

## SPECIAL ISSUE: NATURE'S MICROBIOME

# Correlates of gut community composition across an ant species (*Cephalotes varians*) elucidate causes and consequences of symbiotic variability

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## Abstract

Insect guts are often colonized by multispecies microbial communities that play integral roles in nutrition, digestion and defence. Community composition can differ across host species with increasing dietary and genetic divergence, yet gut microbiota can also vary between conspecific hosts and across an individual's lifespan. Through exploration of such intraspecific variation and its correlates, molecular profiling of microbial communities can generate and test hypotheses on the causes and consequences of symbioses. In this study, we used 454 pyrosequencing and TRFLP to achieve these goals in an herbivorous ant, *Cephalotes varians*, exploring variation in bacterial communities across colonies, populations and workers reared on different diets. *C. varians* bacterial communities were dominated by 16 core species present in over two-thirds of the sampled colonies. Core species comprised multiple genotypes, or strains and hailed from ant-specific clades containing relatives from other *Cephalotes* species. Yet three were detected in environmental samples, suggesting the potential for environmental acquisition. In spite of their prevalence and long-standing relationships with *Cephalotes* ants, the relative abundance and genotypic composition of core species varied across colonies. Diet-induced plasticity is a likely cause, but only pollen-based diets had consistent effects, altering the abundance of two types of bacteria. Additional factors, such as host age, genetics, chance or natural selection, must therefore shape natural variation. Future studies on these possibilities and on bacterial contributions to the use of pollen, a widespread food source across *Cephalotes*, will be important steps in developing *C. varians* as a model for studying widespread social insect-bacteria symbioses.

**Keywords:** bioinformatics/phyloinformatics, insects, microbial biology, next-generation sequencing, species interactions, symbiosis

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## Introduction

Bacterial symbionts are considered to be key innovations behind the evolutionary success and ecological dominance of numerous animals and plants (Buchner 1965; Long 1989; Moran *et al.* 2008; Moya *et al.* 2008; Gibson & Hunter 2010). Among the many animal hosts of influential microbes, insects are the most diverse and arguably the best-studied, harbouring a large number

of symbiotic bacteria with roles in nutrition (Douglas 1998; Akman *et al.* 2002; Feldhaar *et al.* 2007) and protection against natural enemies (Oliver *et al.* 2003, 2009; Kaltenpoth *et al.* 2005; Jaenike *et al.* 2010). Many studies of symbioses between insects and microbes have focused on vertically transmitted endosymbionts, living inside of host cells, tissues and haemolymph. Yet a large number of symbionts exist as members of complex bacterial communities within insect guts (Dillon & Dillon 2004), as seen for other animals, where they shape nutrition, digestion and defence (Ohkuma 2003; Koch & Schmid-Hempel 2011; Engel *et al.* 2012).

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Gut communities can vary across different scales, even within animal species harbouring fairly stable, core gut bacteria (e.g. Roeselers *et al.* 2011). Such variation is often related to differences in the external or internal environment (Schmitt-Wagner *et al.* 2003; Hongoh *et al.* 2006; Behar *et al.* 2008), and experimentation confirms that this may be partially due to community plasticity. Indeed, shifts in gut microbiota can occur in response to changing diet (Kane & Breznak 1991; Turnbaugh *et al.* 2009b), parasite infection (Koch *et al.* 2012) or host ageing (Dillon *et al.* 2010). Importantly, changes in the types and abundance of gut microbiota can have measurable consequences (Hosokawa *et al.* 2007), affecting several physiological processes within the guts of insects and other animals (Santo Domingo *et al.* 1998; Turnbaugh *et al.* 2006; Dillon *et al.* 2010). Thus, findings of natural variation in symbiotic gut communities may indicate important host-level differences in bacterially mediated traits.

Ants number among the many insects engaging in symbioses with bacteria; and in particular, herbivorous ants are hypothesized to depend on the contributions of nutritional symbionts due to the suspected low quality of their diets (Davidson *et al.* 2003; Russell *et al.* 2009). To date, carpenter ants are the only such group known to receive nutritional benefits from internally housed symbionts (Feldhaar *et al.* 2007). But other herbivorous ants engage in symbioses with large masses of gut bacteria, exhibiting novel gut morphologies that have arguably evolved to harbour these microbes (van Borm *et al.* 2002; Cook & Davidson 2006; Stoll *et al.* 2007; Bution & Caetano 2008; Russell *et al.* 2009). These communities have not generally been studied in depth (but see Kautz *et al.* 2013), but broad explorations of ant-associated microbes hint at trophic level and host phylogeny as correlates of gut community composition. Furthermore, phylogenetic analyses indicate that ant gut associates often come from ant-specific lineages on 16S rRNA phylogenies (Russell *et al.* 2009; Anderson *et al.* 2012). Together, these findings suggest herbivorous ants have evolved with a specialized coterie of nutritional gut symbionts.

Proximate impacts of ant diet on gut communities could contribute to the correlation between symbiotic composition and trophic level, and explorations at the intraspecific host level are needed to test this possibility. But to date few studies have explored natural intraspecific variation of symbiotic bacteria from ant guts (see Bution *et al.* 2010 and Lee *et al.* 2008 for exceptions; see also Andersen *et al.* 2013 for an intraspecific study on cuticular microbiomes). To address this, and to therefore elucidate the forces shaping symbioses across these all-important insects, we report in this study on natural variation and plasticity of gut communities within the turtle ant, *C. varians*.

The large masses of gut bacteria and the herbivorous and pollen-feeding habits of *Cephalotes* ants (Baroni-Urbani & de Andrade 1997; de Andrade & Baroni-Urbani 1999; Bution & Caetano 2010) make them intriguing candidates for such studies. This is especially true in the light of similar traits across eusocial bees, whose gut bacteria aid in defence and, probably, pollen digestion (Koch & Schmid-Hempel 2011; Engel *et al.* 2012). Further motivation for *Cephalotes* studies comes from the widespread distributions of potentially ant-specific gut symbionts across this genus (Russell *et al.* 2009; Sanders *et al.* 2014), suggestive of long-standing associations. In spite of this stability, preliminary findings still hint at the potential for subtle variation in gut communities between *C. varians* colonies (Anderson *et al.* 2012), with currently unknown causes. And while limited PCR screening and sequence-based sampling suggest that core gut taxa in *C. varians* are stable in workers reared on artificial diets (Russell *et al.* 2009), the employed molecular techniques have not been sufficient to rule out the gain or loss of rare bacteria, or shifts in the abundance of predominant symbiont strains and species.

Using 454 amplicon pyrosequencing of the 16S rRNA gene, we examined variation in adult worker gut communities across nine colonies of *C. varians* from four different islands in the Florida Keys, USA. In addition, we applied both 454 pyrosequencing and Terminal Restriction Fragment Length Polymorphism (TRFLP) to study plasticity in worker gut communities, comparing those from field-caught vs. laboratory-reared workers and from laboratory-reared worker ants fed on different diets. Our results show how and why these specialized bacterial communities vary, in spite of notable compositional stability across millions of years of *Cephalotes* evolution (Sanders *et al.* 2014). In turn, these findings support hypotheses on the roles of gut bacteria in the use of a pollen diet and on symbiotic variability as a potential source of heritable, adaptive variation.

## Materials and methods

### *Sample collection, rearing conditions and experimental manipulations*

All specimens used in this study were collected by authors CSM or YH from the Florida Keys, USA. *Cephalotes varians* ants were obtained from small, hollow twigs of mangrove trees and either preserved in molecular grade 95–100% ethanol and stored at  $-80^{\circ}\text{C}$  before DNA extraction or kept live for laboratory-rearing or dietary-manipulation experiments. To enable comparisons between ant gut communities and those from the surrounding environment, we also collected leaves,

bark, lichen and nest cavity wood samples from the mangrove tree housing *C. varians* colony YH064. Leaves were stored in molecular grade 100% ethanol and kept at  $-80^{\circ}\text{C}$ , while the remaining samples were stored at  $-80^{\circ}\text{C}$  without ethanol.

Bacterial gut communities were examined for four groups of *C. varians* in this study. (i) Field-caught ants: single workers were sampled from seven wild-caught colonies across five locations on three different islands (see Table S1, Supporting information for details). (ii) Standard laboratory-reared ants: two live colonies of *C. varians* were reared for 6 months on 50% honey water and holidic artificial diet (Straka & Feldhaar 2007) at  $25^{\circ}\text{C}$  under a daily light/dark cycle of 14:10. Fresh diet was provided approximately every 2 days.

(iii) Dietary-manipulation ants (experiment 1): four live colonies of *C. varians* were reared as described previously for at least 4 months before dietary-manipulation experiments. Individual workers were then divided into four groups, feeding on different diets for 3 weeks. Diets used in this experiment were selected, in part, to understand whether varying dietary nitrogen could alter gut communities. Specific treatments were as follows: (i) a synthetic artificial holidic diet containing all amino acids (Straka & Feldhaar 2007); (ii) the same holidic diet with only nonessential amino acids, but the same total amount of dietary nitrogen; (iii) holidic diet without any amino acids; and (iv) 30% sucrose water.

(iv) Dietary-manipulation ants (experiment 2): these ants were reared in the laboratory for at least 4 months before experiments, under the conditions described earlier. Ants from the two colonies subjected to this experiment were divided into three groups and then reared for 2 months on three different diets, including two mimicking food sources consumed by *Cephalotes* in nature. These were specifically: (i) 50% (weight/volume) honey water; (ii) a 50:50 mixture of 50% (w/v) honey water and 50% (w/v) bee pollen solution; (iii) a 50:50 mixture of 50% (w/v) honey water and 50% (w/v) chicken dropping solution. The honey water solution was made by dissolving 12.75 mL of honey in 37.25 mL of water, whereas the chicken dropping and bee pollen solutions were made by homogenizing 3 g of chicken droppings or ground bee pollen (Y.S. Organic Bee Farms, Sheridan, Illinois) in 6 mL of deionized water. Pollen grains used in this study probably contained few bacteria, as indicated by our repeated failure to PCR amplify bacterial 16S rRNA genes using universal primers, in spite of consistent plant COI gene amplification (data not shown).

Before the two dietary-manipulation experiments, we assessed diet consumption by *C. varians* workers reared on 0.1% (w/v) methylene blue-labelled versions of these seven diets. Methylene blue was found in the majority

of mid- and hind-guts from workers 2 weeks after diet administration, confirming consumption and suggesting a time-course for gut community sampling.

Immediately before experiments 1 and 2, two to five individuals were collected from each experimental colony and preserved in molecular grade 100% ethanol and kept at  $-80^{\circ}\text{C}$  for eventual molecular work. Fresh diets were then provided to workers every 2 days for the full experimental durations. For the first experiment, three to five ants from each of four colonies were preserved at week three, while similar numbers of workers from each of two colonies were preserved at one and 2 months for experiment 2.

#### DNA extraction

Adult *C. varians* workers were rinsed in 70% ethanol and sterile deionized water before dissection. Ant guts were aseptically dissected under a microscope using sterile forceps. Between each individual dissection, forceps were washed with a 6% bleach solution and then with sterile deionized water. The dissected mid- and hind-guts were placed into sterile 1.5-mL tubes with 180  $\mu\text{L}$  enzymatic lysis buffer, then ground with sterile pestles. After grinding, the samples were incubated at  $37^{\circ}\text{C}$  for at least 30 min. Extractions then proceeded according to the manufacturer's protocol (i.e. Qiagen DNeasy Kit, protocol for gram-positive bacteria).

Prior to DNA extraction from environmental samples, tissue fragments from mangrove tree leaves, bark from twigs, wood from an internal *C. varians* nest surface, and lichen living on the mangrove tree branches were placed into sterile 1.5-mL tubes. All samples were ground with sterile pestles in liquid nitrogen and subsequently homogenized in 1 mL sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) by vortexing for 1 min. Then, 600  $\mu\text{L}$  of the suspension was transferred into a new sterile 1.5-mL tube and used for DNA isolation, following the protocol used for ants.

#### Molecular analysis of ants and lichen samples

To investigate microbial community composition in light of host genetic distances, we amplified a portion of the cytochrome c oxidase subunit I (COI) gene from one worker per colony. We also amplified a part of the internal transcribed spacer (ITS) rDNA region to identify fungi in the two lichen samples (see Table S2, Supporting information for PCR primers, reaction recipes, and cycling conditions). PCR products were sequenced by Eurofins MWG Operon (Huntsville, AL, USA), and sequence traces were assembled and manually edited using CodonCode Aligner v. 4.02 (CodonCode Corporation, Centerville, MA). A matrix of uncorrected ant COI

distances was generated using the mothur (v.1.29.0) software package (Schloss *et al.* 2009), while BLASTn was used to help identify lichen samples.

### 16S rRNA gene pyrosequencing

Three types of DNA samples were submitted for bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) of 16S rRNA gene amplicons: (i) DNA extracted from the guts of individual field-caught worker ants, (ii) DNA from environmental sources, and (iii) normalized and pooled DNA samples extracted individually from the guts of three workers at the start of experiment 2 and after 2 months of pollen feeding. Amplicon pyrosequencing (performed by Research and Testing Laboratory; Lubbock, TX) targeted the V1-V3 variable regions of the 16S rRNA gene, which were amplified with primers Gray28F (5'GAGTTTGATCN TGGCTCAG) and Gray519R (5'GTNTTACNGCGGCKG CTG).

A total of 233 020 raw sequences from 38 single worker gut samples, four pooled gut samples, and eight environmental samples were initially analysed using mothur (v.1.29.0). The raw sequences were denoised by mothur's implementation of PyroNoise with a default of 450 flows. The remaining 169 974 sequences were trimmed to a minimum length of 200 bp with default parameters in mothur. To speed up the downstream analysis, 18 776 unique sequences were identified and subsequently aligned against the Silva database in mothur. We removed any sequences that did not cover positions 1044–6424 of the full-length SILVA-alignment (V1-V3 hypervariable region of the 16S rRNA gene) and trimmed sequences so that all nucleotides used in our analyses were contained within this region. Our alignment was then filtered by removing all nucleotide positions without sequence data.

As trimming created new duplicate sequences, identical sequences were removed again, leaving 12 774 unique sequences. Chimeric sequences were removed after detection with mothur's implementation of UCHIME (Edgar *et al.* 2011), which used each library as the reference set. The remaining sequences were classified using the Ribosomal Database Project's rdp9 reference set (Cole *et al.* 2009) with a threshold of 80% bootstrap confidence. Based on these results, we removed all chloroplast sequences.

Operational taxonomic units (OTUs) were identified at 97% sequence similarity using the nearest neighbour option. However, we noticed that misaligned sequences in the data matrix seemed to inflate OTU number. At this stage, singletons (i.e. OTUs with only one read in the entire data set) were removed from the alignment. The remaining representative sequences were then manually

adjusted to establish a quality alignment (Appendix S1, Supporting information). Finally, this realigned data set was used for new OTU clustering at 97% sequence similarity.

Using this data set, we produced a table of nonchimeric OTUs, containing information on abundance and taxonomy of bacteria from all samples in our study. This table was converted to a biom-format OTU table in QIIME (1.6.0) (Caporaso *et al.* 2010). It was then utilized for subsequent QIIME-based analyses.

### Taxon-specific bacterial phylogenetics

One trimmed representative sequence (180–250 bp) from each ant-associated OTU was added to an appropriate taxon-specific alignment. Using a manual approach, these 16S rRNA gene sequences were aligned to previously generated alignments (Anderson *et al.* 2012) using MacClade v4.06 (Maddison & Maddison 2003). Data sets consisted of one alignment each from the Alphaproteobacteria, Bacteroidetes, Burkholderiales, Epsilonproteobacteria, Gammaproteobacteria and Opitutales. An additional alignment was generated using the MUSCLE algorithm in SeaView (Edgar 2004; Gouy *et al.* 2010) for all OTUs falling outside of these groups, along with their closest relatives identified in BLASTn searches. Each alignment was uploaded to the CIPRES web portal (Miller *et al.* 2012) for maximum likelihood phylogenetic reconstruction using the RAXML-HPC BlackBox tool (version 7.3.2) (Stamatakis 2006). Proportions of invariant sites were estimated for all runs. For the remaining parameters, we used RAXML default settings.

### Detecting ant-associated bacteria in the environment

To visualize overlap between ant and environmental microbial communities, an OTU network map was generated using QIIME and visualized with Cytoscape (Shannon *et al.* 2003). Environmental sequences from OTUs found at high abundance in *C. varians* guts were characterized through inspection of sequence alignments and BLASTn searches, allowing us to more finely describe their relatedness to known ant symbionts.

### Identifying variable 16S rRNA genotypes

To detect bacterial diversity contained within 97% OTUs, we separately inspected and adjusted alignments for the 19 most abundant ant-associated OTUs. These data sets included raw (i.e. nondenoised) 454 sequence reads, which increased sample sizes along with the lengths of sequences assessed for nucleotide variation. Through careful alignment of these sequences, and

through the application of nucleotide frequency thresholds, we attempted to identify natural 16S rRNA gene variation with minimal influence from alignment-, sequence- and PCR error.

Variable sites in these 19 alignments were defined as those with a minor allele showing  $\geq 1\%$  frequency, as identified with CodonCode Aligner. To further minimize inclusion of spurious variation, we ignored possible length polymorphism in homopolymer tracts of  $\geq 3$  bp, and we only included sites from ambiguously aligned regions if re-alignment of these selected stretches (using the MUSCLE algorithm in SeaView) produced a clear arrangement of nucleotides. Variable sites were concatenated into 16S rRNA genotypes, and the relative abundance of each genotype across samples was calculated separately for each OTU, giving insight into the strain composition for the selected bacterial species. Genotype distributions were illustrated with conditional colour formatting in Microsoft Excel and were also assessed with statistical analyses described below.

#### *Assessment of alpha and beta diversity*

Rarefaction curves were constructed from the estimated number of OTUs in each individual sample using observed species richness in QIIME. Bacterial sequence libraries from individual workers were rarified to 950 reads (i.e. the size of the smallest sequence library included in the analysis) before calculating the Chao1 richness estimator and observed species richness, enabling comparable estimates of alpha diversity across all samples. ANOVA was then used to test differences in OTU richness among colonies and between field-caught vs. laboratory-reared ant workers.

To determine the similarity of communities from different workers and colonies (i.e. beta diversity), we used QIIME to compute Bray–Curtis distances between all libraries based on their OTU composition. Weighted UniFrac was also implemented in QIIME (Lozupone & Knight 2005; Lozupone *et al.* 2007; Hamady *et al.* 2010), enabling us to estimate community similarity based on the fraction of phylogenetic branch length shared by pairs of gut communities. The phylogeny for this latter analysis was inferred from an alignment of all representative sequences using the FastTree algorithm (Price *et al.* 2009). Bray–Curtis and weighted UniFrac distance matrices were then used for principal coordinates analyses (PCoA) in QIIME, and PCoA plots were visualized using R v2.02 ([www.r-project.org](http://www.r-project.org)) or the KiNG graphic program (<http://kinemage.biochem.duke.edu/software/king.php>).

Variation of gut communities (i.e. OTU composition & genotype composition for each OTU) between groups of ants was assessed in R (v2.02) using Adonis

(McArdle & Anderson 2001) from the Vegan package (Oksanen *et al.* 2011), which performed a permutational MANOVA based on Bray–Curtis and/or weighted UniFrac distance matrices (1000 permutations). QIIME-based ANOVA tests were separately used to identify OTUs with differing relative abundance among colonies, and between field-caught vs. laboratory-reared ant workers. To further describe the effects of diet on worker gut communities, we used the RDP Lib Compare algorithm (Cole *et al.* 2009), comparing relative abundance of OTUs in pooled samples of pollen-fed ant guts to those from pooled samples of guts from sibling ants before the shift to pollen feeding.

#### *PCR amplification and terminal restriction fragment length polymorphism*

The 16S rRNA genes from gut bacteria of workers from dietary-manipulation experiments were amplified using the universal bacterial primers 9Fa and 1513R (Russell *et al.* 2009), with the forward primer modified by 6-FAM fluorescent dye labelling at the 5' end. Purified PCR products were digested with the enzyme BstUI. Samples were then submitted to the Medical Genetics DNA Sequencing Facility (University of Pennsylvania) for fragment size estimation. Artefact peaks were minimized by loading  $< 5$  ng/ $\mu$ L per sample, an approach that has been recommended in at least one other study (Yu *et al.* 2005). Sequence libraries of 16S rRNA genes (cloning/Sanger sequencing and 454 amplicon sequencing) were used to predict terminal BstUI fragment sizes, enabling us to assign actual fragments to common gut bacteria. Further detail on Terminal Restriction Fragment Length Polymorphism (TRFLP) protocols and annotation can be found in Appendix S2 and Tables S2–S3 (Supporting information).

#### *Statistical analyses of TRFLP data*

Bray–Curtis distances were generated in R v2.02 using TRFLP data, allowing us to compare gut communities between sibling ants consuming different diets. Principal coordinates analyses (PCoA) were performed on the resulting Bray–Curtis distance matrices using the Vegan package in R. We also performed permutational MANOVA (1000 permutations) with Adonis, assessing whether observed differences between dietary treatments were significant. In addition, the relative abundances of TRFs within each TRFLP profile were converted into an OTU table in QIIME 1.6.0; ANOVA was then used to identify TRFs with varying relative abundance between treatments in both dietary experiments.

For the second dietary experiment, relative abundance values for all TRFs were used to generate

heatmaps (using the heatmap function in Matlab version R2011b). After calculating the weighted average distances between each TRFLP profile, the resulting distance matrices were used to construct dendrograms depicting community similarity.

## Results

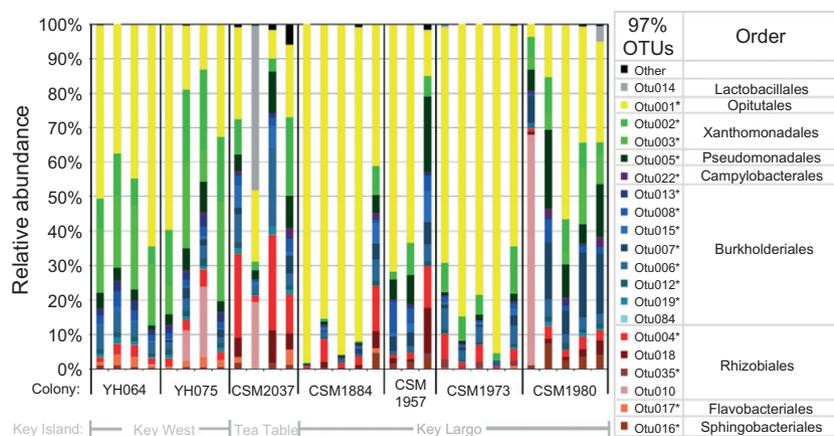
### Core bacterial species from *C. varians* guts and their phylogenetic affinities

Gut communities of 38 ants from seven colonies were characterized using 454 pyrosequencing (115 896 denoized, quality-controlled sequences;  $n = 424\text{--}14\,256$  per library), as were four pools of DNA from workers hailing from two additional colonies (14 430 denoized, quality-controlled sequences;  $n = 2571\text{--}4978$  per library). Seventy-two OTUs (97%) were found across the studied ants (Table S4, Supporting information). Individual libraries contained between 10 and 24 identified OTUs with a mean of 17.9. Five OTUs were present in all libraries (OTU #s 1, 2, 4, 5, and 6), while 11 OTUs were found across all sampled colonies. We designated 'core' bacteria as those found in more than two-thirds of *C. varians* colonies (i.e.  $\geq 7/9$  colonies)—16 OTUs fit this criterion (Fig. 1; Table S5, Supporting information). Together, these core OTUs comprised a median of 99.3% of the sequence reads in each 454 library (range = 32.1–100%) (Fig. S1, Supporting information).

Core OTUs belonged to three phyla, the Verrucomicrobia, the Bacteroidetes and the Proteobacteria. There was one core species in the phylum Verrucomicrobia, representing the order Opitutales. The Bacteroidetes

harboured two core species, one from the Sphingobacteriales and one from the Flavobacteriales. The Proteobacteria harboured the most diverse array of core OTUs: three belonged the Gammaproteobacteria (two from the Xanthomonadales and one from the Pseudomonadales); one came from the Epsilonproteobacteria (order Campylobacteriales); two grouped within the Alphaproteobacteria (order Rhizobiales); and seven classified to the Betaproteobacteria (all within the Burkholderiales) (Fig. 1). BLASTn searches suggested that all core species were most closely related to gut microbes previously discovered from other *C. varians* (Table S5, Supporting information). Phylogenetic analyses using maximum likelihood corroborated these findings, grouping the representative 16S rRNA gene sequences from all core bacteria into ant-specific lineages. These clades typically consisted of microbes from other *Cephalotes* species (Fig. S2, Supporting information).

In addition to core bacteria and microbes with low abundance and low cross-colony incidence, we identified three OTUs showing sporadic distributions yet occasionally high prevalence. One of these, OTU014 from the Lactobacillales (found in 6 of 42 libraries and 4 of 9 colonies), grouped with a previously identified *C. varians* associate on a 16S rRNA phylogeny (Fig. S2g, Supporting information), reaching 47.8% relative abundance in one worker gut community. The remaining two came from the Rhizobiales (OTU010, max within-library abundance = 66.9%,  $n = 10$  libraries,  $n = 5$  colonies; OTU018, max within-library abundance = 13.3%,  $n = 20$  libraries,  $n = 6$  colonies). Like the core Rhizobiales, these two OTUs fell within the broader ant-specific lineage from this order (Fig. S2d, Supporting information).



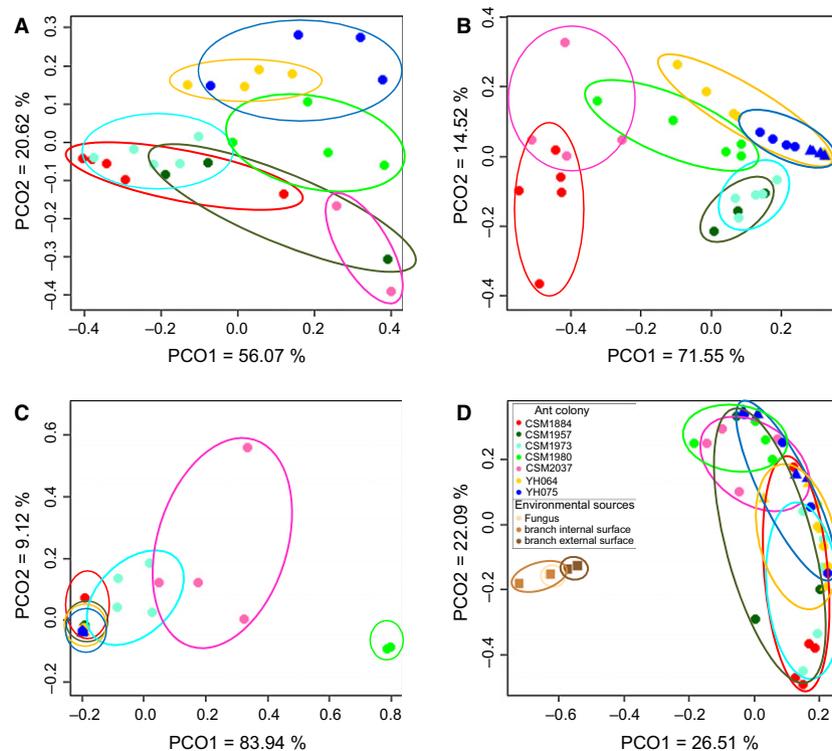
**Fig. 1** Abundance of bacterial species and taxa across *Cephalotes varians* workers and colonies. Bar graphs for each library (one column = gut community from a single field-caught worker) show the percentage of denoized and quality-controlled 454 sequence reads classified to selected 97% OTUs. Rare bacterial OTUs (found in under half of sequence libraries and never exceeding a relative abundance of 4%) were lumped into a single category for simplification. Islands of origin are indicated beneath colony IDs. Bacteria from the same order are represented by different variants of the same colour, and orders to which these OTUs were assigned are indicated in the colour key. Finally, core OTUs/species are indicated with asterisks.

### Variation of gut communities across *C. varians* colonies and populations

Visual evidence indicated that the relative abundance of core species varied across individual field-caught specimens and across seven colonies from three different islands (Fig. 1). To better visualize the variation between gut communities of workers from different colonies, we plotted the results of a principal coordinates analysis performed on Bray–Curtis distances (Fig. 2A; see Fig. S3, Supporting information for PCoA based on weighted UniFrac distances) after removing two outlier libraries enriched with non-core bacteria (CSM1980-1 and CSM2037-3; Fig. S1, Supporting information) and one library with low-sampling depth (CSM2037-5, with  $n = 424$  quality sequences). Separation of gut communities along the first PCoA axis appeared to correlate with differences in relative abundance of OTU001 from

the Opititales. Variation along the second axis was primarily associated with abundance of Rhizobiales core OTU004. Adonis analyses based on Bray–Curtis and weighted UniFrac distances revealed significant differences in the gut bacterial communities among the studied *C. varians* colonies (Bray–Curtis:  $F = 6.857$ ,  $R^2 = 0.673$ ,  $P = 0.001$ ; weighted UniFrac:  $F = 6.141$ ,  $R^2 = 0.648$ ,  $P = 0.001$ ), matching trends seen in the PCoA plot. ANOVA statistics also identified 10 OTUs with significantly differing abundance across colonies, each belonging to a core species (Table S6, Supporting information). Most variation arose due to differences in relative abundance, although three of these variable OTUs appeared absent from a single colony each.

In our studies of alpha diversity, we noted that the average Chao1 richness for gut communities ranged from 15.39–20.42 across field-caught ant workers (Table S7, Supporting information), with no significant



**Fig. 2** Principal coordinates analyses comparing bacterial communities across *C. varians* colonies and environmental sources. (A) PCoA based on Bray–Curtis distance values computed for *C. varians* communities at the 97% OTU level. Only libraries of field-caught workers are included here. Note that three libraries were excluded due to aberrant microbial patterns (i.e. abundant noncore OTUs in two samples) or small sample size. (B) PCoA based on Bray–Curtis distance values computed from the genotype/strain composition of OTU004 (Rhizobiales). (C) PCoA based on Bray–Curtis distance values computed from the genotype/strain composition of OTU008 (Burkholderiales). (D) PCoA based on Bray–Curtis distance values computed for whole communities from *C. varians* colonies and environmental samples (97% OTUs). For panels B and C, libraries of field-caught (circles) and laboratory-reared (triangles) workers are included. Colonies are indicated by colours as indicated in the inset within panel D. Ovals are used to enclose all individual communities from a single colony or sample type. For all panels, the first and second PCoA axes are shown, and the % of Bray–Curtis distance variation explained by each axis is listed next to axis labels. Note the stronger separation of several colonies based on the genotype/strain composition of the two depicted core species. Note also the effect of laboratory rearing on OTU004 composition but not on the composition of OTU008.

differences between colonies (ANOVA,  $P = 0.391$  and  $P = 0.199$  for Chao1 and observed species richness, respectively). Rarefaction curves for a number of communities did not completely plateau (Fig. S4, Supporting information), but their low slopes at high-sampling depth suggested our level of sequencing was sufficient to identify nearly all of the common gut bacteria.

Analysis of COI sequences generated for *C. varians* workers (Appendix S3, Supporting information) showed that those from two colonies collected in Key West, YH064 and YH075, differed from those of five colonies from other Keys islands (CSM1884, CSM1957, CSM1973, CSM1980—Key Largo; CSM2037—Tea Table Key) at 7–8 nucleotides. These Key West COI sequences differed from each other at a single nucleotide out of 996 bp, while those from the latter five colonies had identical sequences. Bray–Curtis distances among gut communities did not strongly correlate with host relatedness when viewed across colonies (Table S8, Supporting information).

#### *Comparison of microbial gut communities between field-caught and laboratory-reared C. varians*

We also used 454 pyrosequencing to compare the gut bacterial communities of four field-caught and four laboratory-reared *C. varians* workers from each of two colonies. Species composition did not change drastically, with the same core OTUs persisting after 6 months of laboratory rearing (Fig. S5, Supporting information). Yet there were subtle shifts in these communities, as summarized in these graphs of OTU abundance and in our PCoA analyses (Figs S6–S7, Supporting information). Adonis analyses indicated that differences between gut communities of field-caught and laboratory-reared workers were significant (Bray–Curtis statistics:  $F = 3.334$ ,  $R^2 = 0.192$ ,  $P = 0.027$ ; weighted UniFrac statistics:  $F = 4.089$ ,  $R^2 = 0.226$ ,  $P = 0.02$ ). Specifically, the abundance of Rhizobiales core OTU004 was, on average, sevenfold higher in laboratory-reared *C. varians* than in field-caught ants (Bonferroni-corrected  $P$ -value = 0.003; Table S9, Supporting information). Contrastingly, Burkholderiales core OTU013 showed a twofold decline in laboratory-reared workers (Bonferroni-corrected  $P$ -value = 0.033; Table S9, Supporting information). In spite of these differences in beta diversity, we did not detect altered alpha diversity between the laboratory and the field (ANOVA,  $P = 0.513$  and  $P = 0.613$  for the Chao1 estimator and observed species richness, respectively).

#### *Are C. varians gut microbes found in the environment?*

We performed 454 pyrosequencing to examine the microbial communities from mangrove tree microhabitats, including the bark of twigs, wood lining a *C. varians*

nest cavity, leaves and lichen ( $n = 2$  for each). Due to extremely shallow sampling of nonchloroplast sequences from leaves (i.e.  $n = 3$ ), and a lack of overlap with ant-associated 97% OTUs, data from leaf samples were excluded from subsequent analyses. Similarities among the remaining environmental bacterial communities and those from 35 worker guts were visualized using PCoA plots (Fig. 2D; Fig. S8, Supporting information). In essence, bacterial communities from ants were clearly distinct from the six environmental communities, with Adonis analyses revealing significant differences (Adonis statistics on Bray–Curtis distances:  $F = 19.596$ ,  $R^2 = 0.329$ ,  $P = 0.001$ ; Adonis statistics on weighted UniFrac stats:  $F = 30.389$ ,  $R^2 = 0.432$ ,  $P = 0.001$ ). This was further illustrated with a network graph, showing how OTUs were partitioned across samples (Fig. S9, Supporting information).

Importantly, of 72 OTUs found across the studied *C. varians*, only eight were also found in environmental samples. However, three of the overlapping OTUs did correspond to core gut microbiota of *C. varians*, including OTU004 (Rhizobiales; seven reads from nest cavity wood), OTU005 (Pseudomonadales; one read from a lichen with an ITS sequence BLAST'ing to *Ramalina*, GenBank Accession #: FJ871076), and OTU006 (Burkholderiales; one read from a lichen with an ITS sequence BLAST'ing to *Lecanora*, GenBank Accession #: AB764071). Given the low read abundance, further work is needed to determine whether this detection signifies alternative lifestyles of ant gut symbionts. Finally, of the other shared OTUs, all were rare within, and sporadically distributed across, ant guts (Table S10, Supporting information). Phylogenetic analyses also indicated that these latter microbes fell outside of the known ant gut bacterial clades (Fig. S2, Supporting information).

#### *Effects of diet on gut bacterial communities of C. varians*

To examine the effects of diet on adult worker gut communities, 16S rRNA TRFLP analysis was performed on gut-extracted DNA from ants in our two experiments. Results from experiment 1 revealed that workers harboured significantly different gut microbiota when fed on different diets (Table S11, Supporting information). Differences were most pronounced between ants feeding on complete holidic diets vs. those on otherwise identical diets without amino acids ( $P < 0.05$  in Adonis tests for three of four colonies). Gut communities from ants fed diets of sugar water also showed some differences compared with those on other diets. However, these differences became nonsignificant after Bonferroni correction, and we failed to find TRFs that differed consistently across diets in the four examined colonies.

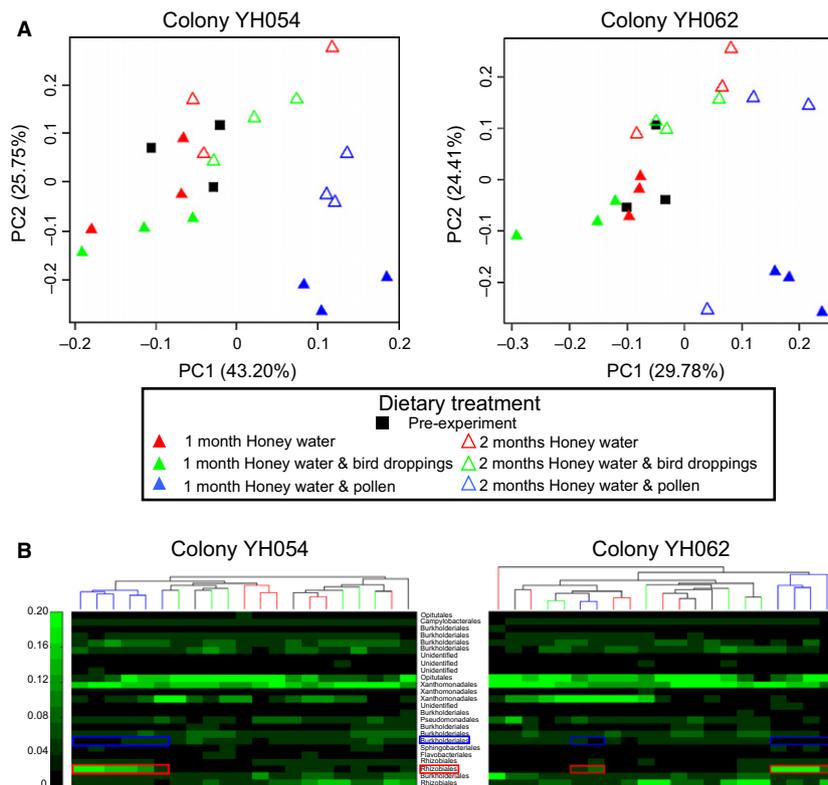
Variation across diets in experiment 2 was, contrastingly, more consistent. Specifically, *C. varians* workers fed on pollen for 1–2 months harboured significantly different communities compared with sibling ants at the start of experiments and siblings fed on other diets (Table S12, Supporting information). This effect was consistent across both experimental colonies, as mirrored in our PCoA analyses (Fig. 3A). ANOVAS and visual heatmap inspections (Fig. 3B) indicated that the gut communities of pollen-fed ants in both colonies were characterized by an increased abundance of one Rhizobiales TRF (93 bp) and by decreased abundance of one Burkholderiales TRF (140 bp) (Table S13, Supporting information). All other altered TRFs had very low abundance within individual profiles and could not be assigned to a particular taxon or OTU.

Analyses of 454 pyrosequencing data sets of pooled pollen-fed ant guts and guts from sibling ants from the start of the dietary experiment revealed that the typical

core microbiota remained in these ants (Fig. S10, Supporting information). However, both comparisons showed proliferation of Rhizobiales OTU004 (2–6% relative abundance increase), coupled with a decrease in Burkholderiales OTU006 (0.3–1.2%) and OTU008 (0.7–0.8%) (Table S14, Supporting information). Correspondence of these core OTUs to the altered TRFs (Table S3, Supporting information) provides a second line of molecular evidence suggesting that pollen consistently alters *C. varians* gut communities.

#### Variation of 16S rRNA genotypes across colonies, locales and rearing conditions

Careful alignment and annotation of all sequences from major ant-associated OTUs revealed hidden variation within 97% 'species' groupings (Table S15, Supporting information). For instance, we detected at least two variable nucleotides in an alignment of Oplitales OTU001



**Fig. 3** Principal coordinates analyses and heatmap figures reveal distinct gut communities in *C. varians* consuming different diets. Results were inferred from two distinct colonies used in the second dietary experiment. (A) PCoA analyses were run on Bray–Curtis distances generated from TRFLP analysis of bacterial 16S rRNA genes. The inset legend shows the symbols used for gut communities of ants from varying experimental classes. (B) Data were generated from the relative abundance of TRFs found in workers from the two experimental colonies. Identities of TRFs were predicted based on *in silico* TRFLP analysis of sequence data sets (Table S2, Supporting information). Colours in each cell of the heatmap reveal the relative abundance of each TRF (row) within each *C. varians* ant gut (column), ranging from 0% (black) to 20% (green). Dendrograms at the tops of each panel were generated from Bray–Curtis distance matrices produced from TRF data. Branch colours use the same convention as that from part A of this figure, showing communities from different dietary treatments. The Rhizobiales TRF with increased abundance in pollen-fed ants is highlighted for pollen-fed communities using red boxes. Similarly, the Burkholderiales TRF with decreased abundance in pollen-fed ants is highlighted in blue.

reads. We confirmed one of these variable sites based on sequence polymorphism from previously generated 16S rRNA clone libraries (Russell *et al.* 2009 and Anderson *et al.* 2012). And after combining previously generated *Opitutaes* 16S rRNA gene sequences with our new data, we detected potential geographic trends. Most notably, three colonies from the southwestern – most sampled Keys (Key West and Sugarloaf Key) showed a predominance of the ‘AA’ genotype, which was extremely rare in ants from more northeasterly locations (Fig. 4).

Assessment of other sequence alignments led to the identification of a number of variable nucleotide sites (i.e. those with the minor allele showing  $\geq 1\%$  frequency) within all but one examined OTU (Table S15, Supporting information). Importantly, 16S rRNA genotypes constructed from sequences at these sites showed colony-specific signatures (Table S16, Supporting information). Like trends seen at the OTU level, these often involved varying relative abundance, although the presence/absence of differences were quite striking in some

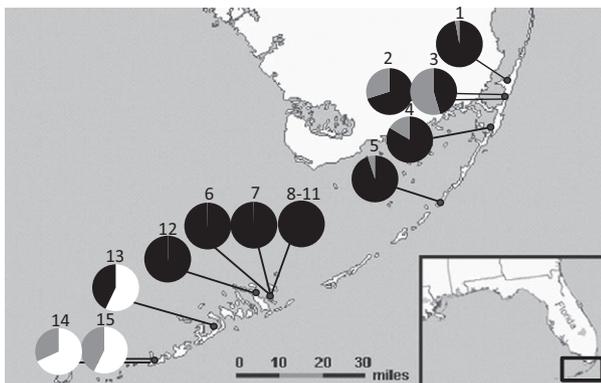
cases (e.g. see OTU007; Table S15, Supporting information). Overall, differences between some colonies were more pronounced when examined at the genotype (vs. 97% OTU) level (Fig. 2B,C vs. 2A). The consistency of several trends across sibling worker gut communities suggests that these are biological trends, rather than sequencing artefacts.

The presence of multiple genotypes within most 97% OTUs also suggested higher alpha diversity than estimated above. When we considered only ‘common’ genotypes (i.e. those found across two or more sequence libraries and constituting at least 3% of the given OTU for at least one library), we found an average of 50.4 bacterial strains per worker, with a range of 24–76 (Table S17, Supporting information). While interperonic variation could explain some of these trends, our potential elimination of real genotypes (e.g. by excluding rare genotypes), the suboptimal nature of 16S rRNA gene for fine-scale strain differentiation, and our modest sampling depth within some OTUs lead us to suspect even greater strain diversity than estimated above.

Analyses of genotype abundance provided several additional insights of relevance. First, genotypes did not commonly appear to be lost from laboratory-reared ants, mirroring our OTU-based assessments of community stability. Within some OTUs, however, there were effects of pollen feeding and laboratory rearing on genotype abundance. This was most notable within Rhizobiales core OTU004. In this case, one genotype consistently fell in relative abundance (i.e. the proportion of OTU004 reads made up by this genotype) among replicate laboratory-reared workers from two colonies (compare yellow and blue triangles vs. circles in Fig. 2b), only to show relative proliferation after ants were switched to 1–2 months of pollen feeding (Table S15, Supporting information). Second, environmental OTUs that overlapped ant core species matched the genotypes of dominant ant-associates (Table S15, Supporting information). Third, several differences between field-caught workers from two colonies remained intact after laboratory-rearing under identical conditions (i.e. in Table S15, Supporting information, compare the genotypic composition of OTU002, OTU012 and OTU013 between colonies YH064 & YH075; see also Fig. 5; in Table S16, Supporting information, see Adonis results with effects of ‘colony’ but not ‘rearing’ in ‘YH064 vs. YH075’ comparisons). Thus, as seen at the species (97% OTU) level, strain composition may be a stable and distinguishing feature across ant colonies.

## Discussion

While the discovery of bacterial function has remained elusive for many gut symbioses (but see Breznak 1982;



**Fig. 4** *Opitutaes* genotype/strain variation across *C. varians* colonies from throughout the Florida Keys. Pie chart colours show the proportions of sequence reads corresponding to each genotype: black = AG genotype; grey = GG genotype; white = AA genotype. Each chart is connected to its collection locale. Numbers above pie charts identify the different *C. varians* colonies sampled here. Colonies sampled with 454 sequencing (range of  $n = 1183$ – $16\,538$  reads mapping to *Opitutaes* OTU001 per colony) were #s 1 (CSM1980,  $n = 5$  workers), 2 (CSM1957,  $n = 3$  workers), 3 (CSM1973,  $n = 5$  workers), 4 (CSM1884,  $n = 5$  workers), 5 (CSM2037,  $n = 4$  workers), 6 (YH054,  $n = 9$  workers), 7 (YH062,  $n = 9$  workers), 13 (YH064,  $n = 8$  workers), and 14 (YH075,  $n = 8$  workers). Colonies sampled in prior studies with Sanger sequencing (range of  $n = 4$ – $81$  reads mapping to *Opitutaes* OTU001 per colony) were #s 8 (CSM1169,  $n = 5$  workers), 9 (CSM1179-2a,  $n = 1$  worker), 10 (CSM1179-2b,  $n = 1$  worker), 11 (CS0543,  $n = 1$  worker), 12 (CSM1235,  $n = 1$  worker), and 13 (CSM1280,  $n = 3$  workers). The identical genotypic compositions (100% AG) for colonies 8–11 are depicted with a single pie chart. Note that all reads were pooled at the colony level before computing average proportions.

Colony	Treatment	Library	OTU002 (genotype GCGTTCGG CG)	OTU012 (genotype TGAACTTC- CATCTTCC)	OTU013 (genotype TGGTCCG)
Key West Colony YH064	Field-caught	YH064-FW2	0.0052	0	0
		YH064-FW3	0.0107	0	0.0273
		YH064-FW4	0.0033	0	0
		YH064-FW5	0.0340	0	0
	Lab-reared	YH064-LW2	0.0871	0	0
		YH064-LW3	0	0	NA
		YH064-LW4	0.0115	0	0
		YH064-LW6	0.0513	0	0
	Key West Colony YH075	Field-caught	YH075-FW1	0	0.0339
YH075-FW2			0	0	0.1475
YH075-FW3			0	0.0667	0.0909
YH075-FW4			0	0.0870	0.1977
Lab-reared		YH075-LW2	0	0.1014	0.0526
		YH075-LW3	0	0.0083	0.2642
		YH075-LW4	0	0.0465	0.1389
		YH075-LW5	0	0.0139	0.3333

**Fig. 5** Genotype/strain stability in field-caught and laboratory-reared workers from two *C. varians* colonies. Heatmap shading from black (low abundance) to light grey (high abundance) shows the proportion of the given core OTUs comprised by the listed genotypes across individual worker ants. 'NA' reveals that 0 sequence reads classified to OTU013 from library YH064-LW3. In all other cases, the given OTUs were present, but the predominant genotypes differed between colonies YH064 and YH075 in the field. After rearing in the laboratory, these differences remained intact, suggesting stable colony-level microbial signatures. The median numbers of sequence reads per OTU per library were 321, 58, and 45 for OTUs 002, 012 and 013, respectively.

Dillon & Dillon 2004; Turnbaugh *et al.* 2006; Muegge *et al.* 2011; Engel *et al.* 2012; Ridley *et al.* 2012), correlations between community composition and host-level traits can be used to infer possible causes and consequences of these interactions (Ley *et al.* 2008; Russell *et al.* 2009, 2012; Sullam *et al.* 2012). Thus, while the gut microbes of *Cephalotes* ants have not yet been characterized in a functional sense, prior findings have hinted at their importance in the evolution of this group (Russell *et al.* 2009; Anderson *et al.* 2012). Our present study on the gut bacteria of *C. varians* expands on that work by: (i) increasing the breadth, depth and replication of sampling within and across individuals, colonies and locations; (ii) examining shifts in gut communities in response to lab-rearing and diet manipulation; (iii) defining core species and systematically characterizing their strain diversity across a range of hosts; and (iv) performing numerous statistical tests to assess community differences and their correlates across multiple scales. As a result of these efforts, we have identified subtle symbiotic variability, the forces behind it, along with the potential implications. We further discuss these findings below, placing our discoveries on the diversity and variability of *C. varians* gut bacteria into the broader context of animal and social insect symbioses.

#### Hidden alpha diversity

Gut bacterial communities of *C. varians* are not particularly diverse, especially when stacked up against those from termites or mammals (Eckburg *et al.* 2005; Hongoh *et al.* 2005; Boucias *et al.* 2013). Instead, these appear comparable with those from other social hymenopterans, such as honeybees (*Apis mellifera*), which harbour a core of approximately eight bacterial phylotypes (Martinson *et al.* 2011). But like *C. varians*, core honeybee gut bacteria are also composed of multiple strains with potentially varying functions (Engel *et al.* 2012; Moran *et al.* 2012). Thus, in spite of the low species-level diversity for these gut communities, functional diversity and lower level taxonomic diversity may be much higher than originally anticipated. Recent findings suggest that hidden strain diversity may characterize the gut microbiota of many animals beyond (Faith *et al.* 2013; Schloissnig *et al.* 2013). Thus, the use of fine-scale molecular and bioinformatics tools will be important in furthering studies of bacterial and symbiont ecology (Eren *et al.* 2013).

#### The implications of long-term symbiont stability

In spite of deeper and broader sampling of *C. varians* gut communities, all identified core microbes belonged

to lineages containing previously identified *C. varians* associates. Most also have close relatives in other *Cephalotes* ants, suggesting long-standing relationships (Sanders *et al.* 2014). Stable core microbiota that dominate host gut communities have not been detected in a number of insects from natural populations (Broderick *et al.* 2004; Dillon *et al.* 2010; Chandler *et al.* 2011; Engel & Moran 2013). Thus, the existence of such a core, especially one with stability across populations, diets, and even species boundaries, suggests specific mechanisms aiding in gut symbiont acquisition and maintenance. Behavioural bacterial transmission may be one such mechanism. Indeed, *C. varians* workers have been shown to engage in oral-anal trophallaxis, which is thought to be a primary means for bacterial transfer in termites and some other herbivorous ants (McMahan 1969; Wilson 1976; Wheeler 1984; Cook & Davidson 2006). Transmission of gut microbiota of eusocial bees also involves a social context, taking place within the hive (Martinson *et al.* 2012; Koch *et al.* 2013).

Alternative means of core gut microbe acquisition in insects involve ingestion of symbionts from maternally deposited capsules or from soil, where some symbionts can dwell (Fukatsu & Hosokawa 2002; Kikuchi *et al.* 2007; Hosokawa *et al.* 2013). Indeed, the detection of DNA from three core *C. varians* microbes in environmental samples suggests potential environmental acquisition, although it is not presently clear whether these bacteria thrive or even survive in such external habitats. Should environmental acquisition prove common, one might expect evolved adult behaviours that target typical symbiont sources or, potentially, specific gut physiologies that select for a very specific range of bacteria.

#### Natural community variability

Combined with other discoveries (Bution *et al.* 2010; Sanders *et al.* 2014), our findings suggest gut community stability is a hallmark of the *Cephalotes* genus. Yet subtle variation exists between colonies in nature. Both trends mirror those from other social insects, with host-specific bacterial lineages showing stable associations, combined with small differences in community composition across various scales (see Sudakaran *et al.* 2012 for an example in a non-social insect). Indeed, honeybees fit this pattern, with the relative abundance of core gut bacteria differing across colonies and possibly geographic locations, and with differences in strain abundance across these same sampling scales (Moran *et al.* 2012). Bumblebee and termite gut microbiota similarly vary between colonies (Minkley *et al.* 2006; Koch *et al.* 2012; Boucias *et al.* 2013), and in the former case, these

colony-specific signatures seem stable, as they remain intact after microbial transfer between host colonies (Koch & Schmid-Hempel 2012).

Small differences among otherwise stable crops of core bacteria raise interesting questions about the mechanisms shaping community dynamics and inheritance. What is clear from this study is that community plasticity, in response to diet, is likely to drive some, but not all, of the natural variation among *C. varians* gut communities.

#### Causes and consequences of diet-induced plasticity

Diet-induced shifts in gut communities have been seen in several other insect systems. For instance, low-protein and high-fibre diets alter the numbers of *Streptococcus* and *Lactobacillus* symbionts in cockroach guts, resulting in a decreased production of lactate and acetate (Kane & Breznak 1991). Similarly, hindgut microbiota of crickets can be altered by changing dietary protein and carbohydrate quantities (Santo Domingo *et al.* 1998). The influence of host diet on the presence and abundance of gut symbionts has also been seen in many vertebrates. Perhaps most famously, obese humans harbour higher ratios of Firmicutes to Bacteroidetes compared with lean individuals. In humans placed on low-calorie diets, this ratio can decrease (Ley *et al.* 2006), and such changes may directly shape obesity due to the known efficiency of Firmicutes-driven caloric extraction (Turnbaugh *et al.* 2006).

While causes and consequences of diet-mediated plasticity are clear in some systems, this is not completely the case for the pollen-driven community shifts in *C. varians*. But given our failure to detect bacterial DNA in the bee pollen used for our study, we can narrow down the list of causes to two possibilities: (i) compounds from pollen favour certain bacteria with the capacity to use them for energy or biomass; or (ii) pollen alters gut physiology in a manner that benefits a limited number of bacteria.

Given the capacity for honeybee-dwelling gut bacteria to break down pectin from pollen cell walls (Engel *et al.* 2012) and the widespread consumption of pollen across the *Cephalotes* genus (Baroni-Urbani & de Andrade 1997; de Andrade & Baroni-Urbani 1999), it is plausible that some *C. varians* gut microbes digest pollen. We hypothesize such a role for the strain of Rhizobiales found to proliferate in the guts of pollen-fed ants. The use of nutrients from recalcitrant pollen cell walls could very well give this, or other candidate pectin digesters, an advantage in the gut environment. Pectin-degrading enzyme activities have indeed been detected from several Rhizobiales bacteria (Hubbell *et al.* 1978; Mateos *et al.* 1992; Fauvert *et al.* 2009), further suggesting the plausibility of such a role.

### Alternative explanations for natural community variability

In our experiments, just one diet promoted consistent shifts in gut communities; additionally, the OTUs shifting in response to pollen were just two of ten with naturally differing abundance across field-collected colonies. When we also consider that several field-observed colony-level differences persisted after extended laboratory rearing, it becomes clear that natural factors aside from diet must shape the composition of gut microflora.

One explanation for stable colony-level differences in gut communities is genetic variability in host ants. Under this scenario, varying host alleles could alter gut physiology, immunology, or the tissues to which microbes adhere, shaping the spectrum of bacteria that are favoured in the gut environment. Currently, there is little evidence available to assess this mechanism, although colony-level genetic differences had only a minor effect on gut communities in a prior study on bumblebees (Koch *et al.* 2012). And though host genotype may play some role in shaping the mammalian gut microbiota (Stewart *et al.* 2005), it has not always been seen as a strong force behind such variation in the humans (Turnbaugh *et al.* 2009a).

History and chance could also play a role in this natural variation in a few conceivable ways. First, mutation and drift could explain why some symbiont strains vary across ants from isolated locales (Fig. 4). Yet in our study, microbial differences were apparent among colonies separated by a few kilometres or even meters, with only a few patterns suggesting broader scale geographic trends. Second, limited bacterial numbers in the inoculum received by queens, or fluctuations within the guts of workers who inoculate sibling queens, could allow for species and genotype fluctuations arising due to chance or within-gut competition. And third, it is possible that local availability of particular microbes could also be of importance should environmental acquisition or horizontal transfer turn out to play roles in gut symbiont transmission.

One last major mechanism behind symbiont variation could involve colony-level natural selection. As gut bacteria are thought to have some heritable component in this system (Wilson 1976), colonies benefiting from their particular blend of gut flora could have higher realized lifetime fitness, promoting the spread of similar communities. Varying selective pressures across spatial scales could drive regional differences in gut communities, but the variation at the local scales seen here could be favoured if gut bacteria were to mediate interactions with natural enemies. In this case, balancing selection could promote the maintenance of diverse community

types within a population (Oliver *et al.* 2013). In the case of bumblebees, microbial communities from different colonies vary in their defensive specificities against strains of a virulent trypanosomatid gut parasite (Koch & Schmid-Hempel 2012), illustrating functional implications of intercolony symbiotic variation and an area of investigation clearly worthy of future study in the *Cephalotes* system.

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Y.H. and J.A.R. conceived of the research performed here along with the study design, with important early contributions from C.S.M. Y.H. performed the experiments, with assistance from P.L. in TRFLP analysis. Y.H. and J.A.R. wrote the manuscript and constructed the figures, with help on figures from P.L. C.S.M. and P.L. assisted greatly in manuscript editing. Y.H. performed most of the data analyses, with contributions from J.A.R. and assistance from P.L. C.S.M. and Y.H. collected all specimens used in this research.

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### Data accessibility

Pyrosequencing reads were deposited in the GenBank Short Read Archive under Accession nos. SRP032758 and SRP032816. COI sequences (KF730266–KF730272), ITS sequences (KF841601–KF841602), and cloned 16S rRNA gene sequences (KF730273–KF730312), have also been deposited in the GenBank database.

### Supporting information

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

**Fig. S1** Frequency histogram showing the degree of core bacterial species domination across libraries.

**Fig. S2** Taxon-specific 16S rRNA phylogenies showing phylogenetic placement for all OTUs in *Cephalotes varians* guts.

**Fig. S3** PCoA of gut microbial communities from field-caught ants showing variation between colonies (weighted UniFrac measure).

**Fig. S4** Rarefaction analyses of microbial communities from individual field-caught workers spanning seven *Cephalotes varians* colonies.

**Fig. S5** Gut community composition from eight field-caught and eight laboratory-reared *Cephalotes varians* from two colonies.

**Fig. S6** Principal coordinates analysis (PCoA) of gut microbial communities of field-caught and laboratory-reared *Cephalotes varians* from two colonies (Bray–Curtis approach).

**Fig. S7** Principal coordinates analysis (PCoA) of gut microbial communities of field-caught and laboratory-reared *Cephalotes varians* from two colonies (weighted UniFrac approach).

**Fig. S8** Principal coordinates analysis (PCoA) of microbial communities from field-caught *Cephalotes varians* and environmental sources (weighted UniFrac approach).

**Fig. S9** Distributions of bacterial OTUs across *Cephalotes varians* workers and the surrounding environment.

**Fig. S10** Taxonomic composition of gut bacteria from pollen-fed ants and their siblings before the shift to pollen feeding (second dietary experiment).

**Table S1** Collection information for the ant colonies utilized in this study.

**Table S2** Information on PCR primers, recipes, and temperatures.

**Table S3** Predicted and observed 16S rRNA TRFs along with their shifts in the second dietary experiment.

**Table S4** OTU table from ant gut community samples and environmental samples.

**Table S5** Information on the core bacterial species (97% OTUs) from *Cephalotes varians* and their detection in the environment.

**Table S6** ANOVA results for bacterial OTUs with varying abundance across field-caught *Cephalotes varians* colonies.

**Table S7** Alpha diversity statistics for gut bacterial communities across *Cephalotes varians* colonies.

**Table S8** COI genetic distances of ants from different colonies (below diagonal) and Bray–Curtis distances of gut bacterial communities from ants across different colonies (above diagonal).

**Table S9** ANOVA results for bacterial OTUs with varying abundance across field-caught vs. laboratory-reared *Cephalotes varians* colonies.

**Table S10** Heat map of eight shared OTUs across ant libraries and six environmental libraries.

**Table S11** Bray-Curtis distances showing differences among gut bacterial communities from *Cephalotes varians* fed on four different diets in first dietary-manipulation experiment.

**Table S12** Bray-Curtis distances showing differences among gut bacterial communities from *Cephalotes varians* workers on three different diets in the second dietary-manipulation experiment.

**Table S13** Terminal restriction fragments (TRFs) with varying abundance across *Cephalotes varians* fed on three diets in second dietary-manipulation experiment.

**Table S14** OTUs with varying abundance between pollen-fed ants and their sibling workers prior to the shift to pollen feeding (second dietary manipulation experiment).

**Table S15** Genotype composition of major OTUs from *Cephalotes varians*.

**Table S16** Adonis statistics assessing effects of colony and rearing on 16S rRNA genotype composition for each OTU.

**Table S17** Diversity of gut communities at the genotype level.

**Appendix S1** Fasta format alignment of representative sequences of pyrosequencing reads.

**Appendix S2** Additional methodologies and full supplementary table and figure legends.

**Appendix S3** Fasta format alignment of COI sequences generated for *C. varians* workers.