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MICROBIOME ANALYSIS OF TWO SYMPATRIC FUNGUS-GARDENING ANTS, *TRACHYMYRMEX SEPTENTRIONALIS* AND *TRACHYMYRMEX TURRIFEX*

by

MATTEA ALLERT

A thesis/ dissertation submitted in partial fulfillment
of the requirements for the degree of
Masters of Science
Department of Biology

Jon Seal, Ph.D., Committee Chair

College of Arts and Sciences

The University of Texas at Tyler
May 2017

The University of Texas at Tyler
Tyler, Texas

This is to certify that the Master's Thesis/Doctoral Dissertation of

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has been approved for the thesis/dissertation requirement on

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Abstract

MICROBIOME ANALYSIS OF TWO SYMPATRIC FUNGUS-GARDENING ANTS, *TRACHYMYRMEX SEPTENTRIONALIS* AND *TRACHYMYRMEX TURRIFEX*

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Thesis/dissertation Chair: Jon Seal, Ph.D.

The University of Texas at Tyler
August 2017

The fungus gardening-ant system is considered a complex, multi-tiered symbiosis between the ants, their fungus, and their corresponding microbes. We examine the bacterial microbiome of *Trachymyrmex septentrionalis* and *Trachymyrmex turrifex* ants and their corresponding fungus, using 16S rRNA, over a large geographical region to determine if horizontal transmission was occurring. The goals of this study was to determine how the ant microbiome was transmitted and how the fungus microbiome was transmitted. We determined that the microbiomes of *T. septentrionalis* and *T. turrifex* ants were different because of the species, while the microbiomes of *T. septentrionalis* and *T. turrifex* fungi were spatially structured and were not determined by the species of ant growing them but the region in which the fungus resided. The most abundant bacterial orders found with *T. septentrionalis* ants were Actinomycetales, Soilrubrobacterales, Xanthomonadales, and Burkholderiales. In *T. turrifex* ants the most abundant bacteria found were Actinomycetales, Entomoplasmatales, and Burkholderiales. The most abundant bacteria associated with the Central Texas fungus gardens, regardless of the ant species growing it, were Entomoplasmatales, Streptophta, and Enterobacteriales. The most abundant bacterial orders in East Texas fungus was Entomoplasmatales and Streptophta.

Chapter 1

Introduction and General Information

Introduction

Microbiomes are a communities of microorganisms existing in the same place at the same time (Robinson et al., 2010). Since the advancement in sequencing technologies, microbiome research has accelerated (Giovannoni et al., 1990; McFall-Ngai et al., 2013; Lloyd-Price et al., 2016). Recently, studies like the Human Microbiome Project have substantially contributed to our understanding of the interactions that are occurring in the human microbiome (Peterson et al., 2009). Over 25,000 studies that looked at various aspects of the microbiome in a variety or taxa have been published (Lloyd-Price et al., 2016). Studying the microbiome enables researchers to gain insights into various organism systems and functions.

Microbiomes are either transmitted vertically or horizontally, meaning organisms acquire it from their parents or from the environment (Berrington et al., 2014; Putignanil et al., 2014). Often it is collected from both transmission methods. For humans, the initial microbiome is obtained from the mother, while the fetus is *in vivo* (Aagaard et al., 2014), with subsequent influences from mother's mile (mother's diet) and the child's diet (Filippos et al., 2010; Ley et al., 2008; Jain et al., 2014). This shows that the development of the human microbiome is complex.

A deviation from the normal microbiome for example, by taking antibiotics or by experiencing a dietary shift, may have negative impacts on human health. Luoto et al. 2011 compared the gastrointestinal microbiome of healthy children to that of obese children, and

found that the obese children had lower levels of *Bifidobacterium* when they were infants compared to their healthy peers, indicating that seemingly minute changes in the microbiome can have important downstream consequences.

Similar to humans, the microbiomes of insects are integral to their health. Insects maintain a stable microbiome, with vital components (Hansen and Moran 2013; Okayama et al., 2016; Raymann et al., 2017; Ahmed et al., 2016). They can acquire their microbiome either horizontally or vertically (Hosokawa et al., 2006; Huang et al., 2013, Cordaux et al., 2001; Ahmed et al., 2016; Kikuchi et al., 2007). Insects demonstrate a variety of interactions with their microbes, and understanding these interactions can help researchers better understand the biological world.

Ants are able to form complex social relationships by interacting with ants in the same caste and with other ant castes, as well as with the external environment. A fundamental question is how ants are able acquire proper nutrition and maintain their abundance (Hunt and Nalepa, 1994). Ants tend to have nutrient poor food sources (Bluthgen et al., 2003; Davidson et al. 2003), yet they are some of the most abundant insects on earth (Pisarski, 1978; Majer, 1990; Stork, 1987; Watanabe and Ruaysoongnern, 1989). This phenomenon can be observed in the *Tetraponera nigra* species group, which has a pouch structure between its midgut and intestine (Borm et al., 2002). The ouch is filled with a dense microbiome that enables the ants to fix nitrogen, which has allowed them to flourish in nutrient poor environments (Borm et al., 2002), noting that not every bacterium found in an organism has a function (Neuvonen et al., 2016). Interestingly, ants are able to harbor host-specific bacteria. A study was conducted that examined *Entomoplasmatales* in Army ants; the researchers found the *Entomoplasmatales* that they harbored were specific

to the Army ant (Funaro et al., 2010). This study indicates that microbiome data can be a tool to discover ancient associations between insects and the bacteria they harbor. It can even give insight into how ants and their microbes coevolved (Anderson et al., 2012). The microbiomes of insects play a role in their defense. For example, attine ants are able to secrete antimicrobial compounds though their metaplural glands to combat parasitic fungus in their fungal garden (Ortius-Lechner et al., 2000; Bot et al., 2002). This ultimately provides the ants with protection because their food source is being protected (Mattoso, Moreira & Samuels, 2012).

Fungus-gardening ants (Hymenoptera: Formicidae: Attini) exhibit a highly integrated symbiosis with certain fungi (Agaricales: mostly Lepiotaceae: Leucocoprineae) which provide them with food (Currie, 2001). When this mutualistic and symbiotic phenomenon was discovered, it was thought to be a one-to-one interaction, where the ants gave fresh leaves to the fungus and the fungus broke the leaves down into a usable energy source for the ants. However, recently, it has been revealed that microbes play a significant role in this interaction (Currie and Scott, 1999; Currie et al., 2003; Currie et al., 2006). However, it is worth noting that not all microbe interactions in the fungus-gardening ant system are beneficial. The mutualistic relationship of fungus gardening-ants has been ongoing for the last 50 million years (Schultz and Brady, 2008; Schultz et al*.,* 2015). The ants provide the fungus with fresh leaves, and the fungus breaks down the leaves providing both themselves and the ants with essential nutrients (Weber, 1972). Not only are these ants a prime example of symbiosis; they are also one of the few organisms that have mastered agriculture (Schultz and Brady, 2008).

Because fungus gardening-ants have been around for the last 50 million years (Schultz and Brady, 2008; Nygaard et al., 2016), they have had time to evolve extensive fungal agricultural systems and foster the intricate relationship between themselves and their fungus. Agriculture in ants only developed once, but is present in five ant systems (Schultz and Brady, 2008). Leaf cutter ants are the most recently evolved (~8-12 mya), yet are the most advanced in terms of their agriculture system (Schultz and Brady, 2008; Nygaard et al., 2016). Because these ants are evolutionarily young, they have genetically similar fungus, most of the fungus being from the same species (Mueller et al., 2010; Schultz and Brady, 2008). There are two major types of fungus gardening-ants: lower attine farmers and higher-attine farmers. Higher attine ants have an obligate symbiotic relationship with their fungus, meaning one cannot survive without the other, while lower attine ants can have fungus that is able to live independently, but the ants are obligately dependent on the fungus (Schultz and Brady, 2008; Nygaard et al., 2016). Most attine ants cultivate a highly specialized fungus. Though it is important to note specific ant species may grow specific fungal genotypes and not every colony in the same species grows the same fungal genotype. *Mycocepurus smitthii* is known to grow many fungal lineages the ants were able to grow different fungal genotypes (Kellner et al., 2013).

Fungus gardening-ants have a complex relationship with their microbiome. It was shown that they play a major role in everyday animal interactions: it was found that microbial communities were mainly responsible for breaking down and converting plant material into usable nutrients for their hosts (Ley et al., 2008; Warnecke et al., 2007; Distel et al, 1997). A study that examined the microbiome of *M. smithii*, a lower attine ant, found the ants and fungus microbiome communities were not distinctly different from each other,

but geographic location appeared to have a profound effect on their microbiome (Kellner et al., 2015). One study showed that different species of higher attine ants have a highly similar microbiome to composting communities which was mainly composed of gram negative bacteria (Scott et al., 2010). Limited evidence suggests that the fungus of the fungus gardening-ants has a core microbe community comprised of the genera's *Enterobacter*, *Pantoea*, *Klebsiella, Citrobacter,* and *Escherichia* (Aylward et al., 2012). Nitrogen is a limiting factor in fungus growing-ant colonies, in order to cope with this shortage, the ants must have an interaction with nitrogen fixing bacteria. Pinto-Tomas et al. 2009 tested this by examining the input of nitrogen (nitrogen from fresh leaves) and comparing it to the nitrogen in the fungus garden and the ant's refuse dump (Pinto-Tomas et al., 2009). This study demonstrates that nitrogen fixing bacteria are present in the ant's fungus-gardens (Pinto-Tomas et al., 2009).

In addition to microbes being in the fungus garden cultivar, microfungal species (fungal microorganisms that live in the fungal cultivar) are found in the fungus as well. Some of these microfungal species can be benign, while others, such as *Escovopsis*, can parasitize the ants' fungus garden (Fernandez-Marin et al., 2009). Ants are able to combat these parasitic micro-fungi by secreting broad-spectrum antibiotics from their metapleural gland (Bot et al., 2002; Fernandez-Marin et al., 2006), grooming the fungus, and using another type of antibiotic, *Pseudonocardia* (Currie et al., 1999a, 2003b). Little and Currie (2007), found black yeast on the the cuticle of fungus gardening-ants, which adds to the already complex symbiotic system (Little and Currie, 2007). Because fungus gardening ants have such a significant influence on the micro-fungal community in their garden, the ants have to potential to be influencing their microbial community as well. Microbes have

been studied in other fungus-gardening ants including *Trachymymex septentrionalis* (Ishak et al., 2011), where Ishak et al. (2011) looked at the microbe community of the same colony of ant over a period of time. These species of ants are found from east and central Texas to Louisiana, and beyond in Florida and the southeastern coastal plain (Seal et al*.,* 2015). Looking at the microbiome communities of two species over a larger geographical scale will give us insight on how they acquire their microbes, how they interact with their environments, and if the different ant species are able to maintain a separate microbiome.

Previous research examined the microbial communities of *Atta colombica* and *Atta cephalotes*, where researchers noted that only a few bacterial genera made up the majority of the microbiome (Aylward et al*.,* 2012). The five main types of bacteria that they found made up 2/3rds of the sampled population, *Enterobacter, Klebsiella, Citrobacter, Escherirchia,* and *Pantoea* (Aylward et al*.,* 2012). This showed that the microbes they found in the fungus have been there throughout the fungus growing season, because of the bacteria that was found in all layers of the fungus (Aylward et al*.,* 2012). This implied that there was constant interaction between the microbes and the ants. One study examined the microbiome of *Atta texana* ants, more specifically looking at the microbiome of the different segments of the ant, the brood, and the fungal inocula (pellets). Most notably, researchers found if fungal inocula contained a high amount of *Mesoplasma*, the colony was more likely to decline (Meirelles et al., 2016). Ishak et al. (2011) examined the microbes present in the fungus gardening ants *Trachymyrmex septentrionalis*; the results indicate that *Pseudonocardia sp.*, *Kribbela sp.*, *Amycolatopsis sp.*, and *Streptomyces sp.* were most abundant in the fungus of *T. septentrionalis*. Ishak et al. (2011) further examined the bacteria found on the body segments of the ants; finding that *Carboxydirorans sp*, a

subgenus of *Pseudonocardia sp,* and *Streptomyces sp* were found on the body of all the ants except the queen and examined the body of the male ants and discovered that their body segments were primarily covered with *Amycolatopsis sp* (Ishak et al., 2011), which shows that even ants within the same colony have distinct microbiomes, and further suggests that different ant species harbor distinct microbial communities. Even though the same species of ants had similar microbial taxa, there were slight differences in their microbe community based on their role within the colony, as well as the body on the ant that was sampled on (Ishak et al., 2011). Although research in fungus gardening-ant microbiomes have been substantial, other than the study by Kellner et al. (2015) and Meirelles et al. (2016), most studies have not extensively examined how geography might influence the microbial communities.

Factors that influence the microbiome are possibly complex, thus making it is necessary to examine many possible factors. Currently, studies tend to emphasize which microbial communities are found in a particular species, but few explore factors that influence them (Ishak et al., 2011; Kellner et al., 2015; Meirelles et al., 2016). By examining how the environment can influence an organism's microbiome, scientists will better understand the underlying mechanisms involved in the transmission of specific bacteria.

To unravel what factors influence the microbiome of *T. septentrionalis* and *T. turrifex* ants and their symbiotic fungus, the following thesis examines the bacterial microbiome of the ants and symbiotic fungi of *T. septentrionalis* and *T. turrifex* across a portion of their shared ranges. Studying this will provide insight into what types of microbes reside in the fungus-gardening ant system and how they are acquired.

In the following thesis, I examined the bacterial microbiome of *T. septentrionalis* and *T. turrifex* ants and their symbiotic fungus. The broad goal of this descriptive study was to describe the ant-associated bacterial microbiome of both ant species and the fungus they grow. The first question asks if each ant species is associated with a distinct bacterial community. The second question investigates the bacterial microbiome of the fungus gardens grown by both species. I specifically tested whether bacterial microbiome composition was explained by 1) the ant species growing it or 2) by the genotype of the fungus. I also investigated whether bacterial microbiomes associated with the ants and fungus varied across geographic distances. Thus, we are able to partition variation in microbiome composition by ant species, fungal species, and region. My first hypothesis is that differences in ant species will drive bacterial microbiome composition in both species. *Trachymyrmex septentrionalis* belongs to the so-called '*septentrionalis'* lineage, which is sister to the high derived leaf-cutting ants, *Atta* and *Acromyrmex*, whereas *T. turrifex* is more distantly related and has close relatives that have tropical distributions (Rabeling et al., 2007; Seal et al., 2015). Furthermore, both of these species have been noticed to exhibit different behaviors in the laboratory and field (Seal and Mueller, 2014; Waller, 1989). My second hypothesis is that ant species in turn will drive (explain) the bacterial community of fungus gardens. Because vertical transmission is considered the general rule in higher fungus gardening ants (Ishak et al., 2011), I tested the hypothesis that ant and fungal microbiomes will exhibit little structure across the geographic range sampled. Nevertheless, regional or location differences in ant or fungal microbiomes may result from variation in soils or local plant communities which provide the substrates (fungus food) the ants are collecting, among other factors. Generally, positive

correlations among ant species and fungal genotype and microbiome composition would suggest vertical transmission of the microbiome. On the other hand, if elements of the fungal and ant microbiome are horizontally transmitted, we would expect correlations between microbial communities and region.

Chapter 2

Methods

Species

This study focuses on two fungus gardening ant species in the genus *Trachymyrmex* that co-occur in the Western Gulf Coastal Plain region of southeastern North America (Seal et al., 2015). *Trachymyrmex septentrionalis* (Hymenoptera: Formicidae: Myrmecine: Attini) is found throughout the Southern United States, from Florida to Texas (McCook, 1880; Rabeling et al., 2007; Seal and Tschinkel; 2006, Seal et al, 2015) as well as extending from Illinois to New Jersey and Long Island (Hölldobler and Wilson, 2010; Morris, 1881; Rabeling et al., 2007; Seal et al., 2015; Wheeler, 1907). *Trachymyrmex turrifex* is thought to have originated from Mexico, and expanded south into Texas and Louisiana (Seal et al., 2015; Rabeling et al., 2007). Both species grow fungus gardens of the species (Agaricales: mostly Lepiotaceae: Leucocoprineae) (Mikheyev et al., 2008; Mueller et al., 1998; Shultz and Brady, 2008; Nygaard et al., 2016) and nest in subterranean chambers (Rabeling et al., 2007). For this study, the ants were initially identified in the field using colony characteristics and behavioral responses. In the lab their identity was confirmed by using morphological methods (Rabeling et al., 2007). Because we cannot visually identify the fungus, samples were identified by DNA fingerprinting (White et al., 1990).

Study area

We collected samples of ants and fungus gardens of both species (*T. septentrionalis* and *T. turrifex*) from sites in two broad locations in central and northeastern Texas, extending from Tyler, Texas (approximately 32.29° N 95.24° W) to Bastrop, Texas (29.39°N 97.32°W). Ants were collected from central Texas (Bastrop and Brazos Counties)

and four sites in northeast Texas (Smith, Cherokee, Henderson, and Upshur Counties). Both species of ants co-occur at many of the same locations, (Figure 1, Table 1).

Sampling Strategy

We collected four to five ants from each colony of both species. We collected four to five ants for analysis because there is potentially considerable heterogeneity among the ants in the colony (Ishak et al., 2011). Ants from each colony were thus pooled to account for individual differences in their microbiomes. Ants were collected directly from inside fungus gardens with ethanol and flame-sterilized forceps, meaning that the ants collected were indoor workers (i.e., not foragers who could pick up bacteria inadvertently while outside the nest). An equal number of *T. septentrionalis* (12) and *T. turrifex* (11) colonies from our samples of East Texas and Central Texas populations were chosen (Table 1). A small sample fungus garden material was collected similarly with flame and ethanol sterilized forceps from same garden chambers where the ants were collected. Furthermore, we collected soil from within the fungus chambers as a negative control (which makes sure that any microbiome difference we find among ants or fungi is not an artifact of soil contamination). All samples were preserved immediately upon collection in 100% ethanol. We collected our samples prior to the mating flight period (May- July), because that is when the fungus gardens are the largest and the bacterial communities are most distinct (Seal & Tschinkel, 2008; Ishak et al., 2011a).

Molecular Methods:

DNA Extraction, PCRs and Sequencing of Microbiomes

DNA extraction and sequencing was performed at MR.DNA in Shallowater, Texas (http://www.mrdnalab.com/). DNA sequences were amplified from whole ants, fungus, and soil using primers Gray28F 5'GAGTTTGATCNTGGCTCAG and Gray519R 5'GTNTTACNGGGCKGCTG that span the V1-V3 hypervariable regions of the 16S rRNA gene. They were processed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After the samples were amplified and checked for adequate genetic yields, the sub-samples were pooled back together and purified using calibrated Ampure XP beads. The purified and pooled PCR product was used to create a DNA library and sequenced using the Illumina MiSeq platform in PEx300 mode.

Fungal Genotyping

Since the ants' fungus gardens cannot be visually identified, representative samples were DNA fingerprinted. Gongylidia (swollen hyphal tips diagnostic feeding structures of higher fungus-gardening ants) (De Fine Licht et al., 2014) were plucked off the fungus with flame-sterilized forceps, placed in an aqueous solution of Chelex, and heated in a thermal cycler (White et al., 1990; Mueller et al., 1998, Sen et al., 2009; Seal et al., 2012; Seal and Mueller, 2014; Seal et al., 2014).

Before PCR amplification, the DNA was diluted (1:10) using nuclease free water (higher concentrations of DNA inhibited PCR reactions). PCR and was preformed using the primers ITS 4 and ITS 5 to amplify the 18S rRNA ITS gene (White at al., 1990; Sen et al., 2009). The PCR products were sent to UT Austin's ICMB Core Facilities for Sanger sequencing.

Sequences were cleaned up and aligned in Geneious 10.1.2 (Kearse et al., 2012), using ClustalW Multiple. Sequencing errors or misreads in the DNA sequences were manually corrected. We analyzed the sequences using both population genetic and phylogenetic approaches (Posada and Crandall, 2001; Freeland, 2006). A Bayesian analysis was performed on the sequences in MrBayes (Huelsenbeck and Ronquist, 2001) to create a phylogenetic tree. We used jModeltest (Posada, 2008) to select the best-fitting model of HKY model using Bayesian Information Criterion. This model was applied in MrBayes (Huelsenbeck and Ronquist, 2001); the analysis was run for 5 million generations with a sampling frequency of 1000 (burnin = 1250). Then the samples were analyzed for population expansion, deviations from neutrality, and diversity comparisons in DnaSP (Rozas et al., 2010).

Microbiome analysis of ants, fungus and soils

Data processing

Initial sequence cleanup was performed by MR. DNA, who removed short sequences with <150 bp, sequences with ambiguous base calls, chimeras, sequences with runs exceeding 6 bp, and singleton sequences (Dowd et al., 2008) (http://www.mrdnalab.com). Sequences were processed using MacQIIME 1.9.1 (Caporaso et al., 2010b), the pipeline used can be found in the appendix. A quality score of 25 was applied and a length limitation of anything outside the bounds of 200 to 1000 was applied. In addition to that, a filter for maximum homopolymer that exceeded 6 was applied. In order to get the sequences ready for processing we had to reorganize the data files because it was a mixture of forward and reverse reads by generating a barcode file (extract_barcodes.py) and by splitting the libraries (split_libraries_fastq.py). A quality

score of 25 was applied and a length limitation of anything outside the bounds of 200 to 1000 was applied. In addition to that, a filter for maximum homopolymer that exceeded 6 was applied. Sequences were clustered based on 97% similarity (pick_open_reference_otus.py). One representative sample was chosen randomly from each OTU (core_diversity_analyses.py) and used to construct an OTU sharing matrix.

Statistical Approaches Community Diversity Analyses of Microbiomes

Taxonomic Diversity

To address taxonomic diversity, we examined the top taxonomic groups in the samples using Micca 1.5.1 (Albanese et al., 2015). The bacterial communities associated with each species was analyzed further using an indicator species analysis (Dufrene and Legendre, 1997), which examines the bacterial community differences between groups, and inform about the bacterial taxa that contribute most toward the overall variation. The VEGAN R package was used to analyze and identify the bacterial taxa and OTUs unique to each ant and fungal species (Kellner et al., 2015; Jari Oksanen et al., 2011).

Alpha Diversity

To address alpha diversity, we performed a rarefraction analysis on the observed OTUs, calculated Simpson's Diversity Index, and ran an Inverse Simpson's Diversity analysis. In addition to performing diversity indices, we performed richness and evenness tests. Each of the tests done was examined separately for the ants, fungus, and soil samples with the exception of the rarefraction analysis.

Beta Diversity

To address beta diversity, we used non-metric multidimensional scaling (NMDS) as an ordination method and used the Bray Curtis approach to look at the differences between the sample types and the ant and fungal species. This method w applies a ranked-based approach based on the dissimilarity of the beta diversity. To analyze the bacterial communities and environmental factors ANOSIM was used. ANOSIM provides an analysis of similarity that uses a distribution free method that analyzes the variation within the beta diversity matrix. The results were confirmed by using a False Discovery Rate (FDR) test (Benjamini and Hochberg, 1994). To further analyze the fungus, we ran a cluster analysis in MacQIIME 1.9.1 (Caporaso et al., 2010b) and visualized it using ETE 3 (Huerta-Cepas et al., 2016).

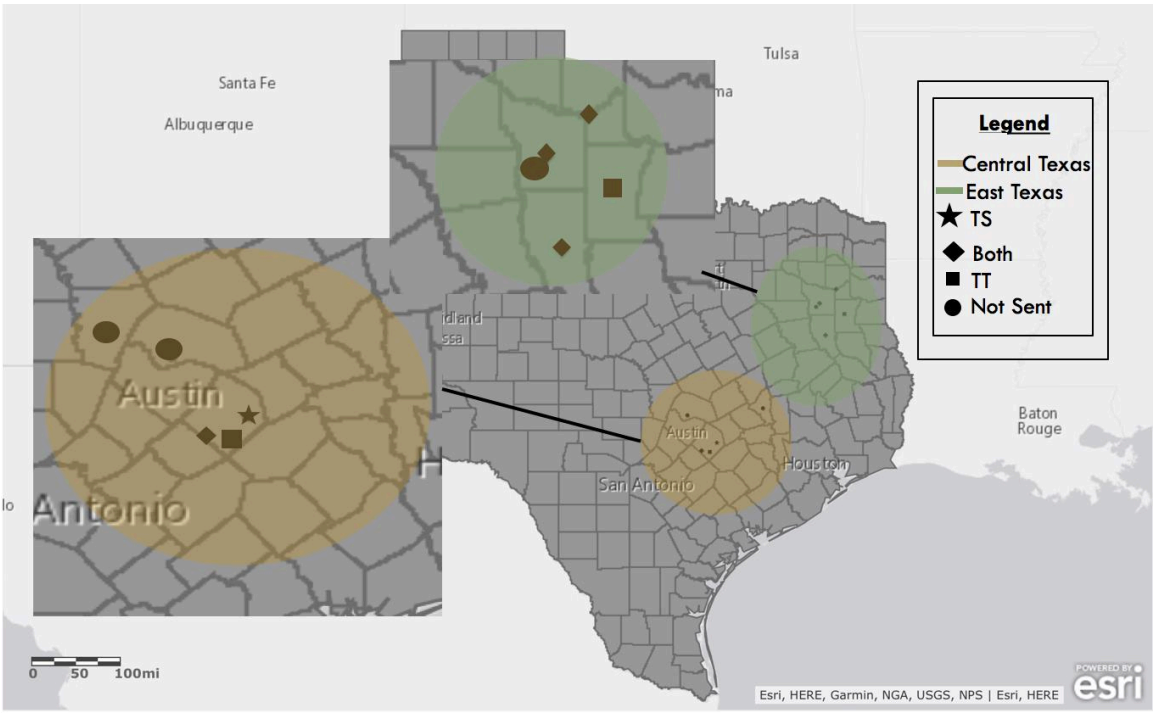

Figure 1: Overview of sampling sites. Displayed here are *T. septentrionalis* (TS), *T. turrifex* (TT) samples, both *T. septentrionalis* and *T. turrifex* samples (Both), and samples that were not sent for processing. In some locations *T. septentrionalis* and *T. turrifex* co-occur, while in others they remain separate.

Table 1: The samples collected according to their location, species, and if they were used for this analyses. For all sequenced samples we analyzed ants (A), fungus (F), and for some we analyzed soil (*).

ID	Location	Species	Sequenced
JNS160520-1	Aggieland	T.S	N
JNS160520-2	Restivo	T.S	N
JNS160520-3	Restivo	T.S	N
JNS160521-1	**Red Rock**	**T.S**	**Y**
JNS160521-2	**Red Rock**	**T.S**	**Y**
JNS160521-3	**Red Rock**	**T.S**	**Y**
JNS160521-4	Rosanky	T.S	N
JNS160521-5	Rosanky	T.S	N
JNS160522-1	Stengl	T.S	N
JNS160522-2*	**Stengl**	**T.S**	**Y**
JNS160522-3	**Stengl**	**T.S**	**Y**
JNS160522-4	Stengl	T.S	N
JNS160522-5	Stengl	T.S	N
JNS160523-1*	**Gladewater**	**T.S**	**Y**
JNS160523-2	Gladewater	T.S	N
JNS160523-3	**Gladewater**	**T.S**	**Y**
JNS160523-4	Gladewater	T.S	N
JNS160523-5	**Gladewater**	**T.S**	**Y**
JNS160531-1*	**UT Tyler Forest**	**T.S**	**Y**
JNS160531-2	**UT Tyler Forest**	**T.S**	**Y**
JNS160531-3	**UT Tyler Forest**	**T.S**	**Y**
JNS160531-5	Faulker	T.S	N
KK160530-1	**Cherokee county**	**T.S**	**Y**
KK160530-2	Cherokee county	T.S	N
JNS160414-1.1	**UT Tyler Forest**	**T.T**	**Y**

JNS160510-1.1	**UT Tyler Forest**	**T.T**	**Y**
JNS160515-1.1	**UT Tyler Forest**	**T.T**	**Y**
JNS160521-1.1*	**Red Rock**	**T.T**	**Y**
JNS160521-2.1*	**Rosanky**	**T.T**	**Y**
JNS160523-1.1	Gladewater	T.T	N
JNS160525-1.1	**Henderson 2**	**T.T**	**Y**
JNS160525-2.1	**Henderson 2**	**T.T**	**Y**
JNS160525-3.1*	**Henderson 2**	**T.T**	**Y**
KK160530-1.1	**Cherokee county**	**T.T**	**Y**
KK160530-2.1	**Cherokee county**	**T.T**	**Y**
KK160530-3.1*	**Cherokee county**	**T.T**	**Y**

Chapter 3

Results

Bacterial Distribution

The total number of raw sequence reads was 4,543,632 with 55 unique samples. The average, unfiltered, sequence length was 518.4 bp. Once all the filters were applied, the total number of sequences was 4,263,815 with an average length of 491.4 bp (mean number of sequences ants: 85,802.375, mean number of sequences fungus: 68,115.458 mean number of sequences soils: 45296.714. Rarefaction analysis (at 97% threshold) was preformed and indicated that the majority of the samples were adequately sampled (Appendix). Sequences were clustered based on 97% similarity (pick_open_reference_otus.py) resulting in 36,713 OTUs (operational taxonomic units) of ants, 33,206 OTUs in fungus and 29,314 OTUs in soil.

Sequences have been deposited in the NCBI Sequence Read Archive under SAR 2680323. Data processing pipeline has been deposited on GitHub (https://github.com/allertm/Microbiome_QIIME).

Ant, Fungus, and Soil Microbiome:

We performed a non-multidimensional scaling (NMDS) analysis on the ant and fungus samples using the Bray Curtis distance of OTU sharing with a stress of 0.1455827, which confirms the grouping of ants, fungus and soil samples in distinct clusters (Figure 2). We found that ants, fungus, and soil microbiomes were significantly different

(ANOSIM, test statistic 0.6818, p-value 0.01). These results were confirmed using a False Discovery Rate test which showed all the sample types to be significantly different from one another (ant vs soil: test statistic 14.348, p-value 0.03; soil vs fungus: test statistic 5.272, p-value 0.0015; ant vs fungus: test statistic -3.386, p-value 0.001). These results confirm that microbial communities of ants and fungus we are analyzing in this study are not contaminants originating from the surrounding soils, which were used as a negative control.

Soil samples had a significantly higher Simpson's Diversity Index when compared to ant and fungus samples (Kruskal-Wallis test: p-value = 0.0014). Ants and fungus samples did not have a significantly different Simpson's Diversity Index (Kruskal-Wallis test: p-value = 0.665).

Ant Microbiome

We performed a non-multidimensional scaling (NMDS) analysis on the ant samples using the Bray Curtis distance of OTU sharing with a stress of 0.1219016 (Figure 3), which confirms the grouping of ant species in distinct clusters. *Trachymyrmex septentrionalis* and *T. turrifex* bacterial communities were significantly different from each other (ANOSIM test statistic 0.50797, p-value 0.01). These results were confirmed using a False Discovery Rate test that showed there was indeed a significant difference between *T. septentrionalis* and *T. turrifex* (test statistic -2.047, p-value 0.043). Region appeared to have no effect on the microbiome of the ant species (test statistic -0.14296, p-value 0.949) using ANOSIM.

Fungal Analysis:

Fungal Microbiome

We performed a non-multidimensional scaling (NMDS) analysis on the fungus samples using the Bray Curtis distance of OTU sharing with a stress of 0.1071379 (Figure 6), which shows the grouping of fungus grown in different regions cluster distinctly. The fungus microbiome was not influenced by the ant species farming it (ANOSIM, test statistic 0.0022609, p-value 0.409). Interestingly, the fungal microbiome was influenced by the geographical region where the colonies were collected (ANOSIM test statistic 0.2428, p-value 0.0299). We then examined whether the clade from which the fungus belonged to had an effect on the microbiome (Figure 6). The fungal genotype had no effect on the microbiome (test statistic -0.04792, p-value 0.589) using ANOSIM.

Fungal phylotyping

The fungus samples were placed into four clades (Figure S5). Clade 1 consisted of primarily of *T. septentrionalis*, Clade 2 consisted of only *T. turrifex*, Clade 3 consisted of solely *T. septentrionalis*, and Clade 4 consisted of both *T. septentrionalis* and *T. turrifex.*. Thus, *T. septentrionalis* is growing a more diverse assemblage of fungi than *T. turrifex*.

Taxonomic analysis:

Taxonomic classification

We examined the top taxonomy orders and genera present in *T. septentrionalis* and *T. turrifex* ant microbiomes (Figure 4 and 5). The most abundant bacterial orders present

in *T. septentrionalis* were Actinomycetales, Soilrubrobacterales, Xanthomonadales, and Burkholderiales. These orders made up more than 79% of the total *T. septentrionalis* microbiome. Similar results were found in *T. turrifex*; the microbiome was mainly composed of the orders Actinomycetales, Entomoplasmatales, and Burkholderiales, which made up more than 79% of the *T. turrifex* ants microbiome. The most abundant bacterial genera found in *T. septentrionalis* were *Soilrubrobacteraceae, Xanthomonadaceae,* and *Propionicimonas* which made up more than 70% of the total taxonomic diversity. The most abundant bacterial families found in *T. turrifex* were *Burkholderiaceae unknown, Amycolatopsis,* and *Microbacteriaceae* which made up more than 60% of the total taxonomic diversity. An indicator species analysis was performed on each ant species and found 30 significant indicator OTUs, showing which bacteria are indicators of that particular ant species (Table S1). We found the majority of the indicator species were from the order Actinomycetales. Finally, we examined the top taxonomic groups of bacteria in *T. septentrionalis* and *T. turrifex* fungal cultivar according to region (Central Texas and East Texas) (Figures 7 and 8). The Central Texas fungal microbiome was primarily comprised of the orders Entomoplasmatales, Streptophta, and Enterobacteriales which made up more than 76% of their microbiome. The most abundant bacterial genera for Central Texas fungus, regardless of the ant species growing it, were *Entomoplasma, Pseudomonadales unknown, Enterobacteriaceae* unknown*, Mesoplasma* and *Streptophyta unknown* which made up more than 55% of the total taxonomic diversity. Similarly, the East Texas fungal microbiomes were also composed of the orders of Entomoplasmatales, Streptophta, Enterobacteriales, and in addition had a large portion of Rickettsiales, which accounted for over 60% of the microbiome. The most abundant bacteria genera for East

Texas fungus, regardless of the ant species growing it, were *Entomoplasma, Entomoplasmatales* unknown, and *Streptophyta* unknown, all of which made up more than 80% of the total microbiome. An indicator species analysis was performed on the geographical regions of the fungus and five significant indicator taxa were found (Table S2), showing which bacteria are significant indicators of the region in which the fungus is grown. The indicator species found where from the orders Acidobacteriales and one was from the order Sphingobacteriales.

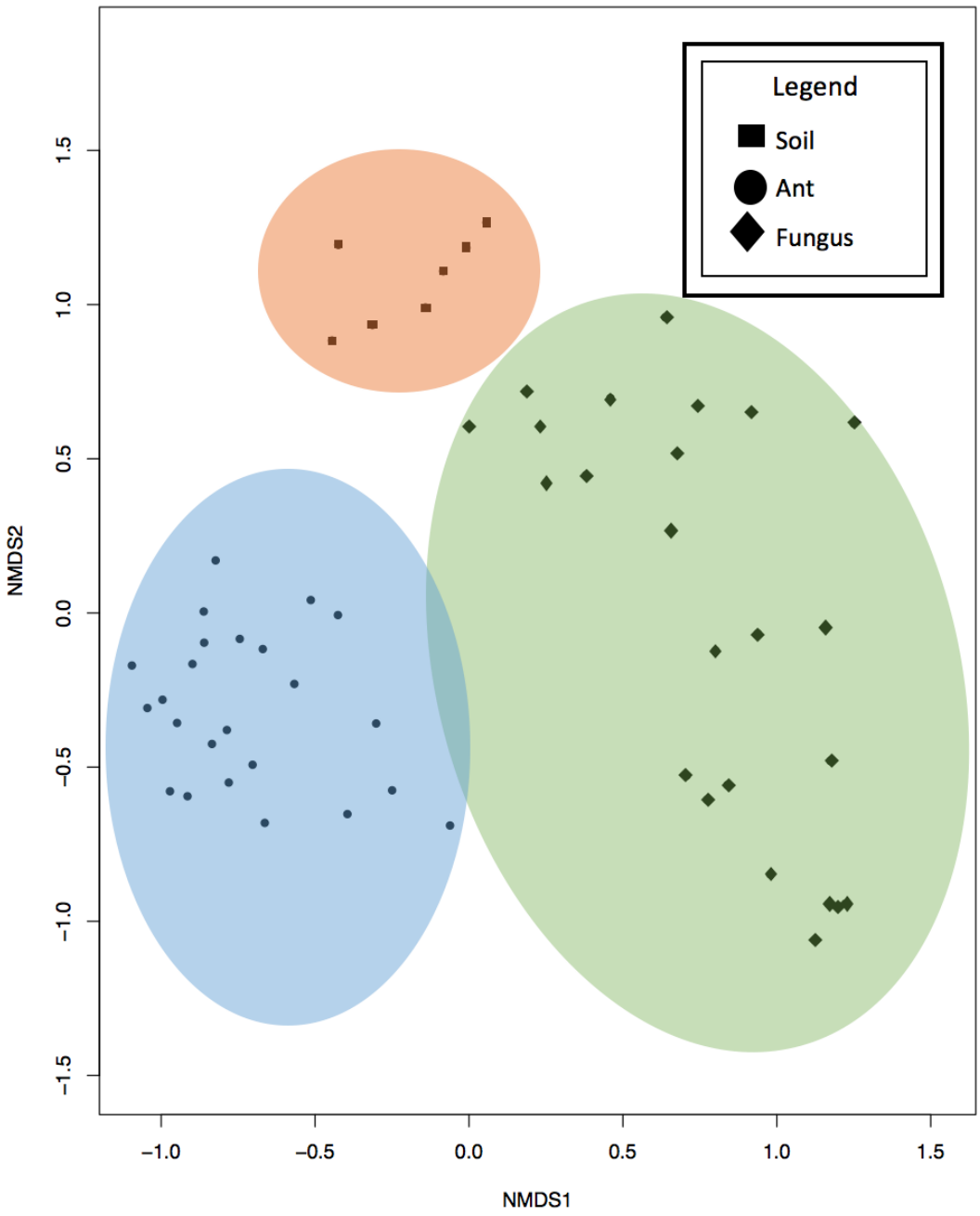

Figure 2: Bray Curtis distance-matrix of all ant and fungus samples using a NMDS model. NMDS was well supported with a stress level of 0.1455827. The ant and fungus samples clusters were distinct (test statistic 0.6818, p-value 0.01 using ANSOIM).

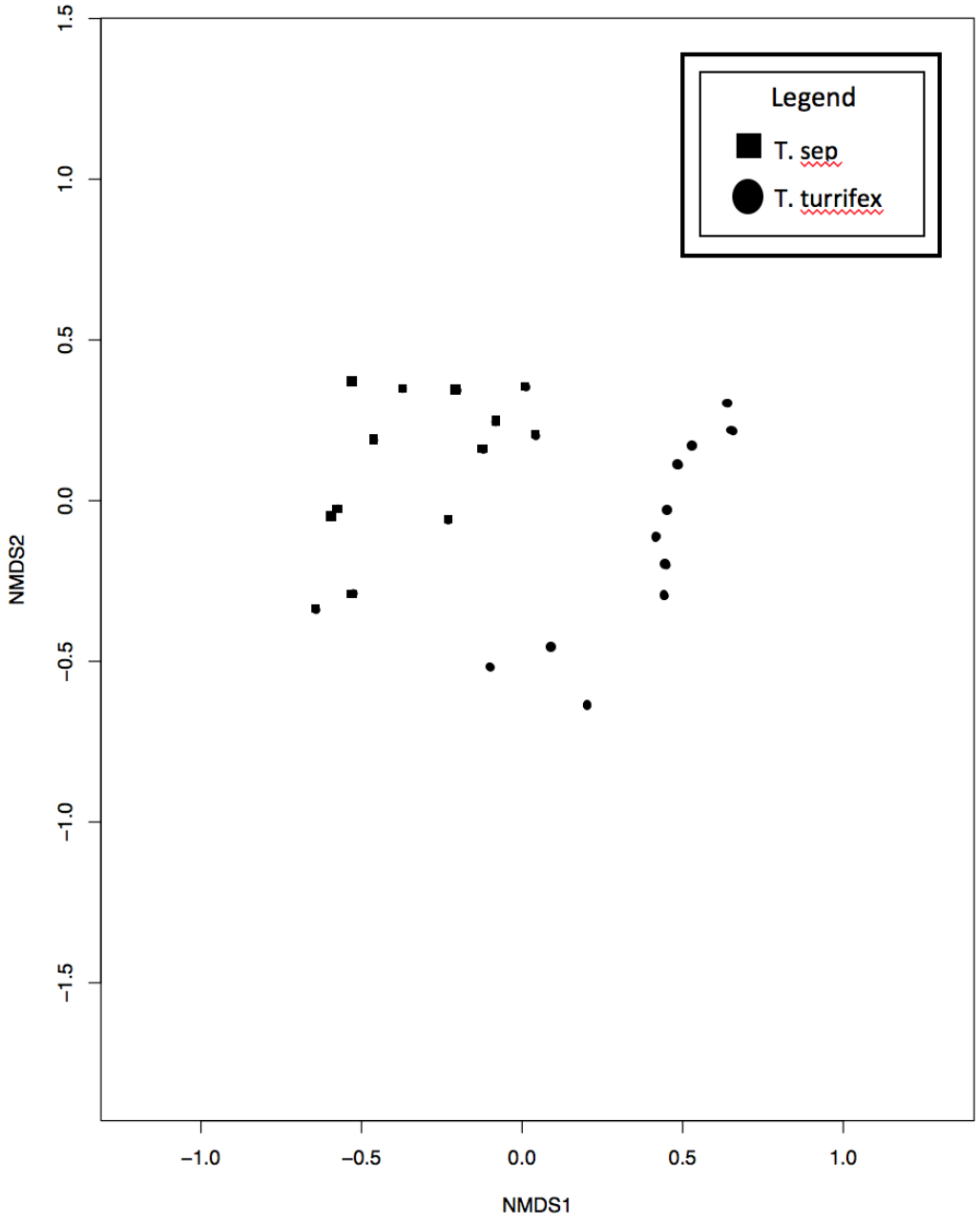

Figure 3: Bray Curtis distance-matrix of ant samples using a NMDS model. NMDS was well supported with a stress level of 0.1219016. The ant species clustered separately from one another (test statistic 0.50797, p-value 0.01 using ANOSIM).

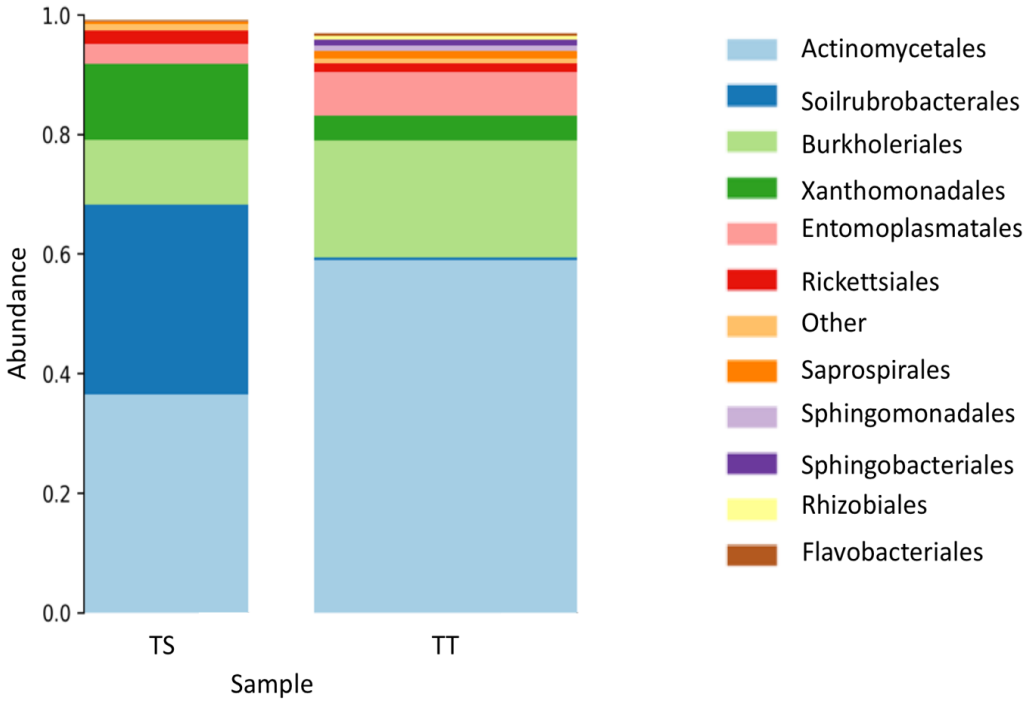

Figure 4: The most abundant bacterial orders found in *T. septentrionalis* (TS) and *T. turrifex* (TT) ant samples. The most abundant bacteria in *T. septentrionalis* were Actinomycetales, Soilrubrobacterales, Xanthomonadales, and Burkholderiales. In *T. turrifex*, the most abundant bacteria were Actinomycetales, Entomoplasmatales, and Burkholderiales.

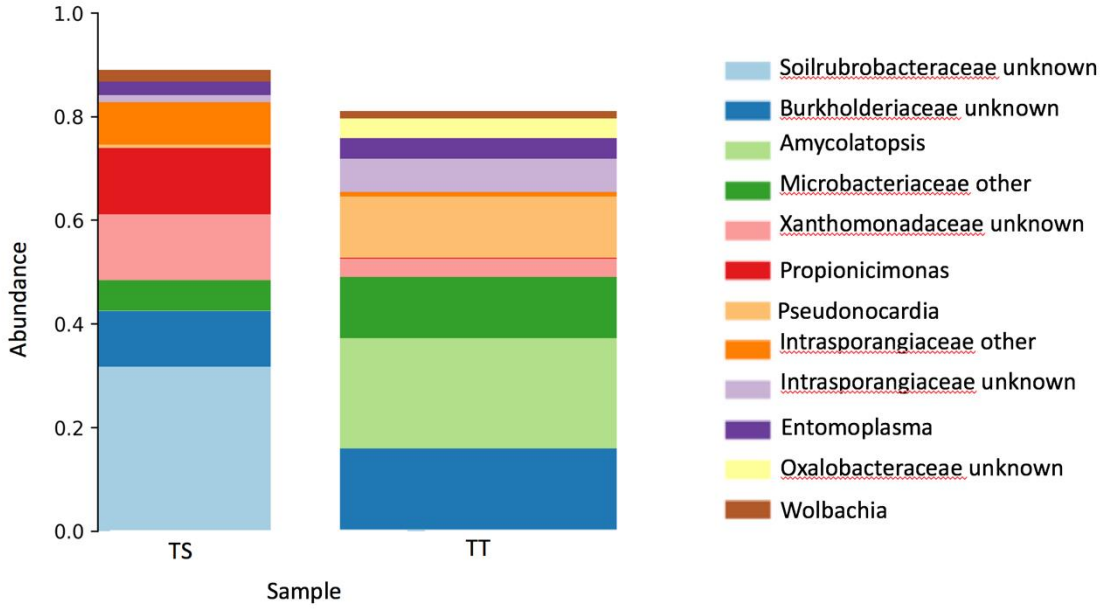

Figure 5: The most abundant bacterial families found in *T. septentrionalis* (TS) and *T. turrifex* (TT) ant samples. The most abundant bacteria in *T. septentrionalis* were Soilrubrobacteraceae, Xanthomonadaceae, and Propionicimonas. The most abundant bacteria found in *T. turrifex* were *Burkholderiaceae unknown, Amycolatopsis,* and *Microbacteriaceae*.

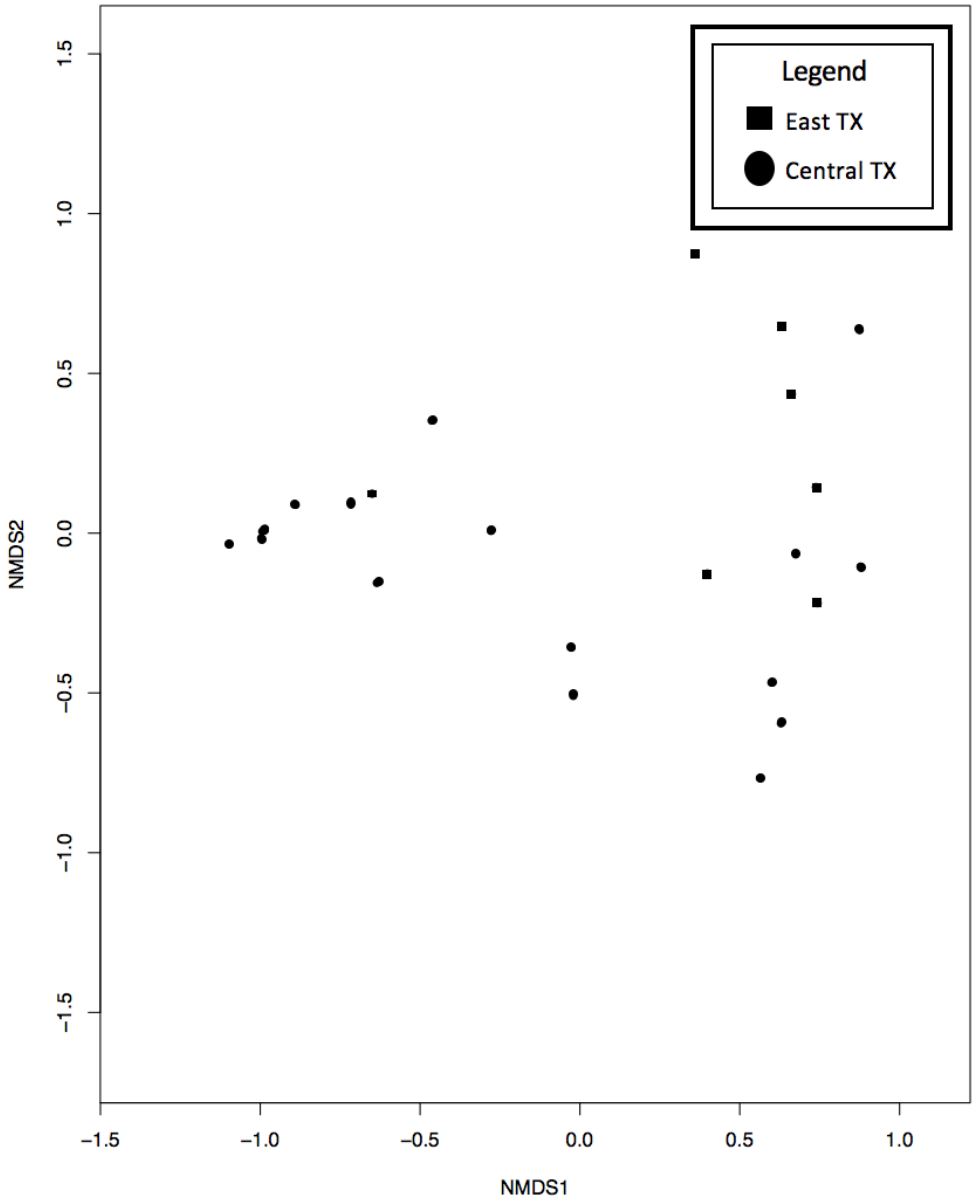

Figure 6: Bray Curtis distance-matrix of fungus samples using a NMDS model. NMDS was well supported with a stress level of 0.1071379. The fungus grown by *T. septentrionalis* and *T. turrifex* do not cluster together (test statistic 0.0022609, p-value 0.409 using ANOSIM), instead, they appear to loosely cluster according to the region in which they were grown (test statistic 0.2428, p-value 0.0299 using ANOSIM) (Squares = East Texas, Circles = Central Texas).

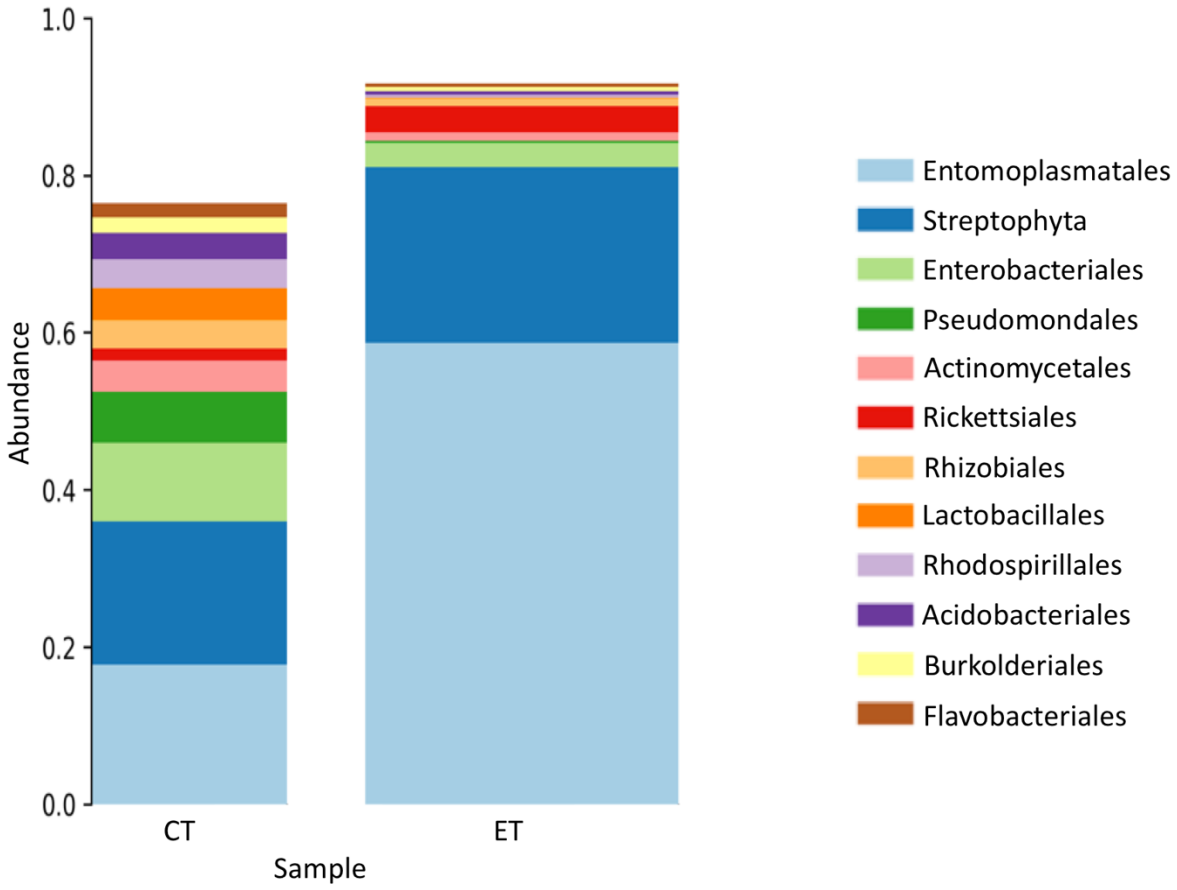

Figure 7: The most abundant bacterial orders in *T. septentrionalis* and *T. turrifex* fungal cultivar according to region, Central Texas (CT) and East Texas (ET). The most abundant bacteria for Central Texas fungus, regardless of the ant species growing it, were in the orders Entomoplasmatales, Streptophta, and Enterobacteriales. The most abundant microbes for East Texas fungus, regardless of ant species, were Entomoplasmatales ant Streptophta. The East Texas fungus had a substantially higher proportion of Entomoplasmatales compared to Central Texas fungus.

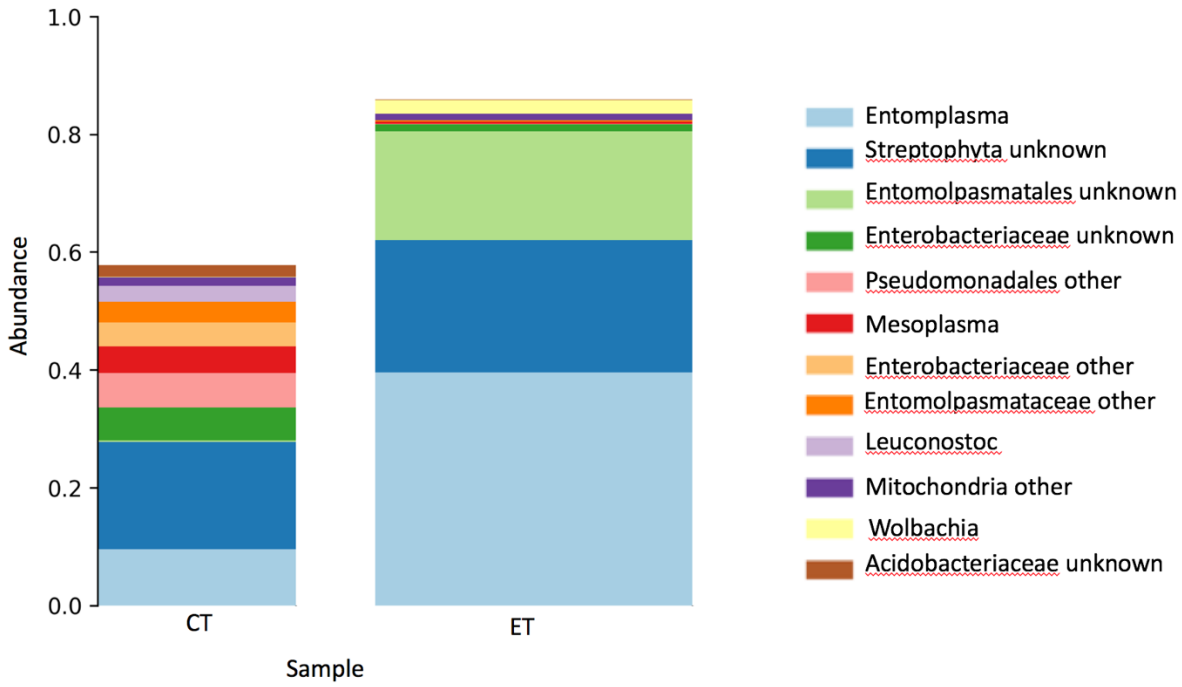

Figure 8: The most abundant bacterial genera in *T. septentrionalis* and *T. turrifex* fungus gardens according to region, Central Texas (CT) and East Texas (ET). The most abundant bacteria for Central Texas fungus, regardless of the ant species growing it, were *Entomplasma* and *Streptophyta unknown*. The most abundant bacteria for East Texas fungus, reguardless of the ant species growing it, were *Entomplasma, Entomolpasmatales unknown,* and Streptophyta *unknown.* East Texas had a higher portion of *Entomolpasmatales unknown*, while Central Texas had a higher proportion of *Pseudomonadales unknown, Enterobacteriaceae unknown,* and *Mesoplasma*.

Chapter 4

Discussion

This was the first study to examine how the bacterial microbiome of two-co-occurring fungus-gardening ants, *T. septentrionalis* and *T. turrifex* may vary spatially. We found that regardless of the ant species, ant, fungus, and soil bacterial samples were significantly different from each other. The ant and fungus samples were more similar than the soil samples, most likely due to the amount of interactions that occur between the ant and fungus. (Figure 2).

The microbiomes of *T. septentrionalis* and *T. turrifex* were also found to be significantly different from one another. The region from where the ants were collected did not have a significant impact on their microbiome. This indicates that the ants do not obtain their microbiome from the external environment; they most likely acquire them from their maternal colony (Figure 3). A similar study was done that looked at the microbiome in *Acromyrmex sp.* gut. It was found that when the ants were raised on a sterile diet they retained four major microbes in their tissues (*Wolbchia,* Rhizobiales*,* and two types of Entomoplasmatale*s* (Sapountzis et al., 2015). This result was similar to what I found in *T. septentrionalis* and *T. turrifex*. They maintained a similar microbiome that had a high abundance of Entomoplasmatales. This indicates that ants can independently maintain a stable microbiome, despite their external environment.

Both *T. septentrionalis* and *T. turrifex* had a high abundance of Actinomycetales as part of their microbiome. Actinomycetales are commonly known to produce antibiotic and is commonly seen in fungus-gardening ants and other insects (Currie et al., 1999; Kaltenpoth, 2009; Kaltenpoth and Engl, 2013). Burkholderiales, which was found in

abundance for both ant species, is thought to help with nitrogen fixation and is known for producing antibiotics against parasitic fungus (Anderson et al., 2012). Not a lot is known about the function and roles Xanthomonadales and Entomoplasmatales (Anderson et al., 2012; Funaro et al., 2010). It is suggested that Entomoplasmatales are closely related to *Mesoplasma* (Funaro et al., 2011). The function of *Mesoplasma* remains unknown, but it might contribute to colony mortality, it might be opportunistic, or it might be a permanent mutualist or a context-dependent mutualist (Sapountzis et al., 2015). Ishak et al. (2011) found a large portion of Solirubrobacteraceae in *T. septentrionalis*, similar to our results. The function of Soilrubrobacteraceae is unknown, however, it has been reported in soil crust (Reddy et al., 2006), agricultural soils (Kim et al., 2007), and earthworm burrows (Singleton et al., 2003). Soilrubrobacteraceae did not appear to be abundant in *T. turrifex* ants. Intrasporangiaceae was abundant in *T. turrifex* but was not as abundant in *T. septentrionalis*. The function of Intrasporangiaceae is relatively unknown, but it has been isolated in mosquitoes, *Anopheles funestus* (Lindh et al., 2005).

The contrasting microbiomes found in *T. septentrionalis* and *T. turrifex* could reflect different evolutionary histories. The finding that *T. turrifex* has a rich assemblage of Actinobacteria suggests that they may have had a longer evolutionary history with pathogens and have evolved a microbiome as a defense measure. However, it is unclear how this might be an adaptive strategy since it lives in the same environment as *T. septentrionalis* which does not appear to have a Actinobacteria-dominated microbiome. *T. turrifex* ants harbor a substantial amount of *Erwinia*, which is a known plant pathogen (Chatterjee and Starr, 1980; Perombelont and Kelman, 1980; Saarilahti et al., 1990). As a

possible response to *Erwinia*, they might have associated themselves with bacteria that produce antibiotics.

The fungus grown by *T. septentrionalis* and *T. turrifex*, which comprised of four different fungal clades, has a very different narrative surrounding its microbiome. The fungal clades do not appear to have a significant impact on their microbiome. In other words, the fungal microbiome is not influenced by the ant species farming it. Rather, fungal microbiomes were possibly influenced by their immediate environment. A possible explanation is that because the fungus functions as an external gut for the ants (De Fine Lincht and Boomsma, 2014; De Fine Licht et al., 2010; Seal et al., 2014), the microbiome is greatly influenced by what the ants feed their garden.

Both *T. septentrionalis* and *T. turrifex* had a high proportion of Enterobacteriwsales present in their fungal cultivar, confirming that it plays a crucial role in maintaining the fungus system. It was found that Enterobacteriales plays a role in metabolizing sugars, meaning they aide in the fungal metabolic processes (Alyward et al., 2012). They also share Streptophyta, which found in plant material they acquire from the ants. Enterobacteriales, which is found in both ant species is attributed for breaking down raw materials (Eilmus and Heil, 2009). Surprisingly, Rickettsiales was detected in *T. turrifex*; it is associated with *Wolbachia,* which is able to manipulate the sex-ratios in a population (Werren et al., 2008). This could indicate that the fungus had ant eggs in it when it was collected and sequenced. Even though *Wolbachia* can be acquired though horizontal transfer (Neuvonen et al., 2016), it is unlikely that this is the sole reason the fungus has such high numbers of *Wolbachia*. The ants might be collecting *Wolbachia* from the external environment while forging, they could be collecting leaf litter that contained *Wolbachia* infected insect frass.

A previous study examined the ant and fungal microbiome of *M. smithii* and discovered that the fungal microbiome was influenced by the environment rather than the fungal genotype (Kellner et al., 2015). This is in line with what I discovered for the fungal microbiome of *T. septentrionalis* and *T. turrifex*.

My study demonstrates the need to use geographic data for studying microbiomes in fungus-gardening ants because it has a profound influence on their fungal microbiome and provides an alternative perspective to the field. The ants are capable of maintaining their own microbiome, which is not significantly influenced by the environment. On the other hand, the ants' fungal microbiome appears to be influenced by the region in which it is located rather than the fungal clade to which it belongs or the ants that farm it. My research suggests that both ant species microbiome is vertically transmitted, while the fungus' microbiome appears to have a strong (environmental) component.

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Appendix A. Figures and Tables

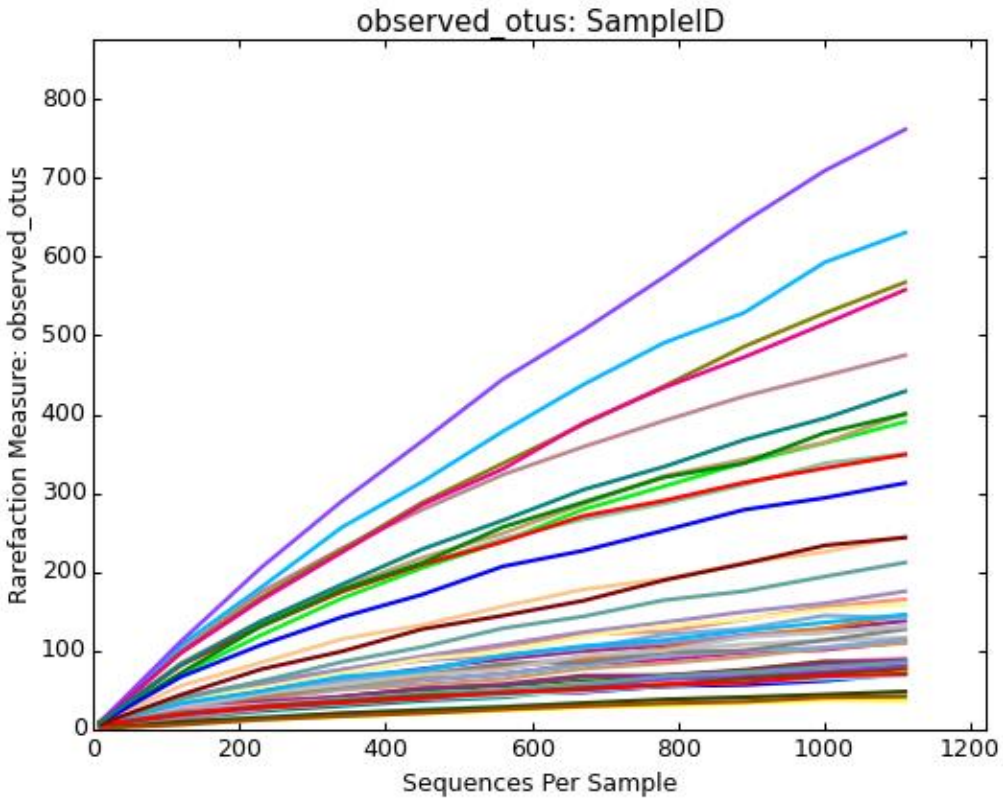

Figure S1: Rarefaction curves for *T. septentrionalis* and *T. turrifex* ant, fungus, and soil samples.

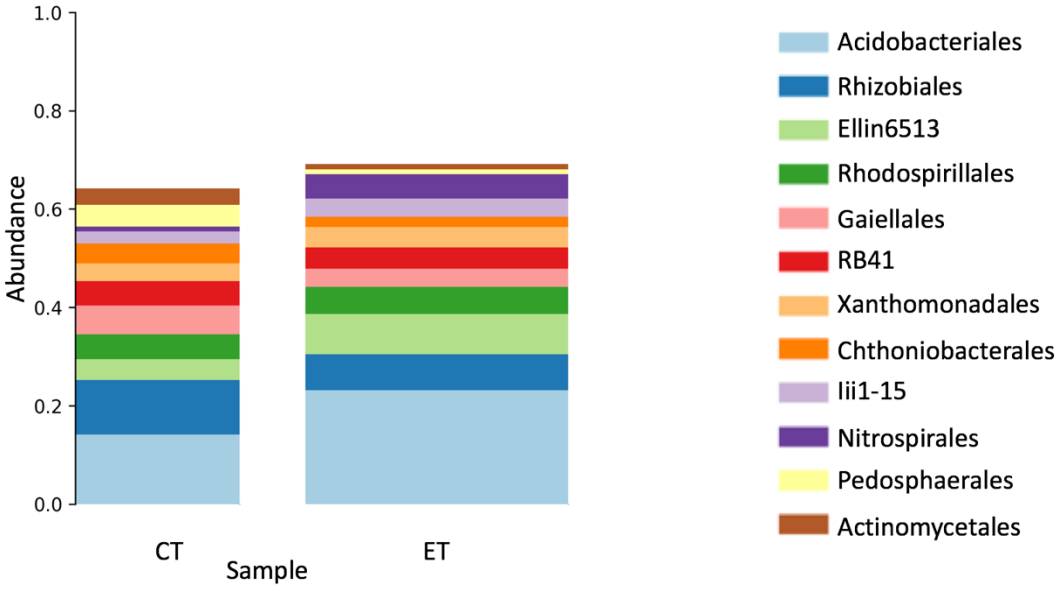

Figure S2: The most abundant bacterial orders of soil in the ants' fungus gardening chambers according to region.

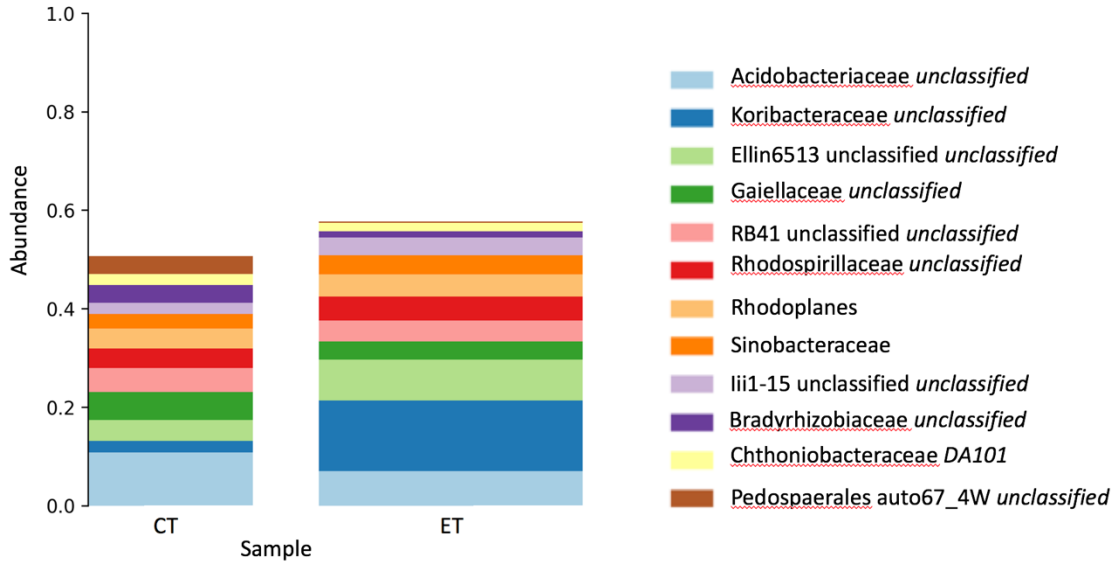

Figure S3: The most abundant bacterial genera of soil in the ants' fungus gardening chambers according to region.

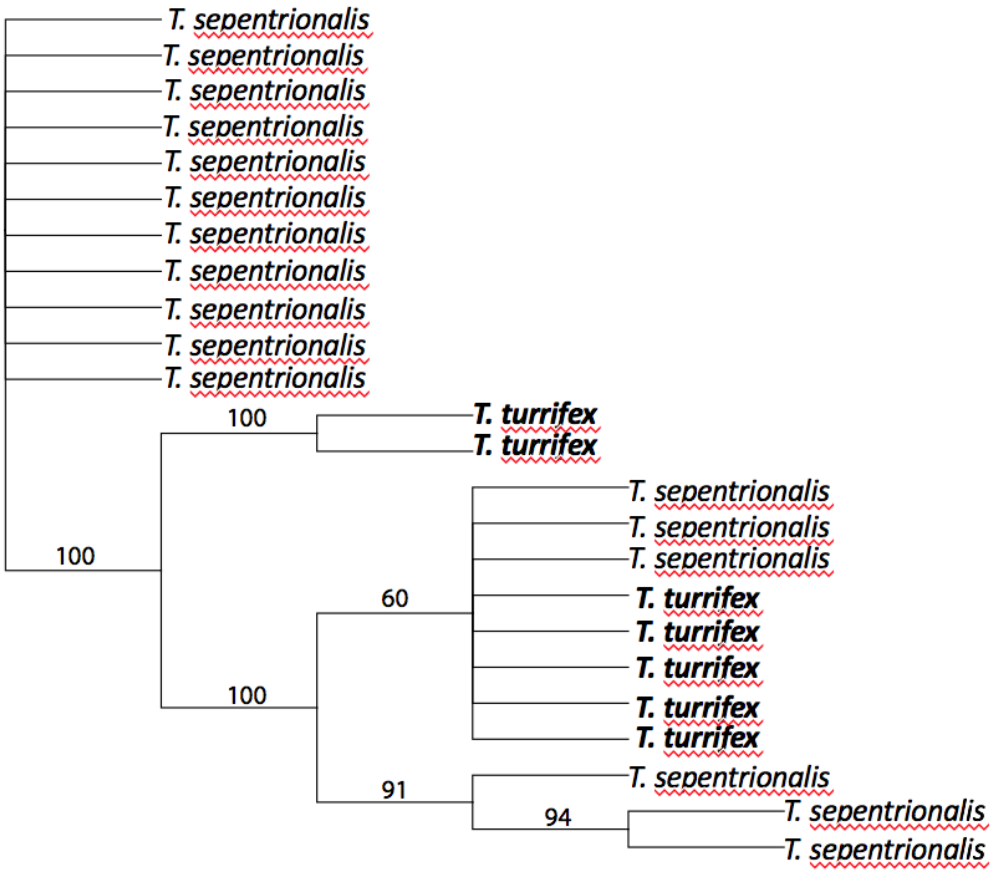

Figure S4: A Bayesian analysis was preformed on fungal genotypes based on the ant species that garden the fungus. Phylogenetic tree of the fungal genotypes grown by *T. septentrionalis* and *T.turrifex*, there are four distinct fungal clades.

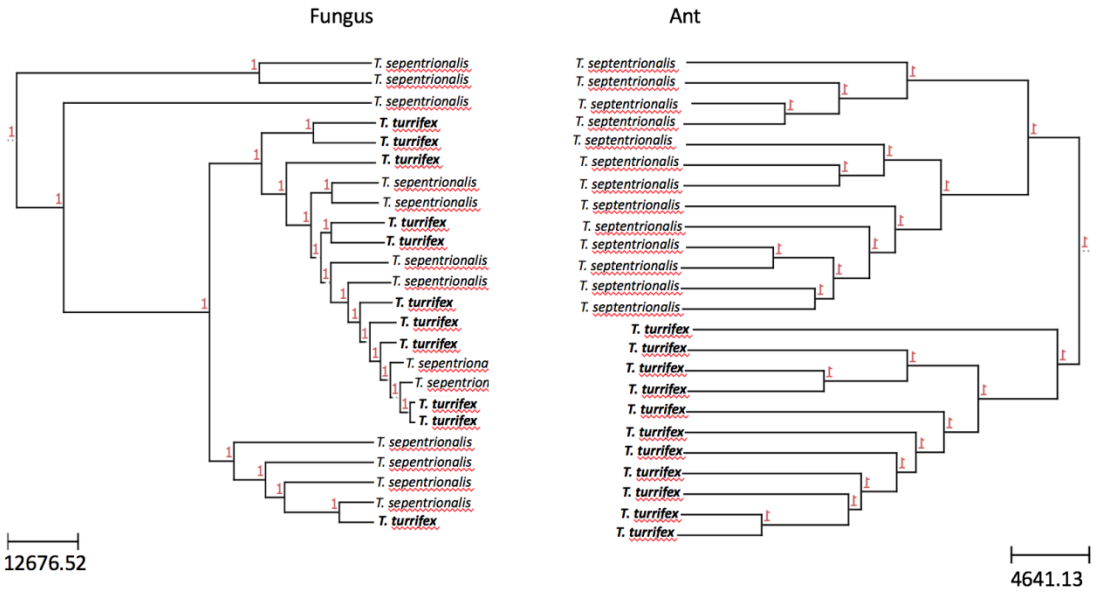

Figure S5: A cluster analysis of the ants and fungus grown by *T. septentrionalis* and *T. turrifex* a beta diversity matrix. There are four main clades and they appear to cluster according to the ant species that are growing the fungus.

Table S1: Significant indicator values (IV) of OTUs in *T. septentrionalis* and *T. turrifex* ant samples according to species. In PC-ORD the indicator species analysis (Dufrene and Legendre, 1997), IV values range from zero to 100%, indicating perfect indication. The p-values were calculated from a Monte Carlo permutation test for each OTU (4999 permutations). The OTUs with an asterisk (*) are significant indicators for *T. turrifex*, while the OTUs without an asterisk (*) are significant indicators for *T. septentrionalis*. This table shows the significant bacterial indicators for *T. septentrionalis* and *T. turrifex* ants.

OTU #	Order	Familie	Genus	IV	SD	P-value
OTU1*	Other	Other	*Other*	57.1	1.5	0.0006
OTU48	SJA-36	unclassified	*unclassified*	91	10.78	0.0174
OTU60	Actinomycetales	Actinospicaceae	*unclassified*	38.5	7.96	0.0368
OTU66	Actinomycetales	Bogoriellaceae	*Georgenia*	77.1	7.85	0.0402
OTU76*	Actinomycetales	Geodermatophilaceae	*Modestobacter*	81.5	6.12	0.0012
OTU77	Actinomycetales	Gordoniaceae	*Gordonia*	85.6	8.85	0.0112
OTU81	Actinomycetales	Intrasporangiaceae	*Janibacter*	93.8	12.41	0.038
OTU88*	Actinomycetales	Kineosporiaceae	*Kineosporia*	70.3	4.91	0.0146
OTU89*	Actinomycetales	Microbacteriaceae	*unclassified*	71.6	7.53	0.013
OTU92	Actinomycetales	Microbacteriaceae	*Candidatus Aquiluna*	83.3	12.33	0.011
OTU100	Actinomycetales	Microbacteriaceae	*Pseudoclavibacter*	58	9.43	0.038
OTU112	Actinomycetales	Micromonosporaceae	*Catellatospora*	89.3	9.57	0.002
OTU114	Actinomycetales	Micromonosporaceae	*Virgisporangium*	45.5	8.38	0.0246
OTU117	Actinomycetales	Nocardiaceae	*unclassified*	99.2	8.63	0.0002
OTU118*	Actinomycetales	Nocardiaceae	*unclassified*	77.2	8.21	0.0312
OTU120*	Actinomycetales	Nocardiaceae	*Rhodococcus*	89.4	9.17	0.0018
OTU121*	Actinomycetales	Nocardioidaceae	*unclassified*	82.7	6.68	0.0008
OTU130	Actinomycetales	Promicromonosporaceae	*unclassified*	97.5	8.69	0.0014
OTU131*	Actinomycetales	Promicromonosporaceae	*Cellulosimicrobium*	76.9	7.74	0.0378
OTU132*	Actinomycetales	Promicromonosporaceae	*Xylanimicrobium*	70.2	8.12	0.0104
OTU133	Actinomycetales	Propionibacteriaceae	*unclassified*	80.4	7.65	0.0038
OTU134	Actinomycetales	Propionibacteriaceae	*unclassified*	90	9.35	0.0006
OTU137	Actinomycetales	Pseudonocardiaceae	*unclassified*	99.2	9.72	0.0002

OTU138	Actinomycetales	Pseudonocardiaceae	*unclassified*	93.8	9.34	0.0006
OTU139	Actinomycetales	Pseudonocardiaceae	*Actinomycetospora*	75.6	9.58	0.0004
OTU143	Actinomycetales	Sporichthyaceae	*unclassified*	83.9	9.56	0.0002
OTU146	Actinomycetales	Streptomycetaceae	*unclassified*	99	10.09	0.0002
OTU163*	Gaiellales	Gaiellaceae	*unclassified*	55.7	8.32	0.0252
OTU168*	Solirubrobacterales	Conexibacteraceae	*Conexibacter*	90.8	6.34	0.0002
OTU180*	unclassified	unclassified	*unclassified*	59.6	9.35	0.0298

Table S2: Significant indicator values (IV) of OTUs in *T. septentrionalis* and *T. turrifex* fungus samples according to regions. In PC-ORD the indicator species analysis (Dufrene and Legendre, 1997), IV values range from zero to 100%, indicating perfect indication. The p-values were calculated from a Monte Carlo permutation test for each OTU (4999 permutations). The OTUs with an asterisk (*) are significant indicators for East Texas. This table shows the significant bacterial indicators for the fungus grown by *T. septentrionalis* and *T.turrifex* ants according to region.

OTU #	**Order**	**Familie**	**Genus**	**IV**	**SD**	**P-value**
OTU21*	Acidobacteriales	Koribacteraceae	*unclasified*	91.4	11.31	0.0202
OTU22*	Acidobacteriales	Koribacteraceae	*Candidatus Koribacter*	97.1	11	0.0042
OTU68*	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	91.1	10.59	0.0164
OTU92*	Actinomycetales	Microbacteriaceae	*Candidatus Aquiluna*	85.5	10.49	0.0488
OTU213*	Sphingobacteriales	Sphingobacteriaceae	*unclasified*	94.1	13.22	0.0314

Microbiome analysis pipeline

Downloaded MacQIIME with the python shell interface

Validated mapping file:

Validate_mapping.py –m map.tsv –o good_map/

Merged fasta and qual files using: (quality filter was already preformed on them by MR.DNA)

convert_fastaqual_fastq.py –f xxx.fasta –q yyy.qual –o xyxy.fastq

Create a barcode file→ generates barcodes.fastq

extract_barcodes.py extract_barcodes.py -f xyxy.fastq -c barcode_single_end --bc1_len 8 -o processed_seqs --rev_comp_bc1

split libraries

split_libraries_fastq.py -o slout/ -i xyxy.fastq -b barcodes.fastq -m map.tsv –-barcode_type 8

count the number the of sequences as a check

count_seqs.py -i slout/seqs.fna

compare to greengenes

pick_open_reference_otus.py -o otus/ -i slout/seqs.fna -p map.tsv

summarize .biom table

biom summarize-table -i otus/otu_table_mc2_w_tax_no_pynast_failures.biom

Run a diversity analysis

core_diversity_analyses.py -o cdout/ -i
otus/otu_table_mc2_w_tax_no_pynast_failures.biom -m map.tsv -t otus/rep_set.tre -e
1114