Received: 29 October 2016 | Revised: 13 March 2017 | Accepted: 3 April 2017

DOI: 10.1111/mec.14140

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

The structured diversity of specialized gut symbionts of the New World army ants

Piotr Łukasik[1] | Justin A. Newton[1] | Jon G. Sanders[2] | Yi Hu[1] | Corrie S. Moreau[3] | Daniel J. C. Kronauer[4] | Sean O'Donnell[1] | Ryuichi Koga[5] | Jacob A. Russell[1]

[1]Department of Biology, Drexel University, Philadelphia, PA, USA

[2]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA

[3]Department of Science and Education, Field Museum of Natural History, Chicago, IL, USA

[4]Laboratory of Social Evolution and Behavior, The Rockefeller University, New York, NY, USA

[5]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

Correspondence
Piotr Łukasik, Division of Biological Sciences, University of Montana, Missoula, MT, USA.
Email: p.lukasik@gmail.com

Funding information
Japan Society for the Promotion of Science, Grant/Award Number: PE13061; Division of Environmental Biology, Grant/Award Number: 1050243, 1050360

Abstract

Symbiotic bacteria play important roles in the biology of their arthropod hosts. Yet the microbiota of many diverse and influential groups remain understudied, resulting in a paucity of information on the fidelities and histories of these associations. Motivated by prior findings from a smaller scale, 16S rRNA-based study, we conducted a broad phylogenetic and geographic survey of microbial communities in the ecologically dominant New World army ants (Formicidae: Dorylinae). Amplicon sequencing of the 16S rRNA gene across 28 species spanning the five New World genera showed that the microbial communities of army ants consist of very few common and abundant bacterial species. The two most abundant microbes, referred to as Unclassified Firmicutes and Unclassified Entomoplasmatales, appear to be specialized army ant associates that dominate microbial communities in the gut lumen of three host genera, *Eciton*, *Labidus* and *Nomamyrmex*. Both are present in other army ant genera, including those from the Old World, suggesting that army ant symbioses date back to the Cretaceous. Extensive sequencing of bacterial protein-coding genes revealed multiple strains of these symbionts coexisting within colonies, but seldom within the same individual ant. Bacterial strains formed multiple host species-specific lineages on phylogenies, which often grouped strains from distant geographic locations. These patterns deviate from those seen in other social insects and raise intriguing questions about the influence of army ant colony swarm-founding and within-colony genetic diversity on strain coexistence, and the effects of hosting a diverse suite of symbiont strains on colony ecology.

KEYWORDS
army ants, codiversification, Dorylinae, *Eciton burchellii*, ectosymbiont, gut bacteria, host specificity, *Labidus praedator*, strain-level diversity

1 | INTRODUCTION

Insects are the world's most diverse group of animals and have come to dominate most terrestrial ecosystems. Growing evidence suggests that this dominance has, in many cases, been facilitated by their microbial associates, which can expand their hosts' metabolic repertoires and ranges of environmental tolerance. Symbionts of insects represent a broad diversity of bacterial and fungal lineages and vary tremendously in their distributions within host bodies, their biological effects, their transmission modes and their co-evolutionary histories with hosts (Douglas, 2015; McFall-Ngai et al., 2013; Moran, McCutcheon, & Nakabachi, 2008).

The often dramatic effects of insect intracellular symbionts (endosymbionts) are particularly well understood. Obligate nutritional endosymbionts have enabled major nutritional transitions onto imbalanced diets, resulting in highly successful and diverse lineages such as Hemiptera (Baumann, 2005; Bennett & Moran, 2015). Facultative endosymbiotic bacteria can also have dramatic effects on life history traits of hosts, including their reproductive biology or susceptibility to natural enemies and abiotic stressors (Feldhaar, 2011; Oliver, Degnan, Burke, & Moran, 2010). This can cause major changes to insect populations (Himler et al., 2011; Jaenike, Unckless, Cockburn, Boelio, & Perlman, 2010) that can resonate across communities (Ferrari & Vavre, 2011; Sanders et al., 2016). However, insects commonly host bacteria outside their haemocoel—on their cuticles or within the digestive tract (Engel & Moran, 2013). The size, composition, location and functions of the communities of these extracellular symbionts (ectosymbionts) vary considerably across host taxa (Engel & Moran, 2013; Sanders et al., 2017). In some cases, bacterial numbers are relatively low, and microbial communities consist of genera commonly found in the environment (Broderick, Buchon, & Lemaitre, 2014; Chandler, Morgan Lang, Bhatnagar, Eisen, & Kopp, 2011). However, in many hosts communities of extracellular bacteria are remarkably stable across individuals, populations and over longer evolutionary timescales, with the key members showing strong host fidelity and, possibly, only rare host switching (Hu, Łukasik, Moreau, & Russell, 2014; Koch, Abrol, Li, & Schmid-Hempel, 2013; Kwong & Moran, 2015; Sanders et al., 2014; Sudakaran, Salem, Kost, & Kaltenpoth, 2012). In at least some cases, these microbes colonize only selected host tissues (Kautz, Rubin, Russell, & Moreau, 2013b; Lanan, Rodrigues, Agellon, Jansma, & Wheeler, 2016; Martinson, Moy, & Moran, 2012) and the microbial communities change in predictable ways as their hosts develop (Sudakaran et al., 2012). Knowledge on the biology and functions of ectosymbionts still lags behind that for endosymbionts (Salem, Florez, Gerardo, & Kaltenpoth, 2015). However, recent advances in the field of genomics, as well as experimental approaches, have revealed diverse effects on numerous aspects of host biology, including digestion, detoxification, nutrition, susceptibility to parasites and pathogens, development, regulation of host physiology and communication with members of the same or other species (Engel & Moran, 2013).

As seen for endosymbionts (Duron et al., 2008; Russell et al., 2012), early studies of ectosymbionts often involved diagnostic PCR screening or Sanger-sequencing-based cloning of small numbers of sequences to characterize microbiota (e.g., Corby-Harris et al., 2007; Russell et al., 2009b). Other efforts focused on cultured representatives of extracellular symbiont communities, raising questions about the impacts of culture bias on our understanding of these symbioses (e.g., Broderick, Raffa, Goodman, & Handelsman, 2004). The adoption of 16S rRNA gene amplicon sequencing on next-generation platforms has greatly expanded the depth of culture-independent community sampling (Jones, Sanchez, & Fierer, 2013; Yun et al., 2014). However, the low rate of evolution of this gene limits our abilities to detect cryptic, strain-level variation (Ellegaard & Engel, 2016). Indeed, the gut bacterial communities of insects such as bees, ants and termites frequently include multiple distinct strains of bacteria that are classified to the same species based on 16S rRNA gene sequence similarity (Ellegaard & Engel, 2016; Engel, Martinson, & Moran, 2012; Engel, Stepanauskas, & Moran, 2014; Hu et al., 2014; Powell, Ratnayeke, & Moran, 2016; Warnecke et al., 2007). And as seen for bacterial endosymbionts of insects (e.g., Oliver, Moran, & Hunter, 2005), the functions of closely related strains of extracellular bacteria can vary (Engel et al., 2012). To truly understand the diversity of gut or cuticular bacteria and the relationship between the microbiota and host biology, researchers should target conserved genes with a history of vertical transfer (i.e., 16S rRNA) to aid in symbiont classification, but also more variable loci to differentiate strains.

The diversity and function of ectosymbiont communities have garnered a good deal of recent attention among the ants (Hymenoptera: Formicidae), a diverse and ecologically dominant group of social insects that colonize a wide range of trophic and habitat niches (Davidson, Cook, Snelling, & Chua, 2003; Holldobler & Wilson, 1990). Specialized symbionts have been implicated as important partners of ants with specialized diets. For example, bacteria colonizing host cuticle function as biocontrol agents in the management of attine ants' fungal gardens (Currie, Scott, Summerbell, & Malloch, 1999), and recent studies have placed an emphasis on the diversity and structure of these communities (Andersen, Hansen, Sapountzis, Sørensen, & Boomsma, 2013). While at least one group of herbivorous ants has evolved a monotypic nutritional symbiosis with an intracellular bacterium (Feldhaar et al., 2007; Wernegreen, Kauppinen, Brady, & Ward, 2009), others harbour substantial populations of bacteria in their gut lumen (Billen & Buschinger, 2000; Roche & Wheeler, 1997; Sanders et al., 2017). Hypothesized to have shaped the ants' adaptation to plant-based diets (Davidson et al., 2003; Russell et al., 2009b), extracellular gut bacteria generally vary across herbivorous ant taxa (Anderson et al., 2012; Sanders et al., 2014; Stoll, Gadau, Gross, & Feldhaar, 2007), exhibiting modest diversity at the species and strain levels (Hu et al., 2014). As seen for the fungus-growing ants (Andersen et al., 2013), strain-level composition of these communities appears similar across sibling workers yet variable between colonies (Hu et al., 2014).

It has not yet been established whether the stereotyped and stable ectosymbiont communities of fungus growers (cuticular bacteria) and liquid-feeding ant herbivores (gut bacteria) represent the norm across this insect family. In fact, some ant taxa appear to consistently harbour few bacterial cells, and others show substantial variability in abundance and composition of their microbiota (Hu et al., 2017; Liberti et al., 2015; Russell, Sanders, & Moreau, 2017; Sanders et al., 2017). It is notable, then, that predatory army ants represent a third guild of trophic specialists engaging in symbioses with specialized bacteria (Anderson et al., 2012; Funaro et al., 2011). Army ants are keystone species in many of the world's tropical forests (Boswell, Britton, & Franks, 1998; Kaspari & O'Donnell, 2003), living in large colonies with one queen and anywhere from tens of thousands to millions of workers (Kronauer, 2009). Unlike fungus growers and herbivorous ants such as *Cephalotes* and *Tetraponera*,

new army ant colonies are always founded by colony fission, where swarms made up by a single, mated queen and tens to hundreds of thousands of accompanying workers depart from the maternal colony (Cronin, Molet, Doums, Monnin, & Peeters, 2013; Kronauer, 2009; Peeters & Ito, 2001). These differences in modes of colony founding across known hosts of specialized symbionts raise questions about the diversity of microbial communities, but also how such diversity is partitioned among vs. within colonies.

The goal of this study was to characterize the composition and structure of bacterial communities of the New World Dorylinae in a comprehensive, multilevel fashion, across individuals, colonies, species, as well as geography. To achieve this, we used multiplex Illumina sequencing of 16S rRNA amplicons for a total of 195 individual workers from 102 New World army ant colonies representing 28 species from the five described genera. Amplicon sequencing of 16S rRNA was also performed for colonies representing two doryline army ant genera from the Old World. For the two most broadly sampled New World species, *Eciton burchellii* and *Labidus praedator*, we targeted multiple workers from replicate colonies collected at different geographic locations. We then characterized strain-level diversity and host specificity of the two dominant symbiont species by sequencing the ribosomal protein L2 (*rplB*) gene for approximately 350 bacterial strains. Finally, we used fluorescent in situ hybridization to visualize specific symbionts within army ant tissues, yielding the first clear insights into their localization.

2 | MATERIALS AND METHODS

2.1 | Ant collections

The 102 Dorylinae colonies used in the study were collected between 2001 and 2013 in six countries in the Americas: Costa Rica, Ecuador, Mexico, Peru, Venezuela and USA, typically from more than one locality within each country (Table S1). Two sites, Henri Pittier National Park (Venezuela) and the Monteverde area (Costa Rica), were the most extensively sampled. The collected ants were identified to the genus or species level based on morphology and colony characteristics, and then preserved in ethanol or acetone and stored at −20°C until further processing.

We also investigated microbial communities of specimens from 15 Old World army ant colonies used by Funaro et al. (2011; Table S2). In some cases, intact ethanol-preserved workers were available, and they were processed the same way as workers from the New World. In other cases, we used DNA extracted following different protocols by Funaro et al. (2011).

2.2 | DNA extraction

From each colony, we aimed to process five medium-sized individual workers whenever possible (Table S3). Insects were surface-sterilized by immersion in 5% bleach for 60 s and then washed three times with molecular-grade water. Next, from each worker we dissected the gaster, the posterior part of the body that contains all sections of the digestive system other than oesophagus. We took care to remove any fragments of the petiole (i.e., the body section immediately anterior to the gaster), and to sterilize forceps between dissections. Individual gasters were flash-frozen in liquid nitrogen, ground with a pestle and then processed using DNeasy Blood and Tissue kits (Qiagen Ltd., Hilden, Germany) following the manufacturer's protocol for Gram-positive bacteria. To control the level of background contamination with bacterial DNA, we included "blank" samples in all extraction batches, typically one for every ten ants processed.

2.3 | Molecular identification of ants

For one worker from each colony, we attempted to amplify and sequence a part of the mitochondrial cytochrome oxidase I (*COI*) gene. We first attempted to amplify a ~1,100-bp fragment using universal primers LCO-1490 and BEN and sequence it with BEN and internal primer HCO-2198 (Table S4). For samples for which we failed to generate product or for which sequence quality was low, we used primers LCO-1490 and HCO-2198 to amplify and sequence ~650 bp of the gene. A range of PCR conditions were used (Table S4). However, for 15 colonies our attempts to generate unambiguous *COI* sequences were unsuccessful. All traces were manually inspected, assembled, edited and aligned using CODONCODE ALIGNER version 4.2.7 (CodonCode Corp., Centerville, MA, USA).

2.4 | Microbial community analysis

All extracted samples, including "extraction blanks," were used for PCRs with universal eubacterial primers for the 16S rRNA gene, 9Fa and 907R, conducted under standardized conditions (Table S4). Concentrations of bacterial DNA in the samples were approximately estimated by running 5 μl of the PCR product on a 2% agarose gel stained with ethidium bromide and comparing the brightness of the bands against the negative controls. Samples yielding strong *COI* bands but with 16S rRNA bands of similar or lesser strength to those for our negative controls were retested with other pairs of eubacterial primers. When such primers failed to amplify, these samples were classified as hosting insufficient numbers of bacteria for reliable microbial community characterization (Table S3) and were not analysed further.

The composition of microbial communities of ants hosting substantial numbers of bacteria was characterized using amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform, conducted following the Earth Microbiome Project protocols (Caporaso et al. 2012). Library preparation and sequencing were conducted at Argonne National Laboratory (Argonne, IL, USA). For New World Dorylinae, we submitted DNA samples from a single worker gaster from each colony, whenever there was at least one exceeding the rough bacterial density threshold specified above. Furthermore, to investigate bacterial distributions at both the colony and species levels, we selected four additional workers from each of 12 colonies of *Labidus praedator* and from each of 15 colonies of *Eciton burchellii*. Our submission also

included 15 samples from the Old World army ants (genera *Aenictus* and *Dorylus*). Finally, we included 13 blanks: either "extraction blanks," or molecular-grade water. Data were analysed using MOTHUR version 1.36.1 (Schloss et al., 2009). Analysis steps are summarized below, and details and specific commands are provided in the Supplementary Material.

After merging quality-filtered and trimmed reads into contigs and then identifying unique genotypes, we used a custom Python script to conduct a custom decontamination procedure, based on the abundance of unique genotypes in experimental and blank libraries. For each unique 16S rRNA genotype identified through sequencing, we compared the maximum relative abundance it attained in any of the blanks and in any of the experimental libraries; we classified sequences as contaminants when their unique genotypes were not at least ten times more abundant in at least one experimental library compared to the maximum abundance seen across the blanks. Subsequently, we removed any "rare" unique genotypes, which did not constitute at least 0.1% of at least one experimental library. Finally, those experimental libraries that lost more than 75% of reads during the previous steps were deemed heavily contaminated and also removed. The decontamination procedure is described in more detail in the Supplementary Material, and its effects on the microbial community composition are summarized in Fig. S1. Reads were then aligned against a custom set of 16S rRNA references, screened for chimaeras with UChime and classified using the RDP TRAINING SET (version 9). All reads that did not classify as Eubacteria were subsequently removed.

The resulting data set was used for OTU clustering at the 97% identity level using the average neighbor algorithm. We then manually processed the output files (i.e., the alignment of all unique sequences, information about taxonomic classification of each, the OTUs to which each unique sequence was assigned, and the number of times each unique sequence appeared in each of the libraries)—using Microsoft Excel and custom scripts written in PYTHON 3.4.2 (Python Software Foundation 2014) and PROCESSING 2.2.1 (Processing Foundation 2014). We used ADONIS, from the VEGAN package (Oksanen et al., 2007) in R version 3.1.3 (R Core Team 2015), to compare the composition of the microbial communities between the two most extensively sampled species, *Eciton burchellii* and *Labidus praedator*, across collection locations, and among colonies of the same species within locations. In case of significant differences, we implemented ANOVA tests in QIIME version 1.9.1 (Caporaso et al., 2010) to identify OTUs that differed in relative abundance among groups in a comparison. We used betadisper() function within the VEGAN package in R to test for differences in dispersion across groups. Principal coordinates analyses (PCoAs) were performed on the Bray–Curtis distance matrices using the LABDSV packages for R.

2.5 | Sequence typing of the dominant symbionts

We investigated the phylogenetic relationships of the two dominant symbionts of army ants, Unclassified Firmicutes and Unclassified Entomoplasmatales. The relationship of Unclassified Firmicutes with other bacterial taxa was assessed using 16S rRNA gene sequences, for which more extensive comparative data were available. For ants whose microbial communities were dominated by a single genotype of Unclassified Firmicutes (as revealed by 16S rRNA amplicon sequencing), we amplified nearly full-length 16S rRNA sequences for direct sequencing using universal eubacterial primers 9Fa and 1513R. Newly designed diagnostic primers UNF-16S-F1 and UNF-16S-R1 were used to amplify Unclassified Firmicutes' 16S rRNA genes from ants hosting more complex bacterial communities (Table S4). PCR products were purified by digestion with Exonuclease I and Antarctic Phosphatase (NEB, Ipswich, MA, USA) and sequenced by Eurofins MWG Operon (Louisville, KY, USA). Sequence traces were manually inspected for the presence of ambiguities, quality-trimmed and aligned using CodonCode Aligner. Using these protocols, we obtained unambiguous 16S rRNA gene sequences from 19 ant DNA samples, including Old World army ants and the two Ponerinae species previously identified to host Unclassified Firmicutes (Tables S2–S3). To characterize the relationships between Unclassified Firmicutes strains, we developed a protocol for amplifying and sequencing the terminal 579-bp fragment of a conserved single-copy gene encoding ribosomal protein L2 (*rplB*; Table S4). We successfully sequenced *rplB* from 252 Unclassified Firmicutes strains, with representation from all Dorylinae genera. Special emphasis was placed on the two most broadly sampled ant species, *Eciton burchellii* and *Labidus praedator*, for which numerous sequences were generated per colony and site (Tables S1, S3).

Phylogenetic relationships of army ant-associated Entomoplasmatales had been inferred previously using the slowly evolving 16S rRNA gene (Funaro et al., 2011). To gain further insight into the strain-level diversity within this group, we developed a protocol for amplifying a 573-bp segment of *rplB* from Entomoplasmatales (Table S4), using the generated amplicons for Sanger sequencing across 120 army ants and a small number of other ants and insects (Table S2).

2.6 | Genotype-level diversity of Unclassified Firmicutes in individual workers

We used 454 pyrosequencing of *rplB* amplicons to assess strain-level diversity of Unclassified Firmicutes in four workers from *Eciton burchellii* colony PL028. Sanger-sequencing results had suggested that each of the selected workers was dominated by a different symbiont strain, or genotype, spurring us to perform deep sequencing to more thoroughly assess within-host strain diversity. Products for pyrosequencing were generated using the same *rplB* primers as for PCRs prior to Sanger sequencing, complete with linkers and barcodes. These products were then sequenced in one direction on a multiplexed lane by Research and Testing Laboratory (Lubbock, TX, USA). The data were analysed using MOTHUR version 1.33 (Schloss et al., 2009). Quality-trimmed reads were aligned against Sanger sequences for the dominant strains in the four samples. We then clustered reads into OTUs at the 98% sequence identity level. This threshold was chosen as the lowest sequence similarity cut-off

allowing us to distinguish between the dominant Unclassified Firmicutes genotypes in the four studied workers (in one of the pairwise comparisons, genotypes had 97.5% identity across 360 bases). Further analysis details are provided in the Supplementary Material.

2.7 | Phylogenetic analyses

Nucleotide sequences were aligned using CLUSTALW in CodonCode Aligner, and alignments of protein-coding genes were manually verified after translation to amino acids. Maximum-likelihood phylogenies of army ant mtDNA (i.e., partial *COI* sequences) and genes from their two dominant symbionts (*rplB* for both, and 16S rRNA for Unclassified Firmicutes) were reconstructed using RAXML 8.2.0 (Stamatakis, 2014). In all cases, we specified the GTR model with the addition of invariant sites and gamma distribution of rates across sites, one of the top three sequence evolution models for each of these data sets according to jmodeltest2 (Darriba, Taboada, Doallo, & Posada, 2012). One thousand bootstrap replicates were run for each search. Host and symbiont phylogenies were illustrated using ITOL version 3.2.1 (Letunic & Bork, 2016).

2.8 | Fluorescence in situ hybridization

We investigated the localization of symbiotic bacteria within the digestive tract of ants using fluorescence microscopy. Guts dissected from 6 to 10 workers from colonies of *Eciton rapax* (PL150), *Nomamyrmex esenbeckii* (PL157) and *Labidus praedator* (PL158) were fixed in 4% formaldehyde. Then, they were either used for whole-mount hybridization, or embedded in glycol methacrylate resin (Technovit 7100—Heraeus Kulzer GmbH, Wehrheim, Germany) and sectioned prior to hybridization. Additionally, digestive tracts dissected from workers of *Eciton burchellii* colony JSC093 were used for whole-mount FISH only. Seven ant species from other subfamilies were processed using the same methods, thus serving as controls (Sanders et al., 2017).

We used a combination of eubacterial probes (EUB338 and EUB897), as well as probes specific to Unclassified Firmicutes and Entomoplasmatales. The specific probes were originally developed as diagnostic and sequencing primers for the two specialized army ant symbionts and tested extensively in a range of PCR conditions. The specificity of fluorescent signal was demonstrated in control experiments, where hybridization was done with no probes, or with probes labelled with the same fluorophores but targeting bacteria that were believed to be absent in the sample. The signal of both probes was visibly increased when they were used in combination with helper probes—unlabeled oligos for the rRNA region immediately adjacent to that targeted by the probe (Fuchs, Glöckner, Wulf, & Amann, 2000). Finally, for the Entomoplasmatales probe, we verified that its signal overlapped with the signal of two other, previously verified probes for a bacterium from that order, *Spiroplasma* (TKSspi and TKSSsp—Fukatsu & Nikoh, 2000), which were a perfect or near-perfect match to our sequences. All probe sequences are provided in Table S5. The detailed FISH protocol is provided as a supplement.

3 | RESULTS

3.1 | Ant species identification and phylogenetics

We obtained reliable *COI* sequence data for workers from 87 of 102 experimental New World Dorylinae colonies. Some of the colonies for which we failed to obtain unambiguous sequence data were confidently classified based on morphology to the same species as colonies with *COI* data from the same site (Table S1). However, reliable data could not be obtained for four species–location combinations. Their approximate position on the *COI* tree was indicated using dashed lines (Figure 1).

The reconstructed *COI* tree provided a good overview of the sampled ant diversity (Figure 1), recapitulating several previously documented relationships recovered with larger sequence data sets. At the same time, several deeper nodes of the tree disagreed with these prior studies, due likely to the insufficiency of a single genetic marker in accurate phylogenetic reconstruction. More specifically, samples classified to the same species based on morphology formed monophyletic clades, typically with high bootstrap support. The reconstructed relationships between species within the genus *Eciton* did not conflict with the results of a recent phylogenomic study (Winston, Kronauer, & Moreau, 2016). However, according to Brady (2003) and Borowiec (2016), the genus *Neivamyrmex* is monophyletic, and thus, we constrained the *COI* tree to enforce *Neivamyrmex* monophyly. Furthermore, recent phylogenetic reconstructions based on multiple genes or genomic data sets (Borowiec, 2016; Brady, Fisher, Schultz, & Ward, 2014; Winston et al., 2016) identified *Nomamyrmex* as the sister genus of *Eciton*, whereas in the *COI* tree the genera *Eciton* and *Labidus* formed a monophyletic clade, with *Nomamyrmex* as an outgroup. While our *COI* tree provides a good framework to understand how microbes vary across groups of related ants, the exact branching patterns do not conform to the exact histories within this group.

3.2 | Microbial community analysis

For 86% of the processed ant workers (424/492), PCRs with eubacterial 16S rRNA primers resulted in amplicon quantities visibly exceeding those for negative controls. Among the specimens failing to yield 16S rRNA amplification, *COI* genes of ants amplified consistently, arguing against template quality or PCR inhibition as an explanation. Interestingly, Dorylinae species differed in the proportions of workers for which 16S rRNA gene amplification succeeded. For example, we successfully amplified bacterial DNA from 98% of *Eciton burchellii* specimens (120/122), 90% of *Labidus praedator* specimens (69/77), but only 15% of *Labidus coecus* specimens (4/26) (Figure 1). For three of the four *L. coecus* workers yielding amplification, large proportions of reads from 16S rRNA amplicon sequencing libraries corresponded to unique genotypes and OTUs found abundantly in negative controls. This indicated that the numbers of bacteria in tissues of those ants were low, relative to the amounts of bacterial DNA present in reagents and laboratory environments

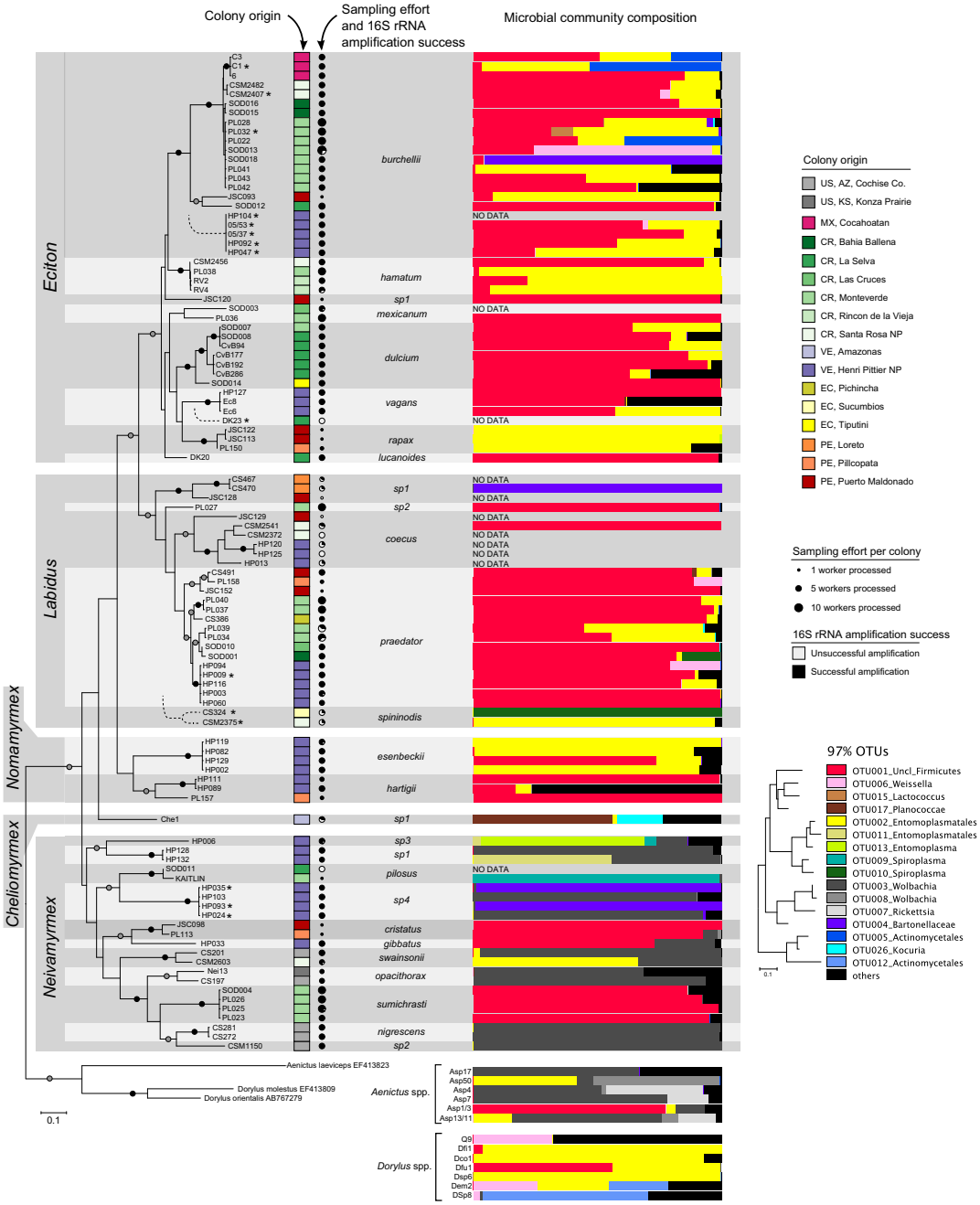

FIGURE 1 The diversity of microbial symbionts across the New World army ants. The ML mtDNA phylogeny of Dorylinae (all genotype/location combinations in our collection) is based on 600-bp sequence of the mitochondrial *COI* gene, and constrained so that *Neivamyrmex* spp. form a clade (following Brady, 2003). Branch lengths represent the mean number of nucleotide substitutions per site. Bootstrap support values between 70% and 94% are represented by grey circles, and between 95% and 100% by black circles on branches. Colonies for which no unambiguous sequence data were obtained (indicated with asterisks) were assumed to be identical at *COI* to morphologically identical colonies from the same location. For four species–location combinations with no available sequence data, their approximate position on the tree is indicated with dashed lines. For all colonies, we indicate their geographic origin, the number of individual workers with DNA extracted, and the proportion of workers for which we succeeded in amplifying 16S rRNA. The relative abundance of bacterial 97% OTUs in ant gasters, based on 16S rRNA amplicon sequencing, is shown for the one worker characterized from each colony, except for those colonies of *E. burchellii* and *L. praedator*, which are featured in Figure 2, where we used an average for all characterized siblings from a single colony. The relationship between symbiont OTUs is demonstrated using a ML phylogeny for the most abundant genotypes from each OTU [Colour figure can be viewed at wileyonlinelibrary.com]

(Salter et al., 2014). These results contrasted sharply with those for amplicon sequence libraries from 55/57 *L. praedator* workers, 78/79 *E. burchellii* workers, 55/55 workers from other New World Dorylinae species and 13/15 for Old World Dorylinae workers. Sequences from these ants were enriched in bacterial taxa found rarely in negative controls; many of these taxa had been known to

colonize ants (Fig. S1). These findings suggest that army ant species differ in the numbers of bacterial cells they contain, although follow-up experiments using more accurate quantitative methods are required to confirm this observation.

We removed from the analysis the eight libraries where the majority of reads represented genotypes and OTUs abundant in negative controls and focused on the remaining 202 libraries (Fig. S1). After contaminant filtering and removal of unaligned, chimaeric or nonbacterial sequences, a total of 3,516,686 reads remained, with 1,399–66,405 (median 15,466) reads per experimental library (Table S6). All calculations below are based on proportions of OTUs, as well as proportions of unique genotypes within OTUs, in individual libraries after the aforementioned quality control steps.

The species-level diversity of army ant-associated bacteria was low. Across all experimental libraries, the median relative abundance of the dominant 97% OTU was 93.9%, the median number of OTUs that reached at least 1% relative abundance in a given library was only two, and only 11% of specimens harboured more than three OTUs exceeding this abundance threshold (Tables S6–S8). Across all 202 libraries, six dominant OTUs accounted for an average of 92.6% reads. The two most abundant, representing the known specialized army ant symbionts Unclassified Firmicutes (OTU001) and Unclassified Entomoplasmatales (OTU002), accounted for an average of 57.8% and 21.6% of reads, respectively. However, there were consistent differences in microbial community composition across libraries representing different ant clades (Figure 1). In three of the genera, *Eciton*, *Labidus* and *Nomamyrmex*, Unclassified Firmicutes- and Unclassified Entomoplasmatales dominated microbial communities of a large majority of the sampled workers. Both of these microbes were also found in some, but not all, species of *Neivamyrmex* and Old World army ants. However, in many characterized ants other bacteria were quite common. For example, in some colonies of *Eciton* and *Labidus*, bacteria identified as Actinomycetales, Bartonellaceae, *Weissella* or *Spiroplasma* were abundant. Microbial communities of *Neivamyrmex* and *Aenictus* species were often dominated by *Wolbachia*, a widespread facultative endosymbiont of insects that was strikingly absent from the other Dorylinae genera.

In addition to variability across higher-level taxa (e.g., genera), we found subtle intraspecific variation in microbiota across several scales within two well-sampled species, *Eciton burchellii* and *Labidus praedator* (Figure 2). Permutational analysis of variance revealed that bacterial communities differed between these two species (ADONIS: $F_{1,123} = 13.96$, $R^2 = .094$, $p = .001$), but also between collection sites ($F_{7,116} = 2.21$, $R^2 = .104$, $p = .005$), although the interaction term was not significant ($F_{2,114} = 2.45$, $R^2 = .033$, $p = .057$; note that colonies of both species were only available from three locations). Furthermore, we found significant differences in microbial community composition among sympatric *E. burchellii* colonies in all cases where at least three colonies were available from a single site ($F_{3,15} = 3.38$, $R^2 = .404$, $p = .002$ in Monteverde, Costa Rica; $F_{3,16} = 4.78$, $R^2 = .473$, $p = .014$ in Henri Pittier NP, Venezuela; and $F_{2,11} = 3.29$, $R^2 = .375$, $p = .024$ in Cocahoatan, Mexico). These differences were largely due to Unclassified Entomoplasmatales (OTU002), Actinomycetales (OTU005) and *Weissella* (OTU006) being abundant in some colonies of *E. burchellii*, thus decreasing the relative abundance of Unclassified Firmicutes (OTU001) (Figure 2, Table S9). We found no significant differences across colonies of *L. praedator* from either Monteverde ($F_{3,13} = 1.83$, $R^2 = .297$, $p = .097$) or from Henri Pittier NP ($F_{3,12} = 0.89$, $R^2 = .182$, $p = .634$). Note that for some of the comparisons above, betadisper() revealed significant differences in dispersion among groups (Table S10), complicating the interpretation of ADONIS results for these very simple microbial communities. Similarly, because of the low bacterial diversity and the dominance of a single OTU in most samples, principal coordinates analysis (PCoA) did not reveal clear structure among samples (Fig. S2).

3.3 | Strain-level symbiont diversity and phylogenetics: Unclassified Firmicutes

A maximum-likelihood phylogeny of Unclassified Firmicutes based on near-full-length 16S rRNA sequences indicated that the symbionts of Dorylinae and two Ponerinae specimens represent a distinct and well-supported clade that belongs to a poorly characterized branch of Firmicutes (Figure 3a). To understand finer patterns of diversity and evolution, we looked at unique 16S rRNA genotype distributions within dominant symbiont OTUs in each sample, using our Illumina amplicon sequencing data (Fig. S3). In the case of Unclassified Firmicutes, a single unique genotype typically made up 75%–80% of that OTU; we believe that in most cases it represented the error-free sequence of the single bacterial strain present in that ant. The remaining 20%–25% of the Unclassified Firmicutes OTU typically consisted of low-abundance genotypes, most of which differed at a single nucleotide position from the dominant genotype; we believe that the vast majority of these rare "unique genotypes" were erroneous sequence reads of the dominant genotype. However, in some cases two distinct unique genotypes were present at high abundance, suggesting the presence of more symbiont variants in a single ant, or perhaps differences between rRNA operons within the genome of a single strain. Several 16S rRNA genotypes from the Unclassified Firmicutes OTU were distributed across multiple host species and geographic locations. At the same time, some *Eciton burchellii* and *Labidus praedator* colonies harboured multiple 16S rRNA genotypes of these bacteria, with different putative strains typically distinguishing individual workers.

Sanger sequencing of the ribosomal protein gene *rplB* enabled finer-scale comparisons of relationships among strains. We obtained clean and unambiguous traces for approximately 90% of *rplB* sequences of Unclassified Firmicutes (253 sequences in total), and about 70% of Entomoplasmatales. Notably, we observed ambiguous peaks in *rplB* chromatograms in a large proportion of samples where 16S rRNA amplicon sequencing indicated the presence of more than one common symbiont genotype, arguing that those indicators of strain coinfection were not likely due to 16S rRNA operon divergence or sequencing error. One of the challenges with inferences of multiple strain coinfection was suboptimal specificity of our primers,

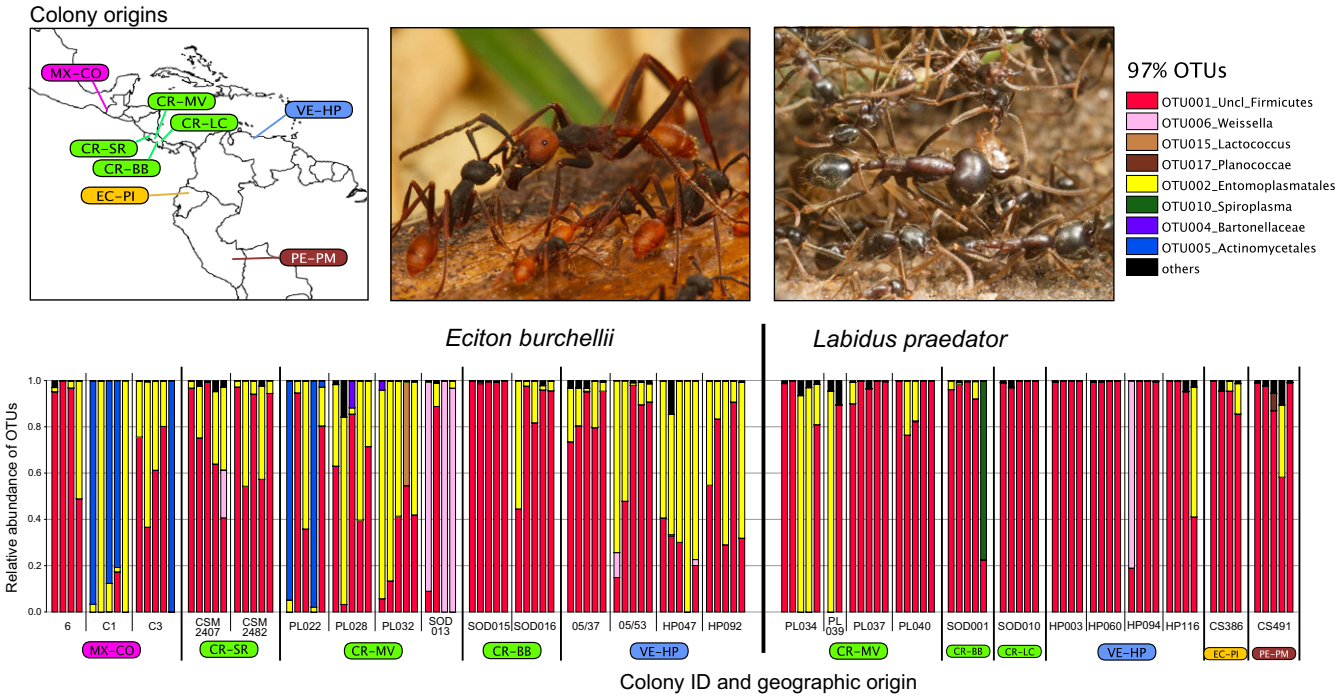

FIGURE 2 Variation in the microbial community composition at the colony and species levels in two broadly sampled ant species. The relative abundance of microbial species (97% OTUs) is shown for individual workers from 15 colonies of *Eciton burchellii* and 12 colonies of *Labidus praedator*. Bars represent individual ants; colonies are separated by thin lines and populations from different locations by thicker lines. Ant photographs were taken by JGS [Colour figure can be viewed at wileyonlinelibrary.com]

developed using genomic sequences of distantly related bacteria. This was evidenced by Entomoplasmatales primers yielding occasional amplification of *rplB* from other Mollicutes, and in a few cases, of Unclassified Firmicutes. As such, while multiple peaks in chromatograms may often imply coexistence of related strains within a single ant, this may not always have been the case.

Using the *rplB* gene for phylogenetics allowed us to address questions of symbiont evolution and specialization. Because of challenges in reconstructing the branching order for deeper parts of the *rplB* tree, we constrained this phylogeny based on deep branch relationships in the 16S rRNA tree, keeping Ponerinae and most *Aenictus* and *Dorylus* symbionts outside the main *Eciton–Labidus* symbiont clade (Figure 3b, see also Fig. S5 for an unconstrained tree). In both the constrained and unconstrained versions of the *rplB* tree, strains originating from the same host species belonged to distinct clades. For example, Unclassified Firmicutes of *Eciton burchellii* formed four clades; all were monophyletic, although some lacked strong bootstrap support. Four such host species-specific clades were identified for *Labidus praedator*, and several host species with less comprehensive sampling recapitulated this trend. Host species-specific clades commonly included strains from distant geographic locations. In many cases, replicate workers from a single colony hosted different symbiont genotypes, belonging to divergent host-specific clades. For example, among eight workers from *Eciton burchellii* colony PL028 we identified four symbiont genotypes representing three divergent clades. Among six workers from *Labidus praedator* colony PL037, we found five genotypes representing three divergent clades (Figure 3b). Unfortunately, low resolution and poor support of deeper nodes in the tree limited our abilities to fully characterize relationships between these disparate lineages.

Next-generation sequencing of *rplB* amplicons (454 pyrosequencing) provided a deeper insight into strain-level diversity of Unclassified Firmicutes in single hosts. Among the four sampled *E. burchellii* workers, all from a single colony, we obtained a total of 11,712 high-quality reads, with a range of 1,360–5,169 per sample. Sequences clustered into 43 OTUs at the 98% similarity level and each OTU was represented in only one worker. Within each library, between 99.54% and 99.58% of reads classified to a single OTU (Table S11; Fig. S4), and in each of these four dominant OTUs, the consensus sequence was identical to the reference Sanger sequence from the same worker (Table S12). Analysis and manual inspection of alignments of reads classified to these four dominant OTUs strongly suggested that virtually all positions with under 99% consensus across reads were the result of sequencing errors at homopolymer sites, or of low sequence quality near the ends of reads (i.e., after the 300th base; Table S12). Alignments of representative sequences from low-abundance OTUs suggested that sequencing error was the most likely driver of their existence: rare OTUs were unique to each sample and differed from sequences in common OTUs by multiple substitutions and indels, mostly in homopolymer tracks. Together, these results suggest that the four studied ants from a single colony each hosted a single and unique strain of Unclassified Firmicutes. Note that this may not always be the case, particularly in specimens where 16S rRNA amplicon sequencing data indicate the presence of different 16S rRNA genotypes in a single sample (Fig. S3) and where *rplB* traces obtained using the Sanger method are ambiguous.

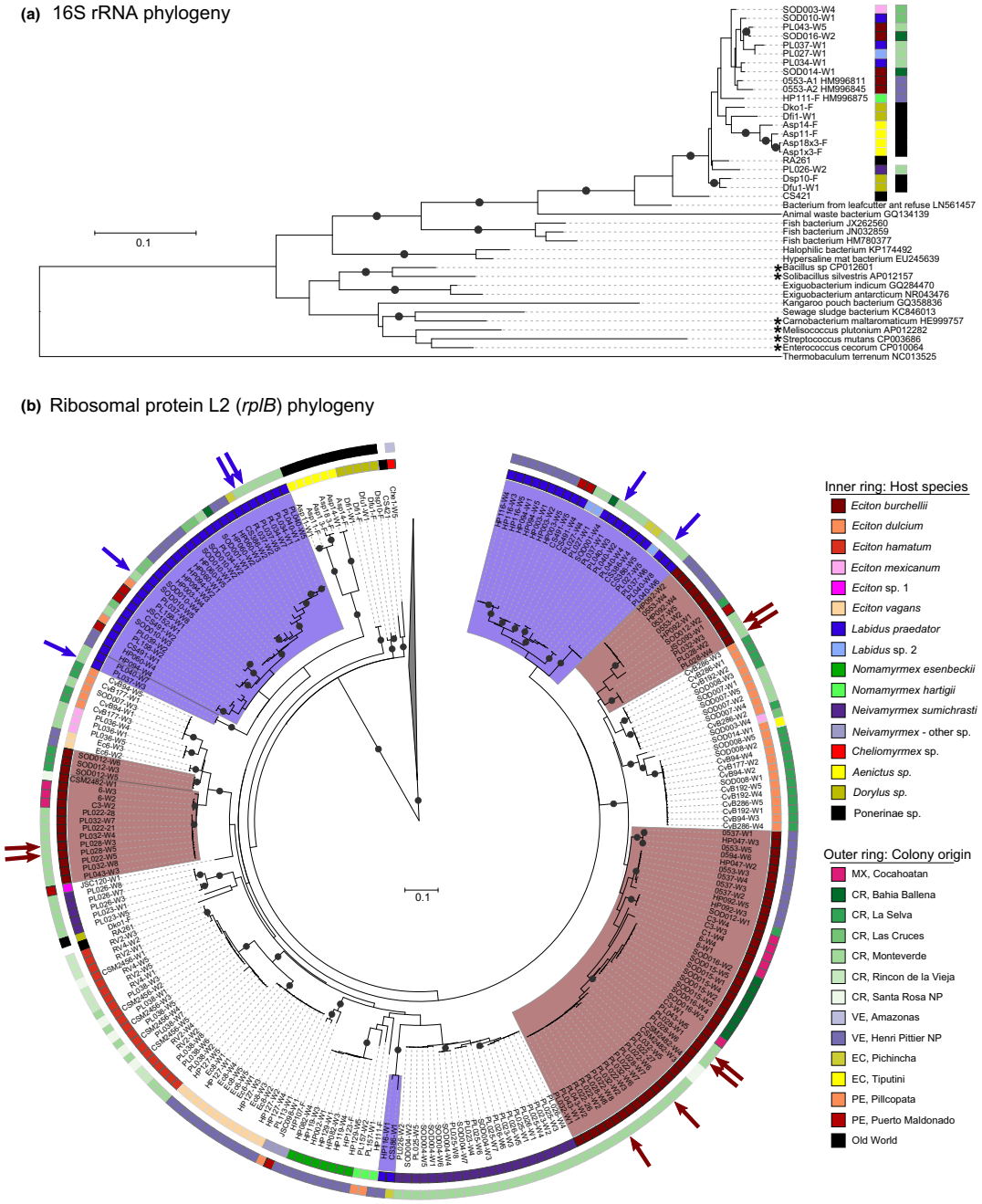

FIGURE 3 Phylogenetic position and relationships between Unclassified Firmicutes strains. (a) ML phylogeny of selected Unclassified Firmicutes from diverse ants, as well as selected outgroups available in GenBank, based on near-full-length sequence of the 16S rRNA gene. (b) ML phylogeny of Unclassified Firmicutes from 252 ant workers, based on a 573-bp sequence of ribosomal protein L2 (*rplB*) gene. Bootstrap support values of 95% or more are represented by black dots on branches. Inner coloured ring indicates host species, and the outer ring indicates colony origin. In the *rplB* tree, we further highlighted clades composed of symbionts of the two most extensively sampled species, *Eciton burchellii* and *Labidus praedator*, and indicated with arrows all specimens from a single colony of each species. The six species indicated with asterisks in the 16S rRNA tree were used as outgroups in the *rplB* tree. Deeper nodes of the *rplB* tree were constrained based on the 16S rRNA tree, as described in Methods [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Strain-level symbiont diversity and phylogenetics—Unclassified Entomoplasmatales

In contrast to the patterns for Unclassified Firmicutes, our analyses of 16S rRNA amplicon sequence data suggested that it was more common for single ants to harbour multiple genotypes of the Unclassified Entomoplasmatales symbiont (i.e., OTU002; Fig. S3C). Noisy sequence traces from our efforts to sequence *rplB* gene fragments after amplification with Entomoplasmatales-specific primers were also consistent with multiple genotypes or strains in single workers. Phylogenetic placement of "clean" *rplB* sequences (Figure 4) revealed that New World Dorylinae host multiple clades from this

bacterial order, showing variable relatedness to named species in the genera *Entomoplasma*, *Mesoplasma*, *Mycoplasma* and *Spiroplasma*. As seen in prior studies that relied on 16S rRNA (Anderson et al., 2012; Funaro et al., 2011), most army ant associates grouped into a single lineage, which in our analysis was made up exclusively of bacteria from army ants. Within this lineage, symbionts typically grouped into host-specific clades. Replicate ant workers from the same colonies commonly hosted distinct symbiont genotypes, sometimes differing at a few nucleotide positions and other times belonging to divergent clades. But again, the fact that noisy sequence traces were commonly obtained when sequencing *rplB* product, combined with the fact that there was often more than one abundant 16S rRNA genotype within the Entomoplasmatales OTU (Fig. S3C) in our amplicon data set, suggests that individual army ant workers may host multiple Entomoplasmatales strains relatively often.

3.5 | Fluorescence in situ hybridization

In all examined Dorylinae species, we observed clear and specific hybridization of fluorescently labelled probes targeting eubacteria and at least one of the two specialized symbionts (Figure 5). Fluorescent signal always localized to the hindgut lumen (Figure 5.1-3, 5.6). In *Eciton burchellii*, we identified several disjunct clusters of bacteria staining with either Entomoplasmatales or Unclassified Firmicutes probes within the pylorus and ileum (Figure 5.3). In the hindgut of *Nomamyrmex hartigii*, most bacterial cells stained with Unclassified Firmicutes probes (Figure 5.8), but we also found clusters staining with Entomoplasmatales probes (Figure 5.5). In the hindgut of *Labidus praedator*, the signal of eubacterial probes overlapped perfectly with the signal of Unclassified Firmicutes probes, and we never detected Entomoplasmatales signal (Figure 5.4, 5.7). Finally, in the hindgut of *Eciton rapax*, we observed no specific signal of Unclassified Firmicutes probes, and the signal of eubacterial probes overlapped with that of the Entomoplasmatales probe (Figure 5.6). Interestingly, in this last species we also found bacterial cells that stained with Entomoplasmatales probes in the foregut, within the crop, attached to what was likely a food pellet (Figure 5.9). These cells formed dense clusters and were ovoid in shape, distinguishing them from rod-shaped Entomoplasmatales cells found in the hindgut.

The results for the four species are consistent with amplicon sequencing data, which revealed: a combination of Entomoplasmatales and Unclassified Firmicutes in most *E. burchellii* colonies, the dominance of Unclassified Firmicutes in guts of *Labidus praedator* and *Nomamyrmex hartigii*, and microbial communities dominated by distinct genotypes of Entomoplasmatales, without Unclassified Firmicutes, in single specimens from three *E. rapax* colonies (Figure 1).

4 | DISCUSSION

Our study combined broad sampling across populations of diverse army ants with deep sampling of two focal species. The specialized and broadly distributed microbes recovered through these efforts match those from prior studies relying on more traditional methods (Anderson et al., 2012; Funaro et al., 2011; Russell et al., 2012). However, the increased scale of sampling and the inclusion of a protein-coding gene in our phylogenetic analysis provide us with much greater insight into host specificity and evolutionary histories of the dominant symbiotic microbes. When combined with symbiont localization through fluorescence microscopy, our study identifies the two dominant, specialized bacteria as gut lumen colonizers, establishing army ants as yet another specialized feeding guild with conserved ectosymbioses.

4.1 | Army ant workers harbour low-diversity symbiont communities

In-depth sequencing of 16S rRNA gene revealed that bacterial communities of the New World Dorylinae are dominated by a small number of species of specialized gut bacteria. While the gut microbiota of arthropods are less complex than those of vertebrates (Engel & Moran, 2013), the low levels of diversity reported here still stand out. In a meta-analysis using cloned 16S rRNA libraries sequenced at varying depth, Colman, Toolson, and Takacs-Vesbach (2012) reported that different insect taxa and functional groups typically harbour 11–103 97% OTUs. Studies from a sample of diverse insects from Hawaii suggested that the average number of 97% OTUs at relative abundance of ≥1% was 7.5 (Jones et al., 2013). Herbivorous ants from the genus *Cephalotes* host 15–20 "core" 97% OTUs (Hu et al., 2014). The number of "core" 97% OTUs in guts of honeybees and bumblebees is approximately eight (Koch & Schmid-Hempel, 2011a; Kwong & Moran, 2015). This is much more than a median of only two 97% OTUs that we observed in army ants. It should be noted that these comparisons may have been somewhat impacted by differences in data analysis methods (Kunin, Engelbrektson, Ochman, & Hugenholtz, 2010; Quince et al., 2009). For example, the custom contaminant filtering used in the current study would have excluded real microbial symbionts of army ants that never exceeded ~0.2% of the community in any sequenced library. However, the relative importance of such rare microbes to the host would be questionable, and proving that they represent real ant associates rather than experimental artefacts would be a daunting task.

4.2 | How ancient and specialized are the gut bacteria of army ants?

Our data suggest that the two symbionts of army ants, Unclassified Firmicutes and Unclassified Entomoplasmatales, have persisted and often dominated the gut microbial communities of their hosts across vast phylogenetic and geographic scales. The Old World and the New World army ants split approximately 87 million years ago (Brady et al., 2014), and it appears that their common ancestor already hosted these two microbes. The phylogenetic relationships among extant strains suggest that the symbionts have codiversified with hosts, but also that they have undergone occasional transfer

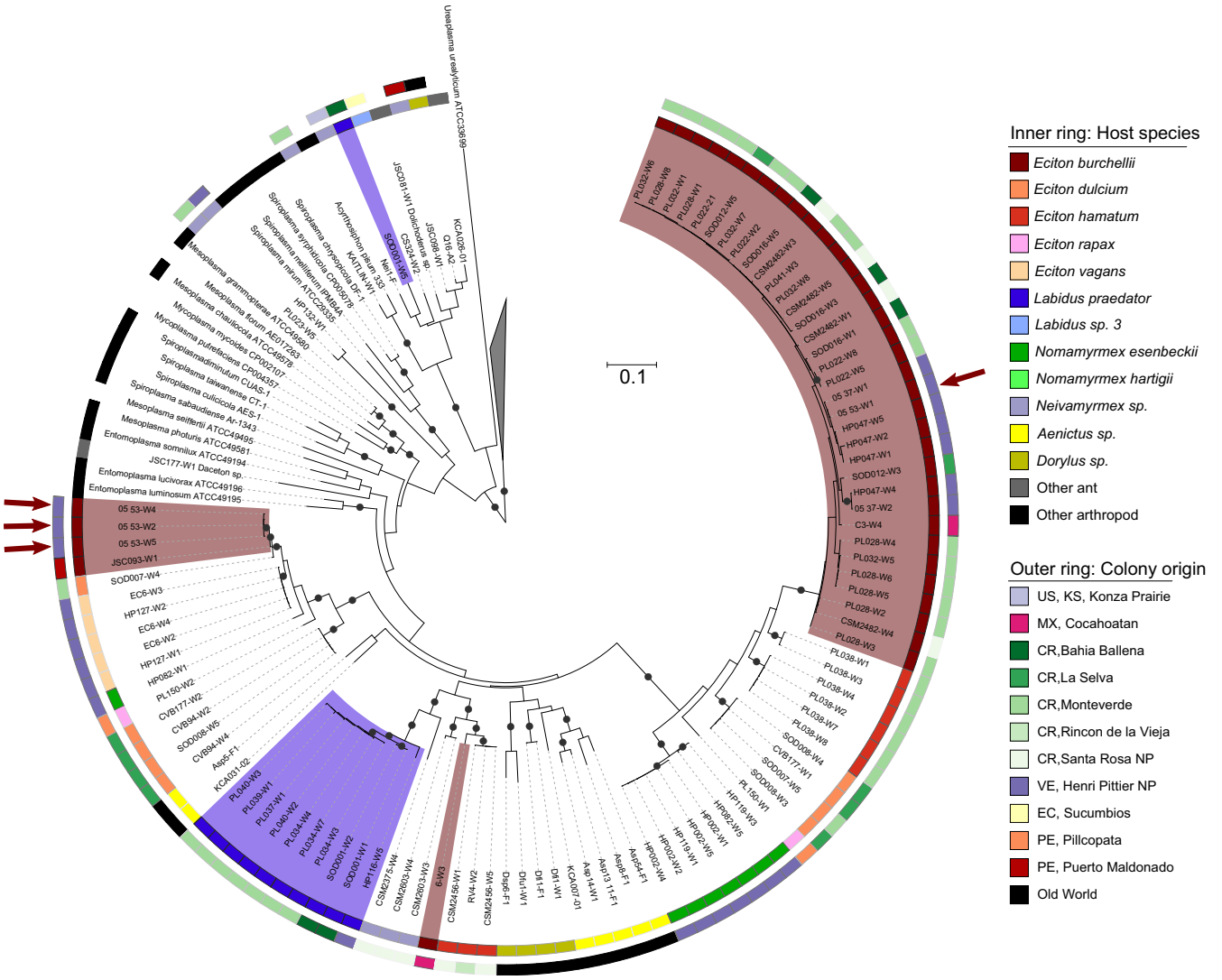

FIGURE 4 Diversity and specificity of Entomoplasmatales from Dorylinae and other ants, based on a 579-bp sequence of ribosomal protein L2 (*rplB*) gene. Bootstrap support values of 95% and over are represented by black dots on branches of ML tree. Inner coloured ring indicates host species and the outer ring—colony origin. Clades comprised of sequences amplified from the two most extensively sampled species, *Eciton burchellii* and *Labidus praedator*, are further highlighted. Arrows indicate all specimens from a single colony of *E. burchellii* [Colour figure can be viewed at wileyonlinelibrary.com]

among army ant species, and perhaps beyond. Understanding the evolutionary history of these associations could provide insights into ant biogeography, as well as into the roles the symbionts play in ant biology. Further testing of this ancient infection model would benefit from examination of Dorylinae genera that are more closely related to the New World army ants than are either *Aenictus* and *Dorylus*, the two genera traditionally lumped within the "Old World" grouping (Borowiec, 2016). Another challenge comes from the findings that some of the predatory ants from the subfamily Ponerinae host divergent strains of Unclassified Firmicutes (Russell et al., 2009b; Figure 3a) and that a microbe with 16S rRNA sequence identity of ~90% to New World Dorylinae symbionts was found in leafcutter ant refuse (Figure 3a). The pairwise similarity at 16S rRNA gene among strains infecting Dorylinae always exceeds 95% (Figure 3a). Similarly, the large majority of Entomoplasmatales strains infecting Dorylinae belonged to army ant-specific clades (Funaro et al., 2011). However, more recent efforts have detected closely related microbes in two Ponerinae species (Kautz, Rubin, & Moreau, 2013a), and the NCBI database contains multiple sequences from other hosts that are at least 94% identical within the V4 region of 16S rRNA gene to the army ant symbionts. As larger amounts of sequence data for symbionts infecting a broader range of hosts become available for phylogenetic comparisons, our understanding of the evolutionary history of this group is likely to improve.

In contrast to these specialized bacteria, *Wolbachia*—the most widely distributed endosymbiont of insects (Zug & Hammerstein, 2012) and the third most abundant microbe in our data set—was prevalent in two distantly related and geographically disparate army ant genera (*Aenictus* and *Neivamyrmex*), yet strikingly absent from other army ants in our study. These patterns suggest more recent

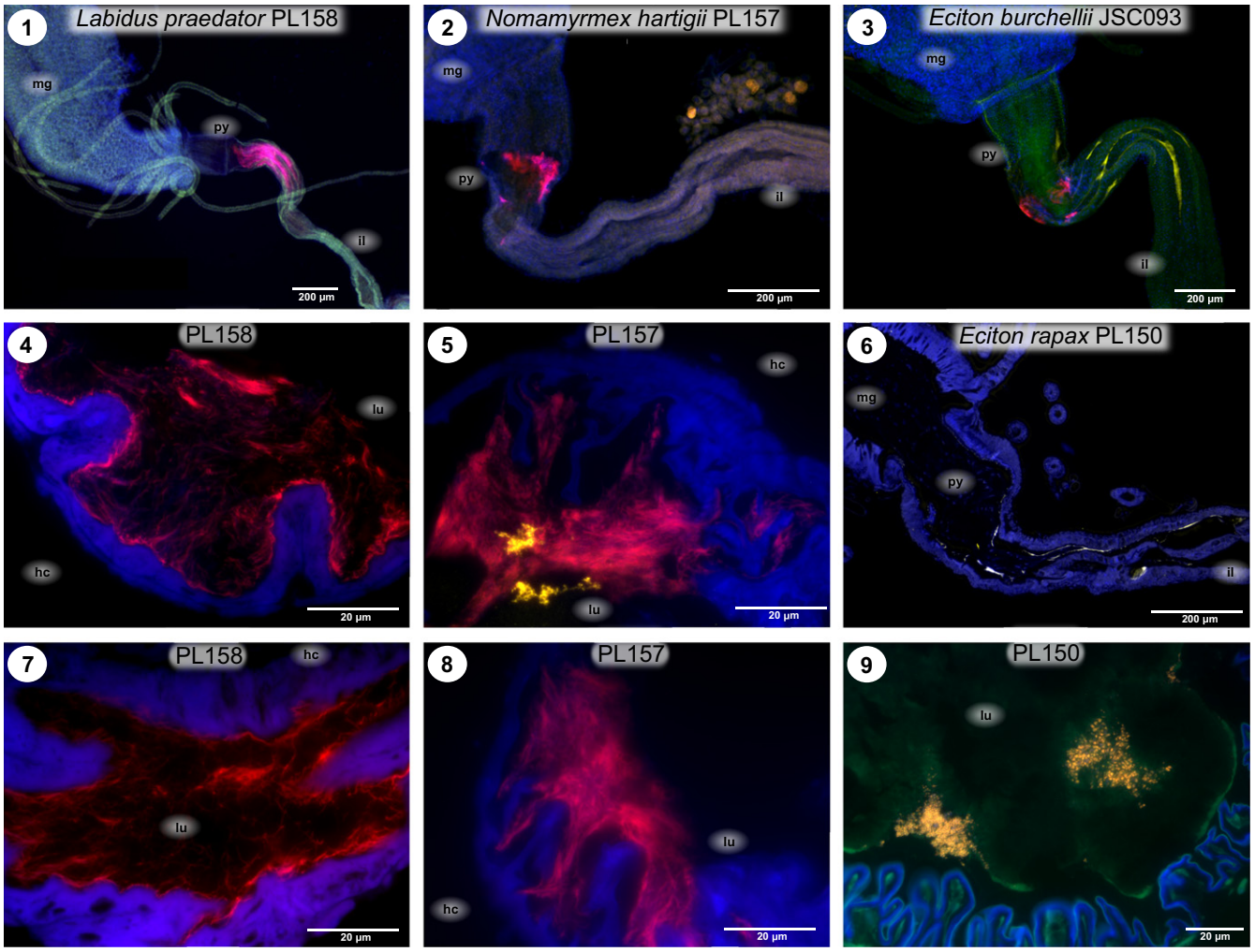

FIGURE 5 Localization of symbiotic bacteria within digestive tracts of workers of four species of Neotropical Dorylinae, demonstrated using fluorescent in situ hybridization (FISH), either of complete dissected guts (whole-mount—images 1–3) or of resin sections. (1–3) Lower midgut and upper hindgut; (4–5) Cross sections of the pylorus; (6) Longitudinal section of lower midgut and upper pylorus; (7–8) Section of ileum; (9) Bacterial colonies on a pellet in the foregut (crop). Blue represents DAPI—universal DNA stain; green—tissue autofluorescence (images 1–3 and 9 only); red—either universal eubacterial probes (images 1, 2, 5) or Unclassified Firmicutes-specific probe (3, 4, 7, 8); yellow—Entomoplasmatales-specific probe (3, 5, 6, 9). Abbreviations: mg—midgut; py—pylorus; il—ileum; lu—gut lumen; hc—haemocoel [Colour figure can be viewed at wileyonlinelibrary.com]

associations between army ants and their current suite of *Wolbachia* associates. Prior findings of close relatedness between *Wolbachia* of these ants and those of other ants and insects argue against strong specialization, at least at the level of army ant genus (Russell et al., 2009a). *Wolbachia* is known to vary in infection prevalence across host clades (Russell, 2012; Russell et al., 2012), and the reasons for this are generally not understood. However, in *Anopheles* mosquitoes, bacteria of the genus *Asaia*, found in guts and other tissues, impede *Wolbachia* transmission (Hughes et al., 2014), raising the possibility that the gut symbionts of army ants could also influence *Wolbachia* distributions.

Beyond these three microbes, other bacteria showed few distributional patterns (Figure 1). For example, deep sampling from two army ant species suggested differing abundance of *Actinomycetales* and *Weissella* species across colonies from the same locales; however, sibling workers from the same colonies showed variability in the relative abundance of these taxa (Figure 2). It is unlikely that these two bacteria have engaged in long-term or highly specialized associations with army ants. For example, BLASTn searches using representatives of the single *Weissella* OTU found in the New World and the Old World army ants revealed 98%–100% identity at the V4 region of 16S rRNA to strains reported from animals, food products and other habitats. Representative sequences from the Actinomycetales OTU were 3% divergent from those of the closest known relatives, reported from corals, sponges and other environments, primarily marine. While abundant in three *E. burchellii* colonies, this microbe was absent from other ant samples, again suggesting a recent origin for this association.

While not unprecedented, broad distributions and dominance by ancient, highly specialized gut ectosymbionts is certainly not typical for insects. Indeed, bacterial gut communities of insects can be highly labile and strongly influenced by diet (Engel & Moran, 2013).

However, a number of social insects exhibit patterns of some resemblance to those in army ants. Honeybees and bumblebees provide an example. These two groups diverged ~87 million years ago (Cardinal & Danforth, 2011), similar to the divergence time between New World and Old World army ants. Like army ants, these bees harbour specialized symbiotic gut bacteria that are thought to have persisted since the time of their common ancestor (Engel et al., 2012; Kwong & Moran, 2015). In this system, two of the most dominant and recurrent bacteria, *Snodgrassella* and *Gilliamella*, seem to have codiversified with their bee hosts, albeit with multiple cases of apparent host switches and occasional losses (Koch et al., 2013). Beyond the corbiculate bees, complex microbial communities of termites also show evidence of codiversification, at least over recent evolutionary timescales (Brune & Dietrich, 2015; Dietrich, Köhler, & Brune, 2014; Mikaelyan et al., 2015). Gut microbial communities from turtle ants in the genus *Cephalotes* also show trends consistent with codiversification. The modestly diverse microbiomes of *Cephalotes* workers consist of ~15–20 bacterial OTUs; and many residents show broad distributions across this 46-million-year-old genus (Hu et al., 2014; Price et al., 2014; Sanders et al., 2014). When OTUs are clustered at appropriate depths (i.e., 99% 16S rRNA identity, given expectations for sequence divergence over 46 million years), the community-level dendrogram for these microbiota shows congruence with the host phylogeny (Sanders et al., 2014). This signal is consistent with codiversification, although more detailed comparisons of phylogenies between specific symbionts and hosts await execution.

The three aforementioned social insect groups feed on recalcitrant and imbalanced plant- or fungus-based diets and rely on microbial associates for digestion, nutritional supplementation (Brune & Dietrich, 2015; Engel et al., 2012; Warnecke et al., 2007), and in at least some cases, for defence (Koch & Schmid-Hempel, 2011b). Omnivorous and carnivorous ants tend to host lower numbers of bacteria in their guts (Sanders et al., 2017), and in the few groups characterized, the microbial communities appear to consist of less specialized associates (Hu et al., 2017; Ishak et al., 2011). While the abundance and importance of bacterial symbionts may vary across army ants, as suggested by differences in our 16S rRNA amplification success for different species (Figure 1; see also Sanders et al., 2017), the recurrent and ancient symbioses of Dorylinae appear exceptional among predatory insects.

4.3 | Community structure emerges at the strain level

While our 16S rRNA amplicon sequence data suggest differences in microbiota composition among army ant genera, many of the differences seen at regional- and colony-level scales were subtle. In addition, bacterial communities of sibling workers were often quite similar, with exceptions being driven by more sporadically occurring microbes. However, our *rplB* sequence data revealed a good deal of genetic variability that was undetected within the V4 region of 16S rRNA gene (Fig. S6A). Similar patterns were recently reported from corbiculate bees (Powell et al., 2016). The presence of distinct and well-supported host species-specific clades (Figure 3b) suggests specialization and stability of host–symbiont associations across evolutionary timescales. These clades commonly consisted of strains from geographically distant locations, revealing further structure in our data set not recovered with 16S rRNA. Furthermore, for each of the comprehensively sampled army ant species except *E. hamatum*, we identified several distinct host-specific symbiont clades, suggesting long-term coexistence of multiple divergent symbiont lineages, extending back for numerous generations within a single matriline.

Among our most curious findings was the discovery that distinct Unclassified Firmicutes strains colonize different workers and that the majority of workers host only a single strain of this bacterium. Thus, while an individual workers' microbiota may not be highly diverse, unexpected symbiont strain diversity is retained at a colony level. While another group of social insects, honeybees, shows similar patterns of multistrain persistence within colonies, different strains commonly infect the same bee workers (Engel et al., 2014; Powell et al., 2016), suggesting a unique type of diversity structuring within the army ants.

Similar to Unclassified Firmicutes, Entomoplasmatales of army ants are represented at the colony level by multiple distinct strains that often monopolize worker individuals (Figures 4, S2C). However, the number of distinct host-specific clades appears to be less than in the case of Unclassified Firmicutes, and coexistence of two or more strains within a single worker seems to be more common. Findings that strain identities (Figures 3 and 4) and the overall microbial community composition (Figure 1) can vary among sibling workers raised the possibility for correlations between these levels of community structure. However, there appear to be no strong relationships between the genotype of the dominant gut symbiont strain(s) and the species compositions of individual workers (Fig. S7A & S7B), leaving the drivers of variation in symbiotic community composition unexplained.

4.4 | For socially transmitted symbionts, modes of host colony founding are likely drivers of diversity and community structure

Sociality provides a unique context for the transfer of surface or gut bacteria (Salem et al., 2015), enabling regular spread of microbes among members of the same colony. Young honeybee and bumblebee workers acquire their specialized gut microbiota through exposure to faeces present in a nest (Powell, Martinson, Urban-Mead, & Moran, 2014). Similarly, oral–anal trophallaxis seems to explain the transfer of gut symbionts in several herbivorous ants including those in the genus *Cephalotes*, whose newly eclosed workers consume faecal fluids from older adults (Cook & Davidson, 2006; Lanan et al., 2016; Wilson, 1976). It is plausible that newly eclosed Dorylinae workers acquire their microbiota through similar mechanisms (i.e., coprophagy or trophallaxis). High microbial strain-level diversity within army ant colonies may, thus, be maintained through a combination of social transmission and (as discussed below) the large numbers of individuals that found new colonies.

Modes of colony founding vary across social insects (Cronin et al., 2013), ranging from independent founding by single queens, to colony fission in which colonies split, so that young queens are accompanied by a substantial work force from the beginning. Army ants as well as honeybees engage in colony fission (Cronin et al., 2013; Peeters & Ito, 2001). In contrast, queens of *Cephalotes*, fungus-growing ants and bumblebees found colonies independently. It is not clear whether the mode of colony founding may play a significant role in determining the diversity of bacterial symbionts colonizing an individual social insect, especially when compared to factors such as diet, host genotype, or the number of social inoculation events solicited by the individual. However, it is plausible that in social insects with dependent colony founding (e.g., fission), socially transmitted symbiont strains unable to coexist within single host individuals could be retained within a colony for multiple worker generations if always found in different host individuals. Hence, in dependent colony founders, the lack of strong bottleneck associated with the founding event could enable greater diversity of socially transmitted symbionts at a colony level than normally found in a single host individual. In contrast, it can be expected that in independent colony founders, only those symbiont strains that can coexist within a founding queen can be passed on to workers. Strain diversity for the ectosymbionts of *Cephalotes* ants (gut symbionts) and attines (residents of laterocervical plates on the cuticle—Mueller, 2012) in some ways fit the expectations resulting from a strong symbiont bottleneck through a single individual. In particular, colonies seem differentiated by the presence of distinct strains within the core symbiont taxa (Anderson et al., 2012; Hu et al., 2014). In army ants, colony-level structuring of strain diversity is less apparent, at least based on our current levels of sampling depth. We note that colony founding differences have also been invoked as the driver behind higher *Snodgrassella* strain-level diversity in colonies of honeybees vs. bumblebees (Ellegaard & Engel, 2016; Powell et al., 2016), suggesting the importance of this factor in structuring social insect microbial communities.

4.5 | Symbiont strain competition, and a model-based approach

Transmission bottlenecks may, indeed, be much less severe for socially transmitted symbionts in social insects that utilize colony fission. But while this could explain the persistence of diverse symbiont strains across individuals within a single social unit, it does not explain why Unclassified Firmicutes strains should show such rare co-occurrence within single workers. A possible explanation is a strong competition or antagonism among strains, frequently resulting in competitive exclusion of all but one strain. Patterns observed in the army ant system could be explained by priority effects: if first colonizers gained a strong competitive advantage, such symbionts could come to dominate their host resources. Host genotype could play a role (Spor, Koren, & Ley, 2011), as could the large differences in behaviour and anatomy among castes. Another plausible explanation is that the initial inoculum received by newly eclosed workers tends to come from a single sibling. Under such a scenario, single strain domination would not require strong within-host microbial interactions as a mechanism behind such structure, especially if host ants become less receptive to colonization by future strains due to changes in behaviour, morphology or physiology (Lanan et al., 2016). Distinguishing among these possibilities will require additional research, as will explaining the contrasting coexistence of related strains within the guts of corbiculate bees or *Cephalotes* ants.

Of further consideration will be theoretical efforts to understand the maintenance of symbiont diversity. For instance, strain distributions and persistence across worker generations could be modelled using population genetics tools, where symbiont strains act like alleles in populations (Jaenike, 2012), represented here by colonies. Over time, as bacterial strains present in a colony monopolize newly emerged workers and old workers die, we would expect shifts in relative abundance of strains, extinction of some, and emergence of new strains through mutation. Horizontal transfer of strains among colonies and perhaps species would, similarly, simulate migration. Finally, colony fission events would have an effect comparable to emergence of geographic barriers. We reason that the large colony size and a lack of transmission bottlenecks suggest that genetic drift-like processes are a weaker force in eroding symbiont diversity in army ant colonies. With occasional novel strain acquisition through horizontal transfer and new strains arising through mutations, one might come to expect a diverse collection of related strains to emerge and be maintained over time. The role of natural selection acting on individuals, colonies or other levels must also be considered in explaining the maintenance of diverse symbiont strains within colonies. However, our current lack of knowledge on symbiont function inhibits our abilities to derive strong predictions. And while we know that Dorylinae species differ in colony sizes, dietary specialization and habitat characteristics (Table S13; Bulova, Purce, Khodak, Sulger, & O'Donnell, 2016; Gotwald, 1995; Rettenmeyer, Chadab-Crepet, Naumann, & Morales, 1983), data are available for only some species, making it difficult to examine connections between host biology and symbiont composition.

4.6 | What functional role do army ant gut microbes play?

Due to their stability over long evolutionary time and presence across multiple species, our data raise the possibility for an important functional role for the most abundant gut microbes of army ants. Army ants are predatory, with diets consisting primarily of other insects (Kronauer, 2009). Nitrogen-recycling symbioses found among some herbivorous ants (Feldhaar et al., 2007) were largely anticipated due to host reliance on nitrogen-poor diets (Cook & Davidson, 2006; Davidson et al., 2003; Russell et al., 2009b), but researchers did not foresee specialized symbioses in predatory ants. The first published genome of a symbiont from a predatory ant, an alphaproteobacterium *Tokpelaia holldobleri*, contained genes involved in protein degradation, urea hydrolysis, as well as amino acid and

vitamin biosynthesis, suggesting roles in nitrogen metabolism and perhaps some aspects of nutrition (Neuvonen et al., 2016). Gut symbionts of insects can also contribute towards detoxification of secondary metabolites, as well as towards development, physiology and immune system development (Broderick et al., 2014; Buchon, Broderick, Chakrabarti, & Lemaitre, 2009; Shin et al., 2011). Furthermore, microbes present on external body surfaces or within the digestive tract of insects can defend hosts against parasites and pathogens (Currie et al., 1999; Kaltenpoth & Engl, 2014; Kaltenpoth, Gottler, Herzner, & Strohm, 2005; Koch & Schmid-Hempel, 2011b). At this point, no genomes of army ant symbionts, or their close relatives, are available for functional analysis. Future metagenomic and, when possible, experimental studies are, hence, needed to reveal the roles of these specialized bacteria. It is worth noting that other carnivorous insects, such as carrion beetles (Kaltenpoth & Steiger, 2014), harbour specialized gut symbionts, making it possible that symbioses involving specialized ectosymbioses could be regular features beyond the herbivores and fungus-growing insects (Cafaro et al., 2011; Hulcr et al., 2012).

5 | CONCLUSIONS

Characterizing the diversity, distribution and functions of microbial symbionts across insects is crucial to understanding the history and present-day biology of the most diverse group of animals on the planet. Ants, in particular, represent one of the most successful groups of insects in terms of their diversity and biomass. Microbial symbionts are thought to have facilitated their dominance of prey-poor rainforest canopies (Davidson et al., 2003). Additionally, symbiotic microbes have been clearly important in the fungus-farming ants, which are among the most influential insects of the tropics (Hölldobler & Wilson, 2011). Our data suggest that ants from a third specialized dietary niche—top predators—are also enriched for highly conserved and specialized symbionts, raising important questions for the roles and impacts of microbial symbionts across insect predators and other carnivorous animals.

ACKNOWLEDGEMENTS

We thank Christoph von Beeren and Kaitlin Baudier for providing ant specimens, and to Ulrich Mueller and two anonymous reviewers for discussion and valuable comments. This project was supported by NSF grants 1050360 to J.A.R. and 1050243 to C.S.M., as well as JSPS Postdoctoral Fellowship (Short-term) PE13061 to P.Ł.

DATA ACCESSIBILITY

Sanger sequences were deposited in GenBank (Accession nos KX982883–KX983349). Amplicon sequencing data were deposited in NCBI Short Read Archive (BioProject: PRJNA341802; 16S rRNA data: SRR4409391–4409626; Unclassified Firmicutes rplB data: SRR4343839–4343842). Colony and worker details are provided in the Supplementary Tables S1–S3. In Tables S1–S3, we also listed the above accession numbers by colony, worker individual and data type. All samples were collected in accordance with national and international laws; collection permit numbers include 122-2009, 192-2012 and R-009-2014-OT-CONAGEBIO (Costa Rica).

AUTHOR CONTRIBUTIONS

P.Ł. and J.A.R. designed the research. P.Ł., J.G.S., C.S.M., D.J.K., S.O.D. and J.A.R. provided specimens. P.Ł., J.A.N. and Y.H. generated molecular data. P.Ł., with contributions from J.G.S., Y.H., C.S.M. and J.A.R., analysed the molecular data and illustrated the results. P.Ł. and R.K. performed fluorescence microscopy. P.Ł. and J.A.R. wrote the manuscript, and all authors contributed to revisions.

REFERENCES

Andersen, S. B., Hansen, L. H., Sapountzis, P., Sørensen, S. J., & Boomsma, J. J. (2013). Specificity and stability of the *Acromyrmex–Pseudonocardia* symbiosis. *Molecular Ecology*, *22*, 4307–4321.

Anderson, K. E., Russell, J. A., Moreau, C. S., Kautz, S., Sullam, K. E., Hu, Y., … Wheeler, D. E. (2012). Highly similar microbial communities are shared among related and trophically similar ant species. *Molecular Ecology*, *21*, 2282–2296.

Baumann, P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology*, *59*, 155–189.

Bennett, G. M., & Moran, N. A. (2015). Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 10169–10176.

Billen, J., & Buschinger, A. (2000). Morphology and ultrastructure of a specialized bacterial pouch in the digestive tract of *Tetraponera* ants (Formicidae, Pseudomyrmecinae). *Arthropod Structure & Development*, *29*, 259–266.

Borowiec, M. (2016). Generic revision of the ant subfamily Dorylinae (Hymenoptera, Formicidae). *ZooKeys*, *608*, 1–280.

Boswell, G. P., Britton, N. F., & Franks, N. R. (1998). Habitat fragmentation, percolation theory and the conservation of a keystone species. *Proceedings of the Royal Society B: Biological Sciences*, *265*, 1921–1925.

Brady, S. G. (2003). Evolution of the army ant syndrome: The origin and long-term evolutionary stasis of a complex of behavioral and reproductive adaptations. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 6575–6579.

Brady, S., Fisher, B., Schultz, T., & Ward, P. (2014). The rise of army ants and their relatives: Diversification of specialized predatory doryline ants. *BMC Evolutionary Biology*, *14*, 93.

Broderick, N. A., Buchon, N., & Lemaitre, B. (2014). Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. *mBio*, *5*, e01117–e01114.

Broderick, N. A., Raffa, K. F., Goodman, R. M., & Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environmental Microbiology*, *70*, 293–300.

Brune, A., & Dietrich, C. (2015). The gut microbiota of termites: Digesting the diversity in the light of ecology and evolution. *Annual Review of Microbiology*, *69*, 145–166.

Buchon, N., Broderick, N. A., Chakrabarti, S., & Lemaitre, B. (2009). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes & Development*, *23*, 2333–2344.

Bulova, S., Purce, K., Khodak, P., Sulger, E., & O'Donnell, S. (2016). Into the black and back: The ecology of brain investment in Neotropical army ants (Formicidae: Dorylinae). *The Science of Nature*, *103*, 31.

Cafaro, M. J., Poulsen, M., Little, A. E. F., Price, S. L., Gerardo, N. M., Wong, B., … Currie, C. R. (2011). Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proceedings of the Royal Society B: Biological Sciences*, *278*, 1814–1822.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*, 335–336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., … Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME Journal*, *6*, 1621–1624.

Cardinal, S., & Danforth, B. N. (2011). The antiquity and evolutionary history of social behavior in bees. *PLoS ONE*, *6*, e21086.

Chandler, J. A., Morgan Lang, J., Bhatnagar, S., Eisen, J. A., & Kopp, A. (2011). Bacterial communities of diverse *Drosophila* species: Ecological context of a host–microbe model system. *PLoS Genetics*, *7*, e1002272.

Colman, D. R., Toolson, E. C., & Takacs-Vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology*, *21*, 5124–5137.

Cook, S., & Davidson, D. (2006). Nutritional and functional biology of exudate-feeding ants. *Entomologia Experimentalis et Applicata*, *118*, 1–10.

Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E., & Promislow, D. E. L. (2007). Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Applied and Environmental Microbiology*, *73*, 3470–3479.

Cronin, A. L., Molet, M., Doums, C., Monnin, T., & Peeters, C. (2013). Recurrent evolution of dependent colony foundation across eusocial insects. *Annual Review of Entomology*, *58*, 37–55.

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, *398*, 701–704.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics and parallel computing. *Nature Methods*, *9*, 772–772.

Davidson, D. W., Cook, S. C., Snelling, R. R., & Chua, T. H. (2003). Explaining the abundance of ants in lowland tropical rainforest canopies. *Science*, *300*, 969–972.

Dietrich, C., Köhler, T., & Brune, A. (2014). The cockroach origin of the termite gut microbiota: Patterns in bacterial community structure reflect major evolutionary events. *Applied and Environmental Microbiology*, *80*, 2261–2269, https://doi.org/10.1128/AEM.04206-13

Douglas, A. E. (2015). Multiorganismal insects: Diversity and function of resident microorganisms. *Annual Review of Entomology*, *60*, 17–34.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L. Q., Engelstadter, J., & Hurst, G. D. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology*, *6*, 27.

Ellegaard, K. M., & Engel, P. (2016). Beyond 16S rRNA community profiling: Intra-species diversity in the gut microbiota. *Frontiers in Microbiology*, *7*, 1475.

Engel, P., Martinson, V. G., & Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 11002–11007.

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Reviews*, *37*, 699–735.

Engel, P., Stepanauskas, R., & Moran, N. A. (2014). Hidden diversity in honey bee gut symbionts detected by single-cell genomics. *PLoS Genetics*, *10*, e1004596.

Feldhaar, H. (2011). Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology*, *36*, 533–543.

Feldhaar, H., Straka, J., Krischke, M., Berthold, K., Stoll, S., Mueller, M., & Gross, R. (2007). Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. *BMC Biology*, *5*, 48.

Ferrari, J., & Vavre, F. (2011). Bacterial symbionts in insects or the story of communities affecting communities. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, *366*, 1389–1400.

Fuchs, B. M., Glöckner, F. O., Wulf, J., & Amann, R. (2000). Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Applied and Environmental Microbiology*, *66*, 3603–3607.

Fukatsu, T., & Nikoh, N. (2000). Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Applied and Environmental Microbiology*, *66*, 643–650.

Funaro, C. F., Kronauer, D. J. C., Moreau, C. S., Goldman-Huertas, B., Pierce, N. E., & Russell, J. A. (2011). Army ants harbor a host-specific clade of Entomoplasmatales bacteria. *Applied and Environmental Microbiology*, *77*, 346–350.

Gotwald, W. H. (1995). *Army ants: The biology of social predation*. Ithaca, NY: Cornell University Press.

Himler, A. G., Adachi-Hagimori, T., Bergen, J. E., Kozuch, A., Kelly, S. E., Tabashnik, B. E., … Hunter, M. S. (2011). Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science*, *332*, 254–256.

Holldobler, B., & Wilson, E. O. (1990). *The Ants*. Cambridge, MA: Belknap Press.

Hölldobler, B., & Wilson, E. O. (2011). *The leafcutter ants: Civilization by instinct*. New York, NY: W. W. Norton & Company.

Hu, Y., Holway, D. A., Łukasik, P., Chau, L., Kay, A. D., LeBrun, E. G., … Russell, J. A. (2017). By their own devices: Invasive Argentine ants have shifted diet without clear aid from symbiotic microbes. *Molecular Ecology*, *26*, 1608–1630.

Hu, Y., Łukasik, P., Moreau, C. S., & Russell, J. A. (2014). Correlates of gut community composition across an ant species (*Cephalotes varians*) elucidate causes and consequences of symbiotic variability. *Molecular Ecology*, *23*, 1284–1300.

Hughes, G. L., Dodson, B. L., Johnson, R. M., Murdock, C. C., Tsujimoto, H., Suzuki, Y., … Rasgon, J. L. (2014). Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *111*, 12498–12503.

Hulcr, J., Rountree, N. R., Diamond, S. E., Stelinski, L. L., Fierer, N., & Dunn, R. R. (2012). Mycangia of ambrosia beetles host communities of bacteria. *Microbial Ecology*, *64*, 784–793.

Ishak, H. D., Plowes, R., Sen, R., Kellner, K., Meyer, E., Estrada, D. A., … Mueller, U. G. (2011). Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminata* ant colonies characterized by 16S amplicon 454 pyrosequencing. *Microbial Ecology*, *61*, 821–831.

Jaenike, J. (2012). Population genetics of beneficial heritable symbionts. *Trends in Ecology & Evolution*, *27*, 226–232.

Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M., & Perlman, S. J. (2010). Adaptation via symbiosis: Recent spread of a *Drosophila* defensive symbiont. *Science*, *329*, 212–215.

Jones, R. T., Sanchez, L. G., & Fierer, N. (2013). A cross-taxon analysis of insect-associated bacterial diversity. *PLoS ONE*, *8*, e61218.

Kaltenpoth, M., & Engl, T. (2014). Defensive microbial symbionts in Hymenoptera. *Functional Ecology*, *28*, 315–327.

Kaltenpoth, M., Gottler, W., Herzner, G., & Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology*, *15*, 475–479.

Kaltenpoth, M., & Steiger, S. (2014). Unearthing carrion beetles' microbiome: Characterization of bacterial and fungal hindgut communities across the Silphidae. *Molecular Ecology*, *23*, 1251–1267.

Kaspari, M., & O'Donnell, S. (2003). High rates of army ant raids in the Neotropics and implications for ant colony and community structure. *Evolutionary Ecology Research*, *5*, 933–939.

Kautz, S., Rubin, B. E. R., & Moreau, C. S. (2013a). Bacterial infections across the ants: Frequency and prevalence of *Wolbachia*, *Spiroplasma*, and *Asaia*. *Psyche*, *2013*, doi:10.1155/2013/936341.

Kautz, S., Rubin, B. E. R., Russell, J. A., & Moreau, C. S. (2013b). Surveying the microbiome of ants: Comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. *Applied and Environmental Microbiology*, *79*, 525–534.

Koch, H., Abrol, D. P., Li, J., & Schmid-Hempel, P. (2013). Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. *Molecular Ecology*, *22*, 2028–2044.

Koch, H., & Schmid-Hempel, P. (2011a). Bacterial communities in central European bumblebees: Low diversity and high specificity. *Microbial Ecology*, *62*, 121–133.

Koch, H., & Schmid-Hempel, P. (2011b). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 19288–19292.

Kronauer, D. J. C. (2009). Recent advances in army ant biology (Hymenoptera: Formicidae). *Myrmecological News*, *12*, 51–65.

Kunin, V., Engelbrektson, A., Ochman, H., & Hugenholtz, P. (2010). Wrinkles in the rare biosphere: Pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental Microbiology*, *12*, 118–123.

Kwong, W. K., & Moran, N. A. (2015). Evolution of host specialization in gut microbes: The bee gut as a model. *Gut Microbes*, *6*, 214–220.

Lanan, M. C., Rodrigues, P. A. P., Agellon, A., Jansma, P., & Wheeler, D. E. (2016). A bacterial filter protects and structures the gut microbiome of an insect. *ISME Journal*, *10*, 1866–1876.

Letunic, I., & Bork, P. (2016). Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, *44*, W242–W245.

Liberti, J., Sapountzis, P., Hansen, L. H., Sørensen, S. J., Adams, R. M. M., & Boomsma, J. J. (2015). Bacterial symbiont sharing in *Megalomyrmex* social parasites and their fungus-growing ant hosts. *Molecular Ecology*, *24*, 3151–3169.

Martinson, V. G., Moy, J., & Moran, N. A. (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology*, *78*, 2830–2840.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 3229–3236.

Mikaelyan, A., Dietrich, C., Köhler, T., Poulsen, M., Sillam-Dussès, D., & Brune, A. (2015). Diet is the primary determinant of bacterial community structure in the guts of higher termites. *Molecular Ecology*, *24*, 5284–5295.

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, *42*, 165–190.

Mueller, U. G. (2012). Symbiont recruitment versus ant-symbiont co-evolution in the attine ant–microbe symbiosis. *Current Opinion in Microbiology*, *15*, 269–277.

Neuvonen, M.-M., Tamarit, D., Näslund, K., Liebig, J., Feldhaar, H., Moran, N. A., … Andersson, S. G. E. (2016). The genome of Rhizobiales bacteria in predatory ants reveals urease gene functions but no genes for nitrogen fixation. *Scientific Reports*, *6*, 39197.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. (2007). The vegan package. *Community ecology package* **10**.

Oliver, K. M., Degnan, P. H., Burke, G. R., & Moran, N. A. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology*, *55*, 247–266.

Oliver, K. M., Moran, N. A., & Hunter, M. S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 12795–12800.

Peeters, C., & Ito, F. (2001). Colony dispersal and the evolution of queen morphology in social hymenoptera. *Annual Review of Entomology*, *46*, 601–630.

Powell, E., Ratnayeke, N., & Moran, N. A. (2016). Strain diversity and host specificity in a specialized gut symbiont of honeybees and bumblebees. *Molecular Ecology*, *25*, 4461–4471.

Powell, J. E., Martinson, V. G., Urban-Mead, K., & Moran, N. A. (2014). Routes of acquisition of the gut microbiota of *Apis mellifera*. *Applied and Environmental Microbiology*, https://doi.org/10.1128/aem.01861-14

Price, S. L., Powell, S., Kronauer, D. J. C., Tran, L. A. P., Pierce, N. E., & Wayne, R. K. (2014). Renewed diversification is associated with new ecological opportunity in the Neotropical turtle ants. *Journal of Evolutionary Biology*, *27*, 242–258.

Processing Foundation (2014). Processing.

Python Software Foundation (2014). Python.

Quince, C., Lanzen, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I. M., … Sloan, W. T. (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods*, *6*, 639–641.

R Core Team (2015). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rettenmeyer, C. W., Chadab-Crepet, R., Naumann, M. G., & Morales, L. (1983). Comparative foraging by neotropical army ants. In P. Jaisson (Ed.), *Social insects in the tropics* (pp. 59–73). Paris: Université Paris-Nord.

Roche, R. K., & Wheeler, D. E. (1997). Morphological specializations of the digestive tract of *Zacryptocerus rohweri* (Hymenoptera: Formicidae). *Journal of Morphology*, *234*, 253–262.

Russell, J. A. (2012). The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent Wolbachia (Alphaproteobacteria) symbionts. *Myrmecological News*, *16*, 7–23.

Russell, J. A., Funaro, C. F., Giraldo, Y. M., Goldman-Huertas, B., Suh, D., Kronauer, D. J. C., … Pierce, N. E. (2012). A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: Broad molecular surveys and a systematic review. *PLoS ONE*, *7*, e51027.

Russell, J. A., Goldman-Huertas, B., Moreau, C. S., Baldo, L., Stahlhut, J. K., Werren, J. H., & Pierce, N. E. (2009a). Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. *Evolution*, *63*, 624–640.

Russell, J. A., Moreau, C. S., Goldman-Huertas, B., Fujiwara, M., Lohman, D. J., & Pierce, N. E. (2009b). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 21236–21241.

Russell, J. A., Sanders, J. G., & Moreau, C. S. (2017). Hotspots for symbiosis: Function, evolution, and specificity of ant-microbe associations from trunk to tips of the ant phylogeny (Hymenoptera: Formicidae). *Myrmecological News*, *24*, 43–69.

Salem, H., Florez, L., Gerardo, N., & Kaltenpoth, M. (2015). An out-of-body experience: The extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society B: Biological Sciences*, *282*, 20142957.

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., … Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, *12*, 1–12.

Sanders, D., Kehoe, R., van Veen, F. J. F., McLean, A., Godfray, H. C. J., Dicke, M., … Frago, E. (2016). Defensive insect symbiont leads to cascading extinctions and community collapse. *Ecology Letters*, *19*, 789–799.

Sanders, J. G., Łukasik, P., Frederickson, M. E., Russell, J. A., Koga, R., Knight, R., & Pierce, N. E. (2017). Dramatic differences in gut bacterial densities help to explain the relationship between diet and habitat in rainforest ants. *bioRxiv*, https://doi.org/10.1101/114512.

Sanders, J. G., Powell, S., Kronauer, D. J. C., Vasconcelos, H. L., Frederickson, M. E., & Pierce, N. E. (2014). Stability and phylogenetic correlation in gut microbiota: Lessons from ants and apes. *Molecular Ecology*, *23*, 1268–1283.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., … Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*, 7537–7541.

Shin, S. C., Kim, S.-H., You, H., Kim, B., Kim, A. C., Lee, K.-A., … Lee, W.-J. (2011). Microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, *334*, 670–674.

Spor, A., Koren, O., & Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews: Microbiology*, *9*, 279–290.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*, 1312–1313.

Stoll, S., Gadau, J., Gross, R., & Feldhaar, H. (2007). Bacterial microbiota associated with ants of the genus Tetraponera. *Biological Journal of the Linnean Society*, *90*, 399–412.

Sudakaran, S., Salem, H., Kost, C., & Kaltenpoth, M. (2012). Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology*, *21*, 6134–6151.

Warnecke, F., Luginbuhl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege, J. T., … Leadbetter, J. R. (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, *450*, 560–565.

Wernegreen, J., Kauppinen, S., Brady, S., & Ward, P. (2009). One nutritional symbiosis begat another: Phylogenetic evidence that the ant tribe Camponotini acquired Blochmannia by tending sap-feeding insects. *BMC Evolutionary Biology*, *9*, 292.

Wilson, E. O. (1976). A social ethogram of the neotropical arboreal ant Zacryptocerus varians (Fr. Smith). *Animal Behaviour*, *24*, 354–363.

Winston, M. E., Kronauer, D. J. C., & Moreau, C. S. (2016). Early and dynamic colonization of Central America drives speciation in Neotropical army ants. *Molecular Ecology*, *26*, 859–870.

Yun, J.-H., Roh, S. W., Whon, T. W., Jung, M.-J., Kim, M.-S., Park, D.-S., … Bae, J.-W. (2014). Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology*, *80*, 5254–5264.

Zug, R., & Hammerstein, P. (2012). Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE*, *7*, e38544.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Łukasik P, Newton JA, Sanders JG, et al. The structured diversity of specialized gut symbionts of the New World army ants. *Mol Ecol*. 2017;26:3808–3825. https://doi.org/10.1111/mec.14140