Metagenomics reveals diet-specific specialization in fungus gardens of grass- and dicot-cutter ants

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Abstract

Leaf-cutter ants are dominant herbivores in the Neotropics. While most leaf-cutter ant species cut dicots to incorporate into their fungus gardens, some species specialize on grasses. Here we examine the bacterial community associated with the fungus gardens of grass- and dicot-cutter ants to elucidate the potential role of bacteria in leaf-cutter ant substrate specialization. We sequenced the metagenomes of 12 *Atta* fungus gardens, across four species of ants, with a total of 5.316 Gbp of sequence data. We show that community composition was significantly different between dicot- and grass-cutter ants, with grass-cutter ant fungus gardens having significantly lower diversity and a significantly higher abundance of *Pantoea*, the most abundant genus overall. Reflecting this difference in community composition, the bacterial functional profiles between the fungus gardens are significantly different. Specifically, grass-cutter ant fungus garden metagenomes are particularly enriched for genes responsible for amino acid, siderophore, and terpenoid biosynthesis while dicot-cutter ant fungus gardens metagenomes are enriched in genes involved in membrane transport. Our results suggest that bacteria in leaf-cutter ant fungus gardens aid in nutrient supplementation, a function especially relevant for the fungus gardens of ants that forage grass, a plant source relatively lower in nutrient value.

Introduction

Understanding the role of microbial symbionts in aiding nutrient acquisition is fundamental to understanding the biology of herbivores. Most herbivores host microbial symbionts that serve as an interface between them and the plants that they consume. These microbes can compensate for the hosts' lack of physiological capacity to obtain energy and nutrients from plants (Hansen & Moran 2013). Herbivore microbial symbionts, often residing in the guts of animals, have been implicated in aiding plant biomass breakdown (Talbot 1977; Kudo 2009; Hess *et al.* 2011; Adams *et al.* 2011), plant defense compound remediation (Wang *et al.* 2010; Adams *et al.* 2013; Boone *et al.* 2013), and nutrient supplementation (Warnecke *et al.* 2007; Hansen & Moran 2011; LeBlanc *et al.* 2013). Microbial communities differ between hosts that specialize on different substrates (Muegge *et al.* 2011) and changes in these communities and their functional capacity are integral to their hosts' transition to utilizing novel substrates (Delsuc *et al.* 2013; Kohl *et al.* 2014; Li *et al.* 2014; Hammer & Bowers 2015; Kohl *et al.* 2016).

Leaf-cutter ants represent a paradigmatic example of the microbial mediation of herbivory. They are dominant herbivores in the Neotropics, consuming an estimated 17% of foliar biomass in the systems in which they live (Costa *et al.* 2008). These ants have a significant impact on their surrounding ecosystems, due to the volume of plant biomass they consume and soil that they excavate in building their underground colonies (Fowler *et al.* 1986; Moutinho *et al.* 2003; Gutiérrez & Jones 2006; Herz *et al.* 2007; Costa *et al.* 2008). Like other metazoans, leaf-cutter ants lack the capacity to break down recalcitrant plant material. Instead, they gain access to the nutrients in plant biomass by farming a fungus, *Leucoagaricus gongylophorus*, which serves as an external gut that enzymatically breaks down recalcitrant biomass in the leaf material that the ants forage (Nagamoto *et al.* 2011; Kooij *et al.* 2011;

Suen *et al.* 2011a; Grell *et al.* 2013; Aylward *et al.* 2013; Khadempour *et al.* 2016). *Leucoagaricus gongylophorus* produces gongylidia, specialized hyphal swellings that contain an abundance of sugars and lipids, that the ants consume and feed to larvae (Bass & Cherrett 1995; North *et al.* 1997).

Recent work has revealed that a community of bacteria reside within leaf-cutter ant fungus gardens (Suen *et al.* 2010; Aylward *et al.* 2012; Moreira-Soto *et al.* 2017). These communities were dominated by Gammaproteobacteria, and consistently contained strains of *Pseudomonas*, *Enterobacter* and either *Rahnella* or *Pantoea* and were consistent with communities of bacteria associated with other fungus-farming insects (Aylward *et al.* 2014). Some garden bacteria are vertically transmitted, as they are present in the fungus pellets that queens use to establish new fungus gardens (Moreira-Soto *et al.* 2017). The community consistency and their vertical transmission, suggest that the bacterial communities are important to the fitness of their hosts. One study, by Pinto-Tomás *et al.* (2009) showed that *Pantoea* and *Klebsiella* bacteria fix nitrogen that supplements the ant diet, which is important for a strict herbivorous system. Nevertheless, the functional role of most garden bacteria remains unknown.

While most leaf-cutter ants use dicots, at least three species of *Atta* are specialized on cutting grass, and another three species cut both grasses and dicots (Fowler *et al.* 1986). All previous studies on the microbial community in leaf-cutter ant fungus gardens have been focused on dicot-cutting ants, likely because dicot-cutters are more common and grass-cutter ants are notoriously difficult to maintain in the lab (Nagamoto *et al.* 2009). In this study, we compare the bacterial communities of fungus gardens from ants that cut grass and dicots. Given that grasses and dicots differ in terms of the cell wall composition (Ding & Himmel 2008; Popper & Tuohy 2010), plant defense compounds (Wetterer 1994; Mariaca *et al.* 1997)

and nutrient availability (Mattson 1980; Winkler & Herbst 2004), we hypothesize that the bacterial community in these fungus gardens will differ in terms of community composition and functional capacity, in response to the different composition of the substrates the ants incorporate into their gardens. To address this, we collected fungus gardens from grass- and dicot-cutter ants and obtained their metagenomes using Illumina sequencing. We analyzed the bacterial community in terms of its taxonomic composition and its functional capacity. We also conducted analyses on the fungus gardens to determine their plant composition, their nutritional composition and their plant defense compound contents.

Methods

Collection of fungus garden

Fungus gardens were collected on the campuses of the University of São Paulo (USP) in Ribeirão Preto, SP, Brazil and the State University of São Paulo (UNESP) in Botucatu, SP, Brazil. Collection dates and GPS coordinates are listed in Table 3.1. We collected fungus gardens from four species of *Atta* leaf-cutter ants: *A. bisphaerica* and *A. capiguara*, which both specialize on grass, *A. laevigata*, which cuts both grasses and dicots, and *A. sexdens*, a dicot-cutter ant (Fowler *et al.* 1986).

To collect the fungus gardens, we identified the ant species by worker morphology then followed the entrance tunnel by digging until we found a fungus garden. Care was taken to enter fungus garden chambers from the side, to avoid damaging the garden with digging tools and to avoid contamination with surrounding soil. Fungus gardens were transported to the laboratory and were aseptically transferred into 50 mL conical tubes. The majority of worker ants were removed from the fungus garden material before being transferred to the tubes. In order to further reduce the chance of soil contamination, only intact fungus garden from the central region of the fungal mass was included in the tubes. Once filled, the tubes

were frozen in liquid nitrogen and stored at -80°C. At least six 50 mL conical tubes were filled from each colony. For each colony, four tubes were used for metagenomics, one tube was used for gas chromatography, and one tube was used for iron content measurements.

DNA extraction

To target the bacteria in the fungus gardens, DNA was extracted by first using a differential centrifugation method (Aylward *et al.* 2012). PBS buffer with 1% tween 80 was added to the tubes and they were shaken for 30 min on a vortex. They were then kept at 4°C for 30 min so that large particles would settle. The liquid portion was decanted and passed through a 40 μm filter. The remaining leaf material from the fungus gardens was photographed after the differential centrifugation, to demonstrate the difference in leaf material consistency (Figure 1). The filtrate was centrifuged for 30 min at 4°C, after which a bacterial cell pellet was formed and the liquid was removed. This process was repeated with the original fungus garden tube. For each fungus garden, cell pellets from four tubes were combined and the DNA was extracted using the Qiagen Plant DNA Extraction Maxi Kit (Qiagen, Hilden, Germany).

DNA sequencing and assembly

All metagenomic sequencing was conducted at the Joint Genome Institute in Walnut Creek, CA. Since some of the DNA concentrations were too low for standard library prep, a low-input prep was completed for all of the samples. Sequencing was performed on an Illumina HiSeq-2500 platform (2 x 151 bp). BBDuk adapter trimming (Bushnell 2017) was used to remove known Illumina adapters. The reads were then processed using BBDuk filtering and trimming. Read ends were trimmed where quality values were less than 12. Read pairs containing more than three ambiguous bases, or with quality scores (before trimming) averaging less than three over the read, or length under 51 bp after trimming, as

well as reads matching Illumina artifact, spike-ins or phiX were discarded. Trimmed, screened, paired-end Illumina reads were assembled using the megahit assembler using with the "--k-list 23,43,63,83,103,123" option. Functional annotation and taxonomic classification were performed using the Integrated Microbial Genomes pipeline.

Plant genus richness

To determine the richness of plant substrate integrated in the fungus gardens of the ants, we used JGI's Integrated Microbial Genomes and Microbiomes (IMG) database "find gene" function to retrieve all genes annotated as *MatK* from the dataset. *MatK* is a widely used chloroplast plant DNA barcode (Hollingsworth *et al.* 2011). Retrieved *MatK* sequences for each metagenome were identified using BLAST. To ensure consistent and reliable certainty with the identified plants, we identified all sequences to the genus level. Because most of the plant biomass was removed from samples before DNA extraction only presence/absence of genera were considered, not abundance.

Bacterial taxonomic analysis

Abundance of bacterial groups (phyla and genera) were determined based on the IMG Phylogenetic Distribution tool, which is part of JGI's standard operating procedure (Huntemann *et al.* 2016). Briefly, IMG uses USEARCH (Edgar 2010) to compare metagenome gene sequences to all identified genomes in their database. One top USEARCH hit per gene is used to assign phylogenetic lineage. To determine relative abundance of bacterial taxonomic groups within each sample, we used the PhyloDist raw data from IMG and first removed all gene sequences that were identified as Eukaryote or Virus. We then matched the PhyloDist data to the gene counts for each gene and normalized them to the total number of genes from Bacteria and Archaea. We used the relative abundances of each phylum and genus to run an non-metric multidimensional analysis (NMDS) using a Bray-

Curtis dissimilarity index with the vegan package in the R statistical programming environment (Oksanen *et al.* 2013; R Core Team 2013). Also using the vegan package, we used ANOSIM and PERMANOVA to determine if groups (grass-cutters vs. dicot-cutters) were significantly different, and we used the Shannon diversity index to compare the diversity of each sample by bacterial genus. To test whether specific genera have significantly different relative abundances between grass- and dicot-cutter ant fungus gardens, we used DESeq2 in the R statistical programming environment (Love *et al.* 2014). Since DESeq2 requires inputs to be integers, we used number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.*).

Bacterial functional analysis

In order to make functional comparisons of the bacteria in grass- and dicot-cutter fungus gardens, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations of the metagenomes through IMG's KEGG Orthology (KO) pipeline, which is part of JGI's standard operating procedure (Huntemann *et al.* 2016). Briefly, genes were associated with KO terms (Kanehisa *et al.* 2014) based on USEARCH 6.0.294 results (Edgar 2010) and were filtered for minimum identity matches and gene sequence coverage. For an overall comparison of functional differences between the fungus gardens, we used the same ordination and statistical methods as for bacterial genus abundance. As with genus group differences, we used DESeq2 to determine what genes are significantly enriched between grass- and dicot-cutter ant fungus gardens, with number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.*).

Iron content

Separate 50 mL tubes of fungus garden material, from the same colonies as above, were used for determination of iron content. All ants were removed from fungus garden

then the remaining material was analyzed at the UW Soil and Forage Lab in Marshfield, WI, using standard methods. Briefly, total iron content was determined by first digesting the fungus garden material in nitric acid/peroxide then analyzing by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Fassel & Kniseley 1974).

Results

Metagenomic statistics

A summary of metagenome statistics is presented in (Table 2). A total of 5.316 Gbp of assembled sequence data was produced in this study, with an average of 443 Mbp per metagenome. The smallest metagenome was from the grass-cutter colony *A. capiguara* 1 at 148.7 Mbp, and the largest metagenome was from the dicot-cutter colony *A. sexdens* 2 at 812.9 Mbp. Maximum scaffold lengths ranged from 61.96 Kbp to 701.42 Kbp, with an average maximum scaffold length of 266.6 Kbp. Between 91.63% and 99.31% of reads were aligned.

Bacterial taxonomic analysis

Proteobacteria (70-99%) were the most abundant bacterial phylum detected in the fungus gardens of *Atta* spp., followed by Actinobacteria (0.13-24%) and Firmicutes (0.096-2.4%) (Supplemental Figure 1). Between fungus gardens, genus-level comparisons showed greater variability than phylum-level comparisons (Figure 2, Figure 3). Overall, *Pantoea* was the most abundant genus in all the fungus gardens (average 37%), followed by *Pseudomonas* (average 17%). The abundance of these two genera was especially pronounced in the grass-cutter ant fungus gardens, where *Pantoea* and *Pseudomonas* averaged 45% and 28%, respectively. The high relative abundance of these two genera contributed to a lower overall diversity in the grass-cutter ant gardens (Shannon diversity index of 1.20-2.44) (Figure 3). While *Pantoea* and *Pseudomonas* were still abundant in fungus gardens of the dicot-cutter ants,

A. laevigata and *A. sexdens*, it accounted for a lower proportion (28% and 6.5%, respectively) of the bacteria in these more diverse gardens (Shannon diversity index of 2.80-4.67). Other dominant bacterial genera included *Enterobacter, Burkholderia, Erwinia, Emticicia, Serratia* and *Klebsiella*. DESeq2 analysis revealed that six bacterial genera (*Entoplasma*, *Flavobacterium*, *Mesoplasma*, *Pantoea*, *Pseudomonas*, and *Spiroplasma*) were significantly different in relative abundance between the fungus gardens. They were all more abundant in the grass-cutter ant fungus gardens.

Bacterial functional analysis

Overall, we found significant differences in the predicted bacterial community functional profiles between grass- and dicot-cutter ant fungus gardens (Figure 4). All individual bacterial genes that were significantly different between grass- and dicot-cutter ant fungus gardens are listed in Supplemental Table 1. In total, 514 predicted bacterial genes were significantly enriched in one group or another, with 313 and 201 genes significantly enriched in grass- and dicot-cutter ant gardens, respectively (Supplemental Table 2, Supplemental Figures 4-6). Grass-cutter ant fungus gardens were enriched for amino acid biosynthesis genes for phenylalanine, tryptophan, tyrosine, histidine, arginine, lysine, cysteine, methionine, glycine, serine and threonine. They were also significantly enriched in terpenoid and siderophore biosynthesis genes (Figure 5) and had a significantly higher abundance of a gene in the nitrogen fixation pathway, nitrogenase molybdenum-iron protein beta chain (Supplementary Table 2). Dicot-cutter ant fungus gardens were particularly enriched in membrane transport genes (Figure 5).

Plant taxonomy and consistency

The incorporated plant material was markedly different in consistency between the fungus gardens. *Atta bisphaerica* and *A. capiguara* gardens both contained material that was

clearly grass, which was not mulched (Figure 1). In contrast, the leaf material in the fungus gardens of *A. laevigata* and *A. sexdens* was mulched to the point of being unrecognizable as plant material (Figure 1). We detected 68 plant species based on the *MatK* gene query in the metagenomes, from 40 genera and 15 families (Table 3). The fungus gardens of dicot-cutter ants had a significantly higher richness of plant genera than those of grass-cutter ants (ANOVA F=9.14, p=0.0128). As expected, the grass-cutter ant fungus gardens all contained grass (*Paspalum,* Poaceae). The dicot-cutter ant fungus gardens contained more genera and families of plants, which were mostly dicots, but three of these fungus gardens also contained some grass (Table 3).

Iron content

The iron content of the fungus gardens is displayed in Figure 6. The grass-cutter ant fungus gardens have lower amounts of iron than the dicot-cutter ant fungus, but this difference is not significant due to the high variability between *A. sexdens* gardens.

Discussion

Understanding how microbial symbiont communities change in relation to host substrate specialization can help inform on animal diet specialization and evolutionary transitions to utilizing novel substrates. *Atta* ants provide a relatively unique opportunity to examine a group of closely related herbivores that have transitioned from specialization on dicots to grasses. Dicots and grasses differ in terms of their cell wall composition, nutrient content and plant defense compounds. Here, using metagenomic sequencing, we examine this transition in the bacterial community in the fungus gardens of ants that are specialized on these different substrates. The results of this study demonstrate that the bacterial community differs depending on type of substrate and likely facilitates the ants' ability to

specialize on grasses, which represents a lower quality of substrate on which to grow their fungal crop.

If bacteria in fungus gardens are responsible for the breakdown of recalcitrant plant biomass, which is found in plant cell walls, we expect that the bacterial communities in the two ant groups examined here would be differentially enriched in the genes necessary for plant biomass breakdown. Grasses have a unique cell wall structure, containing (1→3),(1→4)-β-D-glucan chains and silica, neither of which are present in dicots (Popper & Tuohy 2010). In other systems specialized on grass biomass breakdown, the microbes responsible for this produce specialized enzymes (King *et al.* 2011) and have genomes that are adapted for this function (Wolfe *et al.* 2012). Since we do not observe any changes in abundance of these genes between these two systems, it is unlikely that the bacteria here are contributing to plant biomass breakdown. Indeed, recent work has implicate the fungal cultivar as the primary degrader of plant biomass in leaf-cutter ant fungus gardens (Nagamoto *et al.* 2011; Grell *et al.* 2013; Aylward *et al.* 2013; Khadempour *et al.* 2016).

Leaf-cutter ants, in general, cut an exceptionally broad diversity of plants (Mayhé-Nunes & Jaffe 1998; Solomon 2007) and thus, have the potential to encounter a myriad of plant defense compounds that are toxic to themselves and their fungal cultivar. The ants are not enriched in genes families for plant defense compound detoxification (Rane *et al.* 2016), so they must reduce the intake of these chemicals in other ways. Plant defense compound avoidance occurs in several steps. First, ants avoid cutting plants that contain plant defense compounds that are particularly toxic or abundant (Hubbell *et al.* 1984; Howard 1988; Wirth *et al.* 1997). Second, many plant defense compounds that the ants encounter are volatile chemicals (Howard 1988; Howard *et al.* 1988), and in the time that the ants cut and carry the leaf material back to their colonies, some of the volatiles will have had time to dissipate.

Finally, ants often leave leaf material in caches before they incorporate them into their fungus gardens (Hart & Ratnieks 2000; Roschard & Roces 2003), providing further opportunity for the defense compounds to evaporate. Nevertheless, some amount of volatiles can make their way into the gardens. In this study, using gas chromatography, we were able to detect eucalyptus-related compounds (eucalyptol, α-pinene, β□ pinene, *p*-cymene and γ-terpinene) in the fungus garden of one ant colony (*A. laevigata* 1) that was observed cutting considerable amounts of eucalyptus (Supplemental methods and Supplemental Figure 2).

In order to mitigate the deleterious effects of plant defense compounds, we expect the fungal cultivar *L. gongylophorus* would produce enzymes to degrade them. Indeed, work by De Fine Licht *et al.* (2013) suggests that laccases from the fungal cultivar help detoxify plant defense compounds. Nevertheless, bacteria in the garden may also play a role in mediating plant defense compounds. The bacterial community contains the genes necessary for plant defense compound remediation, including many cytochrome P450s, gluthione S-transferases, and other genes involved in xenobiotic degradation, and aromatic compound degradation, but they are not consistently enriched in the dicot-cutter ant fungus gardens (Supplemental Table 1). We expected that since dicot-cutter ants incorporate a higher diversity of plants into their gardens (Table 3), that the diversity of bacteria would also be higher in these gardens, and that the bacteria would have a higher capacity for the degradation of these defense compounds. While we did observe a greater diversity of bacteria in the dicot-cutter ant fungus gardens (Figure 3) we did not see a significant enrichment of plant defense compound degradation genes in these gardens (Figure 5, Supplemental Table 1). However, we still cannot exclude the possibility that bacteria are taking part in this process. Since each dicot-cutter ant colony cuts a unique set of plants (Table 3), they potentially encounter a

unique set of plant defense compounds. If the bacterial community were to respond in a substrate-specific manner to different plant defense compounds, our analysis in this study would not reveal that. To elucidate the role of bacteria in plant defense compound remediation, closely controlled experiments with particular defense compounds of interest applied to bacterial cultures and to fungus gardens would be necessary.

Pinto-Tomas *et al.* (2009) establishes that *Pantoea* and *Klebsiella* bacteria in Central American leaf-cutter ant fungus gardens are supplementing the ant diet through nitrogen fixation. Plant material, in general, is low in nitrogen, and many herbivores supplement their diets through bacterial nitrogen fixation (Douglas 2009; Hansen & Moran 2013). Grasses are especially low in nitrogen (Mattson 1980; Winkler & Herbst 2004), so we would hypothesize that grass-cutter ant fungus gardens would be enriched in nitrogen-fixing bacteria with a corresponding enrichment of nitrogen-fixing genes. Here we show that *Pantoea* are more abundant in the grass-cutter ant fungus gardens, and that a nitrogenase molybdenum-iron protein beta chain gene is significantly more abundant in grass-cutter ant fungus gardens (Supplemental Table 1). Other genes that are related to nutrient acquisition are also significantly more abundant in the grass-cutter ant fungus gardens (Figure 5), such as genes in amino acid metabolism pathways. While it has been shown that nitrogen fixed by bacteria is incorporated into the bodies of ants (Pinto-Tomás *et al.* 2009), animals cannot simply absorb nitrogen as ammonium or nitrate, they require it to either be in the form of amino acids or other organic nitrogen-containing compounds (White 1993). The enrichment of arginine biosynthesis genes is of particular interest since the genome of *Atta* is deficient in genes in this pathway (Suen *et al.* 2011b), and no evidence has been found that the fungus provides arginine (Aylward *et al.* 2013; Khadempour *et al.* 2016).

Other categories of genes enriched in the grass-cutter ant fungus garden bacteria are those involved in metabolism of terpenoids and secondary metabolites, especially their biosynthesis. Grass-cutter ant fungus gardens are significantly enriched in 67 of these genes. This list includes seven siderophores, which are responsible for iron acquisition (Crosa 1989; Winkelmann 2002). Siderophores are costly to produce so the enrichment of these genes suggests that iron acquisition is important in this system. The grass-cutter and fungus gardens examined in this study contained lower amounts of iron than the dicot-cutter ant fungus gardens (Figure 6). Terpenoids are the most abundant secondary metabolites found in plants, and serve diverse roles (Langenheim 1994; Gershenzon & Dudareva 2007). The majority of research into the connection between plant terpenoids and animal-microbe symbioses are in regards to the detoxification of terpenes that would be deleterious to the animal host (Wang *et al.* 2012; Cheng *et al.* 2013; Adams *et al.* 2013; Boone *et al.* 2013; Raffa 2013). However, not all terpenes are toxic to all organisms (Raffa 2013), and in at least one instance they have been shown to supplement a herbivore's diet after some modification by a gut bacterium (Berasategui *et al.* 2017). Dicots contain higher quantities of terpenoids (Wetterer 1994; Mariaca *et al.* 1997). One possibility is that the bacteria in these fungus gardens are producing terpenes as a nutritional additive, especially in the grass-cutter ant fungus gardens where there are lower terpene inputs and these genes are enriched (Figure 5, Supplemental Figure 2).

It is important to consider this system, not only from the perspective of the bacteria, but also that of the ants. One may ask: if grasses are so poor in nutrients, why do the ants use them as a substrate at all? Grass-cutter ants can be categorized as facultative specialists (Shipley *et al.* 2009), as they have the capacity to use grass, a difficult substrate, as their primary fungus garden input but they are also capable of consuming a wider range of

substrates. The ants themselves are adapted to cutting grass with shorter, wider mandibles than their dicot-cutter counterparts (Fowler *et al.* 1986; Silva *et al.* 2016). They also process leaves differently – they do not mulch the material, likely because the silica contained in grasses would dull their mandibles (Massey & Hartley 2009; Silva *et al.* 2016). The ants' adaptation to grass-cutting, combined with the community response of the bacteria in the gardens, allow grass-cutter ants to use grass as a substrate more efficiently than dicot-cutter ants can. This has allowed grass-cutter ants to exploit a novel niche, presumably reducing the amount of interspecific competition they experience. It should be noted, however, that grass-cutter ants, if given the opportunity to use palatable dicots, actually prefer those over grasses (Nagamoto *et al.* 2009), probably due to their lower recalcitrance and higher nutrient content. This indicates that grass-cutter ants' fundamental niche is broader than their realized one. Not only do grass-cutters cut dicots, when they are available to them, but in this study, we detected grasses in the fungus gardens of some dicot-cutters as well (Table 3) and both *A. laevigata* and *A. sexdens* workers were observed cutting grass (personal observation). Even though there is some grass in these fungus gardens, the majority of species that these ants cut are dicots. As well, *A. laevigata* and *A. sexdens* both process their leaves in the manner consistent with other dicot-cutter ants (Fowler *et al.* 1986)).

Grass-cutter ants forage on a substrate that is lower in nutritional quality than their dicot-cutter counterparts. The grasses they cut are lower in nitrogen and iron. Optimal foraging theory predicts that when the quality of forage is lower, the ants should be cutting a greater diversity of plants (Rockwood & Hubbell 1987). Instead, grass-cutter ants cut a significantly lower diversity of plants than their dicot-cutter counterparts. Grass-cutter ants to do not have access to the diversity of plants necessary to compensate for their low forage quality. Instead, the bacteria in their fungus gardens can provide the necessary nutrition that

a diverse diet provides in dicot-cutter ant fungus gardens, allowing grass-cutter ant species to exploit this novel niche. Not only does our work here provide further evidence of the importance of bacteria in the leaf-cutter ant system, it provides further support that microbial symbionts are important players in novel substrate utilization by animals.

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Table 1 Summary of collection details for leaf-cutter ant colonies used in this study

Leaf-cutter ant colony	Substrate niche	IMG genome number	Collection date	Latitude	Longitude
A. bisphaerica 1	Grass	3300013023	1-Feb-15	S22°50'47.7"	W48°26'.9"
A. bisphaerica 2	Grass	3300013025	3-Feb-15	S22°50'48.4"	W48°26'1.4"
A. bisphaerica 3	Grass	3300013022	3-Feb-15	S22°50'48.4"	W48°26'2.3"
A. capiguara 1	Grass	3300012994	2-Feb-15	S22°54'32.1"	W48°18'28.7"
A. capiguara 2	Grass	3300012996	3-Feb-15	S22°50'47.2"	W48°26'1.3"
A. capiguara 3	Grass	3300012997	3-Feb-15	S22°50'47.6"	W48°26'1.2"
A. laevigata 1	Dicot*	3300013000	20-Jan-15	S21°9'55.5"	W47°50'51.3"
A. laevigata 2	Dicot*	3300012995	17-Jan-15	S21°10'3"	W47°50'47"
A. laevigata 3	Dicot*	3300012998	19-Jan-15	S21°9'56.8"	W47°50'52.7"
A. sexdens 1	Dicot	3300012999	30-Jan-15	S21°9'50"	W47°51'26.9"
A. sexdens 2	Dicot	3300013002	30-Jan-15	S21°9'53.4"	W47°51'10.5"
A. sexdens 3	Dicot	3300013001	31-Jan-15	S21°10'2"	W47°51'5"

*While *A. laevigata* has been described as a grass/dicot-cutter ant (Fowler *et al.* 1986), due to its leaf-processing behavior and fungus garden plant composition observed in this study, we consider it a dicot-cutter.

Table 2 Metagenome sequencing statistics for leaf-cutter ant fungus gardens

Leaf-cutter ant colony	Scaffold total	Scaffold sequence total (Mbp)	Main genome scaffold N/L50	Main genome scaffold N/L90	Max scaffold length (Kbp)	Scaffolds > 50 Kbp	Aligned reads	Protein coding genes
A. bisphaerica 1	628724	390.8	122506/ 740	467228/ 298	249.52	93 (2.07%)	163185122 (98.76%)	607 042 (99.39%)
A. bisphaerica 2	939707	630.9	148370/ 926	680177/ 292	253.54	69 (0.88%)	148406252 (96.49%)	910 609 (99.61%)
A. bisphaerica 3	285649	186.0	29401/ 972	244722/ 247	187.50	49 (1.90%)	167169016 (98.76%)	358 547 (98.19%)
A. capiguara 1	205334	148.7	16608/ 1403	178745/ 247	273.83	37 (2.49%)	199009346 (99.31%)	272 096 (98.99%)
A. capiguara 2	345332	261.2	35330/ 1420	303130/ 247	180.27	34 (1.06%)	204772230 (98.31%)	456 916 (98.70%)
A. capiguara 3	573737	359.5	83958/ 790	508567/ 247	135.51	13 (0.29%)	203079026 (98.77%)	644 865 (98.79%)
A. laevigata 1	853367	517.5	178678/ 686	645038/ 301	274.98	87 (1.53%)	178461364 (96.65%)	871 330 (99.42%)
A. laevigata 2	295365	189.2	32897/ 990	266928/ 247	252.44	92 (4.23%)	189659024 (96.85%)	332 737 (96.95%)
A. laevigata 3	601593	546.3	74824/ 1744	398992/ 340	241.71	17 (0.30%)	209535750 (96.06%)	722 718 (99.01%)
A. sexdens 1	674609	708.4	48220/ 3118	412923/ 341	701.43	167 (2.40%)	156148622 (97.92%)	822 403 (99.46%)
A. sexdens 2	857038	812.9	65552/ 2346	548662/ 328	61.96	17 (0.11%)	150809208 (95.32%)	1 088 719 (99.51%)
A. sexdens 3	1006806	564.7	221493/ 614	772141/ 277	386.55	68 (1.17%)	186976430 (91.63%)	1 029 784 (99.20%)

Table 3 Plant genera detected in each fungus garden sample using *MatK* gene

Sample	Family	Genus	Species *MatK* match %
A. bisphaerica 1	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
	Polygalaceae	*Polygala*	99.3
A. bisphaerica 2	Poaceae	*Paspalum*	99.4
A. bisphaerica 3	Fabaceae	*Chamaecrista*	99.3
	Fabaceae	*Zornia*	100
	Poaceae	*Paspalum*	99.5
	Polygalaceae	*Polygala*	99.0
A. capiguara 1	Poaceae	*Paspalum*	99.7
A. capiguara 2	Poaceae	*Paspalum*	99.6
A. capiguara 3	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
A. laevigata 1	Fabaceae	*Pterogyne*	99.4
	Myrtaceae	*Eucalyptus*	99.9
	Poaceae	*Paspalum*	99.5
	Poaceae	*Urochloa*	100
A. laevigata 2	Asteraceae	*Rensonia*	99.6
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Schizolobium*	100
A. laevigata 3	Anacardiaceae	*Pachycormus*	98.6
	Asteraceae	*Kingianthus*	95.4
	Asteraceae	*Podanthus*	99.5
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Lencaena*	100
	Myrtaceae	*Eucalyptus*	99.8
	Poaceae	*Paspalum*	99.9
A. sexdens 1	Anacardiaceae	*Loxopterygium*	98.4
	Asteraceae	*Cymophora*	98.5
	Bignoniaceae	*Tabebuia*	98.1
	Fabaceae	*Andira*	98.6
	Fabaceae	*Batesia*	98.8
	Fabaceae	*Bussea*	100
	Fabaceae	*Libidibia*	99.8
	Fabaceae	*Pterogyne*	100
	Fabaceae	*Tipuana*	99.9
	Malvaceae	*Pachira*	100
	Myrtaceae	*Eucalyptus*	99.6
	Myrtaceae	*Eugenia*	99.8
	Poaceae	*Scutachne*	98.4
	Rubiaceae	*Genipa*	99.2
	Solanaceae	*Lycianthes*	100
A. sexdens 2	Bignoniaceae	*Tabebuia*	98.3
	Combretaceae	*Lumnitzera*	93.4
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Pterogyne*	99.3
	Fabaceae	*Tipuana*	100
	Lecythidaceae	*Careya*	94.0
	Santalaceae	*Phoradendron*	99.6
A. sexdens 3	Asteraceae	*Echinacea*	99.3
	Asteraceae	*Eclipta*	100
	Asteraceae	*Perymeniopsis*	99.8
	Asteraceae	*Synedrella*	100
	Commelinaceae	*Commelina*	100
	Commelinaceae	*Murdannia*	92.0
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Lencaena*	100
	Malvaceae	*Sida*	99.7
	Myrtaceae	*Eucalyptus*	98.6
	Phyllanthaceae	*Phyllanthus*	100
	Rubiaceae	*Genipa*	100
	Solanaceae	*Acnistus*	99.4

Figure captions

Figure 1 Grass- and dicot-cutter ants differ in the niches that they occupy, and the way that they cut and process leaf material. Field sites in (A) Botucatu, SP and (B) Ribeirão Preto, SP, Brazil. Fungus gardens of (C) grass- and (D) dicot-cutter ants. C. Visual inspection of leaf material from leaf-cutter ant fungus gardens demonstrates the degree of mulching that the different ants complete, with grass-cutters leaving the leaf material more intact (E – *A. bisphaerica* and F – *A. capiguara*), while dicot-cutters mulch to the point of unrecognizable leaf fragments (G – *A. laevigata* and H – *A. sexdens*).

Figure 2 NMDS plot of the relative abundance of bacterial genera in fungus gardens of grass- and dicot-cutter ants. Grass- and dicot-cutter fungus garden bacterial communities are significantly different.

Figure 3 Genus-level bacterial community analysis of leaf-cutter ant fungus gardens from grass- and dicot-cutter ants, demonstrating that dicot-cutter ant fungus gardens have a higher diversity of bacteria. A. Pie charts showing proportions of different bacterial genera in the fungus gardens. B. Shannon diversity index of bacterial genera. C. Bacterial genus richness (for genera that consist of more than 1% of the total normalize gene count).

Figure 4 NMDS plot of KO functional genes from grass- and dicot-cutter ant fungus gardens. The KO profiles are significantly different between the fungus gardens of ants cutting the different substrates.

Figure 5 Particular groups of genes are enriched in either the grass- or dicot-cutter ant fungus gardens. Grass-cutter ant fungus gardens are enriched for genes involved in metabolism of terpenoids and secondary metabolites, as well as genes involved in amino acid metabolism. In contrast, dicot-cutter ant fungus gardens are enriched for genes involved in membrane transport. Bars extending to the left (blue) represent genes that are significantly more abundant in dicot-cutter ant fungus gardens and bars extending to the right (red) represent genes that are significantly more abundant in grass-cutter ant fungus gardens.

Figure 6 Iron content of fungus gardens from this study as measured by inductively coupled plasma optical emission spectroscopy. The iron content in the grass-cutter ant fungus gardens was lower than in the dicot-cutter ant fungus gardens. This difference is not statistically significant, however, since the *A. sexdens* fungus garden iron content is highly variable.

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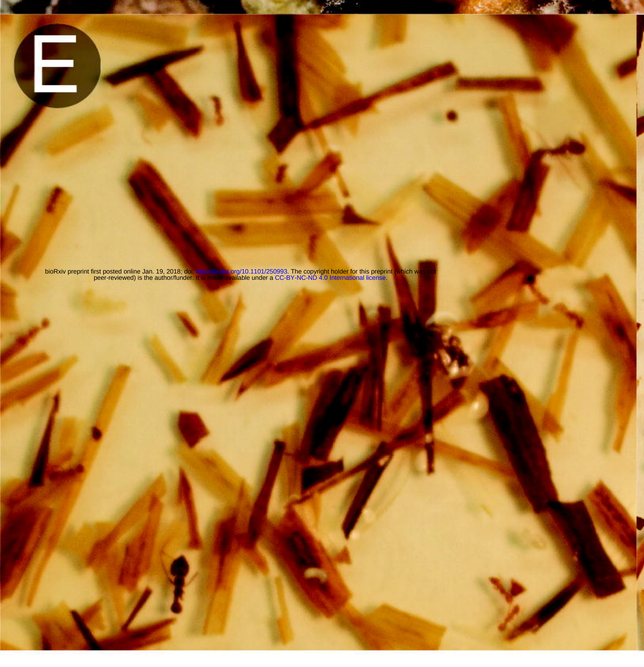
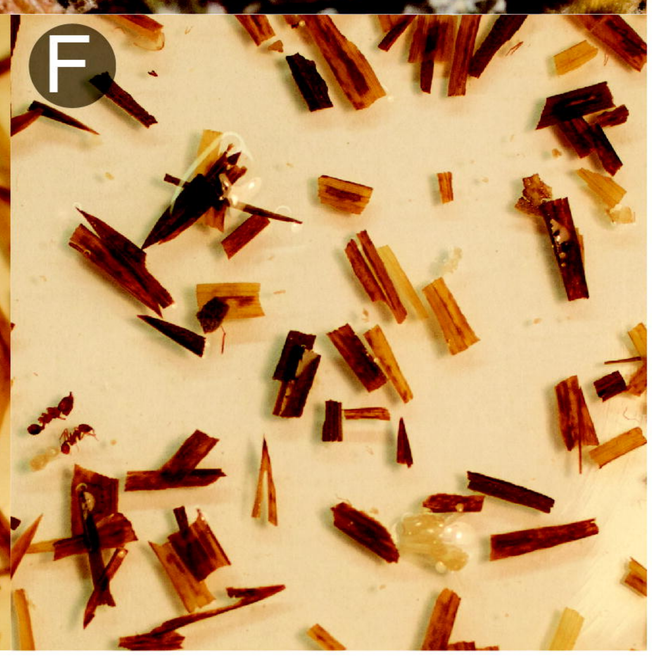
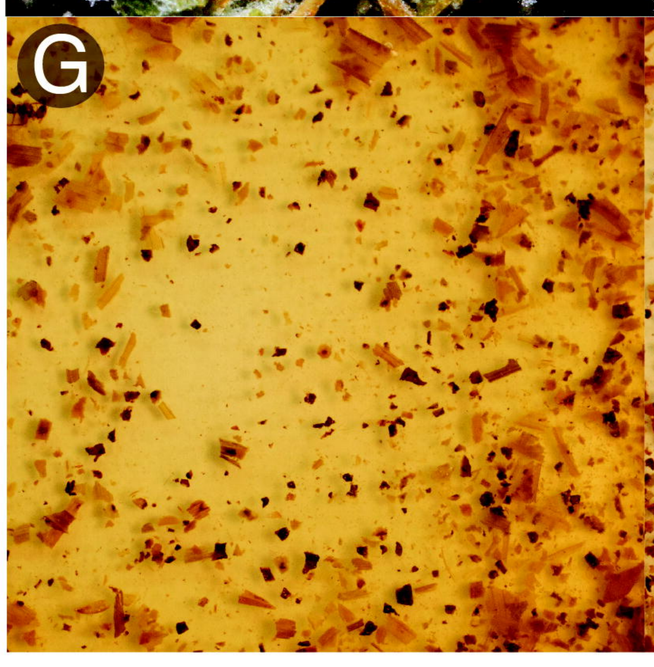
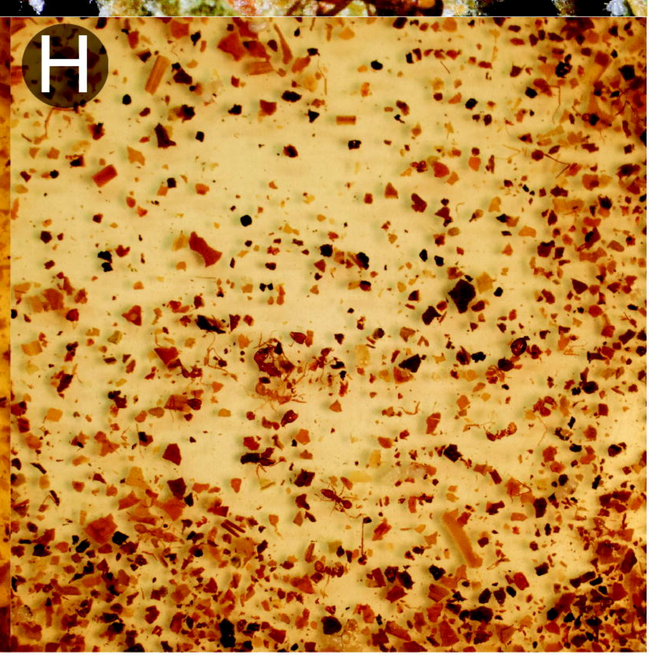


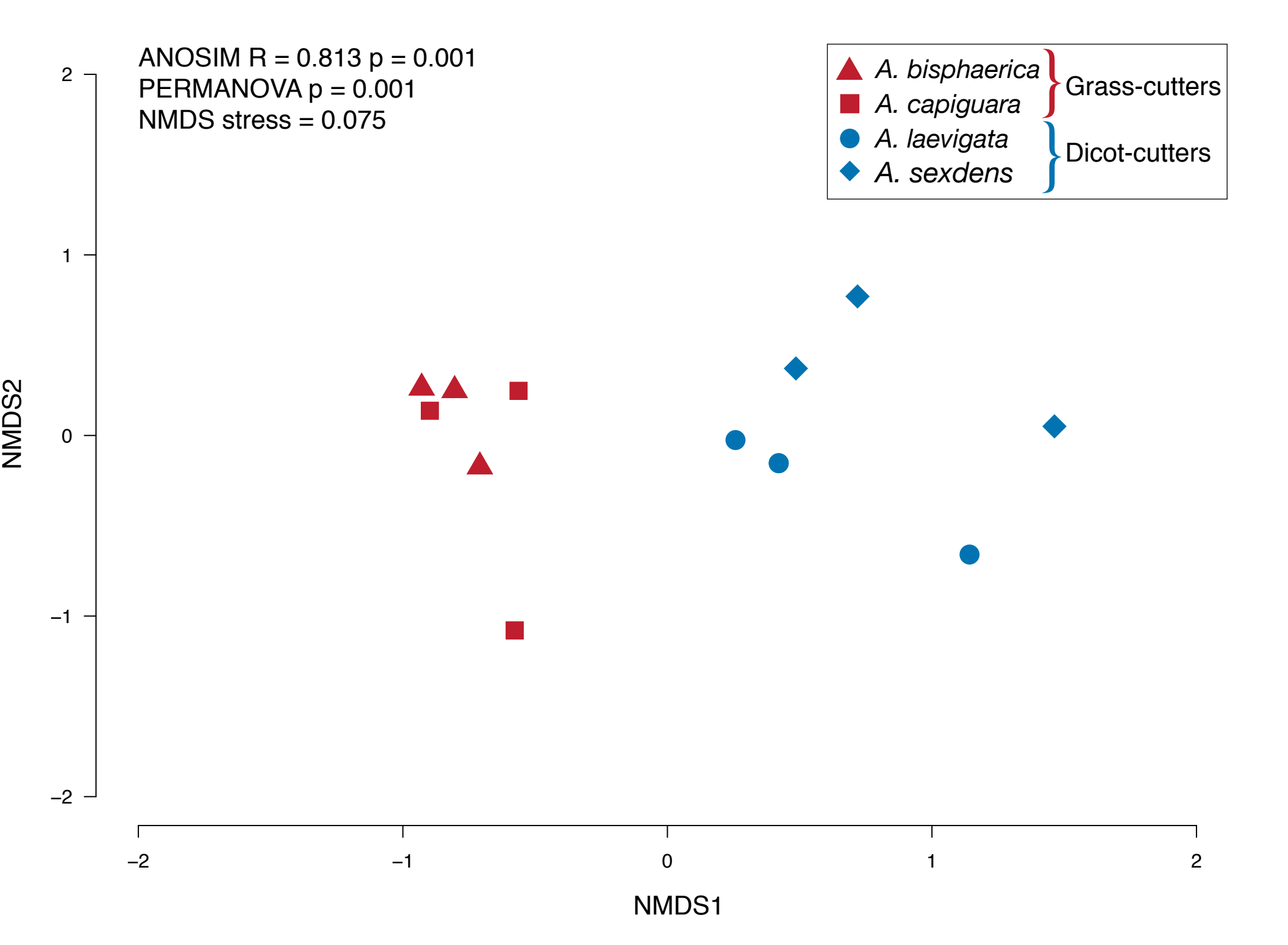


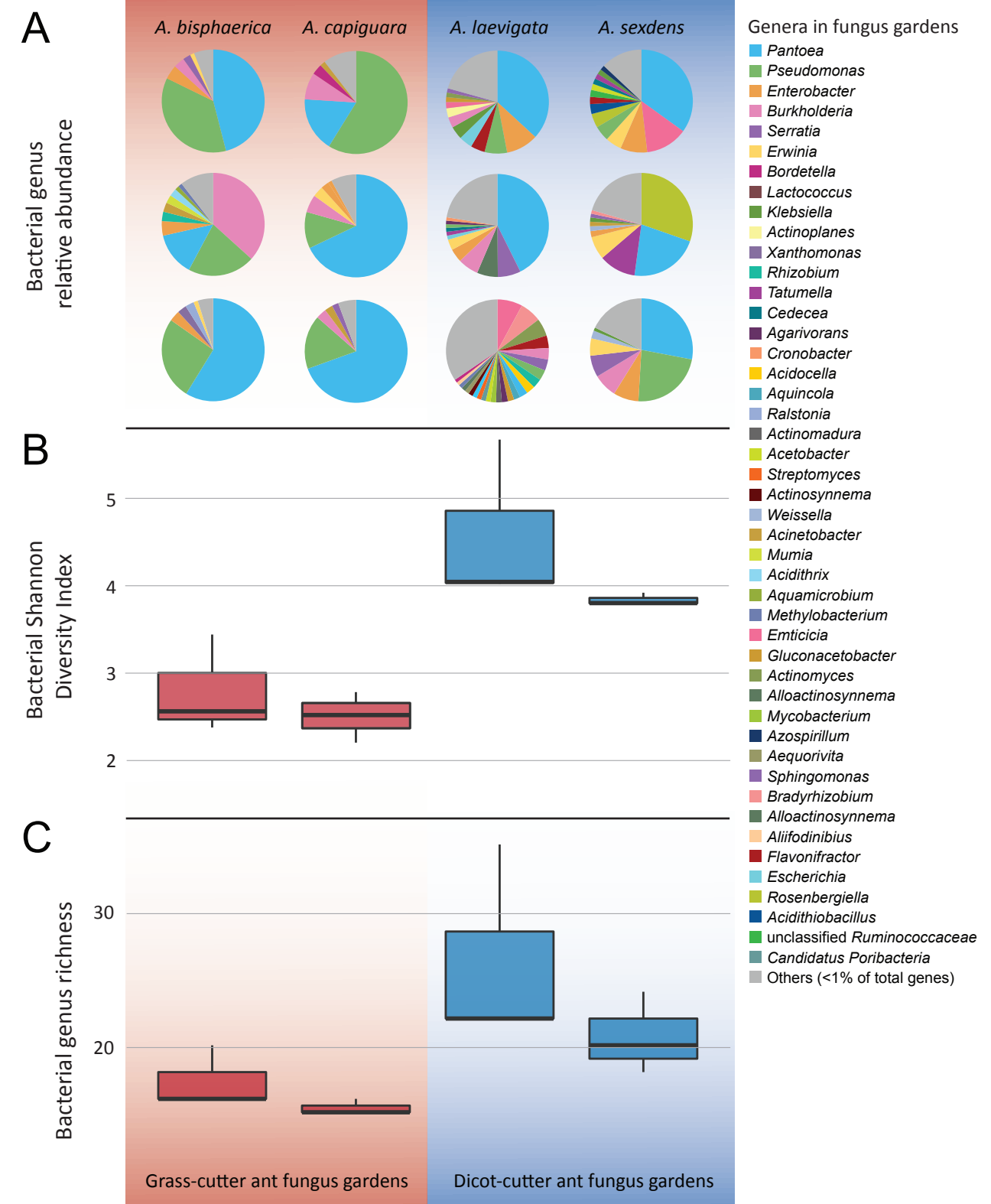


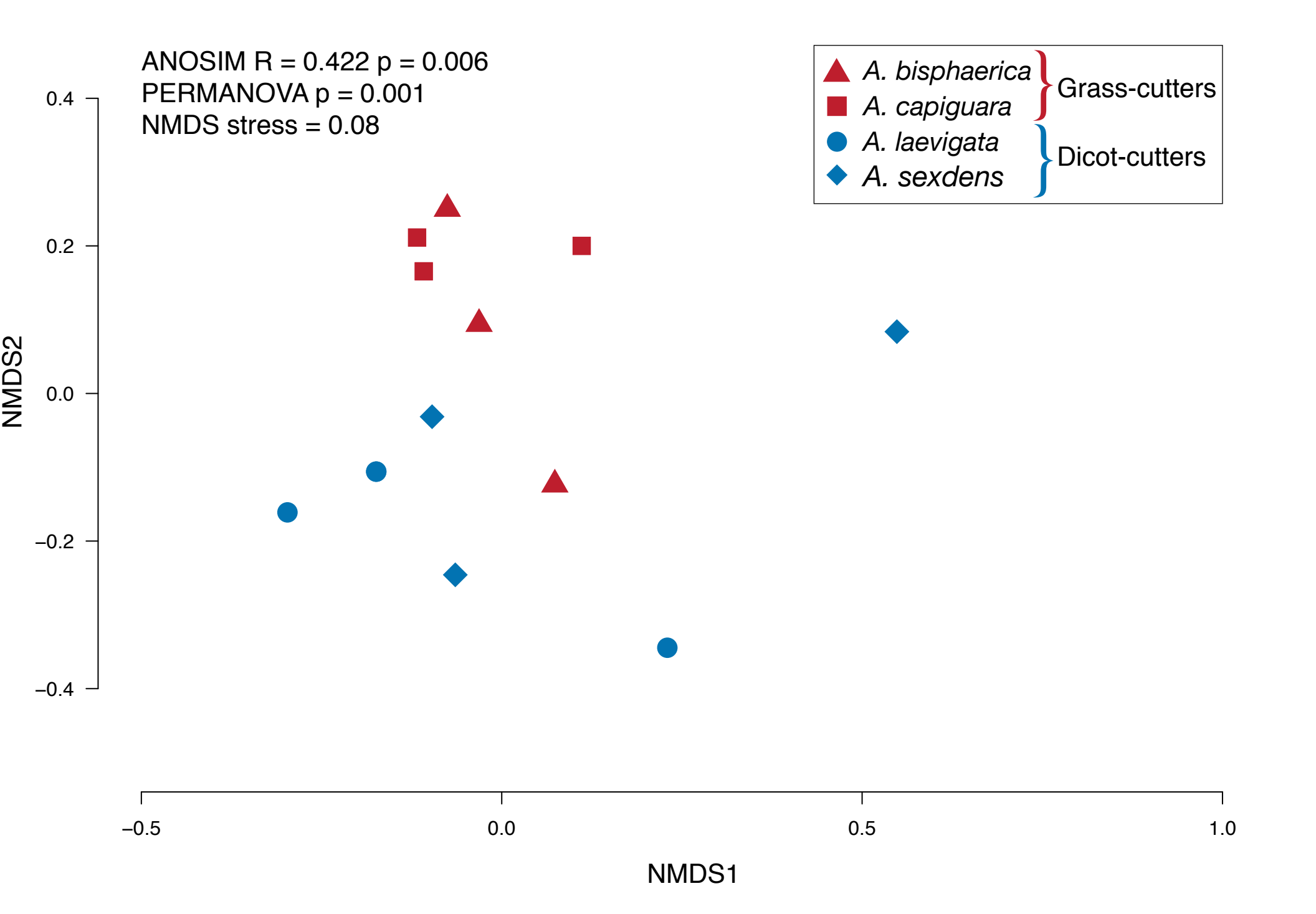


Grass-cutters

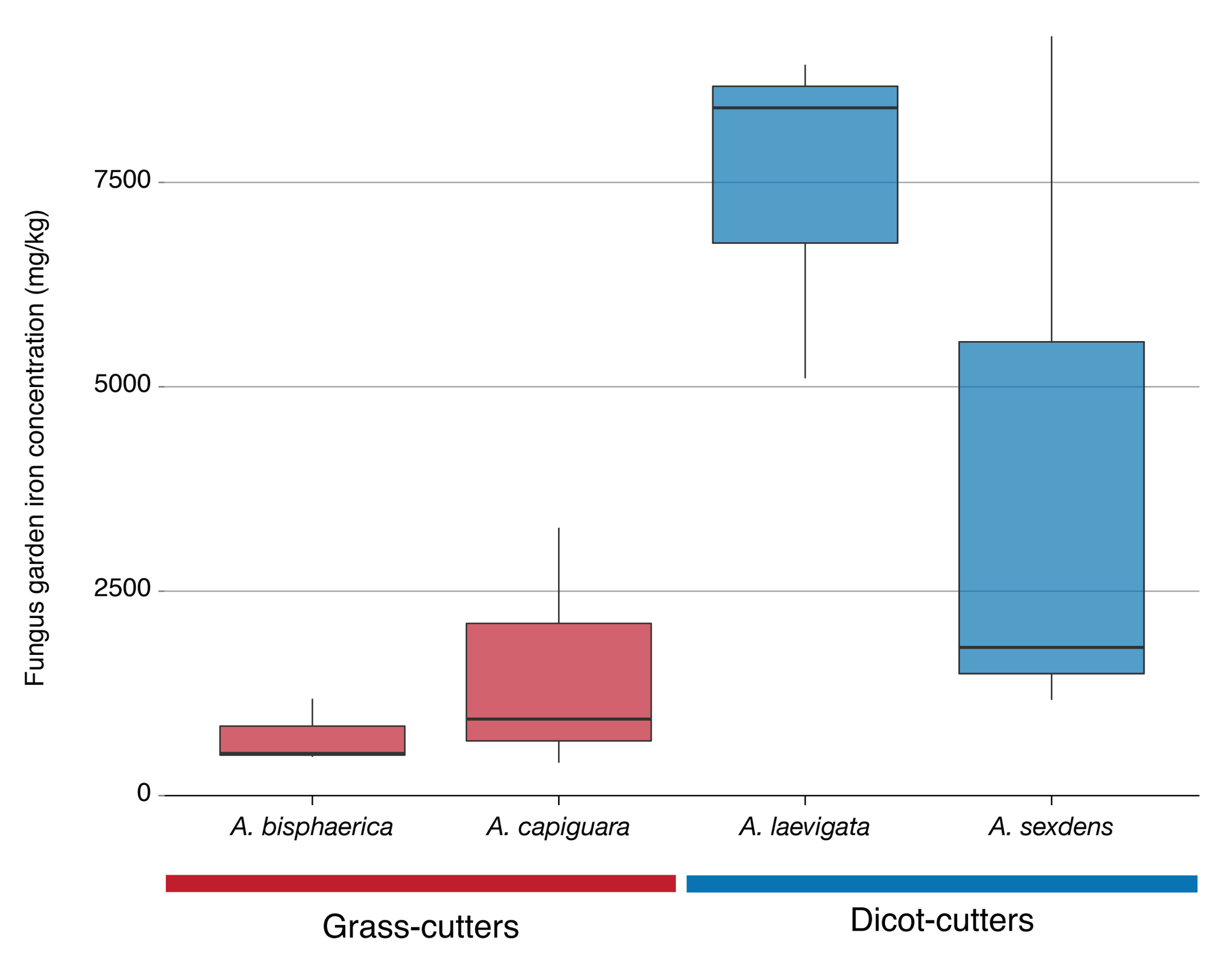
Dicot-cutters


ANOSIM R = 0.813 p = 0.001
PERMANOVA p = 0.001
NMDS stress = 0.075
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters
NMDS1
NMDS2
2
1
0
−1
−2
−2
−1
0
1
2


A
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Bacterial genus relative abundance
B
Bacterial Shannon Diversity Index
5
4
3
2
C
Bacterial genus richness
30
20
Grass-cutter ant fungus gardens
Dicot-cutter ant fungus gardens
Genera in fungus gardens
Pantoea
Pseudomonas
Enterobacter
Burkholderia
Serratia
Erwinia
Bordetella
Lactococcus
Klebsiella
Actinoplanes
Xanthomonas
Rhizobium
Tatumella
Cedecea
Agarivorans
Cronobacter
Acidocella
Aquincola
Ralstonia
Actinomadura
Acetobacter
Streptomyces
Actinosynnema
Weissella
Acinetobacter
Mumia
Acidithrix
Aquamicrobium
Methylobacterium
Emticicia
Gluconacetobacter
Actinomyces
Alloactinosynnema
Mycobacterium
Azospirillum
Aequorivita
Sphingomonas
Bradyrhizobium
Alloactinosynnema
Aliifodinibius
Flavonifractor
Escherichia
Rosenbergiella
Acidithiobacillus
unclassified Ruminococcaceae
Candidatus Poribacteria
Others (<1% of total genes)


ANOSIM R = 0.422 p = 0.006
PERMANOVA p = 0.001
NMDS stress = 0.08
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters
0.4
0.2
0.0
−0.2
−0.4
NMDS2
−0.5
0.0
0.5
1.0
NMDS1


Metabolism of terpenoids and secondary metabolites
Membrane transport
Amino acid metabolism
Biosynthesis of type II polyketide
Biosynthesis of vancomycin group
Nonribosomal peptide structures
Biosynthesis of siderophore group
Type I polyketide structures
Biosynthesis of ansamycins
Sesquiterpenoid and triterpenoid
Zeatin biosynthesis
Carotenoid biosynthesis
Brassinosteroid biosynthesis
Diterpenoid biosynthesis
Terpenoid backbone biosynthesis
Polyketide sugar unit biosynthesis
Biosynthesis of 12-, 14- and 16-membered macrolites
Geraniol degradation
Bacterial secretion system
Phosphotransferase system
ABC transporters
Saccharide, polyol, and lipid transporters
Metalic cation, iron-siderophore and vitamin B12
ABC-2 type and other transporters
Phenylalanine tyrosine and tryptophan metabolism
Tryptophan metabolism
Tyrosine metabolism
Histidine metabolism
Arginine and proline metabolism
Lysine degradation
Cysteine and methionine metabolism
Glycine, serine and threonine metabolism
K02768 tcmO
K02770 elmD
K18893 cepH, sgcC3, mdpC3, kedY3
K18890 dpgD
K17316 bacC
K17240 mxcG
K17238 pchF
K17237 pchE
K17239 pchG
K17215 mbtC
K17214 mbtE
K17213 mbtB
K16299 amphL, nysL, pimD
K15576 rifM, asm45
K15577 rifN, asm22
K15578 rifH
K15554 rifL, asm44
K15553 rifE
K15555 rifK, asm24, asm43
K15496 rifA
K12368 BS, TPS11
K11960 HVS
K11961 TPS21
K11962 CYP71D55
K11963 TPS6_11
K11959 TPS1
K12371 MXPSS1
K12370 EAS
K12369 GERD
K12372 LUP4
K11605 IPT
K11606 UGT76C1_2
K11604 CYP735A
K11607 CISZOG
K10552 ZDS, crtQ
K11073 K10212, crtO
K11075 AOG
K11074 CCS1
K11076 crtX
K11072 crtQ
K10559 crtISO, crtH
K10562 VDE, NPQ1
K10561 ZEP, ABA1
K10545 CYP90D2, D2
K10544 CYP90A1, CPD
K10543 CYP90B1, DWF4
K10539 CPS4
K10538 MAS
K10537 E4.2.3.8
K10117 FOHSDR
K10109 GPS
K10017 hexPS, COQ1
K10015 gcpE, ispG
K10118 FLDH
K09970 dxr
K10014 ispB
K10009 dxs
K10016 ispH, lytB
K09972 FDPS
K10010 ispF
K09815 evaD, eryBVII, aveBV, megDIV, staE
K09816 wbiB
K09817 tll
K06160 tylI, CYP113B
K06858 megY
K05846 eryA
K05847 eryF, CYP107A
K05845 geoB
K05816 atuE
K05813 atuH
K05814 atuF
K05815 atuC
K09523 hlyD, cyaD
K11315 fha1
K03062 impK, ompA, vasF, dotU
K10389 vgrG
K00463 secE
K07326 PTS-Gfr-EIID, gfrD
K12096 PTS-EIIB, sorB-Sor
K12266 PTS-HPR.PTSO, ptsO, npr
K11294 PTS-EIIC, sorA-Sor
K12264 PTS-EI.PTSI, ptsI
K02841 yejB
K03814 ecfA2
K02843 yejE
K19302 iatP
K05286 aguE
K01230 aglG, ggtD
K10661 hisQ
K14026 togB
K10590 ABCA4
K10592 ABCA7
K12757 oleC4
K11090 dasB
K05291 chiG
K05292 gtsA, glcE
K09518 aapJ, bztA
K03850 rbsA
K02739 proV
K09540 modA
K07151 araH
K09580 artQ
K03107 ABC.FEV.S
K01191 alsA
K03361 thiQ
K02735 livF
K00729 ABC.NGC.S
K08496 lptB
K02734 livG
K10967 potI
K02737 livM
K14005 ABC 2.CPSE.P1
K18134 attA1
K02068 ABC.MS.P
K02069 ABC.MS.P1
K02029 ABC.X2.A
K02030 ABC.X2.P
K01992 qrtT
K06148 ABC-2.P
K01990 ABC.CD.P
K01667 quiA
K18383 YUCCA
K02614 ASMT
K05603 E4.1.99.2
K01745 FAHD1
K07008 nagL
K01712 DBH
K18911 hpaI, hpcH
K12256 egtC
K12254 hutH, HAL
K12252 hdc, HDC
K01479 CARNMT1
K12255 hutF
K00673 E4.1.1.19
K09471 spuC
K05526 aruH
K06447 aruI
K10536 L3HYPDH
K01484 puuA
K01585 puuD
K00819 adiA
K01474 astD
K01473 astE
K01470 puo
K03894 SETD2, SET2
K00471 MLL5
K03896 SUV420H
K03895 SETD8
K11419 kdd
K11423 AGPHD1
K05396 DEP1
K00302 GATM
K00613 doeA
K00305 ectA
K00060 CMO
K00304 dsdA
K00303 kbl, GCAT
K00294 soxA
-3 -2 -1 0 1 2 3 4
log2 fold change
Kegg annotation category
KO ID number
Gene name


Fungus garden iron concentration (mg/kg)
7500
5000
2500
0
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters