et al.*, 2003). The carbon

to make these bioweathering compounds comes from the plant photosynthate. The ectomycorrhizae can receive a significant portion of net primary production; up to 22 percent in some studies (Hobbie, 2006). It becomes a more efficient use of that carbon to produce mycorrhizal tissue than plant root tissue because the nutrient uptake per unit of absorbing area is greater (Tinker *et al*., 1994). The fungal hyphae are longer than root hairs and have a smaller diameter that enables exploration of intergranular spaces. This effectively increases the volume of soil available to the plant.

All of the familiar forest mushrooms such as *Russula, Boletus* and *Ganoderma* that are represented in the food web as a major source of nutrition for macrofauna (Maser *et al.*, 1978) are just the sporocarps of ectomycorrhizal fungi. They play a much larger underground role in the ecosystem as primary saprotrophs and plant symbionts (Stamets, 2005). Understanding the diversity and functioning of these fungi is critical to efforts to preserve temperate forest ecosystems (Amaranthus, 1998).

Bacteria also play a role in forest soil food webs as primary decomposers and plant symbionts. In many cases there are syntrophic interactions between bacteria and ectomycorrhizal fungi with each secreting a chemical to supplement bioweathering processes (Adl, 2003). Many plants form symbiotic partnerships with bacteria including the nitrogen fixing *Rhizhobia.* There is new evidence that three-way partnerships exist between plants, fungi, and bacteria (Hoffmann and Arnold, 2010) and in some cases the fungal hyphae rely on assistance from the bacteria to initiate infection (Rigamonte *et al.*, 2010).

Arthropods can have pronounced effects on plant-fungus symbioses. Herbivory can reduce mycorrhizal colonization, and insects can disperse fungal propagules or directly feed on hyphae (Gehring and Bennett, 2009). The mycorrhizae, in turn, can alter the success of herbivores and tend to positively affect specialists and negatively affect generalists. This is, perhaps, due to increased production of secondary metabolites in colonized plants. These defense chemicals have contributed to the high levels of host specialization in insects (Gange *et al.*, 2005). It is becoming clear that multi-trophic interactions are the rule rather than the exception, particularly in temperate forests.

Aphaenogaster spp. as Keystone Mutualists

Ants of the genus *Aphaenogaster* are ubiquitous in Southern Appalachian forests. They are omnivorous foragers that build semi-ephemeral nests just below the surface under rocks or recent deadfall. For the purposes of this study, ants will be indentified to genus level because of cryptic traits that make identification to species level difficult. Regionally, most studies have focused on members of the *fulva-rudis-texana* complex with authors usually identifying *Aphaenogaster rudis* but declining to commit to species-level taxonomy (Warren, 2011).

Aphaenogaster spp. out-competes larger ants through speed and adaptability. It is one of the most common ants collected by researchers in Southern Appalachian forests (Lessard, *et al.,* 2007; Zelokova, 2008). It is distributed across a wider range of elevations than other regional species (Lessard, *et al.,* 2007) and forages at a wider range of temperatures (Dunn, 2007). When compared to other

woodland ants (Fellers, 1987), *Aphaenogaster* spp. arrives at baits first, utilizes a broad range of foodstuffs and displays a variety of dominance behaviors when challenged.

Aphaenogaster ants participate in a unique and asymmetrical symbiotic relationship with the myrmecochore guild of the Southern Appalachian forests. Mymecochores are plants that offer a lipid rich reward, called an elaiosome, attached to the external coat of their seeds. Myrmecochory (seed dispersal by ants) has evolved multiple times worldwide and occurs in 4.5% of species and 17% of plant families (Lengyel, *et al.*, 2010). Regionally it is found in a variety of herbaceous monocots and dicots including *Asarium, Trillium, Hepaticum and Sanguinaria.* Myrmecochores represent up to 40% of Southern Appalachian forest herbaceous species and, in some locations, up to 60% of emergent stems (Beattie *et al.*, 1979). Seeds of myrmecochores present an elaiosome (Fig. 2), for the disperser. Ants will preferentially choose foods with high fatty acid contents as these nutrients are scarce in litter and expensive or impossible for ants to synthesize (Boulay *et al.*, 2006). The elaiosome has evolved to mimic the fatty acid profile of insects and is not similar to that of the seed itself (Hughes *et al.*, 1994). The whole seed, with elaiosome intact, is carried back to the nest and, in most cases, discarded after the elaiosome is fed to the larvae (Fischer *et al.*, 2005). This works out well for plants that “target” *Aphaenogaster* with a particular seed size or timing of seed release. They reap the benefits of predator avoidance and directed dispersal since seeds are collected quickly and carried to nests located in high-light locations (Giladi, 2006). Because *Aphaenogaster* colonies can have overlapping territories that are frequently

relocated (Smallwood, 1979), dispersal area is maximized and parent-offspring and sibling competition are minimized.

Recently, research has been directed at the nature of the mutualism between myrmecochores and *Aphaenogaster* spp. in Southern Appalachian forests (Zelikova *et al.*, 2008; Ness *et al.*, 2009 and Warren *et al.*, 2011). The ants, in this case, are generalists and the mutualism is asymmetrical. *Aphaenogaster* will eat fungus, insect bodies and other diaspores. Other ant species in the Southern Appalachians are known to collect some myrmecochore seeds but not frequently or consistently. *Aphaenogaster* is the primary disperser and collects 74% of the myrmecochorous seeds encountered. It is 50% more likely to be observed foraging within a meter of a myrmecochore (Ness *et al.*, 2009). Species richness and myrmecochore density are both correlated with *Aphaenogaster* density. Even though the plants are not strictly obligated to the mutualism and there is not yet any documented evidence of co-evolution, Ness *et al.* (2009) speculate that *Aphaenogaster* is a keystone species in this ecosystem based on the observation that it provides dispersal services to an entire guild of plants.

Robert Paine (1966) described keystone species as those that are critical to the integrity, structure and diversity of the community. While Ness *et al.* (2009) show this to be the case for the myrmecochore guild in Southern Appalachian forests, to determine whether *Aphaenogaster rudis* complex plays a keystone role for the ecosystem as a whole, the investigation has to be expanded to include other interactions. This must be done in a piecemeal fashion, analyzing one or two interactions at a time because of the impracticality of excluding *Aphaenogaster* in

situ*.* A few other *Aphaenogaster* interactions have been studied in Eastern deciduous forests. *A.rudis* is a merciless predator of the termite, *Reticulitermes flavipes* (Buczkowski and Bennett, 2008). In laboratory tests, it caused 100% mortality in termite colonies. This could indirectly affect microbial diversity and activity if the termite colonies exhibit biological control of microbes in eastern deciduous forest soils as they do elsewhere (Fall, *et al*. 2004; Brauman, 2000). *Aphaenogaster tennesseensis* is a predator of the destructive oak borer, *Enaphalodes rufulusi* (Muilenburg *et al.*, 2008). Interspecies competition with other ants has also been observed (Smallwood, 1982).

Hypotheses: *Aphaenogaster* spp. and soil microbes

Because the microbial soil community plays such large role in the nutrient dynamics of Southern Appalachian forests, it becomes important to examine interactions between *Aphaenogaster* spp. and the soil flora. Frequent colony emigration makes it possible that these ants play a special “Prince Charming” role with respect to microbial activity in soils. Cultivable species of soil microbes have disparate metabolic rates when tested in the field and in the lab. Different metabolic strategies allow a diverse community of microbes to utilize a variety of substrates. Soluble, simple carbon compounds favor r-strategists but as these energy-rich nutrients are used up, k-strategists begin to compete and during late stages of decomposition, become dominant (Blagodatskaya *et al*., 2007). Because starvation in the field is responsible for the slow metabolic rates of r-strategists in oligotrophic

environments, when given the correct nutrient inputs, they will “wake up” and increase metabolic activity. Brown, *et al*. (2000) describe this so called “Sleeping Beauty” effect. In their study, it is earthworms that deliver the priming “kiss” of nutrient-rich mucus.

Bacterial communities in Southern Appalachian soils are highly diverse before disturbance (O’Connell *et al.*, 2007). Nest building by ants can be viewed as a disturbance and if the nests become enriched with easily utilized carbon substrates, the disturbance may result in dominance of r-strategist microbes over the oligotrophic k-strategists, thereby reducing diversity. It is possible that some secretion or fecal material of the ants may activate certain soil flora as the colonies are moved. The middens may also serve as suitable culture medium with enrichment coming from the organic debris. As demonstrated by McGinley, *et al*. (1994), ants create spatial heterogeneity in soil nutrient levels and can increase plant growth.

Fungal communities may be affected similarly by enrichment events. The fungi that employ r-strategies typically focus on rapid host colonization and may be favored initially but might not persist as nutrients are depleted and community succession favors the k-strategists (Lilleskov and Bruns, 2003). If there are biotic interactions between the *Aphaenogaster rudis* complex and soil microorganisms in Southern Appalachian forests, their description will contribute much to the understanding of temperate forest nutrient webs. This study uses molecular methods to compare microbial diversity in *Aphaenogaster* nests to that of bulk soil. Samples from two forest types are analyzed; Albright Grove, a rich cove dominated by mature

Hemlocks in Great Smoky Mountains National Park (GSMNP) and Shope Creek, a mesic oak-hickory community in the Pisgah National Forest in north-eastern Buncombe County, North Carolina, USA. High and low elevation sites were chosen at each location to determine if elevation enhances the effects of any ant-microbe interactions.

Hypothesis 1.

Microbial diversity will be lower in *Aphaenogaster* spp. nests than in bulk soil. Because of the priming effect of additional nutrients in the ant middens, r-strategists will be able to out-compete slower growing, oligotrophic organisms.

Hypothesis 2.

Elevation will increase the effect of ants on microbial diversity. Temperature and moisture regimes can be different at higher elevations. In Southern Appalachian forests, the soil is typically more oligotrophic at high elevation (Garten and Hanson, 2006). Any benefit of nutrient enrichments that ant nests may confer to fast growing organisms will be more pronounced.

Table 1. Carbon stores and residence times for each major biome. The numbers illustrate the extreme differences in carbon turnover rates. The biomes with short carbon residence times are facilitated by microbes. Adapted from Amundson (2001)

Biome	**Area** (10^{-12} m^2)	**Soil C** (kg/m^2)	**C Input** ($kg/m^2/y$)	**Residence Time** (years)
Tundra	8.8	21.8	0.102	213
Boreal moist forest	4.2	11.6	0.190	61
Temperate cool steppe	9.0	13.3	0.300	44
Temperate warm forest	8.6	7.1	0.896	9
Tropical savannah	24.0	5.4	0.479	11
Tropical forest	9.4	14.8	3.038	5
Global (including cultivated)	129.6	10.8	0.585	18.5

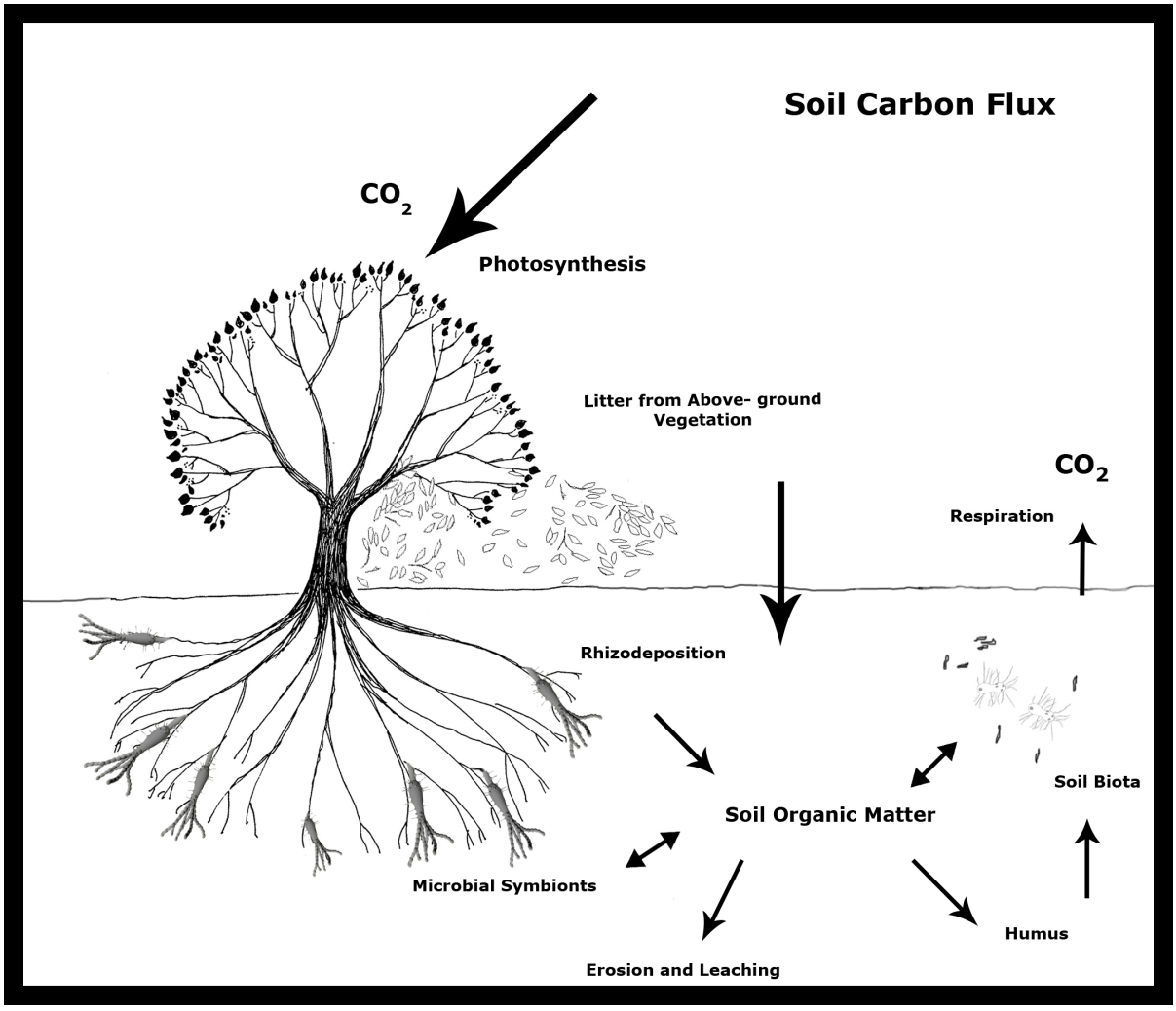


Figure 1. Diagram of carbon flux mechanisms and pools in soil showing that most carbon deposition to soils is from photosynthate. Most respired CO_2 is from microorganisms. The depiction of multiple interactions in this nutrient web, while complex, is still extremely simplified relative to the soil ecosystem. Adapted from Paterson *et al.* (2009)

Figure 2. Foraging *Aphaenogaster* ants encounter a *Sanguinaria* seed. The elaiosome is attached to seed coat. To attract ants, myrmecochorous plants offer a lipid-rich reward that mimics insect bodies. Photo used with permission from Alex Wild, 2011.

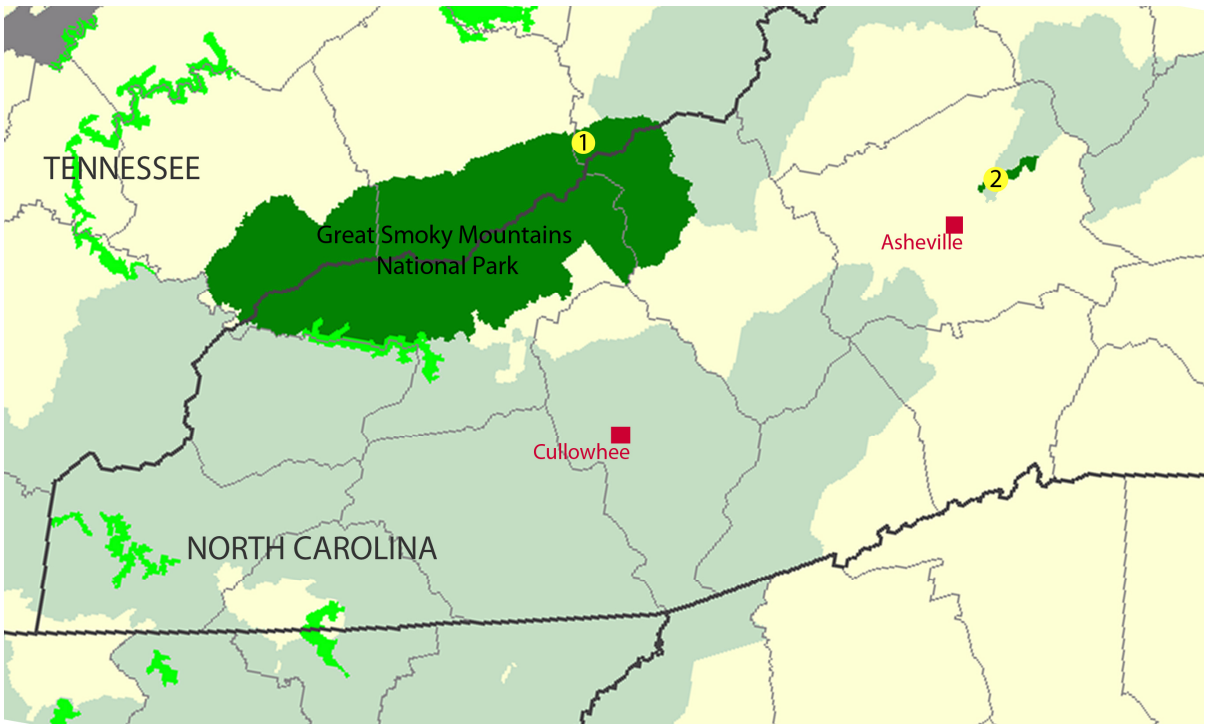


Figure 3. Map of sampling locations, 1, Albright Grove and 2, Shope Creek shown relative to Asheville, NC and Great Smoky Mountains National Park. Base layer imagery from the National Atlas of the United States (2012).

Table 2. Latitude and longitude of sample sites.

	Latitude (decimal degrees)	Longitude (decimal degrees)	Elevation (feet)
Albright Grove			
High Elevation	35.7337 N	83.2802 W	3332
Low Elevation	35.7568 N	83.2715 W	2371
Shope Creek			
High Elevation	35.6529 N	82.4287 W	3850
Low Elevation	35.6607 N	82.4315 W	3092

METHODS

Sample Collection

Soil samples were collected in July 2009 at high and low elevation sites in two forests (Fig. 3). Albright Grove is located near the north-east corner of Great Smoky Mountains National Park in Tennessee. It is classified as old-growth cove hardwood, though the high elevation site at N 35.7337^{o}, W 83.2802^{o} (Table 2) is dominated by Eastern Hemlock (*Tsuga canadensis*). The low elevation samples were collected at N 35.7568^{o}, W 83.2715^{o}, a location that is more representative of cove hardwood forests with large specimens of Tulip Poplar (*Liriodendron tulipifera)*, Magnolia (*Magnolia* acuminata) and Buckeye (*Aesculus octandra*). Both locations had a deep, undisturbed litter layer.

Shope Creek is a 1,600 acre section of the Pisgah National Forest eight miles north-east of Asheville. It is bounded by Asheville's Bee Tree watershed on the east and the Blue Ridge Parkway on the north. Soil samples were acquired in a mesic hickory-oak forest. The higher elevation site, N 35.6529^{o}, W 82.4287^{o} was in transition to a drier, hemlock-oak forest and had a shallow to non-existent litter layer. The low elevation samples from N 35.6607^{o}, W 82.4315^{o} were collected from a location with large hardwood trees and a moderate litter layer.

Samples were collected in triplicate for each of two conditions at all four locations. The variable condition was the presence or absence of *Aphaenogaster* spp. Ant nests were located by turning over rocks. Each replicate was from a

separate nest. The average depth of *Aphaenogaster* nests is 12-24cm (Smallwood, 1982). The chambers are frequently superficial and located just under rocks or logs. All ants collected were identified as *Aphaenogaster rudis* but in deference to Warren (2011) are recorded as members of the *fulva-rudis-texana* complex. Samples from soil with *Aphaenogaster* presence are called “Ant”.

For comparison of microbial diversity of the Ant soil to bulk soil, “Control” samples were collected from under rocks near each nest. Unoccupied soil was observed for two minutes to confirm absence of ants. All samples were obtained by a coring method of pushing a sterile 50 ml Falcon^{TM} tube into the soil to depth of 8cm. Capped sample tubes were immediately placed on dry ice for transport to the laboratory where they were stored at -70^{o} C until processing.

DNA Extraction

Soil samples were homogenized in the Falcon^{TM} storage tubes using a sterile stainless steel spatula. Approximately 0.5 grams of each homogenized sample was processed. Genomic DNA was extracted from all soil samples using the Mo Bio PowerSoil^{TM} DNA Isolation Kit (Mo Bio, Inc. Solano Beach, CA). The alternative lysis method, version 05182007, was followed except for step 5 of the Experienced User Protocol. Instead of vortexing for ten minutes, tubes were agitated in a bead beater for 60s at 2500 rpm.

PCR Amplification

Bacteria

The variable regions V3-V5 of the small subunit of the 16S bacterial ribosomal gene were amplified using the polymerase chain reaction and primer pair 341F (5′-CCT ACG GGA GGC AGC AG-3′) with a GC clamp (40-nucleotide GC-rich sequence added), and 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′, Casamayor *et al*., 2000). The GC-clamp is utilized when downstream applications include denaturing. The double stranded DNA will separate to the point of the clamp but not completely dissociate. Each 50 µl reaction solution contained 23 µl nuclease-free water, 25 µl Promega™ PCR Master Mix 2X (1.25 U *Taq* DNA polymerase, 400µM each: dATP, dGTP, dCTP, dTTP, 3mM $MgCl_2$), 0.25µM 341F/GC, 0.25µM 907R, one µl of DNA solution obtained from the MoBio™ extraction. Additional $MgCl_2$ was added to bring the final reaction concentration to 2 mM.

Thermocycler (Eppendorf Corp., Westbury, NY) protocol was as follows: initial denaturation, 5 min at 94°C then 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute starting at 65° and ending at 55°C, elongation for 3 minutes at 72°C with a final elongation of 7 min at 72°C. Amplification of correctly sized DNA segments vas verified by agarose electrophoresis using a 1KB DNA ladder (Promega, Inc., Madison, WI).

Fungi

The RNA of the small ribosomal subunit in eukaryotes has a Svedberg value of 18 (18S rDNA) and the internal transcribed spacer region (ITS) of the ribosomal DNA is used by researchers for resolving fungal phylogeny (Anderson, *et al*. 2003).

Because the recalcitrant nature of fungal cell walls prevents efficient DNA extraction, a nested PCR approach was used to amplify a large enough quantity of partial ITS gene fragments for DGGE analysis (Anderson *et al.*, 2003). Primer pair EF4 (5′-GGA AGG GAT GTA TTT ATT AG-3′) and ITS4R (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used in the initial round. Fifty microliter reaction solutions consisted of 13 µl nuclease-free water, 10 µl 25 mM $MgCl_2$, 0.25 µM (final concentration) of each primer, 25 µl Promega™ Master Mix 2x and 1 µl of $1x10^{-1}$ diluted DNA solution from the MoBio™ extraction. Cycling parameters were 94°C for 5 minutes then 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, elongation for 2 minutes at 72°C with a final elongation of 5 minutes at 72°C.

The second round of PCR used primer pairs ITS-1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) with a GC clamp and ITS-2R (5′-GCT GCG TTC TTC ATC GAT GC -3′) yielding fragments of ~300bp. Reaction chemistry and thermocycler conditions were the same as in round one except the template DNA (~ 800bp) was the undiluted product of the first reaction.

Separation and Analysis of DNA Fragments

Amplified bacterial and fungal gene fragments were separated using denaturing gradient gel electrophoresis (DGGE). This method employs the variation in G-C content between species to separate the DNA. Samples are loaded onto the top of a thin polyacrylamide gel with increasing concentrations of DNA denaturing urea/formamide. Because G-C base pairs have three hydrogen bonds and A-T base pairs have only two, the G-C rich sequences are more resistant to denaturation. One

millimeter thick acrylamide gels were poured with a concentration gradient of 20% to 60% urea/formamide. The upright 15-well gels were placed into a Bio-Rad DCode™ chamber (Bio-Rad, Life Science Research, Hercules, CA) filled with 1x TAE buffer. 20 µl of PCR product were loaded into each well with a 1kb Promega DNA ladder in each exterior well. 65 V of current was applied to the gels for 15 hours. After electrophoresis, gels were stained with a solution of ethidium bromide which bonds with DNA and fluoresces under UV light allowing visualization of separated bands.

UV photographs of gels were prepared for analysis using Photoshop© graphic software. After the 1kb ladders were used to properly align corresponding gel photos (Fig. 4.a), neon lines were placed across gel images to facilitate the counting of bands in each sample (Fig. 4.b).

The intensity of each band was assigned a discrete number from 0-6 that represented the population density of that species unit in the corresponding sample. Because the same ladder and electrophoresis conditions were used for all gels, comparisons can be made between treatments (Ant vs. Control), elevation and sites.

Statistical Analysis

Shannon Diversity

The Shannon Diversity Index (H') was calculated for each sample using the equation

$$H' = -\sum p_i(\ln p_i)$$

where

$$p_i = B / N$$

or the population density of a species, *i* in the sample of interest (B = band intensity) divided by the total number of individuals (N = total band intensity) in that sample. Evenness was calculated using the equation

$$E = H' / \ln S$$

where S equals the total number of species (DGGE bands) in each sample.

Other Statistical Analyses

Student's t-Test was used to compare the difference in means for evenness and community population size. Because the variances were statistically different for richness and Shannon Diversity between treatments, Welch's t-Test was employed. All were calculated using Microsoft Excel™. PC-ORD™ (MjM Software Design, Gleneden Beach, OR) was used to perform multi-response permutations. Principal components analysis (PCA) was performed in SYSTAT (Systat Software, Inc., Chicago, IL.)

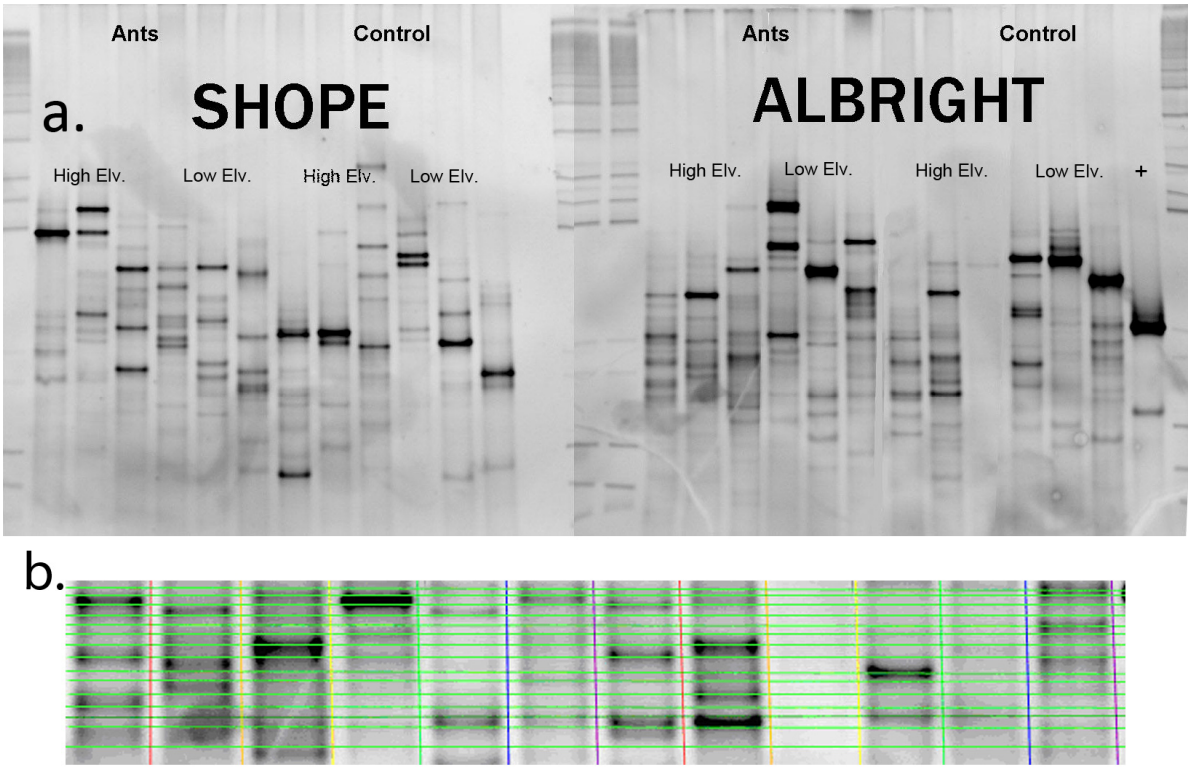


Figure 4. a.) DGGE gels of 300bp fungal ribosomal DNA aligned using exterior ladders. Lanes are labeled according to sample. The far right sample lane is a positive control. b.) Photoshop method of counting population of “species” in soil samples. Each green line identifies bands in different samples with the same G-C content.

RESULTS

Amplification of small subunit ribosomal gene fragments was successful for all 24 samples. DGGE provided separation of fungal DNA fragments in all samples except Albright Control, high elevation sample 3 (Fig. 4). Bacterial fragments were not resolved during denaturing gradient electrophoresis, even after several attempts using fresh concentrated samples (Fig 7). It has been suggested that the forest soil bacteria are so diverse that no species was populous enough for UV detection. This will be discussed in the following section.

Shannon Diversity

Fungal diversity in these Southern Appalachian forest soils increased significantly when ants were present ($p = 0.028$). Species richness was also significantly higher in the ant nest soil ($p = 0.032$). Evenness and total population were higher in the ant nests but not significantly so (Table 3). There is no difference in fungal diversity between overall high and low elevation samples ($p = 0.338$). The effect of increased diversity in ant nests is highly significant among the low elevation samples ($p= 0.003$) but not among the high elevation samples ($p = 0.371$).

Permutation Test

Because the individual data points used in these t-Tests were themselves statistics, a more robust test for determination of significance was desired. A permutation test performed using PC-ORD^{TM} software generated a value of p =

0.022 for the likelihood that the Ant and Control samples came from the same distribution. Also calculated, was the heterogeneity within treatments. The value determined, A = 0.007, reflects the high level of alpha diversity of the soil fungal communities.

Principal Components Analysis

Principal components analysis provided an interesting visual representation of the differences between treatments. Except for the high elevation Shope Creek site, the treatments were not discrete (Fig. 5). The first two factors separate the samples from Shope Creek slighly more than those from Albright Grove (Fig. 6) though the Albright Grove samples were more discrete relative to the variable considered in this study. The first two factors account for greater difference at both high elevation sites than the corresponding low elevation sites (Fig. 7). These components are not directly correlated to alpha diversity differences caused by *Aphaenogaster* and must represent some other factor because statistically, the diversity difference between Ant and Control samples was highly significant at low elevation ($p = 0.003$) but not significant at high elevation ($p = 0.371$). Contrary to the hypotheses, fungal diversity in *Aphaenogaster* spp. nests is significantly higher than in nearby bulk soil and is less strongly affected by ants at high elevation.

Table 3. The differences between Ant and Control samples was significant for diversity and richness but not for population or evenness. p-values are calculated for the diversity measures below based on the separation of fungal DGGE bands. Difference in diversity was highly significant at low elevations but not at high elevations.

	Ants (mean)	Control (mean)	p=
Diversity (H')	1.03	0.96	*0.028
High Elevation Diversity	1.01	0.99	0.371
Low Elevation Diversity	1.06	0.93	**0.003
Evenness (E)	0.75	0.72	0.122
Richness (S)	13.17	11.36	*0.032
Population (N)	24.09	22.45	0.170

* significant ** highly significant

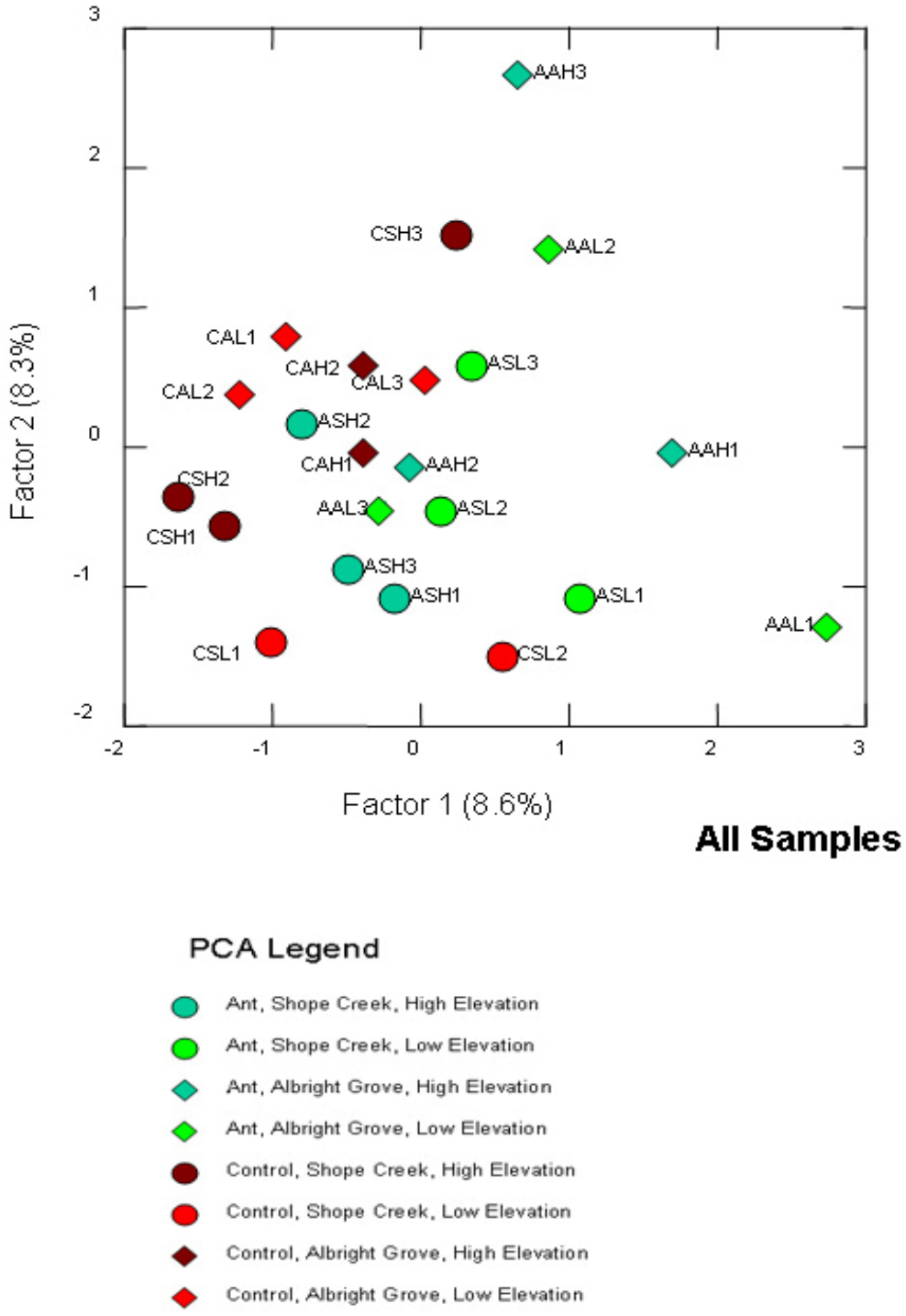
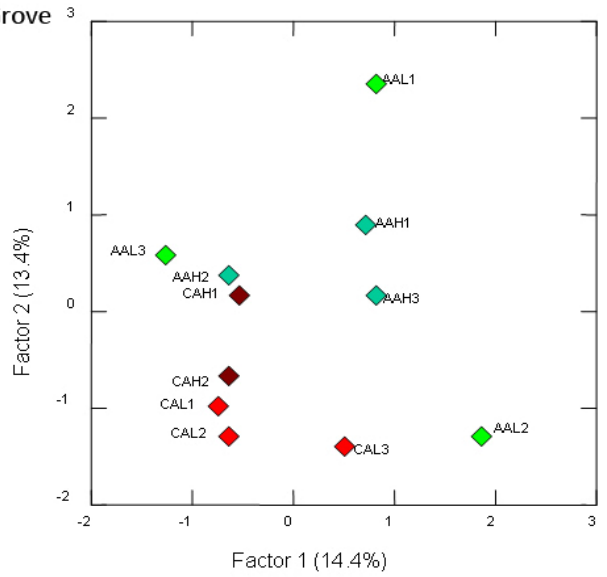


Figure 5. PCA graphs. Sample labels are as follows: 1st letter designates treatment, 2nd letter designates site, 3rd letter designates elevation. The high elevation control sample from Shope Creek would be labeled CSH. There is no data for CAH3. Across all treatments and locations, the first two factors explain only 17% of the difference between samples.

a. Albright Grove



b. Shope Creek

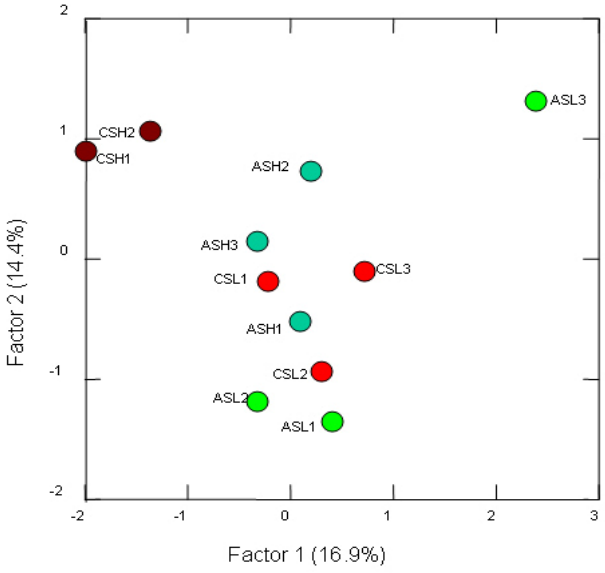
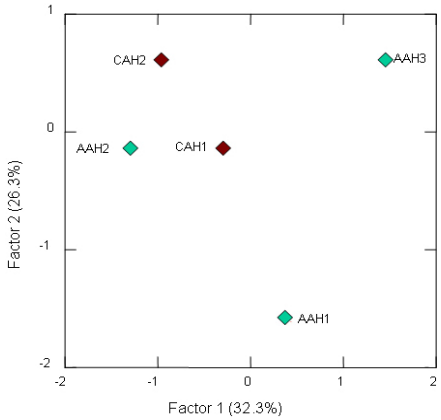
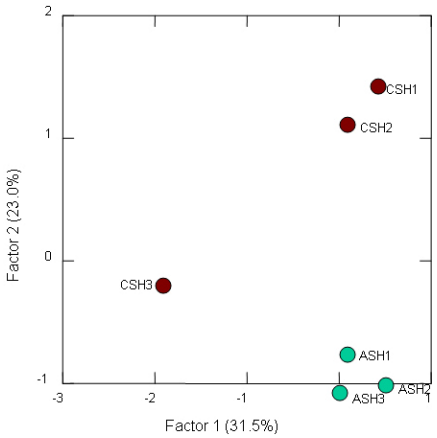


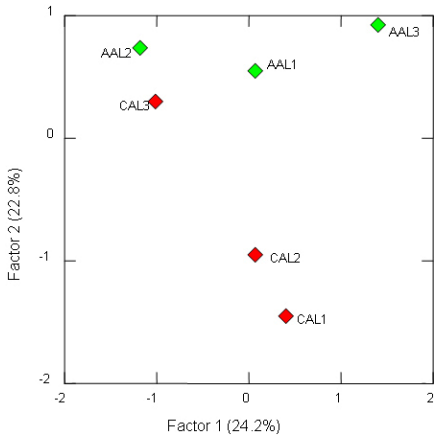
Figure 6. PCA graphs of all Albright Samples (a.) and all Shope Creek samples (b.) except for CSH3 which was an outlier.

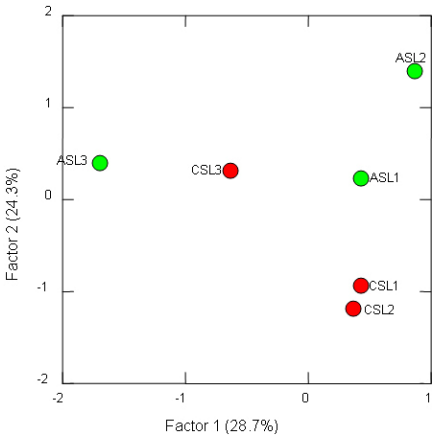
a. High Elevation





b. Low Elevation





Albright Grove

Shope Creek

Figure 7. PCA graphs showing single site grouping of samples. The best separation between samples is at this scale. The significance of the difference in diversity between treatments cannot be calculated at this scale because the number of samples is too few.

DISCUSSION

Keystone Mutualisms

A keystone species is one upon which the functioning and integrity of an ecosystem depend (Caro, 2010). But not all keystone interactions are mutualisms. In the classic case of Paine's (1966) sea star, *Pisaster ochraceus*, predation is the mode of interaction. The sea star is a voracious consumer of *Mytilius polymerus* which is the dominant macroinvertebrate in the intertidal zones of Mukkaw Bay, Washington State, USA. When Paine excluded the sea stars from study areas, the diversity of barnacles and other macroinvertebrates declined relative to the control areas. Because *Mytilius* can out-compete other species for space, the community structure of this intertidal zone is reliant on *P. ochraceus.* There are other examples of keystone predation. *Daphnia* graze on phytoplankton in Zaca Lake, in southern California, USA where they control community structure by increasing evenness among the phytoplankton species (Sarnelle, 2005). The elk of Yellowstone National Park, *Cervus elaphus,* are a major prey species of *Canus lupus* (Beschta, 2003). The elk can decimate cottonwood seedlings if wolves are excluded as they were from Yellowstone during the last century. When wolves are present, they check the elk population, allowing for cottonwood recruitment. This, in turn, stabilizes natural stream topography which is critical to ecosystem functioning (Camporeale and Ridolfe, 2006).

Keystone interactions frequently involve ants and in some ecosystems, the mode of interaction is predation. The army ants of central African rainforests, *Dorylus wilverthi,* migrate while consuming invertebrates in the forest litter. There is also a host of “camp followers”, birds, mammals and invertebrates that prey upon the ants (Peters *et al*., 2011). Plant predation by the leaf cutter ant, *Atta cephalotes*, plays an important role in community dynamics in the lowlands of Costa Rica where ant colonies will clear vegetation from an area then move on, leaving a fertilized canopy gap suitable for new plant recruitment (Perfecto and Vandermeer, 1993).

Most recent descriptions of ant interactions, however, involve mutualisms (Nelson, unpublished). The attine ants mentioned above are obligate mutualists with their food source, a fungus that they cultivate with their collected plant material (Mueller *et al.*, 2001). The ants maintain the axenic purity of their gardens through another mutualism with *Streptomyces*. The bacteria are housed on the ant exoskeleton and produce antibiotics that kill a specialized garden parasite *Escovopsis* (Currie *et al.*, 1999).

Many ant mutualisms involve seed dispersal. In South America, tank bromeliad seeds are collected by ants that build epiphytic carton nests. The ants position the seeds on top of their nests, where they germinate and grow, anchoring the ant nest to the tree (Corbara and Dejean, 1996). Ohnishi and Suzuki (2010) showed that the ant *Tetramorium tsushimae* not only disperses the non-myrmecochorous seeds of the euphorb, *Chamaesyce maculata,* but also protects the plant from herbivory by stinkbugs.

Many ant species involved in mutualisms are considered to be keystone species because they regulate the ecosystem in some important way. Frequently, there are multi-level mutualisms involved. This may also be the case in myrmecochory. This study demonstrates that a myrmecochorous ant increases fungal diversity in Southern Appalachian soils. This additional fungal diversity has benefits for plant diversity outside of the myrmecochore guild (van der Heijden *et al.*, 2008) and can provide a more varied diet for fungivores. Ness *et al.* (2009) argue that *Aphaenogaster* spp. is a keystone with respect to a guild of herbaceous plants. The data offered here broaden the scope of *Aphaenogaster* interactions to a level of ecosystem support that is characteristic of true keystone species.

The eastern temperate forests of North America are a hot spot of myrmecochore diversity. Lengyel *et al.* (2010) estimates that myrmecochory has evolved at least 100 times globally and suggests that the rate of myrmecochore diversification is twice that of non-myrmecochorous sister groups because of access to new habitats through ant dispersal. Warren *et al.* (2010) found no niche expansion provided by *Aphaenogaster* for *Hexastylis arifolia* based on the observation that the ants only utilize soil that is dryer than the optimum (30% soil moisture) for *Hexastylis.* This, however, does not contradict the hypothesis of Lengyal, *et al.* (2010).

Bacterial Diversity

The hypothesis that microbial diversity would be lower in ant nests than bulk soils was based, in part, on an understanding of bacterial metabolism strategies.

This study offers insufficient data to relate bacterial diversity in Southern Appalachian forest soils with the activities of *Aphaenogaster* spp. There has been research indicating that these soils host a very diverse bacterial community (O'Connell *et al.*, 2007 and York, 2008). Internal discussions have focused on the difficulty of statistically resolving the community fingerprints of extremely diverse populations. It has been suggested (O'Connell, personal communication) that the lack of bands resulting from DGGE separation of bacterial 16S DNA is a reflection of the extreme diversity and evenness of the soil community. Theoretically, if there were a very high number of rare species, the quantity of DNA at each band would be too low to detect. Several attempts were made to overcome the lack of visible bands in the bacterial DGGE gels. Double amplification of DNA and increased concentration of denaturing agents in the gels yielded a few bands but nothing sufficient for analysis (Fig 8).

Another study from GSMNP samples used terminal restriction fragment length polymorphism (T-RFLP) to fingerprint community structure (Dillow, 2009). This method, while useful for comparing the dominant species between sites, underestimates diversity because many rare species are lost. Chromatographic peaks that are not large enough to rise above the fluorescent detection of the size standards are discounted in analysis. It would be interesting, in retrospect, to run a T-RFLP analysis on the samples from this study to see if there would be a large number of small peaks among the standards.

Fungal Community Structure

Based on permutation results, the fungal diversity within each treatment is high. This means that many more samples would need to be analyzed before reaching an asymptote of species richness. For instance, of the 67 different species bands detected in the low elevation ant nest samples from Albright Grove, only seven were found in two of the three samples and no species were detected in all three.

R.H. Whittaker (1960) defined alpha diversity as the “richness in species of a particular community”. Beta diversity is the extent of change in community composition related to a change in local environmental factors. The significance value ($p = 0.028$) of the difference in diversity between Ant and Control samples reflects high beta diversity.

While fungal species richness is high in each sample in this study, reflecting high alpha diversity, it is probably grossly underestimated. Any species with small enough numbers to be undetected by DGGE or lost through PCR bias are not counted. Anderson *et al.* (2003) found little PCR bias when analyzing the ITS spacer region of fungal 18S rDNA. Because the same molecular methods were used across all samples in this study, any potential bias does not affect the statistical significance of the finding of difference in fungal diversity between ant nests and bulk soil.

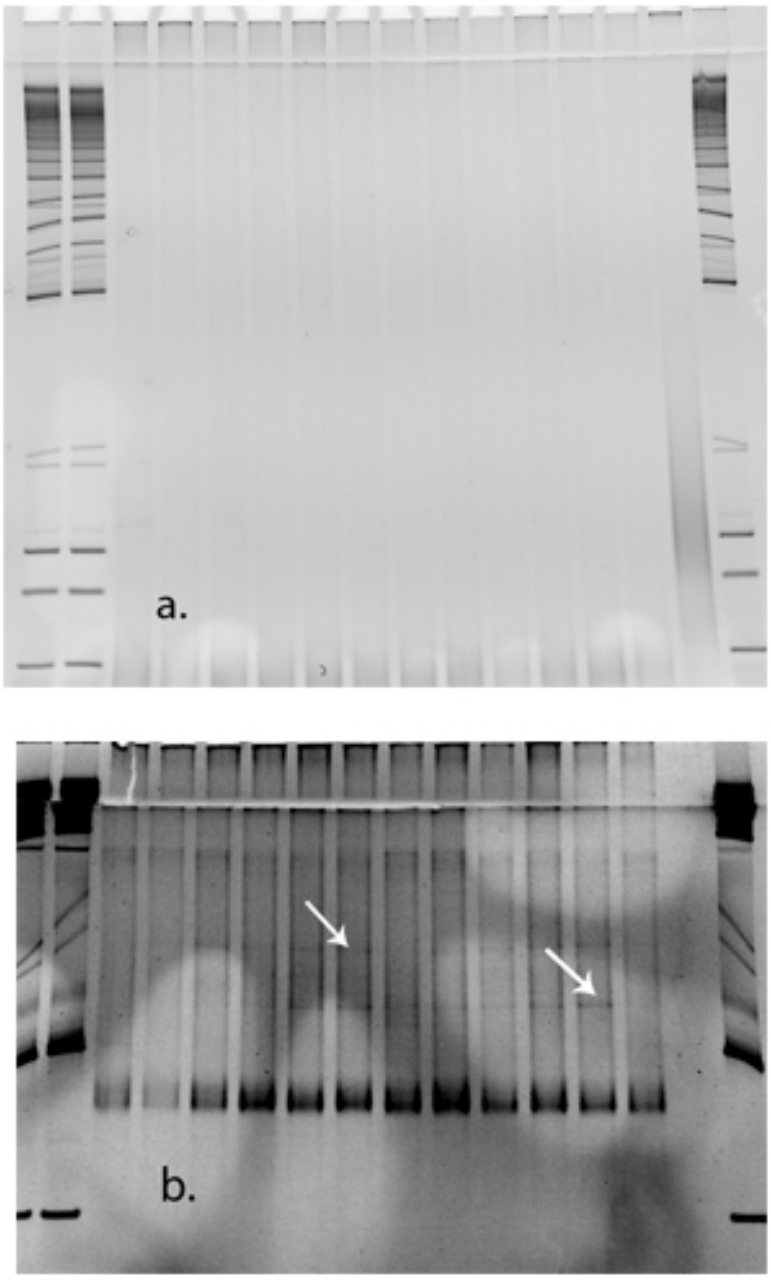


Figure 8. DGGE gels. These attempts to separate bacterial 16S DNA by G-C content were unsuccessful. Most of the gels were similar in appearance to (a). The gel in (b) was poured with a 50 -70 % gradient of urea-formamide. Note the few distinct bands and the large amount of DNA where, apparently, the concentration of urea-formamide was strong enough to break all hydrogen bonds.

CONCLUSION and FUTURE WORK

This study suggests that the activity of *Aphaenogaster* spp. in Southern Appalachian forest soils increases fungal diversity. The finding adds further support to the argument that *Aphaenogaster* is a critical, keystone component of this ecosystem. This study should be replicated with a much larger sample size and additional sites to verify the results. New studies should be conducted to examine possible *Aphaenogaster* interactions with other species and trophic groups.

Because *Aphaenogaster* ants frequently move their nests, temporal studies will determine how long the effect of increased diversity lasts after nest abandonment. This has implications for the level of spatial heterogeneity in forest soil nutrient levels. Community assembly patterns could be ascertained by studies involving placement of sterilized soil in the field. Ants would be excluded from controls but encouraged to build nests in experimental boxes. Consecutive samples from each treatment could elucidate any differences in microbial community succession.

Any future microbial work should take into account the extraordinary diversity of soil flora. Samples should be numerous enough so that species richness reaches an asymptote. The best way to achieve this would be high-throughput cloning or pyrosequencing (Brazelton *et al.*, 2011 and Hong *et al.*, 2011). These methods offer statistical as well as phylogenetic information. Microbial analysis of ant exoskeletons will also be informative. Soil chemistry should be analyzed to supplement molecular data. Functional studies of the microbial taxa affected by *Aphaenogaster* activity can

be performed in the lab if the species are easily cultured. Otherwise, isotope enrichment experiments will have to be conducted in the field to determine how each organism processes carbon and what the interactions contribute to the overall nutrient budgets of the system.

Because of the uncertainty of future environmental conditions, every effort should be made to elucidate the baseline interactions of this keystone ecosystem engineer. *Aphaenogaster* spp. occupies a central role in the food webs found in Southern Appalachian forest soils. Studies involving *Aphaenogaster* will facilitate better understanding of these complex systems.

REFERENCES

A'Bear AD, Boddy L, Raspotnig G, Jones TH. Non-trophic effects of orbatid mites on cord-forming basidiomycetes in soil microcosms. Ecological Entomology 2010;35(4):477-484.

Adl SM. The ecology of soil decomposition. Cambridge (MA): CABI publishing; 2003.

Amaranthus MP. The importance and conservation of ectomycorrhizal fungal diversity in forest ecosystems: lessons from Europe and the Pacific Northwest. 1998. United States Department of Agriculture. General Technical Report PNW-QTR-431.

Amundson R. The carbon budget in soils. Annual Review of Earth and Planetary Sciences 2001;29:535-562.

Anderson IC, Campbell CD, Prosser JI. Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. Environmental Microbiology 2003;5(1):36-47.

Beattie AJ, Culver DC, Pudlo RJ. Interactions between ants and the diaspores of some common spring flowering herbs in West-Virginia. Castanea 1979;44(3):177-186.

Beschta RL. Cottonwoods, elk, and wolves in the Lamar Valley of Yellowstone National Park. Ecological Applications 2003;13(5):1295-1309.

Blagodatskaya EV, Blagodatsky SA, Anderson TH, Kuzyakov Y. Priming effects in Chernozem induced by glucose and N in relation to microbial growth strategies. Applied Soil Ecology 2007;37(1-2):95-105.

Blankinship JC, Niklaus PA, Hungate BA. A meta-analysis of responses of soil biota to global change. Oecologia 2011;165(3):553-565.

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

Boulay R, Coll-Toledano J, Cerda X. Geographic variations in *Helleborus foetidus* elaiosome lipid composition: implications for dispersal by ants. Chemoecology 2006;16(1):1-7.

Brauman A. Effect of gut transit and mound deposit on soil organic matter transformations in the soil feeding termite: A review. European Journal of Soil Biology 2000;36(3-4):117-125.

Brazelton WJ, Sogin ML, Baross JA. Multiple scales of diversification within natural populations of Archaea in hydrothermal chimney biofilms. Environmental Microbiology Reports 2010;2(2):236-242.

Brown GG, Barois I, Lavelle P. Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. European Journal of Soil Biology 2000;36(3-4):177-198.

Bucher M. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytologist 2007;173(1):11-26.

Buczkowski G, Bennett G. Behavioral interactions between *Aphaenogaster rudis* (Hymenoptera : Formicidae) and *Reticulitermes flavipes* (Isoptera : Rhinotermitidae): The importance of physical barriers. Journal of Insect Behavior 2008;21(4):296-305.

Camporeale C, Ridolfi L. Riparian vegetation distribution induced by river flow variability: A stochastic approach. Water Resources Research 2006;42(10):13.

Caro T. Conservation by proxy. Washington (DC): Island Press; 2010.

Casamayor EO, Schafer H, Baneras L, Pedros-Alio C, Muyzer G. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: Comparison by microscopy and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 2000;66(2):499-508.

Corbara B, Dejean A. Arboreal nest building and ant-garden initiation by a ponerine ant. Naturwissenschaften 1996;83(5):227-230.

Currie CR, Scott JA, Summerbell RC, Malloch D. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 1999;398(6729):701-704.

Denison RF, Kiers ET. Life history of symbiotic rhizobia and mycorrhizal fungi. Current Biology 2011;21(18):R775-R785.

Dillow, CT. 2009. Diversity patterns of 16S rDNA of bacteria and *amoa* of Archaea and Bacteria from Eastern Hemlock (*Tsuga canadensis*) rhizosphere soil. [Master's Thesis]. [Cullowhee (NC)]: Western Carolina University.

Dilly O. Regulation of the respiratory quotient of soil microbiota by availability of nutrients. Fems Microbiology Ecology 2003;43(3):375-381.

Drigo B, Van Veen JA, Kowalchuk GA. Specific rhizosphere bacterial and fungal groups respond differently to elevated atmospheric CO_2. Isme Journal 2009;3(10):1204-1217.

Dunn RR, Parker CR, Sanders NJ. Temporal patterns of diversity: Assessing the biotic and abiotic controls on ant assemblages. Biological Journal of the Linnean Society 2007;91(2):191-201.

Elser JJ, Fagan WF, Kerkhoff AJ, Swenson NG, Enquist BJ. Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. New Phytologist 2010;186(3):593-608.

Fall S, Nazaret S, Chotte JL, Brauman A. Bacterial density and community structure associated with aggregate size fractions of soil-feeding termite mounds. Microbial Ecology 2004;48(2):191-199.

Fellers JH. Interference and exploitationin a guild of woodland ants. Ecology 1987;68(5):1466-1478

Ferrol N, Barea JM, Azcon-Aguilar C. The plasma membrane H(+)-ATPase gene family in the arbuscular mycorrhizal fungus *Glomus mosseae*. Current Genetics 2000;37(2):112-118.

Fischer RC, Olzant SM, Wanek W, Mayer V. The fate of *Corydalis cava* elaiosomes within an ant colony of *Myrmica rubra*: elaiosomes are preferentially fed to larvae. Insectes Sociaux 2005;52(1):55-62.

Frey SD, Spatial distribution of soil organisms. In: Paul EA, editor. Soil microbiology, ecology and biochemistry. Burlington (MA): Academic Press; c2007. p. 283-300.

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai ZC, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science 2008;320(5878):889-892.

Gange AC, Brown VK, Aplin DM. Ecological specificity of arbuscular mycorrhizae: evidence from foliar- and seed-feeding insects. Ecology 2005;86(3):603-611.

Garten CT, Hanson PJ. Measured forest soil C stocks and estimated turnover times along an elevation gradient. Geoderma 2006;136(1-2):342-352.

Gehring C, Bennett A. Mycorrhizal fungal-plant-insect interactions: the importance of a community approach. Environmental Entomology 2009;38(1):93-102.

Giladi I. Choosing benefits or partners: a review of the evidence for the evolution of myrmecochory. Oikos 2006;112(3):481-492.

Haselwandter K. Structure and function of siderophores produced by mycorrhizal fungi. Mineralogical Magazine 2008;72(1):61-64.

Henson BJ, Watson LE, Barnum SR. The evolutionary history of nitrogen fixation, as assessed by *Nif*D. Journal of Molecular Evolution 2004; 58(4):390-399.

Hobbie EA. Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. Ecology 2006;87(3):563-569.

Hoffman MT, Arnold AE. Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. Applied and Environmental Microbiology 2010;76(12):4063-4075.

Hong PY, Croix JA, Greenberg E, Gaskins HR, Mackie RI. Pyrosequencing-based analysis of the mucosal microbiota in healthy individuals reveals ubiquitous bacterial groups and micro-heterogeneity. Plos One 2011;6(9).

Horwath W, Carbon cycling and formation of soil organic matter. In: Paul EA, editor. Soil microbiology, ecology and biochemistry. Burlington (MA): Academic Press; c2007. p 303-340

Hughes L, Westoby M, Jurado E. Convergence of elaiosomes and insect prey - evidence from ant foraging behavior and fatty-acid composition. Functional Ecology 1994;8(3):358-365.

Hyodo F, Kohzu A, Tayasu I. Linking aboveground and belowground food webs through carbon and nitrogen stable isotope analyses. Ecological Research 2010;25(4):745-756.

Jansson PE, Berg B. Temporal variation of litter decomposition in relation to simulated soil climate - long-term decomposition in a Scots pine forest. Canadian Journal of Botany-Revue Canadienne De Botanique 1985;63(6):1008-1016.

Johansen A, Jakobsen I, Jensen ES. Hyphal transport by a vesicular-arbuscular mycorrhizal fungus of N applied to the soil as ammonium or nitrate. Biology and Fertility of Soils 1993;16(1):66-70.

Kennedy P. Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. New Phytologist 2010;187(4):895-910.

Lavelle P, Blanchart E, Martin A, Martin S, Spain A, Toutain F, Barois I, Schaefer R. A hierarchical model for decomposition in terrestrial ecosystems - application to soils of the humid tropics. Biotropica 1993;25(2):130-150.

Lengyel S, Gove AD, Latimer AM, Majer JD, Dunn RR. Convergent evolution of seed dispersal by ants, and phylogeny and biogeography in flowering plants: A global survey. Perspectives in Plant Ecology Evolution and Systematics 2010;12(1):43-55.

Lessard JP, Dunn RR, Parker CR, Sanders NJ. Rarity and diversity in forest ant assemblages of Great Smoky Mountains National Park. Southeastern Naturalist 2007;6:215-228.

Lilleskov EA, Bruns TD. Root colonization of two ectomycorrhizal fungi of contrasting life history strategies are mediated by addition of nutrient organic patches. New Phytologist 2003;159(1):141-151.

Long X, Chen CR, Xu ZH, Oren R, He JZ. Abundance and community structure of ammonia-oxidizing bacteria and archaea in a temperate forest ecosystem under ten-years elevated CO2. Soil Biology & Biochemistry 2012;46:163-171.

Maser C, Nussbaum RA, Trappe JM. Fungal small mammal interrelationships with emphasis on Oregon coniferous forests. Ecology 1978;59(4):799-809.

Massicotte HB, Melville LH, Peterson RL, Unestam T. Comparative studies of ectomycorrhiza formation in *Alnus glutinosa* and *Pinus resinosa* with *Paxillus involutus.* Mycorrhiza 1999;8(5):229-240.

McGinley MA, Dhillion SS, Neumann JC. Environmental heterogeneity and seedling establishment - ant plant-microbe interactions. Functional Ecology 1994;8(5):607-615.

Mueller UG, Schultz TR, Currie CR, Adams RMM, Malloch D. The origin of the attine ant-fungus mutualism. Quarterly Review of Biology 2001;76(2):169-197.

Muilenburg VL, Goggin FL, Hebert SL, Jia LL, Stephen FM. Ant predation on red oak borer confirmed by field observation and molecular gut-content analysis. Agricultural and Forest Entomology 2008;10(3):205-213.

Ness JH, Morin DF, Giladi I. Uncommon specialization in a mutualism between a temperate herbaceous plant guild and an ant: are *Aphaenogaster* ants keystone mutualists? Oikos 2009;118(12):1793-1804.

O'Connell SP, York EA, Collins MB, Rosbach DT, Black KR, Haney WB. An initial inventory of bacteria found within the soils and waters of Great Smoky Mountains National Park. Southeastern Naturalist 2007;6:57-72.

Ohnishi YK, Suzuki N. Seed predator deterrence by seed-carrying ants in a dyszoochorous plant, *Chamaesyce maculata* L. Small (Euphorbiaceae). Population Ecology 2001;53(3):441-447.

Paine RT. Food web complexity and species diversity. American Naturalist 1966;100(910):65-75.

Paterson E, Osler G, Dawson LA, Gebbing T, Sim A, Ord B. Labile and recalcitrant plant fractions are utilised by distinct microbial communities in soil: Independent of the presence of roots and mycorrhizal fungi. Soil Biology & Biochemistry 2008;40(5):1103-1113.

Peck RW, Niwa CG. Longer-term effects of selective thinning on microarthropod communities in a late-successional coniferous forest. Environmental Entomology 2005;34(3):646-655.

Perfecto I, Vandermeer J. distribution and turnover rate of a population of atta-cephalotes in a tropical rain-forest in Costa-Rica. Biotropica 1993;25(3):316-321.

Peters MK, Lung T, Schaab G, Wagele JW. Deforestation and the population decline of the army ant *Dorylus wilverthi* in western Kenya over the last century. Journal of Applied Ecology 2011;48(3):697-705.

Rantalainen ML, Haimi J, Fritze H, Pennanen T, Setala H. Soil decomposer community as a model system in studying the effects of habitat fragmentation and habitat corridors. Soil Biology & Biochemistry 2008;40(4):853-863.

Raven PH, Evert RF, Eichhorn SE. Biology of plants. 6th ed. New York (NY): W.H. Freeman and Company; 1999.

Rigamonte TA, Pylro VS, Duarte GF. The role of mycorrhization helper bacteria in the establishment and action of ectomycorrhizae associations. Brazilian Journal of Microbiology 2010;41(4):832-840.

Robertson GP, Groffman PM. Nitrogen transformations. In: Paul EA, editor. Soil microbiology, ecology and biochemistry. Burlington (MA): Academic Press; c2007. p. 341-364.

Sarnelle O. Daphnia as keystone predators: effects on phytoplankton diversity and grazing resistance. Journal of Plankton Research 2005;27(12):1229-1238.

Schussler A, Schwarzott D, Walker C. A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycological Research 2001;105:1413-1421.

Smallwood J. The effect of shade and competition on emigration rate in the ant *Aphaenogaster rudis*. Ecology 1982;63(1):124-134.

Smallwood J, Culver DC. Colony movements of some North-American ants. Journal of Animal Ecology 1979;48(2):373-382.

Smith SE, Read DJ. Mycorrhizal symbiosis.3rd ed. New York (NY): Academic Press; 2008.

Stamets P. Mycelium running, Berkeley (CA): Ten Speed Press; 2005.

Taylor JW, Berbee ML. Dating divergences in the Fungal Tree of Life: review and new analyses. Mycologia 2006;98(6):838-849.

Tinker PB, Durall DM, Jones MD. Carbon use efficiency in mycorrhizas - theory and sample calculations. New Phytologist 1994;128(1):115-122.

van der Heijen MGA. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 2008;11(6):651-651.

van Hees PAW, Rosling A, Essen S, Godbold DL, Jones DL, Finlay RD. Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with *Pinus sylvestris*. New Phytologist 2006;169(2):367-377.

Warren RJ, Giladi I, Bradford MA. Ant-mediated seed dispersal does not facilitate niche expansion. Journal of Ecology 2010;98(5):1178-1185.

Warren RJ, McAfee P, Bahn V. Ecological differentiation among key plant mutualists from a cryptic ant guild. Insectes Sociaux 2011;58(4):505-512.

Whittaker RH. Vegetation of the Siskiyou Mountains, Oregon and California. Ecological Monographs 1960;30(3):280-338.

York EA. 2008. Characterization of bacterial assemblages from the rhizosphere of Eastern Hemlock (*Tsuga canadensis*) using culturing methods and molecular cloning. [Master's Thesis]. [Cullowhee (NC)]: Western Carolina University.

Zelikova TJ, Dunn RR, Sanders NJ. Variation in seed dispersal along an elevational gradient in Great Smoky Mountains National Park. Acta Oecologica-International Journal of Ecology 2008;34(2):155-162.

Zhang WX, Hendrix PF, Snyder BA, Molina M, Li JX, Rao XQ, Siemann E, Fu SL. Dietary flexibility aids Asian earthworm invasion in North American forests. Ecology 2010;91(7):2070-2079.