Summer 8-24-2021

COMPARATIVE MICROBIOME ANALYSIS OF THE FUNFUS GARDENING ANT SPECIES TRACHYMYRMEX ARIZONENSIS

Chase D. Rowan

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by

CHASE ROWAN

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology

Katrin Kellner, Ph.D., Committee Chair
College of Arts and Sciences

The University of Texas at Tyler
July 2021

The University of Texas at Tyler

Tyler, Texas

This is to certify that the Master's Thesis of

CHASE ROWAN

has been approved for the thesis requirement on

July 19th, 2021

for the Master of Biology Degree

Approvals:

Thesis Chair: Katrin Kellner, Ph.D.

Member: Jon Seal, Ph.D.

Member: Brent Bill, Ph.D.

Member: Mathew Greenwold, Ph.D.

Chair, Department of Biology

Dean, College of Arts and Sciences

Acknowledgments

I would like to thank Dr. Katrin Kellner for taking me on as a graduate student and giving me the opportunity to learn about the fascinating mutualistic system of fungus-gardening ants. I would also like the thank Dr. Jon Seal for introducing me to this type of scientific enquiry by allowing me to work and gain experience in his laboratory during my time as an undergraduate at The University of Texas at Tyler. I would also like to extend my thanks to the other students working in our lab who were always very helpful and a joy to work with. Thank you to my committee members: Dr. Jon Seal, Dr. Brent Bill, and Dr. Matthew Greenwold. Everyone involved in my thesis project were gracious and helpful beyond measure. My work was supported by the National Science Foundation (DEB-1354629). I would also like to thank the Southwestern Research Station for their contribution to my work as well as the hospitality that I was shown while there.

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Abstract

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Chase Rowan

Thesis Chair: Katrin Kellner, Ph.D.

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July 2021

Fungus-growing ants (Attini: Formicidae) and their fungal cultivars participate in ant-fungus mutualism that share a 50-million-year-old coevolutionary history. Fungal cultures are grown in gardens alongside ants and a diverse collection of microbes that interact with both species in mutualistic, commensal, and antagonistic relationships. These microbes aid in digestion and detoxification of food, provide essential nutrients, help in nest hygiene, and play a dominant role in defense against pathogens and disease. Microbial communities of many model species have been shown to change in a laboratory setting as compared to their natural environment. High-throughput 16s sequencing of the V4 variable region was used to investigate the bacterial diversity of 32 *Trachymyrmex arizonensis* colonies. Additional changes in colony diversity and structure were analyzed from their initial collection and following 16 weeks of standard laboratory culture. Diversity metrics revealed a shift in the microbial community between each sampling treatment. Approximately 24% (fungal) and 19% (ant) of the variation in distances between fungus and ants collected in the field to those collected in the laboratory was explained by this grouping. Shared taxa between treatments indicated families belonging to Actinobacteria and Proteobacteria to be the main bacterial species shared between the two groups. Indicator species analysis indicated families belonging to *Clostridiales*, *Burkholderiales*, and *Actinomycetales* to be most responsible for driving differences among microbial communities between samples. In addition to the comparative microbiome study, three primers were developed for *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* which make up the core microbiome of *T. septentrionalis* and *T. turrifex.*

Chapter One

Introduction

Fungus-gardening ants

Fungus-gardening ants (Attini: Formicidae) comprise all known ant species participating in ant-fungus mutualism. Higher attine ant genera include leafcutter and non-leafcutter species (such as *Trachymyrmex*) (Mueller et al. 2005). These ants exhibit an obligate mutualistic relationship with the fungal gardens they cultivate. Worker ants forage for a variety of plant materials used to cultivate mycelium of the fungus (Seal et al. 2017). In return for nurture and defense, the fungus provides nutrients for ants (Weber 2012). The nutrients accumulate in specialized hyphal swellings that are rich in lipids and sugars, called gongylidia. The gongylidia serve two main functions: 1) they are the prominent food source for developing larvae within the colony, and 2) they are ingested by worker ants which causes the ants to transmit fungal decomposition enzymes onto the foraged plant materials through fecal fluid (Henrik et al. 2014). In many species, the ants and fungus are completely dependent on each other for survival. In addition to nutrition, the fungal gardens provide a suitable environment for brood care and protection of the queen (Mueller et al. 2005).

Generalized fungus farming in ants belonging to the tribe attine appeared approximately 55-60 million years ago (mya) in South America. It is believed that cultivation of fungal gardens appeared with the domestication of fungus in the family Lepiotaceae (Agaricales: Basidomycotina). Of the over 200 known extant attine species, all are obligate fungus farmers (Mueller et al. 2005). Fungal cultivars are passed between generations via vertical transmission. When new queens leave the colony to establish their own colonies, they store a small piece of fungus in its infrabuccal pouch in their cibarium. The infrabuccal pouch acts as a receptacle by which queens can store and transmit the fungus to a newly found colony (Quinlan et al. 1978).

Microbiome of Fungus-gardening ants

Fungal cultures grow in gardens alongside ants and a diverse collection of microorganisms that interact mutualistically, commensally, or antagonistically with ants, fungus, or with one another (Ishak et al. 2015). The symbiotic microorganisms play critical roles in the shaping of phenotypes, ecology, and evolution of their hosts (Sapountzis et al. 2019). Combined, this collection of microorganisms constitutes the microbiome. The microbiome is defined as the aggregate of genomes of all microbiotas that reside on or within a living organism (Moreau et al. 2020

The fungus gardening ant system is considered a multi-tiered symbiosis between the ants, their fungus, and their corresponding microbes (Allert 2017). Fungus-gardening ants (Hymenoptera: Formicidae, Attini) exhibit one of the most complex microbiomes because both ants and fungus are home to a diverse community of microorganisms (Kellner et al. 2015). Symbiotic bacteria benefit fungus gardening ants in a broad range of ways including digestion and the maintenance of the fungal garden (Mueller et al. 2005). These microbes aid in digestion and detoxification of food by helping ants obtain essential nutrients, help in nest hygiene, and play a dominant role in defense against pathogens and disease (Kellner et al. 2015, Kellner et al. 2018). As such, the microbiota of social insects such as ants, termites, bees, and wasps have become increasingly interesting among researchers because living in close association has been shown to increase the likelihood of disease spread, and group living can drive the evolution of these adaptive strategies to prevent and control the spread of disease. Uniquely, fungus gardening ants must contend with pathogens that affect both the ants themselves as well as the fungal gardens they cultivate (Sapountzis et al. 2019).

These ants have a complex symbiotic relationship with phylogenetically diverse microbes (González et al. 2019). The microbiome of attine ants and their respective fungus is made up of a central core of symbiotic microbes known to produce antimicrobial metabolites and serve other functional roles within a colony (Currie et al. 1999). These microbes that make up the core microbiome include bacteria in the Actinobacteria and Proteobacteria phyla (Sen et al. 2009).

Evolution of mutualism with microbes

The evolution of mutualism between attine ants and antibiotic producing bacteria has been explained in the literature by two prominent theories. The first theory rests on the principle of vertical transmission in which co-evolution between attine ants and antibiotic producing bacteria are locked in an arms race with invading pathogens, such as Escovopsis, to target the pathogen without damaging the fungal garden. The second theory suggests attine ants take up antibiotic producing bacteria from their local soil environment and cultivate them for use in defense through horizontal transmission (Barke et al. 2011). Although there have been recent advancements in research centered around the composition, structure and diversity of bacterial communities living within fungus-growing ants' nest, many details of the rolls various microbes play in protecting the nests remains largely unknown (Little et al. 2006; Sen et al. 2009; Andersen et al. 2013; Woodhams and Brucker 2013). Additional work is also needed to fully understand how environmental factors across landscapes and laboratory settings might alter or influence the composition of microbial communities (Mikheyev et al. 2008; Mueller et al. 2008).

Fungus gardening ant pathogens

The most characterized fungal pathogen associated with attine ants is a pathogenetic microfungus from the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) which is horizontally transmitted between colonies (Currie et al. 2009). *Escovopsis* is a highly virulent fungal pathogen that can affect any of the fungus growing ant species from tribe attine (Mueller et al. 2005). It is thought that fungus gardening ants utilize the antimicrobial metabolites produced by species of Actinobacteria, reared as a biofilm on the cuticle, to help defend their fungal gardens from harmful pathogens such as *Escovopsis.* Failure of this defense can lead to death of worker ants or cause fungal gardens to be abandoned (Heine et al. 2018). These events point to a failure of the antimicrobial defenses imposed by symbiotic microbes and occur most often when colonies are freshly excavated and kept in a laboratory setting without a way for waste to be properly discarded away from the fungal garden (Heine et al. 2018). A suggested mechanism of action of *Escovopsis* involves the production of metabolites from hyphae that affect worker ant behavior which results in colony collapse.

Actinomycetes (Actinobacteria) are Gram-positive bacteria found in aquatic (fresh water and marine environments) and terrestrial habitats. They are, however, most commonly found within samples collected from soils in terrestrial environments. These microbes are well characterized as producers of antimicrobial metabolites and many of the modern antibiotics utilized today were originally isolated from microbes belonging to Actinobacteria (Hussein et al. 2018). Antibiotics are molecules that selectively inhibit bacteria by interfering with cellular machinery essential to bacterial growth and proliferation. Antibiotics target bacterial structures or functions such as cell wall biosynthesis, translation, RNA transcription, DNA replication and synthesis, membrane and to inhibit bacterial growth (Ivanova et al. 2016).

Aims of the study

In this study, we utilized 16s sequencing of the rRNA V4 variable region to explore how the microbial communities of the fungus gardening, non-leafcutter higher attine ant species *Trachymyrmex arizonensis* change in a laboratory setting compared to their natural habitat. Samples of both ants and their corresponding fungus were collected in the field. Samples were subsequently collected from the same colonies after spending 16 weeks in a laboratory setting. By comparing the microbial composition of both ants and fungus from the field to those collected after 16 weeks in a laboratory setting, we hope to elucidate whether this change in environment significantly changes the microbiome of the system. Shared and divergent bacterial species between sampling treatments were also identified. If the microbiomes are inherited vertically, then we expect microbiome structure in field colonies to persist in the laboratory. Alternatively, if microbiomes obtain most of their structure from the environment (horizontal transmission), then there should be significant changes in the microbiome structure when ants and fungus are placed in the laboratory. There is also the possibility that different components of the microbiome will show different patterns, which would then represent a mixture of transmission models.

The secondary focus of this study is the design of primers for genera that make up the core microbiome of *T. septentrionalis* and *Mycetomoellerius* (formerly *Trachymyrmex*, Solomon et al. 2019) *turrifex*. Bacteria in genera *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* of phylum Actinobacteria are the most commonly found microbes in these two species of ants. Primers were designed for the purpose

of screening ants and fungal gardens to further study this relationship. We also validated these primers for future studies.

Chapter Two

Methods

Study species

This research project is focused on the North American non-leafcutter higher-attine ant species *Trachymyrmex arizonensis. T.arizonensis* are typically found at mid elevations (1000-2000 m) in mountainous areas within the Chihuahuan and Sonoran Deserts in central and southern Arizona, western New Mexico, and the states of Chihuahua and Sonora in Mexico (Rabeling et al. 2007). This species of ant occurs in a variety of habitats including arid Ocotillo- and Acacia-dominated scrub in mountain foothills, oak-juniper-pine woodlands, and relatively mesic mid-elevation creek valley forests. (Rabeling et al. 2007). Nests can be found under rocks or logs as well as in open soil and are predominantly found in areas with partial shade. Established colonies have approximately 3-5 fungal garden chambers and can have over 1000 workers (Mueller et al. 2005).

Collection of *T. arizonensis*

Colony fragments and samples (ants and fungus) were collected in and around the Southwestern Research Station (SWRS) near Portal, Arizona, part of the Chiricahua Mountain range in the Colorado National Forest. Areas around the research station (within approximately 6000 m) were surveyed for the presence of *T. arizonensis*. Surveyed locations were chosen if they appeared to have appropriate habitat, including foliage and elevation, to support colonies of our target species. Colonies of *T. arizonensis* were located by searching for recognizable features of the nest entrance. These features include a messy soil crater around the nest entrance, along with a distinctive yellowish-gray colored external refuse midden located nearby (Rabeling et al. 2007)

T. arizonensis colonies were non-destructively sampled by careful excavation by using shovels, spoons, putty knives, and trowels to dig through soil to expose the fungal garden and ants below. Excavation was initiated by digging an approximately 0.5 meter deep trench a short distance from the entrance of the nest. Once the trench had been excavated, it was carefully extended horizontally toward the nest entrance using small excavation tools including shovels, trowels, and spoons. The loose soil

accumulating in the bottom of the trench was periodically removed and discarded as to not lose the initial depth of the trench. As the wall of the trench approached the entrance of the nest, a putty knife was utilized to carefully shave away the soil to expose the tunnels, chambers, and fungal garden of the colony. Any ants that escaped the tunnel entrance during the excavation process were collected using a handheld aspirator and stored in a plastic container lined with fluon. Once the fungal garden of the colony was exposed, sterile field samples of approximately 5-10 worker ants and their corresponding fungus were collected using flame sterilized forceps and separately preserved in sterile vials containing 100% molecular grade ethanol. Sterile vials containing 100% molecular grade ethanol were pre prepared in a biocontainment hood at the University of Texas at Tyler. Sterility of the samples were insured by submerging forceps into 95% ethanol and flaming with a torch until they glowed red. Forceps were sterilized in this manner in between each ant or piece of fungus collected. Live ants and fungus (colony fragments) were also collected and stored in plastic nest boxes with plaster substrate (for moisture retention) before being translocated to the laboratory at the SWRS facility. Preserved sterile samples of ants and fungus in 100% ethanol were kept in -20 °C between July 26th and August 14th. Colony fragments and preserved sterile samples (ants and fungus) were transported to University of Texas at Tyler and stored in -80 °C upon arrival at the University of Texas at Tyler. Colony fragments were carefully maintained with minimal human contact and were fed a restricted diet of polenta to avoid the introduction of exogenous microbes. Sixteen weeks after the date of excavation, sterile samples of both ants and fungus were collected from the colony fragments maintained in the laboratory and stored in -80 °C. In summary, worker ants and fungus were collected from each colony at the time of excavation (field samples) and again 16 weeks after housed in a laboratory setting (lab samples).

Microbial DNA extraction and 16s sequencing

Microbial DNA extraction and 16S sequencing were performed by MrR DNA laboratories in Shallowater, Tx, USA. The 16S rRNA gene V4 variable region PCR primers 515/808 with barcode on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 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 amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Samples were multiplexed using unique dual indices and then pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. The pooled and purified PCR products were used to prepare an Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a IlluminaMiSeq following the manufacturer's guidelines. Raw sequence data were uploaded to GenBank and are available under accession numbers SAMN20295924 – SAMN20295931, SAMN20295916 – SAMN20295923, SAMN20296297 - SAMN20296304, SAMN20296306 - SAMN20296313.

Microbiome bioinformatics

Microbiome sequencing data was analyzed using Quantitative Insights Into Microbial Ecology version 2 (Qiime2). (Caporaso et al. 2010). Demultiplexing and the removal of non-biological sequences (barcodes and primers) was facilitated with FASTQ processor software provided by Mr. DNA laboratories (https://www.mrdnalab.com/mrdnafreesoftware/fastq-processor.html). Demultiplexed, paired-end sequence reads were imported into Qiime2 using the Casava 1.8 qiime2 import tool. Forward and reverse 16s rRNA sequences were trimmed to 231 base pairs due to quality score dropping below Q25 beyond this position. Dada2 in Qiime2 was used to filter, denoise, merge, and remove chimeras of demultiplexed reads. Operational Taxonomic Units (OTUs) were defined by clustering of 3% divergence (97% similarity). Final OTUs were taxonomically classified using Greengenes classifier pre-trained for forward and reverse primers 515 and 806 respectively (DeSantis et al. 2020).

Statistics

All statistics were performed using the R packages phyloseq version 1.36.0 (McMurdie 2013) and indicspecies version 1.7.9 (De Caceres et al. 2009) with graphics generated with the R package ggplot2. version 3.3.3 (Wickham H 2016) Bray-Curtis and Unweighted unifrac distances were calculated using the phyloseq package in R (McMurdie 2013). Rarefaction curves were calculated using the R package phyloseq version 1.36.0 (McMurdie 2013). Alpha diversity metrics, including Shannon's, Simpson diversity index, were calculated to determine the microbial diversity within each sample as well as

significance in diversity between samples collected in the field and in the laboratory. A Wilcoxon signed-rank test was implemented to determine if the observed number of OTUs differed significantly between sampling treatments. An Adonis statistical test, with 9999 permutations, was performed to calculate the significance between field and laboratory samples. Hierarchical cluster analyses (HCL) were generated of ant and fungal microbial community structures based on Bray-Curtis distance matrix. Indicator species analysis determined which OTUs were responsible for observed differences between study groups by using the multiplatt function of the 'indicspecies' package in R. To further explore the difference in microbial communities between laboratory and field collected samples, Venn diagrams were constructed to display the overlap of OTUs between different sample types (ants and fungus). All OTUs present in each sample type (ants and fungus) and sampling treatment (laboratory and field) were plotted using BIOINFOGP software version 2.1.0 (Oliveros 2007).

Primer development

Sequence data from the microbiome of *Trachymyrmex. septentrionalis* and *Mycetomoellerius turrifex* were obtained from a previous study (Allert 2017). The microbes that make up the core microbiome include bacteria in the *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* genera of phylum Actinobacteria. OTUs associated with *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* were extracted from the data set using a faidx script (Mathews 2014) before a multiple sequence alignment (Pseudonocardia: 68 OTUs, 480 bp per OTU; Solirubrobacter: 231 OTUs, 512 bp per OTU; Amycolatopsis: 38 OTUs, 488 bp per OTU) was performed using Clustalw (Thompson 1994). Consensus sequences for each of the three genera were constructed using EMBOSS (https://www.bioinformatics.nl/cgi-bin/emboss/cons). Consensus sequences were used to develop primers for the genera listed above. Primers were designed using PrimerQuest (Integrated DNA Technology) (Owczarzy et al. 2008).

Microbial DNA extraction

Worker ants (1-3) were homogenized in 480 uL of pre-heated CTAB at 60° C and 20 µL of proteinase k added. The contents were vortexed and incubated for 30 min at 60 °C. The mixture was centrifuged at 13000 rpm at room temperature for 8 min. The supernatant was extracted and 500 uL of

Chloroform-isoamyl-alcohol (24:1) was added. The solution was gently mixed with a micropipette for 1 min, and centrifuged at 13000 rpm at room temperature for 8 min. The top aqueous phase was extracted with care taken to avoid pipetting the intermediate phase. Five hundred milliliters of -20° C isopropanol was added and gently mixed for 1 min, prior to centrifugation at 4° C at 13000 rpm for 5 min. The ethanol was removed, and pellet dried in a heat block at 35° C. The dry pellet was resuspended in 30 mL of sterile nuclease free H2O and incubated at 60° C for 1 hr (suspension was gently mixed after 15 min with micropipette). Quality and concentration were assessed using a Thermo scientific NanoDrop 2000 spectrophotometer.

PCR

Primers designed for *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* were validated using a standard thermal cycler PCR test. Two primer sets (sets 1 & 2) were developed for each of the genera for amplification. The forward primer for *Amycolatopsis* (set 1) was 5'- CAATCTGCCCTGTACTT-3' and the reverse primer sequence was 5'- CTGGCGAAAGAGGTTTA-3' with an amplicon length of 324 base pairs. The forward primer for *Amycolatopsis* (set 2) was 5'- GGGAAACTGGGTCTAATA-3' and the reverse primer sequence was 5'- AGGTTTACAACCCGAAG-3' with an amplicon length of 285 base pairs. The forward primer sequence for *Solirubrobacter* (set 1) was 5'-GTGCTTAACACATGCAA-3' and the reverse primer sequence was 5'- CCACCAACAAGCTAATG-3' with an amplicon length of 234 base pairs. The forward primer sequence for *Solirubrobacter* (set 2) was 5'- GGAAACTCGGGCTAATAC-3' and the reverse primer sequence was 5'- GGTTTACAACCCGAAGG-3' with an amplicon length of 291 base pairs. The forward primer sequence for *Pseudonocardia* (set 1) was 5'-GGGAAACTGGGTCTAATA-3' and the reverse primer sequence was 5'-AGGTTTACAACCCGAAG-3' with an amplicon length of 297 base pairs. The forward primer sequence for *Pseudonocardia* (set 2) was 5'- GTGCTTAACACATGCAA-3' and the reverse primer sequence was 5'- CCACCAACAAGCTGATA-3' with an amplicon length of 235 base pairs (Table 1).

The PCR protocol for *Solirubrobacter* (set 1 & 2) and *Pseudonocardia* (set 1 & 2) are as follows: 1.) initial denaturation temperature 94ºC for two minutes, 2.) 38 cycles of denaturation at 94ºC for 1 minute, annealing at 50.4ºC for 1 minute, and elongation at 68ºC for two minutes, then 3.) extension at

72ºC for five minutes. PCR products were held at 12 ºC until removed from the thermocycler. The PCR protocol for *Amycolatopsis* (set 1 & 2) is as follows: 1.) initial denaturation temperature 94ºC for two minutes, 2.) 38 cycles of denaturation at 94ºC for 1 minute, annealing at 50.0ºC for 1 minute, and elongation at 68ºC for two minutes, then 3.) extension at 72ºC for five minutes. PCR products were held at 12 ºC until removed from the thermocycler. (brand/model)

Gel electrophoresis

Gel electrophoresis was performed on amplified *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* PCR products to validate of each primer set (Table 1) Agarose (1.5%) gels were made by combining 150 mL 1X TAE buffer and 2.25g agarose before microwaving mixture for approximately 1 minute. SYBR safe (15 µL) was added to the mixture once it had cooled to slightly above room temperature. Gel electrophoresis was run at 90V for 25 minutes. Agarose gel was visualized and imaged under UV light.

Chapter Three

Results

Collection results

Sixteen colonies were found within 6000 m of the Southwestern Research Station near Portal, AZ in oak-juniper woodlands in higher elevations and ocotillo- and Acacia-dominated areas found in lower elevations (Figure1A, Figure 1B) Sixteen weeks after the date of excavation sterile samples of both ants and fungus were collected from colony fragments maintained in the laboratory on a restricted diet of polenta. Final samples included ants (8) and fungus (8) from sixteen colonies collected in the field and laboratory for a total of 32 samples (16 ants + 16 fungus = 32 samples).

Sequencing results

The average read length of 16s rRNA gene sequences was 254.7 base pairs with a minimum length of 232 base pairs and maximum length of 439 base pairs. (Illumina MiSeq platform). After raw reads were filtered and trimmed based on quality score and length, there was an average of 404,913.5 sequence reads per sample. Among the filtered reads, 6088 reads were taxonomically classified using Greengenes classifier pre-trained for forward and reverse primers 515 and 806 respectively. Taxonomic classification retrieved 6088 bacterial Operational Taxonomic Units (OTUs). No Archaeal sequences were classified using this classifier. The two most abundant phyla in all samples were Bacteroidetes and Firmicutes which were found in fungus samples collected in the field and lab, respectively. The most abundant phylum found in both ants collected in the field and lab was Proteobacteria. Rarefaction curves for each sample were constructed showing sample size in relation to species. The rarefaction reached a plateau which demonstrated that all OTUs of the microbial community were satisfactorily represented within the data (see supplemental figure 1 and 2).

Alpha diversity

Alpha diversity is the mean diversity in sites at a local scale. In the present study, alpha diversity represents richness and evenness of microbial species distribution within individual samples. The microbial alpha diversity within each sample was analyzed using Shannon's and Simpsons' diversity indexes (Figure

2A, Figure 2B). The average diversity value for laboratory collected fungal samples were H= 2.502221 and D = 0.734002703 while the average diversity value for field collected samples were H= 1.166079198 and D= 0.40344382. Shannon's and Simpsons' values exhibited wide variation between workers collected in the in the laboratory and in the field. The average diversity value for laboratory collected workers were H=2.50222104 and D= 0.734002703. The average Shannon's and Simpsons' diversity values for workers collected in the lab were H= 1.166079198 and D= 0.40344382. A larger decline in diversity was observed between laboratory and field collected fungus than was seen in workers when comparing between times (Figure 3A, Figure 3B).

A Wilcoxon signed-rank test was implemented to determine if the observed number of OTUs differed significantly between sampling treatments (lab and field) for ants and fungus. The number of observed microbial OTUs were significantly more abundant in ants collected in the field to those collected in the laboratory ($p = 0.00031$). The number of observed microbial OTUs also significantly different between fungus collected in the field to those collected in the laboratory ($p = 0.00062$) with field collected samples showing higher abundance.

Beta diversity

To compare the change in bacterial diversity between samples collected in the field to those collected in the laboratory, ordination non-metric multidimensional scaling (**NMDS**) and hierarchical cluster analyses (HCA) were performed based on Bray-Curtis and unweighted unifrac dissimilarity matrix using the R package phyloseq version 1.36.0 (McMurdie 2013). The bacterial microbial diversity of ants and fungus were found to be significantly different in both sampling treatments (laboratory and field). with higher diversity being observed in laboratory collected samples A clear shift in the bacterial community at the OTU level was observed in ordination maps between samples collected in the field to those collected after spending 16 weeks in the laboratory (Figure 4A, Figure 4B). An Adonis, permutational multivariate analysis of variance nonparametric statistical test showed that the microbial composition between each sampling sites (field and lab) were statistically different from one another for both ants and fungus. Approximately 24% of the variation in distances between fungus collected in the field to fungus collected in the laboratory was explained by this grouping ($R^2 = 0.2377$, $p = 0.001$) (Table 3). Approximately 19% of

the variation in distances between workers collected in the field to workers collected in the laboratory was explained by the grouping observed in the ordination map ($R^2 = 0.1867$, $p = 0.023$) (Table 2). Hierarchical cluster analysis (HCL) of ant and fungal microbial community structures based on Bray-Curtis distance matrix showed strong clustering of bacterial communities between laboratory and field collected samples for both organisms. Clustering was not driven by the association of the same colonies sampled under different treatments (Figure 5A, Figure 5B).

Indicator species analysis

Performing an indicator species analysis (R package - indicspecies) revealed a total of 112 indicator species for samples collected from fungus and 23 indicator species from samples collected from workers. From the 6088 OTUs found in fungus samples, 112 were significant indicators, with 106 OTUs being significant indicators for field fungus and 6 for lab fungus. The most significant OTUs for field fungus were species from families Ruminococcaceae and Lachnospiraceae (indicator value = 0.385, $p = 0.0003$; indicator value = 0.529, $p = 0.0003$, $\alpha = 0.05$) from the order Clostridiales. The most significant OTUs for laboratory fungus were species from family Comamonadaceae (indicator value = 0.505, $p = 0.0094$, $\alpha = 0.05$) from order *Burkholderiales*. Of the 6088 OTUs found in worker ant samples, 23 were significant indicators, with 14 OTUs being significant indicators for field worker ants and 9 for lab worker ants. The most significant OTUs for field worker ants were species from families Nocardioidaceae and Pseudonocardiaceae (indicator value = 0.687, $p = 0.0015$; indicator value = 0.645; $p = 0.0068$; $\alpha = 0.05$) from order Actinomycetales. The most significant OTUs for laboratory worker ants were species from families Dermacoccaceae and Comamonadaceae from orders Actinomycetales and Burkholderiales respectively (indicator value = 0.581, $p = 0.0092$; indicator value = 0.527, $p = 0.0025$; $\alpha = 0.05$) (see table 2 in appendix)

Venn diagram comparison

To show a more complete survey of the OTU distribution between samples (ants and fungus) collected in the field and laboratory, shared and unique OTUs between sampling treatments were calculated and Venn diagrams were produced. Of the 6088 unique OTUs present in all samples, 1456 were found in workers collected in the field and 564 were found in workers collected in the laboratory. The microbiome

of worker samples collected in the field shared 114 OTUs to those collected in the laboratory. Field workers contained 1342 unique OTUs while laboratory workers contained 450 unique OTUs (Figure 6A). The majority of the most abundant OTUs shared between workers collected in the field to those collected in the laboratory were Actinobacteria species with a small percentage coming from Proteobacteria and Firmicutes. Families included are as follows: *Nocardioidaceae*, *Solirubrobacteraceae*, *Intrasporangiaceae*, *Rickettsiaceae*, *Tsukamurellaceae*, *Xanthomonadaceae*, *Pseudonocardiaceae*, *Moraxellaceae*, and *Dermacoccaceae*. Of the 6088 unique OTUs present in all samples, 4146 were found in fungus collected in the field and 545 were found in laboratory collected fungus. Field fungus samples contained 4073 unique OTUs while laboratory fungus samples contained 472 unique OTUs. The microbiome of fungus samples collected in the field shared 73 OTUs to those collected in the laboratory (Figure 6B). The most abundant phyla shared between fungus collected in the laboratory and field included bacteria from Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes. Families included among these are as follows: Porphyromonadaceae, Sporolactobacillaceae, Entomoplasmataceae, Burkholderiaceae, Enterococcaceae, Lachnospiraceae, and Enterobacteriaceae.

Primers

Primers for *Pseudonocardia*, *Amycolatopsis*, and *Solirubrobacter* successfully amplified DNA segments for their given taxa. Sets 1 and 2 of primers for *Pseudonocardia* and *Solirubrobacter* resulted in single bands after gel electrophoresis. Primer sets 1 and 2 for *Amycolatopsis* resulted in two bands after gel electrophoresis (Figure 8).

Chapter Four

Discussion

The aim of the study was to determine how much, if any, the microbial composition of ants and fungus changed while contending with laboratory variables as compared to samples collected in their natural environment. It is currently unknown by which mechanism of transmission, vertical or horizontal, the microbiome of fungus gardening ants is inherited. If the microbiome is inherited vertically, we expected to see very little change in the microbiome structure between the two treatments. However, if microbiomes are predominately derived from their environment, laboratory collected samples should significantly differ from those collected in the field. Alternatively, it was possible that the microbiome structure of ants and their respective fungus are derived by a mixture of transmission modes and would react independently over the course of the study.

The data collected on the diversity of bacterial communities associated with ants and fungus of *Trachymyrmex arizonensis* show that fungal gardens undergo a more dramatic shift than worker ants while contending with laboratory variables. These changes were observed despite that significant care was taken to minimize the introduction of exogenous microbes from outside sources into the system. In addition to microbial diversity between treatments, several potentially structurally important bacterial species were identified for both ants and fungus. The importance of a core microbiome in fending off harmful pathogens from destroying fungal gardens, the primary food source of fungus gardening ants, has been elucidated in previous studies.

Comparative analysis of *Trachymyrmex arizonensis* microbiome

To determine whether ants or fungus were a sole driving factor in a shift in microbial diversity, we analyzed a data set consisting of OTUs present in worker ant samples as well as OTUs present in fungus samples. The observed microbiome variation between sampling treatments suggests the core microbiome of ants may be vertically transmitted while the microbiome of fungus may be horizontally transmitted from their environment. Although a closer look into the relationship between microbes and hosts will be needed to verify these findings, the decrease in microbial diversity in laboratory collected fungal samples suggests

much of its structure is taken up from the environment. Additionally, the retention of bacterial diversity between treatment groups leads us to believe that vertical transmission is the most likely mode of transmission for ants. If the microbiome of worker ants is indeed inherited (vertically transmitted), we would expect to see be a correlation between genetic variation of hosts (ants or fungus) with those of the bacteria. Phylosymbiosis is the phenomenon in which host microbial communities tend to recapitulate the phylogeny of their host (Lim et al. 2020). Further experimentation into the phylogeny of both host and their bacterial symbionts would help to clarify these claims.

Diversity between sampling treatments

Diversity metrics within fungal and worker samples in each treatment group reported varied results (Figure 2A, Figure 2B). Microbial diversity within fungal samples (alpha diversity) exhibited a significant shift between those collected in the field and laboratory while workers did not. However, samples collected from the laboratory tended to have lower diversity values than those collected within the field in both treatment groups A larger discrepancy in the diversity between treatments (lab and field) was observed in fungal samples as compared to worker samples (Figure 3A, Figure 3B). This discrepancy indicates a larger loss in diversity within fungal gardens of colonies after spending 16 weeks in a laboratory setting as opposed to workers. As revealed by beta diversity indexes, microbial diversity was found to be significantly different between each treatment group (field and lab). Observing ordination maps from Bray-Curtis distance matrices showed diversity between samples also varied significantly between treatments (Table 2, Table 3). Beta diversity metrics between samples revealed a similar trend as alpha diversity metrics. Although both ant and fungal diversity between sampling treatments were significant, ordination maps showed a clearer separation in fungus than was seen in ants (Figure 4A, Figure 4B). This shift in microbial diversity could be in response to a number of variables including, most notably, diet and environment. It has been theorized that many symbiotic microbes are taken up from their environment through horizontal transmission either through food sources or living environment (Woodhams et al. 2013). Changes in living conditions that heavily affected both variables were most likely the most important factors driving the changes observed in the microbiome. Hierarchical cluster analysis (HCL) of ant and fungal microbial community structures based on Bray-Curtis distance matrix showed strong clustering of

bacterial communities between laboratory and field collected samples for both organisms (Figure 5A, Figure 5B).

Microbial composition

Indicator species analysis and Venn diagrams (Figure 9, Figure 10) were utilized to further investigate the taxa associated with the observed shift in microbial composition. In relation to total OTUs present, few were shared between field and laboratory collected samples with just 1.59% shared between fungus and 5.98% shared between workers. (Figure 7A, Figure 7B) The most abundant OTUs shared between workers collected in the field to those collected in the laboratory were *Actinobacteria* species with a small percentage coming from Proteobacteria and Firmicutes. Families included within these phyla are as follows: *Nocardioidaceae*, *Solirubrobacteraceae*, *Intrasporangiaceae*, *Rickettsiaceae*, *Tsukamurellaceae*, *Xanthomonadaceae*, *Pseudonocardiaceae*, *Moraxellaceae*, and *Dermacoccaceae*. Families *Intrasporangiaceae, Dermacoccaceae, Nocardioidaceae, Tsukamurellaceae,* and *Pseudonocardiaceae* belong to the order *Actinomycetales*, a large order of bacteria in which some species are known to produce secondary metabolites with antibiotic properties. These bacteria are thought to serve in defense from pathogens within the fungus gardening system (cite). The preservation of these bacteria within the microbiome of workers after a significant shift in composition further supports the growing body of evidence that bacteria present on the cuticle of worker ants serve a functional role in maintaining microbial homeostasis. The most abundant OTUs shared between field and laboratory collected fungus were much more diverse than those shared between laboratory and field collected worker ants. While worker samples were predominantly made up of *Actinobacteria* species, the most abundant OTUs shared between fungus collected in the laboratory and field included bacteria from *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Tenericutes*. Families present include *Porphyromonadaceae*, *Sporolactobacillaceae*, *Entomoplasmataceae*, *Burkholderiaceae*, *Enterococcaceae*, *Lachnospiraceae,* and *Enterobacteriaceae*.

Actinobacteria and Proteobacteria are frequently present in the fungal gardens of leaf cutters such as *Acromyrmex echinatior*, *Atta cephalotes,* and *Atta colombica* (Ronque et al. 2020). Ronque et al. suggested that these bacteria play roles in nutrient cycling in the fungal garden in addition to the antimicrobial properties discussed above (Ronque et al. 2020). Li et al. demonstrated that Actinobacteria

are the predominant microbial phyla found on the exoskeleton of fungus gardening ants. The prevalence of *Actinobacteria* in samples collected from workers in the field and in the lab reinforce these findings. This association suggests a protective role by these microorganisms that, in conjunction with regular grooming and hygienic behavior, could reduce pathogenic infection in the fungal garden (Ronque et al. 2020).

The main drivers for significance between laboratory and field collected fungus came from orders Burkholderiales (family: *Comamonadaceae*) and Clostridiales (families: *Ruminococcaceae* and *Lachnospiraceae*) respectively. Significance was mainly driven between laboratory and field collected workers by orders *Burkholderiales* (family: Dermacoccaceae) and *Actinomycetales* (families: Nocardioidaceae and Pseudonocardiaceae) respectively. Further analysis of the function and properties of these important bacterium in future studies could elucidate their functional role within the system.

***Pseudonocardia, Amycolatopsis,* and *Solirubrobacter* primers**

The main goal of this portion of the study was to design primers for use in future experimentation. The consensus sequences used to design these primer sets could be used in future studies to design real time PCR primers to determine the quantity of each genus present in samples under various experimental variables. The ability to screen colonies for bacterium that make up their core microbiome would enable researchers to easily and cheaply determine if the abundance of these critical microbes were decreasing or remaining level at various time positions after relocation to the laboratory. If microbiome structure is transmitted horizontally, we would expect to see a steady decrease in the quantity of these bacteria. If the microbiome structure is transmitted vertically, we would expect to see the quantity remain relatively level throughout multiple points in time.

Conclusions

Due to the complexity and dynamic nature of the microbiome, studies involving the care and maintenance of study species within a laboratory have the potential for non-reproducibility or erroneous results. The microbiome of animal models across multiple taxa have been shown to be significantly affected by both diet and environment variables. Given the broad interdisciplinary developments in microbiome research within the past decade, insights into the external pressures driving microbiome

diversity are of overarching importance to the scientific community. Primers and indicator species produced in this study can aid in future investigations into the presence or absence of key microbial species that may be of importance to the fungus gardening ant species *Trachymyrmex arizonensis.*

Figures

Figure 1A: Photo of ocotillo-Acacia habitat. This type of habitat is where *T. arizonensis* have been found in previous studies

Figure 1B: Photo of oak-juniper habitat. This type of habitat is typically where *T. arizonensis* were found

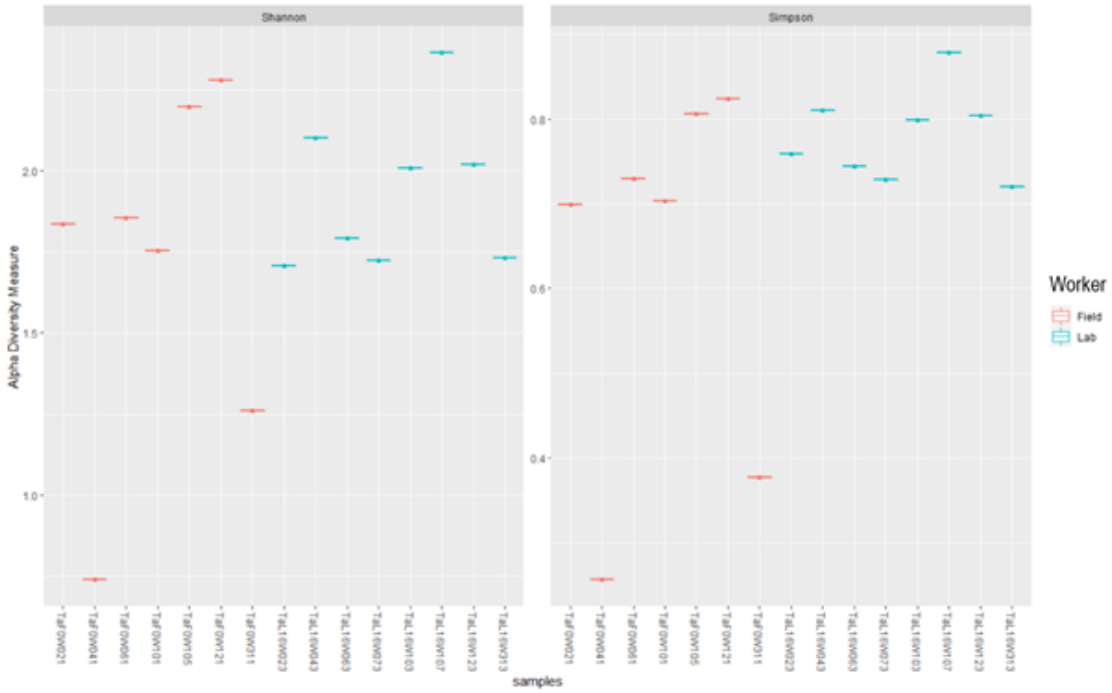

Figure 2A: Microbial Shannon's and Simpsons' alpha diversity indexes within each worker ant sample

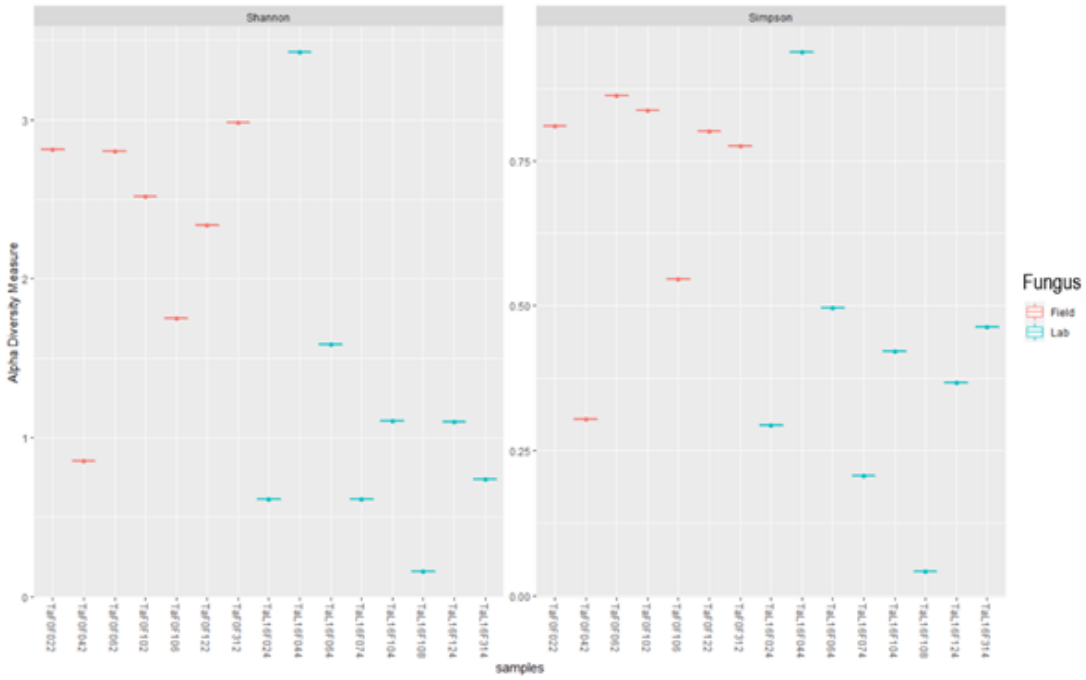

Figure 2B: Microbial Shannon's and Simpsons' alpha diversity indexes within each fungus sample

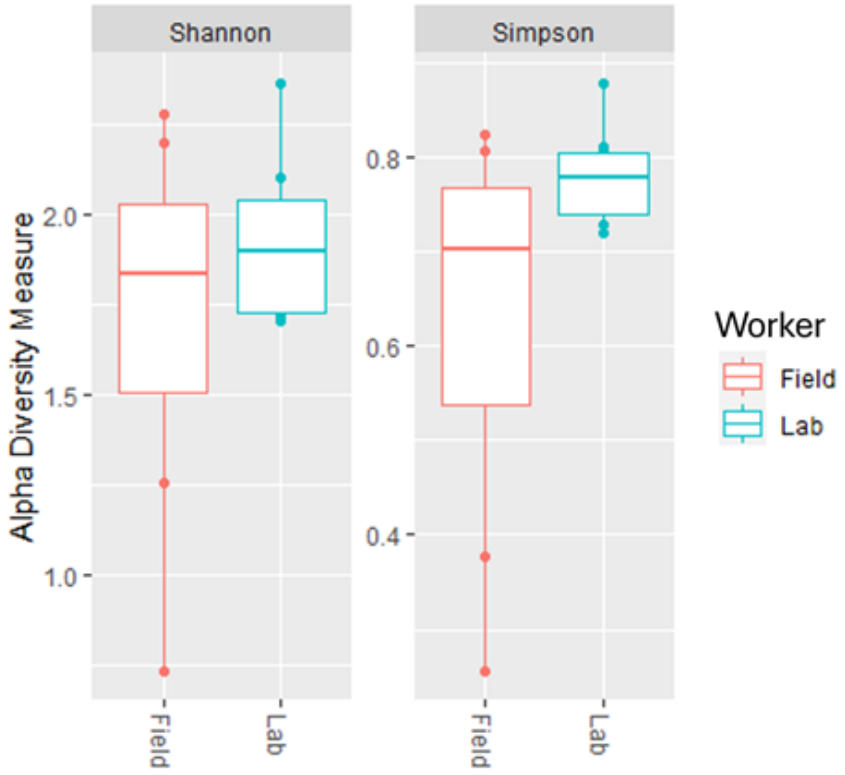

Figure 3A: Shannon's and Simpsons alpha diversity indexes compared between laboratory and field collected worker ants

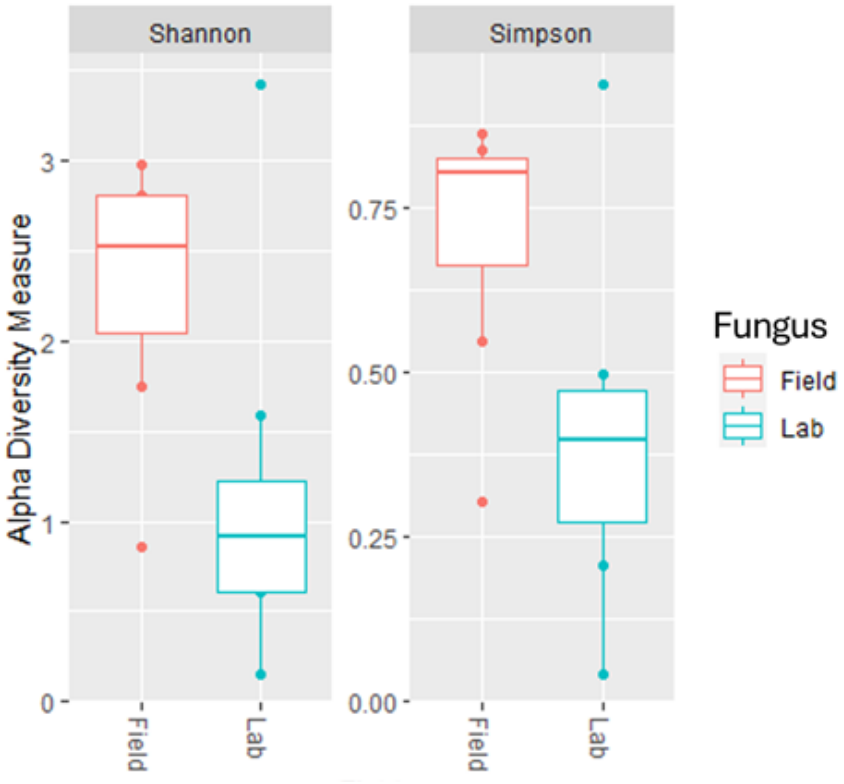

Figure 3B: Shannon's and Simpsons alpha diversity indexes compared between laboratory and field collected fungus

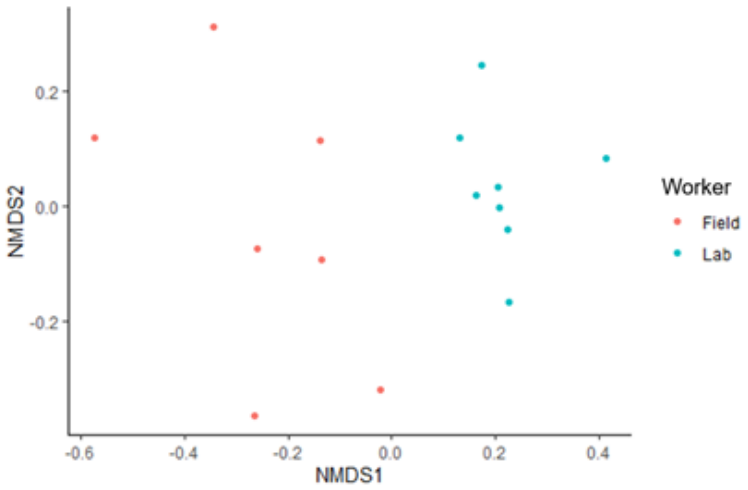

Figure 4A: Ordination non-metric multidimensional scaling (**NMDS**) based on Bray-Curtis dissimilarity matrix for microbiome of worker ants

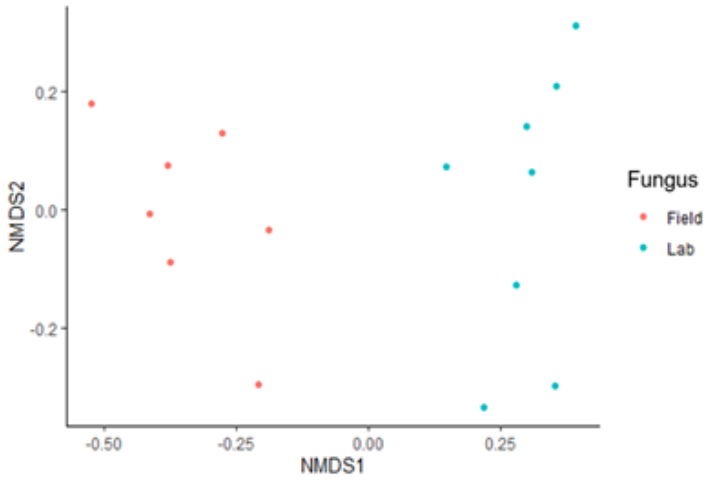

Figure 4B: Ordination non-metric multidimensional scaling (**NMDS**) based on Bray-Curtis dissimilarity matrix for microbiome of fungus

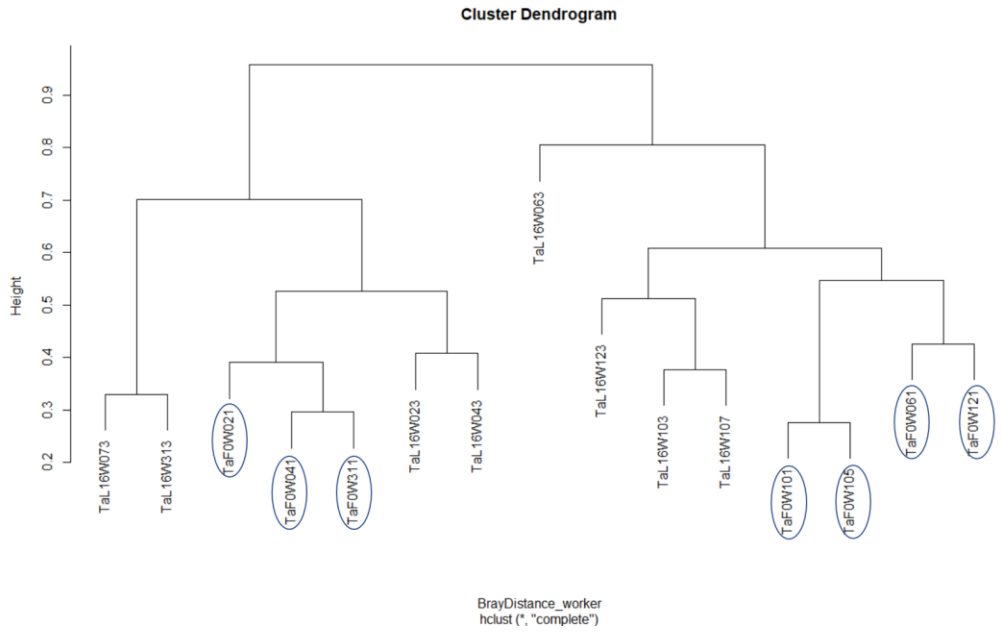

Figure 5B: Hierarchical cluster analysis (HCL) of worker ant microbial community structures based on Bray-Curtis distance matrix (F0 = Field collected samples; L16 = Lab collected samples; Circles indicate field collected samples).

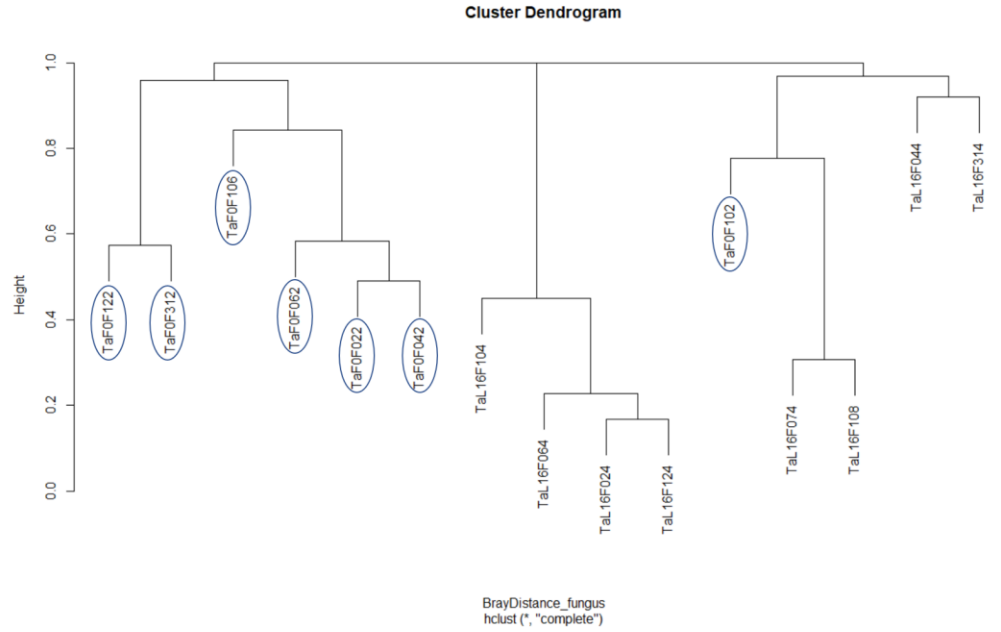

Figure 5A: Hierarchical cluster analysis (HCL) of fungal microbial community structures based on Bray-Curtis distance matrix (F0 = Field collected samples; L16 = Lab collected samples; Circles indicate field collected samples).

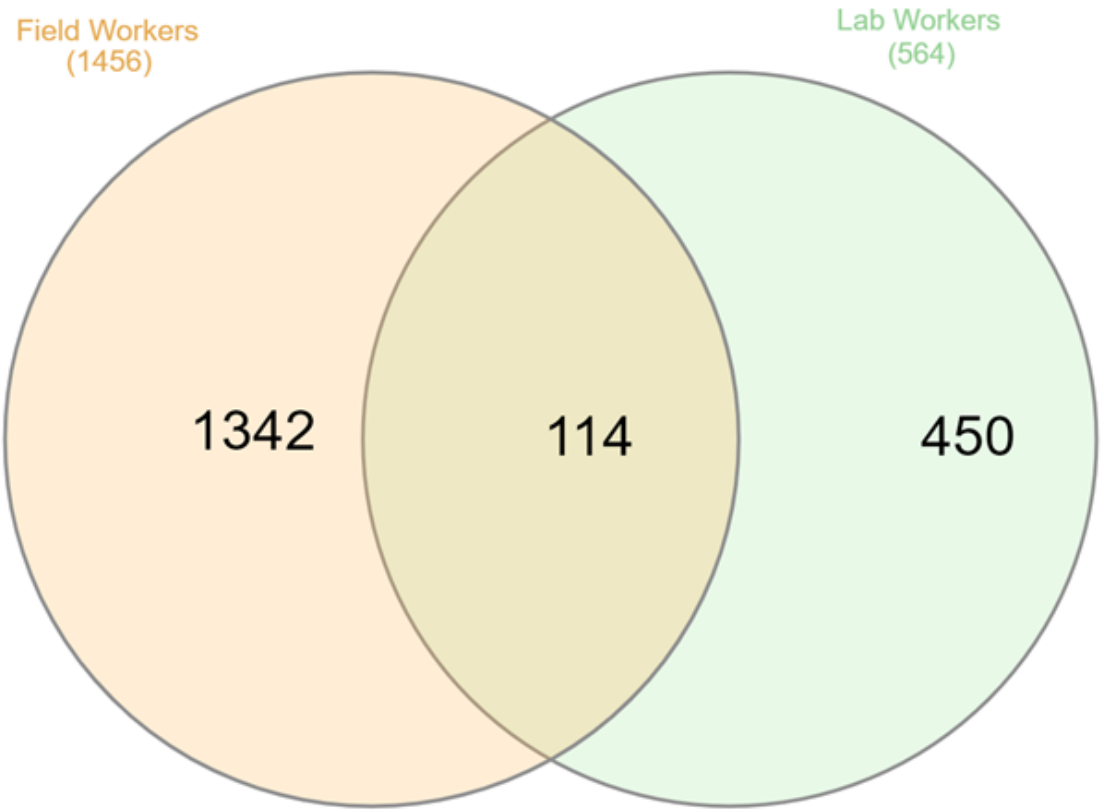

Figure 6A: Venn diagram representing all operational taxonomic units present in worker ant samples collected in the field and laboratory. Of the 6088 unique OTUs present in all samples, 1456 were found in workers collected in the field and 564 were found in workers collected in the laboratory. The microbiome of worker samples collected in the field shared 114 OTUs to those collected in the laboratory. Field workers contained 1342 unique OTUs while laboratory workers contained 450 unique OTUs.

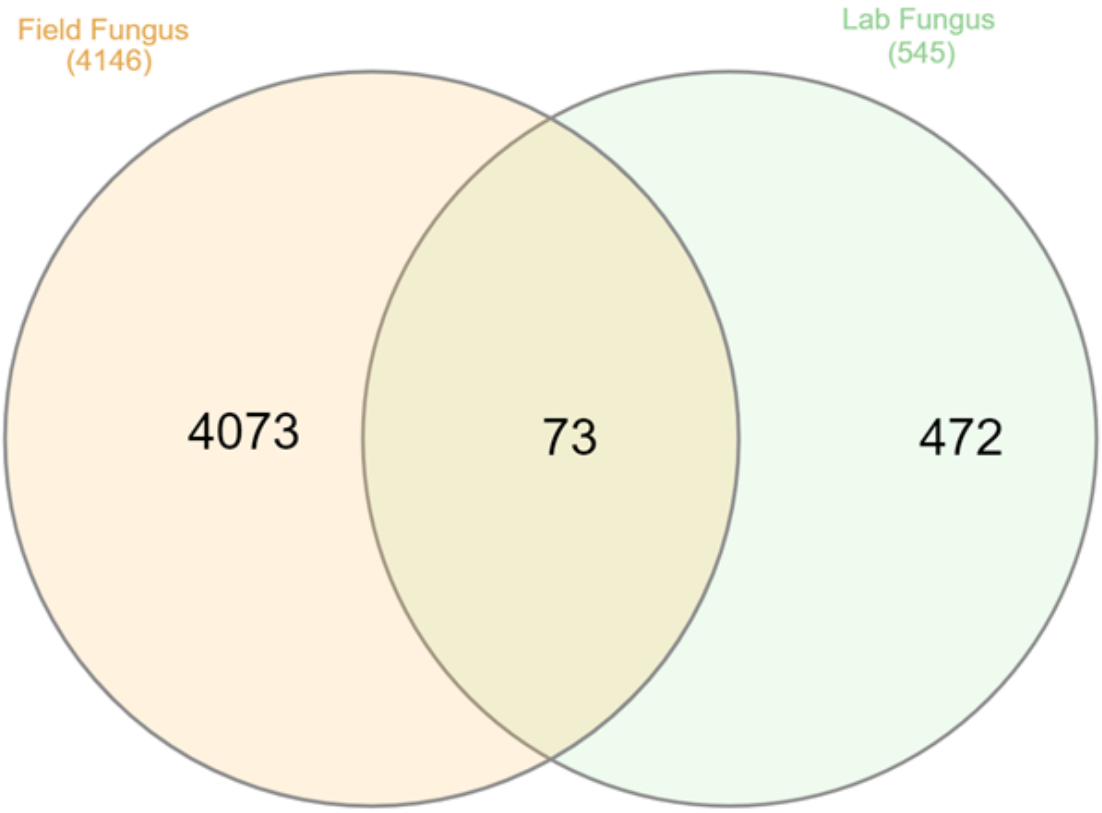

Figure 6B: Venn diagram representing all operational taxonomic units present in fungal samples collected in the field and laboratory. Of the 6088 unique OTUs present in all samples, 4146 were found in fungus collected in the field and 545 were found in fungus collected in the laboratory. Field fungus samples contained 4073 unique OTUs while laboratory fungus samples contained 472 unique OTUs. The microbiome of fungus samples collected in the field shared 73 OTUs to those collected in the laboratory.

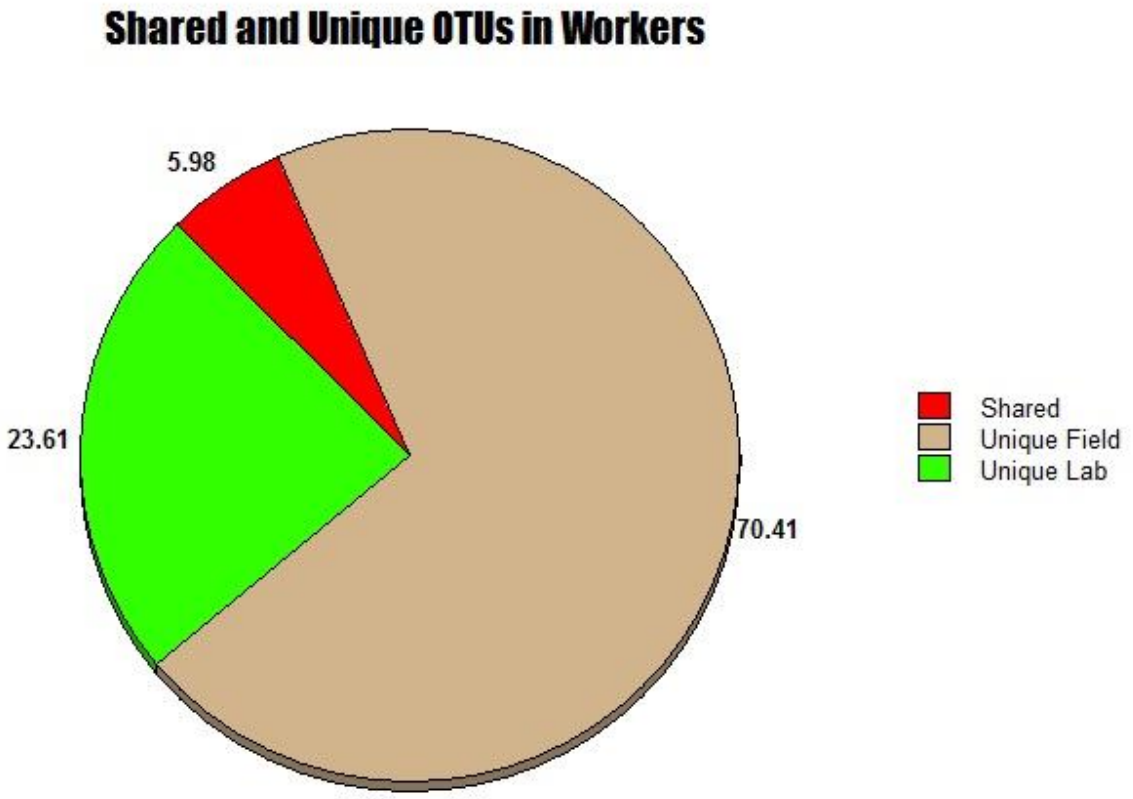

Figure 7A: Pie chart depicting the ratio of shared and unique OTUs between laboratory and field collected worker ants

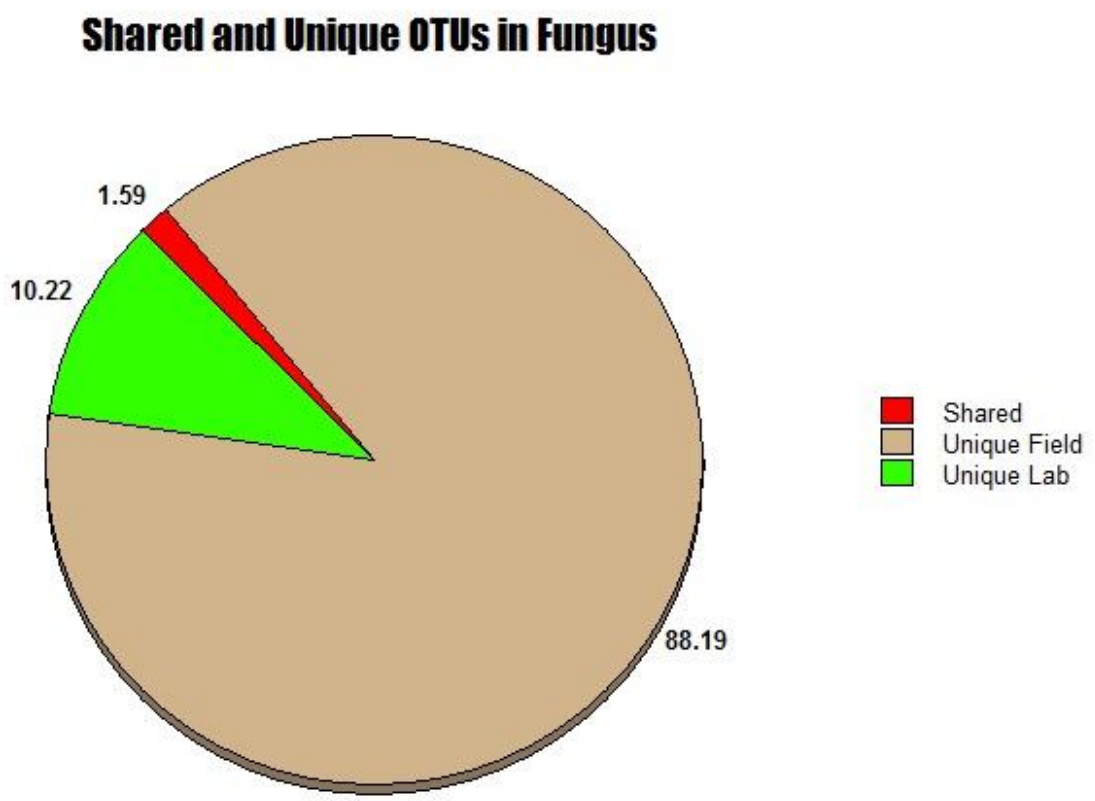

Figure 7B: Figure 7A: Pie chart depicting the ratio of shared and unique OTUs between laboratory and field collected fungus

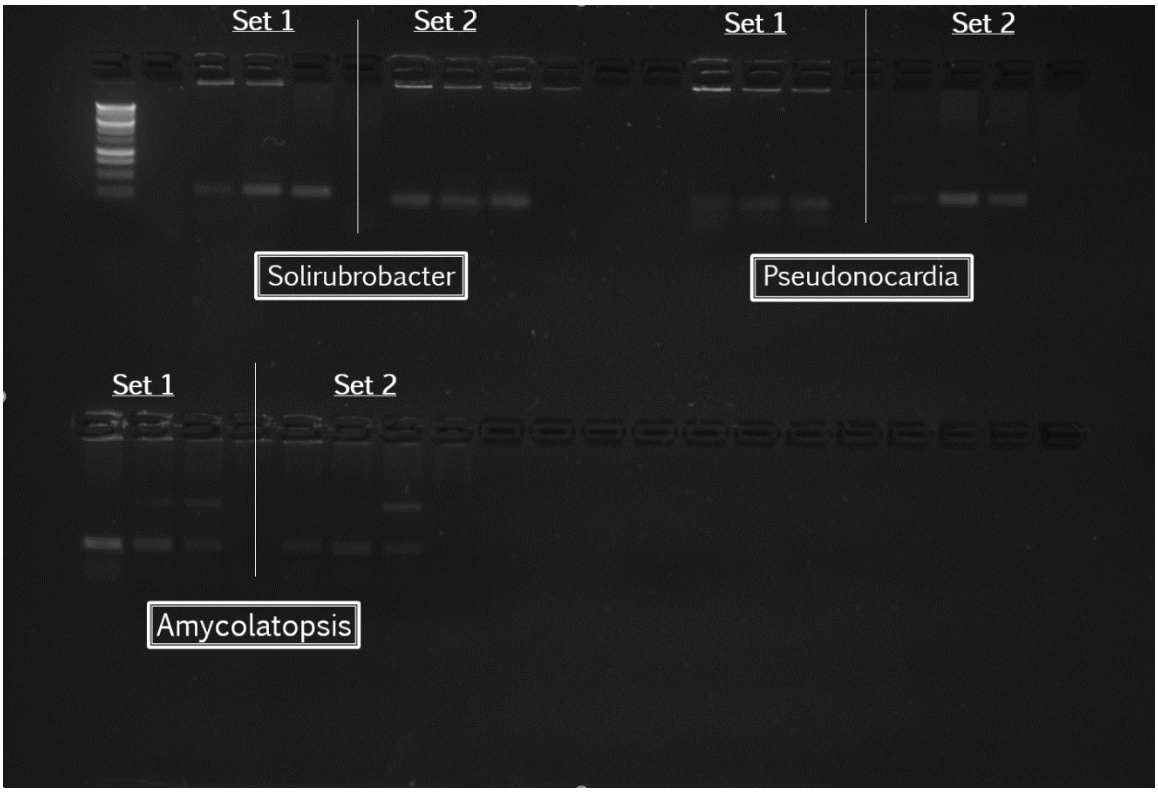

Figure 8: Gel electrophoresis of primers for *Pseudonocardia* (sets 1 & 2), *Solirubrobacter* (sets 1 & 2), and *Amycolatopsis* (sets 1 & 2). Top left: *Pseudonocardia* (sets 1 & 2)*;* Top right: *Solirubrobacter* (sets 1 & 2); Bottom left: *Amycolatopsis* (sets 1 & 2)

Forward Primer	*Reverse Primer*	*Amplicon Size (bp)*	*Genus targeted*	*Annealing Temp*
CAATCTGCCCTGTACTT	CTGGCGAAAGAGGTTTA	324	Amycolatopsis	50.4
GGGAAACTGGGTCTAATA	AGGTTTACAACCCGAAG	285	Amycolatopsis	50.4
GTGCTTAACACATGCAA	CCACCAACAAGCTAATG	234	Solirubrobacter	50.4
GGAAACTCGGGCTAATAC	GGTTTACAACCCGAAGG	291	Solirubrobacter	50.4
GGGAAACTGGGTCTAATA	AGGTTTACAACCCGAAG	297	Pseudonocardia	50.0
GTGCTTAACACATGCAA	CCACCAACAAGCTGATA	235	Pseudonocardia	50.0

Table 1: Primer sequence, annealing temperature, amplicon size, target genus and annealing temperature for primers designed in this study

	Df	*Sum of Squares*	*Mean of Squares*	*F Model*	*R^2*	*Pr(>F)*
Worker ant data	1	0.47114	0.47114	2.9851	0.18674	0.023
Residuals	13	2.05178	0.15783		0.81326	
Total	14	2.52292			1.00000	
Significant codes	**0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

Table 2: Adonis permutational multivariate analysis of variance nonparametric statistical test for worker ant data

	Df	*Sum of Squares*	*Mean of Squares*	*F Model*	*R^2*	*Pr(>F)*
Fungus data	1	1.3767	1.37671	3.9442	0.23277	0.001
Residuals	13	4.5377	0.34905		0.76723	
Total	14	5.9144			1.00000	
Significant codes	**0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

Table 3: Adonis permutational multivariate analysis of variance nonparametric statistical test for fungal data

References

Allert, M. (2017). *Microbiome analysis of two sympatric fungus-gardening ants*, Trachymrmex septentrionalis *and* Trachymyrmex turrifex. https://scholarworks.uttyler.edu/biology_grad/47/

Barke, J., Seipke, R. F., Yu, D. W., & Hutchings, M. I. (2011). A mutualistic microbiome. Communicative & Integrative Biology, 4(1), 41–43. https://doi.org/10.4161/cib.13552

Caporaso, J., Kuczynski, J., Stombaugh, J. et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Currie, C. R., Mueller, U. G., & Malloch, D. (1999). The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Sciences of the United States of America, 96(14), 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scottt, J. A., Summerbell, R. C., & Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature, 398(6729), 701–704. https://doi.org/10.1038/19519

De Caceres, M., Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. http://sites.google.com/site/miqueldecaceres/.

De Fine Licht, H., Boomsma, J. & Tunlid, A. (2014). Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nat Commun 5, 5675. https://doi.org/10.1038/ncomms6675

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., & Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology, 72(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

González, C. T., Saltonstall, K., & Fernández-Marín, H. (2019). Garden microbiomes of Apterostigma dentigerum and Apterostigma pilosum fungus-growing ants (Hymenoptera: Formicidae). Journal of Microbiology, 57(10), 842–851. https://doi.org/10.1007/s12275-019-8639-0

Heine, D., Holmes, N.A., Worsley, S.F. et al. (2018). Chemical warfare between leafcutter ant symbionts and a co-evolved pathogen. Nat Commun 9, 2208. https://doi.org/10.1038/s41467-018-04520-1

Ishak, H. D., Miller, J. L., Sen, R., Dowd, S. E., Meyer, E., & Mueller, U. G. (2011). Microbiomes of ant castes implicate new microbial roles in the fungus-growing ant Trachymyrmex septentrionalis. Scientific Reports, 1, 20–22. https://doi.org/10.1038/srep00204

Ivanova, N., Gugleva, V., Dobreva, M., Pehlivanov, I., Stefanov, S., & Andonova, V. (2016). We are IntechOpen , the world ' s leading publisher of Open Access books Built by scientists , for scientists TOP 1 %. Intech, i(tourism), 13.

Kellner, K., Ishak, H. D., Linksvayer, T. A., & Mueller, U. G. (2015). Bacterial community composition and diversity in an ancestral ant fungus symbiosis. FEMS Microbiology Ecology, 91(7), 1–10. https://doi.org/10.1093/femsec/fiv073

Kellner, K., Kardish, M. R., Seal, J. N., Linksvayer, T. A., & Mueller, U. G. (2018). Symbiont-Mediated Host-Parasite Dynamics in a Fungus-Gardening Ant. Microbial Ecology, 76(2), 530–543. https://doi.org/10.1007/s00248-017-1124-6

Klaus, A., Zimmermann, E., Röper, K. M., Radespiel, U., Nathan, S., Goossens, B., & Strube, C. (2017). Co-infection patterns of intestinal parasites in arboreal primates (proboscis monkeys, Nasalis larvatus) in Borneo. International Journal for Parasitology: Parasites and Wildlife, 6(3), 320–329. https://doi.org/10.1016/j.ijppaw.2017.09.005

Little, A. E. F., Murakami, T., Mueller, U. G., & Currie, C. R. (2006). Defending against parasites: Fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biology Letters, 2(1), 12–16. https://doi.org/10.1098/rsbl.2005.0371

McMurdie, P.J., Holmes, S. (2013). "phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data." PLoS ONE, 8(4), e61217. http://dx.plos.org/10.1371/journal.pone.0061217.

Mikheyev, A. S., Vo, T., & Mueller, U. G. (2008). Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists. Molecular Ecology, 17(20), 4480–4488. https://doi.org/10.1111/j.1365-294X.2008.03940.x

Moreau, C. S. (2020). Symbioses among ants and microbes. Current Opinion in Insect Science, 39, 1–5. https://doi.org/10.1016/j.cois.2020.01.002

Mueller, U. G. (2012). Symbiont recruitment versus ant-symbiont co-evolution in the attine ant-microbe symbiosis. Current Opinion in Microbiology, 15(3), 269–277. https://doi.org/10.1016/j.mib.2012.03.001

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L., & Schultz, T. R. (2005). The evolution of agriculture in insects. Annual Review of Ecology, Evolution, and Systematics, 36(September), 563–595. https://doi.org/10.1146/annurev.ecolsys.36.102003.152626

Oliveros, J.C. (2007). VENNY. An interactive tool for comparing lists with Venn Diagrams.

Owczarzy, Richard et al. (2008). "IDT SciTools: a suite for analysis and design of nucleic acid oligomers." Nucleic acids research vol. 36, Web Server issue: W163-9. doi:10.1093/nar/gkn198

Ronque, M. U. V., Lyra, M. L., Migliorini, G. H., Bacci, M., & Oliveira, P. S. (2020). Symbiotic bacterial communities in rainforest fungus-farming ants: evidence for species and colony specificity. Scientific Reports, 10(1), 1–12. https://doi.org/10.1038/s41598-020-66772-6

Ronque, M.U.V., Lyra, M.L., Migliorini, G.H. et al. (2020). Symbiotic bacterial communities in rainforest fungus-farming ants: evidence for species and colony specificity. Sci Rep 10, 10172. https://doi.org/10.1038/s41598-020-66772-6

Sapountzis, P., Nash, D. R., Schiøtt, M., & Boomsma, J. J. (2019). The evolution of abdominal microbiomes in fungus-growing ants. Molecular Ecology, 28(4), 879–899. https://doi.org/10.1111/mec.14931

Sapountzis, P., Nash, D. R., Schiøtt, M., & Boomsma, J. J. (2019). The evolution of abdominal microbiomes in fungus-growing ants. Molecular Ecology, 28(4), 879–899. https://doi.org/10.1111/mec.14931

Sen, R., Ishak, H. D., Estrada, D., Dowd, S. E., Hong, E., & Mueller, U. G. (2009). Generalized antifungal activity and 454-screening of Pseudonocardia and Amycolatopsis bacteria in nests of fungus-growing ants. Proceedings of the National Academy of Sciences of the United States of America, 106(42), 17805–17810. https://doi.org/10.1073/pnas.0904827106

Trevelline, B. K., Sosa, J., Hartup, B. K., & Kohl, K. D. (2020). A bird's-eye view of phylosymbiosis: Weak signatures of phylosymbiosis among all 15 species of cranes. Proceedings of the Royal Society B: Biological Sciences, 287(1923). https://doi.org/10.1098/rspb.2019.2988

Weber, N. A. (2012). Fungus-Growing Ants A symbiotic relationship exists between an insect and a. Science, 153(3736), 587–604.

Wickham, H., (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Woodhams, D. C., & Brucker, R. M. (2013). Disease defence through generations: Leaf-cutter ants and their symbiotic bacteria. Molecular Ecology, 22(16), 4141–4143. https://doi.org/10.1111/mec.12431

Appendix and Supplemental Figures

List of supplemental figures

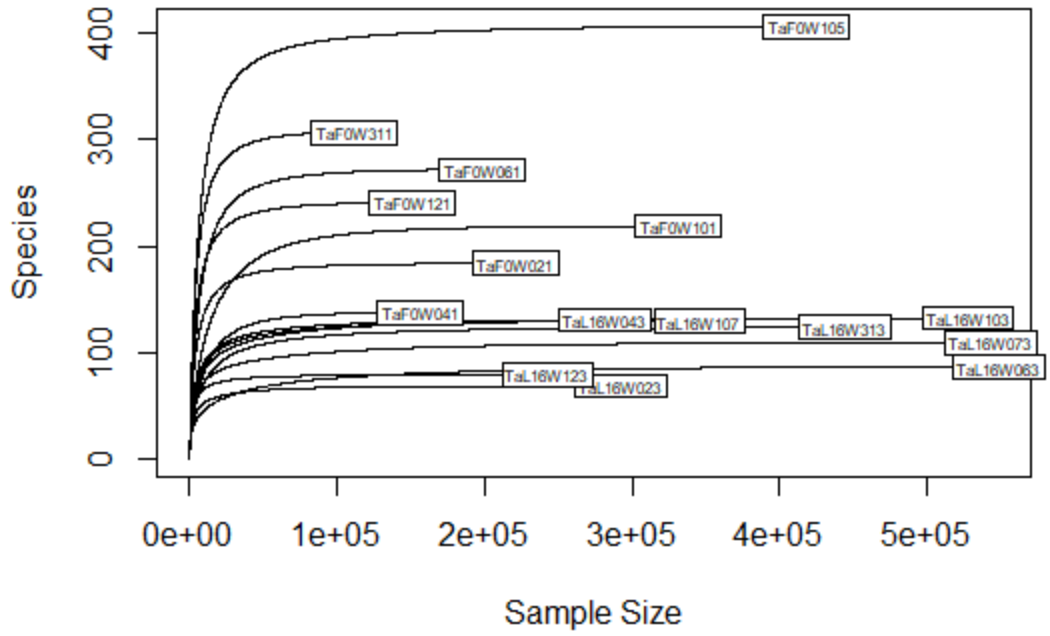

Figure S1: Rarefaction curves for all worker samples

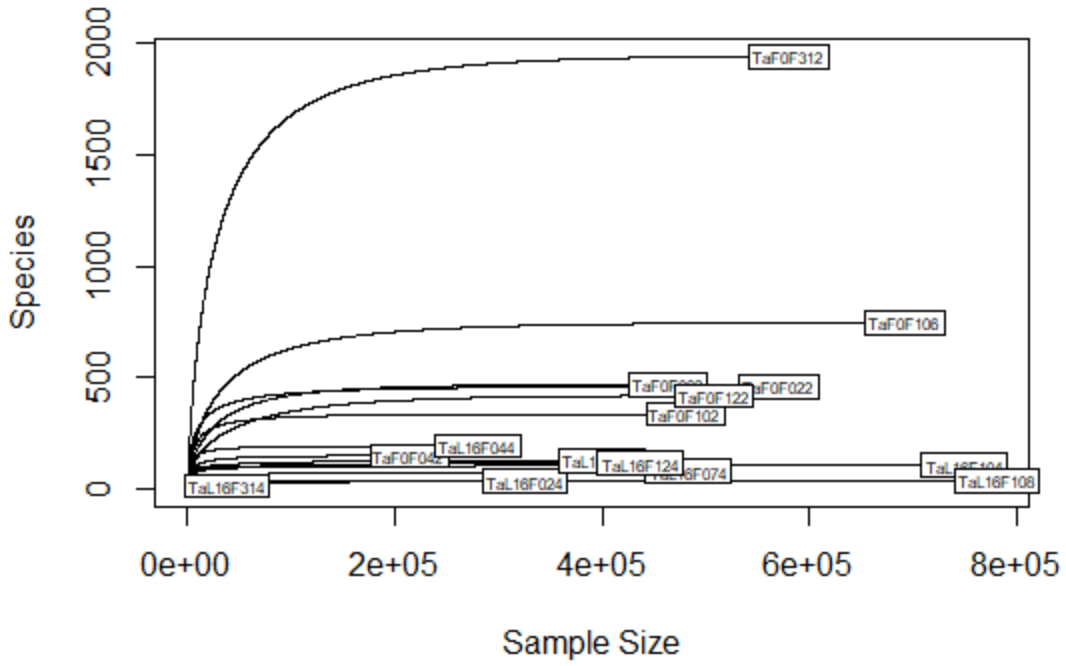

Figure S2: Rarefaction curves for all fungal samples

Indicator Species analysis for Fungus:		
Multilevel pattern analysis		

Association function: r.g		
Significance level (alpha): 0.05		
Total number of species: 6088		
Selected number of species: 112		
Number of species associated to 1 group: 112		
List of species associated to each combination:		
Group Field #sps. 106		
OTU ID	Stat	p.value
4a0b292ba716582f9af46694458c0b9b	0.743	0.0003 ***
0e5ed0b239d77fd4282c265aa1706563	0.673	0.0018 **
f52cc1224cac807cd42d9a901ed53c9b	0.663	0.0003 ***
4091b2eca756083b664b9882ba43d6ce	0.653	0.0003 ***
5b43bbcd7c2bdc0910e5d01a5a29561d	0.627	0.0268 *
c884e8c6371788af363784067981b5f6	0.621	0.0088 **
0be942c91e1b9fa91f102a9ec03124cf	0.607	0.0261 *
f5b678ca37728e48fbb1256b7fbb3f03	0.604	0.0068 **
3e748e7e76b86e44f0d87d1d2b06f244	0.602	0.0268 *
a896b9e68c2283cce1e8e96cb7117320	0.596	0.0263 *
926fe892b1de6a368095dee7cee0e60b	0.595	0.0080 **
f7778d609f13c9161758fdba93cced0e	0.594	0.0268 *
a89ffd50a5b876895f0e71e20036c835	0.587	0.0068 **
cd7ca9595215c090759344743ecee519	0.581	0.0068 **
1d145835e7f0188562322f4eefb02657	0.573	0.0283 *
f3946e858034e0bcfe41bb1200a06a46	0.571	0.0018 **
77f7c652cf10c10e64ac477241844198	0.57	0.0268 *
6ce0ae19978fa5c55ff165cfe21d84a8	0.563	0.0068 **
148e6e49e0d28c6aad0077c63566de2b	0.561	0.0268 *
2e2a87e12f98bae286f184e0a70dc491	0.553	0.0268 *
aba462141ecd869e6c9dbcad5570a628	0.552	0.0272 *
cbdb7f03664d7c0a540776a5fb0862a9	0.548	0.0018 **
1a523f18ecb4d5caf58b9ed76d316b32	0.547	0.0268 *
770f6e4062ec4ed604139c6579e1e0ab	0.541	0.0080 **
97c7d8159d6563e77fdcd8fb71ceb9d3	0.537	0.0068 **
9db8a93f490332ef5aa4ae6662f22d9e	0.532	0.0277 *
dcaa8f764159582ddb72226b265ab846	0.532	0.0268 *

9a32612072bca04a9ea4b1ef0f857c19	0.53	0.0268 *
e847443cf4da33a633b6ccd1d2c399cb	0.529	0.0003 ***
d28d858fc73af6346cfab1f1bbc74859	0.529	0.0018 **
198d3ed9599a8b04708b51cdbf662c2f	0.529	0.0268 *
bc7a67999cc192f4c4a021880636ef97	0.528	0.0068 **
9cf53136196df5d8f6e9fc36ee6c7b98	0.524	0.0068 **
f1419463895bdd44b6d6df3c03bd4912	0.524	0.0080 **
b8c4ed03d7d91546b9f6ad80b80135a3	0.521	0.0248 *
945184b6386c192c0066e0a98a154780	0.519	0.0245 *
6c8975847c187bd396fcff8eeb635546	0.519	0.0080 **
ba07f8f65a3be6de8b77cafc0e6c2969	0.514	0.0277 *
9d127ac8ec1a7b03befe92229847b811	0.511	0.0273 *
12d2e427a519dd7b0bab6285d5c3a14b	0.51	0.0068 **
eb92e0ad022465eb56915a21b47063a0	0.504	0.0274 *
808e3d4013b86e8dfe5d52d69eb83e98	0.503	0.0018 **
2994d2236904a8ecdd1e91a52d1106cd	0.5	0.0260 *
9bd22e89b48709dbb22cd2a70496678e	0.5	0.0260 *
4ec5fb2b78571eb63b7b7f8a4cf32845	0.499	0.0018 **
35e868287a98939ba23dc261e5158678	0.492	0.0273 *
004dec8027e01f2b317bc5b97b806aef	0.492	0.0274 *
0dd4b2e6a4cf2060686834bd6330ee43	0.487	0.0026 **
69647c700456d86b3b23e11b3ca89514	0.485	0.0268 *
1c5b5646ffe080b6c4814d682dfa2f03	0.48	0.0273 *
a7bc7a114d44fc504e29440deaa177d4	0.479	0.0084 **
5bc77cdc5d8a4313bef53afda6eee5f1	0.479	0.0268 *
04ecfad5772d2e09a84a0f5ef460536c	0.475	0.0267 *
e917912913b002dbb511f9376750cbf4	0.47	0.0070 **
a443e786f67086749ef29ce0ab494c9e	0.47	0.0018 **
8bae7f7b348a6a3a856cc14cc2a22cda	0.469	0.0268 *
37795ec070b1846b8e8d58a0ff3362b2	0.469	0.0268 *
dc014a4e4980dbb46080c39eaf8fe9fb	0.453	0.0068 **
cc99805bb66da5b890114fb628e5f69d	0.453	0.0277 *
a285136eb52ed887013aaa7917ce5051	0.45	0.0260 *
cc53cb9c6fdf0d743c27ae2c72e9ba73	0.447	0.0068 **
3290a8b008cc935f53af9dfcf5ee019a	0.442	0.0268 *
b1e0c9d1fa04a8d85ea9c8512255ad71	0.436	0.0277 *
f5236f96db38a1fdf4db6aa762c613fb	0.435	0.0081 **
6d6483fe9e2705b0a299080fcf7bb295	0.43	0.0277 *
b0b56033755860b4f4bed90f06f042d1	0.429	0.0068 **
489f985b6e87a04363fc9df4f34081ba	0.427	0.0243 *
fc8a0d0609361d13fe869d3776b666a9	0.425	0.0268 *
c0e69dcfa51d442b19751104d64f57d5	0.423	0.0076 **
b4bb370a59f2637d406ccc0f5a1b2a1e	0.419	0.0080 **

0a8076572299fb51e0ca4f64509eaa7c	0.419	0.0274 *
0a3cf58d4ca062c13d42c9db4ebcbc53	0.418	0.0068 **
232427755d62fac53784240c1e5ff853	0.415	0.0268 *
ac28d66567623750eeed68db3b549bbd	0.406	0.0077 **
8d48b7928984830a57e5bc688633ccce	0.403	0.0077 **
b0546a8c6a38415c52d2dc916319d3fd	0.398	0.0283 *
822676861967cdbbe970f83a96c9b165	0.396	0.0260 *
6169bf3edec89fe72f5dd8a0e07b7c26	0.394	0.0268 *
e8a4c6aeadbe980de2b8d5b91d443c99	0.387	0.0268 *
b0d9211620cec0895e6d05edbc9d7c8f	0.385	0.0003 ***
1ec9fd06c88669eb15764e0b441ade46	0.379	0.0283 *
cf7f488ed8661d55ec4be7e5ce5caff2	0.376	0.0268 *
0ffe5fc7a5d1f5c69fd106cfb7a2d6f5	0.372	0.0261 *
d61f695cdb91385cacc8ed72af53930b	0.365	0.0274 *
e29e174c907405b145aef4584faefbf6	0.358	0.0018 **
9b10bd7cdd77daa62539c2fa2f2f8e13	0.358	0.0274 *
91ebfad40d1b4eba610e0130ea576fd5	0.358	0.0084 **
0792c52491986770305bd5af157aff78	0.356	0.0274 *
d817c1c9b1a55b6af451568e6e5fa39e	0.341	0.0018 **
71faf4d5097713c03d4620a57cb704df	0.341	0.0281 *
ff9b90e63f58ee65cffc39d1b3c6d8a7	0.336	0.0077 **
9cfb89b1a4f17a815dd1c36557343fde	0.333	0.0076 **
4cf3f039d6616c17440c25eab8df8b00	0.324	0.0018 **
01078ee576629d71440aa2282ed0feaf	0.323	0.0088 **
adf6d94ef71aef02ab94bc7f2a6d150a	0.316	0.0018 **
8d8b6930e6da1de492cb4039f43e14ce	0.311	0.0277 *
22030bcb4ae43cf6a6499c6344d8df44	0.308	0.0274 *
4ca3aaf851d66bb85de3eec3a40759da	0.307	0.0019 **
abe24e2949833423d053070436f01b3e	0.302	0.0084 **
c0488696135bd32c62260be9b4184494	0.302	0.0068 **
019245104ed4e1a751a760748c8a5205	0.296	0.0260 *
ca5870e02541a61555da871e5419f732	0.292	0.0277 *
f204937d706f891ce032454c2b914768	0.291	0.0088 **
25b24da6ce1fa022f865d934ff5a4da0	0.289	0.0288 *
e36d7fd043eb6ab2baf09e0d03207af4	0.282	0.0068 **
42ad4ab63682287b8bb1ce7f4562b7ad	0.279	0.0088 **
Group Lab #sps. 6		
OTU ID	stat	p.value
1d3799a88585210d48ce1dc25f052642	0.58	0.0453 *
5b92978f4d848dd5c1f7b193fdd67b30	0.561	0.0451 *
7911816f5e81f650f769aba0d5c708cb	0.548	0.0327 *
c0c6dea060404ea17972053bc9e98ae7	0.508	0.0094 **

c0d5395792eadbf5f62e8ffb14fa0262	0.496	0.0370 *
741280f21e803ddc0af08372de797025	0.401	0.0332 *

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1		

Supplemental Table 1: Indicator species analysis showing significant OTUs for fungal samples

Indicator Species analysis for Workers:

Multilevel pattern analysis

Association function: r.g

Significance level (alpha): 0.05

Total number of species: 6088

Selected number of species: 23

Number of species associated to 1 group: 23

List of species associated to each combination:

Group Field #sps. 14

OTU ID	stat	p.value
6ef30ad70950c7994898405da4aa1f2a	0.687	0.0015 **
fcd80f0cf279eff3c50b66a78d98beeb	0.645	0.0068 **
e553d8cfc9db67cf92a834ed05576ac8	0.545	0.0324 *
512755705129dbbc8da713be369b3984	0.543	0.0424 *
22dbdb2168e098ebe80b1a233166d38e	0.533	0.0456 *
e388cd6373ff5c0e883461e6241df4a8	0.53	0.0298 *
9a11b7e15474f55602c8e8771556c67f	0.521	0.0400 *
21652c29c1c975e08f2f5ecf90b7bc22	0.518	0.0366 *
48e74d63da54607ae8d0acb389c7acf	0.508	0.0269 *
3cc57e349da9029c661003c0058362e5	0.491	0.0441 *
099b8a07b6b73755cad5d2df763bd3c	0.483	0.0256 *
d829bee4984f82ffc2453212157caf96	0.45	0.0149 *
2d16d001e21717f960cf771ae879b1dd	0.391	0.0261 *
7ef6a6f0dfd650a93138c85eebf1b5c0	0.38	0.0065 **

Group Lab #sps. 9

OTU ID	stat	p.value
7911816f5e81f650f769aba0d5c708cb	0.611	0.0083 **
9f4ca9a5f425cda0094a04261d6b2fc6	0.61	0.0084 **
4579146d375e989d45276198ad07904d	0.585	0.0125 *
36073aa00911cbb7a90be813530d8e20	0.581	0.0092 **
d15bc449222795a9ff230013aa633686	0.543	0.0170 *
c0c6dea060404ea17972053bc9e98ae7	0.527	0.0025 **
c0d5395792eadbf5f62e8ffb14fa0262	0.475	0.0460 *
8ce12e88f6b59bb09494567f0d678092	0.471	0.0423 *
20b3df08cd5fac2be26a2928155bab69	0.361	0.0195 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Supplemental Table 2: Indicator species analysis showing significant OTUs for fungal samples