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Highly similar microbial communities are shared among related and trophically similar ant species

KIRK E. ANDERSON,*†[1] JACOB A. RUSSELL,‡[1] CORRIE S. MOREAU,§ STEFANIE KAUTZ,§ KAREN E. SULLAM,‡ YI HU,‡ URSULA BASINGER,* BRENDON M. MOTT,† NORMAN BUCK* and DIANA E. WHEELER*

*Department of Entomology, University of Arizona, Tucson, AZ 85721, USA, †USDA-ARS Carl Hayden Bee Research Center, Tucson, AZ 85719, USA, ‡Department of Biology, Drexel University, Philadelphia, PA 19104, USA, §Department of Zoology, Field Museum of Natural History, Chicago, IL 60605, USA

Abstract

Ants dominate many terrestrial ecosystems, yet we know little about their nutritional physiology and ecology. While traditionally viewed as predators and scavengers, recent isotopic studies revealed that many dominant ant species are functional herbivores. As with other insects with nitrogen-poor diets, it is hypothesized that these ants rely on symbiotic bacteria for nutritional supplementation. In this study, we used cloning and 16S sequencing to further characterize the bacterial flora of several herbivorous ants, while also examining the beta diversity of bacterial communities within and between ant species from different trophic levels. Through estimating phylogenetic overlap between these communities, we tested the hypothesis that ecologically or phylogenetically similar groups of ants harbor similar microbial flora. Our findings reveal: (i) clear differences in bacterial communities harbored by predatory and herbivorous ants; (ii) notable similarities among communities from distantly related herbivorous ants and (iii) similar communities shared by different predatory army ant species. Focusing on one herbivorous ant tribe, the Cephalotini, we detected five major bacterial taxa that likely represent the core microbiota. Metabolic functions of bacterial relatives suggest that these microbes may play roles in fixing, recycling, or upgrading nitrogen. Overall, our findings reveal that similar microbial communities are harbored by ants from similar trophic niches and, to a greater extent, by related ants from the same colonies, species, genera, and tribes. These trends hint at coevolved histories between ants and microbes, suggesting new possibilities for roles of bacteria in the evolution of both herbivores and carnivores from the ant family Formicidae.

Keywords: bacteria, coevolution, Formicidae, herbivores, symbiosis, trophic ecology

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Introduction

While documented across the eukaryotic tree, the diversity of symbionts is especially impressive and well-studied across the insects (Douglas 1998; Moran *et al.* 2008; Gibson & Hunter 2010). Early morphological studies firmly established that many of these hexapods with specialized diets had microbial residents in their bodies, long hypothesized to compensate for deficiencies in the host diet (Buchner 1965). More recently, the development of molecular and statistical tools for identifying microbial function and characterizing microbial communities has revolutionized our understanding of symbiosis between microbes and insects. For example, many species of phloem-sap feeding Hemiptera have carbohydrate-rich diets deficient in many amino acids. Obligate endosymbionts play a critical role in host fitness by

Correspondence: Kirk E. Anderson, Fax: +1 520 670 6493; E-mail: kirk.anderson@ars.usda.gov

[1]Authors contributed equally.

synthesizing essential amino acids lacking in the host diet (Douglas 2003; Moran & Degnan 2006; Wu *et al.* 2006). In addition to nutritional benefits, facultative symbionts in the Hemiptera provide resistance to parasitoids, heat tolerance, and defense against pathogens (Montllor *et al.* 2002; Scarborough *et al.* 2005; Oliver *et al.* 2006).

While identifying the roles of symbionts in ants has been slow relative to that for Hemiptera, symbiotic bacterial communities have been described for several ant groups (Yurman & Dominguez-Bello 1993; Schröder *et al.* 1996; van Borm *et al.* 2002; Stoll *et al.* 2007; Russell *et al.* 2009; Funaro *et al.* 2011; Ishak *et al.* 2011). Ants play an integral role in the trophic ecology of most terrestrial ecosystems (Hölldobler & Wilson 1998). For example, one colony of the carnivorous army ant, *Eciton burchellii*, can retrieve up to 30,000 prey items daily from the tropical canopy floor. This ant has 557 recorded associate species, approximately 300 of which depend to some degree on the ants for their survival (Rettenmeyer *et al.* 2011). A broad survey of army ant microbiota revealed ant-specific clades of bacteria presumably associated with their predatory life-style, but not suspected to provide essential nutritional benefits (Funaro *et al.* 2011). In contrast, the nutritional ecology of non-predaceous ants is often cryptic, involving unseen or limited sources of nutrition and putative contributions from resident microbial communities (Feldhaar *et al.* 2007). Another recent survey showed that many herbivorous ant species contain ant-specific bacterial taxa that could provide valuable nutritional contributions to their hosts (Russell *et al.* 2009). This finding complements ecological studies demonstrating that many ants, particularly in the tropics, are not generalists as previously assumed, but functional herbivores with restricted diets (Blüthgen *et al.* 2003; Davidson *et al.* 2003). This transition to herbivory is associated with extreme ant abundance and diversity in tropical forests and tropical canopies in particular, where they far outnumber potential prey organisms (Davidson *et al.* 2003; Cook & Davidson 2006). Thus, it is intriguing to consider the nutritional role of microbial symbioses for the evolutionary success of ants.

One major taxon of herbivorous ants with a particularly rich community of bacteria is Cephalotini, the largest tribe of ants restricted to the Neotropics (160 sp.) (antweb.org). The tribe contains just two genera, *Cephalotes* and *Procryptocerus*, which nest inside live or dead plant stems. Their diet appears to be primarily herbivorous, including sugar-rich plant exudates or honeydew gathered from phloem-sap feeding Hemiptera. Many lines of evidence suggest the intimate association of Cephalotini with symbiotic bacteria. *Cephalotes pusillus* fed antibiotics suffered higher mortality, suggesting that microbial symbionts are a necessary component of fitness (Jaffe *et al.* 2001). In three species of *Cephalotes*, the ileum represents the largest portion of the digestive tract, and electron micrographs reveal bacterial biofilms apparently adhering to the hindgut intima via pilli-like extensions, suggesting that bacteria utilize the ant excretions (Roche & Wheeler 1997; Bution & Caetano 2010a). When we also consider that some of these bacteria were undergoing binary fission, it would appear that they are adapted to a lifestyle in the gut environment (Bution *et al.* 2010). The transmission modes of these microbes may resemble those seen in cockroaches and termites. In particular, larval microbes are purged during pupation. Bacteria are then acquired when newly emerged adults lick the abdominal tip of older workers (Wilson 1976; Wheeler 1984; Roche & Wheeler 1997). This behaviour (abdominal, or proctodeal trophallaxis) is rare in ants (Hölldobler & Wilson 1990) and consistent with recent results wherein newly emerged individuals (callow workers, males and queens) contained no gut bacteria or a reduced community of symbionts (Russell *et al.* 2009).

In the present study, we use cloning and 16S sequencing to further characterize the bacterial communities of cephalotine species. We then compare the cephalotine gut flora to bacterial communities from several other ant species, testing the hypothesis that related ants and different ant taxa from similar trophic niches harbor similar gut flora (Stoll *et al.* 2007; Russell *et al.* 2009). This work provides the first statistical, sequence-based analysis of beta diversity across bacterial communities of ants. It is also one of the most comprehensive studies to date on variation of microbial communities within and across insect species. Our findings reveal that similar microbial communities are harbored by ants from similar trophic niches and, to a greater extent, by related ants from the same colonies, species, genera, and tribes.

Materials and methods

Comparing cephalotines to other ecologically defined groups of ants

In this study, we compare the gut flora of herbivorous cephalotine ants (*C. varians*, *C. rohweri*, *C. atratus* and *Procryptocerus batsei*) to the occurrence and abundance of bacteria from a spectrum of ant taxa with different nesting habitats and previously defined trophic levels (Blüthgen *et al.* 2003; Davidson *et al.* 2003; Russell *et al.* 2009). As strict predators, we included the neotropical army ants *Cheliomyrmex* sp. *E. burchellii*, *Labidus praedator* and *Nomamyrmex hartigii*. These ants lack a permanent nesting site, and hunt cooperatively for large prey items on the forest floor. We also included *Pheidole* sp.

and *Cryptopone gilva*, ants that nest in soil or decaying plant matter and function as more generalist scavengers/predators. Finally we included two non-cephalotine species classified as herbivores. Like the cephalotines, *Dolichoderus* sp. and *Tetraponera attenuata* belong to taxa that nest in tree cavities and consume hemipteran honeydew but may also scavenge for live or dead arthropods and cryptic sources of nutrition from the surface of the host plant.

Specimens

Cephalotes varians were collected from the Florida Keys, USA, and *C. rohweri* were collected near Tucson, Arizona, USA. Ants were either immediately preserved in 95% ethanol or reared in the lab on artificial diets of sugar water (see Table S1 in Supporting information for details). One worker from a *Dolichoderus* species – preserved in 95% ethanol after collection from Thailand (by David Lohman) – was also newly included in this study. For extractions of gut tissues, dissections were performed under sterile conditions with a dissecting microscope. Data from other ant taxa were included from previously published studies (Russell *et al.* 2009; Funaro *et al.* 2011) that utilized identical protocols for collections, extractions, dissections and molecular analyses.

PCR and DNA sequencing

DNA extractions from *C. varians, C. rohweri* (whole ants and guts) and the unidentified *Dolichoderus* species were performed using the Qiagen DNeasy kit according to the manufacturer's protocol. We PCR amplified 16S rRNA genes from bacterial communities using universal primers 9Fa and 1513R or 10F and 1507R, according to protocols published previously in Russell *et al.* (2009). Products were then cloned using the Invitrogen TopoTA system (vector pCR2.1), using One Shot chemically competent *E. coli* cells for transformation (Invitrogen, Carlsbad, CA, USA) and blue-white colony screening on LB plates with Kanamycin. White colonies were picked and grown overnight in LB media. These cultures served as the sources of DNA (extracted via boiling) used for insert-size assays, in which vector primers M13F and M13R were utilized to amplify the cloned fragments. Properly sized PCR products were then purified using an 'Exo-Ap' procedure and sequenced with both universal and vector primers (see Russell *et al.* 2009).

Sequence analyses: ant specific dataset

After editing and assembling sequences in Sequencher (version 4.2) (Gene Codes Corporation, Ann Arbor, MI, USA), newly generated 16S rRNA sequences ($n = 686$) were combined with data published in association with Russell *et al.* (2009; two *Cephalotes atratus* libraries from the guts of two separate workers, one gut community from a *C. varians* worker, a *C. gilva* library from a one whole worker, one gut community from a *Pheidole* sp. worker, a *Procryptocerus batesi* library from a whole worker, and one *T. attenuata* library from a single worker) and Funaro *et al.* (2011; one library from a whole *Cheliomyrmex* sp. worker, two libraries from separate *E. burchellii* workers and two from two separate larvae, one library from a whole *N. hartigii* worker, and one library from a whole *L. praedator* worker). Chimeric sequences were removed from the resulting file after analyses with Bellerophon (version 3) (Huber *et al.* 2004) on the Greengenes website (DeSantis *et al.* 2006). The 959 remaining sequences (from 39 clone libraries spanning 27 colonies across 13 species) were uploaded to the Ribosomal Database Project website, where they were aligned and classified (Cole *et al.* 2009). Aligned sequences were used to reconstruct a maximum likelihood phylogeny using RAxML 7.2.8 Black Box (Stamatakis 2006) on the CIPRES web portal (Miller *et al.* 2010).

The topology with highest likelihood score was edited on the interactive tree of life (iTOL) website (Letunic & Bork 2007), where branch coloration and colour strips were added for visualization of host libraries and bacterial taxonomy. This phylogeny was also used for subsequent analyses on Fast UniFrac (Hamady *et al.* 2009), a program that compares bacterial communities by assessing the fraction of phylogenetic branch length that is unique to each. Environmental files assigning sequences in the phylogeny to their respective hosts were uploaded along with the RAxML-generated phylogeny. We then implemented weighted, normalized UniFrac analyses to compare the composition of communities based on phylogenetic overlap (or lack thereof) between them.

UniFrac distances generated through this approach were used for Principle coordinates analysis (PCoA), cluster analysis with jackknifing, and UniFrac significance tests. While the aforementioned analyses focused on individual libraries with $n \geq 10$ sequences, we also performed separate analyses on pooled libraries for all species with a total of $n \geq 15$ sampled 16S rRNA sequences. In these cases, we ran UniFrac significance tests and PCoA analyses to determine whether different ant species harbor different bacterial communities.

Sequence analyses: taxon-specific datasets

To elucidate the taxa responsible for variation between microbial communities while providing insight into the

evolutionary context of the identified microbes, we performed phylogenetic analyses on particular clades of interest. To break sequences up into smaller taxon-specific datasets we utilized results from RDP classification and phylogenetic analyses. Specifically, trees were built for all taxa containing monophyletic lineages with at least five closely related bacteria from cephalotine ants (as ascertained through our initial phylogenetic analyses). This approach led to a total of seven separate datasets representing the Bacteroidetes, Burkholderiales, Entomoplasmatales, Epsilonproteobacteria, Gammaproteobacteria, Rhizobiales and Opitutales. Each taxon-specific dataset was then uploaded to RDP for sequence alignment along with top BLASTn hits and representative outgroup sequences (mostly from phylogenies in a previous study – see ST-6 of Russell *et al.* 2009). Maximum likelihood analyses for these smaller alignment datasets were then performed using GARLI (Zwickl 2006) on the CIPRES web portal. Each analysis utilized a GTR + G + I model of nucleotide substitution, with the parameters for this model being estimated during the run. Individual phylogenies were visualized on the iTOL website after adding branch coloration and colour strips to identify bacteria from particular ant hosts (vs. those from other arthropods or environments). Ant-specific clades were then detected by visual inspection.

Using these phylogenies, all sequences from clone libraries with $n \geq 10$ sequences were assigned to the identified ant-specific bacterial clades and sub-clades contained within (with $n \geq 4$ members) or to bacterial taxa with ≥80% RDP bootstrap support (if they did not group into a host-specific lineage). These data were then utilized to generate a heatmap, whereby the proportional representation of each bacterial lineage or taxon for each library was indicated with colour-coding. The resulting figure illustrates the finer scale patterns behind inter-individual, inter-colony, and interspecific variation in bacterial communities.

Sequence analyses: OTU calculations and rarefaction

To examine sequence divergence and the diversity of the sampled communities we computed uncorrected (p) pairwise distances for sequences in *Cephalotes* libraries using PAUP* v4.0b10. Due to insufficient sequence overlap between some sequences from different libraries, distance matrices were computed separately for *C. atratus*, *C. rohweri* and *C. varians* (species-wide OTU analysis). In separate analyses, distance matrices were constructed for *C. varians* libraries with $n \geq 30$ sequence reads from individual workers (*C. varians* individual OTU analysis) or for ant colonies with at least four sampled individuals (larvae and/or workers) and $n \geq 30$ sequence reads (ant colony OTU analysis). Distance matrices were used by the program Mothur v.1.21.1 (Schloss *et al.* 2009) to assign sequences to 97% OTU's using the average neighbour method. OTU's from the species-wide analysis were deposited into Table S1 (Supporting information) and also used to construct rarefaction curves for each of the three *Cephalotes* species in Mothur. Those from the individual and colony-level OTU analyses were also used to generate rarefaction curves. In doing so, we estimated the diversity and coverage of bacterial communities from species, colonies, and individuals belonging to the *Cephalotes* genus.

Results

Bacterial taxonomy and distributions

A total of 753 bacterial 16S rRNA universal sequences from the genus *Cephalotes* were generated and classified to several phyla, classes, and orders (Genbank accession numbers will be obtained following submission). The majority of these had previously been found as gut associated microbes from related ants (Table S1, Supporting information). Most abundant and ubiquitous among these taxa were the Opitutales (comprising an average of 31.0%, 21.3% and 27% of the sequence reads from *C. varians*, *C. rohweri*, and *C. atratus* libraries, respectively), Xanthomonadales (comprising an average of 23.7%, 12.8% and 5.4% of the sequence reads from *C. varians*, *C. rohweri* and *C. atratus* libraries respectively), Burkholderiales (comprising an average of 13.9%, 24.5% and 32.4% of the sequence reads from *C. varians*, *C. rohweri* and *C. atratus* libraries respectively), and Pseudomonadales (comprising an average of 2.8%, 24.8% and 23.7% of the sequence reads from *C. varians*, *C. rohweri* and *C. atratus* libraries, respectively). Rhizobiales were found in clone libraries of *C. varians* and *C. atratrus* (found in an average of 7.3% and 11.6% of sequence reads from libraries of these two respective species), but not *C. rohweri*. However, this microbe was detected in the lone worker that was profiled with diagnostic screening (Table S1, Supporting information). In spite of the apparently low titers of this bacterium, previous studies have found Rhizobiales to be ubiquitous within *C. varians* and possibly across the Cephalotini. The other aforementioned orders were similarly found at high incidence across *Cephalotes* species and colonies of *C. varians* (Russell *et al.* 2009). However, it should be noted that the one male from this study (from *C. rohweri*) did not appear to harbor any of these typical bacterial groups. And while it did contain Opitutales bacteria, the modestly sampled community from a *C. rohweri* queen that had no contact with workers after eclosion was also different from those of conspecific workers of this species.

In addition to the above-mentioned abundant microbes, rarer microbes from other bacterial taxa were found in *Cephalotes* ants, adding to the repertoire reported previously (Russell *et al.* 2009). For instance, *C. varians* harbored Campylobacterales (detected in the libraries of 5/13 surveyed workers, with 8.7% average prevalence per worker), Flavobacteriales (detected in the libraries of 8/13 surveyed workers, with 3.0% average prevalence per worker), Sphingobacteriales (detected in the libraries of 9/13 workers, with 2.9% average prevalence per worker) and *Spiroplasma* (detected in the libraries of 2/13 workers, with 5.7% average prevalence per worker). These were not detected in the other examined *Cephalotes* species, and no rare microbes from these other cephalotines were found in more than one worker.

Examination of the clone library from *P. batesi*, another cephalotine, revealed the presence of Burkholderiales (5.9% of sequence reads), Pseudomonadales (5.9% of sequence reads), Rhizobiales (35.3% of sequence reads), Sphingobacteriales (5.9% of sequence reads) and Xanthomonadales (11.8% of sequence reads). Subsequent analyses (detailed below) revealed that these were closely related to microbes from the same taxa in *Cephalotes* ants. The sampled *P. batesi* worker also harbored microbes that classified with low bootstrap support to the Epsilonproteobacteria (29.4% of the sequence reads), which were unrelated to microbes from other cephalotines.

Microbes from the above mentioned groups were rare or absent in the other studied ant species, with the exceptions of Rhizobiales in a single *Pheidole* species and both Rhizobiales and Burkholderiales in two herbivorous ant species – *T. attenuata* and *Dolichoderus* sp. Aside from these patterns of broadly distributed bacterial groups, two other notable trends were evident, as first reported in Funaro *et al.* (2011). First, carnivorous army ants from three species in the tribe Ecitonini (*Cheliomyrmex* sp., *E. burchellii* and *N. hartigii*) shared bacteria from the Entomoplasmatales. Similarly, adult army ant workers from three genera (*E. burchellii*, *L. praedator* and *N. hartigii*) harbored related bacteria from an unclassified division of the Firmicutes.

Phylogenetic and UniFrac analyses

The 16S rRNA phylogeny of all 959 sequences (from 39 clone libraries spanning 27 colonies across 13 species) is presented in Fig. 1 while the same tree, with branch lengths drawn to scale, is presented in Fig. S1 (Supporting information). Visible on this tree were several clades of bacteria that were enriched among the Cephalotini, including two that were also shared with unrelated herbivorous ants. Bacteria from predatory army ant species were found in completely different parts of this tree, with those from the Entomplasmatales and an unclassified group within the Firmicutes exhibiting remarkable prevalence among the sampled libraries. We used this topology (with branch lengths) for analyses in Fast UniFrac, initially comparing communities between individual ants. PCoA results from weighted and normalized UniFrac analyses are presented in Fig. S2 (Supporting information). Bacterial communities from ants of the genus *Cephalotes* grouped together along the first PCoA axis. An exception to this trend was seen for a community from three pooled adult *C. varians* workers from a single colony (CSM1323, magenta triangle), which was mostly comprised of *Spiroplasma*. Also grouping with *Cephalotes* communities on the first PCoA axis were communities from *T. attenuata* (an unrelated herbivore), a *Dolichoderus* species (an unrelated herbivore), and *P. batesi* (another cephalotine). At the other end of this axis were communities from army ants, reflecting findings that their microbes come from different taxonomic groups than bacteria from the cephalotines.

Along the second PCoA axis we observed separation between two moderately sampled *C. varians* colonies (CSM1169 – red hexagons, and CSM1280 – gold circles). ANOVA analyses utilizing the raw PCoA data indicated that the communities from these species were significantly different ($P < 0.001$; data not shown). This finding was supported by a UniFrac significance test that revealed highly significant differentiation among these two colonies ($P < 0.001$). In addition, three out of four communities from *C. rohweri* grouped near the upper reaches of the second axis – above those from other *Cephalotes*. However, communities from all species in this genus showed at least some overlap on this axis and both others.

To better visualize the differences between communities of different species we pooled data from all conspecific adult workers (and larvae, for *E. burchellii*), running weighted and normalized UniFrac analyses for all resulting species libraries with $n \geq 15$ sequence reads. The results are presented in Fig. 2. Communities from the three *Cephalotes* species showed strong clustering along each of the first three PCoA axes. These, in turn, grouped with communities from *P. batesi* and *T. attenuata* along the first axis, which explained 69.25% of the variation. Communities from three army ants in the Ecitoninae grouped at the opposite end of axis 1, clustering along axis 2 as well.

To determine the significance of the observed differences we subsequently performed UniFrac significance tests on pooled communities from these ant species. *P*-values from these analyses are shown in Table 1. After Bonferroni corrections (values shown below the diagonal), only comparisons between species grouping

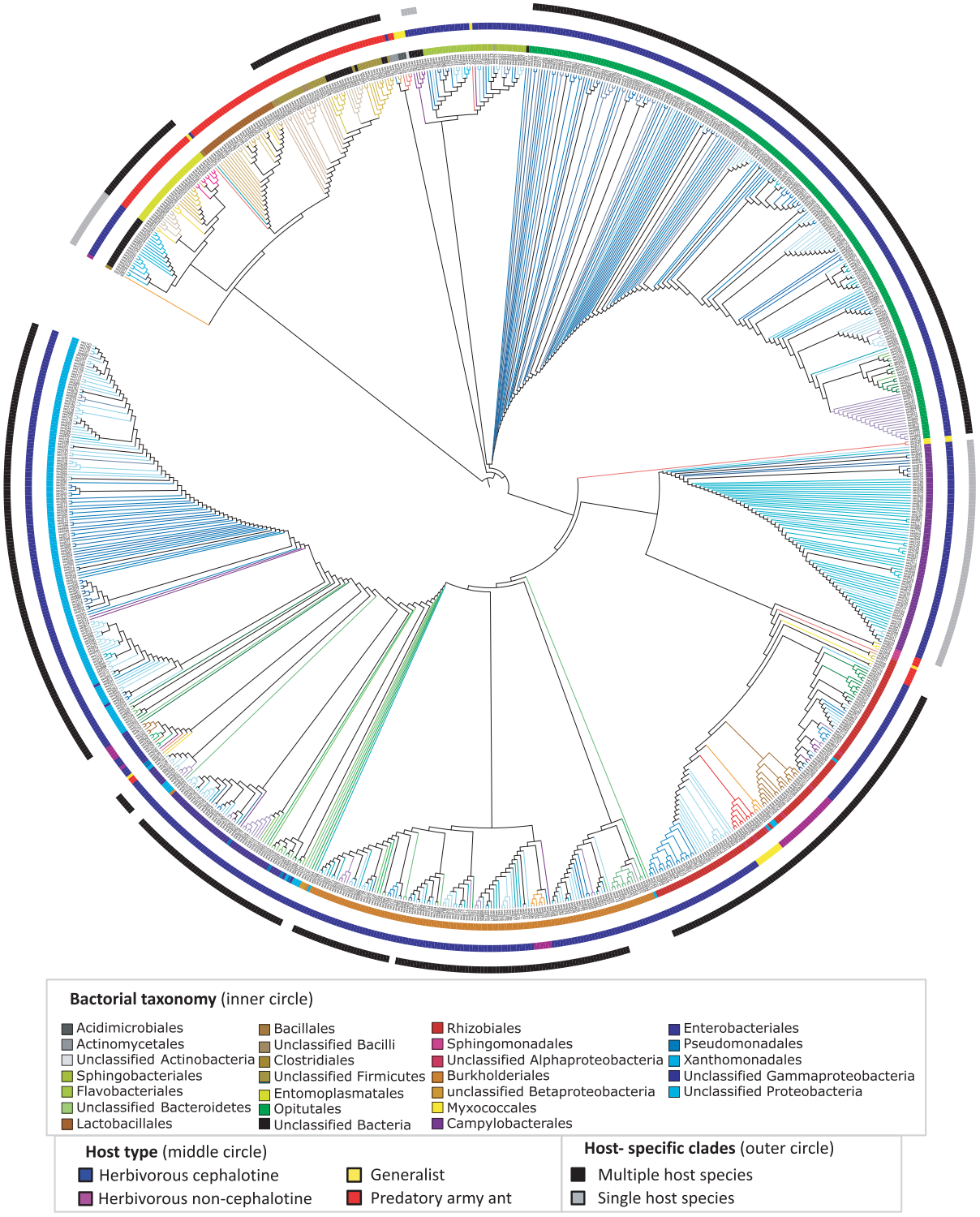

Fig. 1 Phylogeny of bacteria from ants. Rooted maximum likelihood phylogeny reveals host-specificity and relatedness among bacteria from several studied ant species, most notable for microbes from related and trophically similar ants. All bacteria in this tree were sampled via universal PCR, cloning, and Sanger sequencing. Branch colors (see Fig. S1 for full color key) illustrate host taxonomy, colony ID's, and worker ID's for each bacterium in our study. Colors within the inner circle illustrate the taxonomic classification for the bacterial OTUs. The taxonomic/trophic level category for the studied ant hosts is indicated by colors in the middle concentric circle. Black and grey shading within the outer circle is used to highlight ant-specific clades of bacteria (see Fig. S3 for full detail). Note that the branch lengths are not drawn to scale here to facilitate viewing.

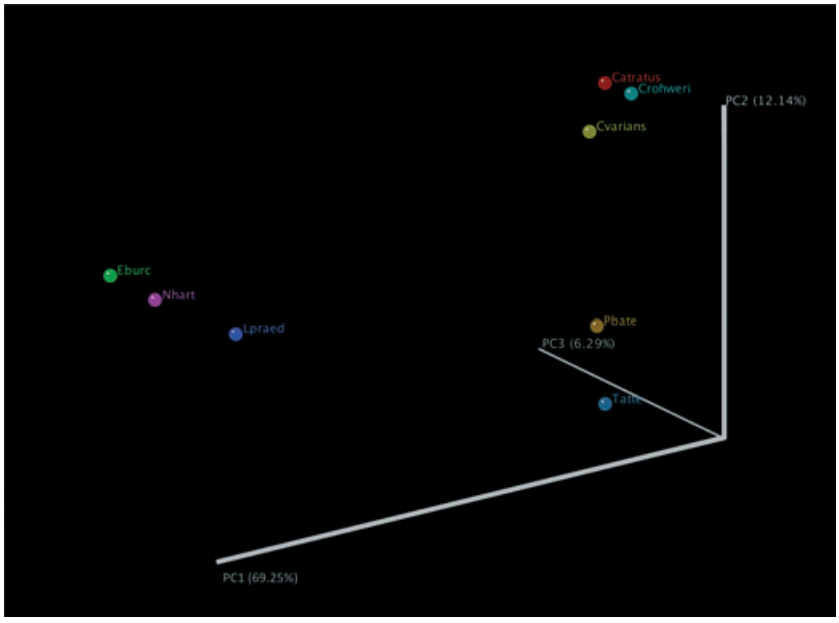

Fig. 2 PCoA analysis of bacterial communities from different ant species. Libraries from individual ants were pooled and included in this analysis for all species with $n \geq 15$ sequences. Positions of the bacterial communities for each species along the three first principal component axes are illustrated in this figure, along with the percentage of variation explained by each axis.

at opposite ends of PCoA axis 1 remained significant, further underscoring the similarities of communities from cephalotines (along with *T. attenuata*).

Interpreting the finer-scale trends of variation between microbial communities

In assessing relatedness among the identified bacteria, it was previously reported that the Entomoplasmatales from Ecitoninae ants formed a monophyletic clade with those from related Aenictinae and Dorylinae army ants (Funaro *et al.* 2011). Similarly, bacteria from the unclassified Firmicutes group found in *E. burchellii*, *L. praedator*, and *N. hartigii* formed a lineage that was sister to a bacterium from an unrelated predatory ant (*Leptogenys* sp.) (Funaro *et al.* 2011).

A previous study had also suggested that many bacteria from cephalotines belonged to ant-specific clades (Russell *et al.* 2009). To determine whether this was true for the microbes identified in this study, we performed seven separate phylogenetic analyses using these sequences, along with previously identified ant associates from this group, selected outgroup sequences, and top BLASTn hits. The resulting maximum likelihood phylogenies for these seven groups (Bacteroidetes, Burkholderiales, Entomoplasmatales, Epsilonproteobacteria, Gammaproteobacteria, Rhizobiales and Opitutales) are presented in Fig. S3 (Supporting information).

One to four major clades exclusively consisting of ant associates were identified within each of the above bacterial taxa. These were divided into subclades in cases when substantial proportions of sequences fell into distinct lineages within these larger clusters. Separate subclades typically consisted of sequences from different 97% OTU's, hinting that they were comprised of distinct species according to accepted definitions

Table 1 UniFrac distances (above diagonal) and Bonferroni-corrected *p*-values (from UniFrac significance tests; below diagonal) based on weighted, normalized pairwise comparisons between bacterial communities from different ant species.

	C. atratus	*C. rohweri*	*C. varians*	*E. burchellii*	*L. praedator*	*N. hartigii*	*P. batesi*	*T. attenuata*
C. atratus	–	0.271	0.313	0.927	1	1	0.532	0.585
C. rohweri	1	–	0.351	0.952	1	1	0.542	0.585
C. varians	1	0.308	–	0.911	0.994	0.987	0.438	0.557
E. burchellii	*≤0.001*	*≤0.001*	*≤0.001*	–	0.521	0.444	0.925	0.923
L. praedator	*≤0.001*	*≤0.001*	*≤0.001*	1	–	0.421	1	1
N. hartigii	*≤0.001*	*≤0.001*	*≤0.001*	1	1	–	1	1
P. batesi	1	1	1	*≤0.001*	*≤0.001*	0.084	–	0.455
T. attenuata	0.98	0.7	0.224	*≤0.001*	*≤0.001*	*≤0.001*	1	–

(Stackebrandt & Goebel 1994). OTU assignment and designation of clade and subclade membership, where applicable, are presented in Table S1 (Supporting information).

While clades identified in a previous study (Russell *et al*. 2009) were, again, found to be ant-specific (see Fig. 1 for an overview), we also detected novel ant-specific lineages in the Burkholderiales, Entomoplasmatales, Campylobacterales, Flavobacteriales, unclassified Epsilonproteobacteria and Sphingobacteriales (Fig. S3, Supporting information). All but one of these consisted of microbes from just a single host species, typically *C. varians*. However, with just one exception (the unclassified Epsilonproteobacteria), these microbes were found in workers originating from multiple colonies, suggesting that they may be common members of microbial communities from the studied ants.

After assigning relevant sequences to ant-specific clades (Table S1, Supporting information), all remaining sequences not binned into such lineages were grouped into orders or to the next highest taxonomic level with at least 80% bootstrap support in RDP analyses. The proportional representation of each clade or taxon was then assessed for libraries with ten or more sequence reads. Results were presented as a heatmap in Fig. 3. To illustrate relatedness among the gut communities,

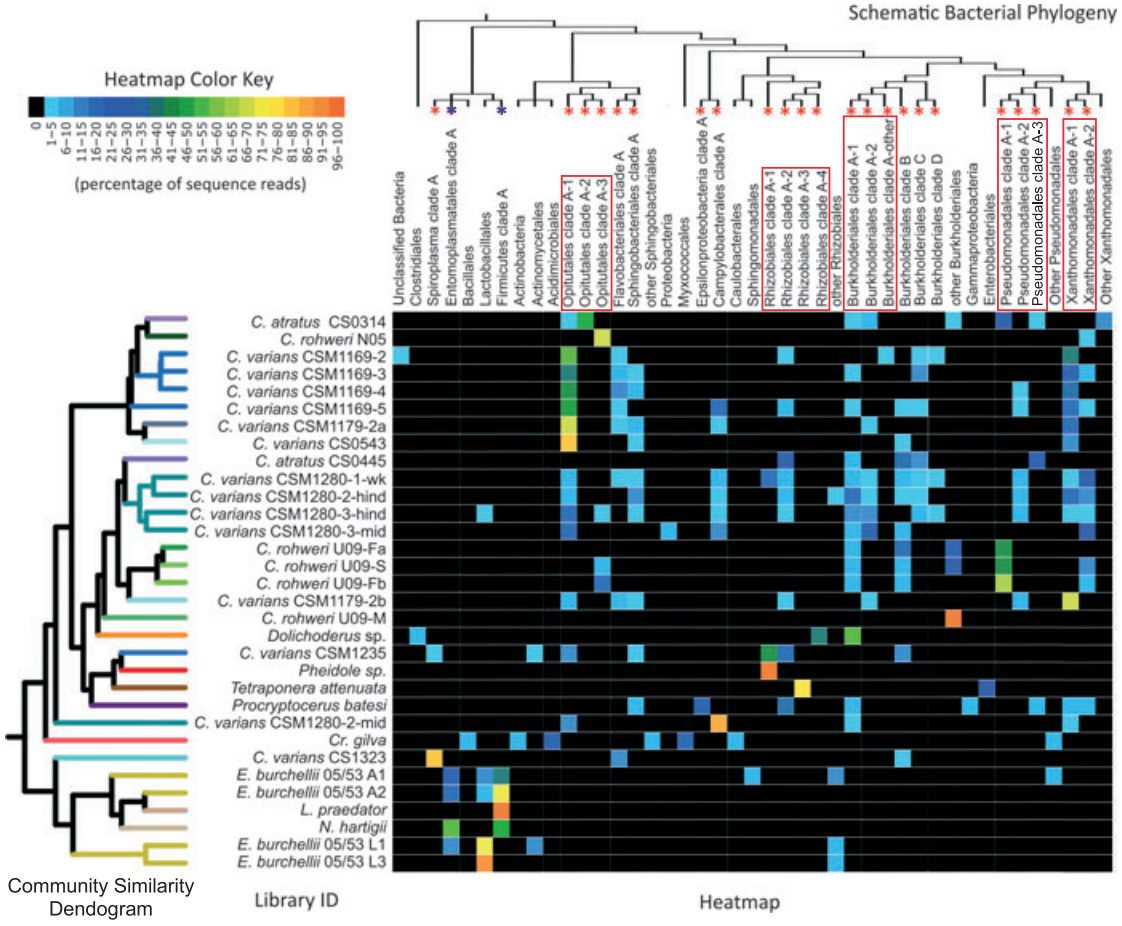

Fig. 3 Variation in the microflora harbored by ants revealed for whole libraries and individual bacterial taxa. Colors in each cell of the heatmap reveal the proportion of sequence reads from each clone library (rows) within each of the identified bacterial taxa or clades (columns; see methods and Table S1 for further details). All identified ant-specific clades (see Fig. S3) are indicated with asterisks on the schematic bacterial tree at the top of the figure (red = clade or sub-clade consisting of bacteria from Cephalotini ants; purple = clade consisting only of bacteria from non-Cephalotine ants). The dendrogram on the left illustrates microbial community similarity based on the results of a UniFrac jackknife cluster analysis. Note that branches of this dendrogram are colored to indicate colony of origin. With regard to the bacterial tree at the top of this figure, it should be noted: 1) that lineages, or ''sub-clades'', contained within open red boxes were found to cluster together within larger ant-exclusive clades on our trees, and 2) that listed taxonomic groupings that were not monophyletic in relation to other listed clades/taxa are not connected to the schematic bacterial tree (based on Ciccarelli et al. 2006). Finally, the profiled communities came from various ant tissues (i.e. various portions of the gut or whole workers—see Table S1 for a detailed description). All but three sampled ant hosts (*C. rohweri* U09Ms—a male; *E. burchellii* 05/53 L1 & L2s—both larvae) were adult workers.

we combined the heatmap image with a dendrogram generated through cluster analysis in Fast UniFrac. This tree was constructed with a jackknifing analysis, in which each replicate utilized pairwise, weighted, and normalized UniFrac distances generated with 10 randomly selected sequences from each library.

Patterns of community level variation among cephalotine species were related to the differential presence or abundance of microbes within ant-specific clades and subclades. For instance, Rhizobiales were not detected in the universal libraries of *C. rohweri*, while these bacteria were represented in at least some *C. atratus*, *C. varians* and *P. batesi* workers. Additionally, *C. varians* workers harboring Opitutales were all colonized by members of subclade A-1, with just one instance of colonization by a member of subclade A-3. In contrast all Opitutales from *C. rohweri* grouped into subclade A-3, while *C. atratus*-associated Opitutales grouped into a separate cluster (subclade A-2). Along similar lines, only *C. atratus* and *P. batesi* harbored members of Pseudomonadales subclade A-3, while three of four *C. rohweri* workers, plus one worker from *C. atratus*, harbored members of subclade A-1. *C. varians* microbes from this ant specific Pseudomonadales lineage all belonged to subclade A-2. Finally, Xanthomonadales bacteria from an ant-specific clade were found in 12 of 13 *C. varians* and three of four *C. rohweri* workers. Those from *C. rohweri* came exclusively from subclade 2, in contrast to *C. varians*, which harbored members of subclades 1 and 2 – sometimes within the same individuals. Although prior diagnostic PCR screens and phylogenetic analyses (Fig. S3, Supporting information) detected these Xanthomonadales in *C. atratus* (Russell *et al.* 2009), they were not found in universal 16S rRNA libraries from this species, suggesting that they were rare members of their hosts' microbial communities.

Variation among two moderately-sampled *C. varians* colonies that harbored significantly different bacterial communities was driven, in part, by the enrichment of Opitutales (from clade A-1) within workers from colony CSM1169. Specifically, bacteria from this group comprised 39.4–52% of the sequence reads from the four studied workers, compared to workers of colony CSM1280, for which Opitutales comprised from 5.9 to 11.6% of clone library sequence reads. Workers from CSM1169 also harbored higher levels of Xanthomonadales – with a median of 35.1% vs. 23.5% for those of CSM1280. The communities from workers in this latter colony were comparatively balanced, consisting of bacteria from numerous clades and subclades found at similar levels. However, the combined representation of Burkholderiales (from clades A-D) was notably greater in CSM1280 (median = 29.6% of sequence reads) than in CSM1169 (median = 6.9% of sequence reads).

Discussion

The diversity and variation of bacterial communities

Our findings reveal clear differences in the bacterial communities harbored by different ant species. They also highlight the similarity of microflora harbored by: (i) predatory ants from the army ant tribe Ecitonini; (ii) herbivorous ants of the tribe Cephalotini and (iii) to a lesser extent, distantly related herbivorous ants (Table 1, Figs 1 and 2). Although only some of the studied communities were isolated from ant guts, it is interesting to note that *C. varians* communities from whole ants showed no major differences related to sampling of mid-gut, hind-gut, whole-gut, or whole ant tissues. This finding, combined with previous localization of many of the studied *Cephalotes* bacteria to the gut environment (Roche & Wheeler 1997; Russell *et al.* 2009; Bution & Caetano 2010a,b), suggests that the elucidated cephalotine communities are largely gut associated. This is likely true for several bacteria identified from other ants in this study. Indeed, some of the bacteria detected from *Dolichoderus* and *Tetraponera* have been localized to the guts of species from these genera (Stoll *et al.* 2007; Russell *et al.* 2009).

In spite of the different primers used to sample *Cephalotes* communities (*C. rohweri* vs. those from *C. varians* and *C. atratus*), we still found no significant differences in the bacterial communities harbored by workers of different cephalotine species (Table 1). And while the use of different primer sets could have led to some more subtle systematic differences between these species, they are unlikely to have yielded the trends of different subclade prevalence seen for *C. rohweri* vs. *C. varians.* As such, it appears that these species show a tendency to harbor microbes from related, but nevertheless distinct lineages within the Burkholderiales, Opitutales, Pseudomonadales, Rhizobiales and Xanthomonadales (Fig. 1). A large majority of microbes sampled from the cephalotine tribe fell into predominantly cephalotine-specific clades with representatives from multiple colonies, if not multiple species; this was true for *C. atratus* (90% of 50 reads), *C. rohweri* (87.3% of 55 reads), *C. varians* (99.2% of 622 reads) and *P. batesi* (64.7% of 17 reads). Two of these clades also harbored microbes from known herbivorous ants, including those from the genera *Tetraponera* and *Dolichoderus*.

Similar to this finding, an average of 90.5% of the sequence reads from adult *E. burchellii*, *L. praedator* and *N. hartigii* workers fell into one of two army-ant specific clades. One of these was the sister clade to a bacterium previously found in an unrelated, predatory ant from the genus *Leptogenys*. It should be noted that some of the army ants considered in this study were selected a

priori due to the detected presence of Entomoplasmatales. This makes it possible that these trends are not reflective of the group at large. And, furthermore, limited sampling across the army ants makes it difficult to draw firm conclusions for this group. However, our findings of related microbes in taxonomically and geographically distant cephalotines suggest the existence of a core microbiome from *Cephalotes* and *Procryptocerus* ants. They also indicate that the core gut microbes are typically represented by lineages that are either specialized on, or enriched for a lifestyle in the guts of cephalotines and possibly other herbivorous ants. Previous findings have similarly hinted at the presence of a specialized, core gut flora from other herbivorous *Cephalotes*, *Pseudomyrmex* and *Tetraponera* species (Billen & Buschinger 2000; Stoll *et al.* 2007; Bution & Caetano 2008; Eilmus & Heil 2009; Russell *et al.* 2009). In light of these trends it seems plausible that many ecologically similar and related groups of ants could harbor their own varieties of domesticated microbes.

While we have likely identified much of the core cephalotine microbiota, it remains possible that other microbes will be uncovered in other species of Cephalotini or as rare members of their hosts' gut communities. Relating to this latter point, rarefaction curves on the studied communities did not reach an asymptote, revealing that we have not yet identified the full diversity of bacteria within the sampled workers, colonies, or species (Fig. S4, Supporting information). While next generation sequencing methods could potentially reveal additional bacterial lineages, much of the uncovered diversity appears to belong to ant-specific clades already identified. For instance, eight out of the twelve singleton OTU representatives from the *C. varians* species library were from one of the aforementioned ant-specific clades. This was true for 11/13 singletons from *C. rohweri* and 6/7 from *C. atratus*. Given the 97% cut-off for OTU designation and the fact that ambiguous base pairs were not factored into distance calculations, it does not seem likely that such rare reads are simply a reflection of sequencing or PCR error. Instead, they likely extend from the presence of rare microbes.

Habitat and dietary differences across the Cephalotini suggest a core gut microbiota

No single group of bacteria showed phylogenetic trends with unequivocal support for codiversification. However, bacterial lineages harboring subclades that were exlclusive (or nearly so) to one or two host species (Opitutales clade A, Pseudomondales clade A) suggest the potential for codiversification and long-term maintenance through phoresis and trophallaxis (Fig. S3a,b, Supporting information). Although bacteria from other lineages may also codiversify with ants (i.e. Burkholderiales clade A, Rhizobiales clade A, Xanthomonadales clade A), close relatedness between microbes from different ant species suggests the possibility of occasional horizontal transfer or environmental acquisition. Conceivable sources for such acquisition include soil, fungi, food, and the host tree phyllosphere.

Despite the potential for environmental acquisition, several lines of evidence suggest that this factor is not driving the trends of similar gut communities across the Cephalotini. For instance, *C. varians* occurs in the Florida Keys, nesting primarily in mangroves; *C. atratus* is widespread throughout the mainland Neotropics, occurring in a wide variety of host trees; and *C. rohweri* is found in the Sonoran desert, nesting primarily in Palo Verde trees (de Andrade & Baroni Urbani 1999). The lack of geographic overlap and the known differences among microbial phyllosphere communities from different tree species (Redford *et al.* 2010) makes it unlikely that similar microbes are acquired from such disparate environments.

Additionally, the dietary variation seen across the Cephalotini (Creighton & Gregg 1954; Creighton & Nutting 1965; Creighton 1967; de Andrade & Baroni Urbani 1999) suggests a need to invoke alternative explanations for the observed bacterial distributions. Trophic levels inferred from stable isotope analysis indicate that several exudate-feeding arboreal ant species are herbivores with carbohydrate-rich and nitrogen-poor diets (Blüthgen *et al.* 2003; Davidson *et al.* 2003). Given the sugar-rich resource of extrafloral nectaries and hemipteran honeydew hypothesized to support most aboreal ants, including some cephalotines, it is interesting that we did not discover lactic or acetic acid bacteria among the microbiota. This suggests that ephemeral access to, or a complete lack of sugars on many host trees has likely contributed to the evolution of the core cephalotine microbiota.

Cephalotines are likely leaf foragers (*sensu* Cook & Davidson 2006), scouring the plant surface for pollen, fungi, excrement, as well as other more cryptic sources of nutrition. As such, dietary differences among these arboreal ants are likely governed by the host trees, which can directly or indirectly alter the available sources of nutrition. Directly, the trees provide sap, pollen, and possibly nectaries; indirectly, host trees attract hemipteran and avian species that produce honeydew and nitrogen rich excrement respectively, and wind-blown pollen from other plants accumulates on their leaves and branches. Since the tree species inhabited by *C. atratus*, *C. rohweri* and *C. varians* exhibit drastic differences in their habitats, taxonomy, and ecology, it would seem that the range of available nutritional resources must differ between these ants. Accordingly, in contrast

to *C. rohweri*, both *C. atratus* and *C. varians* are thought to (i) have greater access to plant-derived carbohydrates and (ii) to scavenge for fresh insect remains (Wilson 1976; Corn 1980; Blüthgen *et al.* 2000). When considering the differences between these ant species it appears that the remarkable similarity of their gut communities is not a reflection of ecological overlap favoring the acquisition of similar microbes. Instead, the compendium of data is most consistent with a long-term, coevolved relationship between the cephalotines and a core gut microbiota.

Metabolic properties of related bacteria suggest putative functions for the members of the core cephalotine community

Although we do not yet have functional data on the roles of gut microbes in cephalotine ants, the detrimental effects of antibiotic treatment (Jaffe *et al.* 2001) and the apparent widespread distribution of a core microbiota hint strongly at their importance. Thus, to derive hypotheses on the roles for specific gut symbionts, we discuss below the known metabolic attributes of the identified core microbes.

Opitutales have the greatest frequency of occurrence in cephalotines, both within and across individuals (Figs 3 and S3a, Supporting information). This bacterial group is aerobic or facultatively aerobic, and some produce external structures (fimbriae and prosthecae) to facilitate host attachment and generate greater surface area for nutrient uptake (Hedlund *et al.* 1997). *Opitutus* can reduce nitrate to nitrite so they may be involved in microbial recycling of nitrogen for reincorporation by the ant. They ferment the mono-, di-, and polysaccharides abundant in plant nectar and insect honeydew, generating propionate and acetate as end products (Chin & Janssen 2002), which may be used by other microbial residents (e.g. *Xanthomonadaceae*). However, during periods of nutrient dearth they are capable of surviving on minimal substrate and growing very slowly (Chin *et al.* 2001). Their growth can be accelerated in the presence of other bacterial species (Chin & Janssen 2002), suggesting they may be well suited to the gut biofilm environment (Roche & Wheeler 1997; Bution & Caetano 2010a).

Xanthomonadales were the next most prevalent group (Fig. S3b, Supporting information), and BLASTn results suggest relatedness with species of *Lysobacter* and *Luteimonas*. *Luteimonas composti* can utilize a few amino acids as carbon substrates as well as acetate and propionate (Young *et al.* 2007), which are end products of *Opitutus* metabolism. The genus *Lysobacter* is incredibly diverse, including species antagonistic to pathogenic fungi, thermophilic representatives from hydrothermal vents, and chemolithotrophs that derive energy from inorganic substances like rock (Emerson & Moyer 1997). As newly emerged workers of *Cephalotes* are colonized by microbes, their ileum gradually darkens, which may be due to the accumulation of byproducts associated with chemolithotrophic metabolism (Caetano & da Cruz-Landim 1985).

While third in overall representation, the Burkholderiales appear to harbor a much wider diversity of taxa (Fig. S3c, Supporting information). According to BLASTn results, sequences were similar to *Pusillimonas* and *Castellianiella*, bacterial genera often associated with the phylosphere and soil. Related *Acaligenaceae* are typically obligate aerobes that utilize organic and amino acids as carbon sources, but not carbohydrates. Some can grow anaerobically using denitrification enzymes, with nitrate or nitrite as the terminal electron acceptor (De Ley *et al.* 1986). Additionally, some *Pusillimonas* can degrade salicylates, compounds found in many pollen types (Stolz *et al.* 2005). From the same phylogenetic neighbourhood, some *Burkholderia* sp. can fix atmospheric nitrogen, while others occur as endophytes of many plants, producing a wide variety of antibiotics with activity against entomopathogenic fungi (Santos *et al.* 2004). Such fungi (i.e. *Aspergillus* sp.) are common and tend to invade the tree where it has been damaged by insects (Boyd & Cotty 2001), the same sites chosen for nesting by cephalotines. The metabolic versatility of *Burkholderia cepacia* allows it to colonize a wide range of ecological niches, including the Hymenoptera, where it is well-represented (Martinsen *et al.* 2010).

A broad range of unrelated ants, in addition to cephalotines, play host to related Rhizobiales (Fig. S3d, Supporting information; Stoll *et al.* 2007; Russell *et al.* 2009), suggesting a deep ant-symbiont history. Within this group, *Bartonella* (the genus containing the ant-specific Rhizobiales clade) are composed of facultative intracellular parasites, considered versatile opportunistic pathogens of animals. Arthropods serve as the vectors, but little is known about the interactions between *Bartonella* and insects (Minnick & Anderson 2000). *Bartonella* occurs as part of the core microbiota of the honey bee, but does not appear to be associated with pathogenicity (Cox-Foster *et al.* 2007; Martinsen *et al.* 2010). Considerable microscopy data reveals no intracellular bacteria in *Cephalotes*, although some bacteria are intimately associated with the hind gut, and others occupy the endo-peritrophic space of the mid-gut (Roche & Wheeler 1997; Bution & Caetano 2008, 2010a). *Bartonella* are well adapted to the arthropod gut environment and a lack of carbohydrates, and can derive carbon and energy from the catabolism of amino acids rather than glucose (Chenoweth *et al.* 2004).

Sequences corresponding to the Pseudomonadales (Fig. S3b, Supporting information) include distant (90%) BLASTn hits to many *Pseudomonas* spp. and *Azot-*

obactor spp. now considered a synonym, or at least sister genus to *Pseudomonas* (Young & Park 2007). This group is metabolically diverse, composed of members capable of colonizing a wide range of eukaryotic and environmental niches and some that fix nitrogen under aerobic conditions. The ileum of *Cephalotes* spp. is inundated with both bacteria and tracheoles suggesting that this niche could support aerobic metabolism (Bution & Caetano 2010a). As an example of metabolic diversity, *Pseudomonas* can respire using nitrate as an electron acceptor rather than oxygen, releasing nitrogen gas as a byproduct into the gut microenvironment under anaerobic conditions. *Pseudomonas aeruginosa* can flourish in a biofilm environment and survive entirely on a diet of uric acid, utilizing it as a sole source of carbon, nitrogen, and energy (Rouf & Lomprey 1968). Although enzymatic assays from cultivable bacteria revealed no urocolytic activity in *Cephalotes* (Yurman & Dominguez-Bello 1993) we hypothesize that the processing of uric acid into usable forms of nitrogen may be one role played by the microbial flora.

Conclusions and future directions

Many factors may affect the presence and abundance of gut symbionts including diet, age, disease state and behaviour (Dillon & Dillon 2004). In social insects, the rates of trophallaxis and microbial succession following adult emergence are likely important variables affecting colony health (Anderson *et al.* 2011). Our results suggest differences in microbial communities among workers within colonies, although these were typically smaller in magnitude compared to inter-colony differences. Nevertheless, future investigations are needed to determine whether these were simply due to limited sampling, random artifacts of individual PCR reactions, or whether they represent actual differences. In the latter case, it would be interesting to ascertain if this inter-worker variation relates to stochastic shifts in the starting communities (i.e. inocula received through oral-anal trophallaxis), different worker ages or different roles for workers in colony behaviour, or slight differences in diet. The newly eclosed *Cephalotes rohweri* queen intentionally denied trophallaxis with workers contained potentially pathogenic species related to *Escherichia* and *Erwinia* (Table S1, Supporting information). The absence of these bacteria in other colony members that performed trophallaxis suggests that early colonization is important for pathogen protection. In vertebrates, early colonization can dramatically alter community assembly, and the ability of a climax community to inhibit pathogen colonization is considered the most important function of the indigenous insect gut microbiota (Dillon & Dillon 2004).

The larval stage was not sampled from cephalotines in this study, but previous investigations of social insects indicate that larval microbial communities can differ drastically from those of adults (Gilliam 1997; Evans & Armstrong 2006; Ishak *et al.* 2011). Adults possess a highly specialized proventricular valve (Eisner 1957), restricting the flow of particulate matter to the midgut where digestion occurs (Roche & Wheeler 1997). Larvae have a very different digestive tract, and unlike adults, can typically digest solid food, resulting in liquid nutrients that are redistributed to adults in glandular and hemolymph fluid secretions via oral trophallaxis (Wheeler 1994). This form of ''group digestion'' is thought to be common throughout higher ants (Vinson & Sorensen 1986; Cassill *et al.* 2005). Future investigations of *Cephalotes* should detail the microbiota harbored by different larval instars, and the role each may play in colony-level nutrient acquisition and processing.

The compendium of microscopy data from *Cephalotes* suggests that the adult symbiotic microbial community is primarily adapted to life in the distal mid-gut and hind-gut (Caetano & da Cruz-Landim 1985; Roche & Wheeler 1997). Some members of the bacterial communities reported here may play a role in nitrogen recycling, occurring in a specialized hindgut structure (Bution & Caetano 2008, 2010a; Russell *et al.* 2009). In terms of general function, the core microbial community of *Cephalotes* is composed of bacteria that can subsist without carbohydrates, on minimal media, and tend to occur in a biofilm environment. The bacterial taxa potentially serve in pathogen protection, denitrification, uric acid metabolism, nitrogen fixation, and the preferential catabolism of amino or organic acids. Indeed, the ubiquitous distributions of several cephalotine-specific bacterial lineages with 60 million years since common ancestry of their hosts (Moreau *et al.* 2006) suggests that gut bacteria have contributed significantly to cephalotine evolution.

When we combine findings from cephalotines with those from other herbivorous and predatory ants (Stoll *et al.* 2007; Caetano *et al.* 2009; Eilmus & Heil 2009), the emerging trends are consistent with the presence of host-specific microbes across many ant groups, and the sharing of related microbes by trophically similar ants. Clearly, the time is right to pursue the roles, costs, and benefits of this diverse collection of mysterious microbes, piecing together their contributions to the ecology and evolution of the ant family Formicidae.

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K.E.A. is broadly interested in the ecology and evolution of social species, cooperation and mutualisms. J.A.R. studies the ecology and evolution of symbioses between insects and microbes. C.S.M. studies the origin and evolution of species, particularly patterns of diversification in the ants. S.K. is interested in how interspecific interactions shape the ecology and evolution of ants. K.E.S. is primarily interested in the trophic ecology, digestive physiology, and gut bacterial communities of fish. Y.H. performs research on gut symbionts of ants and their roles in ant nutrition. U.B, B.M.M. and N.B. research many aspects of Hymenopteran molecular and microbial ecology. D.W. studies caste related phenomena, ant digestive morphology and microbial symbioses.

Data accessibility

DNA sequences: GenBank accessions JQ254100 – JQ254882, See Table S2 (Supporting information) for further accession numbers and related information.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogeny of 16S rRNA sequence libraries with branch lengths drawn to scale.

Fig. S2 UniFrac PCoA analyses on each library with $n \geq 10$ sequences.

Fig. S3 Phylogenies of bacterial taxa harboring ant-specific clades.

Fig. S4 Rarefaction analyses of microbial communities from individual ants, colonies and species.

Table S1 Information on the sequences utilized in this study.

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