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 with non-leafcutting fungus gardening ants, making diet-related ecological and life history questions difficult to study. To address this limitation, we utilized dietary DNA metabarcoding on excavated ant fungus gardens to generate forage diversity metrics for the two co-occurring species *Trachymyrmex septentrionalis* and *Mycetomoellerius turrifex*. Ten fungus garden samples from each species were collected from a 60x70 m plot in East Texas. Each of the colonies we sampled was paired with a colony from the other species within 3 m of it. Plant forage diversity was assessed with chloroplast *trnL* primers, and insect frass forage diversity was assessed with mitochondria *COI* primers. DNA metabarcoding identified a total of 44 plant taxa across all samples, but performed poorly when characterizing foraged insect frass. Plant beta diversity was significantly different between the gardens of *T. septentrionalis* and *M. turrifex* colonies, as well as paired colonies. Colony pairs also had significantly different plant alpha diversity. This indicates that diet preference is likely driven both by ant species-specific plant preference, and colony location-specific plant resource availability. Overall, our results show that dietary DNA techniques are a promising tool for the identification of plant forage 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]. The foragers of non-leafcutting fungus gardening ants are also usually smaller and more cryptic than those of leafcutting ants. Additionally, some foragers may collect items that are unsuitable, if not toxic, and deposit them in their nests but not the fungus

71 gardens [41]. Thus observations on forager behavior alone are not likely to depict a
72 comprehensive overview of what ants are collecting and incorporating into their fungus garden.
73 These factors have contributed to a deficit of literature regarding the diet composition of non-
74 leafcutting fungus gardening ants, with the little information available primarily consisting of a
75 handful of forage material observations, often originally published in conjunction with the original
76 natural history descriptions of the ants [1,2]. Clearly, relying solely on visual identification of
77 forage material is insufficient for the study of non-leafcutting fungus gardening ants.
78
79 **Dietary DNA**
80 Dietary DNA (dDNA) techniques present a possible alternative to visual forage identification.
81 Metabarcoding techniques based on dDNA, DNA extracted from an organism's gut, stomach, or
82 fecal matter, have emerged as an accurate and resource-effective way to determine animal
83 diets [7,13]. Previous studies, in multiple animal groups, have successfully utilized dDNA
84 analysis to determine diet composition when direct observations of feeding behavior and/or
85 morphological identification of forage material was insufficient [7,13].
86
87 Dietary DNA analysis has only been used to examine ant diet composition in a handful of
88 studies, with even fewer of these studies having attempted to determine broad diet composition
89 [8,14,15]. One factor complicating dDNA ant research is a yet unidentified compound in the
90 gaster of adult ants that has been found to inhibit PCR, potentially impacting gut content dDNA
91 analysis in adult ants [15,16]. As an additional complication, in many species adult ants are
92 unable to digest solid food, instead feeding it to their brood. The adult ants then consume the
93 digested liquid nutrients via trophallaxis from their larvae [8,17]. Wulff et al. (2021) leveraged
94 this adult-larvae digestion dynamic to their advantage when using dDNA analysis to investigate
95 the diet composition of *Solenopsis invicta* [8]. They did so by sequencing the gut content of *S.*

96 *invicta* larvae instead of adults, as the larvae act as a centralized depository of colony prey and
97 are not known to produce PCR inhibitors [8,15,16].
98
99 A similarly innovative approach could be used to analyze the diet composition of fungus
100 gardening ants, since their fungus gardens also act as a centralized depository for colony
101 forage. The fungus gardens cultivated by fungus gardening ants function as an external
102 ancillary digestive system for the colony [2,17–19]. Within this ancillary digestive system, forage
103 materials used as fungal substrate become highly degraded, due both to physical mastication
104 by the ants and enzymatic breakdown by the fungus. This degradation necessitates the use of
105 specialized genetic techniques, like dDNA metabarcoding, that are designed to work with highly
106 degraded staples [2,5,20].
107
108 **Evaluation of Diet and Study Species**
109 Using dDNA metabarcoding techniques to identify fungal substrate in ant gardens could be
110 used to answer a number of ecological and natural history questions. Dietary DNA
111 metabarcoding has been successfully used to detect niche partitioning, and overlap, in several
112 animal species [21–25]. These techniques have also been used to understand seasonal and/or
113 biogeographic based shifts in animal diets [26–32].
114
115 Dietary differences have been investigated (without the use of dDNA methods) in several
116 species of fungus gardening leafcutting ants. In a dry tropical forest in Costa Rica, the co-
117 occurring leafcutter species *Atta cephalotes* and *Acromyrmex octospinosus* were found to
118 forage almost entirely different resources, with the only exception being they both foraged
119 flowers [33]. A similar trend was observed in Panama, with *Atta* species foraging primarily on
120 tree leaves and co-occurring *Acromyrmex* species foraging primarily on flowers and herbal

leaves [34]. To our knowledge, no previous studies have examined differences in diet between 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 and New York; while the range of *M. turrifex* extends from Texas to northeastern Mexico and western Louisiana [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 in non-leafcutting fungus gardening ants.

The primary objective of this study was to explore whether dDNA metabarcoding could be used to identify and describe the diversity of plant material and/or insect frass used as substrate in the fungus gardens of co-occuring *T. septentrionalis* and *M. turrifex* colonies. The secondary objective of this study was to evaluate whether diversity metrics based on identified fungal substrate were sufficient to detect differences in diet between *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 an approximately 60x70 m plot on the University of Texas at Tyler campus in Tyler, Texas, USA.

147 Sampling occurred from May 16-26, 2023. Each of the ten *M. turrifex* colonies we sampled was
148 paired with a *T. septentrionalis* colony within 3 m of it. These colony pairs, composed of one *M.*
149 *turrifex* colony and one nearby *T. septentrionalis* colony, were sampled concurrently. Each
150 colony was only sampled once.
151
152 Fungus gardens were non-destructively sampled from each colony by careful excavation of the
153 topmost chamber containing an appreciable amount of fungus. Sampling was focused on
154 chambers closer to the surface since deeper chambers were unlikely to contain fungus until
155 later in the season when the upper chambers are too hot for fungus garden growth. Sterilized
156 tools were used when removing fungus gardens from the chamber to minimize DNA
157 contamination from environmental sources and previously sampled colonies. Excavated fungus
158 garden samples were placed in sterile vials containing 95% ethanol and stored in a -80°C
159 freezer.
160
161 **Sample Metabarcoding**
162 Metabarcoding was conducted by Jonah Ventures (https://jonahventures.com/) using
163 chloroplast *trnL* (forward primer c: 5' CGAAATCGGTAGACGCTACG 3', reverse primer h: 5'
164 CCATTGAGTCTCTGCACCTATC 3') and mitochondrial cytochrome c oxidase subunit I (*COI*)
165 (forward primer ZBJ-ArtF1c: 5' AGATATTGGAACWTTATATTTTATTTTTGG 3', reverse primer
166 ZBJ-ArtR2c: 5' WACTAATCAATTWCCAAATCCTCC 3') primers [43,44]. Individual fungus
167 garden samples were shipped overnight in an ice box to Jonah Ventures in Boulder, CO.
168
169 At Jonah Ventures, frozen samples were thawed for 1-2 hours before processing. Sample
170 barcodes were recorded and assigned a well within the 96 well plate or numbered extraction
171 tube. Under a laminar flow hood, sterile cotton swabs (Fisher, Catalog No. 22-363-173) were
172 coated with sediment matter, and the swabs were placed in the corresponding extraction plate

173 or tube. Sterile tweezers and pliers were used to handle cotton swabs and remove the wooden
174 ends of the cotton swab before extraction. Plates or tubes were immediately processed or
175 stored in -20°C until the extraction process could be performed.
176
177 Genomic DNA from each fungus garden sample was extracted using the DNeasy 96 PowerSoil
178 Pro Kit (384) (Catalog No. 47017) according to the manufacturer's protocol. Genomic DNA was
179 eluted into 100 µl and frozen at -20°C.
180
181 For *trnL* genetic analysis, a portion of the chloroplast *trnL* intron was PCR amplified from each
182 genomic DNA sample using the c and h trnL primers. Both forward and reverse primers also
183 contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing.
184 Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications
185 (Promega, Catalog No. M5133, Madison, WI) which included 0.4 µM of each primer and 1 µl of
186 gDNA. DNA was PCR amplified using the following conditions: initial denaturation at 94°C for 3
187 minutes, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at
188 72°C, and a final elongation at 72°C for 10 minutes.
189
190 For *COI* genetic analysis, a portion of the *COI* gene was PCR amplified from each genomic
191 DNA sample using the ZBJ-ArtF1c and ZBJ-ArtR2c primers. Both forward and reverse primers
192 also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing.
193 Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications
194 (Promega, Catalog No. M5133, Madison, WI) which included 12.5 µl Master Mix, 1.0 µM of each
195 primer, 3.0 µl of gDNA, and 7.5 µl DNase/RNase-free H2O. DNA was PCR amplified using the
196 following conditions: initial denaturation at 94°C for 5 minutes, followed by 45 cycles of 30 s at
197 94°C, 45 s at 45°C, and 45 s at 72°C, and a final elongation at 72°C for 10 minutes.
198

199 To determine amplicon size and PCR efficiency, each reaction was visually inspected using a
200 2% agarose gel with 5 µl of each sample as input. Amplicons were then cleaned by incubating
201 amplicons with Exo1/SAP for 30 minutes at 37°C followed by inactivation at 95°C for 5 minutes
202 and stored at -20°C.
203
204 A second round of PCR was performed to complete the sequencing library construct, appending
205 with the final Illumina sequencing adapters and integrating a custom sample-specific 12-
206 nucleotide index sequence (one index read per amplicon) generated by Jonah Ventures. The
207 indexing PCR included Promega Master mix, 0.5 µM of each primer and 2 µl of template DNA
208 (cleaned amplicon from the first PCR reaction) and consisted of an initial denaturation of 95°C
209 for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30
210 seconds.
211
212 Final indexed amplicons from each sample were cleaned and normalized using SequalPrep
213 Normalization Plates (Life Technologies, Carlsbad, CA). 25 µl of PCR amplicon was purified and
214 normalized using the Life Technologies SequalPrep Normalization kit (Catalog No. A10510-01)
215 according to the manufacturer's protocol. Samples were then pooled together by adding 5µl of
216 each normalized sample to the pool.
217
218 Sample library pools were sent for sequencing on an Illumina MiSeq (San Diego, CA) at the
219 Texas A&M Agrilife Genomics and Bioinformatics Sequencing Core facility using the v2 500-
220 cycle kit (Catalog No. MS-102-2003). A PhiX concentration of 15% was used. As a quality
221 control measure, a bioanalyzer chip was used for concentration adjustment at the sequencing
222 center prior to sequencing.
223
224 Raw sequence data were demultiplexed using pheniqs v2.1.0, enforcing strict matching of

225 sample barcode indices (i.e, no errors) [45].

226

227 **Sequence Processing**

228 After receiving the demultiplexed data in the form of paired end FASTQ files from Jonah

229 Ventures, the files were imported into Qiime2 for further processing (Figure 1) [46].

230

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

232 **metrics.**

233

234 The Qiime2 plugin "cutadapt" was used to remove primers from the sequences. The Qiime2

235 plugin "dada2" was used to denoise the *trnL* and *COI* sequences, truncate the *COI* sequences,

236 and merge the forward and reverse *COI* sequence pairs [47]. Only the forward *trnL* sequences

237 were used because of the short *trnL* amplicon length (143 bp). The *trnL* sequences were not

238 truncated because their untruncated median quality scores were 30 or greater. The *COI*

239 sequences were truncated where their median quality scores dropped below 30 (forward: 100

240 bp, reverse: 87 bp). Rarefaction of the sequences was conducted using Qiime2's 'feature-table

241 rarefy' command. A sampling depth of 9677 was used for *trnL* sequences (Figure 2). Due to

242 low *COI* sequencing depth (<1000) in the majority of samples, downstream analysis was not

243 conducted for *COI* outside of taxonomic classification (Figure 3).

244

245 **Fig 2. Rarefaction curves for *trnL* sequences.** The dashed vertical line shows the chosen

246 *trnL* rarefaction depth of 9677.

247

248 **Fig 3. Rarefaction curves for *COI* sequences.** The lower left corner of the plot has been

249 magnified to show greater detail.

250

251 Qiime2's "RESCRIPt" plugin was used to create two reference databases from NCBI; one
252 specific to *trnL* and a second specific to arthropod *COI* [48,49]. Initial reference pools were
253 constructed from these downloaded databases via primer-pair search using Qiime2's 'feature-
254 classifier extract-reads' command. These initial pools were both expanded twice using
255 RESCRIPt's 'extract-seq-segments' command. During reference pool expansions of the *trnL*
256 specific database a '--p-perc-identity' value of 0.7 was used. During reference pool expansions
257 of the *COI* specific database a '--p-perc-identity' value of 0.9 was used. These '--p-perc-identity'
258 values were chosen because at the end of their respective reference pool expansions they had
259 extracted sequence segments from ~70% of their original query sequences [49]. Dereplication
260 during each of these steps was performed using the '--p-mode super' modifier in RESCRIPt's
261 'dereplicate' command. These expanded reference pools were then used to construct two
262 classifiers, one for *trnL* and one for *COI*, using RESCRIPt's 'evaluate-fit-classifier' command
263 (Figure S1).
264
265 Taxonomic assignment for *trnL* and *COI* sequences was conducted using the custom
266 RESCRIPt classifiers described above, in combination with Qiime2's 'feature-classifier classify-
267 sklearn' command.
268
269 **Statistical Analysis**
270 Once taxonomic assignment was completed, the Qiime2 feature and metadata files were
271 imported into R and used to create two phyloseq objects using the R packages "qiime2R" and
272 "phyloseq" [50–52]. An independent phylogenetic tree was constructed by grafting our classified
273 *trnL* sequences onto the GBOTB.extended.WP.tre megatree, reported by Jin and Qian (2022),
274 using the 'phylo.maker' command from the R package "U.PhyloMaker" [53,54]. Six classified
275 *trnL* sequences failed to graft to the tree due to low taxonomic resolution and were discarded.
276 The resulting phylogenetic tree was converted to a phylo class object using the

277 ‘phyloseq::read_tree’ command; multichotomies were then resolved using the ‘multi2di’
278 command from the R package “ape” [55]. This tree was merged with our phyloseq object using
279 the ‘phyloseq::merge_phyloseq’ command. This updated phyloseq object was used to calculate
280 alpha diversity for each colony, in the form of Faith’s phylogenetic diversity (Faith’s PD) scores
281 and species richness, using the ‘calculatePD’ command in the R package “biomeUtils” [56].
282 Beta diversity between each colony was also calculated from the updated phlyoseq object, in
283 the form of unweighted unique fraction metric (uUniFrac) dissimilarity distances, using the
284 ‘phyloseq::distance’ command.
285
286 These specific diversity metrics were chosen for two primary reasons: 1) their calculations are
287 based on relative frequency of occurrence (binary presence/absence) of the sequenced taxa,
288 and 2) their calculations take into account the relative relatedness of the sequenced taxa.
289
290 The number of sequencing reads from dDNA often does not accurately represent the biomass
291 of consumed material [6,13]. This is particularly relevant in studies like ours, where sequenced
292 material consists of a broad diversity of partially digested multi-cellular samples. It is likely that
293 digestive processes, the order items are consumed, primer biases, and taxonomic biases in the
294 reference database influence both the sequencing outcomes and taxonomic classification
295 [6,57,58]. Due to the known impact of these factors on estimations of abundance, only metrics
296 based on binary presence/absence of the sequenced taxa (i.e. uUniFrac and Faith’s PD) were
297 used.
298
299 Another issue arises from the various states of DNA degradation present in our samples; as
300 sequences originating from the same species, or even the same individual, may end up
301 classified at different taxonomic levels. For example, if one group of sequences are classified as
302 *Fagaceae* and another group of sequences from the same sample are classified as *Fagaceae*

303 *Quercus*, it is not currently possible to determine if this indicates the presence of two separate
304 genera of *Fagaceae*, or if both sets of sequences come from the same genera. This can result
305 in the artificial inflation of identified taxa, as sequences from a single taxon could be functionally
306 double counted. Some previous dDNA studies have attempted to account for this issue by
307 compressing their post-classified dDNA taxa into diet events: instances where there is
308 unequivocal evidence from dDNA analysis that an organism consumed at least one individual of
309 a species [8,59]. While this approach does minimize double counting of taxa, it increases the
310 risk of undercounting dietary events and can obscure dietary events of taxa unable to be
311 identified to the species level [59]. To avoid the drawbacks of using a diet event based
312 approach, we instead utilized metrics that included the phylogenetic relationship of classified
313 taxa in their calculations (i.e. uUniFrac and Faith's PD). Using these phylogeny-weighted
314 metrics allows sequences classified at different taxonomic levels to be assessed as separate
315 taxa, while minimizing the impact of possible double counts.
316
317 Our analysis incorporated two main predictor variables: colony ant species (*T. septentrionalis*
318 and *M. turrifex*) and colony pair ID (Pair-2, Pair-8, Pair-12, Pair-14, Pair-17, Pair-20, Pair-28,
319 Pair-30, Pair-44, and Pair-50). We tested for differences in fungal substrate plant taxa alpha
320 diversity (Faith's PD and species richness) and beta diversity (uUniFrac dissimilarity distance).
321
322 Faith's PD and species richness distributions were tested for normality via the Shapiro-Wilk
323 normality tests by using the 'shapiro.test' command in R. To analyze Faith's PD and species
324 richness against each of our predictor variables, several analysis of variance (ANOVA) analyses
325 were conducted using the 'aov' command in R.
326
327 Permutational analysis of variance (PerMANOVA) analyses were conducted using the 'adonis2'
328 commands in the R package "vegan" [60]. Our PerMANOVA analyses used 9,999 permutations.

329

330 Data visualization was performed with the R packages “tidyverse”, “ggtree”, and “ggrepel” [61–

331 63].

332

333 ## Results

334 **Plant Taxa Identified from Fungus Garden Samples**

335 Across all sampled fungus gardens, a total of 44 plant classifications were identified from *trnL*

336 sequences (Figure 4). Fungal substrate plant taxa composition was derived from 32 plant

337 classifications for *T. septentrionalis* and 33 plant classifications for *M. turrifex*. There was

338 considerable overlap in plant taxa composition between the two species, with 21 plant

339 classifications present in both *T. septentrionalis* and *M. turrifex* colonies. 11 plant classifications

340 were identified only from *T. septentrionalis* colonies and 12 plant classifications were identified

341 only from *M. turrifex* colonies.

342

343 **Fig 4. Phylogenetic tree and binary heat map of plant taxa identified from fungus garden**

344 **samples.** Each branch end is linked to a row in the binary heat map, showing

345 presence/absence of each respective plant classification identified in the *M. turrifex* (Mt) and *T.*

346 *septentrionalis* (Ts) fungus garden samples. Cells with a black dot indicate the plant

347 classification was present in both colonies from that pair.

348

349 In *T. septentrionalis* colonies, the most frequently occurring plant classifications (occurred in

350 ≥50% of *T. septentrionalis* samples) were: *Fagaceae*, *Magnoliopsida*, *Rubiaceae*, *Asteraceae*,

351 and *Fagales.* For *M. turrifex* colonies, the most frequently occurring plant classifications

352 (occurred in ≥50% of *M. turrifex* samples) were: *Fagaceae*, *Oxalis* (*Oxalidaceae*),

353 *Magnoliopsida*, *Rubiaceae*, *Fagales*, *Acalypha* (*Euphorbiaceae*), and *Poaceae*.

354

355 **Plant Taxa Alpha Diversity**

356 ANOVA analyses were used to determine if Faith's PD or species richness of fungal substrate
357 plant taxa differed significantly between colonies based on colony ant species or colony pair ID.
358 *Trachymyrmex septentrionalis* and *M. turrifex* fungus gardens did not have significantly different
359 Faith's PD ($F_{1,18}$ = 0.045, p = 0.834), nor did they have significantly different species richness
360 ($F_{1,18}$ = 0.223, p = 0.643). There were, however, significant differences in Faith's PD ($F_{9,10}$ =
361 3.921, p = 0.022) and species richness ($F_{9,10}$ = 3.105, p = 0.046) between colony pairs.

362

363 **Plant Taxa Beta Diversity**

364 A PerMANOVA analysis was used to determine if the uUniFrac dissimilarity distances of fungal
365 substrate plant taxa were significantly different between colonies based on colony ant species
366 or colony pair ID. There was a significant difference found between colony ant species and the
367 uUniFrac dissimilarity distances of their plant taxa (p = 0.046) (Figure 5). There was also a
368 significant difference between colony pairs and the uUniFrac dissimilarity distances of their plant
369 taxa (p = 0.014).

370

371 **Fig 5. NMDS plot showing the uUniFrac dissimilarity of plant taxa identified from fungus**
372 **garden samples.** Colony pairs are connected by grey lines.

373

374 **Arthropod Taxa Identified from Fungus Garden Samples**

375 A total of six arthropod classifications were identified from *COI* sequences. All colony fungus
376 garden samples contained *Insecta* sequences. Only four samples contained sequences
377 identified to a lower taxonomic level than *Insecta*: *M. turrifex* colony 44's fungus garden
378 contained *Carabidae* (*Coleoptera*) and *Lepidoptera* sequences, *M. turrifex* colony 14's fungus
379 garden contained *Anomoea laticlavia* (*Coleoptera Chrysomelidae*) sequences, *M. turrifex* colony

380 20's fungus garden contained *Coleoptera* sequences, and *T. septentrionalis* colony 112's
381 fungus garden contained *Hemiptera* sequences.
382
383 **Discussion**
384 Dietary DNA techniques successfully identified plant and arthropod taxa present in the fungus
385 gardens of *T. septentrionalis* and *M. turrifex*. The dDNA analysis was particularly successful
386 with the *trnL* primer, as it identified a relatively large number of plant taxa, many of them to a
387 relatively high level of taxonomic resolution. These results could be particularly impactful
388 considering the literature deficit of specific plant taxa known to be foraged by non-leafcutting
389 fungus gardening ants. Only a handful of previous studies make any mention of specific foraged
390 plant taxa, with most only describing the general type of material the ants were collecting such
391 as flower petals or dead leaves [1,2,40-42,64–68].
392
393 Colony ant species appeared to be a significant factor in beta diversity metrics, but not alpha
394 diversity metrics, of fungal substrate plant taxa. Similarly, Colony pair appeared to be a
395 significant factor in both plant taxa beta diversity metrics and alpha diversity metrics. These
396 results suggest that diet preference is likely driven both by ant species-specific plant preference
397 and colony location-specific plant resource availability. The presence of dietary differences
398 between *T. septentrionalis* and *M. turrifex* colonies on our site appears similar to short-term
399 observations of dietary differences made on co-occurring *Atta* and *Acromyrmex* colonies
400 [33,34].
401
402 The plant taxa we identified from *T. septentrionalis* and *M. turrifex* fungus garden sequences
403 were consistent with the limited existing descriptions of forage material collected by these ant
404 species. One previous study observed *M. turrifex* collecting *Sorghum halepense* and *Solidago*
405 *sp.*, both of whose families, *Poaceae* and *Asteraceae* respectively, were present in our samples

406 [40]. Another previous study suggested that *T. septentrionalis* collects *Quercus* (*Fagaceae*),
407 which was also present in our samples [41].
408
409 If we include descriptions of known forage material from other higher agriculture non-leafcutting
410 ants, we find additional overlap between our identified plant taxa and that of previous studies.
411 *Lauraceae*, *Rosaceae*, *Rubiaceae*, *Poaceae*, and *Euphorbiaceae* are all plant families which
412 were identified in our samples, and are known to be collected by or, in the case of lab colonies,
413 accepted by higher agriculture non-leafcutting ants [64,66]. Leafcutting species like *Atta texana*
414 are known to forage on *Pinus* (*Pinaceae*) needles, but our study is the first published record of a
415 non-leafcutting species collecting *Pinus* [69]. The ants may be collecting pieces of male pine
416 cones, as this has been observed in the field and laboratory during early spring (March-April)
417 (JN Seal, unpublished observations).
418
419 A notable drawback of only using dDNA analysis on sampled fungus gardens is that dDNA
420 analysis alone cannot determine the state plant material was in when it was collected by the
421 ants. Some higher agriculture fungus gardeners outside of *Atta* and *Acromyrmex*, like *T.*
422 *septentrionalis*, are known to cut fresh vegetative material to use as fungus garden substrate,
423 while others do not [2,41]. In our study, we could not determine whether the detected plant
424 sequences originated from the ants foraging fresh cut plant material, plant detritus, or frass. We
425 also could not determine which part of the plant they collected (i.e. leaves, flowers, or sticks).
426 The inability to detect which state plant material was collected in could obscure differences in
427 foraging behavior between these two ant species. For example, one ant species may forage on
428 *Quercus* (*Fagaceae*) catkins, while a second ant species may forage caterpillar frass that
429 contains *Quercus* leaves. Similarly, one ant species may cut soft *Quercus* vegetation, whereas
430 a second ant species might only take dead vegetation that has fallen from the tree. In all of
431 these examples the ant's fungus gardens would contain *Quercus* sequences, but dDNA

432 analysis would be unable to differentiate the state the material was collected in. Additionally,
433 since the number of sequencing reads cannot be reliably used as a quantitative measure of the
434 biomass of digested material, we cannot use dDNA to determine if colonies are foraging
435 different amounts of the same material [6].
436
437 While our study establishes dDNA analysis as a potential tool for identification of fungus garden
438 substrate, we also identified several limitations that should be accounted for in future studies
439 attempting to use similar techniques. One issue was the low taxonomic resolution of some plant
440 sequences. While we were able to successfully identify 44 plant classifications in the fungus
441 gardens using our selected *trnL* primer, unidentified *Magnoliopsida* had the sixth highest
442 absolute read abundance post-rarefaction, indicating that a number of plant species may be
443 present in the fungus gardens that our *trnL* primer was unable to identify to an informative
444 taxonomic level. In future studies this could be mitigated, at least in part, by using multiple plant
445 primers designed for different target regions [23,70,71]. Another issue was the misidentification
446 of plant sequences at the species level. While some identified plant species, like *Desmodium*
447 *ciliare* (*Fabaceae*), are known to occur on our study site; other species, such as *Salix hukaoana*
448 (*Salicaceae*), are not (though other species of *Salix* are certainly in the general vicinity). These
449 errors were likely the result of our classifier misidentifying sequences as related taxa due to an
450 incomplete reference database. Ideally, future studies could construct a region-specific
451 taxonomic library of plant sequences to be used when training their classifier [72]. Barcoding
452 insect species present in a study site would be more difficult, as they are more mobile and can
453 inhabit above both and below ground areas during different life stages.
454
455 Dietary DNA analysis alone is not capable of distinguishing *trnL* sequences from plant material
456 foraged directly by the ants and *trnL* sequences from insect frass foraged by the ants. Since
457 *Trachymyrmex septentrionalis* and *M. turrifex* both readily forage insect frass containing plant

458 material, plant DNA sequences from foraged frass were potentially detected by the *trnL* primer
459 we used [13,40,42]. We had hoped to use the *COI* primer results to gain insight into what
460 arthropod taxa the ants were collecting frass from, but the primer performed too poorly to draw
461 any meaningful conclusions. Other studies attempting to determine *Lepidoptera* species from
462 their frass found that amounts of dry frass less than 5 mg may be too small to detect insect DNA
463 sequences [73]. This insect sequence detectability cutoff was likely higher in our samples due to
464 the physical and enzymatic breakdown of foraged material occurring in the fungus gardens,
465 making it difficult to detect frass-producing-insect DNA [2]. Another potential confounding issue
466 could be a decrease in PCR efficiency due to our use of a 1-step PCR approach versus a 2-step
467 PCR approach for sequencing (See Methods Bohmann et al. (2022) for a discussion on the
468 topic) [74].
469
470 Many of the issues we discussed can be mitigated by taking the limitations of dDNA analysis
471 into account when designing future studies. Our recommendations for future studies include: 1)
472 using multiple plant primers to maximize taxonomic coverage, 2) constructing a region-specific
473 taxonomic library of plant sequences, 3) sampling at several different time points to allow
474 detection of temporal changes in fungal substrate plant composition, and 4) incorporating a
475 supplemental observational component to gain an understanding of what state forage material
476 is being collected in. Overall, we have demonstrated the efficacy of using dDNA analysis as a
477 tool for the taxonomic identification of forage material in fungus-gardening ants, and as a
478 possible tool to detect dietary differences between species. Dietary DNA analysis offers an
479 opportunity to improve our understanding of plant taxa foraged by non-leafcutting fungus
480 gardening ants, and, with appropriate supporting methods, can be applied to gain new insight
481 into their ecology and life history.
482

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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.

Supplemental

Fig S1. The F-measure of our classifiers being used on their own training dataset.

References

1. Leal IR, Oliveira PS. Foraging ecology of attine ants in a Neotropical savanna: seasonal use of fungal substrate in the cerrado vegetation of Brazil. Insectes sociaux. 2000;47: 376–382. doi:10.1007/PL00001734

2. De Fine Licht HH, Boomsma JJ. Forage collection, substrate preparation, and diet composition in fungus-growing ants. Ecological Entomology. 2010;35: 259–269. doi:10.1111/j.1365-2311.2010.01193.x

3. Solomon SE, Rabeling C, Sosa-Calvo J, Lopes CT, Rodrigues A, Vasconcelos HL, et al. The molecular phylogenetics of *Trachymyrmex Forel* ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine' ant agriculture. Systematic entomology. 2019;44: 939–956. doi:10.1111/syen.12370

4. Schultz TR, Brady SG. Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences of the United States of America. 2008;105: 5435–5440. doi:10.1073/pnas.0711024105

5. De Fine Licht HH, Schiøtt M, Mueller UG, Boomsma JJ. Evolutionary transitions in enzyme

513 activity of ant fungus gardens. Evolution. 2010;64: 2055–2069. doi:10.1111/j.1558-
514 5646.2010.00948.x

515 6. Nielsen JM, Clare EL, Hayden B, Brett MT, Kratina P. Diet tracing in ecology: Method
516 comparison and selection. Methods Ecol Evol. 2018;9: 278–291. doi:10.1111/2041-
517 210X.12869

518 7. Sousa LL, Silva SM, Xavier R. DNA metabarcoding in diet studies: Unveiling ecological
519 aspects in aquatic and terrestrial ecosystems. Environmental DNA. 2019;1: 199–214.
520 doi:10.1002/edn3.27

521 8. Wulff JA, Kjeldgaard MK, Eubanks MD, Sword GA. From the bellies of babes: a larval-
522 based approach to ant molecular gut content analysis. Insectes sociaux. 2021;68: 245–
523 253. doi:10.1007/s00040-021-00822-8

524 9. Mintzer A. Foraging activity of the Mexican leafcutting ant *Atta mexicana* (F. Smith), in a
525 sonoran desert habitat (*Hymenoptera*, *Formicidae*). Insectes sociaux. 1979;26: 364–372.
526 doi:10.1007/BF02223555

527 10. Berish CW. Leaf-Cutting Ants (*Atta cephalotes*) Select Nitrogen-Rich Forage. American
528 Midland Naturalist. 1986;115: 268. doi:10.2307/2425863

529 11. Mintzer A. Diet of the Leafcutting Ant, *Atta Mexicana* (*Hymenoptera*: *Formicidae*), in a
530 Sonoran Desert Habitat. Journal of the Arizona-Nevada Academy of Science. 1995.

531 12. Weber NA. Gardening ants the attines. American Philosophical Society. 1972; 146.

532 13. Ando H, Mukai H, Komura T, Dewi T, Ando M, Isagi Y. Methodological trends and
533 perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding.
534 Environmental DNA. 2020;2: 391–406. doi:10.1002/edn3.117

535 14. Muilenburg VL, Goggin FL, Hebert SL, Jia L, Stephen FM. Ant predation on red oak borer
536 confirmed by field observation and molecular gut-content analysis. Agricultural and forest
537 entomology. 2008;10: 205–213. doi:10.1111/j.1461-9563.2008.00371.x

538 15. Penn HJ, Chapman EG, Harwood JD. Overcoming PCR Inhibition During DNA-Based Gut
539 Content Analysis of Ants. Environmental Entomology. 2016;45: 1255–1261.
540 doi:10.1093/ee/nvw090

541 16. Gadau J. DNA isolation from ants. Cold Spring Harbor Protocols. 2009;2009:
542 pdb.prot5245. doi:10.1101/pdb.prot5245

543 17. Holldobler B, Wilson EO. The Ants. 1st ed. Cambridge, Mass: Belknap Press: An Imprint of
544 Harvard University Press; 1990.

545 18. De Fine Licht HH, Boomsma JJ, Tunlid A. Symbiotic adaptations in the fungal cultivar of
546 leaf-cutting ants. Nature Communications. 2014;5: 5675. doi:10.1038/ncomms6675

547 19. Seal JN, Schiøtt M, Mueller UG. Ant-fungus species combinations engineer physiological
548 activity of fungus gardens. Journal of Experimental Biology. 2014;217: 2540–2547.
549 doi:10.1242/jeb.098483

550 20. Moller IE, De Fine Licht HH, Harholt J, Willats WGT, Boomsma JJ. The dynamics of plant
551 cell-wall polysaccharide decomposition in leaf-cutting ant fungus gardens. Plos One.
552 2011;6: e17506. doi:10.1371/journal.pone.0017506

553 21. Burgar JM, Murray DC, Craig MD, Haile J, Houston J, Stokes V, et al. Who's for dinner?
554 High-throughput sequencing reveals bat dietary differentiation in a biodiversity hotspot
555 where prey taxonomy is largely undescribed. Mol Ecol. 2014;23: 3605–3617.
556 doi:10.1111/mec.12531

557 22. Kartzinel TR, Chen PA, Coverdale TC, Erickson DL, Kress WJ, Kuzmina ML, et al. DNA
558 metabarcoding illuminates dietary niche partitioning by African large herbivores. Proc Natl
559 Acad Sci USA. 2015;112: 8019–8024. doi:10.1073/pnas.1503283112

560 23. Soininen EM, Gauthier G, Bilodeau F, Berteaux D, Gielly L, Taberlet P, et al. Highly
561 overlapping winter diet in two sympatric lemming species revealed by DNA metabarcoding.
562 PLoS ONE. 2015;10: e0115335. doi:10.1371/journal.pone.0115335

563 24. Sato JJ, Shimada T, Kyogoku D, Komura T, Uemura S, Saitoh T, et al. Dietary niche
564 partitioning between sympatric wood mouse species (*Muridae*: *Apodemus*) revealed by
565 DNA meta-barcoding analysis. J Mammal. 2018;99: 952–964.
566 doi:10.1093/jmammal/gyy063

567 25. Smith JA, Thomas AC, Levi T, Wang Y, Wilmers CC. Human activity reduces niche
568 partitioning among three widespread mesocarnivores. Oikos. 2018 [cited 21 Oct 2023].
569 doi:10.1111/oik.04592

570 26. Aizpurua O, Budinski I, Georgiakakis P, Gopalakrishnan S, Ibañez C, Mata V, et al.
571 Agriculture shapes the trophic niche of a bat preying on multiple pest arthropods across
572 Europe: Evidence from DNA metabarcoding. Mol Ecol. 2018;27: 815–825.
573 doi:10.1111/mec.14474

574 27. Komura T, Ando H, Horikoshi K, Suzuki H, Isagi Y. DNA barcoding reveals seasonal shifts
575 in diet and consumption of deep-sea fishes in wedge-tailed shearwaters. PLoS ONE.
576 2018;13: e0195385. doi:10.1371/journal.pone.0195385

577 28. Forsman AM, Hoenig BD, Gaspar SA, Fischer JD, Siegrist J, Fraser K. Evaluating the
578 impacts of metabarcoding primer selection on DNA characterization of diet in an aerial
579 insectivore, the Purple Martin. Ornithology. 2021 [cited 21 Oct 2023].
580 doi:10.1093/ornithology/ukab075

581 29. Palumbo F, Squartini A, Barcaccia G, Macolino S, Pornaro C, Pindo M, et al. A multi-
582 kingdom metabarcoding study on cattle grazing Alpine pastures discloses intra-seasonal
583 shifts in plant selection and faecal microbiota. Sci Rep. 2021;11: 889. doi:10.1038/s41598-
584 020-79474-w

585 30. Roffler GH, Allen JM, Massey A, Levi T. Metabarcoding of fecal DNA shows dietary
586 diversification in wolves substitutes for ungulates in an island archipelago. Ecosphere.
587 2021;12. doi:10.1002/ecs2.3297

588 31. Brun L, Schneider J, Carrió EM, Dongre P, Taberlet P, van de Waal, et al. Focal vs. fecal:
589 Seasonal variation in the diet of wild vervet monkeys from observational and DNA

590 metabarcoding data. Ecol Evol. 2022;12: e9358. doi:10.1002/ece3.9358

591 32. Wang B, Li Y, Zhang G, Yang J, Deng C, Hu H, et al. Seasonal variations in the plant diet
592 of the Chinese Monal revealed by fecal DNA metabarcoding analysis. Avian Res. 2022;13:
593 100034. doi:10.1016/j.avrs.2022.100034

594 33. Wetterer J. Foraging Ecology of the Leaf-Cutting Ant Acromyrmex Octospinosus in a Costa
595 Rican Rain Forest. Psyche. 1991;98: 361–371. doi:10.1155/1991/46737

596 34. Kooij PW, Liberti J, Giampoudakis K, Schiøtt M, Boomsma JJ. Differences in forage-
597 acquisition and fungal enzyme activity contribute to niche segregation in Panamanian leaf-
598 cutting ants. Plos One. 2014;9: e94284. doi:10.1371/journal.pone.0094284

599 35. Seal JN, Brown L, Ontiveros C, Thiebaud J, Mueller UG. Gone to Texas: phylogeography
600 of two (*Hymenoptera*: *Formicidae*) species along the southeastern coastal plain of North
601 America. Biological Journal of the Linnean Society. 2015;114: 689–698.
602 doi:https://doi.org/10.1111/bij.12426

603 36. Senula SF, Scavetta JT, Banta JA, Mueller UG, Seal JN, Kellner K. Potential Distribution of
604 Six North American Higher-Attine Fungus-Farming Ant (*Hymenoptera*: *Formicidae*)
605 Species. Journal of Insect Science (Online). 2019;19. doi:10.1093/jisesa/iez118

606 37. Rabeling C, Cover SP, Johnson RA, Mueller, G U. A review of the North American species
607 of the fungus-gardening ant genus *Trachymyrmex (Hymenoptera*: *Formicidae*). Zenodo.
608 2007 [cited 7 Sep 2023]. doi:10.5281/zenodo.180014

609 38. Sanchez-Peña SR. Some Fungus-Growing Ants (*Hymenoptera*: *Formicidae*) from
610 Northeastern Mexico. Florida Entomologist. 2010;93: 501–504. doi:10.1653/024.093.0404

611 39. Wheeler WM. The fungus-growing ants of North America. 1907.

612 40. Waller DA. Foraging Behavior of *Trachymyrmex turrifex* Wheeler (*Formicidae*: *Attini*).
613 Source: The Southwestern Naturalist. 1989;34: 271–275.

614 41. Seal JN, Tschinkel WR. Complexity in an obligate mutualism: do fungus-gardening ants
615 know what makes their garden grow? Behavioral Ecology and Sociobiology. 2007;61:
616 1151–1160. doi:10.1007/s00265-006-0328-4

617 42. Seal JN, Tschinkel WR. Food limitation in the fungus-gardening ant, *Trachymyrmex*
618 *septentrionalis*. Ecological Entomology. 2008;33: 597–607. doi:10.1111/j.1365-
619 2311.2008.01010.x

620 43. Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, et al. Power and
621 limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Res.
622 2007;35: e14. doi:10.1093/nar/gkl938

623 44. Razgour O, Clare EL, Zeale MRK, Hanmer J, Schnell IB, Rasmussen M, et al. High-
624 throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat
625 species. Ecol Evol. 2011;1: 556–570. doi:10.1002/ece3.49

626 45. Galanti L, Shasha D, Gunsalus KC. Pheniqs 2.0: accurate, high-performance Bayesian

627 decoding and confidence estimation for combinatorial barcode indexing. BMC
628 Bioinformatics. 2021;22: 359. doi:10.1186/s12859-021-04267-5

629 46. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al.
630 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.
631 Nature Biotechnology. 2019;37: 852–857. doi:10.1038/s41587-019-0209-9

632 47. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-
633 resolution sample inference from Illumina amplicon data. Nature Methods. 2016;13: 581–
634 583. doi:10.1038/nmeth.3869

635 48. Robeson MS, O'Rourke DR, Kaehler BD, Ziemski M, Dillon MR, Foster JT, et al.
636 RESCRIPt: Reproducible sequence taxonomy reference database management. PLoS
637 Computational Biology. 2021;17: e1009581. doi:10.1371/journal.pcbi.1009581

638 49. Robeson M. Using RESCRIPt's "extract-seq-segments" to extract reference sequences
639 without PCR primer pairs. 2022. Available: https://forum.qiime2.org/t/using-rescripts-
640 extract-seq-segments-to-extract-reference-sequences-without-pcr-primer-pairs/23618

641 50. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and
642 graphics of microbiome census data. Plos One. 2013;8: e61217.
643 doi:10.1371/journal.pone.0061217

644 51. Bisanz JE. qiime2R: Importing QIIME2 artifacts and associated data into R sessions. 2018.
645 Available: https://github.com/jbisanz/{qiime2R}

646 52. Team RC. R: A Language and Environment for Statistical Computing. Vienna, Austria;
647 2023. Available: https://www.R-project.org/

648 53. Jin Y, Qian H. V.PhyloMaker2: An updated and enlarged R package that can generate
649 very large phylogenies for vascular plants. Plant Diversity. 2022;44: 335–339.
650 doi:10.1016/j.pld.2022.05.005

651 54. Jin Y, Qian H. U.PhyloMaker: An R package that can generate large phylogenetic trees for
652 plants and animals. Plant Diversity. 2023;45: 347–352. doi:10.1016/j.pld.2022.12.007

653 55. Paradis E, Schliep K. ape 5.0: an environment for modern phylogenetics and evolutionary
654 analyses in R. Schwartz R, editor. Bioinformatics. 2019;35: 526–528.
655 doi:10.1093/bioinformatics/bty633

656 56. Shetty S, Person P, Person P. biomeUtils: Utilities for microbiome analytics. 2024.

657 57. Deagle BE, Thomas AC, Shaffer AK, Trites AW, Jarman SN. Quantifying sequence
658 proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: which
659 counts count? Mol Ecol Resour. 2013;13: 620–633. doi:10.1111/1755-0998.12103

660 58. Clare EL, Chain FJJ, Littlefair JE, Cristescu ME. The effects of parameter choice on
661 defining molecular operational taxonomic units and resulting ecological analyses of
662 metabarcoding data. Genome. 2016;59: 981–990. doi:10.1139/gen-2015-0184

663 59. Hambäck PA, Weingartner E, Dalén L, Wirta H, Roslin T. Spatial subsidies in spider diets

vary with shoreline structure: Complementary evidence from molecular diet analysis and stable isotopes. Ecology and Evolution. 2016;6: 8431–8439. doi:10.1002/ece3.2536

60. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. vegan: Community Ecology Package. 2024. Available: https://vegandevs.github.io/vegan/

61. Yu G, Smith DK, Zhu H, Guan Y, Lam TT. Ggtree: An R Package for Visualization and Annotation of Phylogenetic Trees With Their Covariates and Other Associated Data. Methods in Ecology and Evolution. 2016;8: 28–36. doi:10.1111/2041-210x.12628

62. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. Welcome to the Tidyverse. JOSS. 2019;4: 1686. doi:10.21105/joss.01686

63. Slowikowski K. ggrepel: Automatically Position Non-Overlapping Text Labels with "ggplot2." 2024. Available: https://{CRAN}.R-project.org/package=ggrepel

64. Jaffé K, Villegas G. On the communication systems of the fungus-growing ant *trachymyrmex urichi*. Insectes sociaux. 1985;32: 257–274. doi:10.1007/BF02224915

65. Kaspari M. Removal of seeds from Neotropical frugivore droppings. Oecologia. 1993;95: 81–88. doi:10.1007/BF00649510

66. Leal LR, Oliveira PS. Interactions between Fungus-Growing Ants (*Attini*), Fruits and Seeds in Cerrado Vegetation in Southeast Brazil'. 1998 pp. 170–178.

67. Pizo MA, Oliveira PS. The Use of Fruits and Seeds by Ants in the Atlantic Forest of Southeast Brazil [1]. Biotropica. 2000;32: 851–861. doi:10.1111/j.1744-7429.2000.tb00623.x

68. Moreira AA, Bueno FC, Oliveira CG, Diniz EA, Garcez DL, Campos MCG, et al. Foraging activity of *Trachymyrmex fuscus* (*hymenoptera*: *formicidae*) in a degraded area. Sociobiology. 2007;50: 409–417.

69. Kulhavy D, Smith L, Ross W. Impact of the Texas Leaf-Cutting Ant (*Atta texana* (Buckley)) (Order *Hymenoptera*, Family *Formicidae*) on a Forested Landscape. 2001.

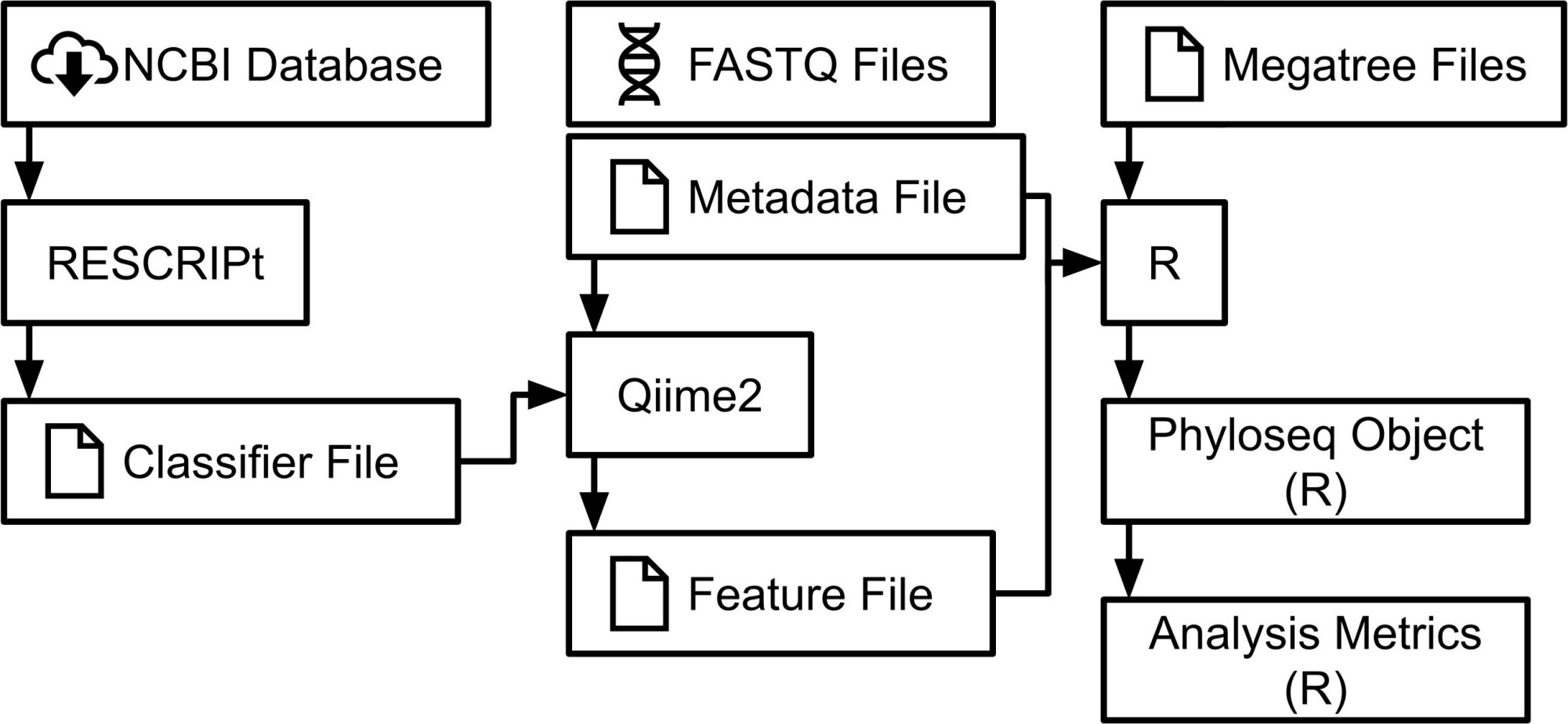
70. Hibert F, Taberlet P, Chave J, Scotti-Saintagne C, Sabatier D, Richard-Hansen C. Unveiling the diet of elusive rainforest herbivores in next generation sequencing era? The tapir as a case study. PLoS ONE. 2013;8: e60799. doi:10.1371/journal.pone.0060799

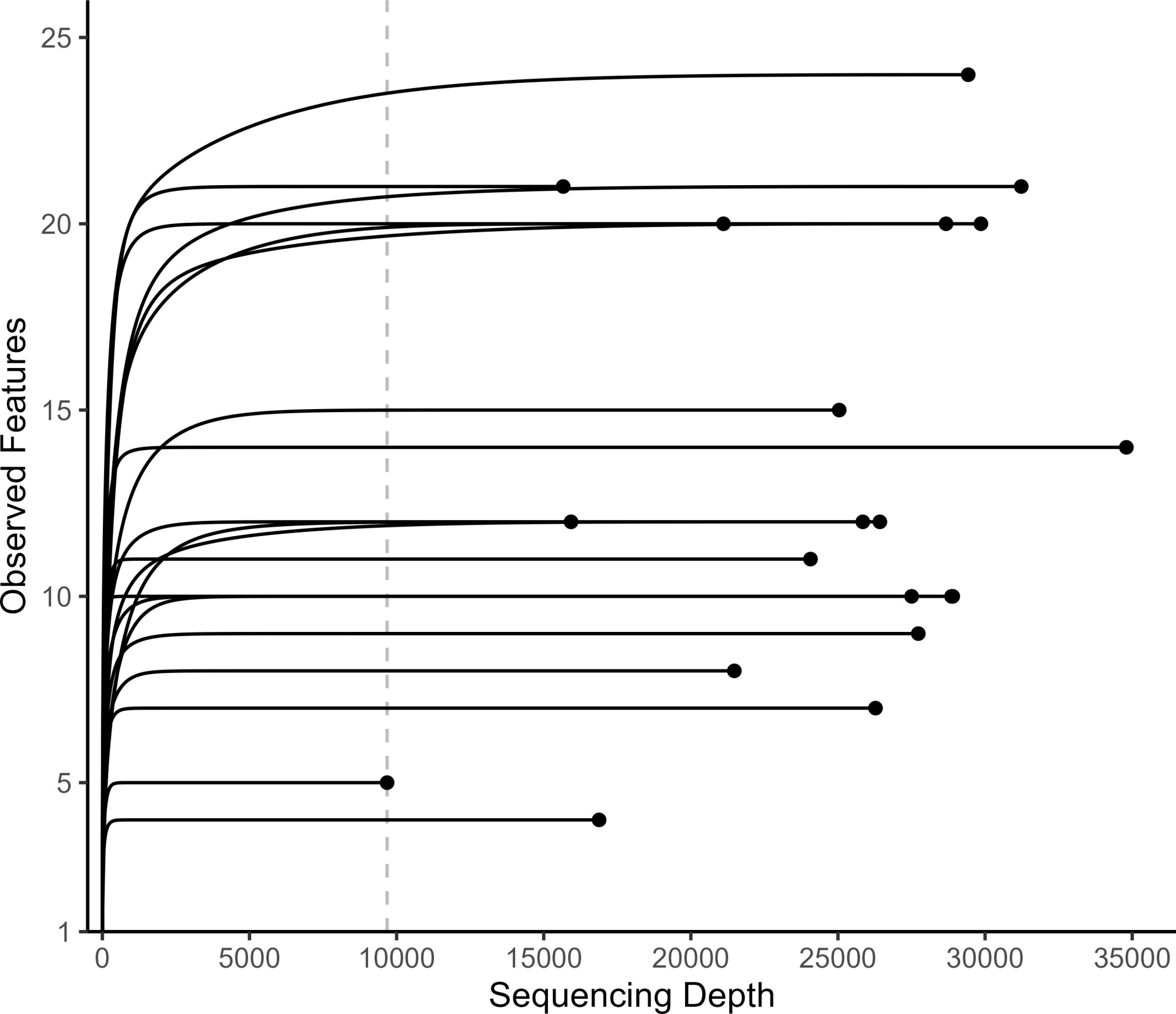
71. Clare EL. Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications. Evol Appl. 2014;7: 1144–1157. doi:10.1111/eva.12225

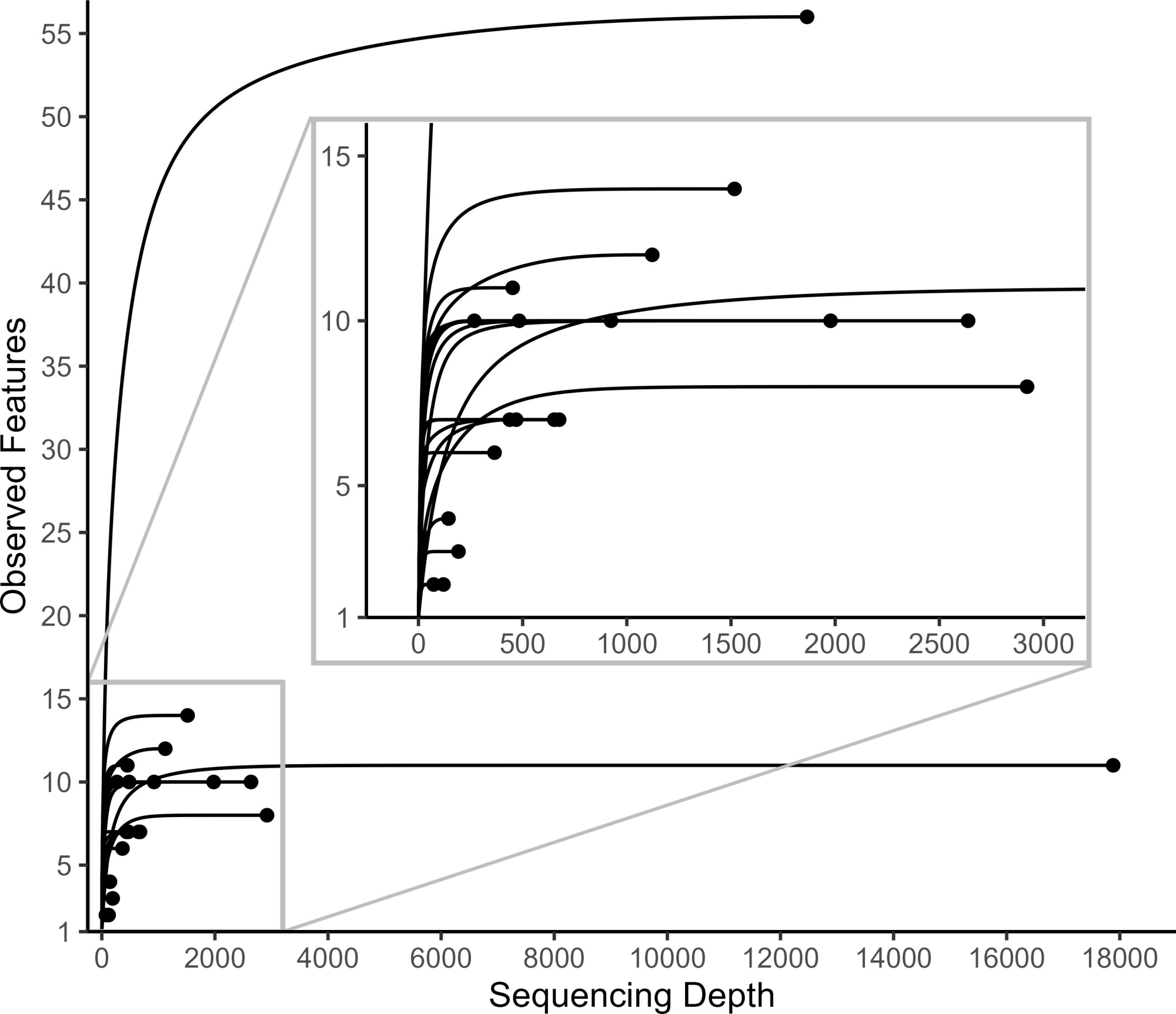
72. Furlan EM, Davis J, Duncan RP. Identifying error and accurately interpreting environmental DNA metabarcoding results: A case study to detect vertebrates at arid zone waterholes. Molecular Ecology Resources. 2020;20: 1259–1276. doi:10.1111/1755-0998.13170

73. Rytkönen S, Vesterinen EJ, Westerduin C, Leviäkangas T, Vatka E, Mutanen M, et al. From feces to data: A metabarcoding method for analyzing consumed and available prey in a bird-insect food web. Ecol Evol. 2019;9: 631–639. doi:10.1002/ece3.4787

700 74. Bohmann K, Elbrecht V, Carøe C, Bista I, Leese F, Bunce M, et al. Strategies for sample
701 labelling and library preparation in DNA metabarcoding studies. Molecular ecology
702 resources. 2022;22: 1231-1246. doi:10.1111/1755-0998.13512
703


NCBI Database
FASTQ Files
Megatree Files
RESCRIPt
Metadata File
R
Qiime2
Classifier File
Phyloseq Object (R)
Feature File
Analysis Metrics (R)


Observed Features
25
20
15
10
5
1
0
5000
10000
15000
20000
25000
30000
35000
Sequencing Depth


Observed Features
Sequencing Depth
55
50
45
40
35
30
25
20
15
10
5
1
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
15
10
5
1
0
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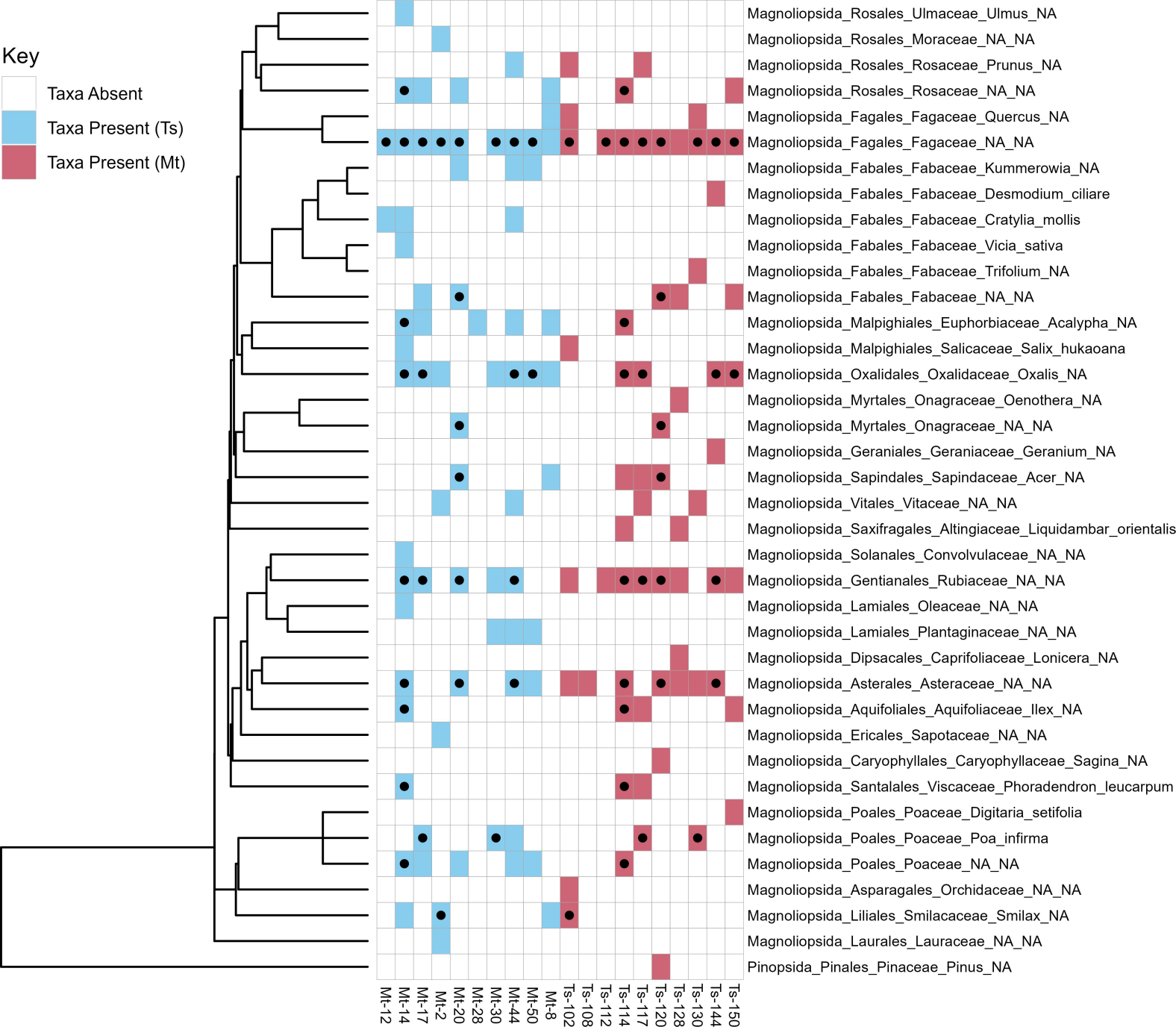
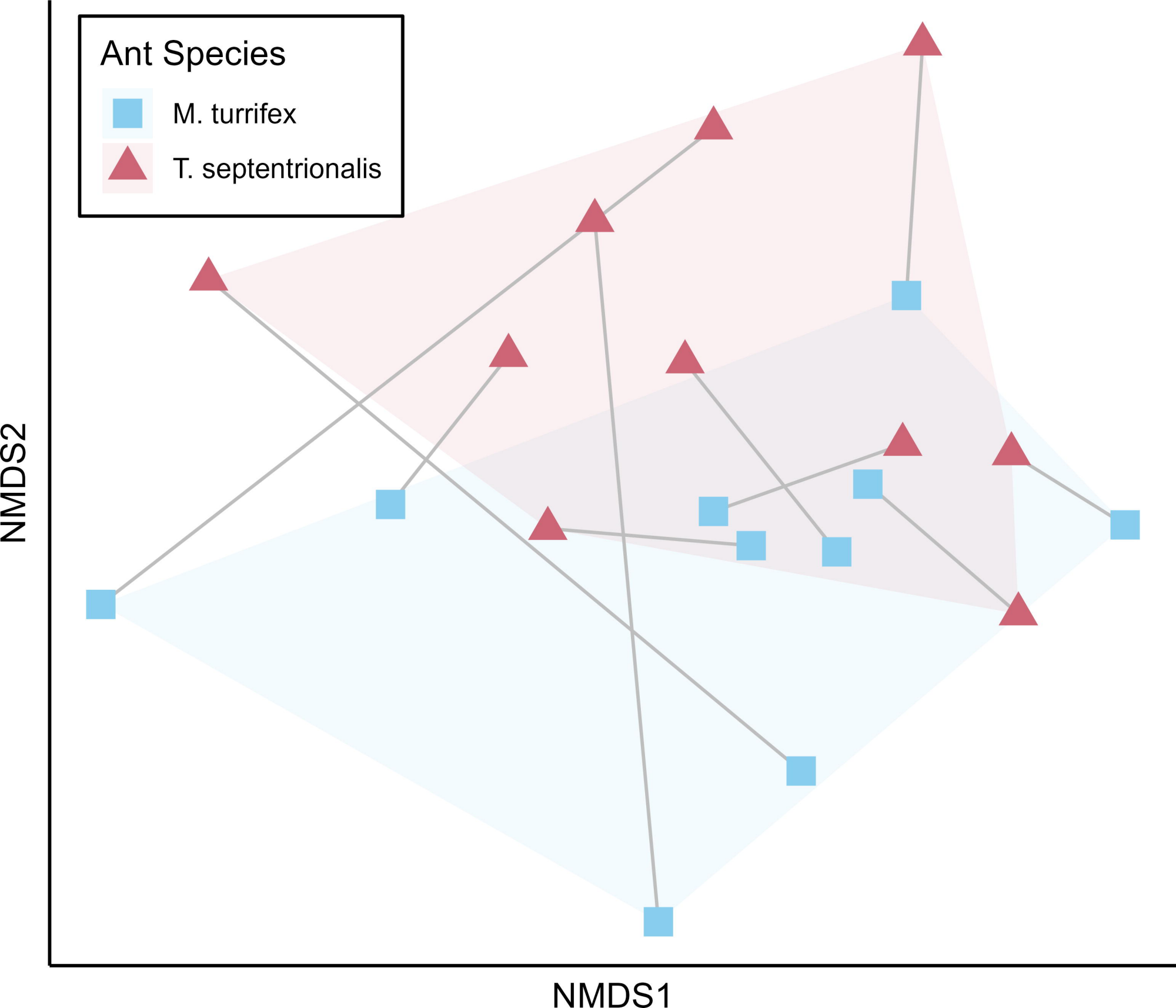
1000
1500
2000
2500
3000


Key
Taxa Absent
Taxa Present (Ts)
Taxa Present (Mt)
Magnoliopsida_Rosales_Ulmaceae_Ulmus_NA
Magnoliopsida_Rosales_Moraceae_NA_NA
Magnoliopsida_Rosales_Rosaceae_Prunus_NA
Magnoliopsida_Rosales_Rosaceae_NA_NA
Magnoliopsida_Fagales_Fagaceae_Quercus_NA
Magnoliopsida_Fagales_Fagaceae_NA_NA
Magnoliopsida_Fabales_Fabaceae_Kummerowia_NA
Magnoliopsida_Fabales_Fabaceae_Desmodium_ciliare
Magnoliopsida_Fabales_Fabaceae_Cratylia_mollis
Magnoliopsida_Fabales_Fabaceae_Vicia_sativa
Magnoliopsida_Fabales_Fabaceae_Trifolium_NA
Magnoliopsida_Fabales_Fabaceae_NA_NA
Magnoliopsida_Malpighiales_Euphorbiaceae_Acalypha_NA
Magnoliopsida_Malpighiales_Salicaceae_Salix_hukaoana
Magnoliopsida_Oxalidales_Oxalidaceae_Oxalis_NA
Magnoliopsida_Myrtales_Onagraceae_Oenothera_NA
Magnoliopsida_Myrtales_Onagraceae_NA_NA
Magnoliopsida_Geraniales_Geraniaceae_Geranium_NA
Magnoliopsida_Sapindales_Sapindaceae_Acer_NA
Magnoliopsida_Vitales_Vitaceae_NA_NA
Magnoliopsida_Saxifragales_Altingiaceae_Liquidambar_orientalis
Magnoliopsida_Solanales_Convolvulaceae_NA_NA
Magnoliopsida_Gentianales_Rubiaceae_NA_NA
Magnoliopsida_Lamiales_Oleaceae_NA_NA
Magnoliopsida_Lamiales_Plantaginaceae_NA_NA
Magnoliopsida_Dipsacales_Caprifoliaceae_Lonicera_NA
Magnoliopsida_Asterales_Asteraceae_NA_NA
Magnoliopsida_Aquifoliales_Aquifoliaceae_Ilex_NA
Magnoliopsida_Ericales_Sapotaceae_NA_NA
Magnoliopsida_Caryophyllales_Caryophyllaceae_Sagina_NA
Magnoliopsida_Santalales_Viscaceae_Phoradendron_leucarpum
Magnoliopsida_Poales_Poaceae_Digitaria_setifolia
Magnoliopsida_Poales_Poaceae_Poa_infirma
Magnoliopsida_Poales_Poaceae_NA_NA
Magnoliopsida_Asparagales_Orchidaceae_NA_NA
Magnoliopsida_Liliales_Smilacaceae_Smilax_NA
Magnoliopsida_Laurales_Lauraceae_NA_NA
Pinopsida_Pinales_Pinaceae_Pinus_NA
Mt-12
Mt-14
Mt-17
Mt-2
Mt-20
Mt-28
Mt-30
Mt-44
Mt-50
Mt-8
Ts-102
Ts-108
Ts-112
Ts-114
Ts-117
Ts-120
Ts-128
Ts-130
Ts-144
Ts-150


Ant Species
M. turrifex
T. septentrionalis
NMDS2
NMDS1