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Author for correspondence:
Elizabeth G. Pringle
e-mail: epringle@unr.edu

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Community analysis of microbial sharing and specialization in a Costa Rican ant–plant–hemipteran symbiosis

Elizabeth G. Pringle[1,2] and Corrie S. Moreau[3]

[1]Department of Biology, Program in Ecology, Evolution, and Conservation Biology, University of Nevada, Reno, NV 89557, USA
[2]Michigan Society of Fellows, University of Michigan, Ann Arbor, MI 48109, USA
[3]Department of Science and Education, Field Museum of Natural History, 1400 South Lake Shore Drive, Chicago, IL 60605, USA

EGP, 0000-0002-4398-9272

Ants have long been renowned for their intimate mutualisms with trophobionts and plants and more recently appreciated for their widespread and diverse interactions with microbes. An open question in symbiosis research is the extent to which environmental influence, including the exchange of microbes between interacting macroorganisms, affects the composition and function of symbiotic microbial communities. Here we approached this question by investigating symbiosis within symbiosis. Ant–plant–hemipteran symbioses are hallmarks of tropical ecosystems that produce persistent close contact among the macroorganism partners, which then have substantial opportunity to exchange symbiotic microbes. We used metabarcoding and quantitative PCR to examine community structure of both bacteria and fungi in a Neotropical ant–plant–scale-insect symbiosis. Both phloem-feeding scale insects and honeydew-feeding ants make use of microbial symbionts to subsist on phloem-derived diets of suboptimal nutritional quality. Among the insects examined here, *Cephalotes* ants and pseudococcid scale insects had the most specialized bacterial symbionts, whereas *Azteca* ants appeared to consume or associate with more fungi than bacteria, and coccid scale insects were associated with unusually diverse bacterial communities. Despite these differences, we also identified apparent sharing of microbes among the macro-partners. How microbial exchanges affect the consumer-resource interactions that shape the evolution of ant–plant–hemipteran symbioses is an exciting question that awaits further research.

1. Introduction

Mutualistic symbioses, i.e. mutually beneficial interactions where the partners live in prolonged physical contact, have been a major driver of the evolution of life on Earth, from the origin of eukaryotes to coral-reef diversity hotspots. Mutualistic symbioses are currently receiving unprecedented attention across biological subdisciplines as the use of high-throughput sequencing begins to reveal the ubiquitous and dynamic associations between microbes and macroorganisms. These associations provide vital functions for both partners, including increased metabolic capacity, nutrition and protection, but have so far been examined mostly as two-way host–microbe interactions. A central question in mutualism biology is how mutualistic interactions shape and are shaped by their surrounding ecological communities [1].

Symbioses can be classified as 'open' or 'closed', where open symbioses have symbionts that can be gained or lost from the environment and are thus typically subject to greater influence from the outside community than are symbionts in closed systems [2]. Closed symbioses, by contrast, are usually transmitted vertically from parent to offspring and tend to show higher partner fidelity, which may lead more easily to coevolution between partners [2].

Although the distinction between open and closed symbioses provides a bird's-eye view of mutualistic symbiosis in its ecological context, the complete framework will probably require 'open' and 'closed' at two ends of a continuum of possible environmental interactions. What constitutes the space between requires investigation into how organisms' evolutionary history and present ecology simultaneously influence the extent of interaction between a given mutualistic symbiosis and its environment.

Both coevolution and the key role of the ecological community in shaping a mutualistic symbiosis were first demonstrated in a single pioneering experiment: Janzen [3] separated tropical acacia plants that provide housing and food for ant symbionts from those ant symbionts and showed that herbivore damage to plants increased, strongly reducing plant fitness. Ant–plant protective mutualisms, including acacia-like 'ant-plants' (myrmecophytes) with hollow cavities (domatia) that host symbiotic ant defenders, have been central to developing our understanding of mutualism ever since [4]. The diversity of ant–plant interactions, as well as the sheer abundance of ants that is maintained by them, may have been driven primarily by the evolution of protective-nutritional mutualism between ants and honeydew-producing hemipteran insects (e.g. scale insects and aphids) [5,6]. Hemipterans feed on plant sap and metabolize it into carbohydrate-rich honeydew, which allows honeydew-feeding ants to take on primarily arboreal lifestyles as 'cryptic herbivores' [6]. Indeed, the vast majority of myrmecophytes feed ants in part via the honeydew of hemipteran scale insects [7].

Although both ants and hemipterans can obtain their required energy from plant phloem sap, several key nutrients, and essential amino acids in particular, are in much shorter supply [8]. Hemipterans were early and enduring models for studying how animals can obtain such key nutrients from microbes [9], but the broad relevance of bacteria and fungi as potential sources of nutrition for ants, which are typically omnivorous, was recognized only recently [10,11]. Most phloem-feeding hemipterans are associated with just one or a few highly specialized intracellular bacteria that are vertically transmitted, i.e. 'closed' symbiosis, whereas ants are more typically associated with gut bacterial symbionts, i.e. 'open' symbiosis. Nevertheless, evidence to date suggests that these associations may provide good examples of the continuum of ecological interaction with symbiosis: some ants exhibit core microbiota that, though subject to dietary influence, vary little through evolutionary time [12–14], and hemipteran honeydew can contain proteins derived from their bacterial symbionts as well as even some of the bacteria themselves [15,16]. Microbial symbionts have in fact recently been suggested to mediate ant–hemipteran mutualisms [17]. Because there is a potentially strong feedback loop among plant chemistry, hemipteran honeydew, and the quantity and quality of ant defensive behaviour [18], which may be modified by the insect-associated microbes, microbial symbionts may also play key roles in the eco-evolutionary outcomes of ant–plant protective mutualisms.

To investigate the extent of environmental influence on patterns of microbial communities in an ant–plant–hemipteran symbiosis, we investigated the abundance and composition of bacteria and fungi in the myrmecophytic tree *Cordia alliodora* in Costa Rica. *Cordia alliodora* is a widespread Neotropical tree that forms hollow domatia at stem nodes, where ant symbionts nest and tend several species of scale insects. In Costa Rica, *Co. alliodora* trees are commonly inhabited by both *Azteca* spp. (Dolichoderinae) and *Cephalotes setulifer* Emery (Myrmicinae) ants, either in separate trees or in different domatia on the same tree [19]. Whereas *Azteca* ants appear to be omnivorous and to host very few specialized gut symbionts, *Cephalotes* is the quintessential cryptic herbivore, and this habit may be facilitated by a core gut bacterial microbiome that is maintained throughout the genus [10,12–14]. In *Co. alliodora*, however, both *Azteca* and *Cephalotes* share a very similar environment and diet: individual tree domatia can transition between ant occupants over time, and both ants tend large numbers of honeydew-producing scale insects in two subfamilies: Pseudococcidae : Pseudococcinae and Coccidae : Myzolecaniinae (E. G. Pringle 2007, personal observation). The vertically transmitted intracellular symbionts of a major clade of Pseudococcinae species have been well studied and are unusual in that the metabolic pathways are shared between two nested bacteria—a Gammaproteobacteria within the cytoplasm of the Betaproteobacteria *Tremblaya*—and the host insect genome [20,21]. To our knowledge, the microbial symbionts of Myzolecanniinae remain virtually unstudied. Relatives in Coccidae : Coccinae were reported to have abundant fungal symbionts [9].

Using high-throughput sequencing of 16S rRNA from bacteria and internal transcribed spacer (ITS) region rDNA from fungi, we tested the hypothesis that the persistent physical contact created by the symbiosis among the tree, ants and scale insects would influence the composition of the insects' microbial associates and symbionts. For bacteria, we predicted that environmental influence would be strongest in *Azteca*, weaker in *Cephalotes* and weakest for the coccoid scale insects, based on the expected differences in localization, transmission and partner fidelity of the associated bacteria. For fungi, we predicted that *Azteca* ants would consistently associate with domatia-derived fungi: these associations have been previously reported from other *Azteca*-plant symbioses [22], and we have observed black, fungal patches within the ant domatia. We had few predictions for the fungal associations of the rest of the insect taxa, which have been very little studied.

2. Material and methods

(a) Sample collection

Samples were collected in June 2012 in the Area de Conservación Guanacaste, Sector Santa Rosa, Costa Rica (10°50′ N, 85°36′ W). Two species of *Azteca* commonly occupy *Co. alliodora* trees at the site (*Azteca pittieri* and *Azteca beltii*; [23]), and they are difficult to distinguish in the field. We therefore chose study trees based on which ant genera were present and subsequently identified *Azteca* to species level with molecular barcoding (electronic supplementary material, text S1). We collected samples from five trees (approx. 9 cm average diameter at breast height) (electronic supplementary material, table S1). From each tree, we collected ants (Dolichoderinae: *Azteca* spp. and/or Myrmicinae: *Ce. setulifer*), hereafter *Azteca* and *Ce. setulifer*, scale insects (Coccidae: Myzolecaniinae: *Cryptostigma* spp. and/or Pseudococcidae: Pseudococcinae: *Paraputo* cf. *larai*), hereafter *Cryptostigma* and *Paraputo*, and environmental samples from the ant domatia and tree leaves. Domatia samples consisted of 2 mm^2 scrapings of an interior domatium wall occupied by each ant species per tree; leaf samples consisted of approximately 4 cm^2 per tree from each of two leaves from

separate whorls. Stable-isotope samples were transferred to a freezer within 1 h of collection and frozen for less than 12 h before they were dried for ≥48 h at 60°C. Microbial-survey samples were stored in 100% ethanol until processing.

(b) Stable isotopes

To test whether there was a relationship between the trophic position of the insects and the abundance of their internal microbes, we measured $\partial^{15}N$ in the ants and scale insects. Sets of approximately 20 ant worker bodies (head and thorax) ($n = 6$ *Azteca* and $n = 6$ *Ce. setulifer*) and entire scale insects ($n = 3$ *Cryptostigma* and $n = 3$ *Paraputo*) were ground with a mortar and pestle. Four larval leaf-chewing herbivores, three spiders, and nine leaves were analysed for comparison. Analyses were performed at the UC Davis Stable Isotope Facility. We tested for differences in $\partial^{15}N$ among sample types using a generalized linear model because of the unbalanced sampling design and conducted Tukey *post hoc* comparisons in the *multcomp* package in R v. 3.3.1 [24,25].

(c) DNA extraction

Insects were surface sterilized prior to DNA extraction. Ant gasters and whole scale insects were dipped in 95% ethanol, soaked for 1 min in 5% bleach and rinsed in sterile water. We clipped gasters (i.e. posterior abdomens, which contain the entire digestive tract apart from the mouth and oesophagus) from ant bodies and pooled 1–3 gasters for each ant sample ($n = 15$ *Azteca* samples, $n = 15$ *Ce. setulifer* samples). We dissected the midgut from one additional *Ce. setulifer* ant and extracted it separately. Ethanol was evaporated from the domatia ($n = 10$) and leaf ($n = 6$) samples prior to extraction. DNA was extracted using a modified version of the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) (see [26]).

(d) Quantitative PCR to determine microbe copy numbers

To estimate the abundance of bacteria and fungi in our samples, we performed real-time quantitative PCR (qPCR) of the bacterial 16S rRNA gene and fungal ITS rDNA gene (see also the electronic supplementary material, text S1). For 16S rRNA, we used the universal bacterial primers 515F and 806R [27]. For ITS rDNA, we used ITS1-F and 5.8S primers [28,29]. All qPCRs were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) using SsoAdvanced 2X SYBR green supermix (Biorad) and 2 µl of DNA extract. For bacterial 16S rRNA, standard curves were generated from the *Escherichia coli* 16S rRNA gene. For fungal rDNA, standard curves were generated from the ITS rDNA gene of *Pleurotus* sp., obtained from store-bought oyster mushrooms (electronic supplementary material, text S1). To determine the number of gene copies per microgram of DNA, we measured the DNA concentration of each sample on a Qubit (Life Technologies, Grand Island, NY, USA) (electronic supplementary material, text S1).

We tested for differences among sample types in the abundance of 16S rRNA and ITS rDNA separately using the *nparcomp* package in R v. 3.3.1 [25,30] because the sampling was unbalanced and the data exhibited a strong right skew. We used two-sided tests on a multivariate *t*-distribution with a Satterthwaite approximation. We tested for a relationship between the abundance of bacteria in our ant and scale-insect samples and their $\partial^{15}N$ signatures using a general linear model in R.

(e) Sequencing of bacterial and fungal communities

We first sequenced both 16S bacterial rRNA and ITS fungal rDNA amplicons using tag-encoded 454 FLX-titanium amplicon pyrosequencing (Research and Testing Laboratory, Lubbock, TX, USA). 16S rRNA from bacteria was amplified in the V1–3 region using primers 28F and 519R [31]. ITS rDNA from fungi was amplified using fungal-specific primers ITS1-F and ITS4 [28]. Two blank negative controls from the PowerSoil DNA extraction kit produced no sequences. The 454 sequences were extracted and processed in MOTHUR v. 1.36.0 [32], following the standard operating procedure (accessed 2 February 2015; [33]) with some modifications.

For ITS sequences, after running sff.multiple with minflows = 360, we trimmed all sequences at both ends to 250 total bases, discarding shorter sequences. This trimming scheme produced a good match to a mock community that we sequenced for quality control (electronic supplementary material, text S1). We detected chimeras using the uchime algorithm implemented in MOTHUR. Operational taxonomic units (OTUs) were then determined by calculating pairwise distances between sequences and clustering using the average neighbour method and a 0.03 distance. Although the 97% OTU cut-off may approximate species imprecisely in some taxa [34], it is currently the most common threshold for community analysis of both bacteria and fungi and allowed us to compare within and between these hyperdiverse kingdoms. Representative OTUs and phylotypes were classified using a recent UNITE database (UNITEv6_sh_dynamic) [35]. We included singletons in the analyses presented here; analyses excluding singletons were run in parallel (see the electronic supplementary material).

Because our preliminary analyses of the 454 16S rRNA sequences indicated unusually high diversity among the bacteria associated with our two *Cryptostigma* (Coccidae: Myzolecaniinae) samples, we resequenced all of our samples for bacterial 16S rRNA, including five additional *Cryptostigma* spp. samples (electronic supplementary material, table S1) and 13 additional Myzolecaniinae (electronic supplementary material, text S4), on an Illumina MiSeq platform (Argonne National Laboratory, Lemont, IL, USA), amplifying the V4 region using primers 515F and 806R [27]. Because these MiSeq 16S rRNA sequences produced very similar results and more total reads after filtering (241 077 from MiSeq versus 201 246 from 454), here we focus on the MiSeq results for 16S rRNA. MiSeq sequences were processed in MOTHUR v. 1.37.0 [32], following the standard operating procedure (accessed 1 April 2016; [36]). We again detected and removed chimeras using the uchime algorithm. Sequences were classified using the SILVA database (www.arb-silva.de). We removed sequences classified as Archaea, chloroplasts, or mitochondria, and clustered sequences into OTUs using the dist.seqs and cluster commands. OTUs were subsequently classified at 97% (see above). Sequences from four blank negative controls from the PowerSoil DNA extraction kit were analysed in parallel to test for possible reagent- or platform-derived contamination.

(f) Analysis of bacterial and fungal communities

To investigate the alpha diversity of our samples, we compared composition based on phylotypes, conducted rarefaction analysis and calculated diversity. We calculated phylotypes from the SILVA and UNITE database for bacteria and fungi, respectively. Rarefaction curves were calculated from data binned at the OTU level with the rarefaction.single command in MOTHUR. To compare diversity, we calculated an inverse Simpson index for all replicates subsampled at 1100 sequences for bacteria and 902 sequences for fungi (see below) using the summary.single command in MOTHUR. Because the distributions of diversity estimates were right-skewed, we assessed differences in diversity among samples using the *nparcomp* package in R.

Before beta-diversity analyses, the MOTHUR sub.sample command was used to subsample the data. Bacteria were subsampled at 1100 sequences, which excluded nine samples. Fungi were subsampled at 902 sequences, which excluded three samples. Community composition was compared in two ways. First, we used nonmetric multidimensional scaling (NMDS) to visualize differences in community composition among all of our sample types. The *vegan* package in R v. 3.3.1 was used to calculate Bray–Curtis distances (vegdist function), and stable solutions to the first two NMDS axes were calculated using the metaMDS function [25,37]. Second, we visualized the extent to which each ant shared bacterial and fungal OTUs with their scale insects and domatia environment using Venn diagrams. We searched for overlap among the *Azteca*, *Azteca* domatia and *Azteca*-tended scale insects and among the *Ce. setulifer*, *Ce. setulifer* domatia and *Ce. setulifer*-tended scale insects (electronic supplementary material, figure S1). The merge.groups and venn commands in MOTHUR were used to visualize OTU overlap.

(g) Functional inference from inferred hemipteran metagenomes

To examine whether the two genera of hemipteran scale insects (*Cryptostigma* coccids and *Paraputo* pseudococcids) harboured bacterial endosymbionts with similar predicted functional activity, we used the online Galaxy version of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v. 1.0.0) to predict metagenome function from 16S rRNA sequences [38] (electronic supplementary material, text S1). To compare the relative abundance of predicted gene families from PICRUSt, we calculated the relative abundance of each gene family per sample and tested for differences between *Cryptostigma* and *Paraputo* using *t*-tests with unequal variances and a Bonferroni correction for multiple comparisons.

3. Results

(a) Trophic position and microbial abundance

Among our insects, there was no relationship between bacterial abundance and ∂^{15}N natural abundance (figure 1). Unexpectedly, the *Paraputo* pseudococcids, which feed on tree phloem, exhibited ∂^{15}N levels significantly higher than tree leaves and not significantly different than the *Azteca* ants or the spiders (electronic supplementary material, figure S2*a*). By contrast, the *Cryptostigma* coccids, which also feed on tree phloem, exhibited ∂^{15}N levels similar to leaf-chewing herbivores and not significantly different than tree leaves. *Cryptostigma* coccids and *Paraputo* pseudococcids contained similar abundances of bacteria, and both contained low abundances of fungi (electronic supplementary material, figure S2*b*). *Cephalotes setulifer* ants exhibited much lower ∂^{15}N than *Azteca* ants (figure 1). The posterior abdomens of the *Ce. setulifer* ants contained the highest bacterial abundance of any of our samples (electronic supplementary material, figure S2*b*), consistent with the indication that these bacteria play a role in *Cephalotes* nutrition [10]. By contrast, the posterior abdomens of *Azteca* ants contained very few bacteria but a higher abundance of fungi, suggesting that perhaps fungi are an important source of nutrition for these ants, as has been shown in other symbiotic ant–plant mutualisms [11].

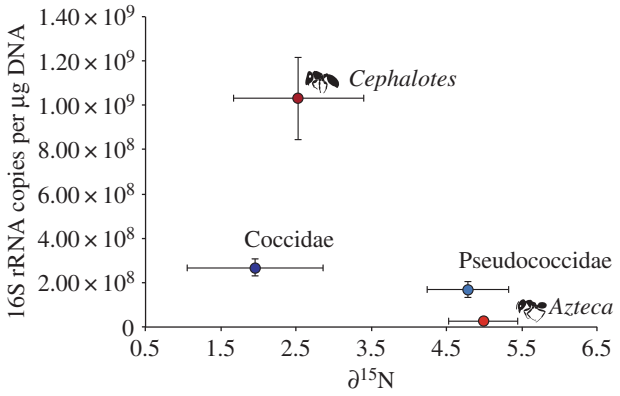

Figure 1. Relationship between the abundance of bacteria in ants (posterior abdomens; red dots) and whole scale insects (blue dots) and their ∂^{15}N signatures. Error bars indicate s.e. Sample sizes are as follows: *Azteca* $n = 4$; *Cephalotes* $n = 5$; Coccidae $n = 3$; Pseudococcidae $n = 3$. (Online version in colour.)

(b) Alpha diversity of communities

(i) Bacteria

There were 20 identifiable bacterial phyla among all of our sample types, including ants, scale insects, domatia and leaves, with Proteobacteria, Actinobacteria, Bacteroidetes and Verrucomicrobia representing nearly 92% of all 241 077 high-quality sequences in the MiSeq dataset. Nearly 7% of sequences were not classified at the phylum level. Classification by phylotype produced 50 classes, 90 orders and 203 families. A class-level barplot indicated 15 abundant classes and particular abundance of Alphaproteobacteria and Gammaproteobacteria in all sample types (figure 2*a*). Surprisingly, at least 11 classes were present in high abundance in the *Cryptostigma* coccids, compared to only two classes in the *Paraputo* pseudococcids (see §3d, below).

The 16S rRNA sequences were binned at 97% similarity into 5069 OTUs, including 1555 non-singletons. 0.6% of these OTUs clustered with sequences from the blank negative controls and represented potential reagent- or platform-derived contaminants (electronic supplementary material, table S2). Rarefaction analysis indicated that our sequence coverage was exhaustive in the case of non-singletons (electronic supplementary material, figure S3*b*)—except in the case of leaves and *Cephalotes* domatia, probably owing to low bacterial sequence counts and/or abundance, respectively (electronic supplementary material, figure S2*a*)—and the relative richness of the different sample types was very similar when singletons were included (electronic supplementary material, figure S3*a*; see also the electronic supplementary material, figure S4*a*). Bacteria found in *Azteca* ants and *Cryptostigma* coccids exhibited higher OTU richness that was less stable across individual replicates than the bacteria in *Ce. setulifer* ants or *Paraputo* pseudococcids (electronic supplementary material, figure S5). Indeed, *Paraputo* pseudococcids exhibited the lowest bacterial diversity among our sample types, whereas *Azteca* domatia exhibited particularly high diversity (electronic supplementary material, figure S6*a*). The core microbiome of *Ce. setulifer* ants strongly mirrored previous reports of core bacterial species common to the genus (electronic supplementary material, table S3; [10,12,13]).

(ii) Fungi

There were 17 identifiable fungal classes among all of our samples, with Dothideomycetes, Sordariomycetes, Agaricomycetes and Eurotiomycetes representing approximately

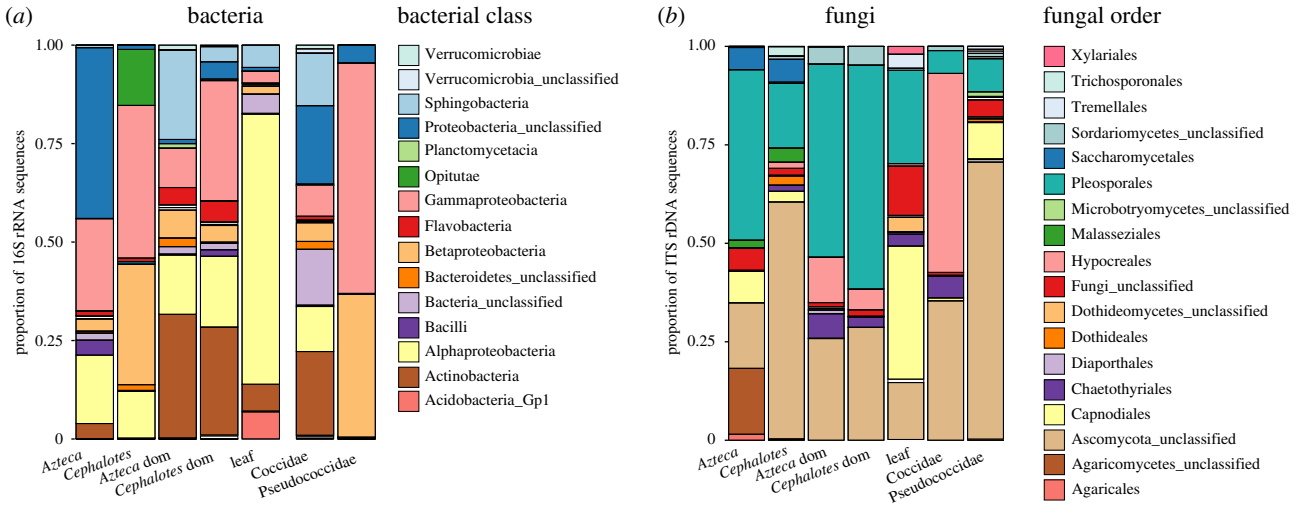

Figure 2. Taxonomic composition of (*a*) bacterial and (*b*) fungal communities for all sample types. Each bar corresponds to the pooled sequence counts for all replicates within a sample type. Coccidae (Myzolecaniinae: *Cryptostigma*) and Pseudococcidae (Pseudococcinae: *Paraputo*) are set apart in (*a*) for easier comparison of their distinct alpha diversity (see also the electronic supplementary material, figure S5). Note that nearly all of the sequences in the insects and approximately 50% of the sequences in the domatia that are classified by UNITE as 'Ascomycota_unclassified' provide good BLASTn hits to Chaetothyriales.

50% of all 139 646 high-quality sequences. Unclassified Ascomycota represented another approximately 28% of sequences, and nearly 18% of sequences were unclassified at the phylum level. Phylotypes at the order and family level produced 56 and 112 operational units, respectively. An order-level barplot indicated 18 abundant orders and particularly high abundance of Pleosporales and Chaetothyriales in all sample types, and in ants and ant domatia especially (figure 2*b*).

The fungal ITS rDNA sequences were binned at 97% similarity into 1602 OTUs, which included 1398 non-singletons. Rarefaction analysis indicated that our sequence coverage was exhaustive for all sample types, including for coccids, which had the fewest total sequences (electronic supplementary material, figure S3*c*,*d*). Fungal OTUs were more variable than bacteria among replicates within sample types (electronic supplementary material, figure S7). Leaves exhibited particularly high fungal diversity compared to the rest of the samples (electronic supplementary material, figure S6*b*).

Chaetothyriales comprised three of the five fungal OTUs with the highest relative abundance. These three OTUs produced BLAST hits at approximately 97% sequence identity to the so-called 'domatia symbiont clade' [39] (E-values: e-97). They were present in the domatia and the gasters of both *Azteca* and *Ce. setulifer*. The ninth most abundant fungal OTU was also Chaetothyriales but produced a BLAST hit at 100% sequence identity to a previously described domatia-carton-associated OTU from an ant-plant in Thailand [39]. This OTU was virtually absent from *Azteca* and *Ce. setulifer* gasters.

(c) Beta diversity of communities

Beta-diversity analyses were conducted on 2026 bacterial OTUs and 1092 fungal OTUs after subsampling at 1100 and 902 sequences per replicate, respectively. The NMDS ordinations revealed that the different sample types grouped much more tightly by their communities of bacteria (stress = 0.12) than by their communities of fungi (stress = 0.19) (figure 3; electronic supplementary material, figures S4*b* and S8). In fact, in the bacterial communities, the only two samples that exhibited any considerable overlap were the *Cryptostigma* coccids and the *Azteca* domatia. The *Cephalotes* ants and the *Paraputo* pseudococcids grouped the farthest apart from the rest of the samples, consistent with a role for highly specialized bacterial endosymbionts in these taxa. By contrast, there was substantial NMDS overlap among all samples in their fungal communities, with the sole exception of leaves. The leaves were composed of a very distinct community of endophytes and leaf pathogens (electronic supplementary material, table S4).

The Venn diagrams of OTU overlap between a given ant taxon and its environment suggested that, despite the overall separation in bacterial communities indicated in the NMDS analysis, there is leakage of individual bacterial OTUs among ants, their tended scale insects, and their domatia (figure 4*a*; electronic supplementary material, table S5 and figure S4*c*,*d*). The fungal Venn diagrams, by contrast, showed that despite extensive leakage between sample types of the most common fungal OTUs, which was also suggested by the NMDS, these shared OTUs comprised a relatively small proportion of the overall OTU richness: only 22% of all fungal OTUs were shared among samples for *Azteca* and 18% for *Ce. setulifer* (figure 4*b*; electronic supplementary material, table S6). Many fungal OTUs were unique to ants or their domatia, as well as, somewhat surprisingly, to the *Paraputo* pseudococcids tended by *Ce. setulifer* ants.

(d) Bacterial diversity in *Cryptostigma* coccids

The bacterial diversity in the *Cryptostigma* spp. coccids was unusually high for an insect in the Sternorrhyncha suborder of Hemiptera (figure 2*a*; electronic supplementary material, figure S5*c*). To probe this unexpected result further, we explored the diversity and function of these bacteria. In our dataset, the *Paraputo* pseudococcids exhibited much more typical diversity for Sternorrhyncha—93.5% of all sequences came from only two OTUs (figure 2*a*; electronic supplementary material, figure S5*d*). A search of the NCBI database revealed good BLAST hits to known pseudococcid endosymbionts, *Tremblaya princeps* (E-value e-121; Betaproteobacteria

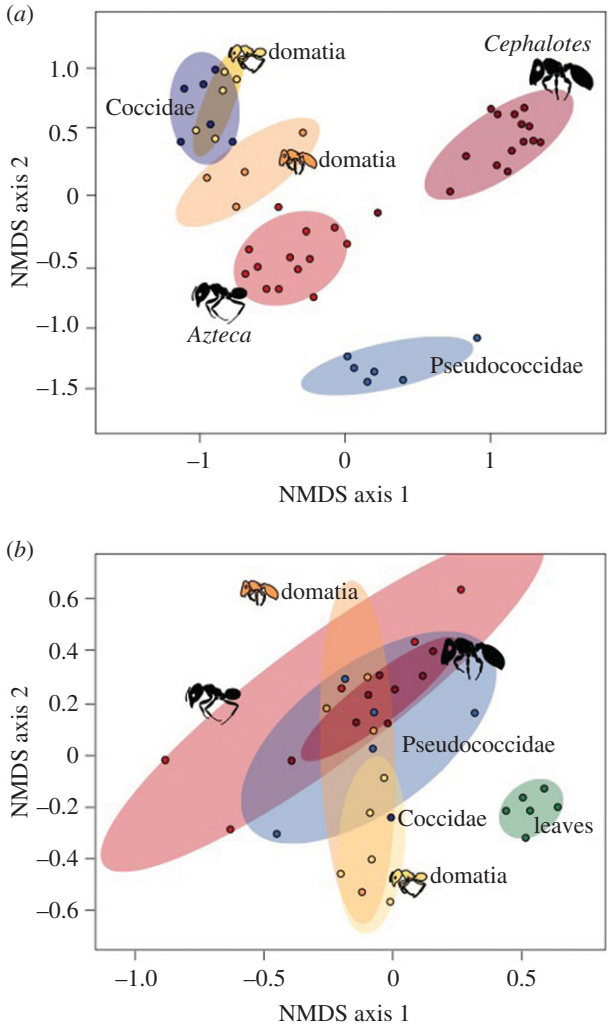

Figure 3. Nonmetric multidimensional scaling for (*a*) bacteria (stress = 0.12) and (*b*) fungi (stress = 0.19). Ellipses indicate 80% confidence intervals (CI). Leaves are not included in (*a*) because no leaf samples included ≥1100 bacterial sequences after chloroplast sequences were discarded. Note that the only *Cephalotes* bacterial sample that falls outside of its 80% CI (halfway towards *Azteca*) was the sample where DNA was extracted from only the midgut.

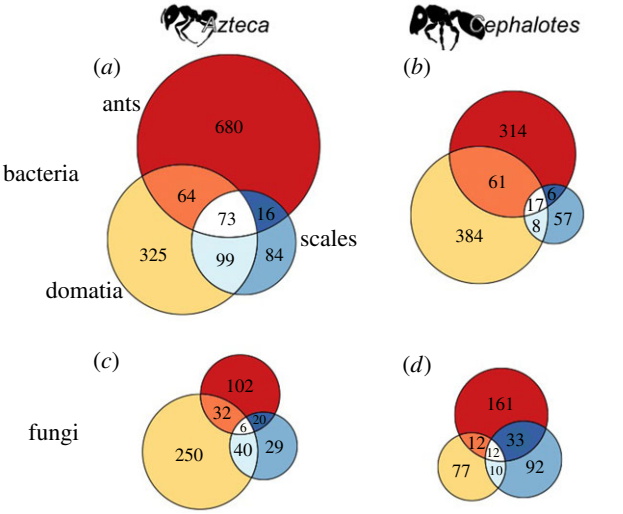

Figure 4. Venn diagrams of OTU overlap calculated separately for (*a*,*c*) *Azteca* and (*b*,*d*) *Cephalotes* microbial communities (see also the electronic supplementary material, tables S5 and S6). Ants are the top circles (red), domatia are the lefthand circles (yellow) and scale insects (*Cryptostigma* coccids and *Paraputo* pseudococcids) are the righthand circles (blue). For both bacteria (*a*,*b*) and fungi (*c*,*d*), the size of the circle is proportional to the number of OTUs present in the sample type. (Online version in colour.)

in figure 2*a*) and a Gammaproteobacteria from *Dysmicoccus neobrevipes* (E-value e-128; Gammaproteobacteria in figure 2*a*) [20,40] that presumably comes from the *Sodalis*-allied bacteria that have repeatedly replaced the symbiont within *Tremblaya* [21]. A third OTU (OTU00031) that made up 3.2% of sequences from our *Paraputo* pseudococcids (Proteobacteria_unclassified in figure 2*a*) did not produce BLAST hits to pseudococcid symbionts but instead to *Sodalis*-like uncultured symbionts from stinkbugs and beetles. In contrast to this low bacterial diversity in the *Paraputo* pseudococcids, our six samples of *Cryptostigma* spp. coccids contained 1175 bacterial OTUs, of which 192 were present in 50% or more of our samples (89% of sequences) and 41 were present in all six of our samples (31% of sequences) (electronic supplementary material, table S7).

Despite the striking differences in the composition of the *Cryptostigma* coccid and *Paraputo* pseudococcid bacterial communities, the functional characteristics of the bacterial metagenomes predicted to Level 2 KEGG orthologs by PICRUSt were remarkably similar (electronic supplementary material, figure S9). NSTI values ranged from 0.03 to 0.12. Well represented gene categories included those of presumed symbiotic importance, with prominent roles for amino acid and carbohydrate metabolism. Genes involved in the metabolism of secondary plant compounds were significantly more abundant in the *Cryptostigma* coccids, whereas genes involved in cellular and environmental processes were significantly more abundant in the *Paraputo* pseudococcids (electronic supplementary material, figure S9 and table S8).

4. Discussion

In this study, we examined how the overlap in environment and natural history of the insects in an ant–plant–hemipteran symbiosis affected the community composition of their associated symbiotic microbes. Consistent with our predictions, *Ce. setulifer* ants and *Paraputo* pseudococcids exhibited distinct, apparently specialized bacterial communities that were comparatively closed to environmental influence. In addition, based on the qPCR results, *Azteca* ants consumed (or associated with) significantly more fungus than *Ce. setulifer* ants, which may provide the *Azteca* ants with their required nitrogen in the absence of a specialized bacterial microbiome. Counter to our predictions, the *Cryptostigma* coccids exhibited an unexpectedly diverse bacterial community, and the *Ce. setulifer* ants were associated with many of the same fungi as the *Azteca* ants.

The bacterial communities of *Azteca* and *Ce. setulifer* posterior abdomens were very different despite the ants' similar ecology (sharing the same individual trees and subsisting at least in part on similar honeydew diets) and convergent adaptation to the *Co. alliodora* host tree (both *A. pittieri* and *Ce. setulifer* are *Co. alliodora* specialists [41,42]). Substantial evidence now suggests that *Cephalotes* ants have coevolved with their gut microbiome [10,12,13,43], and our *Ce. setulifer* ants reflected these previously reported patterns in gut-bacteria alpha-diversity (electronic supplementary material, table S3). The variance in bacterial diversity among *Azteca* replicates was also higher (electronic supplementary material, figures S5*a*,*b* and S6*a*) and the overall abundance of bacteria

was lower (electronic supplementary material, figure S2*b*) than in *Ce. setulifer*, consistent with the hypothesis that the *Azteca* gut bacteria represent a more transient and less functionally important community than in *Cephalotes* [12].

What the *Co. alliodora*-associated *Azteca* lack in a stable bacterial gut community, however, may be compensated for by their consumption of (or association with) fungi, as indicated by the high abundance of fungal ITS sequences in *Azteca* posterior abdomens (electronic supplementary material, figure S2*b*). Recent studies have found intimate co-feeding relationships between Chaetothyriales fungi and plant-ants, in which the ants fertilize the fungi and also consume it [11,44]. In other ant–plant symbioses, there has been some evidence for distinct fungal communities associated with different ant species (e.g. [39]), although a recent study found evidence against codiversification between *Azteca* and their associated Chaetothyriales [22]. Here we found that *Ce. setulifer* and both *Azteca* species were associated with what appeared to be the same strains of Chaetothyriales at the 97% OTU level, showing a potentially strong effect of the ants' shared environment (host tree) on the identity of their fungal associations (electronic supplementary material, text S2). Consistent with the suggestion that the thinner cell walls of domatia-associated Chaetothyriales make them easier to digest than carton-associated taxa [39,45], our ant posterior abdomens contained OTUs from the 'domatia-symbiont clade' [39] but not from a carton-associated OTU. In all of our sample types, we also found abundant Pleosporales fungi, which, like Chaetothyriales, is thought to be saprotrophic in other contexts; the potential role of Pleosporales in ant–plant symbioses remains to be explored.

Despite the distinct compositions of the insects' symbiotic bacterial communities, several bacterial OTUs appeared to be shared among the ants, their tended scales and/or their domatia (figure 4*a*,*b*; electronic supplementary material, table S5). Although the levels of sharing of diverse OTUs supports this result overall (electronic supplementary material, tables S5 and S6), individual cases of shared OTUs need to be verified because of the possibility for cross-talk among multiplexed samples [46]. Two potential ecological pathways for microbes to be shared between ants and scale insects are: (i) if the ants are 'farming' the scale insects for meat, or (ii) if these bacteria are passed to the ants in low abundance via scale-insect honeydew, as has been shown for some gut-associated bacteria in aphids [15]. This kind of trophic microbial sharing could have nutritional or other effects on the consumers in ant–plant–hemipteran interactions [17].

Some surprising results emerged from our investigation of the *Co. alliodora* scale insects. First, the *Paraputo* pseudococcids exhibited unusually high ∂^{15}N for herbivores (electronic supplementary material, text S3). Another unexpected result was that our *Paraputo* pseudococcids contained non-negligible quantities of fungi (electronic supplementary material, figure S2*b*) and a surprisingly high diversity of fungal OTUs (figure 4*d*; electronic supplementary material, figure S7*d*). It is not clear how the pseudococcids acquire these fungi or whether they play a functional role. Unlike the *Paraputo* pseudococcids, our *Cryptostigma* coccids contained few fungi, suggesting that these insects have very different microbial symbionts than their relatives in the Coccinae subfamily, which contain dense aggregations of lymph-associated and intracellular fungi [9]. Even more surprisingly, our *Cryptostigma* insects had very diverse bacterial associates, which, if accurate (electronic supplementary material, text S4 and figure S10), has not been documented for any other insect in the Sternorrhyncha, the suborder of Hemiptera whose members include scale insects, whiteflies, psyllids and aphids [47]. Two caveats, however, are: (i) that we used adult female coccids in all cases, and it can be difficult to know whether these insects are still alive without careful dissection (P. J. Gullan 2016, personal communication); and (ii) we do not know where these symbionts are located within the insect except that they were present after surface sterilization.

Although the metagenome prediction analysis conducted with PiCRUST is a coarse tool that should be interpreted with some caution, it too suggested possible symbiotic function of the *Cryptostigma* coccid bacteria. It is tempting to speculate that if the Myzolecaniinae are associated with diverse bacterial symbionts, this unusual symbiosis could be related to the insects' long, immobile period as adults. Immobility creates little if any chance to feed on different plant phloem sieve-tubes over time, and this stationary lifestyle may require the Myzolecaniinae to process many unusual and possibly toxic metabolites from the plant in its lifetime [48]. Indulging in this speculation, we note that some of the few gene functions that were more abundant in the *Cryptostigma* coccids compared to in the *Paraputo* pseudococcids were those related to the metabolism of plant secondary metabolites (electronic supplementary material, figure S9).

This study represents a first step towards a holistic understanding of how microbial symbionts are integrated with ecological interactions among macroorganisms. The enormous diversity of microbes in this ant–plant–hemipteran symbiosis (like, presumably, in most interactions among macroorganisms) presents a challenge for determining the interactions of functional relevance, but such complexity may be of profound importance to organismal ecology and evolution. Ants are emerging models for microbial study that also have wide-ranging effects on the functions of entire ecosystems, many of which are threatened by global change. Elucidating how ants interact in a microbial world is thus a necessary challenge.

Data accessibility. DNA sequences are available in GenBank (accessions: SRP095765). Code, OTU tables, taxonomy and representative sequences of 97% OTUs are deposited in Dryad (accession URL: http://dx.doi.org/10.5061/dryad.16830) [49].

Authors' contributions. E.G.P. and C.S.M. conceived the study; E.G.P. and C.S.M. designed the experiments; E.G.P. performed the experiments and analysed the data. E.G.P. wrote the initial manuscript, and C.S.M. contributed to revising the manuscript.

Competing interests. We declare we have no competing interests.

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