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Received: 11 May 2023 | Accepted: 31 October 2023

DOI: 10.1111/1462-2920.16544

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Microbial symbionts are shared between ants and their associated beetles

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Funding information
"Excellence Initiative - Research University" at the Faculty of Biology of the Jagiellonian University in Kraków, Poland; Narodowa Agencja Wymiany Akademickiej, Grant/Award Number: PPN/PPO/2018/1/00015; Narodowe Centrum Nauki, Grant/Award Number: 2018/31/B/NZ8/01158; National Science Foundation, Grant/Award Number: 1050360

Abstract

The transmission of microbial symbionts across animal species could strongly affect their biology and evolution, but our understanding of transmission patterns and dynamics is limited. Army ants (Formicidae: Dorylinae) and their hundreds of closely associated insect guest species (myrmecophiles) can provide unique insights into interspecific microbial symbiont sharing. Here, we compared the microbiota of workers and larvae of the army ant *Eciton burchellii* with those of 13 myrmecophile beetle species using 16S rRNA amplicon sequencing. We found that the previously characterized specialized bacterial symbionts of army ant workers were largely absent from ant larvae and myrmecophiles, whose microbial communities were usually dominated by *Rickettsia*, *Wolbachia*, *Rickettsiella* and/or *Weissella*. Strikingly, different species of myrmecophiles and ant larvae often shared identical 16S rRNA genotypes of these common bacteria. Protein-coding gene sequences confirmed the close relationship of *Weissella* strains colonizing army ant larvae, some workers and several myrmecophile species. Unexpectedly, these strains were also similar to strains infecting dissimilar animals inhabiting very different habitats: trout and whales. Together, our data show that closely interacting species can share much of their microbiota, and some versatile microbial species can inhabit and possibly transmit across a diverse range of hosts and environments.

INTRODUCTION

Host-associated microbiota have played important roles in animal ecology and evolution (McFall-Ngai et al., 2013). Symbiotic microbes have enabled the emergence and evolutionary success of multiple host clades and species and strongly affected individual host fitness and population dynamics (Fisher et al., 2017). The effects of symbiotic microbial strains on hosts range from positive nutritive or defensive interactions to harmful parasitic or pathogenic relationships (Berg et al., 2020). These effects vary across host and microbial symbiont genotypes and environmental conditions (Sze et al., 2020).

There are relatively clear differences among the functional categories of symbiotic microbes: closed,

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mixed and open (Perreau & Moran, 2021). Closed symbioses include microbes transmitted strictly vertically (maternally) across host generations. They include obligate intracellular symbionts of insects that feed on nutritionally incomplete diets and require supplementation with essential amino acids and vitamins (Bennett & Moran, 2015; Rio et al., 2016). Microbes that form mixed symbioses, in addition to utilizing vertical transmission, can also transmit horizontally, that is, move among non-relatives within and across host species. This symbiont category includes facultative endosymbiotic bacteria such as *Wolbachia* (Kaur et al., 2021), ranging in their effects on insects from beneficial to deleterious, depending on conditions. In the third major category, open symbioses, exemplified by most gut microbes, symbiont cells are generally transmitted through the environment. While primarily linked to nutrition, gut microbiota also play other critical roles, such as protection against parasites and pathogens or influencing development (Engel & Moran, 2013).

The factors shaping the establishment of microbial symbioses across different host species are not well understood, with research strongly biased toward vertebrate and especially mammalian systems and their 'open' symbioses (Petersen & Osvatic, 2018). However, in taxa such as insects, it may be more common to find microbes that establish mixed or closed relationships. How the microbial symbionts are transmitted across individuals of a species largely determines opportunities for their interspecific transfer. In mammals, the acquisition of microbes needed for development or survival mostly occurs during birth and early life through a shared environment with family members (Campos-Cerda & Bohannan, 2020; Ferretti et al., 2018; Moeller et al., 2018). In insects, mechanisms for symbiont maternal transmission have evolved repeatedly and include transovarial transmission, the deposition of symbiont-containing capsules, egg smearing with faecal matter (Ohbayashi et al., 2020) or manipulating nest materials (Shukla et al., 2018). On the other hand, social behaviour is also important, particularly for the transmission of gut microbiota. Through shared environment, shared food sources or feeding habits (e.g., coprophagy or trophallaxis), microbial communities of social animals more closely resemble those of the other members of their own social group (colony, family, herd, etc.) than those of other groups (Bo et al., 2020; Brito et al., 2019; Engel & Moran, 2013; Sarkar et al., 2020).

Sharing the same environment by different species may provide opportunities for interspecific transmission of gut microbes. The similarities in microbial community composition between humans and their pet dogs (Song et al., 2013) serve as a good example. Likewise, there are reports of microbe sharing across interacting insect species. The similarities in gut microbiota composition between fungus-growing ants and their social parasites in another ant genus were explained by nest space and food sharing or predatory behaviour (Liberti et al., 2015). Also, it has been shown that velvety tree ants and their myrmecophiles have similarities in microbiota composition (Perry et al., 2021). On the other hand, many insects lack abundant gut microbiota, and many of the microbes found in their digestive tracts may belong to the 'transient' category, not forming stable associations with hosts (Hammer et al., 2019). In at least some of these cases, facultative endosymbionts dominate microbial community profiles. The horizontal transmission of these mixed symbionts may be more challenging, as they are often unable to survive outside of the host environment; at the same time, due to their often high abundance in insect tissues, they may be easier to study. There are reports of apparent transmission of *Wolbachia* from *Drosophila simulans* to the parasitic wasp *Leptopilina boulardi* (Heath et al., 1999), from prey to predator in the case of mites and terrestrial isopods (Clec'h et al., 2013; Hoy & Jeyaprakash, 2005) and from ants to kleptoparasitic ant crickets (Orthoptera: Myrmecophilidae) (Tseng et al., 2020). Unfortunately, the limited resolution provided by most studies listed—that of 16S rRNA OTUs, which can group strains separated by tens of millions of years of evolution (Ochman et al., 1999), complicates conclusions. Nevertheless, it appears that the likelihood of interspecific transmission should correlate with the frequency and intensity of the interactions among host insect species.

Army ants (Formicidae: Dorylinae) in the genus *Eciton* are a particularly interesting group from the social interaction perspective (Kronauer, 2020). With each colony numbering thousands to millions of workers, these nomadic ants roam terrestrial habitats in search of animal prey. They reproduce by dependent colony founding: new colonies form by fission when a large colony splits in two, each retaining a single queen (Gotwald, 1995; Peeters & Ito, 2001; Schneirla, 1971). These colonies host a diverse set of closely associated invertebrate species collectively known as myrmecophiles (Gotwald, 1995; Kronauer, 2020; Rettenmeyer et al., 2011; von Beeren et al., 2021a). Myrmecophile insects, which include beetles, especially rove beetles (family Staphylinidae) (Parker, 2016; von Beeren et al., 2021a), vary in their level of integration and roles in army ant colonies (Akre & Rettenmeyer, 1968; Rettenmeyer, 1961; von Beeren et al., 2011, 2018, 2021b). Some myrmecophiles show elaborate adaptations to cope with their predatory army ant hosts. For instance, many inquiline species mimic the cuticular hydrocarbon profile of their host ants to facilitate peaceful interactions (von Beeren et al., 2011, 2018, 2021b). Furthermore, some species possess protective morphologies against occasional ant attacks (Gotwald, 1995; von Beeren et al., 2021b), while others mimic the ants' body shape to achieve a high level of social integration into the army ant society

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(Gotwald, 1995; Maruyama & Parker, 2017; von Beeren et al., 2018). Other species usually show no apparent adaptations to life with army ants and rather resemble their free-living relatives. Typically, they either inhabit the army ants' refuse deposits (von Beeren et al., 2023), are found during the ants' raids or are found loosely scattered in the leaf litter around army ant bivouacs—mobile nests made of living ant workers' bodies (Rettenmeyer, 1961).

These diverse interactions create opportunities for the transmission of microbes among community members, either directly between species or through their shared social environment. However, despite the ecological importance of army ants as keystone species in tropical forests (Hoenle et al., 2019; Kronauer, 2020; Pérez-Espona et al., 2018), we know relatively little about the dynamics, specificity or sharing of their microbial symbionts. It was shown that the microbiota of New World army ant workers consist primarily of two specialized bacteria, Unclassified Firmicutes and Unclassified Entomoplasmatales (Anderson et al., 2012; Funaro et al., 2011; Łukasik et al., 2017; Mendoza-Guido et al., 2022), likely transmitted socially across worker generations (Łukasik et al., 2017). However, little is known about the microbial associations of other community members, including army ant larvae and myrmecophiles.

The goal of this project was to assess the degree of microbial overlap across unrelated but cohabiting species within colonies of the army ant *Eciton burchellii parvispinum* Forel, 1899. Specifically, we compared the microbiome of two beetle groups: (1) inquilines, representing species that live inside the temporary bivouac nests of army ants and thus have frequent contact with host ants; (2) outskirt inhabitants, consisting of scavengers and predators that are found outside the bivouac and have less contact with ants. We expected the first group to have a more similar microbiome to host ants due to their frequent physical contact with host workers. We addressed this by sequencing 16S rRNA gene amplicons for 105 beetles representing 13 species, from eight different colonies from a Costa Rican montane forest site, in addition to ant workers and larvae. We then tracked microbial clades and genotypes across species and colonies. For the most broadly distributed microbial taxon, the genus *Weissella* (Lactobacillales), we increased the resolution of strain association analysis by sequencing a protein-coding gene. By combining these data, we show substantial overlap in microbial composition among different hosts.

EXPERIMENTAL PROCEDURES

Insect collection, identification and classification

We sampled insects in July 2012 in the rainforest of Monteverde, Costa Rica (Figure 1A, Table S2). From raiding or emigration columns of eight *Eciton burchellii parvispinum* colonies, we collected medium-sized ant workers, myrmecophile beetles, and, in two cases, army ant larvae. We also captured six presumably free-living staphylinid beetles in leaf litter away from the sampled army ant colonies. Upon collection, specimens were immediately preserved in 95% ethanol and stored at −20°C until processing. Nine myrmecophiles from two morpho-species were starved for 24 h prior to preservation in order to test the persistence of microbes within myrmecophiles.

We had previously identified all ant colonies based on the morphological characters of workers (Longino, 2010) and the partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene (Łukasik et al., 2017). Morphological characters and DNA barcodes were also used to identify myrmecophile beetles (Appendix S1, Figure 1C). In short, specimens were individually mounted and z-stack images were produced using a Leica Z16 APO stereomicroscope equipped with a light dome, a Leica DFC450 camera and the processing software Leica application suite (version 4) at the Rockefeller University. Within a few hours, specimens were placed back into ethanol and stored at −20°C until DNA extraction. Using the latest species identification keys for each morphospecies and two recently published *COI* reference databases of *Eciton* associates (von Beeren et al., 2021a; von Beeren et al., 2023), we identified all myrmecophiles to the lowest taxonomic level possible. In cases where we were not able to identify the species, we added a morphotype designation to the taxon name to provide unique identifiers (e.g., *Ecitodonia* ST-F). The species distribution across the sampled colonies is shown in Figure 1B.

We categorized the collected myrmecophiles either as inquilines or as outskirt inhabitants (Figure 1C). The following species were categorized as inquilines as they are known inhabitants of army ant bivouacs (Akre & Rettenmeyer, 1966, 1968; Rettenmeyer, 1961; von Beeren et al., 2021a; von Beeren & Tishechkin, 2017): *Ecitophya* cf. *simulans* (ant-mimicking rove beetle), *Ecitomorpha* cf. *melanotica* (ant-mimicking rove beetle), *Ecitomorpha* cf. *nevermanni* (ant-mimicking rove beetle), *Cephaloplectus mus* (feather-winged beetle, protective morphology), *Euxenister* cf. *caroli* (histerid beetle, protective morphology) and *Symphilister* cf. *hamati* (histerid beetle, protective morphology). The species *Colonides* cf. *collegii* (histerid beetle, protective morphology) was also classified as a bivouac inhabitant, although no direct observation exists. This is because histerid beetles that participate as hitchhikers in army ant colony emigrations are usually also present within the army ants' bivouacs (von Beeren et al., 2021a; von Beeren & Tishechkin, 2017).

Outskirt inhabitants were those myrmecophiles that usually had no access to the inner part of army ant

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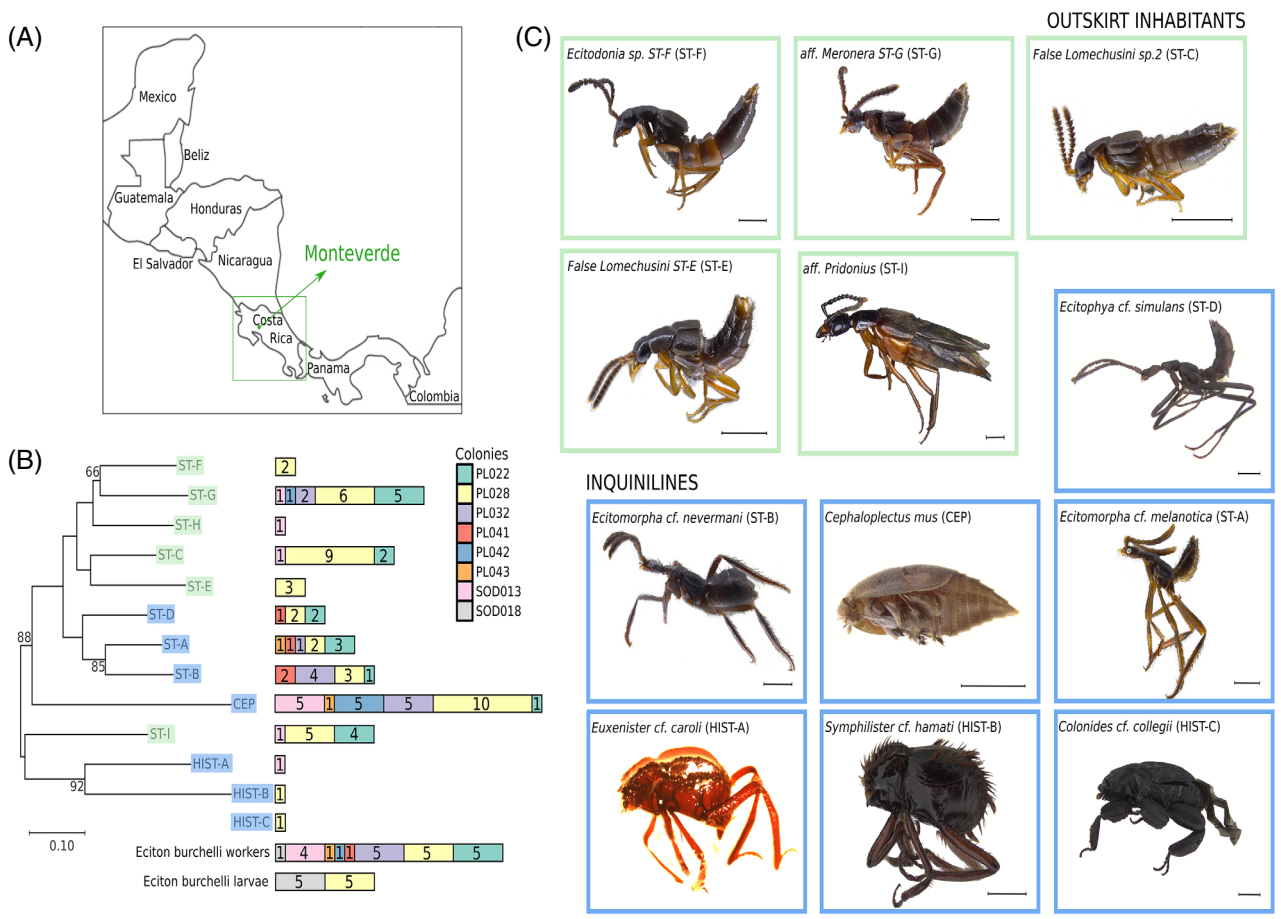

FIGURE 1 The origin and diversity of the studied myrmecophiles. (A) The sampling location; (B) A maximum-likelihood tree for myrmecophile beetle species used in this study, based on a 658 bp portion of the mitochondrial *cytochrome oxidase I* (*COI*) gene. Bars represent the number of specimens per colony, which is represented by different colours, representing 13 species or ant workers/larvae, obtained from different *E. burchellii* colonies. In the tree, bootstrap support values >60% are shown. We did not obtain a clean sequence for species HIST-C, hence it is not included in the tree. Colours of species labels in the tree represent the functional category the insects belong to. (C) Representative specimens of 12 myrmecophile species. Scale bars represent 1 mm. We did not obtain a picture of the species labelled ST-H.

bivouacs. For the herein-studied species, little to no information on their basic biology is available. We thus inferred their loose association with host ants using information on related species, often of the same genus and/or we additionally used unpublished data about the beetle fauna inhabiting *Eciton burchellii foreli* refuse deposits (von Beeren et al., 2023). As a result, we categorized the following species as outskirt inhabitants: *Ecitodonia* ST-F (rove beetle, genus includes refuse visitors; Akre & Rettenmeyer, 1966), aff. *Meronera* ST-G (rove beetle, genus includes refuse visitors; unpublished data, von Beeren et al., 2023), False Lomechusini ST-C (rove beetle, related species is a refuse visitor; von Beeren et al., 2023), False Lomechusini ST-E (rove beetle, related species is a refuse visitor; von Beeren et al., 2023), aff. *Pridonius* ST-I (rove beetle, the genus includes refuse visitors; von Beeren et al., 2023).

Microbial symbiont screens and amplicon library preparation

Prior to DNA extraction, all insects were surface-sterilised through 1-min immersion in 1% bleach, followed by rinsing with molecular-grade water. We extracted DNA from dissected gasters (for ant workers) or whole specimens (larvae, beetles) using DNeasy Blood and Tissue kits (Qiagen Ltd.), following the protocol for Gram-positive bacteria. The DNA extractions were used for PCR reactions with the universal primers 9Fa and 907R (Łukasik et al., 2017; Russell et al., 2009) for the bacterial *16S rRNA* gene and LCO-1490 and HCO-2198 (Folmer et al., 1994) for the *COI* gene of ants and myrmecophiles. PCR reaction conditions were described previously (Łukasik et al., 2017). The 16S rRNA gene amplification success, assumed to correlate with the bacterial abundance in the sample,

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was estimated by comparing the brightness of bands in an agarose gel against negative extraction controls. If the brightness of 16S rRNA bands was comparable to or lower than that of these negative controls, such samples were not processed further.

DNA samples that were classified as having substantial bacterial load were submitted, to Argonne National Laboratory for the preparation of amplicon libraries and subsequent sequencing of the V4 hypervariable region of the *16S rRNA* gene (primers: 515F-806R), following the Earth Microbiome Project protocols (Caporaso et al., 2012). Paired-end 150 bp sequencing was performed on an Illumina MiSeq platform. Along with insect DNA samples, six extraction blanks and three blanks consisting of molecular-grade water were sequenced to aid in identifying contaminants. Most of the samples used in this study (107/144) were submitted at the same time, but a subset, including all workers, were sequenced across four separate batches (Table S1). The analytical workflow used, including careful contamination filtering (described further) and our focus on high-abundance OTUs and genotypes, should have limited the batch effect (Salter et al., 2014).

Microbiota data analysis

The amplicon data were analysed using a custom protocol combining vsearch and usearch with custom Python scripts, described in detail at https://github.com/catesval/army_ant_myrmecophiles. All steps up to sequence clustering (inclusive) were performed individually for each library, given our previous findings that unoise3 can erroneously exclude genotypes abundant in some individuals but rare in the whole dataset (Prodan et al., 2020). Briefly, after extracting reads corresponding to the experimental libraries from the sequencing runs and adding data for previously characterized ant specimens (Łukasik et al., 2017), we quality-filtered and merged overlapping reads from each pair into contigs using PEAR v0.9.11 (Zhang et al., 2014). We performed dereplication using vsearch v2.15.2_linux_x86_64 (Rognes et al., 2016) and denoising with usearch v11.0.667_i86linux32 (Edgar, 2010; Edgar et al., 2011). Samples were denoised individually, given our previous findings that unoise3 can erroneously exclude genotypes abundant in some individuals but rare in the whole dataset. In this step, we used a lower minisize parameter than recommended (1 instead of 8) in order to conserve the diversity found in low-total-read-count negative controls, needed for subsequent decontamination, and because we focused analyses on abundant genotypes anyway. Then, the resulting lists of unique sequence variants (further referred to as zero-radius Operational Taxonomic Units or zOTUs) for each library were merged. We then performed OTU picking and chimera removal using the uparse algorithm incorporated in the usearch software, with default parameters. Next, taxonomy was assigned using the syntax function in vsearch (with a cutoff of 0.80), using the SILVA SSU 138 database as a reference (Quast et al., 2013). Finally, we used custom scripts to merge the outputs of OTU picking and taxonomy assignment to create the OTU and zOTU tables. The parameters of all analyses and all scripts are provided in the GitHub repository linked above.

As shown previously, contamination during sample processing can strongly alter the microbial community profiles of organisms with less abundant microbiota, including some army ants (Łukasik et al., 2017; Salter et al., 2014). Because of this, we screened and filtered putative contaminant genotypes, identified based on their relative abundance in libraries representing insect samples and negative controls. We adapted and expanded the custom approach explained previously (Łukasik et al., 2017), as described in detail in the GitHub repository. While this approach should effectively eliminate contaminants, its downside is the possible exclusion of rare symbiotic microbes. However, in our analyses, we focused on abundant and widespread microbes. Specifically, we selected for more detailed investigation those 97% OTUs that fulfilled at least two of the following three criteria: the average relative abundance of the OTU was equal to or higher than 0.0001 in at least 20 samples; its relative abundance in at least one sample was ≥0.05; or its average relative abundance was 0.01.

For the calculation of the genotype-level composition (zOTU) of the OTUs, we calculated what percentage of each 97% OTU was represented by different zOTUs, and then filtered zOTUs to keep only those represented in the dataset by at least 100 reads and representing at least 5% of reads classified to an OTU in at least one sample. The final data, managed using Microsoft Excel, were visualized using the pheatmap library (Kolde, 2019) in R version 3.6.3 (2020-02-29). The degree of similarity in the microbiome community of inquilines and outskirt inhabitants to the microbiome of their host ants was explored using a principal coordinates analysis based on compositional microbiome data. Differences between four categories (ant worker, ant larvae, inquilines and outskirt inhabitants) were assessed using PERMANOVA with 1000 permutations and testing the nested effect of species in the case of myrmecophiles. Statistical analyses were performed using the Vegan package version 2.5.7 (Oksanen et al., 2020).

We did not rarefy the data before comparisons among samples, given our strong focus on taxa abundant in the dataset and samples, strict relative abundance thresholds applied and demonstrated limited effects of the procedure on diversity comparisons (McMurdie & Holmes, 2014; Willis, 2019).

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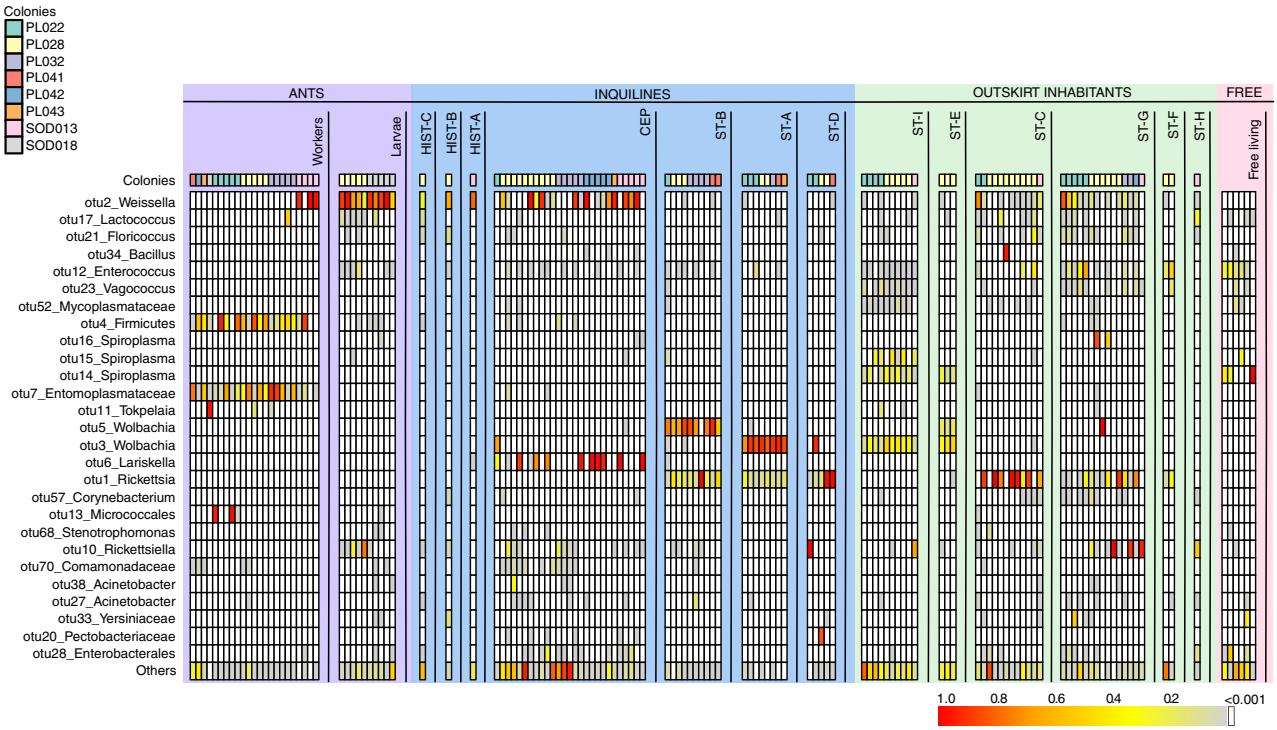

FIGURE 2 The relative abundance of the bacterial 97% OTUs in specimens of *E. burchellii* workers, larvae and 13 myrmecophile species from eight ant colonies from Monteverde, and a few free-living staphylinids. Columns represent insect specimens divided into species, ordered based on *COI* ML tree and labelled on top with the abbreviation assigned in Figure 1; background shading represents the functional categories. Rows represent bacterial OTUs sorted according to ML phylogeny for their representative genotypes. The colour gradient represents the relative abundance of each OTU in each of the insect specimens.

rplB genotype-level diversity of *Weissella*

To obtain additional insights into the diversity of *Weissella*, one of the most broadly distributed microbial symbionts in our dataset, we amplified and sequenced a portion of the 50S ribosomal protein L2 (*rplB*) gene from 51 Monteverde specimens in which the microbe was present (Figure 2, Figure 3A and Table S4). Additionally, 31 samples from other army ant species and collection sites that were not a part of the primary dataset but tested positive for *Weissella* based on specific PCR primers or amplicon data (Łukasik et al., 2017), were included for *rplB* sequencing. To achieve this, we used newly designed primers within the *rplB* and the adjacent *rpsS* gene: ArWei_rplB_F3: GGTCGTCGTAATATGACTGGT, Leu_rpsS_R3: TGAACGACGTGACCATGTCTTG and Wei_RpsS_Rseq: CTTCAACCTTCTTCTTCAACAACAAGYKRGC. The PCR program was: 94°C for 1 min, 25 cycles of 95°C for 15 s, 70°C–>62.8°C (decreasing by 0.3°C each cycle) for 15 s, 72°C for 20 s; 35 cycles of 94°C for 15 s, 58°C for 15 s, 72°C for 20 s; 70°C for 2 min. Purified PCR products were Sanger-sequenced by Eurofins Genomics LLC. The *rplB* sequences obtained after trimming traces immediately after the stop codon, thus removing intergenic regions and short *rpsS* fragments had a length of 510-bp. They were aligned against homologues identified in the NCBI database through BLASTn searches (Table S6).

Phylogenetic analyses

We quality-checked and aligned all insect *COI* sequences as well as all *Weissella rplB* sequences in CodonCode Aligner v. 9.0.1 (CodonCode Corporation, Centerville, MA, U.S.A.) and manually curated the alignments. Then, we performed Maximum Likelihood phylogenetic analysis in MEGAX (Kumar et al., 2017), utilizing the GTR model with gamma-distributed rates and invariant sites (G + I), 5 discrete gamma categories and 1000 bootstrap replicates. Trees were visualized using TreeGraph (Stöver & Müller, 2010). The insect *COI* tree is unrooted, while the *Weissella rplB* tree was rooted using *Leuconostoc carnosum* and *Leuconostoc paramesenteroides* as outgroups.

RESULTS

Species identification and phylogeny

We combined previously obtained *COI* sequences with new high-quality *COI* sequences for all ant larvae, for

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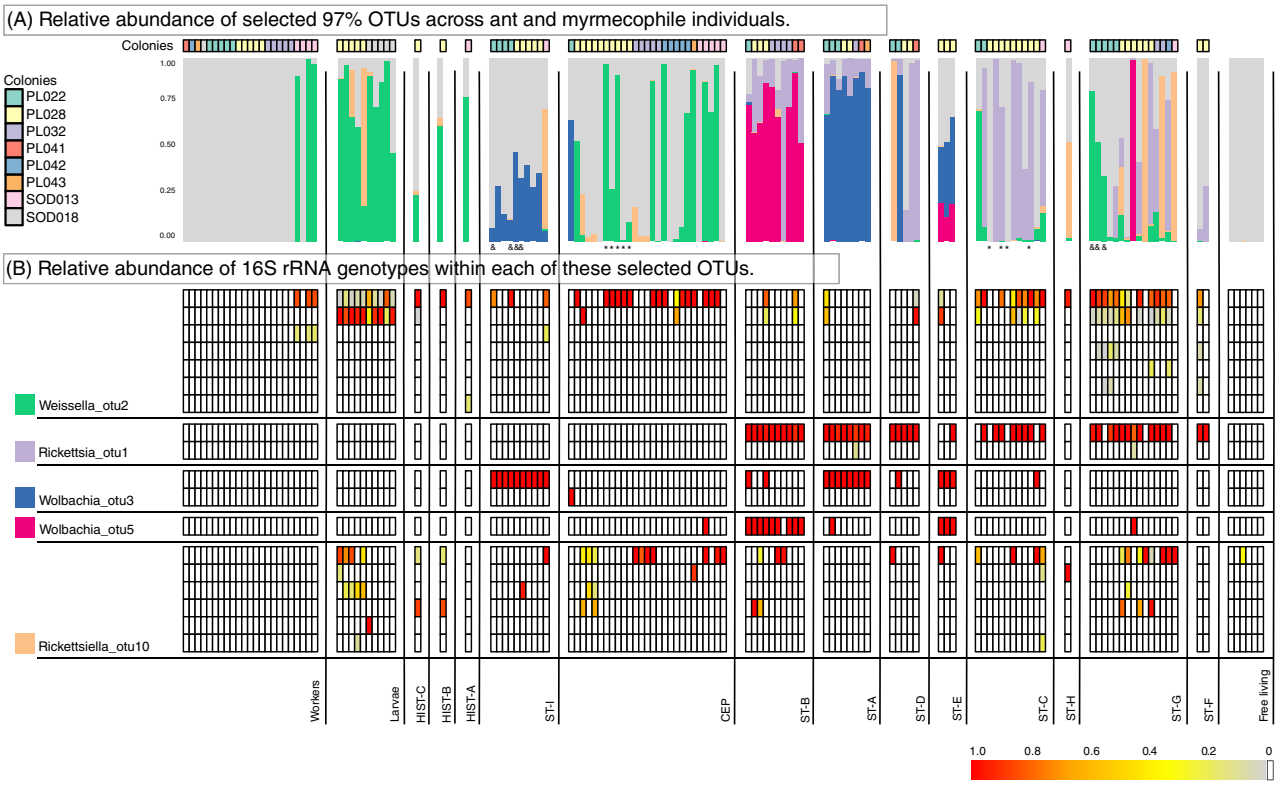

FIGURE 3 (A) The relative abundance of selected, broadly distributed and abundant bacterial 97% OTUs across ant and myrmecophile individuals, relative to the total number of reads in a sample. Associations between colours and bacterial OTUs are shown in panel B. B) Relative abundance of 16S rRNA genotypes within each of these selected OTUs. In both panels, individual insects are represented by columns and sorted by species, as in Figure 2. Data for a given OTU is shown only for those individuals where the relative abundance of that OTU in an individual is at least 0.001 (the same threshold was applied in Figure 2), helping reduce the impact of potential cross-contamination. In panel B, only the genotypes with a total of at least 100 reads and a relative abundance >0.05 of the OTU in at least one sample are shown, and others were ignored for the purpose of this comparison; consequently, the cumulative relative abundance of all genotypes shown is 1.00 for each OTU in each individual.

89 of the 96 myrmecophile beetles and for 5 out of 6 free-living beetle specimens. In most of the remaining cases, the noisier barcode sequences closely resembled some of the clean sequences, allowing for specimen classification. Seven sequences matched either amphipod or nematode sequences, presumed food or parasites. We made a note of this to check if these *COI* signals had some influence on the results from the 16S rRNA sequence analysis. These specimens were classified based on morphology (see Tables S1 and S3 and Appendix S1, for details).

Based on these data, we concluded that our collection comprised 13 myrmecophile species from three families: Staphylinidae, Ptiliidae and Histeridae (Figure 1). The four free-living beetle species belonged to the family Staphylinidae. High-quality, unambiguous sequences of representative specimens from each of the species were used for the phylogenetic reconstruction of the relationships among myrmecophiles, with the exception of HIST-C, for which we were not able to obtain a high-quality sequence (Figure 1B). Overall, by combining barcoding and morphology information we obtained the critical framework for correlating microbiota similarity and distribution across multi-species ant-myrmecophile communities. However, the taxonomic identification of the myrmecophile species bins was challenging. In the Appendix S1, we explain how we assigned taxonomic IDs through the comparison of *COI* barcode sequences and morphological features with myrmecophiles of the more comprehensively studied *E. burchellii foreli* subspecies.

Microbial community composition

After quality filtering, decontamination and removal of negative controls, the 16S rRNA amplicon sequencing dataset comprised 135 libraries, with a median of 20,346 reads (range 1367–69,222). After denoising and decontamination, we obtained 5603 microbial genotypes (zOTUs), which were grouped into 1656 Operational Taxonomic Units (OTUs) at 97% identity. Of those, 28 OTUs, comprising a summed average of 85% of filtered reads per sequence library, were selected for more detailed analysis according to the criteria specified in the Methods (Figure 2).

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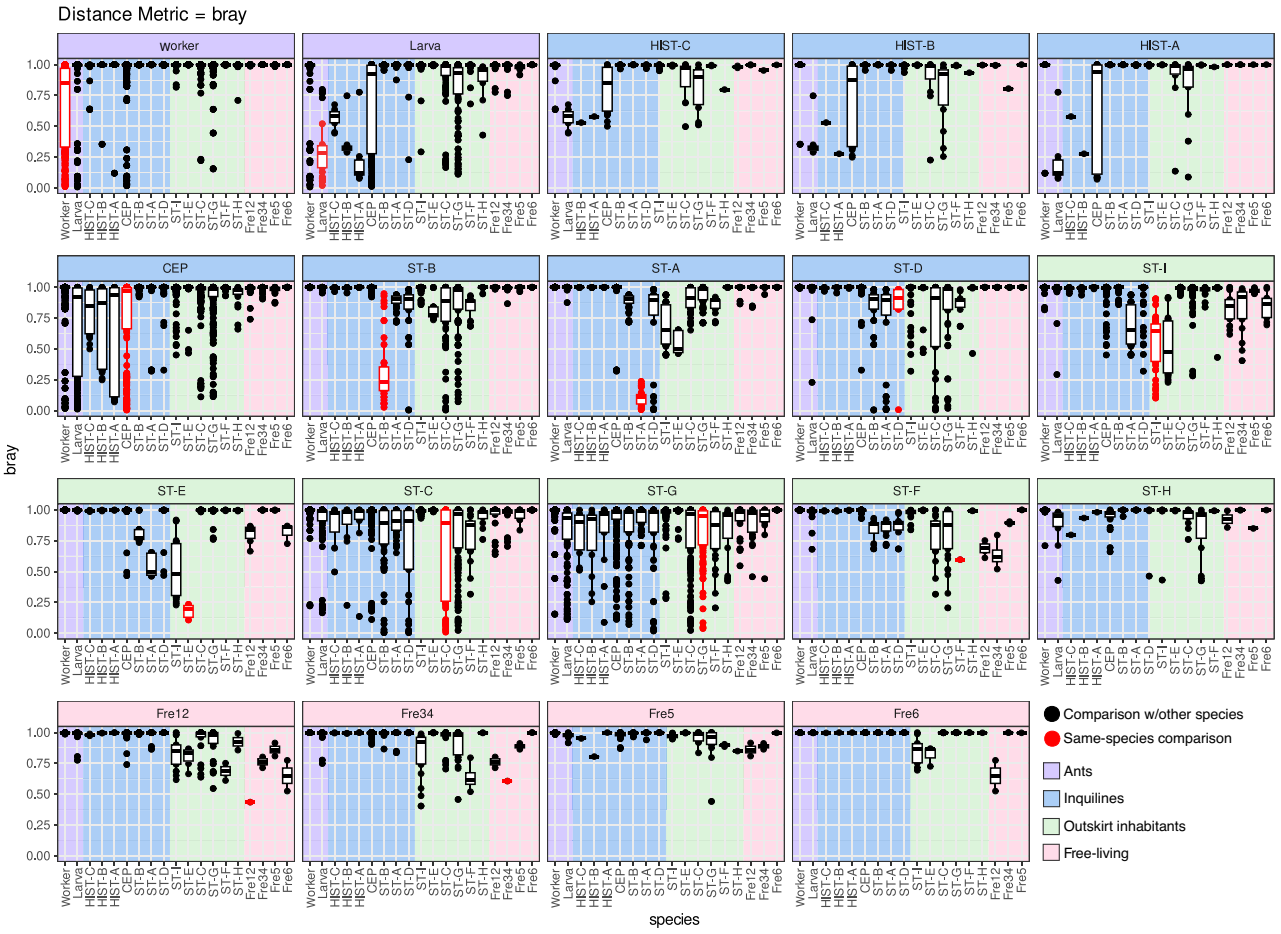

FIGURE 4 Bray–Curtis dissimilarity pairwise comparison, at the level of 97% bacterial OTUs, among samples from each species versus all other samples, from all colonies and species. Each dot represents the distance between two samples, of the same (red) or different species (black), and the title and background colour indicate the functional group they belong to. Self-comparisons are not shown, and so the within-species comparisons are lacking for some species. The boxes show 25%, 50% (median) and 75% inter-quartile ranges. Metrics were calculated using the Phyloseq 1.30.0 and Vegan 2.6-2 packages and visualized using ggplot2 3.4.0, in R version 3.6.3.

Results for *Eciton burchellii* workers—based on a partially overlapping sample set—resembled prior observations (Łukasik et al., 2017). Their microbiota were dominated by Unclassified Firmicutes (OTU4) and Unclassified Entomoplasmataceae (OTU7), two bacterial clades identified as stable residents of army ant gut habitats (Funaro et al., 2011; Łukasik et al., 2017). The OTUs corresponding to these bacteria comprised, on average, 35% and 32% of *E. burchellii* worker sequence libraries, being present in 19 or 22 of the 23 characterized workers, respectively. However, both these bacteria were rare across larvae, with Unclassified Firmicutes (OTU4) present in four of 10 individuals, with an average relative abundance of 0.29% for those infected (Figure 2). Further, reads assigned to these OTUs were rare across myrmecophiles, generally not exceeding the level expected from cross-contamination in MiSeq lanes dominated by army ant libraries (Illumina, 2017). Few other microbes were abundant/common within or among *E. burchellii* worker libraries. Among the exceptions were *Weissella* (Lactobacillales) (OTU2), *Tokpelaia* (Rhizobiales) (OTU11), and an unclassified clade in the order Micrococcales (OTU13), all found sporadically across workers.

The symbiotic microbiota of *Eciton burchellii* larvae were clearly different from worker-associated microbiota (ADONIS, $F_{(1,31)} = 17.146$, $p < 0.001$; Figures 2 and 4; note that in the comparison we did not use colony information, as both workers and larvae were available from one colony only). The dominant 97% OTU, *Weissella* (OTU2), accounted for 73% of reads on average across the larval libraries. The second most abundant member of the larval microbiota was *Rickettsiella* (Gammaproteobacteria: Legionellales) (OTU10), present in 6 out of 10 larvae, with an average relative abundance of 10% for those infected. OTUs representing the genera *Enterococcus* (OTU12) and *Lactococcus* (OTU17) numbered among those with sporadic presence and low abundance in *E. burchellii* larvae.

Microbial communities of myrmecophile beetles showed species-specific patterns (Figure 2). Across all species, the bacterial 97% OTU that was most

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abundant on average among samples where it was present corresponded to the alphaproteobacterial genus *Rickettsia* (OTU1), which represented an average of 15% of the reads in those libraries where it was present and had a relative abundance >0.1% in 46 of 96 libraries. Other abundant and widespread bacteria included the aforementioned *Weissella* OTU2, comprising 14% of the total reads on average among libraries where it was found and present in 51 libraries. Reads clustering within a single *Rickettsiella* OTU (OTU10, the one also found in army ant larvae) made up 6% of reads per library on average where present and was found in 34 libraries. *Wolbachia* was represented by two OTUs; OTU5 was present in 15 libraries with an average relative abundance of 8% among them and OTU3—found at 12% average relative abundance where present, and appearing in 26 libraries. Other bacteria exceeding 1% average relative abundance in the dataset included *Spiroplasma* (Mollicutes) (OTU14, OTU15, OTU16), Firmicutes: *Floricoccus* (OTU21), *Enterococcus* (OTU12) and Pseudomonadota: *Candidatus Lariskella* (OTU6), Yersiniaceae (OTU33) and Enterobacterales (OTU28).

Some of these myrmecophile-associated microbes were highly species-specific. For example, OTU6 classified as *Lariskella* was abundant in about half of the *Cephaloplectus mus* (CEP) beetle specimens but virtually absent in all other samples. However, other microbial OTUs abundant in the dataset were observed in multiple host species. This was particularly clear for *Weissella* OTU2, occurring in all 12 myrmecophile species, in addition to army ant worker and larvae libraries. In eight of these 12 myrmecophile species, this OTU was present with a relative abundance of 5% or higher in at least one individual. Likewise, *Rickettsiella* OTU10 was present in 11 myrmecophile species in addition to army ant larvae. Many other bacteria were found in more than one beetle species but not in army ant workers or larvae. Among them were a 97% OTU1 classified as *Rickettsia*, present in seven myrmecophile species and two *Wolbachia* OTUs, present in seven (OTU3) and five (OTU5) myrmecophile species (Figure 4 and Figure 3A). Overall, we observed significant differences in microbial community composition between workers and myrmecophiles (ADONIS, $F_{(1,116)} = 14.521$, $p < 0.001$, Figure 4) and between larvae and myrmecophiles (ADONIS, $F_{(1,103)} = 7.615$, $p < 0.001$), when only using category as a variable, without considering colony information. The differences among myrmecophile species and between the two functional categories they were grouped in, independent of colony, were also significant (ADONIS, with the effect of species nested within category: $F_{(1,93)} = 6.576$, $p < 0.001$ for category, $F_{(10,83)} = 5.972$, $p < 0.001$ for species). Note that inquiline and outskirt staphylinids form separate clades and we cannot rule out phylogenetic position driving some of the observed differences. Nevertheless, communities of *Eciton burchellii* larvae and some myrmecophile beetle species often grouped together in the PCoA plots (Figure S1) and had typically lower Bray–Curtis dissimilarity values (Figure 4) when compared against each other rather than when compared with workers, being dominated by the same microbial OTUs (Figure 2). We found no difference in microbiome composition among individuals of two myrmecophile species that were immediately preserved or starved for 24 h prior to preservation (PERMANOVA, $F_{(1,11)} = 1.3296$, $p < 0.217$ for False Lomechusini sp.2 (ST-C) and $F_{(1,26)} = 0.64155$, $p < 0.636$, Figure S2A, B). Likewise, the microbial composition of individuals whose COI barcode matched putative prey or parasites did not stand out from other representatives of the same morphologically identified species (Figure 2).

Some of the microorganisms detected in myrmecophile beetles were also found in free-living beetles. Among these, a single *Enterococcus* OTU (OTU12) was the most prevalent, being present in five out of six specimens in the latter category, with an average abundance of 14% among the myrmecophile and free-living beetle samples where it was present. This OTU was also found, at low abundance, in six *Eciton burchellii* larvae and 41 myrmecophile beetles from seven species—primarily, colony outskirt inhabitants (Figure 2). Aside from *Enterococcus*, *Spiroplasma* (OTU14) was also found in three free-living specimens and two myrmecophile species inhabiting colony outskirts ($n = 12$ specimens). These patterns resulted in relatively greater similarity in microbial communities between free-living staphylinids and those that inhabit colony outskirts, relative to ants or inquiline species (Figure 4). However, despite such trends for these broadly distributed insect associates (Paniagua Voirol et al., 2018; Russell et al., 2012), the abundant OTUs shared among army ants and myrmecophiles were generally not present in free-living, sympatric beetles.

16S rRNA genotype-level microbial associations

Genotype-level 16S rRNA (zOTU) data provided more detailed information about the diversity and distribution of the symbiont strains within and across species (Figure 3B), suggesting possible cases of recent transfer of symbionts among host ants and their myrmecophiles. Across the 28 selected OTUs, we identified between 1 and 13 genotypes that fulfilled the abundance criteria that were likely to exclude sequencing errors and cross-contaminants. These genotypes could represent genomes of different strains or alternatively, sequence variation among operons within a single genome (Větrovský & Baldrian, 2013) and can provide valuable novel insights into host-symbiont interactions

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(Kolasa et al., 2023). Two or more distinct genotypes from a single OTU were often found in the same individual. At the same time, the same genotypes were found in different species (Table S5) in all 24 97% OTUs shared across two or more species. Within a single host species, we observed up to eight different genotypes of the same OTU (Table S4), but with no clear distribution patterns across colonies (Figure 3A and Figure 3B).

Genotype diversity varied among the most abundant broadly distributed 97% OTUs (Figure 3B). *Weissella* OTU2 had seven detected genotypes, followed by *Ricketsiella* OTU10 with six genotypes, *Rickettsia* OTU1 and *Wolbachia* OTU3 with two each, and *Wolbachia* OTU5, with one. Genotype diversity also varied among host species. For example, in most myrmecophile species, we identified two *Weissella* genotypes, but aff. *Meronera* ST-G and *Ecitodonia* sp. ST-F specimens possessed up to four. In most individual insects, one genotype of *Rickettsia* was present, but in two *Ecitomorpha* cf. *melanotica* (ST-A) specimens and one aff. *Meronera* ST-G specimen, we detected additional, low-abundance genotypes. In the case of *Wolbachia* OTU3, the same genotype was detected in almost all infected insects, but one specimen of *Cephaloplectus mus* (CEP) and one of False *Lomechusini* sp. *2* (ST-C) hosted an alternative genotype. Finally, for *Rickettsiella* (OTU10), most of the insects where this OTU was present harboured only one of the six genotypes, but a subset possessed two or three.

rplB genotype-level diversity of *Weissella*

Sanger-sequencing of the protein-coding gene *rplB* for *Weissella*-positive specimens yielded 31 high-quality and unambiguous sequences. For phylogenetic analysis, we combined this dataset with newly generated sequences for 11 workers and larvae from previously characterized ant species or locations (from Łukasik et al., 2017) and sequences extracted from 25 reference genomes for other *Weissella* and other Leuconostoceae/Lactobacillales strains. The resulting maximum likelihood phylogeny provided highly supported information on the relationships among the newly characterized strains, despite known limitations of single genes relative to genome-level datasets in resolving deeper nodes of the phylogeny (Fanelli et al., 2022). Sequences from army ants and myrmecophiles fell into two divergent broad clades (Figure 5). The more abundant clade comprised two sub-clades. One sub-clade included *Weissella ceti*, a species originally isolated from a beaked whale carcass (Vela et al., 2011), several isolates from diseased rainbow trout cultures that were recently assigned to a separate species, *W. tructae* (Figueiredo et al., 2015; Pereira et al., 2022), as well as a single sequence from a myrmecophile specimen classified as *False Lomechusini* sp. *2* (ST-C). The myrmecophile sequence differed by only 3 bp (0.59%) from the *W. ceti* type strain. However, most of the newly obtained *rplB* sequences from ants and myrmecophiles belonged to the second sub-clade, ca. 3% distinct from these previously described *Weissella* isolates. The other, divergent *Weissella* clade comprised sequences from myrmecophiles and ant larvae exclusively.

Within these two broad clades, the sequences from *E. burchellii* army ants and different myrmecophile species were highly similar and often identical. The clade that included *W. ceti* also included sequences from nine myrmecophile species as well as army ant workers and larvae. The two most abundant *rplB* genotypes within this clade were both represented by strains from Monteverde *E. burchellii* workers and several myrmecophile species. Interestingly, one of these *rplB* genotypes was also found in *E. burchellii* workers and larvae from Venezuela (Łukasik et al., 2017). The other clade in our *rplB* phylogeny included a genotype represented by sequences from four myrmecophile species and from *E. burchellii* larvae from Monteverde and Venezuela. Three identical sequences from Venezuelan *Nomamyrmex esenbeckii* army ant larvae represented a second, divergent genotype within that second clade.

DISCUSSION

We have shown that phylogenetically distant and biologically different insects living together within army ant colonies share substantial portions of their microbiota: they frequently harbour microbes with identical genotypes at the V4 region of the 16S rRNA gene and, for *Weissella*, also at a portion of the *rplB* gene. When checking if these similarities were related to colony or level of integration of the myrmecophiles with the ants (inquilines vs. outskirt inhabitants), we did not find any discernible patterns. We also found an overlap at the *Weissella* 16S-V4 and *rplB* genotype level between different *Eciton burchellii parvispinum* colonies from Costa Rica and *Eciton burchellii foreli* from Venezuela—separated by ca. 2000 km and an estimated four to seven million years of evolution (Łukasik et al., 2017; Winston et al., 2017). As a reference, the specialized and putatively worker-to-worker-transmitted Firmicutes and Entomoplasmatales symbionts generally differ among these ant colonies by about 1% within the same *rplB* gene (Łukasik et al., 2017). These patterns strongly suggest that some microbes, and *Weissella* in particular, are shared across interacting species within colonies, and across geographically distant colonies of a species, at relatively short timescales. The surprising relatedness of *Weissella* strains from army ant colonies, and those isolated from divergent

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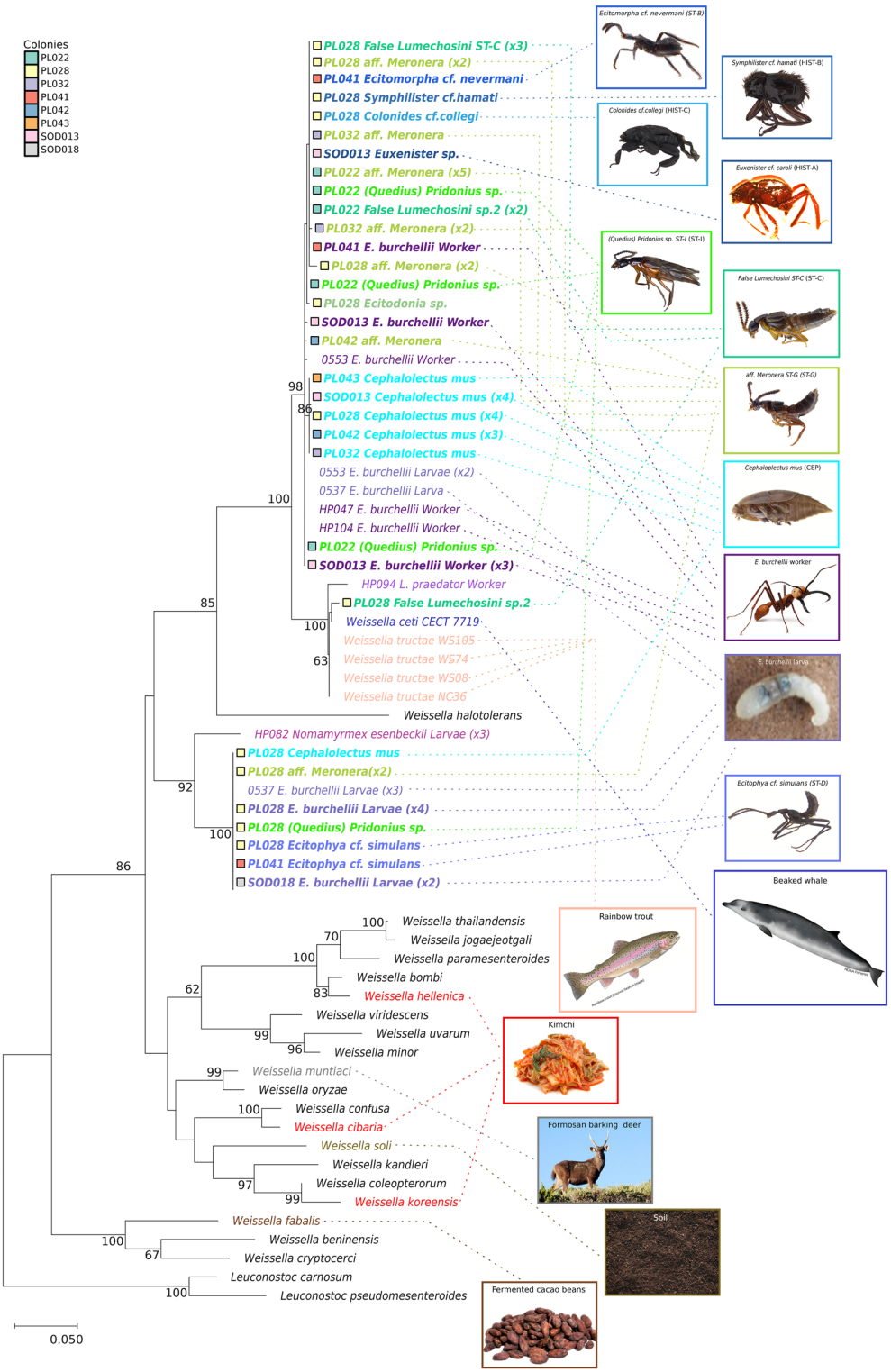

FIGURE 5 Maximum Likelihood phylogeny of *Weissella* genotypes from different hosts based on the partial 510-bp sequence of the ribosomal protein L2 (*rplB*) gene. The colour of the labels represents the environment or host from which the given *Weissella* isolate was derived, and it is the same colour as the frame of its picture. Labels in bold letters indicate ant and myrmecophile strains from Monteverde (the core samples from this project). Bootstrap values higher than 60 are shown. Identical sequences from different insects belonging to the same species and colony are represented only once in the tree, with the number of samples indicated between parenthesis next to the label.

and physiologically dissimilar animals from completely different environments—beaked whale and rainbow trout (Figueiredo et al., 2015; Vela et al., 2011) suggests its much broader distribution across host organisms and environments. Then, our work highlights the versatility of some microbial clades and strains and

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the extent and likely significance of microbial strain transmission at multiple scales.

Army ant workers and larvae differ substantially in their microbial community composition

In our collection, we found little microbial symbiont sharing between ant developmental stages. However, there was only one colony for which we had samples from both workers and larvae (PL028). For this colony, the microbial communities of army ant larvae were distinct from those of adults. The ancient bacterial symbionts that dominate worker communities, Unclassified Firmicutes and Unclassified Entomoplasmatales (Funaro et al., 2011; Łukasik et al., 2017), were present in only one larva, at low abundance. Likewise, the dominant larval symbionts, *Weissella* and *Rickettsiella*, were uncommon in workers: *Weissella* was only present in some specimens from a colony with no larvae sampled and *Rickettsiella* was never detected. However, the consistent presence and high relative abundance of these microbes in *E. burchellii* larvae from Costa Rica, but also the presence of closely related *Weissella* in *E. burchellii* and *Nomamyrmex* army ant larvae from Venezuela, suggests persistent association and likely importance, in larval biology (Łukasik et al., 2017). In other social Hymenoptera, larval microbiota also differ from those of adults and can play important roles. For example, in *Cephalotes* turtle ants, microbiota change substantially as the larvae develop (Hu et al., 2023), exhibiting a consistent successional pattern of unknown functional importance. Likewise, honeybee larval microbiota are very different from those of adult bees and can play important roles (Anderson et al., 2018; Kapheim et al., 2015; Martinson et al., 2012). For example, a novel lactic acid bacterium was shown to inhibit the growth of the pathogen *Paenibacillus larvae* in honeybee larvae (Forsgren et al., 2010). On the other hand, there is evidence that microbes can be transmitted from adults to larvae through social interactions, as shown for *Atta* and *Acromyrmex* leaf-cutter ants (Sapountzis et al., 2018; Zhukova et al., 2017). More systematic sampling and surveys of larval instars are necessary to clarify the significance of microbes in ant developmental biology.

Microbial sharing among army ant colony members

The striking degree of overlap in bacterial associations among different myrmecophile species and between myrmecophiles and army ant larvae suggests extensive microbial sharing. In particular, *Weissella* and *Rickettsiella*, two dominant larval associates, were both present in a large share of myrmecophile specimens—representing 13 and 12 species, respectively—both inquilines and colony outskirt inhabitants. The identity of 16S rRNA and (in the case of *Weissella*) *rplB* sequences among strains from different hosts conflicts with their strong specialization on a particular host species and instead, is highly suggestive of their ongoing or recent horizontal transmission. At the same time, it is likely that closely related strains found within army ant colonies differ in their level of host-specificity and other biological characteristics. For example, one of the myrmecophile species, *Cephaloplectus mus* (CEP), consistently and uniquely hosted *Weissella* strains with about 0.5% *rplB* gene nucleotide sequence divergence, compared to strains that colonized a wider range of species (Figure 5). Several 16S rRNA genotypes of the facultative endosymbionts *Wolbachia* and *Rickettsia* are also broadly distributed across different myrmecophile species, and the same patterns seem to apply to most other abundant bacterial OTUs in our dataset. However, it is important to emphasize that bacterial strains identical to the sequenced 253-bp fragment of 16S rRNA may still be separated by millions of years of evolution and differ dramatically in genome contents and the range of functions (Hassler et al., 2022; Ochman et al., 1999). Conserved protein-coding genes such as *rplB* provide greater phylogenetic resolution. However, despite near-identity at the *rplB* gene among strains previously classified as *W. ceti*, higher divergence elsewhere within the genomes combined with biochemical differences were recently used to justify the delimitation of *W. tructae* (Pereira et al., 2022). Unfortunately, we currently lack the resolution to resolve the relationships among strains detected in different myrmecophile species or estimate transmission timing. While transmission is likely to be ongoing in many cases, whole-genome comparison among isolates from different host species would provide the ultimate evidence.

Army ant colonies as arenas for interspecific exchange of microbial symbionts

Close and intensive interactions among army ants and their diverse myrmecophile beetles could facilitate microbial strain transmission across these species, resulting in the observed patterns indicative of broad host distribution of the dominant microbial strains. On the other hand, we found no consistent patterns when comparing the two functional categories (inquilines and colony outskirt inhabitants) despite observed major differences among species. While some OTUs found in myrmecophiles and ants were also present in the few sampled free-living staphylinids, the primary OTUs that we identified as being shared among the community members, *Weissella*, *Rickettsiella, Wolbachia* and

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Rickettsia, were not (Figure 2, Figure 3A and Figure 3B). This is also evidenced when comparing the microbial composition similarity among samples grouped by species, where we see that Bray–Curtis dissimilarity indexes for free-living beetles are generally high (Figure 4). Thus, with their hundreds of associated insect species and constant interactions among individuals within bivouacs, army ant colonies appear to serve as excellent arenas for interspecific microbial symbiont exchange. Similarly, in velvety tree ant (*Liometopum occidentale*) communities, ants and myrmecophiles show similarities in their microbiota composition, depending on their level of interaction (Perry et al., 2021). As myrmecophiles can be found in nests of several ant species (Danoff-Burg, 2008; Kronauer & Pierce, 2011), the study of these relationships and their influence on the microbiota of the species involved could be of great importance to understanding microbe sharing among insects and beyond.

The mechanisms of transmission among ant workers, larvae, and their associated beetles are likely to vary substantially among bacterial symbiont categories (Perreau & Moran, 2021). Bacteria that form 'open' symbioses, which include most gut symbionts, are commonly acquired from or through the environment—creating opportunities for different cohabiting insects to acquire the same microbes from the same sources within a colony. Sharing food sources, predation, grooming and other social interactions create opportunities for direct transfer of microbes from one host insect to another. In the cases of *Weissella* and *Rickettsiella*, it is tempting to assume that larvae are the primary sources of these microbes for other insects within a colony. On the other hand, in *Eciton burchellii* army ants, batches of larvae are synchronized and separated by periods when no larvae are present within colonies, preventing direct transmission from older to younger larvae (Kronauer, 2020). Given the scarce presence of these two microbes in workers, it becomes, alternatively, tempting to postulate beetles as the source of *Weissella* for new generations of larvae, despite limited evidence for direct interactions between larvae and most myrmecophile species. It is also possible that army ant larvae are repeatedly inoculated with symbiotic microbes of prey insects, which in the case of *E. burchellii* comprises primarily *Camponotus* brood (Hoenle et al., 2019; Rettenmeyer et al., 1983); future studies of prey species' microbiota may verify this. Whichever the ultimate source, through their close integration into colony biology, many myrmecophiles are plausibly exposed to similar microbial inocula as those encountered by army ant larvae, and it seems likely that inoculation is not unidirectional.

Interspecific transmission of facultative endosymbionts such as *Wolbachia* and *Rickettsia* is likely more complicated than that of gut symbionts but still probably facilitated within large colonies inhabited by multiple potentially suitable hosts. To establish a novel infection, heritable endosymbionts need to be physically transferred from body fluids of one insect to another, avoid the immune system, establish means of transmission to host reproductive tissue and across generations and affect host fitness in ways that would prevent the rapid clearing of the infection by natural selection (Bright & Bulgheresi, 2010). It can be argued that at least the first step in the process—opportunity for acquiring the new infection—is facilitated among species that live closely together and interact frequently and are exposed to shared pools of external parasites or perhaps preying on each other (Ahmed et al., 2015; Clec'h et al., 2013). *Wolbachia* has been found in extracellular environments of attine ants (Andersen et al., 2012; Frost et al., 2014), *Drosophila melanogaster* (Pietri et al., 2016) and *Nasutitermes arborum* termites (Diouf et al., 2018), among others, and while the viability of the cells was not established, this might facilitate their transmission. This could also be true for other microbes thought to exist strictly as intracellular symbionts. There is also evidence that the environment insects live in might act as a reservoir of some microbes, with the *Wolbachia* signal being detected in plant matter and fungi (Li et al., 2017). However, other studies show that cohabitation does not always result in microbiota similarities, as is the case of some ant species and their trophobiont mealybugs and aphids (Ivens et al., 2018).

Broad distributions of bacterial clades that infect ants and myrmecophiles

Several of the microbes abundant in army ant colonies are distributed much more broadly. *Wolbachia* is estimated to infect approximately half of all insect species, many nematodes and other invertebrates (Kaur et al., 2021). Likewise, *Rickettsia* infects diverse insects, often forming persistent associations—although some strains are only vectored by arthropods, with plants or vertebrates as definitive hosts (McGinn & Lamason, 2021). *Rickettsiella* is also known from diverse arthropods, although the nature of these associations is often unclear (Zchori-Fein & Bourtzis, 2012).

In contrast, the genus *Weissella* is not well-known as an insect associate, despite some of the named species being originally isolated from insects (*Weissella bombi* from a bumblebee, *Weissella cryptocerci* from a cockroach—Heo et al., 2019; Praet et al., 2015). Better-known species come from vertebrates (e.g., *Weissella confusa*, human pathogen—Fairfax et al., 2014; Kamboj et al., 2015) and fermented foods (*Weissella koreensis* in kimchi, *Weissella fabaris* in fermented cacao beans—Lee et al., 2002; Snauwaert et al., 2013). The broad distribution of the genus *Weissella*'s indicates its metabolic versatility, which, combined with abundant opportunities for bacterial

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transmission across cohabiting species, may explain its broad distribution in army ant colonies.

Explaining the close similarity of ant and myrmecophile microbes to previously described strains of *Weissella ceti*/*tructae*, isolated from beaked whale (Vela et al., 2011) and farmed rainbow trout (Castrejón-Nájera et al., 2018; Figueiredo et al., 2015; Pereira et al., 2022), respectively, is more challenging. The latter is a well-documented virulent pathogen, but we have no information on the biology of the former. Regardless, the vertebrate species they were isolated from are physiologically dissimilar and inhabit completely different environments than army ants, and opportunities for direct microbial exchange among them must be extremely limited. Still, the close similarity within the *rplB* protein-coding gene between strains infecting fish, whales, ant larvae and some myrmecophile beetles does indicate that the interspecific transmission, likely through a long chain of other hosts or habitats, must have occurred relatively recently or is perhaps ongoing.

Such versatility may not be an unusual characteristic of a bacterial genus. Species from the genus *Lactobacillus*, for example, can be found in a wide range of vertebrate and invertebrate hosts and also in fermented plant and milk products (Zheng et al., 2020). Members of the genera *Enterobacter* and *Pseudomonas* can also be found in a broad spectrum of habitats, such as plants, soil, aerosol and water, in addition to being opportunistic pathogens or more commensal members of the gut microbiota of vertebrates and invertebrates (Grimont & Grimont, 2006; Silby et al., 2011). The Earth Microbiome Project—the broadest microbial survey to date—has reported many other bacterial clades broadly distributed and abundant across environments, including *Bacillus, Enterobacteriaceae and Streptococcus* (Thompson et al., 2017); https://earthmicrobiome.org/). But, we are far from understanding their ecological and evolutionary relevance in different environments.

Biological properties and fitness effects as a critical aspect of microbial transmission and distribution

Depending on the nature of the association with their hosts, symbiotic microorganisms vary in their fitness effects, transmission propensity and evolutionary potential. Facultative endosymbionts such as *Rickettsia*, *Wolbachia and Spiroplasma* have been traditionally regarded as reproductive parasites, but more recent research has revealed a range of functions that can clearly benefit hosts, including the biosynthesis of nutrients and protection against natural enemies (Kaur et al., 2021; Łukasik et al., 2013; Nikoh et al., 2014; Sapountzis et al., 2018). Through the combination of reproductive manipulation and fitness benefits, new infections with these microbes, likely initially acquired from other species, have sometimes swept through host populations (Himler et al., 2011; Jaenike et al., 2010; Kriesner et al., 2013), with effects likely reverberating in multi-species communities (Ferrari & Vavre, 2011). At short timescales, such infections could enable rapid response and adaptation to environmental challenges, particularly relevant in the rapidly changing world of the Anthropocene (Lemoine et al., 2020). At longer timescales, they may facilitate and speed up speciation (Janson et al., 2008; Moran, 2007). The patterns and processes relevant to the distribution and transmission of these microbes are increasingly recognized as an essential component of their hosts' biology.

For members of the microbiota thought to form open symbioses, represented by *Weissella* and likely *Rickettsiella* clades, we are only starting to unravel their distributions, the spectra of their functional diversity, details of their associations with host organisms, and ecological and evolutionary significance. With highly fragmented and biased data, we are far from understanding any of these processes in non-model organisms, including millions of insect species that have not yet been formally described (Adis, 1990; Stork, 2018), and which are increasingly threatened by extinction as climate change and other anthropogenic disturbances intensify (Raven & Wagner, 2021). Adding to the challenge, microbes identified in a wild-collected insect individual may not necessarily form stable associations, instead originating from food or other environmental sources. Such transient microbes may form a significant portion of the microbial community profile in some host species (Hammer et al., 2019). Then, a single observation of a host-microbe combination should not be regarded as proof of stable association. However, multiple such observations indicate, at the very least, that abundant opportunities exist for interaction among organisms and the establishment of such symbiosis. Hence, our data suggest that army ant colonies serve as convenient arenas for the interspecific exchange of various microbes. It is likely that such microbial exchange is common in other environments, for example, where animals share food resources (Stahlhut et al., 2010) or in predator–prey interactions (Clec'h et al., 2013; Kennedy et al., 2020). To fully understand the processes and patterns related to microbial transmission across species, their dynamics and significance, it is clear that future broad surveys of microbiota across diverse wild insect communities will need to include a comprehensive analysis of their ecology and interactions with other organisms.

AUTHOR CONTRIBUTIONS

Catalina Valdivia: Conceptualization (equal); data curation (equal); formal analysis (lead); investigation (equal); methodology (equal); software (lead); validation (lead); visualization (lead); writing – original draft

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(equal); writing – review and editing (equal). **Justin A. Newton:** Investigation (equal). **Christoph von Beeren:** Data curation (equal); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Sean O'Donnell:** Resources (equal); writing – review and editing (supporting). **Daniel J. C. Kronauer:** Resources (equal); writing – review and editing (supporting). **Jacob A. Russell:** Conceptualization (equal); funding acquisition (equal); methodology (equal); resources (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (supporting). **Piotr Łukasik:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (lead); resources (equal); software (supporting); supervision (equal); validation (supporting); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGEMENTS

We thank Minoru Moriyama, Alfred F. Newton and Mariana Chani-Posse for help with specimen identification, Szymon Drobniak for statistical advice, and two anonymous reviewers for valuable comments. We also thank the Symbiosis Evolution Group at Jagiellonian University, especially Diago Castillo-Franco, Sandra Ahlen Mullio and Junchen Deng, for their participation in discussions about the results and how to present them. The project was supported by the U.S. National Science Foundation grant 1050360 to Jacob A. Russell, Polish National Agency for Academic Exchange grant PPN/PPO/2018/1/00015 and Polish National Science Centre grant 2018/31/B/NZ8/01158 to Piotr Łukasik. The open-access publication of this article was funded by the program 'Excellence Initiative—Research University' at the Faculty of Biology of the Jagiellonian University in Kraków, Poland.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sanger sequences were deposited in GenBank (Accession nos OP850275-OP850288). Amplicon sequencing data were deposited in NCBI Sequence Read Archive (BioProject: PRJNA900236; 16S rRNA data: SAMN31683713–SAMN31683857). Colony and insect details are provided in Tables S1 and S2. In Tables S1 and S6, we listed the above accession numbers by individual.

ETHICS STATEMENT

All samples were collected following national and international laws; collection permit numbers include 122-2009, 192-2012 and R-009-2014-OT-CONAGEBIO (Costa Rica).

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How to cite this article: Valdivia, C., Newton, J.A., von Beeren, C., O'Donnell, S., Kronauer, D.J.C., Russell, J.A. et al. (2023) Microbial symbionts are shared between ants and their associated beetles. *Environmental Microbiology*, 25(12), 3466–3483. Available from: https://doi.org/10.1111/1462-2920.16544

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