Full Title:

Forage preference in two geographically co-occurring fungus gardening ants: a dietary DNA approach

Short Title:

Dietary DNA techniques for forage identification in fungus gardening ants

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Abstract

Traditional methods of forage identification are impractical to use with non-leafcutting fungus gardening ants, making diet-related ecological and life history questions difficult to study. In an attempt to address this limitation, we utilized dietary DNA metabarcoding as a method for directly identifying foraged substrate from the ant's fungus gardens. We then attempted to use metrics based off of these dietary DNA results to determine if two co-occurring fungus gardening ant species were niche separated by their preference in forage material. Fungus gardens from the co-occurring ant species *Trachymyrmex septentrionalis* and *Mycetomoellerius turrifex* were collected from a plot in East Texas and sequenced using chloroplast *trnL* and mitochondria *COI* primers. Results of this analysis indicate that dietary DNA metabarcoding was successful in identifying and describing a diversity of plant material present in the fungus gardens, with a total of 42 plant taxa identified. When fungal substrate plant taxa alpha and beta diversity metrics were compared between colonies of *T. septentrionalis* and *M. turrifex*, no evidence of diet-based niche separation was detected. Overall, our results show that dietary DNA techniques represent a promising tool that can be used to identify foraged plant material in ant fungus gardens, enabling the study of future diet-based ecological and natural history questions.

Introduction

Fungus Gardening Ants

Fungus-gardening ants are a New World monophyletic group characterized by their cultivation, and obligate consumption, of fungi. These ants use material foraged by their colony as a substrate to grow their fungus garden, feeding off the fungus instead of directly consuming the forage themselves. The more basal fungus-gardening ants forage insect-feces, dry plant debris, leaf-litter, and seed fragments [1,2]. More derived fungus gardening ants practice "higher agriculture" and can be subdivided into two main groups: the leafcutting ants, and the

non-leafcutting higher agriculture ants [3,4]. The most specialized of these two groups, the leafcutting ants, are known to forage extensively on freshly cut vegetative material; while the non-leafcutting higher agriculture ants often forage considerable amounts of freshly fallen flower petals, soft leaf tissue, and fruit, in addition to material similar to that collected by more basal fungus gardening species [2,5].

Traditional Methods of Diet Identification

Visual identification of material carried by foraging workers is a commonly utilized technique in determining ant diet and forage preferences. However, reliance on this technique presents several issues. Visual forage identification is time consuming, relies heavily on taxonomic expertise, and requires foragers to be conspicuous enough for observation [6,7]. In addition, observing individual foragers only offers a small snapshot of total colony dietary breadth, as a single colony may send out hundreds or even thousands of workers to forage [8].

Among fungus gardening ants, visual identification of forage material is most practical with the leafcutting genera *Atta* and *Acromyrmex*, as their foraging strategies predispose them to easy observation. The characteristic foraging trails created by *Atta* and *Acromyrmex* colonies can often be cleanly followed from the vegetation they are cutting to their nest/tunnel entrances, allowing for species level identification of their forage material [9–11]. Unfortunately, visual identification of forage material falls short with fungus gardening ant species outside of *Atta* and *Acromyrmex*. Most non-leafcutting fungus gardening ants do not create true foraging trails, making it difficult and very time consuming to accurately trace the species of vegetation that they are foraging from [12]. Additionally, the foragers of non-leafcutting fungus gardening ants are usually smaller and more cryptic than those of leafcutting ants. These factors have contributed to a deficit of literature regarding the diet composition of non-leafcutting fungus gardening ants, with the little information available primarily consisting of a handful of forage

material observations, often originally published in conjunction with the original natural history descriptions of the ants [1,2]. Clearly, relying solely on visual identification of forage material is insufficient for the study of non-leafcutting fungus gardening ants.

Dietary DNA

Dietary DNA (dDNA) techniques present a possible alternative to visual forage identification. Metabarcoding techniques based on dDNA, DNA extracted from an organism's gut, stomach, or fecal matter, have emerged as an accurate and resource-effective way to determine animal diets [7,13]. Previous studies, in multiple animal groups, have successfully utilized dDNA analysis to determine diet composition when direct observations of feeding behavior and/or morphological identification of forage material was insufficient [7,13].

Dietary DNA analysis has only been used to examine ant diet composition in a handful of studies, with even fewer of these studies having attempted to determine broad diet composition [8,14,15]. One factor complicating dDNA ant research is a yet unidentified compound in the gaster of adult ants that has been found to inhibit PCR, potentially impacting gut content dDNA analysis in adult ants [15,16]. As an additional complication, in many species adult ants are unable to digest solid food, instead feeding it to their brood. The adult ants then consume the digested liquid nutrients via trophallaxis from their larvae [8,17]. Wulff et al. (2021) cleverly leveraged this adult-larvae digestion dynamic to their advantage when using dDNA analysis to investigate the diet composition of *Solenopsis invicta*. They did so by sequencing the gut content of *S. invicta* larvae instead of adults, as the larvae act as a centralized depository of colony prey and are not known to produce PCR inhibitors [8,15,16].

A similarly innovative approach could be used to analyze the diet composition of fungus gardening ants, since their fungus gardens also act as a centralized depository for colony

forage. The fungus gardens cultivated by fungus gardening ants function as an external ancillary digestive system for the colony [2,17–19]. Within this ancillary digestive system, forage materials used as fungal substrate become highly degraded, due both to physical mastication by the ants and enzymatic breakdown by the fungus. This degradation necessitates the use of specialized genetic techniques, like dDNA metabarcoding, that are designed to work with highly degraded staples [2,5,20].

Niche Partitioning and Study Species

Using dDNA metabarcoding techniques to identify fungal substrate in ant gardens could be used to answer a number of ecological and natural history questions. Dietary DNA metabarcoding has been successfully used to detect niche partitioning, and overlap, in several animal species [21–25]. These techniques have also been used to understand seasonal and/or biogeographic based shifts in animal diets [26–32].

Diet-based niche separation has been investigated (without the use of dDNA methods) in several species of fungus gardening leafcutting ants. In a dry tropical forest in Costa Rica, the co-occurring leafcutter species *Atta cephalotes* and *Acromyrmex octospinosus* were found to forage almost entirely different resources, with the only exception being they both foraged flowers [33]. A similar trend was observed in Panama, with *Atta* species foraging primarily on tree leaves and co-occurring *Acromyrmex* species foraging primarily on flowers and herbal leaves [34]. To our knowledge, no previous studies have examined diet-based niche separation in non-leafcutting fungus gardening ants.

Trachymyrmex septentrionalis and *Mycetomoellerius turrifex* are two higher agriculture non-leafcutting fungus gardening ant species, with a partial overlap of their geographic distribution occurring in East Texas [35,36]. The range of *T. septentrionalis* stretches from Texas

to Florida, and as far north as central Illinois; while the range of *M. turrifex* extends from west to east Texas, northeastern Mexico, western Louisiana, and the southern edge of Oklahoma [36–38]. Like most non-leafcutting fungus gardeners, specific information on the preferred forage material of *T. septentrionalis* and *M. turrifex* is highly limited [2]. What documentation does exist indicates that they both forage insect frass and plant material, but only *T. septentrionalis* has been observed cutting/foraging fresh vegetation [2,39–42]. Their geographic range overlap and the similarity of their documented forage material makes these two species excellent subjects to test the efficacy of using dDNA analysis to study diet-based niche partitioning in non-leafcutting fungus gardening ants.

The primary object of this study was to determine if dDNA metabarcoding can be used to identify and describe the diversity of plant material and/or insect frass used as substrate in *T. septentrionalis* and *M. turrifex* fungus gardens. The secondary object of this study was to compare diversity metrics of identified fungal substrate material in an attempt to detect diet-based niche partitioning in co-occurring *T. septentrionalis* and *M. turrifex* colonies.

Materials and Methods

Sample Collection

The fungus gardens of ten *T. septentrionalis* and ten *M. turrifex* colonies were sampled from a plot on the University of Texas at Tyler campus in Tyler, Texas, USA. Sampling occurred from May 16-26, 2023. Each of the ten *M. turrifex* colonies we sampled was paired with a *T. septentrionalis* colony within 3 m of it. These colony pairs, composed of one *M. turrifex* colony and one nearby *T. septentrionalis* colony, were sampled concurrently. Each colony was only sampled once.

Fungus gardens were non-destructively sampled from each colony by careful excavation of the topmost chamber containing an appreciable amount of fungus. Sterilized tools were used when removing fungus gardens from the chamber to minimize DNA contamination from environmental sources and previously sampled colonies. Excavated fungus garden samples were placed in sterile vials containing 95% ethanol and stored in a -80°C freezer.

Sample Metabarcoding

Metabarcoding was conducted by Jonah Ventures (https://jonahventures.com/) using chloroplast *trnL* (forward primer c: 5' CGAAATCGGTAGACGCTACG 3', reverse primer h: 5' CCATTGAGTCTCTGCACCTATC 3') and mitochondrial cytochrome c oxidase subunit I (*COI*) (forward primer ZBJ-ArtF1c: 5' AGATATTGGAACWTTATATTTTATTTTTGG 3', reverse primer ZBJ-ArtR2c: 5' WACTAATCAATTWCCAAATCCTCC 3') primers [43,44]. Individual fungus garden samples were shipped overnight in an ice box to Jonah Ventures in Boulder, CO.

At Jonah Ventures, frozen samples were thawed for 1-2 hours before processing. Sample barcodes were recorded and assigned a well within the 96 well plate or numbered extraction tube. Under a laminar flow hood, sterile cotton swabs (Fisher, Catalog No. 22-363-173) were coated with sediment matter, and the swabs were placed in the corresponding extraction plate or tube. Sterile tweezers and pliers were used to handle cotton swabs and remove the wooden ends of the cotton swab before extraction. Plates or tubes were immediately processed or stored in -20°C until the extraction process could be performed.

Genomic DNA from each fungus garden sample was extracted using the DNeasy 96 PowerSoil Pro Kit (384) (Catalog No. 47017) according to the manufacturer's protocol. Genomic DNA was eluted into 100 µl and frozen at -20°C.

For *trnL* genetic analysis, a portion of the chloroplast *trnL* intron was PCR amplified from each genomic DNA sample using the c and h trnL primers. Both forward and reverse primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega, Catalog No. M5133, Madison, WI) which included 0.4 µM of each primer and 1 µl of gDNA. DNA was PCR amplified using the following conditions: initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, and a final elongation at 72°C for 10 minutes.

For *COI* genetic analysis, a portion of the *COI* gene was PCR amplified from each genomic DNA sample using the ZBJ-ArtF1c and ZBJ-ArtR2c primers. Both forward and reverse primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega, Catalog No. M5133, Madison, WI) which included 12.5 µl Master Mix, 1.0 µM of each primer, 3.0 µl of gDNA, and 7.5 µl DNase/RNase-free H2O. DNA was PCR amplified using the following conditions: initial denaturation at 94°C for 5 minutes, followed by 45 cycles of 30 s at 94°C, 45 s at 45°C, and 45 s at 72°C, and a final elongation at 72°C for 10 minutes.

To determine amplicon size and PCR efficiency, each reaction was visually inspected using a 2% agarose gel with 5 µl of each sample as input. Amplicons were then cleaned by incubating amplicons with Exo1/SAP for 30 minutes at 37°C followed by inactivation at 95°C for 5 minutes and stored at -20°C.

A second round of PCR was performed to complete the sequencing library construct, appending with the final Illumina sequencing adapters and integrating a sample-specific,12-nucleotide index sequence. The indexing PCR included Promega Master mix, 0.5 µM of each primer and 2

µl of template DNA (cleaned amplicon from the first PCR reaction) and consisted of an initial denaturation of 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

Final indexed amplicons from each sample were cleaned and normalized using SequalPrep Normalization Plates (Life Technologies, Carlsbad, CA). 25 µl of PCR amplicon was purified and normalized using the Life Technologies SequalPrep Normalization kit (Catalog No. A10510-01) according to the manufacturer's protocol. Samples were then pooled together by adding 5µl of each normalized sample to the pool.

Sample library pools were sent for sequencing on an Illumina MiSeq (San Diego, CA) at the Texas A&M Agrilife Genomics and Bioinformatics Sequencing Core facility using the v2 500-cycle kit (Catalog No. MS-102-2003). Necessary quality control measures were performed at the sequencing center prior to sequencing.

Raw sequence data were demultiplexed using pheniqs v2.1.0, enforcing strict matching of sample barcode indices (i.e, no errors) [45].

Sequence Processing

After receiving the demultiplexed data in the form of paired end FASTQ files from Jonah Ventures, the files were imported into Qiime2 for further processing (Figure 1) [46].

Fig 1. Flowchart of each major bioinformatic step in the development of our analysis metrics.

The Qiime2 plugin “cutadapt” was used to remove primers from the sequences. The Qiime2 plugin “dada2” was used to denoise the sequences, truncate the sequences, and merge the forward and reverse sequence pairs [47]. The *trnL* sequences were truncated where the 25th percentile of the quality scores dropped below 20, or 10 base pairs from the end of the sequences, whichever came first (forward: 220 bp, reverse 213 bp). The *COI* sequences were truncated where the 25th percentile of the quality scores dropped below 20 (forward: 100 bp, reverse: 73 bp). Rarefaction of the sequences was conducted using Qiime2’s ‘feature-table rarefy’ command. A sampling depth of 9623 was used for *trnL* sequences (with no samples removed during rarefaction), and a sampling depth of 267 was used for *COI* sequences (with four samples with sequence counts under 267 removed during rarefaction).

Qiime2’s “RESCRIPt” plugin was used to create two reference databases from NCBI; one specific to *trnL* and a second specific to arthropod *COI* [48,49]. Initial reference pools were constructed from these downloaded databases via primer-pair search using Qiime2’s ‘feature-classifier extract-reads’ command. These initial pools were both expanded twice using RESCRIPt’s ‘extract-seq-segments’ command. During reference pool expansions of the *trnL* specific database a ‘--p-perc-identity’ value of 0.7 was used. During reference pool expansions of the *COI* specific database a ‘--p-perc-identity’ value of 0.9 was used. These ‘--p-perc-identity’ values were chosen because at the end of their respective reference pool expansions they had extracted sequence segments from ~70% of their original query sequences [49]. Dereplication during each of these steps was performed using the ‘--p-mode super’ modifier in RESCRIPt’s ‘dereplicate’ command. These expanded reference pools were then used to construct two classifiers, one for *trnL* and one for *COI*, using RESCRIPt’s ‘evaluate-fit-classifier’ command.

Taxonomic assignment for *trnL* and *COI* sequences was conducted using the custom RESCRIPt classifiers described above, in combination with Qiime2’s ‘feature-classifier classify-sklearn’

command. After taxonomic assignment, a rooted phylogenetic tree was created using the Qiime2 plugin “phylogeny” and its ‘align-to-tree-mafft-fasttree’ command.

Statistical Analysis

Once taxonomic assignment was completed, the Qiime2 feature, tree, and metadata files were imported into R and used to create two phyloseq objects using the R packages “qiime2R” and “phyloseq” [50–52]. These phyloseq objects were used to calculate alpha diversity for each colony, in the form of Faith’s phylogenetic diversity (Faith’s PD) scores and species richness, using the ‘calculatePD’ command in the R package “biomeUtils” [53]. Beta diversity between each colony was also calculated from the phlyoseq objects, in the form of unweighted unique fraction metric (uUniFrac) dissimilarity distances, using the ‘phyloseq::distance’ command.

These specific diversity metrics were chosen for two primary reasons: 1) their calculations are based on relative frequency of occurrence (binary presence/absence) of the sequenced taxa, and 2) their calculations take into account the relative relatedness of the sequenced taxa.

Dietary DNA studies almost never utilize quantitative estimates of abundance, as the number of sequencing reads from dDNA does not accurately represent the biomass of consumed material [6]. This is particularly relevant in studies like ours, where sequenced material consists of a broad diversity of partially digested multi-cellular samples. It is likely that digestive processes, the order items are consumed, primer biases, and taxonomic biases in the reference database influence both the sequencing outcomes and taxonomic classification [6,54,55]. To mitigate these issues, metrics incorporating abundance estimates were not used in our analysis. Instead, only metrics based on binary presence/absence of the sequenced taxa (i.e. uUniFrac and Faith’s PD) were used.

Another issue arises from the various states of DNA degradation present in our samples; as sequences originating from the same species, or even the same individual, may end up classified at different taxonomic levels. For example, if one group of sequences are classified as *Fagaceae* and another group of sequences from the same sample are classified as *Fagaceae Quercus*, it is not currently possible to determine if this indicates the presence of two separate genera of *Fagaceae*, or if both sets of sequences come from the same genera. This can result in the artificial inflation of identified taxa, as sequences from a single taxon could be functionally double counted. Some previous dDNA studies have attempted to account for this issue by compressing their post-classified dDNA taxa into diet events: instances where there is unequivocal evidence from dDNA analysis that an organism consumed at least one individual of a species [8,56]. While this approach does minimize double counting of taxa, it increases the risk of undercounting dietary events and can obscure dietary events of taxa unable to be identified to the species level [56]. To avoid the drawbacks of using a diet event based approach, we instead utilized metrics that included the phylogenetic relationship of classified taxa in their calculations (i.e. uUniFrac and Faith's PD). Using these phylogeny-weighted metrics allows sequences classified at different taxonomic levels to be assessed as separate taxa, while minimizing the impact of possible double counts.

Our analysis incorporated two main predictor variables: colony ant species (*T. septentrionalis* and *M. turrifex*) and colony pair ID (Pair-2, Pair-8, Pair-12, Pair-14, Pair-17, Pair-20, Pair-28, Pair-30, Pair-44, and Pair-50). We tested for differences in fungal substrate plant taxa alpha diversity (Faith's PD and species richness) and beta diversity (uUniFrac dissimilarity distance).

For both the *trnL* and *COI* datasets, Faith's PD and species richness distributions were tested for normality via the Shapiro-Wilk normality tests by using the 'shapiro.test' command in R. To analyze Faith's PD and species richness on datasets with normal distributions (*trnL* dataset)

against each of our predictor variables, several analysis of variance (ANOVA) analyses were conducted using the 'aov' command in R. To analyze Faith's PD and species richness on datasets with non-normal distributions (*COI* dataset) Kruskal-Wallis rank sum tests were conducted using the 'kruskal.test' command in R.

Permutational analysis of variance (PerMANOVA) and Procrustes analyses were conducted using the 'adonis2' and 'protest' commands respectively in the R package "vegan" [57]. Our PerMANOVA analyses used 9,999 permutations.

The R packages "ggplot2", "ggtree", and "dplyr" were used to create Figures 2-4 [58–60]. The R package "conflicted" was used to identify possible conflicts when running multiple R packages [61]. The 'devtools::install_github' command in the R package "devtools" was used to download and install several of the previously discussed R packages [62].

Results

Plant Taxa Identified from Fungus Garden Samples

Across all sampled fungus gardens, a total of 42 plant taxa were identified from *trnL* sequences (Figure 2). Fungal substrate plant taxa composition was derived from 34 plant taxa for *T. septentrionalis* and 31 plant taxa for *M. turrifex*. There was considerable overlap in plant taxa composition between the two species, with 22 plant taxa present in both *T. septentrionalis* and *M. turrifex* colonies. 12 plant taxa were identified only from *T. septentrionalis* colonies and 11 plant taxa were identified only from *M. turrifex* colonies.

Fig 2. Phylogenetic tree and binary heat map of plant taxa identified from fungus garden samples. Tree nodes are labeled with bootstrap values. Each branch end is linked to a row in

the binary heat map, showing presence/absence of each respective plant taxon identified in the *M. turrifex* (Mt) and *T. septentrionalis* (Ts) fungus garden samples.

In *T. septentrionalis* colonies, the most frequently occurring plant taxa (occurred in ≥50% of *T. septentrionalis* samples) were: *Magnoliopsida*, *Quercus* (*Fagaceae*), *Asteraceae*, and *Rubiaceae.* For *M. turrifex* colonies, the most frequently occurring plant taxa (occurred in ≥50% of *M*. *turrifex* samples) were: *Magnoliopsida*, *Quercus* (*Fagaceae*), *Poaceae*, and *Acalypha* (*Euphorbiaceae*).

Arthropod Taxa Identified from Fungus Garden Samples

A total of 6 arthropod taxa were identified from *COI* sequences post rarefaction. All rarified colony fungus garden samples contained *Insecta* sequences. Only three samples contained sequences identified to a lower taxonomic level than *Insecta*: *M*. *turrifex* colony 44's fungus garden contained *Carabidae* (*Coleoptera*) and *Lepidoptera* sequences; *M*. *turrifex* colony 14's fungus garden contained *Anomoea laticlavia* (*Coleoptera Chrysomelidae*) sequences; and *M*. *turrifex* colony 20's fungus garden contained *Coleoptera* sequences.

Plant Taxa Alpha Diversity

ANOVA analyses were used to determine if Faith's PD or species richness of fungal substrate plant taxa differed significantly between colonies based on colony ant species or colony pair ID. *Trachymyrmex septentrionalis* and *M. turrifex* fungus gardens did not have significantly different Faith's PD ($F_{1,18}$ = 0.285, p = 0.6), nor did they have significantly different species richness ($F_{1,18}$ = 0.169, p = 0.686). There were, however, significant differences in Faith's PD ($F_{9,10}$ = 5.563, p = 0.007) (Figure 3) and species richness ($F_{9,10}$ = 5.615, p = 0.006) between colony pairs (Figure 4).

Fig 3. Boxplot depicting Faith's PD scores of fungal substrate plant taxa for each colony pair.

Fig 4. Boxplot depicting species richness of fungal substrate plant taxa for each colony pair.

Plant Taxa Beta Diversity

A PerMANOVA analysis was used to determine if the uUniFrac dissimilarity distances of fungal substrate plant taxa were significantly different between colonies based on colony ant species or colony pair ID. There was no significant difference found between colony ant species and the uUniFrac dissimilarity distances of their plant taxa ($p = 0.089$). There was also no significant difference between colony pairs and the uUniFrac dissimilarity distances of their plant taxa ($p = 0.471$).

Arthropod Taxa Alpha Diversity and Beta Diversity

No significant alpha diversity or beta diversity differences in detected fungus garden arthropod sequences were found between colonies based on ant species or colony pair ID ($p \geq 0.134$).

Discussion

Dietary DNA techniques successfully identified plant and arthropod taxa present in the fungus gardens of *T. septentrionalis* and *M. turrifex*. The dDNA analysis was particularly successful with the *trnL* primer, as it identified a relatively large number of plant taxa, many of them to a high level of taxonomic resolution. These results are particularly impactful considering the literature deficit of specific plant taxa known to be foraged by non-leafcutting fungus gardening ants. Only a handful of previous studies make any mention of specific foraged plant taxa, with most only

describing the general type of material the ants were collecting such as flower petals or dead leaves [1,2,40,42,63–67].

When it came to fungal substrate plant taxa, colony ant species was not a significant factor in alpha or beta diversity metrics. This indicates that *T. septentrionalis* and *M*. *turrifex* were not collecting significantly different plant material. In contrast, colony pair was found to be a significant factor when it came to plant taxa alpha diversity metrics. Taken together, this could indicate that diet preference is driven by colony location-specific plant resource availability, rather than ant species-specific plant preference.

The plant taxa we identified from *T. septentrionalis* and *M. turrifex* fungus garden sequences were consistent with the limited existing descriptions of forage material collected by these ant species. One previous study observed *M. turrifex* collecting *Sorghum halepense* and *Solidago sp.*, both of whose families, *Poaceae* and *Asteraceae* respectively, were present in our samples [40]. Another previous study suggested that *T. septentrionalis* collects *Quercus* (*Fagaceae*), which was also present in our samples [42].

If we include descriptions of known forage material from other higher agriculture non-leafcutting ants, we find additional overlap between our identified plant taxa and that of previous studies. *Lauraceae*, *Rosaceae*, *Rubiaceae*, *Poaceae*, and *Euphorbiaceae* are all plant families which were identified in our samples, and are known to be collected by or, in the case of lab colonies, accepted by higher agriculture non-leafcutting ants [63,65]. Leafcutting species like *Atta texana* are known to forage *Pinus* (*Pinaceae*), but our study is the first to demonstrate that the non-leacutting higher agriculture species *T. septentrionalis* will also forage on *Pinus* [68].

The lack of dDNA evidence for diet-based niche separation between *T. septentrionalis* and *M. turrifex* leads to two possible conclusions regarding the efficacy of using dDNA in this context. The first conclusion would be that dDNA techniques, at least as implemented in this study, give insufficient data to demonstrate diet-based niche separation occurring between our study species. A notable drawback of only using dDNA analysis on sampled fungus gardens is that dDNA analysis alone cannot determine the state plant material was in when it was collected by the ants. Some higher agriculture fungus gardeners outside of *Atta* and *Acromyrmex*, like *T. septentrionalis*, are known to cut fresh vegetative material to use as fungus garden substrate, while others do not [2,42]. In our study, we could not determine whether the detected plant sequences originated from the ants foraging fresh cut plant material, plant detritus, or frass. We also could not determine which part of the plant they collected (i.e. leaves, flowers, or sticks). The inability to detect which state plant material was collected in could be obscuring differences in foraging niche between these two ant species. One ant species may forage on *Quercus* (*Fagaceae*) catkins, while a second ant species may forage caterpillar frass that contains *Quercus* leaves. Similarly, one ant species may cut soft *Quercus* vegetation, whereas a second ant species might only take dead vegetation that has fallen from the tree. In all of these examples the ant's fungus gardens would contain *Quercus* sequences, but dDNA analysis would be unable to differentiate the state the material was collected in. Additionally, since the number of sequencing reads cannot be used as a quantitative measure of the biomass of digested material, we cannot use dDNA to determine if colonies are foraging different amounts of the same material [6].

Another, related, pitfall of using fungus garden dDNA was discovered during colony excavation when fungus garden samples were extracted. We noted more often than not that fungus gardens were built around thin tree roots that crossed the ant's fungus chambers. While attempts were made to minimize the amount of root material in collected fungus garden

samples, DNA contamination from rootlets and microscopic root remnants is likely. This problem is exacerbated by the fact that both ant species in our study are known to accept forage material from these same tree species (i.e. *Quercus* catkins), meaning that any removal of these tree sequences from analysis would almost certainly remove taxa that the ants were foraging.

The second possible conclusion would be that dDNA techniques, as implemented in this study, are sufficient at identifying diet-based niche separation (or lack thereof), but the ants are separated by another dimension and/or outside influences are impacting normal niche partitioning. Our study did not measure if *T. septentrionalis* and *M. turrifex* were niche separated by a dimension other than forage material such as preferred foraging temperature. Niche partitioning via temperature/temporal separation is known to occur between co-occurring leafcutter species such as *Acromyrmex lobicornis* and *Acromyrmex striatus* in the Monte desert of Argentina, as well as *Acromyrmex balzani* and *Atta opaciceps* in the grasslands of Brazil [69–71]. This phenomenon has also been well documented in non-fungus gardening ant species [72–74].

With both species near the limit of their respective abiotic tolerances in Texas, it is also possible that climate could be eroding the advantage of whichever is normally the more competitive of the two species, allowing coexistence [36,75]. Alternatively, in heavily human-modified locations where these two species co-occur, like our sampling site which had a history of being mowed, human disturbance could be what is inhibiting competitive displacement that may normally occur [75]. Supporting this view, the co-occurring leafcutter species *Atta sexdens* and *Atta colombia* have been shown to display less pronounced habitat segregation, and will often overlap, in park-like and man-made habitats [34]. Also noteworthy is the fact that *T. septentrionalis* workers were observed to emerge and begin foraging several weeks before *M.*

turrifex colonies became active, which could result in resource preemption by *T. septentrionalis* that may not be visible during our single sampling point [75].

While our study establishes dDNA analysis as a powerful tool for identification of fungus garden substrate, we also identified several limitations that should be accounted for in future studies attempting to use similar techniques. One issue was the low taxonomic resolution of many plant sequences. While we were able to successfully identify 42 plant taxa in the fungus gardens using our selected *trnL* primer, unidentified *Magnoliopsida* had the highest absolute read abundance post-rarefaction, indicating that a number of plant species may be present in the fungus gardens that our *trnL* primer was unable to identify to an informative taxonomic level. In future studies this could be mitigated, at least in part, by using multiple plant primers designed for different target regions [23,76,77].

Dietary DNA analysis alone is not capable of distinguishing *trnL* sequences from plant material foraged directly by the ants and *trnL* sequences from insect frass foraged by the ants. Since *Trachymyrmex septentrionalis* and *M. turrifex* both readily forage insect frass containing plant material, plant DNA sequences from foraged frass were potentially detected by the *trnL* primer we used [13,40,42]. We had hoped to use the *COI* primer results to gain insight into what arthropod taxa the ants were collecting frass from, but the primer performed too poorly to draw any meaningful conclusions. Other studies attempting to determine *Lepidoptera* species from their frass found that amounts of dry frass less than 5 mg may be too small to detect insect DNA sequences [78]. This insect sequence detectability cutoff was likely higher in our samples due to the physical and enzymatic breakdown of foraged material occurring in the fungus gardens, making it difficult to detect frass-producing-insect DNA [2].

Many of the issues we discussed can be mitigated by taking the limitations of dDNA analysis into account when designing future studies. Our recommendations for future studies include: using multiple plant primers to maximize taxonomic coverage, sampling at several different time points to allow detection of temporal changes in fungal substrate plant composition, and incorporating a supplemental observational component to gain an understanding of what state forage material is being collected in. Overall, we have demonstrated that dDNA analysis is a powerful tool for the taxonomic identification of forage material in fungus-gardening ants, as it is capable of identifying large numbers of plant taxa to a high level of taxonomic resolution. Dietary DNA analysis offers an opportunity to improve our understanding of plant taxa foraged by non-leafcutting fungus gardening ants, and, with appropriate supporting methods, can be applied to gain new insight into their ecology and life history.

Acknowledgments

We would like to thank Christine Bays, Dillon Flowers, Gabriel McDanield, and Grant Rook, all of whom took time out of their lives to help with colony excavation and sampling. Funding provided by the National Science Foundation (IOS-1552822).

Data Availability

The raw sequences for all samples have been deposited with links to BioProject accession number PRJNA1124954 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1124954). The Qiime2, RESCRIPt, and R code used during this project can be found at https://github.com/mrichardsperhatchv/FORAGE-PREFERENCE-IN-TWO-GEOGRAPHICALLY-CO-OCCURRING-FUNGUS-GARDENING-ANTS.

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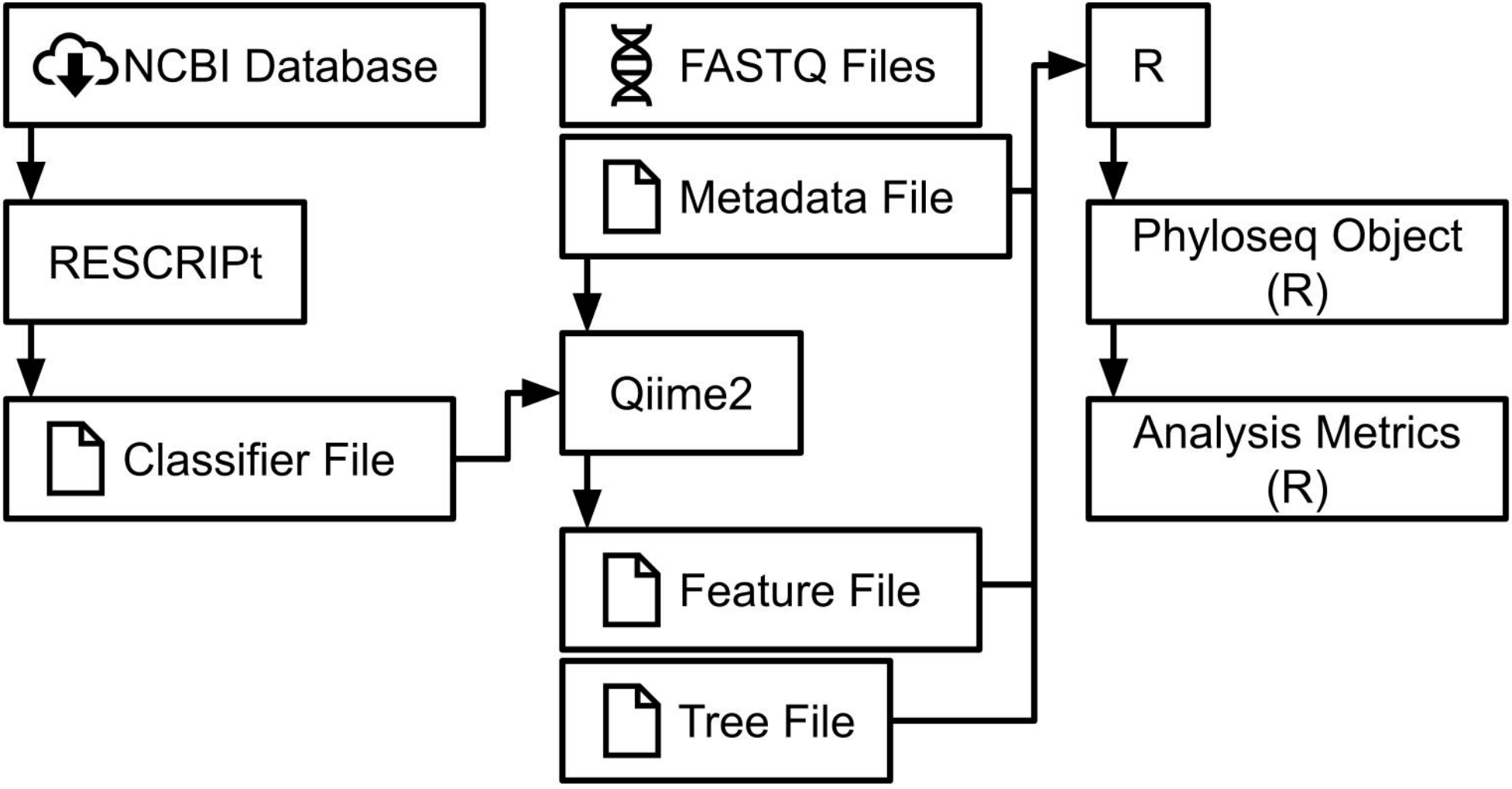
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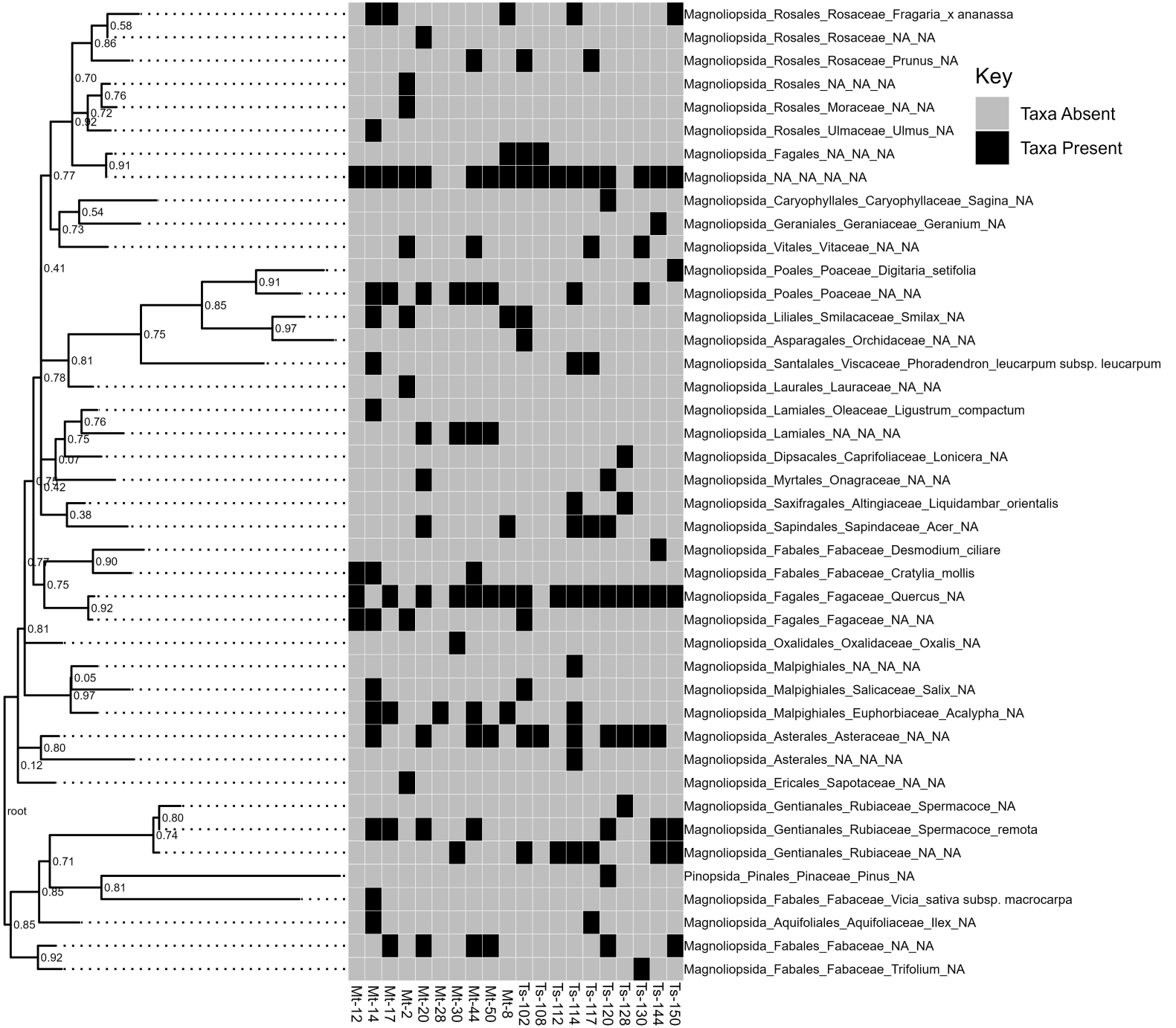
Key
Taxa Absent
Taxa Present
Magnoliopsida_Rosales_Rosaceae_Fragaria_x ananassa
Magnoliopsida_Rosales_Rosaceae_NA_NA
Magnoliopsida_Rosales_Rosaceae_Prunus_NA
Magnoliopsida_Rosales_NA_NA_NA
Magnoliopsida_Rosales_Moraceae_NA_NA
Magnoliopsida_Rosales_Ulmaceae_Ulmus_NA
Magnoliopsida_Fagales_NA_NA_NA
Magnoliopsida_NA_NA_NA_NA
Magnoliopsida_Caryophyllales_Caryophyllaceae_Sagina_NA
Magnoliopsida_Geraniales_Geraniaceae_Geranium_NA
Magnoliopsida_Vitales_Vitaceae_NA_NA
Magnoliopsida_Poales_Poaceae_Digitaria_setifolia
Magnoliopsida_Poales_Poaceae_NA_NA
Magnoliopsida_Liliales_Smilacaceae_Smilax_NA
Magnoliopsida_Asparagales_Orchidaceae_NA_NA
Magnoliopsida_Santalales_Viscaceae_Phoradendron_leucarpum subsp. leucarpum
Magnoliopsida_Laurales_Lauraceae_NA_NA
Magnoliopsida_Lamiales_Oleaceae_Ligustrum_compactum
Magnoliopsida_Lamiales_NA_NA_NA
Magnoliopsida_Dipsacales_Caprifoliaceae_Lonicera_NA
Magnoliopsida_Myrtales_Onagraceae_NA_NA
Magnoliopsida_Saxifragales_Altingiaceae_Liquidambar_orientalis
Magnoliopsida_Sapindales_Sapindaceae_Acer_NA
Magnoliopsida_Fabales_Fabaceae_Desmodium_ciliare
Magnoliopsida_Fabales_Fabaceae_Cratylia_mollis
Magnoliopsida_Fagales_Fagaceae_Quercus_NA
Magnoliopsida_Fagales_Fagaceae_NA_NA
Magnoliopsida_Oxalidales_Oxalidaceae_Oxalis_NA
Magnoliopsida_Malpighiales_NA_NA_NA
Magnoliopsida_Malpighiales_Salicaceae_Salix_NA
Magnoliopsida_Malpighiales_Euphorbiaceae_Acalypha_NA
Magnoliopsida_Asterales_Asteraceae_NA_NA
Magnoliopsida_Asterales_NA_NA_NA
Magnoliopsida_Ericales_Sapotaceae_NA_NA
Magnoliopsida_Gentianales_Rubiaceae_Spermacoce_NA
Magnoliopsida_Gentianales_Rubiaceae_Spermacoce_remota
Magnoliopsida_Gentianales_Rubiaceae_NA_NA
Pinopsida_Pinales_Pinaceae_Pinus_NA
Magnoliopsida_Fabales_Fabaceae_Vicia_sativa subsp. macrocarpa
Magnoliopsida_Aquifoliales_Aquifoliaceae_Ilex_NA
Magnoliopsida_Fabales_Fabaceae_NA_NA
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Mt-12
Mt-14
Mt-17
Mt-2
Mt-20
Mt-28
Mt-30
Mt-44
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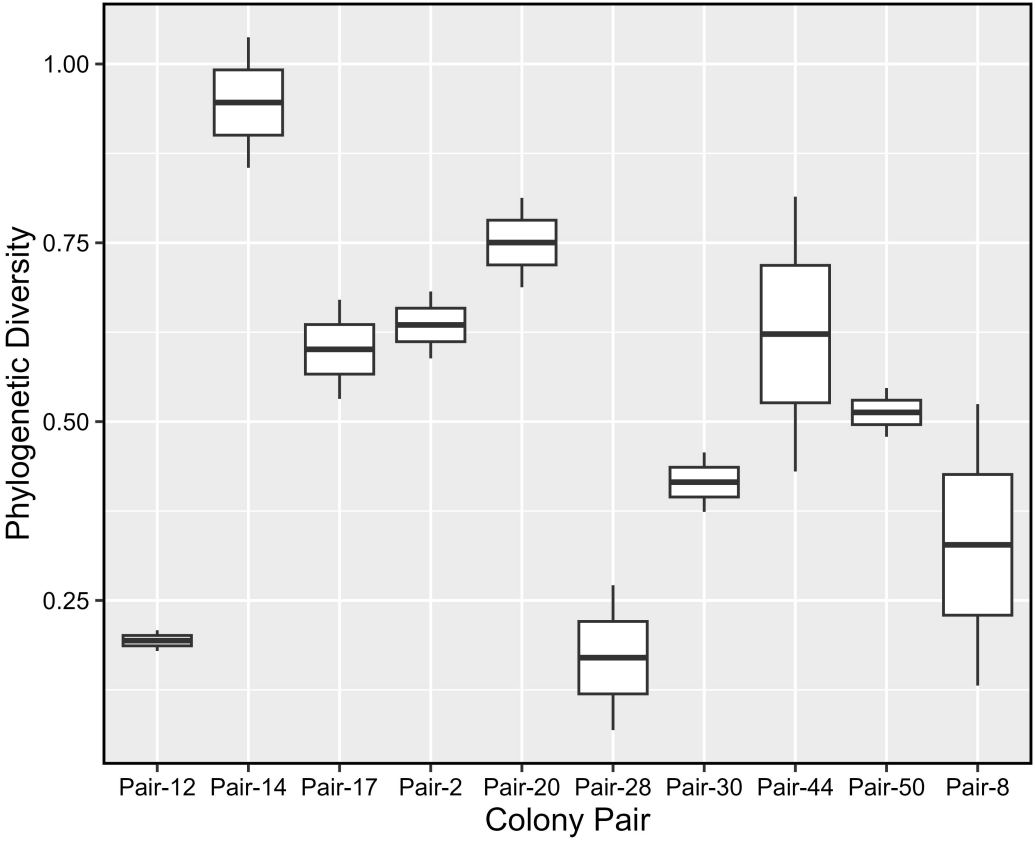
Ts-150
root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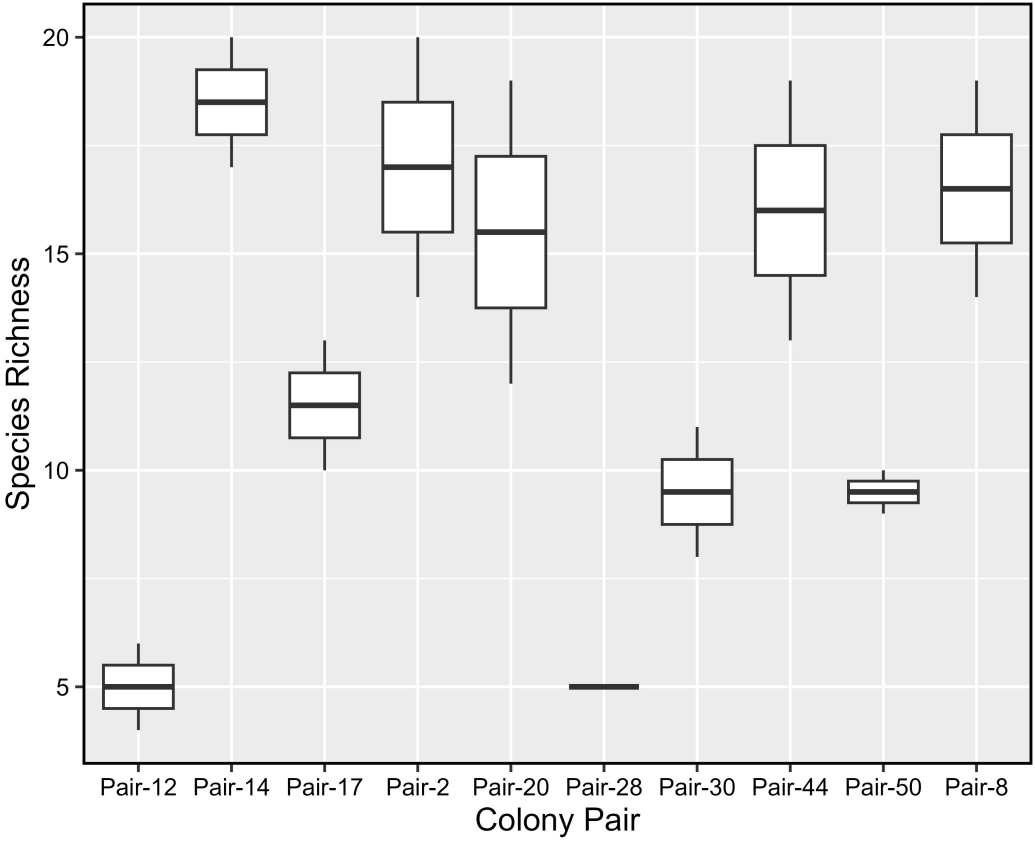
1.00
0.75
0.50
0.25
Pair-12
Pair-14
Pair-17
Pair-2
Pair-20
Pair-28
Pair-30
Pair-44
Pair-50
Pair-8
Colony Pair


Species Richness
20
15
10
5
Pair-12
Pair-14
Pair-17
Pair-2
Pair-20
Pair-28
Pair-30
Pair-44
Pair-50
Pair-8
Colony Pair