Environments And Hosts Structure The Bacterial Microbiomes Of Fungus-Gardening Ants And Their Symbiotic Fungus Gardens.

Blake Bringhurst
University of Texas at Tyler
Mattea Allert
University of Texas at Tyler
Matthew Greenwold
University of Texas at Tyler
Katrin Kellner
University of Texas at Tyler
Jon Nicholas Seal (trachymyrmex@gmail.com)
University of Texas at Tyler https://orcid.org/0000-0001-8013-1438

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Abstract

The fungus gardening-ant system is considered a complex, multi-tiered symbiosis, as it is composed of ants, their fungus, and microorganisms associated with either ants or fungus. We examine the bacterial microbiome of *Trachymyrmex septentrionalis* and *Mycetomoellerius turrifex* ants and their symbiotic fungus gardens, using 16S rRNA Illumina sequencing, over a large geographical region (east and central Texas). Typically microorganisms can be acquired from a parent colony (vertical transmission) or from the environment (horizontal transmission). Because the symbiosis is characterized by co-dispersal of the ants and fungus, elements of both ant and fungus garden microbiome could be characterized by vertical transmission. The goals of this study were to explore how both the ant and fungus garden bacterial microbiome are acquired. The main findings were that different mechanisms appear to explain the structure the microbiomes of ants and their symbiotic fungus gardens. Ant associated microbiomes had a strong host ant signature, which suggests vertical inheritance of the ant associated bacterial microbiome. On the other hand, the bacterial microbiome of the fungus garden was more complex in that some bacterial taxa appear to be structured by the ant host species, whereas others by fungal lineage or the environment (geographic region). Thus bacteria in fungus gardens appear to be acquired both horizontally and vertically.

Introduction

Symbioses with microorganisms have been crucial to the success of insects, if not eukaryotic life in general (Klepzig *et al.*, 2009; McFall-Ngai *et al.*, 2013; Douglas, 2014, 2015; Parfrey *et al.*, 2018). One of the most striking discoveries is that many symbioses often consist of more than a single host and a single symbiont, and are best viewed as a community of macro- and microorganisms (Moran, 2001; Woyke *et al.*, 2006; Douglas, 2010; Johansson *et al.*, 2013; Klassen, 2019; Brown *et al.*, 2020). One of the central issues facing symbiology (the study of symbioses) is understanding how these complex entities are organized and function in a dynamic world, especially with regard to the mechanisms that maintain specificity and homeostasis between hosts and microbial symbionts (Douglas, 2010; Silverstein *et al.*, 2012; Garcia & Gerardo, 2014; Hambleton *et al.*, 2014; Dheilly *et al.*, 2015; Parfrey *et al.*, 2018). For example, insects often have a microbiome that is structured toward particular life history, e.g., with microorganisms often supplying key nutrients or resources that essentially ‘upgrade’ specialized but otherwise poor diets (Douglas, 2011; Feldhaar, 2011; Douglas, 2015) or employing symbionts as defense against pathogens (Kaltenpoth & Engl, 2013; Clay, 2014; Van Arnam *et al.*, 2018). Ants are among the most ecologically important insects, which can be attributed to their large colonies, population sizes and diversity in diets (King *et al.*, 2013; Parr *et al.*, 2016; Farji-Brener & Werenkraut, 2017; Swanson *et al.*, 2019). While ants have a digestive system similar to most omnivorous insects, they may feed on a variety of food sources and employ symbionts to upgrade their diets (Blüthgen *et al.*, 2003; Davidson *et al.*, 2003). For example, some ants may harbor bacteria that fix nitrogen or involved in N cycling generally (Borm *et al.*, 2002; Neuvonen *et al.*, 2016; Hu *et al.*, 2018).

The fungus gardening ant (tribe Attini) symbiosis has long been known as a complex system of interacting macro- and microsymbionts. For example, Actinobacteria cultivated via exocrine glands on some attine species' cuticle produce secondary metabolites that appear to have a defensive function against predatory fungal species (*Escovopsis* spp.) (Meirelles *et al.*, 2014; Li *et al.*, 2018; Montoya *et al.*, 2021). The relationship between Actinobacteria and *Escovopsis* spp. appears to have a long coevolutionary history with the fungus-gardening ant symbiosis (Cafaro *et al.*, 2011; Osti & Rodrigues, 2018), though some Actinobacteria may be environmentally acquired (Mueller *et al.*, 2010). Likewise, *Burkholderia* bacteria associated with fungus gardens also appear to have antimicrobial function (Francoeur *et al.*, 2021). Certain yeasts may be antagonists towards Actinobacteria and nitrogen fixing bacteria may provide amino acids to the ants (Little & Currie, 2008; Pinto-Tomás *et al.*, 2009; Rodrigues *et al.*, 2009). Microfungi may also serve important nutritional, detoxification and defensive functions against specialist and general competitors and predators of the fungus garden (Rodrigues *et al.*, 2009). Much of our understanding has focused on the species found in the tropics, which may or may not be representative of the interactions occurring in all fungus-gardening symbioses. For instance, the specialized predatory fungus (*Escovopsis*) is not thought to be very common in temperate attines (Rodrigues *et al.*, 2011) and other bacterial taxa may be just as important if not more important than Actinobacteria (Ishak *et al.*, 2011). As a result, detailed intraspecific natural history studies of microbiomes of ants and their symbiotic fungi may increase our understanding of the roles of microbial associates (Kaltenpoth & Engl, 2013).

With the advent of next-generation methods, the structure of the microbial communities of fungus-gardening ants is only beginning to be understood. The bacterial communities of fungus-gardens typically are characterized by few genera that make up the vast majority of the microbiome; for example, *Atta colombica* and *Atta cephalotes* fungus gardens contained primarily *Enterobacter, Klebsiella, Citrobacter, Escherirchia,* and *Pantoea*, i.e., bacteria that are often found in herbivore digestive systems (Aylward *et al.*, 2012). Probably because attine ants have outsourced most digestion to the external fungal symbiont, the gut microbiome appears to be less diverse and may have a relatively minor role with regard to digestion (Sapountzis *et al.*, 2015; Sapountzis *et al.*, 2019). The function of many bacterial taxa residing in fungus gardens are poorly understood; for instance, notably, researchers found that fungal inocula contained a high amount of *Mesoplasma*, the colony was more likely to decline, though it was unclear whether this was correlative or causal (Meirelles *et al.*, 2016). Ishak et al. (2011) examined the microbes present in the fungus gardening ants *Trachymyrmex septentrionalis*; the results indicate that Actinobacteria (e.g., *Pseudonocardia sp.*, *Kribbela sp.*, *Amycolatopsis sp.*, and *Streptomyces sp*) and Mollicutes were the most abundant bacterial taxa in the fungus of *T. septentrionalis*. Ishak et al (2011) suggested that ants within the same colony and colonies have distinct microbiomes. A recent comparative study illustrated that five different attine species and their colonies harbor distinct microbial communities from one another (Ronque *et al.*, 2020).

Like many symbioses, attine symbionts are obtained from a mix of horizontal and vertical transmission. For example, the fungus garden is typically vertically transmitted from parent colony to gynes (new queens) (Mikheyev *et al.*, 2007; Schultz & Brady, 2008; Mueller *et al.*, 2018; Beigel *et al.*, 2021).

Actinobacteria, are also thought to be primarily vertically transmitted, since clades of ants typically grow similar strains of *Pseudonocardia* (Mikheyev *et al.*, 2008; Cafaro *et al.*, 2011). On the other hand, some studies have documented sharing of fungal symbionts among ant species and environmental acquisition of bacteria (Kellner *et al.*, 2015; Schultz *et al.*, 2015; Mueller *et al.*, 2018; Beigel *et al.*, 2021). Unfortunately, there have been very few studies that have taken a comparative approach to determine how the microbiomes of these symbioses are acquired.

Although research is accumulating on fungus gardening-ant microbiomes, not many studies have examined how geography, host species or type of fungal symbiont may influence the structure of microbial communities (Kellner *et al.*, 2015; Meirelles *et al.*, 2016; Ronque *et al.*, 2020). For example, ants in the 'higher attine genera' (*Mycetomoellerius*, *Paratrachymyrmex*, *Seriocomyrmex* and *Trachymyrmex*), grow conservatively five lineages of fungi and may host switch to varying degrees (Mikheyev *et al.*, 2008; De Fine Licht & Boomsma, 2014; Ješovnik *et al.*, 2017; Solomon *et al.*, 2019; Luiso *et al.*, 2020; Beigel *et al.*, 2021). It remains unknown how the microbiome may be altered as members of the symbiosis are added or lost, such as by fungal symbiont switching observed among many host ant species (Mikheyev *et al.*, 2007; Mikheyev *et al.*, 2008; De Fine Licht & Boomsma, 2014; Schultz *et al.*, 2015; Mueller *et al.*, 2018; Luiso *et al.*, 2020; Beigel *et al.*, 2021). Unfortunately, there have been very few microbiome studies that examine both ants and fungus gardens across ecological scales (Ishak *et al.*, 2011; Kellner *et al.*, 2015). Studies have focused on fungus garden bacterial microbiome structure (Aylward *et al.*, 2012; Meirelles *et al.*, 2016) or solely on ants (Sapountzis *et al.*, 2015; Sapountzis *et al.*, 2019; Ronque *et al.*, 2020). Similarly, Ishak et al (2011) examined temporal patterns of microbiome structure, whereas Kellner et al (2015) examined spatial components. Groundbreaking - yet limited - studies like the latter are needed because the ants, fungus and many of their bacterial associates are clearly interacting with one another across ecological and evolutionary scales (Schultz & Brady, 2008; Seal & Mueller, 2014; Seal *et al.*, 2014; Mueller *et al.*, 2018; Beigel *et al.*, 2021). Therefore conclusions drawn from the microbiome of only one partner of the symbiosis are limited.

To unravel what factors influence the microbiome of *T. septentrionalis* and *M. turrifex* ants and their symbiotic fungus, we examined the bacterial microbiome of the ants and symbiotic fungi of *T. septentrionalis* and *M. turrifex* across a portion of their shared ranges. The broad goal of this study was to characterize the bacterial microbiome of *T. septentrionalis* and *M. turrifex* and their symbiotic fungus garden and determine potential drivers of this variation. Specifically, we had four main questions: **1)** Do the ant species harbor different bacterial communities? **2)** Do the two ant species influence the bacterial community structure of the fungus gardens? **3)** Are fungal lineages characterized by different microbiomes? **4)** Are there geographical differences in the structure of ant or fungal bacterial microbiomes?

Materials And Methods

Study area

Samples of both ants and fungus garden materials were obtained during May and June, 2016 from field colonies from sites in two broad regions in east-central Texas. Samples were obtained from four sites in northeast Texas in close proximity to Tyler, Texas ('East Texas') (approximately 32.29° N 95.24° W) and from three sites approximately 300 km away in Bastrop, Texas (approximately 29.39°N 97.32°W) ('Central Texas') (Fig. 1). Both species of ants co-occur at most of the same locations (Fig. 1, Table 1). Although all of these sites occur in the Post Oak Savanna Ecoregion, which is characterized by sandy soils and an overstorey of post oaks (*Quercus stellata*) and occasionally loblolly or shortleaf pine (*Pinus taeda* and *P.echinata*, respectively) and understory shrubs such as yaupon holly (*Ilex vomitoria*) and eastern redcedar (*Juniperus virginiana*), East Texas tends to be wetter than Central Texas (115 cm vs. 90 cm of precipitation per year, respectively) (Diggs *et al.*, 2006). The latter is especially known for extreme droughts where less than 30 cm of rain may fall in a year (Moore *et al.*, 2016). Central Texas is also the western limit of *T. septentrionalis* whereas East Texas (and western Louisiana) is the eastern limit of *M. turrifex* (Rabeling *et al.*, 2007; Seal *et al.*, 2015; Senula *et al.*, 2019; Luiso *et al.*, 2020).

Sampling Methods

We collected up to five adult ants from each colony of both species; these ants were pooled into a single DNA extract to account for heterogeneity among individual ants in the colony (Ishak et al., 2011). Ants were collected directly from inside fungus gardens with ethanol and flame-sterilized forceps, meaning that the ants collected were likely garden workers (i.e., not foragers who could pick up bacteria inadvertently while outside the nest). Roughly an equal number of *T. septentrionalis* and *M. turrifex* colonies from our samples of the East Texas and Central Texas populations were utilized (N = 13 for *T. septentrionalis* and N = 11 for *M. turrifex*) (Table 1). A small sample of fungus garden material was collected similarly with flame and ethanol sterilized forceps from the same garden chambers where the ants were collected. Lastly, seven soil samples were taken from within the nest fungal chambers (N = 4 from East Texas and N = 3 from Central Texas) to act as a negative control, and make sure the microbe communities observed with the ant or fungal samples were not a relic of soil contamination. All samples were preserved immediately upon collection in 100% ethanol.

Fungal Genotyping

Each fungus garden was genetically identified to phylotype (clade) using the terminology and methods of Luiso et al. (2020). Briefly, gongylidia (swollen hyphal tips diagnostic feeding structures of higher fungus-gardening ants) were plucked off the fungus with flame-sterilized forceps, placed in an aqueous solution of Chelex, and heated in a thermal cycler (Luiso *et al.*, 2020). Before PCR amplification, the DNA extract was diluted (1:10) using nuclease free water (higher concentrations of DNA inhibited PCR reactions). The PCR was performed using the ITS 4 and ITS 5 primers to amplify the 18S rRNA ITS gene (White *et al.*, 1990; Luiso *et al.*, 2020). The resulting PCR products were sent to the DNA Sequencing Facility at the University of Texas at Austin for Sanger sequencing on an Applied Biosystems 3730 DNA Analyzer. The resulting ITS sequences were cleaned up and aligned in Geneious 10.1.2 (Kearse *et al.*,

2012). Sequencing errors or misreads in the DNA sequences were manually corrected. Sequences were identified using BLAST and personal databases of ITS sequences (Table 1) (Luiso *et al.*, 2020).

DNA Extraction, PCRs and Sequencing of Microbiomes

DNA extraction and sequencing of the 16S samples was performed at MRDNA in Shallowater, Texas (http://www.mrdnalab.com/). DNA sequences were amplified from whole ants and fungus 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. Sequences have been deposited in the NCBI Sequence Read Archive under the BioProject PRJNA789907.

Microbiome Bioinformatics

Initial sequence cleanup was performed by removing short sequences with <150 bp, sequences with ambiguous base calls, chimeras, sequences with runs exceeding 6 bp, and singleton sequences (Dowd *et al.*, 2008). The resulting sequences were then inputted into Qiime2-2020.6, after having their barcodes and linker and reverse primers removed (Bolyen *et al.*, 2019). Sequences were then demultiplexed using the demux plugin https://github.com/qiime2/q2-demux and went through quality control using the dada2 plugin (Callahan *et al.*, 2016). When using the dada2 plugin, sequences were truncated down to 260 base pairs as the average quality score dipped below 20 beyond this point. Taxonomy classification with 99% OTU similarity was performed utilizing the SILVA 132_QIIME database (Quast *et al.*, 2013; Yilmaz *et al.*, 2014). To do this, we created our own taxonomic classifier using the "feature-classifier fit-classifier-naive-bayes" command and the SILVA database. This classifier was used to assign sequences a taxonomic classification using the "feature-classifier" plugin (Bokulich *et al.*, 2018) with the "classify-sklearn" command. An OTU table was created by inputting a tabulated taxonomic bar plot, created using the "taxa barplot" command, of our sequences into Qiime2 View (https://view.qiime2.org). OTUs that matched with chloroplast or mitochondrial sequences were manually removed from the OTU table prior to any further analyses.

This OTU table can be found on Dryad Digital Repository *https://doi.org/10.5061/dryad.7wm37pvv1*. The Qiime2 processing pipeline has been deposited on GitHub (https://github.com/bsbringhurst/TS-and-MT-Microbiome-Files).

Statistical Methods

The VEGAN R package was used to calculate the Shannon's Diversity Index (H) for every ant, fungal garden, and soil sample (Oksanen *et al.*, 2018). The respective average Shannon's Diversity Index values were then compared among groups (ant host, fungal clade or region), using either Welch's two sample t-tests or ANOVAs, for the ant and fungal garden samples. A Tukey's Honest Significant Difference test was performed using the multcomp package in R as the post-hoc analysis to compare Shannon's Diversity Index values among groupings with three different groups (Hothorn *et al.*, 2008). EstimateS v9.1.0 was used to create rarefaction curves to compare OTU richness among groups of ant and fungal garden samples as well as compare sampling adequacy (Colwell, 2013). Rarefaction curves were plotted along with their respective 95% confidence interval. These rarefaction curves were additionally extrapolated out to 20 samples if a variable category did not contain over 20 samples to further illuminate differences between the variable categories due to their small sample sizes. Finally, indicspecies R package was used to conduct an indicator species analysis (ISA) (with each analysis having 9,999 permutations) to examine which bacterial taxa contribute most toward the overall variation in microbiome structure of the ant and fungal garden samples (Cáceres & Legendre, 2009).

Multivariate Statistics

Since our ant and fungal samples were confounded by multiple variables (host ant species (N = 2), fungal garden clade (N = 3) and geographic region (N = 2)), we explored how much variation in microbiome structure was explained by these variables. To see any initial patterns in the data, we first constructed non-metric multidimensional scaling (NMDS) plots based off of Bray Curtis distances using the VEGAN R package to visualize patterns between the ant and fungal microbiomes (Oksanen *et al.*, 2018. To look for statistical significance in these patterns, we then created OTU tables with a subset of either the ant or fungal samples. A detrended correspondence analysis (DCA) was performed on each of the two OTU tables (ants or fungus) utilizing the VEGAN R package that then determined whether a canonical correspondence analysis (CCA) or redundancy analysis (RDA) was best tool (Oksanen *et al.*, 2018). A threshold value of 4 for the DCA Axis 1 was used during the DCA to denote whether a CCA or RDA was to be used, with a value greater than 4 denoting that a CCA was optimal (Oksanen *et al.*, 2018). Each CCA or RDA was performed under a reduced model and used 10,000 permutations.

To further quantify differences between the microbial communities of the ants and their fungus gardens, CCAs or RDAs were performed on major bacterial classes identified by the significant OTUs in the ISAs to determine whether host ant, fungal lineage or region played any role in community structure of these groupings. These tests were performed on OTU tables consisting of the subset of OTUs corresponding to significant taxonomic groups revealed by the ISA. Further ISAs were performed on all the OTUs within these bacterial classes to highlight which taxa might be driving the overall patterns.

Results

Bacterial Distribution

After the raw data was processed and filtered through Qiime2, the dataset consisted of 1,003,775 sequences, with 481,514 sequences associated with the 24 ant samples, 470,669 sequences associated with the 24 fungal samples, and 51,592 sequences associated with the 7 soil samples. From these resulting sequences, 1,065 OTUs were detected, with 334 of these OTUs found within the ant samples, 775 OTUs found within the fungal samples, and 513 OTUs found the soil samples.

Regional Analysis of Ant, Fungus, and Soil Microbiome:

Initial multivariate analysis of the ant and fungal samples indicated that ants, fungus and soil samples formed visually distinct clusters (Fig. S1; stress = 0.184). Moreover, ants, fungus, and soil bacterial microbiomes were significantly different from one another (CCA; $F = 3.1581$, $df = 2$, $p < 0.0001$). The ant ($H = 1.946$), fungus garden ($H = 1.947$), and soil samples ($H = 4.019$) had significantly different Shannon's Diversity Index values ($F = 12.68$, $df = 2$, $p = 3.27 \times 10^{-5}$), with the soil samples having a significantly higher average in comparison to the ant and fungus garden samples (Fig. S2). These results confirm that microbial communities of ants and fungus we were analyzing in this study are not contaminants originating from the surrounding soils. In all subsequent analyses, soils were removed since they were of no primary interest.

Ant Microbiome Composition

The two ant species were the primary driver of their microbiome (CCA; $F = 3.8141$, $df = 1$, $p < 0.0001$), whereas fungus garden clade ($F = 0.8464$, $df = 2$, $p = 0.6684$) and region ($F = 0.6298$, $df = 1$, $p = 0.8807$) did not appear to have significant roles in structuring the bacterial microbiome of ants. These findings were corroborated with NMDS plots showing that the ant samples formed distinct clusters based on species (Fig. 2a; stress = 0.190), and not region (Fig. 2b; stress = 0.190). Additionally, NMDS plots showed that *T. septentrionalis* had a less variable microbiome than *M. turrifex*, since *T. septentrionalis* ant samples were more clustered together while *M. turrifex* ant samples were more variable (Fig. 2a).

Ant microbiomes appeared to be structured by different bacterial taxa. The three most common bacterial taxa in the microbiome of *T. septentrionalis* ants were one strain of *Solirubrobacter* (29.409%), *Luteimonas* (15.8%), and an unknown member of Burkholderiaceae (9.209%) (Table 2a). In contrast, the three most common taxa in the microbiome of *M. turrifex* ants were *Amycolatopsis* (19.814%), an unknown member of Burkholderiaceae (14.633%), and an undescribed member of Microbacteriaceae (10.530%) (Table 2b). Additionally, *Pseudonocardia* strains consisted of 5.760% of the *M. turrifex* ant microbiome whereas it was nearly absent in (<1%) *T. septentrionalis* ants, (Table 2a,b). The indicator species analysis (ISA) of these two ant species also suggested different core microbiomes; with the Actinobacteria, *Amycolatopsis* (indicator value (IV) = 0.539, $p = 0.0001$) and *Pseudonocardia* (IV = 0.500, $p = 0.001$) strains were the top two significant contributors to overall variation in the *M. turrifex* ant microbiome whereas two strains of *Solirubrobacter* (IV = 0.894, $p = 0.0001$; IV = 0.816, $p = 0.0001$) were the top significant contributors to overall variation within the *T. septentrionalis* microbiome (Table 3a,b, Table S1). As a next step, we probed whether bacterial diversity in the classes identified as significant in

the ISA were explained by 1) geographic region, 2) ant species or 3) fungal garden clade (Table S1). The ISA reported that four bacterial classes (Actinobacteria, Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria) were important in describing the variation among ants. The Actinobacteria community of the ants was primarily explained by the ant species (CCA; Table 4a). Similarly, the Alphaproteobacteria and the Gammaproteobacteria communities of the ants were explained by ant species (CCA; Table 4a). However, the Bacteroidia community for the ants was not explained by any variable (RDA; Table 4a). The ISA found 15 significant OTUs within the Actinobacteria community that differed between the two ant species. OTUs of *Pseudonocardia* and *Amycolatopsis* associated with *M. turrifex* and OTUs of *Naumannella*, an undescribed Intrasporangiaceae, and *Aeromicrobium* within *T. septentrionalis* were important drivers in overall discrimination between the two ant associated Actinobacteria communities (Fig. 3a,b). Other significant OTUs driving the differentiation in the Alphaproteobacteria between the two ant species included a *Sphingomonas* OTU in *M. turrifex* and within the Gammaproteobacteria, a *Lutimonas* OTU in *T. septentrionalis* (Fig. S3a,b).

Despite community level differences in taxa comprising the bacterial microbiomes of the two ant species, overall diversity of the two species was similar. Although *M. turrifex* was projected to have significantly more OTUs than *T. septentrionalis* (Fig. 2c), there was not a statistical difference in the average Shannon's Diversity Index of bacteria between *T. septentrionalis* (H = 1.902) and *M. turrifex* (H = 1.998) ants (t = 0.70803, df = 22, p = 0.4864).

Fungal Microbiome Composition

Unlike ant bacterial microbiomes, geographic region appeared a greater role in structuring the bacterial microbiome of the fungus garden, though this was not statistically significant (CCA; F = 1.3466, df = 1, p = 0.1065) than fungal clade (F = 0.9482, df = 2, p = 0.5223) or host ant species (F = 1.0390, df = 1, p = 0.3347). The three most common taxa in the microbiome of East Texas fungus were *Mesoplasma* (57.891%) and two strains of *Spiroplasma* (21.857%, 5.020%) (Table 5a). The three most common taxa in the microbiome of Central Texas fungus were *Mesoplasma* (31.797%), *Pseudomonas* (8.750%), and *Tyzzerella* (4.880%) (Table 5b).

The ISA found that two OTUs defined East Texas fungus while 47 OTUs defined the Central Texas fungi; with most of these indicator OTUs found in the families Acetobacteraceae, Acidobacteriaceae, Burkholderiaceae, Cthoniobacteraceae, Enterobacteriaceae, Sphingobacteriaceae, and Weeksellaceae (Table 3c,d, Table S2). Except for a single undescribed OTU in the genus *Entomoplasma*, no members of the Entomoplasmataceae (the family of the most common taxa, *Mesoplasma*) were significant members of the fungus garden bacterial microbiomes (Tables 3,S2).

Since *Mesoplasma* was very common in the fungus garden samples, yet seemed to poorly characterize fungus garden microbiome structure (per the ISA), sequences of *Mesoplasma* were removed to examine for patterns among the other bacterial taxa. Without *Mesoplasma*, the fungus microbiome was significantly determined by region (CCA; F = 1.5921, df = 1, p = 0.0106), whereas fungal clade (F = 0.8532, df = 2, p = 0.7251) and host ant species (F = 1.0982, df = 1, p = 0.2832) were not significant factors in

structuring fungus garden microbiomes. These findings were corroborated with an NMDS plot showing that the fungal samples formed clusters based around their region (Fig. 4, stress = 0.145).

The ISA reported that five bacterial classes (Acidobacteriia, Actinobacteria, Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria) were important in describing the variation among the fungus gardens (Table 3 and S2). As with the ants, the following analyses probed whether variation in the OTUs in these classes were explained by 1) geographic region, 2) host ant species or 3) fungal clade. The Acidobacteria community of the fungus gardens was explained by both fungal clade and the region (RDA; Table 4b). The Actinobacteria community of fungus gardens was significantly explained by the host ant species, but the fungal clade may play a role in determining community variation, though the test was not statistically significant (CCA; Table 4b), but low enough to warrant concern about a Type II error. The Alphaproteobacteria community of fungus gardens was explained by host ant species and by fungal clade (CCA; Table 4b). The Gammaproteobacterial community of fungus gardens was explained by ant species (CCA; Table 4b). The Bacteroidia community for fungus gardens was not explained by any variable (RDA; Table 4b). The ISAs within the Acidobacteriia community found that OTUs of *Granulicella, Edaphorbacter,* and 2 undescribed OTUs from the Acidobacteria subgroup 2 being significant to Central Texas fungal samples (Fig. 5a). Similarly, regional differences among the Actinobacteria were driven by five OTUs, with *Nocardioides, Mycobacterium,* and *Corynebacterium* in Central Texas fungus gardens and Intrasporangiaceae and *Naumannella* significant for East Texas fungal samples (Fig. 5b). Nine OTUs within Alphaproteobacteria were found to drive the differentiation of this community based on region, with some of these significant OTUs for the Central Texas fungal samples being associated with Caulobacteracea, Acetobacteraceae, Xanthobacteraceae, and Micropepsaceae (Fig. 5c). 4 OTUs within Gammaproteobacteria were found to drive regional community differentation, with OTUs within *Ideonella, Enhydrobacter*, and the *Burkholderia-Caballeronia-Paraburkholderia* complex significant for Central Texas fungal samples (Fig. 5d). Additionally, OTUs within Acidobacteriia and Alphaproteobacteria were found to significantly drive community differences of the three fungal lineages, but this significance was due to these driver OTUs being found primarily within the Clade B3 samples, which was relatively undersampled (n=3) (Fig. S4a,b).

Comparing extrapolated rarefaction curves for fungus garden microbiomes (*Mesoplasma* excluded) show that Central Texas fungi contain significantly more OTUs in comparison to East Texas fungi (Fig. 6a); the average Shannon's Diversity Index for Central Texas (H = 3.155) fungus was greater than that of East Texas (H = 2.151) fungus (t = -2.1604, df = 13.524, p = 0.049). Rarefaction curves of fungus garden microbiome based on host ant species showed no significant differences (Fig. 6b). Rarefaction curves based on fungal clade showed significant differences; with Clade B4 fungus having more expected OTUs than Clade B5 fungus (Fig. 6c). The similarity between the rarefaction curves of fungus grown by the different ant host species was also reflected in the Shannon's Diversity Index values for fungus grown by *T. septentrionalis* (H = 2.430) and *M. turrifex* (H = 2.459) (t = 0.5936, df = 21.908, p = 0.9532) (Fig. 6d). However, there was not a statistical difference in the average Shannon's Diversity Index between Clade B3 (H = 3.033), Clade B4 (H = 2.581), and Clade B5 (H = 2.205) fungal samples (F = 0.608, df = 2, p = 0.554) (Fig. 6e).

Comparing extrapolated rarefaction curves for fungus garden microbiomes (*Mesoplasma* included) show that Central Texas fungi contain significantly more OTUs in comparison to East Texas fungi (Fig. S5a); the average Shannon's Diversity Index for Central Texas (H = 3.058) fungus was greater than that of East Texas (H = 1.489) fungus (t = -2.6417, df = 22, p = 0.0149). Rarefaction curves of fungus garden microbiome based on host ant species showed no significant differences (Fig. S5b). Rarefaction curves based on fungal clade showed significant differences; with Clade B4 fungus having more expected OTUs than Clade B5 fungus (Fig. S5c). The similarity between the rarefaction curves of fungus grown by the different ant host species was also reflected in the Shannon's Diversity Index values for fungus grown by *T. septentrionalis* (H = 1.675) and *M. turrifex* (H = 2.268) (t = 0.97297, df = 22, p = 0.3411) (Fig. S5d). However, there was not a statistical difference in the average Shannon's Diversity Index between Clade B3 (H = 3.056), Clade B4 (H = 2.191), and Clade B5 (H = 1.659) fungal samples (F = 1.188, df = 2, p = 0.325) (Fig. S5e). In summary, *Mesoplasma* did not appear to influence overall diversity patterns.

Discussion

The most significant finding in this study is that different processes appear to structure the bacterial microbiomes of the fungus-gardening ant symbiosis. Each ant species had a unique bacterial microbiome, whereas fungal lineage appeared to have little to no role in structuring the bacterial microbiome of the fungus garden. This study, along with another that compared five additional attine species (Ronque et al., 2020) provides more evidence that individual ant species possess unique bacterial microbiomes. Unlike an earlier report that found similarities of bacterial microbiome structure in the ants and fungus gardens of the attine *Mycocepurus smithii*, fungus gardens of *T. septentrionalis* and *M. turrifex* possess bacterial microbiomes that are very distinct from their host ant species (Kellner et al., 2015). Other than OTUs of two bacterial classes (Acidobacteria and Alphaproteobacteria) associated with fungus gardens that appeared to segregate among fungal lineages and the two ant species, there appeared to be few if any bacteria that could be codispersed with the fungus (Table 4). The bacterial microbiome of the fungus garden had a lesser defined structure that may in part be determined by the environment.

The ant associated bacterial microbiomes were very distinct. While *T. septentrionalis* and *M. turrifex* had a high abundance of Actinobacteria as part of their microbiome, the taxonomic identity of these bacteria differed between the two species. *Mycetomoellerius turrifex* ant microbiome was characterized by Actinobacteria in the genera *Pseudonocardia* and *Amycolatopsis*, whereas the ant microbiome of *T. septentrionalis* was characterized by *Ponticoccus* and *Microlunatus*. Additionally, *T. septentrionalis* appeared to have an extensive coverage of of *Solirubrobacter*. The function of *Solirubrobacter* is unknown, however, it has been reported in soil crust (Reddy & Garcia-Pichel, 2009), agricultural soils (Kim et al., 2007), and earthworm burrows (Singleton et al., 2003). Actinobacteria are commonly known to produce antibiotic and are commonly seen in fungus-gardening ants and other insects where they are thought to serve as defensive symbionts (Kaltenpoth et al., 2005; Fernández-Marín et al., 2009; Kaltenpoth, 2009). For example, Actinobacteria are thought to be part of a generalized defense against *Escovopsis* pathogens (Currie et al., 2003; Li et al., 2018). Considering the overall differences in ant

microbiome structure and the differences in indicator species, it would appear that each species has either 1) a different strategy of dealing with pathogens or 2) experiences different pathogens altogether. The identity of pathogens in either population is largely unknown and unexplored, though studies on other attines have shown that different attine taxa can share the same or similar pathogens (Gerardo et al., 2006; Kellner et al., 2018).

Assuming that the significant ant species effect of ant bacterial microbiomes is an outcome of vertical transmission, the contrasting microbiomes found in *T. septentrionalis* and *M. turrifex* could reflect different phylogeographic histories. The relative uniformity of bacterial communities associated with *T. septentrionalis* ants could reflect low diversity from recent population expansion or strong purifying selection of microbial consortia, whereas the high variation among *M. turrifex* ant microbiomes could be due to relaxed selection on bacterial consortia and/or high diversity due to an ant population expansion that happened in the more distant past. The genetic diversity of *T. septentrionalis* ants is generally low in Texas (and lowest in Central Texas), which likely represents recent expansion, a conclusion supported by mtDNA, microsatellites and genotyping-by-sequencing approaches (Seal et al., 2015; Matthews et al., 2020).

Variation in ant species microbiomes may involve responses to stress, since stress is thought to structure microbiomes (Zaneveld et al., 2017; Howe-Kerr et al., 2020; Medina et al., 2022). For example, microbiomes may become more variable in stressful environments as hosts lose control over their symbionts or variable microbiome structure may be a mechanism to deal with environmental stress (Medina et al., 2022). The region studied (east and central Texas) encompasses the eastern and western range limits of *M. turrifex* and *T. septentrionalis*. Recent distribution models indicated that different variables predicted their respective distributions; for example, the *M. turrifex* distribution was explained by annual mean temperature whereas *T. septentrionalis* was predicted by winter precipitation (Senula et al., 2019). How the microbiomes react to these environmental variables remains unknown, but could be explored experimentally by exposing ant colonies to temperature or humidity stress.

Interestingly, we found very little support that each ant species are structuring the microbiome of their fungus gardens, since the gardens of each species were not different from one another in terms of their associated bacterial community. This is surprising, considering the differences in ant-associated Actinobacteria, which hypothetically would produce different secondary metabolites (van der Meij et al., 2017; Batey et al., 2020; Goldstein & Klassen, 2020) that could in turn influence the abundances of other bacterial taxa. One possible explanation is that regions could differ in plant communities and thus the types of substrates the ants are feeding the garden (de Fine Licht & Boomsma, 2010; De Fine Licht & Boomsma, 2014; Seal et al., 2014). While there were significant differences among the OTU's encountered in each region, most belonged to the same bacterial taxa (e.g., Actinobacteria or Acidobacteria, Table 3, S2), which suggests common mechanisms in the acquisition of these bacteria and some genetic differences among bacteria that could be explained by dispersal limitation. 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 lineage, which suggested that the ants were actively acquiring

bacteria from the environment (Kellner et al., 2015). As a result, most of the impact of ant species or fungal lineage could be obscured by fungal substrates and environments.

This study along with others (Sapountzis et al., 2015; Meirelles et al., 2016; Sapountzis et al., 2019) revealed that *Mesoplasma* to be common in ants and fungus gardens, yet variation in Mesoplasma (or Enterbacteriales, generally) was not a significant predictor or microbiome structure or ant host, fungus garden or region. Phylogenetic analyses suggest that there are attine specialized *Mesoplasma* distinct from army ant *Mesoplasma*, but the resolution of standard Illumina reads appears to be too low to warrant interspecific comparisons among attine species (Meirelles et al., 2016). 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).

Conclusion

This study has shown that two distantly related, co-occurring fungus gardening ant species possess complex microbiomes that are acquired by different mechanisms. Much of the ant associated microbiome is presumably inherited due to the strong ant-host signal, whereas the most of the bacterial microbiome of the fungus garden appears to be acquired from the environment. A limitation of the current study is that it did not incorporate intraspecific molecular markers (i.e., within host or fungus garden lineage); as a result, this study did not possess the power to explicitly test for possible 'phylosymbiosis' of ant or ant-fungus and bacterial symbionts, which predicts phylogenetic concordance among hosts and symbionts (Lim & Bordenstein, 2020; Trevelline *et al.*, 2020). Two distinct processes could determine the apparent species-specific ant microbiome. Bacteria could be inherited vertically via founding queens, which would explain the strong cophylogenetic signal between ants and *Pseudonocardia* bacteria (Cafaro *et al.*, 2011). On the other hand, the species specific effect we report here could also be explained by horizontal transmission if ants are actively acquiring bacteria in a species specific manner (Mueller *et al.*, 2010). As a result, while there is a strong ant species effect driving microbiome structure, some of this pattern could result from behavioral differences where the ants selectively acquire bacterial lineages over others. Therefore, the comparison of *M. turrifex* and *T. septentrionalis* may be somewhat crude and may overemphasize coevolutionary signals, considering the large genetic distances between these two species (Solomon et al 2019). Future studies should compare microbiomes of closely related species, such as those found in western North America and the northern Neotropics (Solomon *et al.*, 2019; Beigel *et al.*, 2021) and employ intraspecific genetic markers (Matthews *et al.*, 2020; Beigel *et al.*, 2021; Matthews *et al.*, 2021). Moreover, co-phylogenetic concordance does not necessarily imply coevolution/coadaptation (Douglas, 2010). To discriminate among the latter, cross-fostering experiments (switching host ants onto novel fungi; (Seal & Mueller, 2014; Seal *et al.*, 2014)) could demonstrate whether fungal associated (*e.g.*, Acidobacteria and Alphaproteobacteria, Table 4) are adapted to certain hosts.

Statements And Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mattea Allert, Blake Bringhurst, Matthew Greenwold, Katrin Kellner, and Jon Seal. The manuscript was written by Mattea Allert, Blake Bringhurst, and Jon Seal. All authors read and approved the final manuscript.

Data Availability

Raw microbiome sequences for all samples have been uploaded to NCBI under the BioProject PRJNA789907. Fungal ITS sequences from all applicable fungal garden samples have been uploaded to GenBank, with accession numbers found in Table 1. Our Qiime2 pipeline, along with sample R script used during the post-processing analysis, can be found at *https://github.com/bsbringhurst/TS-and-MT-Microbiome-Files*. The resulting OTU table output by our Qiime2 pipeline can be found in the DRYAD Digital Repository at https://doi.org/10.5061/dryad.7wm37pvv1.

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Tables

Table 1 Collection locations, species and fungal clade and Genbank Accession numbers of the fungus garden genotypes used in this study.

ID	Locality Name	Texas Region	Ant Species	Fungal Clade	Genbank Accession
JNS160521-1	Red Rock	Central	*T.septentrionalis*	B3	MK142454
JNS160521-2	Red Rock	Central	*T.septentrionalis*	B4	MK142349
JNS160521-3	Red Rock	Central	*T.septentrionalis*	B4	MK142350
JNS160522-2	Stengl	Central	*T.septentrionalis*	B4	MK142369
JNS160522-3	Stengl	Central	*T.septentrionalis*	B4	MK142371
JNS160523-1	Gladewater	Eastern	*T.septentrionalis*	B4	MK142354
JNS160523-3	Gladewater	Eastern	*T.septentrionalis*	B4	MK142347
JNS160524	Gladewater	Eastern	*T.septentrionalis*	*	Not Genotyped
JNS160531-1	UT Tyler Forest	Eastern	*T.septentrionalis*	B4	MK142355
JNS160531-2	UT Tyler Forest	Eastern	*T.septentrionalis*	B5	MK142365
JNS160531-3	UT Tyler Forest	Eastern	*T.septentrionalis*	B5	MK142366
KK160530-4	Cherokee county	Eastern	*T.septentrionalis*	B5	MK142353
KK160530-2	Brownsboro	Eastern	*T.septentrionalis*	B5	MK142360
JNS160414-1	UT Tyler Forest	Eastern	*M. turrifex*	B5	MK142458
JNS160510-1	UT Tyler Forest	Eastern	*M. turrifex*	B5	MK142475
JNS160515-1	UT Tyler Forest	Eastern	*M. turrifex*	B5	MK142474
JNS160521-1	Red Rock	Central	*M. turrifex*	B3	MK142454
JNS160521-2	Rosanky	Central	*M. turrifex*	B4	MK142373
JNS160525-	Henderson 2	Eastern	*M. turrifex*	B5	MK142459

1					
JNS160525-2	Henderson 2	Eastern	*M. turrifex*	B5	MK142455
JNS160525-3	Henderson 2	Eastern	*M. turrifex*	B5	MK142460
KK160530-1	Cherokee county	Eastern	*M. turrifex*	B5	MK142456
KK160530-2	Cherokee county	Eastern	*M. turrifex*	B5	MK142457
KK160530-3	Cherokee county	Eastern	*M. turrifex*	B3	MK142361

Table 2 OTUs with greater than 1% frequency for (A.) *T. septentrionalis*, and (B.) *M. turrifex* ant samples

A

Sample Type	OTU (Class – OTU)	Percentage
T. septentrionalis Ants	Thermoleophilia - *Solirubrobacter* Strain 1	29.409%
	Gammaproteobacteria - *Luteimonas*	15.811%
	Gammaproteobacteria - Unknown Burkholderiaceae	9.209%
	Actinobacteria - *Naumannella*	8.454%
	Actinobacteria - Unknown Intrasporangiaceae	8.312%
	Mollicutes - *Mesoplasma*	5.609%
	Alphaproteobacteria - *Wolbachia*	5.102%
	Actinobacteria - Unknown Microbacteriaceae	4.337%
	Thermoleophilia - *Solirubrobacter* Strain 2	3.938%
	Actinobacteria - *Microlunatus*	2.213%
	Actinobacteria - *Aeromicrobium*	1.816%
	Actinobacteria - *Luteipulveratus*	1.330%

B

Sample Type	OTU (Class – OTU)	Percentage
M. turrifex Ants	Actinobacteria - *Amycolatopsis*	19.814%
	Gammaproteobacteria - Unknown Burkholderiaceae	14.633%
	Actinobacteria - Unknown Microbacteriaceae	10.530%
	Mollicutes - *Mesoplasma*	7.988%
	Actinobacteria - *Luteipulveratus*	7.145%
	Mollicutes - *Spiroplasma*	6.277%
	Gammaproteobacteria - *Noviherbaspirillum*	5.505%
	Actinobacteria - *Pseudonocardia* Strain 1	4.143%
	Alphaproteobacteria - *Wolbachia*	3.372%
	Gammaproteobacteria - *Luteimonas*	3.197%
	Actinobacteria - Unknown Intrasporangiaceae	2.695%
	Actinobacteria - *Pseudonocardia* Strain 2	1.617%
	Bacteroidia - *Niabella*	1.019%

Table 3 The top 5 OTUs with the highest indicator values (IV) for (A.) *T. septentrionalis* ant, (B.) *M. turrifex* ant, (C.) East Texas fungus garden, and (D.) Central Texas fungus garden samples found using the indicator species analysis

A

Sample Type	OTU (Class - OTU)	IV	p-value
T. septentrionalis Ants	Thermoleophilia - *Solirubrobacter* Strain 1	0.894	0.0001
	Thermoleophilia - *Solirubrobacter* Strain 2	0.816	0.0001
	Actinobacteria - *Ponticoccus*	0.806	0.0001
	Actinobacteria - Unknown Intrasporangiaceae	0.691	0.0005
	Actinobacteria - *Naumannella*	0.617	0.0001

B

Sample Type	OTU (Class - OTU)	IV	p-value
M. turrifex Ants	Actinobacteria - *Amycolatopsis*	0.539	0.0001
	Actinobacteria - *Pseudonocardia*	0.500	0.0001
	Alphaproteobacteria - Unknown Sphingomonadaceae	0.457	0.0300
	Actinobacteria - *Cutibacterium*	0.456	0.0274
	Actinobacteria - *Corynebacterium*	0.443	0.0287

C

Sample Type	OTU (Class - OTU)	IV	p-value
East Texas Fungus	Actinobacteria - *Naumannella*	0.554	0.0119
	Actinobacteria - Unknown Intrasporangiaceae	0.517	0.0239

D

Sample Type	OTU (Class (if applicable) - OTU)	IV	p-value
Central Texas Fungus	Gammaproteobacteria - *Enhydrobacter*	0.610	0.0028
	Actinobacteria - *Corynebacterium*	0.588	0.0073
	Acidobacteriia - Unknown Acidobacteria	0.575	0.0026
	Saccharimonadia - Unknown Saccharimonadales	0.556	0.0035
	Verrucomicrobiae - Unknown Opitutaceae	0.547	0.0033

Table 4 Tests of significance of variation with bacterial classes associated with (A.) ant and (B.) fungus garden bacterial microbiomes. Significant ($\alpha < 0.05$) are in bold. Degrees of freedom of ant species and region = 1 whereas df =2 in Fungal clade. CCA was performed on Actinobacteria, Alphaproteobacteria and Gammaproteobacteria whereas RDA was performed on Bacteroidia and Acidobacteria (See methods)

A.

Taxon	Ant Species	Fungal Clade	Region
Acidobacteria	F = 1.846, p = 0.055	F = 2.318, p = 0.113	F = 1.126, p = 0.329
Actinobacteria	**F = 7.933, p <0.0001**	F = 0.5810, p = 0.913	F= 0.697 p = 0.715
Alphaproteobacteria	**F = 3.420, p <0.0001**	F = 1.5537, p = 0.181	F = 1.0697 p = 0.383
Bacteroidia	F= 0.6217, p = 0.539	F = 0.7599 p = 0.525	F = 0.3367 p = 0.796
Gammaproteobacteria	**F = 3.144, p = 0.0004**	F= 0.529, p = 0.784	F = 0.2872, p = 0.887

B.

Taxon	Ant Species	Fungal Clade	Region
Acidobacteria	F= 1.456, p = 0.1500	**F = 3.2213, p = 0.0162**	**F = 2.8395, p = 0.0373**
Actinobacteria	**F= 1.5228, p = 0.0315**	F = 1.4886, p= 0.058	F = 0.9488, p = 0.461
Alphaproteobacteria	**F = 2.7829, p < 0.0001**	**F= 1.9127, p = 0.0006**	F= 1.2292, p = 0.235
Bacteroidia	F= 0.9383, p = 0.443	F= 0.2981, p = 0.898	F= 1.3256, p = 0.253
Gammaproteobacteria	**F= 2.0707, p = 0.0003**	F= 0.9232, p = 0.539	F= 0.5533, p = 0.810

Figures

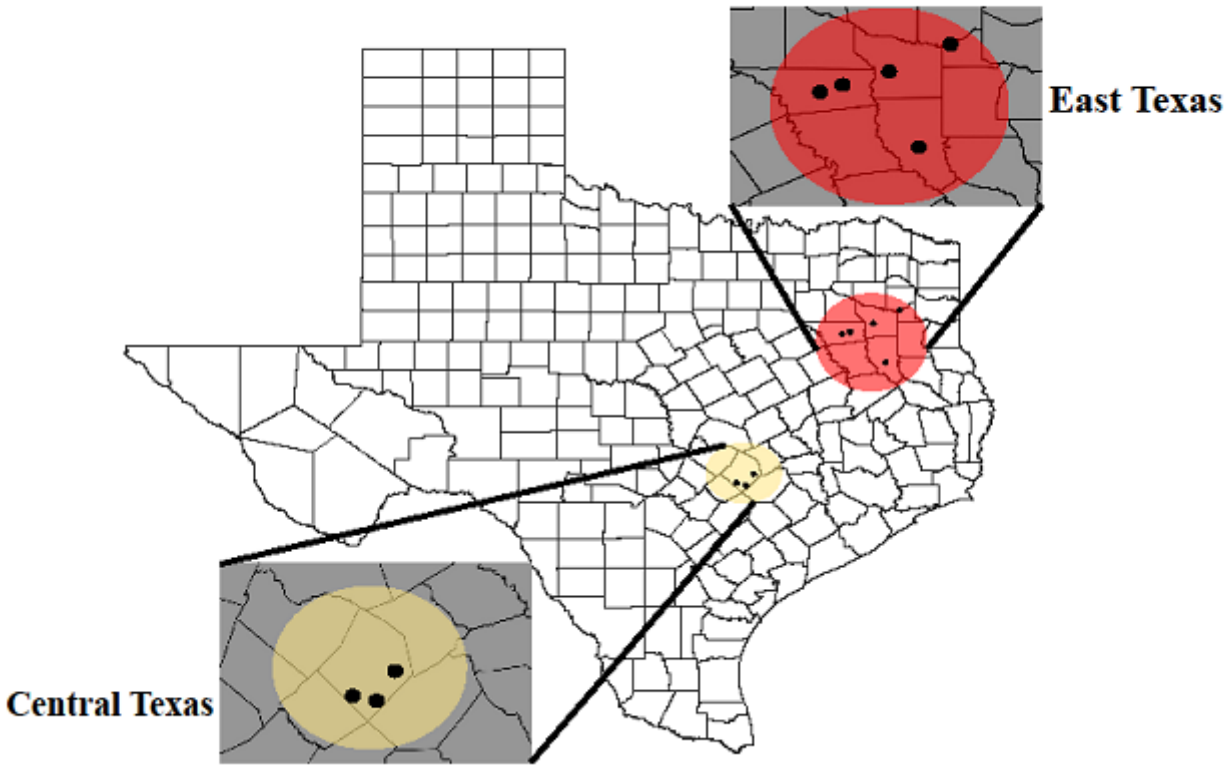

Figure 1

Map of Texas displaying the East Texas (red) and Central Texas (yellow) sample collection sites

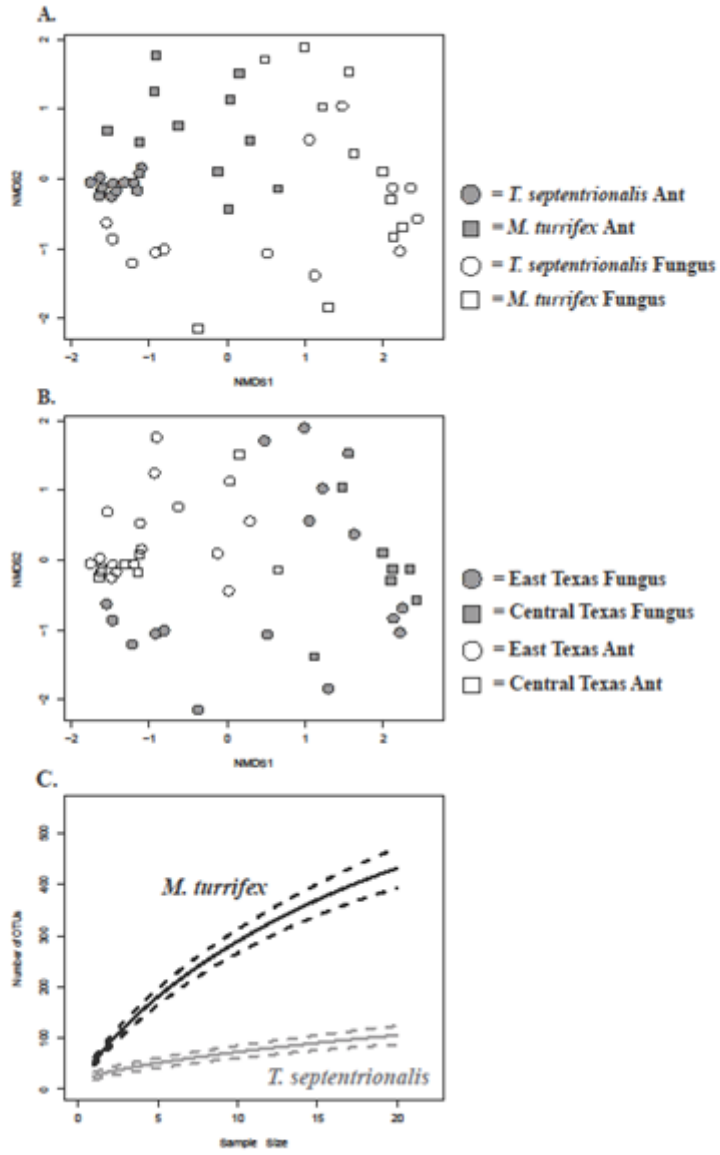

Figure 2

Non-metric dimensional scaling (NMDS) plots depicting (A.) both ant and fungal samples, with symbols reflecting the samples' associated ant species, and (B.) both ant and fungal samples, with symbols reflecting the samples' associated region. Rarefaction curves (C.) were performed to illuminate any differences in predicted OTU richness between *M. turrifex* (black) and *T. septentrionalis* ant samples

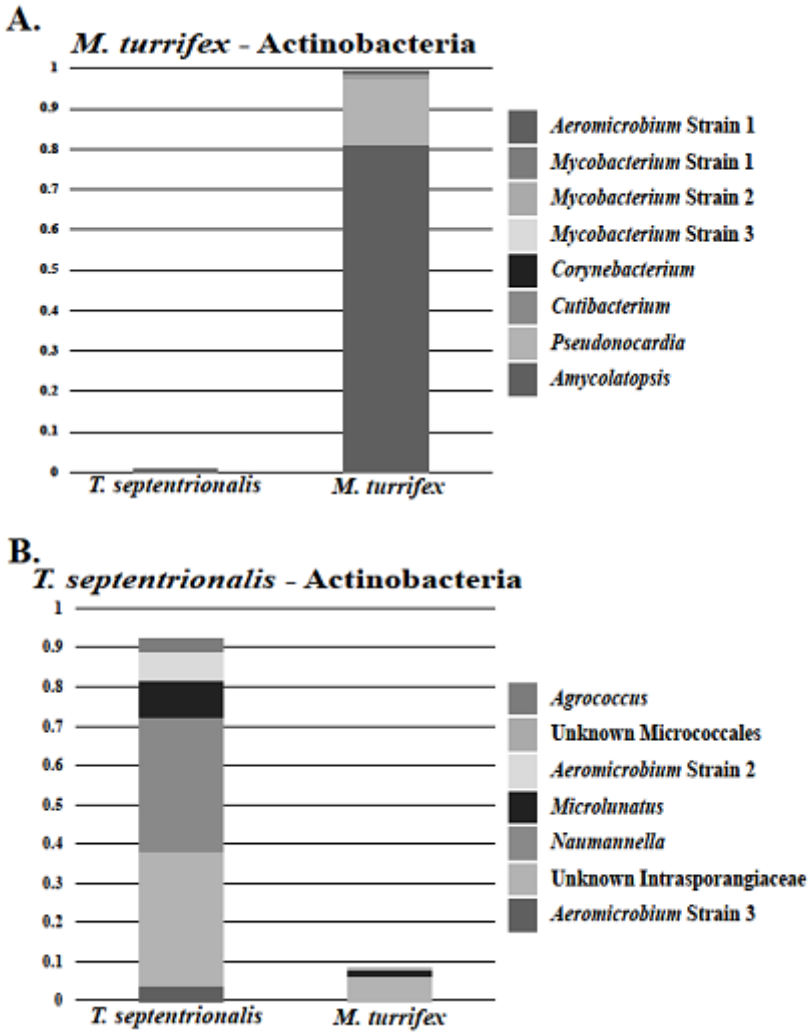

Figure 3

Bar plots depicting the proportion of the significant OTU abundances within the Actinobacteria community (identified using an indicator species analysis) of ant samples that are driving the Actinobacteria community differences between the two ant species, with separate plots made for the significant taxa corresponding to (A.) *M. turrifex* and (B.) *T. septentrionalis* ants.

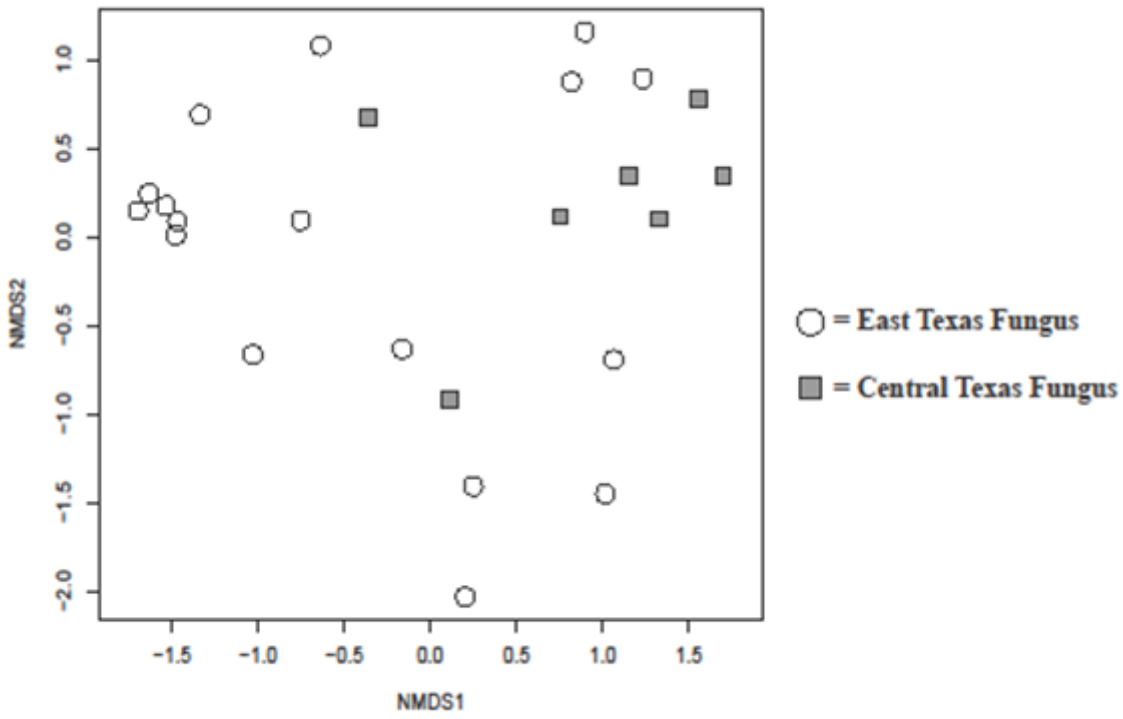

Figure 4

Non-metric dimensional scaling (NMDS) plot depicting the regionality of fungal samples, with OTUs assigned as *Mesoplasma* excluded.

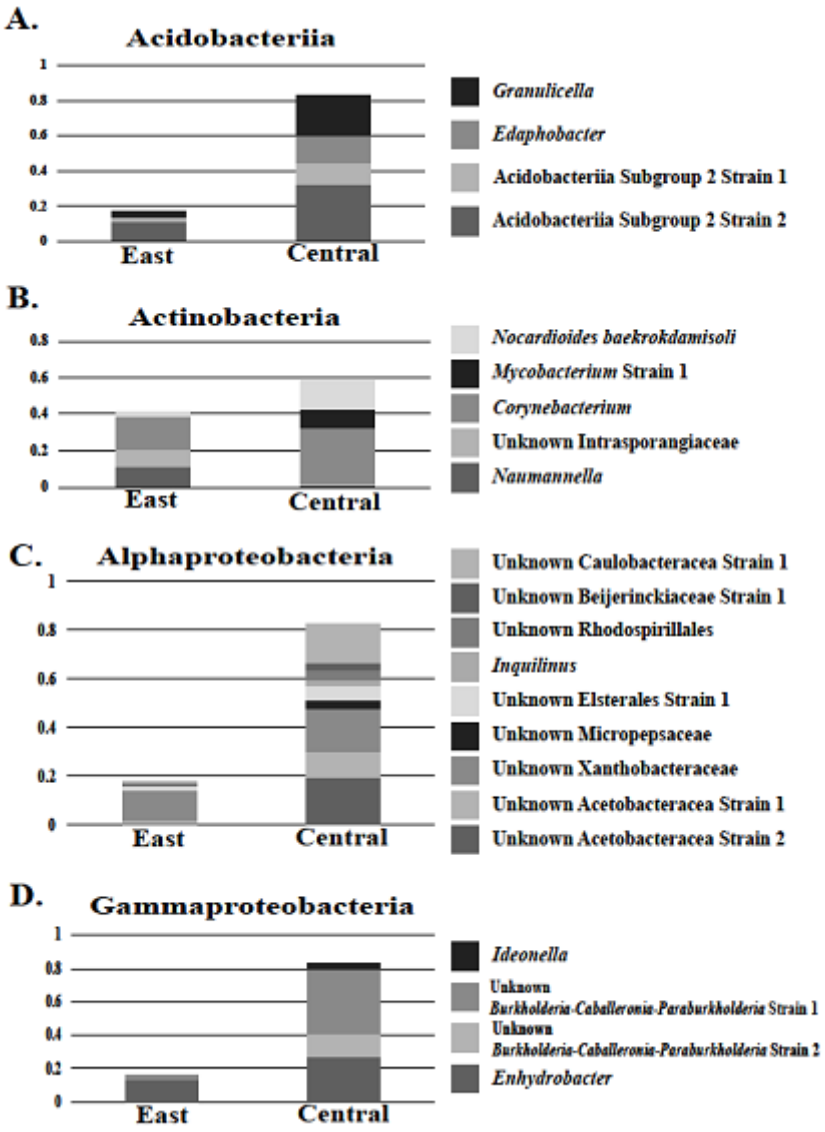

Figure 5

Bar plots depicting the proportion of the significant OTU abundances within the (A.) Acidobacteriia, (B.) Actinobacteria, (C.) Alphaproteobacteria, and (D.) Gammaproteobacteria communities (identified using an indicator species analysis) of fungal samples that are driving the community differences between the two collection regions, east (East) and central (Central) Texas.

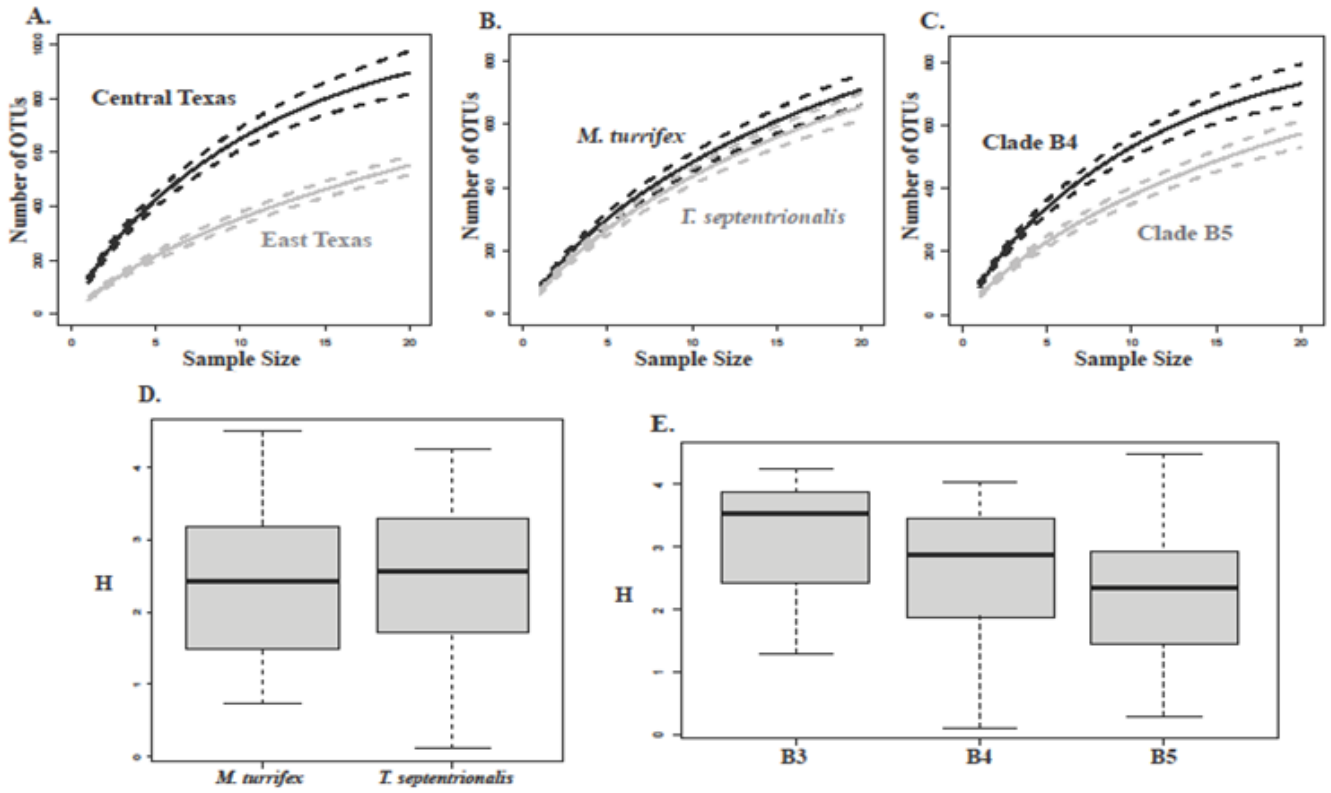

Figure 6

Rarefaction curves between bacterial OTU richness in (A.) Central Texas (black) and East Texas (gray) fungus garden samples, (B.) fungal garden samples associated with *M. turrifex* (black) and *T. septentrionalis* (gray) ant hosts, and (C.) fungus garden samples genotyped as Clade B4 (black) and Clade B5 (gray). Box plots were made depicting the average Shannon's Diversity Index (H) for (D.) fungal gardens grown by either *M. turrifex* (mean H = 2.459) or *T. septentrionalis* (mean H = 2.430) and (E.) Clade B3 (mean H = 3.033), Clade B4 (mean H = 2.581), and Clade B5 (mean H = 2.205). All figures were made without *Mesoplasma* assigned OTUs included.

Supplementary Files

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