Low diversity and host specificity in the gut microbiome community in species of *Eciton* army ants (Formicidae: Dorylinae) in a Costa Rican rainforest

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Keywords: Dorylinae, symbiosis, microbial diversity, host specificity, Costa Rica, Firmicutes, Entomoplasmatales

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Number of pages: 37

Number of figures: 7

Number of tables:7

Abstract.

Neotropical army ants of the genus *Eciton* are top arthropod predators in tropical rainforests. Microbial symbionts, including Unclassified Firmicutes (UF) and Unclassified Entomoplasmatales (UE), are associated with this genus and likely play a significant role in the biology of these ants. While previous work focused on army ant-gut microbe associations across large geographic scales, here we report a community survey of the gut microbes colonizing the six sympatric *Eciton* army ant species in a single Costa Rican location. Furthermore, we characterized the gut microbiota associated with different army ant castes in the swarm-raiding species *E. burchellii*. We employed a combination of 16S rRNA amplicon sequencing, fluorescent and electron microscopy to identify gut microbes and to verify their presence in ant guts. We also measured the diversity and interaction specificity of the ant–gut microbe interaction network. The two most dominant OTU phylotypes in all species were related to UF and UE previously found in army ants, followed by OTUs assigned to the genus *Weissella*. Furthermore, the worker castes of *E. burchellii* shared a similar gut microbiota, also dominated by these two potential symbionts. Overall, we found a low diversity of gut microbes and a low interaction specificity between army ants and microbes at the community level, mainly because most microbe strains were detected in various *Eciton* species. These results were confirmed by microscopy techniques, as FISH analyses documented the presence of the two dominant phylotypes within ant guts and electron microscopy located bacterial biofilms in the hindgut near the microvilli, whose morphology suggest that these bacteria probably belong to the dominant phylotypes UE and UF. Taken together, our results confirm that the *Eciton*'s gut microbiome is consistently dominated by a few species of specialized bacteria that may improve nutrient uptake efficiency of host ants. Further research should employ multi-omics and culture-dependent strategies to fully understand the role of these potential symbionts in ant ecophysiology.

Introduction

Insects are among the most diverse and abundant animal groups on Earth, occupying all types of habitats (Bahrndorff *&* al. 2016). The diversification and evolutionary success of insects depend in part on symbiotic interactions (Poulsen & Sapoutzis 2012), particularly with microorganisms (Weiss & Aksoy 2011). Bacterial symbionts can fulfill different functions in insects, where they can contribute to host nutrition, digestion, reproduction, and defense (Ohkuma 2003, Dillon & Dillon 2004, Engel & Moran 2013).

Many of the bacterial communities known to be associated with insects inhabit the digestive tract, where they can perform key nutrition-related functions such as nutrient recycling or upgrading (Engel & Moran 2013, Moran & al. 2019), but also contribute to the defense against pathogens (Koch & Schmid-Hempel 2011) and other aspects of host health. The gut microbiome composition differs according to host phylogeny (Anderson & al. 2012, Sanders & al. 2014) and environmental factors (Amato 2013, Kaltenpoth & Engl 2014). Large differences in microbiome composition and abundance have been described among different taxa and even among individuals within the same taxon, and it has been suggested that certain species may harbor virtually no gut microbes (Hammer & al. 2017, Sanders & al. 2017, Hammer & al. 2019). In some insects, the hindgut harbors large bacterial populations due to favorable pH and redox conditions (Dillon & Dillon 2004, Douglas 2015), while lower bacterial loads are found in the midgut, which tends to be a hostile environment for microorganisms because the epithelium secretes digestive enzymes and is immunologically active. For example, the *D. melanogaster* midgut produces various antimicrobial peptides, digestive enzymes (including lysozymes) and a dual oxidase (DUOX: NADPH oxidase) that generates microbicidal reactive oxygen species (Douglas 2015).

Several ant clades have important symbiotic relationships with their gut microbiome (Boursaux-Eude & Gross 2000, Douglas 2015, Moreau 2020). In herbivorous ants, bacteria can help their hosts by synthesizing essential amino acids, unfolding lignocellulosic components, supplying vitamins and

cofactors, and detoxifying harmful plant subcomponents (RUSSELL & *a*l. 2009, DOUGLAS 2009, SUEN & *a*l. 2010). A well-known example is the symbiosis between carpenter ants and their endosymbiont, *Blochmannia floridanus*, which recycles nitrogenous waste and upgrades its host's diet by providing essential amino acids during metamorphosis and is maternally transmitted across generations by oocytes infection (KUPPER & al. 2016, FELDHAA*R* & *a*l. 2007, ZIENT*Z* & *a*l. 2006). Likewise, in turtle ants of the genus *Cephalotes*, abundant, relatively diverse, and highly specific gut microbial communities recycle common nitrogenous waste products such as urea and uric acid to synthesize essential amino acids that are then provided to host workers in substantial quantities (HU & al. 2018, DUPLAIS & al. 2021). Similarly, herbivorous ants of the genus *Dolichoderus* host *Tokpelaia* bacteria that encode largely complete pathways for nitrogen recycling and biosynthesis of all essential amino acids and different vitamins (BISCH & al. 2018).

Recent studies have characterized the gut microbiome of a few clades of predatory ants (FUNARO & al. 2011, NEUVONEN & *al*. 2016, ŁUKASIK & *a*l. 2017, IVENS & al. 2018, BISCH & al. 2018). The gut microbiome of a conspicuous predatory ant, the Giant Neotropical Bullet Ant *Paraponera clavata*, is dominated mainly by two bacterial operational taxonomic units (OTUs) in the genus *Tumebacillus* (Firmicutes), known to associate with a diversity of hosts (MOREAU & RUBIN 2017). In at least some instances, the microbial symbionts of predatory ants appear to play significant roles, such as in the case of *Tokpelaia* in the gut of the ant *Harpegnathos saltator,* which seems to import and degrade amino acids such as histidine and arginine, as well as urea, to produce glutamate from these nitrogen sources (NEUVONEN & *al*. 2016). This contrasts with the role of *Tokpelaia* in the herbivorous ant *Dolichoderus* spp., which harbors genes for the biosynthesis of histidine and arginine. These differences are likely the result of a metabolic adaptation to the diet of their hosts: protein-rich diets in predatory ants select for microbes involved in amino acid degradation, while carbohydrate-rich diets in herbivorous ants promote symbionts specialized in amino acid biosynthesis (BISCH & al. 2018).

Army ants are considered the ultimate predators in the ant world (SCHNEIRLA 1971, KRONAUER 2020) and are characterized by mass raiding, nomadism, and colony fission, collectively known as the army ant adaptive syndrome (GOTWALD, 1995). One of the best-studied species is the swarm-raider *Eciton burchellii*, which is a main predator of arthropods in Neotropical humid forests (FRANKS 1982, RETTENMEYER 1983; GOTWALD 1995, HOENLE & al. 2019), and has an extraordinarily rich fauna of over 300 associated animal species, from mites to birds (RETTENMEYER & *a*l. 2011).

Unlike for most predatory ants, there are several studies focusing on the gut microbiomes and possible symbionts of army ants (FUNARO & al. 2011, ANDERSON & al. 2012, ŁUKASIK & *a*l. 2017). In general, these studies have shown that these ants consistently harbor relatively abundant but low-diversity microbial communities. Specifically, microbiomes of all New World army ants seem to be dominated by few taxa, including 'Unclassified Entomoplasmatales' (UE), 'Unclassified Firmicutes' (UF), Actinomycetales, and *Weissella*. Particularly, UE and UF, are the dominant bacteria in the digestive tracts of *Eciton* workers (ŁUKASIK & al. 2017).

Interestingly, there is evidence that different species or even different colonies, as well as different individuals within the same colony, harbor different strains of these bacteria (FUNARO & al. 2011, ANDERSON & al. 2012, ŁUKASIK & al. 2017). However, previous studies characterized army ants from different geographic locations, despite the known geographic variation in microbiome composition. Consequently, microbiome diversity and host-microbe specificity patterns across different army ant species living in the same population and across ant castes within the same colony have not yet been addressed systematically.

To bridge this knowledge gap, we systematically evaluated differences in gut microbiome composition between *Eciton* species, colonies, and castes at a single site: La Selva Biological Station in Costa Rica. The goals of the present study were threefold: (1) to characterize the composition and structure of the gut bacterial communities of sympatric *Eciton* species, (2) to test for caste differences in microbiome composition within a single species, *E. burchellii*, and (3) to localize bacteria in *E. burchellii* gut tissue to confirm the results from the broader sequencing-based surveys. We addressed these goals by characterizing the gut microbiomes of workers of different colonies from all locally occurring *Eciton* species at La Selva, i.e., *E. burchellii*, *E. dulcium*, *E. hamatum*, *E. lucanoides*, *E. mexicanum,* and *E. vagans*, using *16S rRNA* amplicon sequencing. In addition, we compared bacterial communities of different castes from two colonies of *E. burchellii*. Lastly, we employed scanning and transmission electron microscopy (SEM and TEM) and fluorescence *in situ* hybridization (FISH) to visualize and localize bacteria within the gut tissue of *E. burchellii* ants. This study shows that *Eciton* army ants harbor a relatively simple and unspecific gut microbiome at the community level, adding evidence to the current general view that this is the case with most predatory ants.

Materials and methods

Specimen Collection: Army ants of the species *E. burchellii*, *E. dulcium*, *E. hamatum*, *E. lucanoides*, *E. mexicanum* and *E. vagans*, including regular workers, submajors, majors and males, were collected from February to April 2013 and March to April 2014 at La Selva Biological Station (LSBS) in Costa Rica (N10 25.847 W84 00.404, altitude 67 m; Tab. S1). Regular workers and submajors can easily be distinguished based on the coloration and the mandible shape, the latter of which are still triangular but more stretched in length in the submajors (RETTENMEYER 1961). Both worker types are easily distinguishable from majors, which possess the *Eciton*-typical hook-shaped mandibles (RETTENMEYER 1961, POWELL & FRANKS 2006). The workers were collected during nocturnal colony emigrations and colony raids (for more information see VON BEEREN & al. 2016, VON BEEREN & al. 2018). Ant species were identified employing the taxonomic keys of John T. Longino (LONGINO 2010). Regular workers from one additional *E. burchellii* colony were collected in September 2017 at LSBS. Regular workers were further divided into two size classes, which were previously denoted as minors and media (FRANKS 1985).

The microbial diversity and distribution were analyzed using pools of eight specimens for each worker type per *Eciton* colony (one pooled sample per colony), from twelve colonies of *E. burchellii*, nine of *E. dulcium*, eleven of *E. hamatum*, two of *E. lucanoides*, nine of *E. mexicanum* and six of *E. vagans. Eciton* workers of all studied species are deposited at the Technical University Darmstadt Insect Collection and are either mounted on insect cardboards or preserved in absolute ethanol in 50mL vials and stored in a -30°C freezer. To compare microbiomes across *E. burchellii* worker size classes and males, three individuals were chosen from each of the following categories: minors, medias, submajors, majors and males, from two of the colonies characterized in the previous step. Symbionts were localized within ant gut tissues using three individuals for each of the microscopy techniques: SEM, TEM, and FISH.

DNA extraction and sequencing: As a first approximation, the bacterial DNA for gut microbiome profiling was extracted from the whole gaster, the body part that includes most of the ant digestive system. We are aware that separating the intestinal tract into different parts would have given us additional information about the microbes' preferred locations within the workers but performing such an analysis was beyond the scope of this study. Our aim was to screen for gut microbe differences between sympatric species and between *Eciton burchellii* castes. To prevent contamination with environmental bacteria, all extractions were performed under sterile conditions in a laminar flow hood. To remove bacteria from the ants' cuticles, all insects were surface-sterilized by immersing them individually for 30 seconds in 5%

bleach and then in 1x phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) for 30 seconds. A pretreatment was applied to the samples that consisted of placing gasters, previously cut with sterile disposable razors, inside 1.5 ml tubes with stainless steel beads (5mm) and 180 µl of the enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM sodium EDTA, 1.2 % Triton X100 and 20 mg/ml lysozyme). Then, samples were homogenized in a QIAGEN TissueLyzer II for 3 min. at 30 Hz, and the homogenate was processed, following the manufacturer's modified extraction protocol from the QIAGEN DNeasy Blood & Tissue kit. Each DNA extraction batch had its own extraction control (blank sample) that was used as a negative control throughout the procedure (including sequencing). As a single positive control, a gaster of a *Cephalotes* sp. worker was used.

The ant gut bacterial communities were characterized by sequencing amplicons of the V4 region of bacterial *16S rRNA* (CAPORASO & *a*l. 2011, CAPORASO & *a*l. 2012). The V4 region was amplified using universal bacterial primers (515F: 5' GTGCCAGCMGCCGCGGTAA 3', 806R: 5' GGACTACHVGGGTWTCTAAT 3') (KOZICH & al. 2013) with adapter stubs. The PCR reactions consisted of 2.5 µl of sample DNA, 0.5 µl of 10 µM primers, 12.5 µL of 2X KAPA HiFi Hotstart Readymix, and water to the total reaction volume of 25 µl. PCRs ran at 95 °C for 3 min, followed by 26 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 30s, with a final extension step at 72 °C for 5 min. A second PCR reaction was performed to attach Illumina sequencing adapters and barcodes. The reaction employed 5 µl of the amplification product of the first PCR, 5 µl of Nextera Index barcodes (forward and reverse) with 25 µl of 2X KAPA HiFi Hotstart Readymix and water up to a total volume of 50 µl. The indexing reaction conditions were the same as above, except for fewer cycles: 8 instead of 26. Indexed PCR products were then pooled with eight samples each and purified together using 0.6 X Agencourt AMPure XP beads (Beckman Coulter). DNA was quantified in each purified product with Qubit™ (ThermoFisher Scientific) and equimolarly pooled to create a single sample. Libraries from DNA samples and controls were sequenced on an Illumina MiSeq platform at the Rockefeller University Genomics Resource Center to obtain 2x250bp paired-end sequencing reads.

Sequence processing and bioinformatic analyses: The sequences of both datasets were processed with the software R (R Core Team 2017) using the package DADA2 (version 1.14) through a modified pipeline (CALLAHAN & al. 2016a). First, the paired-end reads were joined into contigs. Then, sequences were clustered into Amplicon Sequence Variants (ASVs) and screened for sequencing errors: ASVs with less than 248 bp and sequences with more than 254 bp, singletons and chimeras were removed. The remaining sequences were aligned to the SILVA reference database (version 132) (QUAST & al. 2013) with a 97% nucleotide similarity level. Taxonomy assignment was performed with the naive Bayesian classifier method (WANG & al. 2007). Sequences not matching any records in the database were denoted as "Unclassified". Subsequently, to minimize the artifacts created in the ASV designation, all sequences were aligned with the package Biostrings (PAGÈS & al. 2019), and then clustered into OTUs with 99% sequence similarity applying the neighbor joining clustering method with the package DECIPHER (WRIGHT 2016).

Finally, all the sequences that were not assigned to Bacteria were removed, along with those classified as mitochondria and chloroplast, employing the package phyloseq (version 1.22.3) (MCMURDIE & HOLMES 2013, CALLAHAN & al. 2016b). Potentially contaminating sequences were identified based on their prevalence in experimental samples and blanks using the decontam R package with a 0.5 threshold (DAVIS & al. 2018). Sequences with higher prevalence in negative control samples than in ant samples were defined as potential contaminants and then removed from the analysis. Moreover, four samples (EB16-Media1, EB16-Male2, EB16-Male3, EB16-Male1) were excluded from the analyses because they had fewer than 1,000 sequence reads.

Data Availability: Raw data can be accessed at the National Center for Biotechnology Information Sequence Read Archive under Bioproject ID PRJNA818015 and accession numbers SAMN26814244 - SAMN26814318.

Interaction specificity, modularity, and diversity of the ant gut microbe network: A quantitative contingency matrix summarizing the interactions of *Eciton* species (rows) and OTUs (columns) was established to quantify the interaction specificity of the *Eciton*-gut microbe network at LSBS at the species/OTU- and at the network-level. The interaction matrix was based on incidences, which represent the number of times different OTUs were detected in different army ant colonies. Accordingly, link strengths among pairs represent the number of colonies of a given *Eciton* species that contained representatives of a given OTU in ant guts (see also IVENS & al. 2016). The network approach was thus based on spatiotemporally independent collection events, yielding a conservative estimate of microbe specialization, because microbes belonging to the same OTU collected from the same colony are only represented by a single incidence (BLÜTHGEN 2010). The species-level specificity was quantified with the standardized Kullback-Leibler distance d' and the network-level specificity with the standardized two-dimensional Shannon entropy H_2' (BLÜTHGEN & al. 2006). Both metrics are normalized relative to minimum and maximum possible values and consequently range from 0 (random, unspecific interactions) to 1 (highly specific interactions) (BLÜTHGEN & al. 2006). The H_2' of the actual ant-microbe network was compared with H_2' values of 10,000 randomized networks to test whether the observed network pattern deviates from random interactions using the null model algorithm for contingency matrices developed by Patefield (PATEFIELD 1981, BLÜTHGEN & al. 2006). Modularity, which describes the extent to which species interactions are clustered into distinct network subgroups, was evaluated using the quantitative modularity metric *Q* (DORMANN & STRAUSS 2014). This metric is also normalized and ranges from 0 (minimum modularity) to 1 (maximum modularity).

In addition, several diversity metrics were calculated to assess the diversity of OTUs between *Eciton* species within the network. Species richness *S* gives the total number of OTUs detected in different *Eciton* colonies. The diversity metric e^H represents the exponential Shannon diversity of network links (IVENS & al. 2016, JOST 2006), serving here as an intuitive measure of microbial diversity per host species. In addition, comparability between *S* and e^H measurements were improved by randomly sub-setting network incidences per army ant species to the lowest network incidence number of 13 incidences in *Eciton lucanoides*. Rarefaction was performed 100 times and the mean and standard deviation of *S* and e^H were calculated for the network subsets (S_{rare} and $e^H{}_{rare}$). The *Eciton* army ant-gut microbe network visualizing the host specificity of gut microbes was arranged to minimize the number of crosses. The same network specificity and diversity calculations were performed for different *Eciton* castes/worker types within the species *E. burchellii*.

To analyze beta diversity, a non-metric multidimensional scaling plot (NMDS) was created based on Bray-Curtis dissimilarities (BRAY & CURTIS, 1957) to compare microbial diversity between the different *E. burchellii* castes and sexes, as well as between *Eciton* species. PERMANOVA and ANOSIM (ANDERSON & WALSH, 2013) analyses were performed employing the Bray-Curtis dissimilarity matrix with the R packages phyloseq (version 1.22.3) and vegan (2.5-6) (OKSANEN & et al., 2019). To complement the analysis, a heat map was created by plotting the relative abundance of OTUs from the two datasets. In this analysis, only OTUs that had a relative abundance ≥ 1% of the total reads in at least one sample were included. The databases were rearranged with the R package tidyverse (WICKHAM & al. 2019), and plots were created using the R packages ggplot2 (WICKHAM 2016), pheatmap (KOLDE 2019) and phyloseq (version 1.22.3) (MCMURDIE & HOLMES 2013). The heatmap of the *Eciton* species dataset was reordered according to their phylogenetic relationships. Also, a bar plot of the relative abundance of the OTUs of the different *Eciton* species was included as a supplementary material.

Scanning Electron Microscopy: The localization of symbiotic bacteria within the digestive tract of *E. burchellii* workers was visualized by SEM, with a special focus on biofilm formations. Digestive tracts of three collected *E. burchellii* media workers were dissected in sterile conditions in 1x PBS, then fixed in modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) (KARNOVSKY 1965) for at least one hour and stored in fixative at 4 °C until processing. To begin processing, samples were washed with a 0.1M phosphate buffer. Then, samples were post-fixed with 1% osmium tetroxide (OsO_4) solution and washed with distilled water. Subsequently, samples were dehydrated in an ethanol gradient (30, 50, 70, 80, 90, 95 and 2 x 100 %), washed with xylene and embedded in paraffin (Paraplast, Leica) (BANCROFT & al. 2018). Sections (30 µm) were obtained using a PT-PC PowerTome ultramicrotome (RMC products), mounted on aluminum bases and coated with gold with an Ion Coater (IB-5, Giko). Finally, samples were observed under a Hitachi S-3700N scanning electron microscope.

Transmission Electron Microscopy: The aseptically dissected gut of three *E. burchellii* workers were used for in depth ultrastructural exploration using TEM. The samples were fixed with a modified Karnovsky solution and washed with a 0.1M phosphate buffer. Subsequently, a 1% solution of Osmium tetroxide (OsO_4) was employed for post-fixation, after which the samples were washed with distilled water. For dehydration, an acetone gradient (30, 50, 70, 90 and 3x 100%) was applied. Samples were placed in Spurr medium-viscosity resin to make cuts of different thicknesses (50 to 80 nm) with the Powertome microtome. Finally, samples were contrasted with uranyl acetate and lead and observed in a Hitachi H7100 transmission electron microscope.

Fluorescence in situ hybridization: The presence of symbiotic bacteria within the digestive tract of ants was determined by fluorescence microscopy. Digestive tracts were dissected from three workers of *E. burchellii* as described above and then fixed in 4% formaldehyde. Subsequently, intestinal contents were filtered employing nucleopore polycarbonate filters (Whatman, United Kingdom) to avoid, among other issues, auto-fluorescence. FISH was performed over the filters using a combination of eubacterial probes (EUB338 and EUB897, label CY3) and probes specific to UF (UNF16SF1 - 5' GAGTTGTCCTCGTCTTATCGG '3, label CY5) and UE (cute493R - 5' AGAAAGCCACGGCNAACTAT '3, label CY5) (ŁUKASIK & al. 2017).

Filter sections were incubated with 1 ml hybridization buffer (5 M NaCl, 1 M Tris–HCl, 1% (v/v) SDS), formamide (30% FA (v/v), and H_2O) and 3 µl (10µM) probe working solution for 120 min at 46 °C in a hybridization oven. The filter sections were then washed in prewarmed buffer (10 M Tris, 5 mM EDTA, a probe dependent amount of 5 M NaCl, H_2O) for 15 min at 48 °C. Then, samples were washed in ice cold H_2O for 20 sec and mounted on glass slides with antifade solution that includes DAPI (ProLong™ Gold Antifade Mountant with DAPI) and finally they were observed with a confocal microscope Olympus FV1000. For negative controls, probes without their competitors were used to verify the presence of nonspecific labeling, and no fluorescence was observed in either case (Fig. S2).

Results

Microbiome composition across *Eciton* species: We obtained a total of 6,823,185 sequencing reads from 49 gut samples of the six *Eciton* species, with a median of 77,225 reads per sample (range: 3,134-1,117,978 reads per sample). ASV sequence clustering at 99% identity cutoff resulted in a total of 534 OTUs (for details, see Tab. S2).

The ant microbial communities were dominated by multiple OTUs associated with Unclassified Entomoplasmatales (UE) (GenBank Accession: HM996853.1) and Unclassified Firmicutes (UF) taxa (GenBank Accession: KX983349.1). Five bacterial OTUs representing UE and six OTUs belonging to UF were abundant across samples, together accounting for 91.8% of all reads in the dataset (38.41% for UE and 52.77% for UF). In the case of UE OTUs, UE-01 was most abundant in all *Eciton* species except *E.*

mexicanum, where the host-specific OTU UE-02 dominated instead (Fig. 1A, Fig. S1). Regarding UF OTUs, two phylotypes were abundant in all *Eciton* species (UF-01 and UF-03). In contrast, UF-02 was mainly present in *E. hamatum* and *E. vagans* (Fig. 1A, Fig. S1). Besides these two dominant symbionts, other OTUs belonging to the genera *Weissella* (1.96%), *Tokpelaia* (1.29%), *Serratia* (0.67%) and *Acinetobacter* (0.20%) were also present in some of the *Eciton* samples (>1% of relative abundance in at least one sample).

Microbiome composition in *Eciton burchellii* worker castes and males: Across *E. burchellii* worker castes and males, a total of 1,621,829 sequencing reads were obtained, with a median of 49,714 reads per sample (range: 12,344-185,448 reads per sample), which clustered into a total of 247 OTUs (for details see Tab. S3). The gut microbiomes of *E. burchellii* castes and males were dominated by the two bacterial phylotypes: UE and UF. Four OTUs belonging to the phylotype UE and two belonging to UF were abundant in different samples, accounting for 89.54% of all reads (62.86% by UE and 26.68% by UF). Additionally, in one male sample and one submajor sample, we observed high relative abundance of *Weissella* OTUs (Fig. 2A).

Interaction specificity and diversity of the host-gut microbe network: The army ant-gut microbe network showed a low degree of specificity (H_2' = 0.13), demonstrating that the community was dominated by non-specific associations. Yet, network specificity differed from purely random network models (H_2' tested against null models, p = 0.001), mainly because few bacterial OTUs showed clear host preferences (see UE-02, UE-05, CT-02 and AC-01 in Fig. 1B). Host specificity at the microbe OTU level was also generally low (mean *d':* 0.132; range d': 0.000-0.389). Similarly, interaction specificity was low at the army ant species-level (all *d'* values ≤ 0.18). Not surprisingly, modularity of the network was also low (Q = 0.16), showing that there is little partitioning of hosts between microbial OTUs. Gut microbe diversity was similar in all *Eciton* species (S_{rare} range: 8.35-11.00 OTUs, eH_{rare} range: 7.35-10.50 OTUs, Fig. 3A). Beta diversity metrics showed significant differences in the composition of the microbial communities of the different *Eciton* species (Fig. 3B; ANOSIM, p<0.001, R=0.445; PERMANOVA, F=5.264, p<0.001; stress=0.212). Mainly, the NMDS plot showed that the microbial communities of *E. hamatum* and *E. vagans* are more similar *t*han the other species.

The network between *E. burchellii* castes and gut microbes also showed a low degree of specificity (H_2' = 0.04) and it did not differ from random network models (H_2' tested against null models, p= 0.156). As expected then, specificity was also low at the microbe OTU level (mean *d'*: 0.054; range *d'*: 0.000-0.263, Fig. 2B) and at the *E. burchellii* castes level (all *d'* values ≤ 0.15), and the network showed no sign of modularity (*Q* = 0.06). Gut microbe diversity was similar in all *E. burchellii* castes (S_{rare} range: 5.91-7.54 OTUs, eH_{rare} range: 5.55-6.73 OTUs, Fig. 4A). However, considering beta diversity, the gut microbial communities in male samples are separated from the worker castes and they also show a high dispersion as indicated by the NMDS plot (Fig. 4B). The differences were not statistically significant (ANOSIM, p=0.111, R=0.086; PERMANOVA, F=1,2117, p=0.221; stress=0.154).

Microscopic analyses of *E. burchellii* worker digestive tracts: Microscopic analysis of gut tissues from media workers revealed different types of intestinal cavities, especially in the hindgut. Inside one of these hindgut cavities (Fig. 5A), SEM images show a possible biofilm formation. This biofilm consists mainly of coccoid-shaped bacteria (Fig. 5B) with sizes between 200-700 nm. These observations could indicate the presence of UE, one of the most abundant microorganisms detected in our culture-independent analyses. TEM observations showed microorganisms near midgut microvilli (Fig. 5C) with the presence of bacilli bacteria (Fig. 5D). These intracellular bacteria range in size from 0.60 µm to 1 µm, with dense cytoplasm. Some of them can be observed undergoing cell divisions, suggesting that they are metabolically active (Fig. 5E).

FISH results confirmed our *16S rRNA* sequencing results, showing an abundance of bacteria in the gut (Fig. 5F). The use of specific probes further confirmed the presence of the most abundant bacteria detected in the gut of *E. burchellii* in our culture-independent analysis: UF (Fig. 5G) and UE (Fig. 5H). FISH visualizations showed that Firmicutes are bacilliform bacteria, and Entomoplasmatales have a coccoid shape, which is consistent with our SEM observations of the *Eciton* digestive tract.

Discussion

Gut bacterial communities in *Eciton* army ants: We found that the gut bacterial communities of six sympatric *Eciton* species were dominated by a small number of bacterial taxa, which is consistent with previous reports (FUNARO & al. 2011, ANDERSON & al. 2012, ŁUKASIK & al. 2017). As mentioned before, the intestinal microbiome of different predatory ants is often dominated by one to two bacterial taxa (ŁUKASIK & al. 2017, MOREAU & RUBIN 2017), while herbivorous ants may harbor a more conserved and diverse microbiome (HU & *a*l. 2014, HU & *a*l. 2018). However, other herbivorous ants such as *Dolichoderus* and *Pseudomyrmex* also harbor relatively few bacterial OTUs, indicating that diet alone is not sufficient to explain the diversity of gut microbial communities (SANDERS & al 2017, BISCH & al. 2018).

The dominant phylotypes in the *Eciton* gut community identified in this study, UE and UF, have been detected in other army ant species from both the New World (e.g., *Labidus praedator* and *Nomamyrmex hartigii*) and the Old World (e.g., *Aenictus* spp. and *Dorylus* spp.), and they represent lineages highly specific to army ants (FUNARO & al. 2011, ANDERSON & al. 2012, ŁUKASIK & al. 2017). In general, the Entomoplasmatales order remains poorly understood, with the exceptions of the intracellular human pathogen *Mycoplasma* (DALEY & al. 2014) and plant- and insect-associated *Spiroplasma* (BOVÉ & al. 2003, BALLINGER & PERLMAN 2019). Different lineages of Entomoplasmatales have been associated with diverse insect taxa including bees (MEEUS & al. 2012), beetles (LUNDGREN & al. 2007), fruit flies (ANBUTSU & FUKATSU 2011) and ants (SAPOUNTZIS & al. 2015, DE OLIVEIRA & al. 2016). Bacteria from the phylum Firmicutes have been detected in a wide variety of insects, such as Coleoptera, Hemiptera, Lepidoptera and Diptera (YUN & al. 2014), as well as in the giant bullet ant *Paraponera clavata* (MOREAU & RUBIN 2017) and the edible ant *Liometopum apiculatum* (GONZÁLEZ-ESCOBAR & al. 2018).

A previous phylogenetic analysis, based on near-full 16S rRNA, placed the UF as a well-supported, divergent, and undescribed clade, representing a potential new order within the phylum Firmicutes that also includes symbionts of other predatory ants (ŁUKASIK & al. 2017). Recently, an unclassified core gut microorganism of the ponerine ant *Diacamma cf. indicum* was included in this clade, adding evidence for a novel evolutionary origin independent from other insect-associated Firmicutes bacteria, such as *Lactobacillus, Apilactobacillus* and *Bombilactobacillus* (SHIMOJI & al. 2021).

Even though these two dominant phylotypes in *Eciton* microbiomes represent lineages with a specific association, their role remains unknown (FUNARO & al. 2011, ANDERSON & al. 2012, ŁUKASIK & al. 2017). It has been proposed that they participate in the degradation of chitin forming the cuticle of the ants' prey, although there is no experimental evidence yet to support this possibility (SAPOUNTZIS & al. 2015). In other predatory ants such as *H. saltator*, known microbial symbionts contribute to nitrogen metabolism, including the conversion of histidine, arginine, and urea into glutamine, a more suitable source of energy that could be even involved in insect fecundity by activating cell growth via the TOR pathway (BISCH & al. 2018). Furthermore, it has been speculated that UE bacteria are not essential for growth or development because they are absent in eggs and larvae (FUNARO & al. 2011). Neither UE nor UF bacteria appear to be pathogenic since they are ubiquitous across healthy *Eciton* individuals, consistent with previous reports from healthy *Acromyrmex* leaf-cutter ants and their dominant Entomoplasmatales symbiont (SAPOUNTZIS

& al. 2015). Therefore, further research is required to fully elucidate the role of the UE and UF symbionts in army ant physiology.

In addition to UE and UF, the genus *Weissella* (Phylum Firmicutes; class Bacillii) was an abundant taxon across army ant samples. This genus has been detected in a wide range of habitats (FUSCO & al. 2015), including the gut of different insects like the lepidopteran *Ostrinia nubilalis*, an important European pest of maize (BELDA & al. 2011), the cockroach *Cryptocercus kyebangensis* (HEO & al. 2019), the ant *Pseudomyrmex ferrugineus* (EILMUS & HEIL 2009) and other army ants such as *L. praedator* (ŁUKASIK & al. 2017). In *E. burchellii*, workers from the same colony show variability in the relative abundance of this taxon and it was suggested that the association with this bacterium is unlikely to be ancient (ŁUKASIK & al. 2017). Members of the genus *Weissella* are facultatively anaerobic bacteria, and several species present an obligate fermentative metabolism, being able to produce lactic acid and acetic acid from different sugar substrates (FUSCO & al. 2015, PRAET & al. 2015). This suggests that *Weissella* could potentially play a fermentative role in army ant guts, processing simple sugars that result from digestion. Nonetheless, several strains of *Weissella ceti* have been reported as pathogens in fish and whales, showing different metabolic adaptations such as the presence of genes associated with virulence factors and antibiotic resistance (ABRIOUEL & al. 2015). To fully understand the functional interactions of these potential symbionts with their host, further experiments employing metagenomics and metatranscriptomics are needed, as well as culture-dependent strategies to isolate *Weissella* and other army ants' symbionts (mainly UE and UF) to evaluate their enzymatic and secondary metabolism capabilities by performing different types of bioassays (e.g., MOREAU 2020).

It is currently challenging to contextualize these results with those from other predatory ants, since studies are limited. One possible trend is that Firmicutes are often associated with predatory ants. For example, the bullet ant, *Paraponera clavata*, hosts the Firmicutes taxon *Tumebacillus*, which is consistently present across individuals. However, the role of these microbes is still unknown (MOREAU & RUBIN 2017). Another study of the gut microbiome of predatory ants in the genus *Odontomachus* found a dominant Unclassified Firmicutes bacteria. As in our study, different Unclassified Firmicutes variants (ASVs) appear to be one of the main components of the reported differences between *Odontomachus chelifer* and *Odontomachus hastatus* microbiomes, suggesting species-specific microbe patterns in these species (ROCHA & al. 2022).

Gut bacterial community in worker castes and males of *E. burchellii*: It has been proposed that the presence of microorganisms in individual guts might correlate with the host's genotype as well as with its behavior and anatomy (SPOR & al. 2011). All these characteristics can differ between ant worker castes (JAFFÉ & al. 2007). Yet, in our analyses' minors, medias, submajors, majors, and males of *E. burchellii* harbor a similar gut bacterial community, dominated by UF and UE phylotypes. Similar results were recently found in the ponerine ant *Diacamma cf. indicum*, where workers share a similar gut microbiome, dominated by an Unclassified Firmicutes bacterium, although this bacterium has very low abundance within the reproductive castes (SHIMOJI & al. 2021). Furthermore, other adult workers from social insects, such as bees, have a relatively stable set of intestinal bacterial species, but distinct from the queen's microbiome (KWONG & MORAN 2015, MILLER & al. 2019).

Notwithstanding that we found a low diversity microbiome with similar dominant OTUs across *E. burchellii* worker types and males, a previous study employing more diverse genetic markers showed that even individuals from the same colony can harbor different UF and UE genotypes (ŁUKASIK & al. 2017). This supports the need of further phylogenomic and population genetic analyses to obtain a better understanding of the distribution patterns of these potential symbionts across army ant individuals, castes, and species.

In addition, the low gut bacterial abundance detected in the removed male samples (EB16-Male2, EB16-Male3, EB16-Male1) might be attributed to the fact that these males must have emerged from their pupae just a few days before their collection, during the first nomadic colony emigrations (personal observation, CvB; see SCHNEIRLA 1971, GOTWALD 1995, KRONAUER 2020). This pattern can be the result of limited social interactions between males and workers, decreasing the opportunities for symbiont transmission. This is consistent with previous results regarding bee-associated microbes, where newly hatched individuals (callow workers, males, and queens) harbored no gut bacteria or a reduced community of symbionts (KEŠNEROVÁ & al. 2016). Moreover, this agrees with previous studies with army ants that found a low incidence of gut bacteria across eggs, larvae, and pupae, suggesting that they are not required for growth and development and therefore are unlikely to be maternally transmitted (FUNARO & al. 2011), but rather socially transferred (ŁUKASIK & al. 2017). Microbiomes of army ant queens have never been studied, partly because collecting queens' results in the collapse of the colony.

Host-Microbe Specificity: Our results for both UE and UF suggest that most bacterial OTUs show little host preference and only a few appear to show a certain level of host specificity (d'>0.20), in line with previous findings for a larger number of New World army ant species across the Americas (ŁUKASIK & al. 2017). Our experimental setup, with all species originating from the same location, allowed us to separate the effects of phylogenetic relationship from the effect of geographic locations. As expected, we found a relatively homogeneous community of gut microbes, which can be explained by the dominance of host generalists accompanied with host co-occurrence at a single site. A few UE and UF OTUs were nonetheless differentially distributed across *Eciton* species, supporting previous results, and expanding the finding of such differences even in sympatric, closely related species that form a single community. Overall, these findings support the hypothesis of the specialization and stability between army ants and both bacterial phylotypes (UE & UF), which seems to have originated well before the diversification of the genus *Eciton* and perhaps all Dorylinae (ŁUKASIK & al. 2017). Despite this, there does not appear to be a relationship between the phylogeny of *Eciton* ants and the distribution of their symbionts, indicating that other factors are likely to be determining the observed patterns of specificity (Fig. 1A). However, to conclusively resolve the phylogenetic and evolutionary relationships between army ants and their microbial symbionts it is critical to sequence and characterize their genomes.

Bacterial localization in the *E. burchellii* digestive tract: The combination of different microscopic techniques allowed us to detect and localize intestinal bacteria. By using FISH microscopy with specific probes, we confirmed the presence of the most abundant OTUs detected in our culture-independent analysis inside *Eciton* ant guts: UE and UF. The morphology and size of the bacteria detected by FISH is consistent with observations made by electron microscopy: UE cells have a coccoid shape, like bacteria (0.20 µm to 0.70 µm) observed with SEM; UF cells are bacilliform, like the intracellular bacteria detected by TEM (0.60 µm to 1.00 µm).

The intestinal bacteria in *E. burchellii* observed by SEM and TEM are compartmentalized in certain regions of the gut, such as hindgut cavities or near midgut microvilli. The bacterial community of *Cephalotes* ants has been studied in detail in recent years. These ants feed mainly on extrafloral nectar, fungi, pollen, and their guts are divided into sections: the foregut (esophagus and crop), the midgut, and the hindgut (ileum and rectum). It has been shown that these sections present anatomical and physiological differences that influence the composition and abundance of the microbial communities, that contribute to the function of each section in the digestion process (Lanan & al. 2016, Flynn & al. 2021). Furthermore, employing TEM, we observed unknown intracellular bacteria near the microvilli in healthy ants, which reinforces the argument that they do not have a direct pathological impact on the health of their host (SAPOUNTZIS & al. 2015), and their function could be related to nutrition.

Regarding the potential role of the two dominant phylotypes detected in army ant digestive tracts, bacteria in the order *Entomoplasmatales* (Mollicutes) have been reported also as one of the dominant groups in the gut community of another Neotropical ant genus, *Acromyrmex*, where they are described as cocci with an approximate diameter of 0.7 µm (SAPOUNTZIS & al. 2015), which is consistent with our findings in army ants. However, in *Acromyrmex* ants, these microorganisms are found extracellularly as well as intracellularly in the fat body tissues, with the presence of an extra plasma membrane that helps them survive in the host's cytoplasm (SAPOUNTZIS & al. 2015, SAPOUNTZIS & al. 2018, SAPOUNTZIS & al. 2019). In contrast, in army ants, these bacteria appear to live in the gut lumen, specifically within the pylorus and ileum (ŁUKASIK & al. 2017). On the other hand, Firmicutes bacteria have been widely reported in insect gut microbiomes (YUN & al. 2014, MOREAU & RUBIN 2017, GONZÁLEZ-ESCOBAR & al. 2018) and some of them share morphology and size range with UF (TEGTMEIER & al. 2016, AUDISIO &. al 2011).

Different clades of ants, and especially other insects, differ dramatically in the abundance, taxonomic and functional diversity of their microbiomes (SANDERS & al. 2017). Many ant clades host very low bacterial numbers, while the microbial communities of others are dominated by nutritional or facultative endosymbionts. Two clades of Hymenoptera whose microbiomes have been characterized quite comprehensively, herbivorous *Cephalotes* ants and social bees, host abundant, specialized, socially transmitted gut microbiota comprising multiple bacterial taxa and diverse strains, an association dating back at least 46 million years in *Cephalotes* ants (HU & *a*l. 2018) and about 80 million years in social bees (KWONG & *a*l. 2017). In contrast, the microbiome of army ants comprises fewer microbial clades, and given dietary differences, likely fulfills different functions. As noted by ŁUKASIK et al (2017), another interesting difference among these hymenopteran insects is how strain-level diversity is distributed: in *Eciton* species, the pool of strains present at the colony level is partitioned so that different workers tend to harbor distinct strains each, whereas in bees, many distinct strains are present in each worker. Therefore, more systematic research on other ants and other social insects is required to elucidate the role of colony characteristics (size, nutrition, dependent or independent colony founding) in the composition of the microbial community across and within species, populations, and colonies. However, to address these questions, we require studies including phylogenetic markers with higher taxonomic resolution than the short fragments of the conserved 16S rRNA gene. Simultaneously, we should address another critical question: how the genotype-level differences among symbionts correspond to functions, and ultimately, what the role of the microbiome in the biology of the hosts is.

In the case of *Eciton* ants, these two categories of information can be provided by the rapidly improving 'omics' approaches, specifically, metagenomics and metatranscriptomics, coupled with cultivation efforts of UF and UE bacteria to sequence and annotate their genomes to characterize their metabolic potential and thus identify their specific functions within the host. This strategy has been applied successfully in turtle ants (CHANSON & al. 2021), fungus-growing ants (SAPOUNTZIS & al. 2015, SAPOUNTZIS & al. 2018, SAPOUNTZIS & al. 2019) and honeybees (ZHENG & al. 2016), and thus has the potential to allow us to fully understand the biological importance and the evolution of the microbiota associated with army ants and how it influences the host.

Conclusions: In the present work we combined microbiome composition analyses with imaging techniques to supplement information on host-microbe associations in army ants. Our results confirmed that gut communities of the army ants are composed of few bacterial OTUs and are similar between the sympatric species studied, as well as between *E. burchellii* worker castes and males, with a clear dominance of two bacterial phylotypes: UF and UE. Microscopy techniques confirmed our sequencing results, demonstrating the presence of UF and UE bacteria in *E. burchellii* guts. Our community-based

evaluation of host-microbe associations unveiled a low degree of specificity, with only a few bacterial OTUs showing clear host preferences. Possible functions of these microorganisms in army ant guts remain unknown and offer plenty of opportunities for future work.

Acknowledgments

Collecting permits were granted by the "Comisión Institucional de Biodiversidad" (Institutional Biodiversity Committee, University of Costa Rica; Resolution Number 023, 048 and 094); and authorized by La Selva Biological Station. We are grateful to the staff of the Research Center for Microscopic Structures (CIEMic) of the University of Costa Rica (UCR), for their support in electron microscopy, especially to Manuel Jiménez. Finally, we thank Dr. Dagmar Woekben, Dr. Max Nepel and Florian Strasser for their assistance implementing FISH techniques at UCR. A.B.F.I. was supported by the European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement 624145. This research was supported by University of Costa Rica projects 810-B3-273 and 810-C1-080. We thank the staff members of La Selva Biological Station for their generous support during field work.

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Table and figure captions

Fig.1: OTU abundance and host specificity. (A) Heatmap showing the abundance of OTUs in each colony (x-axis). Order of x-axis is based on phylogenetic relationships between *Eciton* species, which was recovered from Winston & al. 2016. (B) *Eciton* army ant-gut microbe network visualizing the host specificity of gut microbes. Link widths correspond to the number of times an OTU was detected in different army ant colonies. Link colors depict the microbes' species-level specificity measured as Kullback-Leibler distance (d'). Abbreviations: Ac: *Acinetobacter*; CT: *Tokpelaia*; EB: *E. burchellii*; ED: *E. dulcium*; EH: *E. hamatum*; EL: *E. lucanoides*; EM: *E. mexicanum*; En: Enterobacteriaceae; EV: *E. vagans*; RA: Relative abundance; Se: *Serratia;* UE: Unclassified Entomoplasmatales; UF: Unclassified Firmicutes; We: *Weissella*.

Fig. 2: OTU abundance in digestive tracts of *E. burchellii* worker castes and males. (A) Heatmap showing the abundance of different OTUs in each worker caste and male sample. (B) *E. burchellii* caste-gut microbe network visualizing the host specificity of gut microbes. Link widths correspond to the number of times an OTU was detected in different army ant castes. Abbreviations: Ac: *Acinetobacter*, Bur: Burkholderiaceae, Ce: *Cedecea*, Cl: *Cloacibacterium*, Em: *Empedobacter*, En: Enterobacteriaceae, Ent: *Enterococcus*, Mi: Micrococcales, RA: Relative abundance; Rho: *Rhodanobacter*, UE: Unclassified Entomoplasmatales, UF: Unclassified Firmicutes, We: *Weissella*. Link colors depict the microbes' species-level specificity measured as Kullback-Leibler distance (d'). Images of *E. burchellii* workers and male were taken from (KRONAUER, 2020).

Fig. 3: Alpha and beta diversity plots of the different *Eciton* species. A) Non-multimetric dimensional scaling (NMDS) plot based on a Bray Curtis dissimilarity matrix of the gut microbiome of different *Eciton* species. Each point represents the gut microbiome of an individual sampled, and the color represents the species to which it belongs. There is a significant difference in the composition of microbial community between the species (ANOSIM, $p<0.001$, $R=0.447$; PERMANOVA, $F=5.235$, $p<0.001$; stress=0.224). The stress value obtained does not clearly reflect in 2D the significant differences observed between the *Eciton* species 2D. B) Barplots showing the number of OTUs observed (*S*), and the exponential form of Shannon diversity (*eH*) and their standard deviation. *S* and *eH* are values obtained from 100 rarefied networks. Order of x-axis is based on phylogenetic relationships between *Eciton* species, which was recovered from Winston & al. 2016.

Fig. 4: Alpha and beta diversity plots of different castes and sexes of *E. burchellii*. A) Non-multimetric dimensional scaling (NMDS) based on a Bray Curtis dissimilarity matrix of the gut microbiome of different castes and sexes of *E. burchellii*. Each point represents the gut microbiome of an individual sampled, and the color represents its caste. There is no significant difference in the composition of microbial communities between the different castes and sexes (ANOSIM, $p=0.257$, $R=0.040$; PERMANOVA, $F=0.930$, $p=0.551$; stress=0.176). B) Barplots showing the number of OTUs observed (S), and the exponential form of Shannon diversity (*eH*) and their standard deviation. S and *eH* are values obtained from 100 rarefied networks.

Fig. 5: **Localization of bacteria inside the gut of *Eciton. burchellii* media workers.** A) Ultrastructure of cavities in the hindgut. B) Close-up of a cavity pictured in A, showing a possible biofilm formation with the presence of coccoid-shaped bacteria. C) Ultrastructure of the hindgut showing biofilm formations (arrows). D) Close view of biofilm formation near gut microvilli. E) Bacteria fission (arrow). Filtered bacterial cells from ant worker digestive tracts employing F) a universal probe for bacteria (red-EUB338+EUB897) and DAPI (blue), G) a specific probe for Unclassified Firmicutes bacteria (orange-

UNF16SF1) and DAPI (blue) and H) a specific probe for Unclassified Entomoplasmatales (green- cute493R) and DAPI (blue).

Supplementary figure and tables captions

Fig. S1: Bar plot showing the abundance of OTUs in each colony (x-axis). Order of x-axis is based on phylogenetic relationships between *Eciton* species. Order of x-axis is based on phylogenetic relationships between Eciton species, which was recovered from Winston & al. 2016. Abbreviations: Ac: *Acinetobacter*; CT: *Tokpelaia*; EB: *E. burchellii*; ED: E. dulcium; EH: *E. hamatum*; EL: *E. lucanoides*; EM: *E. mexicanum*; EV: *E. vagans*. Se: *Serratia*; UE: Unclassified Entomoplasmatales; UF: Unclassified Firmicutes; We: *Weissella.*

Fig. S2: Negative controls for symbiont-specific probes. A) Negative control (Cy5- UNF16SF1 without competitors, color: orange) and DAPI (blue). B) Negative control (Cy5- cute493R without competitors, green) and DAPI (blue).

Tab. S1: Specimen collection data for *Eciton* species and *E. burchellii* worker castes and males employed in this study.

Tab. S2: Total, average, minimum and maximum number of sequences across *Eciton* species.

Tab. S3: Number of sequences recovered from *E. burchellii* worker castes and male samples.

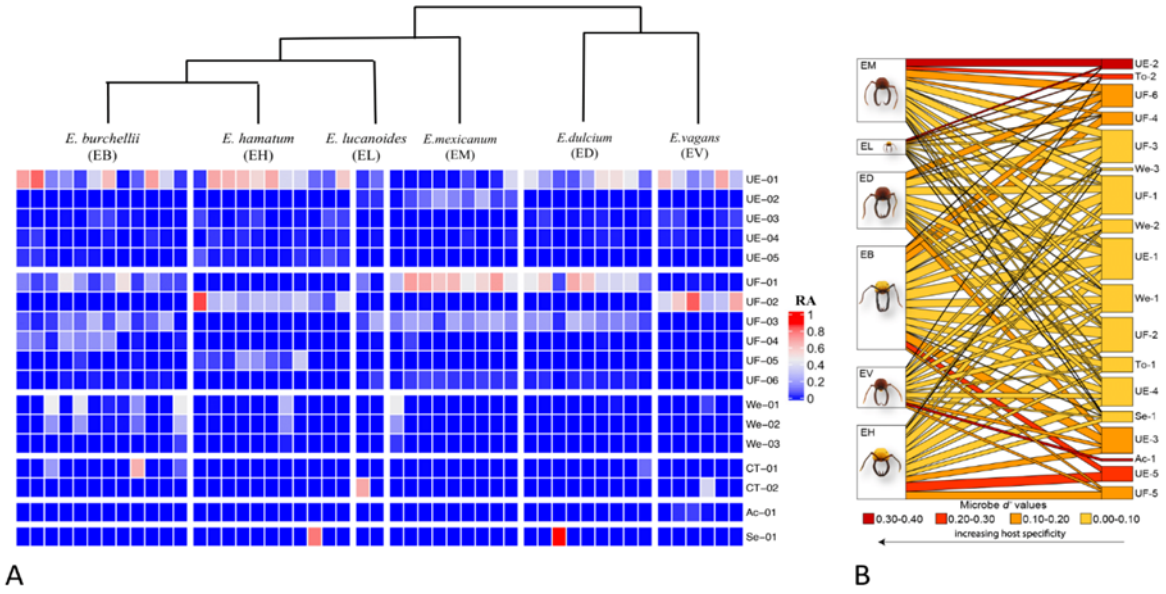
Tab. S4: Diversity metrics for each *Eciton* species. Incidences represent the number of times we found bacterial OTUs in army ant species, *S* represents the number of OTUs observed (species richness) and *eH* represents the exponential form of Shannon diversity. S_{rare} and eH_{rare} are values obtained from 100 rarefied networks.

Tab. S5: Diversity metrics for each *E. burchellii* caste and males. Incidences represent the number of times we found bacterial OTUs in each sample type, *S* represents the number of OTUs observed (species richness), and *eH* represents the exponential form of Shannon diversity. S_{rare} and eH_{rare} are values obtained from 100 rarefied networks.

Tab. S6: Operational Taxonomic Units (OTUs) sequences, taxonomy and relative abundance detected across all samples within the six *Eciton* species dataset.

Tab. S7: Operational Taxonomic Units (OTUs) sequences, taxonomy and relative abundance detected across all samples within the *Eciton burchellii* castes and male's dataset.

884 Fig 1:


E. burchellii (EB)
E. hamatum (EH)
E. lucanoides (EL)
E.mexicanum (EM)
E.dulcium (ED)
E.vagans (EV)
UE-01
UE-02
UE-03
UE-04
UE-05
UF-01
UF-02
UF-03
UF-04
UF-05
UF-06
We-01
We-02
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Ac-01
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UF-6
UF-4
UF-3
We-3
UF-1
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UF-2
To-1
UE-4
Se-1
UE-3
Ac-1
UE-5
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Microbe d' values
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increasing host specificity
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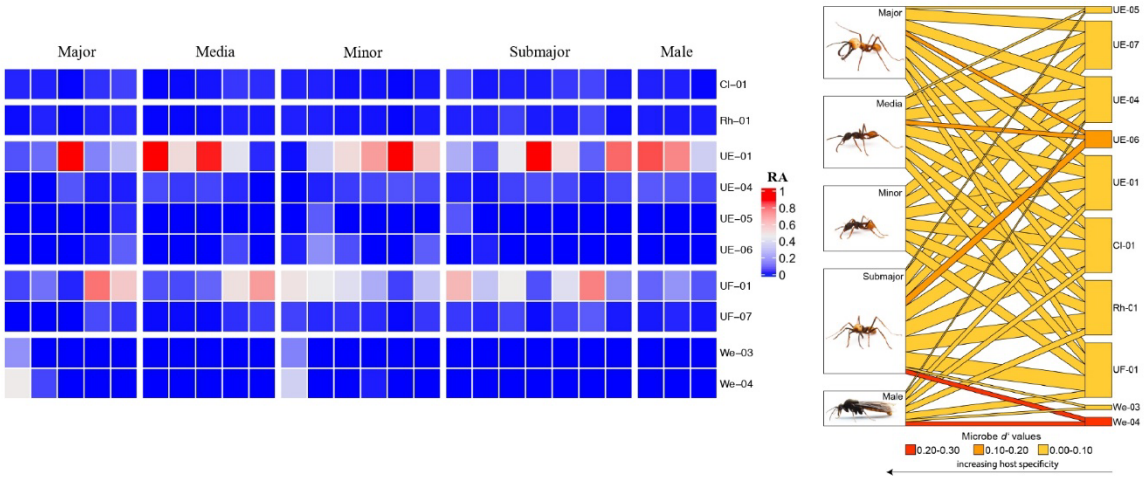

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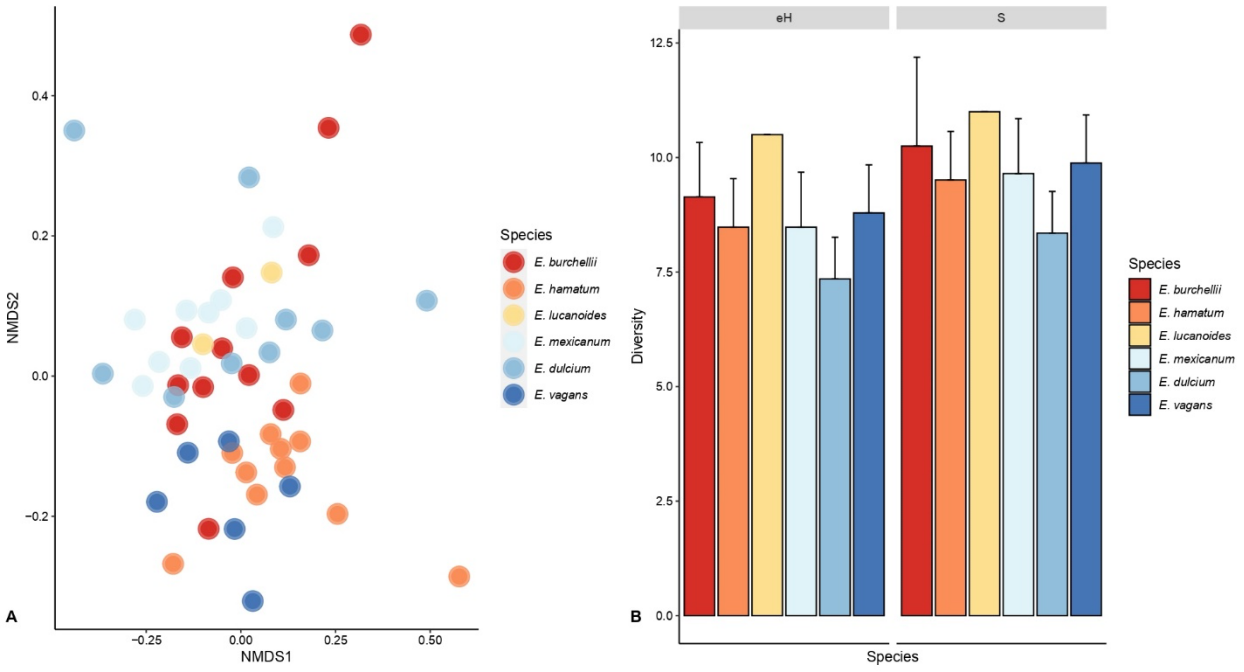

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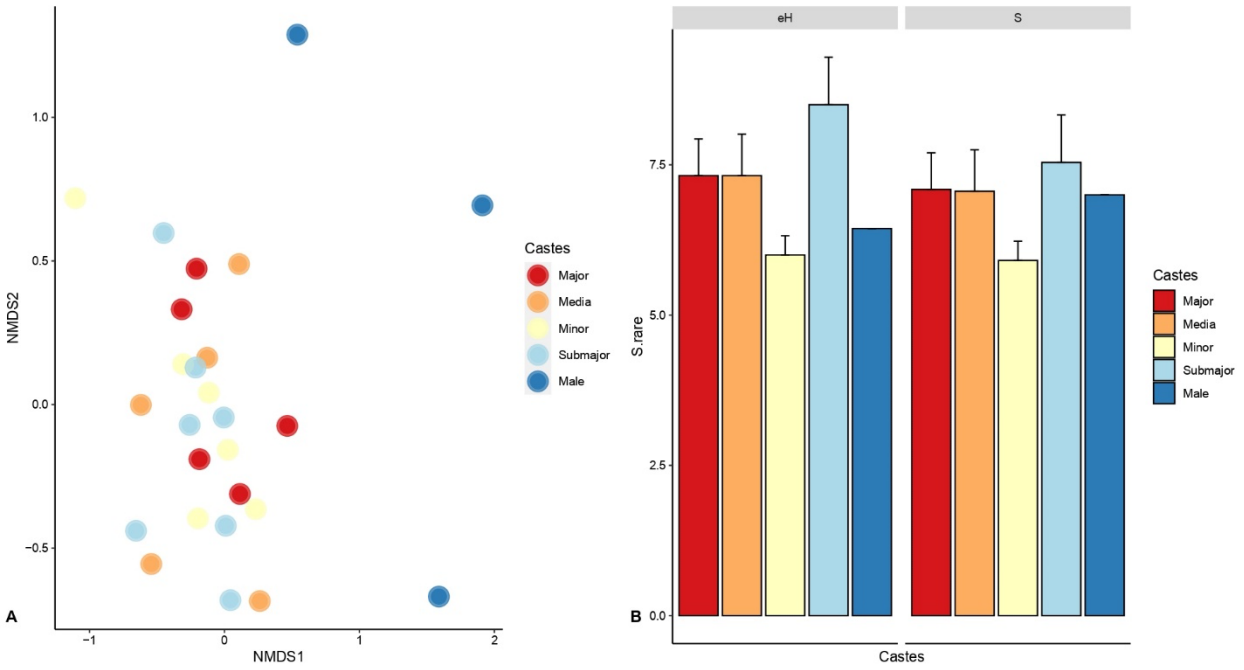

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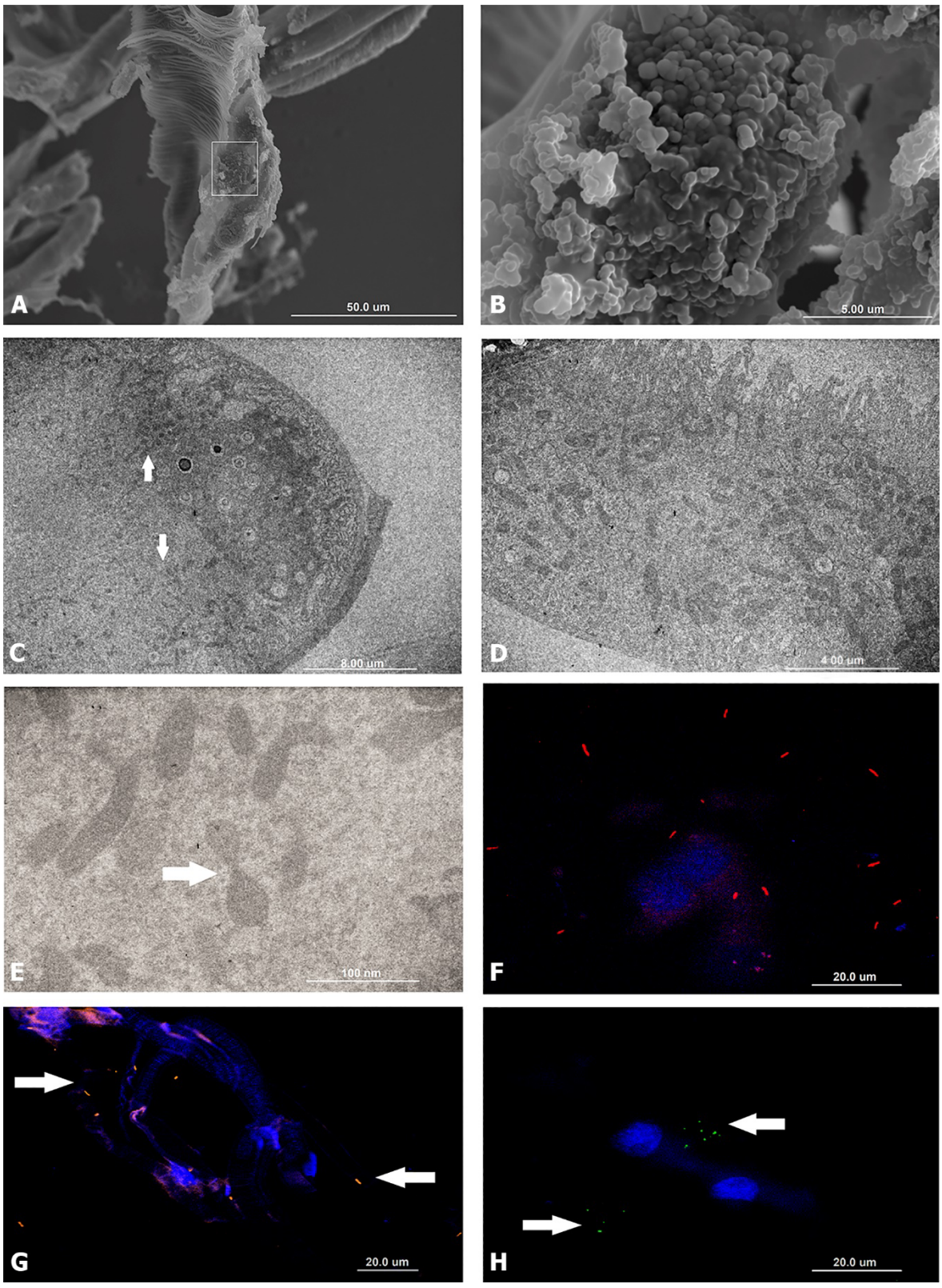

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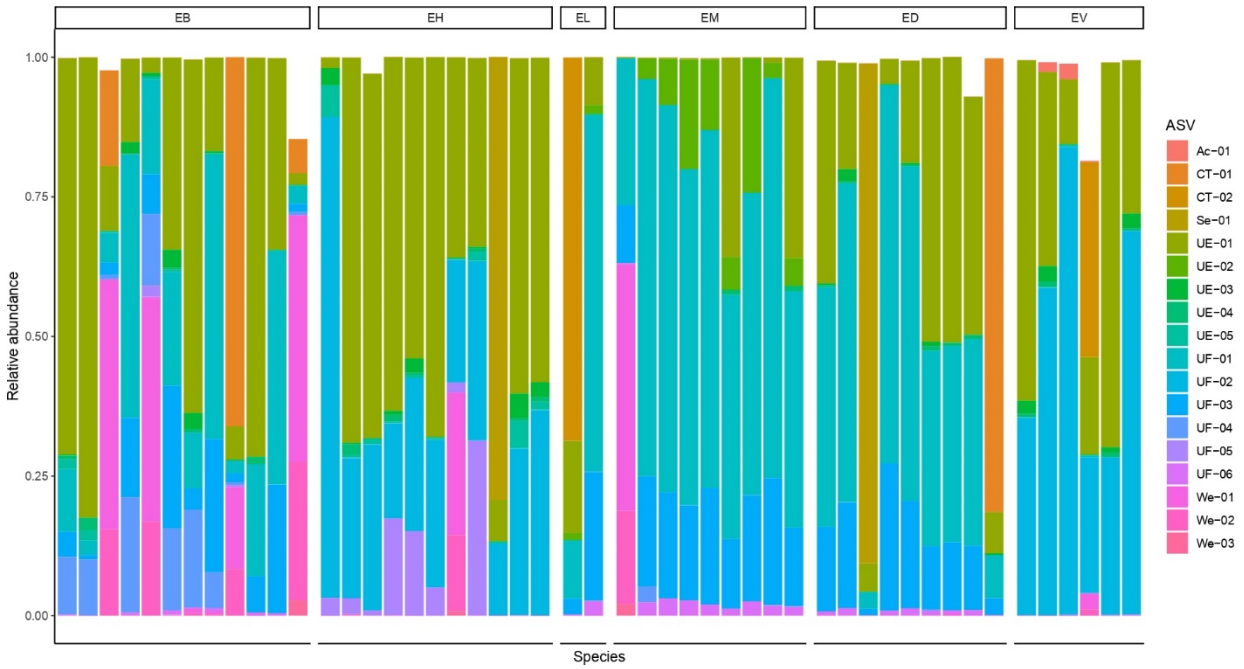

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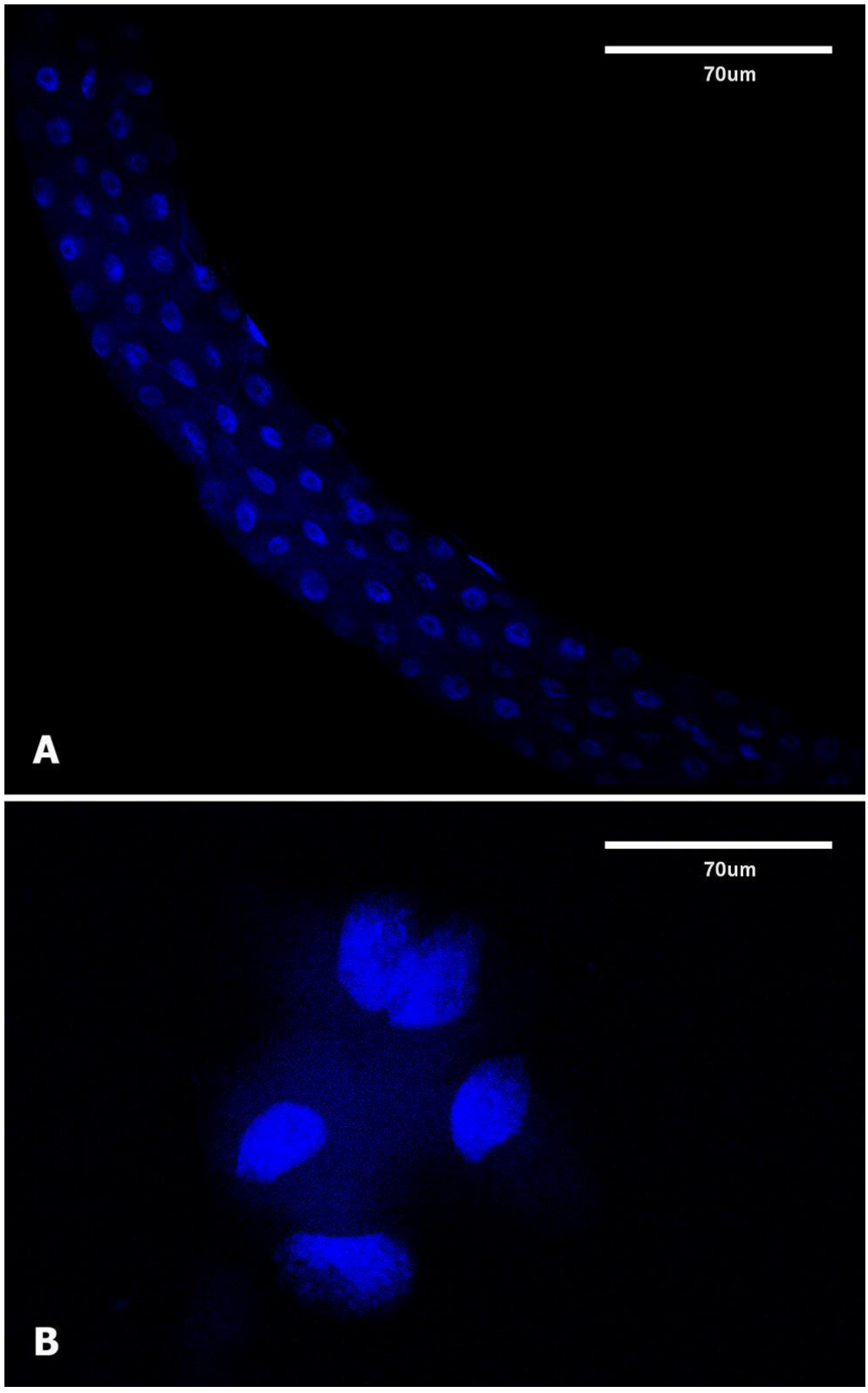

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905 Tab. S1:

ID	Specie	Sample	Location	Date
EB1	*E. burchellii*	Pooled adult workers	Emigration	March, 2013
EB3	*E. burchellii*	Pooled adult workers	Emigration	February, 2013
EB4	*E. burchellii*	Pooled adult workers	Bivouac collection	February, 2013
EB5	*E. burchellii*	Pooled adult workers	Emigration	March, 2013
EB6	*E. burchellii*	Pooled adult workers	Emigration	March, 2013
EB7	*E. burchellii*	Pooled callows	Emigration	March, 2013
EB8	*E. burchellii*	Pooled adult workers	Emigration	March, 2013
EB9	*E. burchellii*	Pooled adult workers	Refuse deposit	April, 2013
EB10	*E. burchellii*	Pooled callows	Emigration	March, 2013
EB11	*E. burchellii*	Pooled adult workers	Refuse deposit	April, 2013
EB16	*E. burchellii*	Pooled adult workers	Emigration	February, 2014
EB17	*E. burchellii*	Pooled adult workers	Emigration	April, 2014
ED1	*E. dulcium*	Pooled adult workers	Emigration	February, 2013
ED3	*E. dulcium*	Pooled adult workers	Emigration	March, 2013
ED4	*E. dulcium*	Pooled adult workers	Emigration	March, 2013
ED5	*E. dulcium*	Pooled adult workers	Emigration	April, 2013
ED6	*E. dulcium*	Pooled adult workers	Emigration	April, 2014
ED8	*E. dulcium*	Pooled adult workers	Emigration	March, 2014
ED9	*E. dulcium*	Pooled adult workers	Emigration	April, 2014
ED10	*E. dulcium*	Pooled adult workers	Emigration	April, 2014
ED11	*E. dulcium*	Pooled adult workers	Emigration	April, 2014
EH3	*E. hamatum*	Pooled adult workers	Emigration	February, 2013
EH4	*E. hamatum*	Pooled adult workers	Emigration	March, 2013
EH5	*E. hamatum*	Pooled adult workers	Raiding	March, 2013
EH6	*E. hamatum*	Pooled adult workers	Emigration	March, 2013
EH7	*E. hamatum*	Pooled adult workers	Emigration	March, 2013
EH8	*E. hamatum*	Pooled adult workers	Emigration	March, 2013
EH9	*E. hamatum*	Pooled adult workers	Emigration	April, 2013
EH10	*E. hamatum*	Pooled adult workers	Emigration	April, 2013
EH11	*E. hamatum*	Pooled adult workers	Raiding	March, 2014
EH12	*E. hamatum*	Pooled adult workers	Emigration	March, 2014
EH14	*E. hamatum*	Pooled adult workers	Emigration	April, 2014
EL1	*E. lucanoides*	Pooled adult workers	Emigration	February, 2013
EL3	*E. lucanoides*	Pooled adult workers	Raiding	April, 2014
EM4	*E. mexicanum*	Pooled adult workers	Emigration	March, 2013
EM6	*E. mexicanum*	Pooled adult workers	Emigration	March, 2014
EM7	*E. mexicanum*	Pooled adult workers	Emigration	March, 2014
EM8	*E. mexicanum*	Pooled adult workers	Emigration	March, 2014
EM9	*E. mexicanum*	Pooled adult workers	Emigration	May, 2014
EM10	*E. mexicanum*	Pooled adult workers	Emigration	April, 2014

EM11	*E. mexicanum*	Pooled adult workers	Emigration	April, 2014
EM12	*E. mexicanum*	Pooled adult workers	Emigration	April, 2014
EM13	*E. mexicanum*	Pooled adult workers	Emigration	April, 2014
EV1	*E. vagans*	Pooled adult workers	Emigration	February, 2013
EV3	*E. vagans*	Pooled adult workers	Emigration	March, 2013
EV5	*E. vagans*	Pooled adult workers	Emigration	March, 2013
EV6	*E. vagans*	Pooled adult workers	Emigration	April, 2014
EV7	*E. vagans*	Pooled adult workers	Emigration	April, 2014
EV8	*E. vagans*	Pooled adult workers	Emigration	April, 2014
EB16_Major1	*E. burchellii*	Major	Emigration	February, 2014
EB16_Major2	*E. burchellii*	Major	Emigration	February, 2014
EB16_Media2	*E. burchellii*	Media	Emigration	February, 2014
EB16_Media3	*E. burchellii*	Media	Emigration	February, 2014
EB16_Minor1	*E. burchellii*	Minor	Emigration	February, 2014
EB16_Minor2	*E. burchellii*	Minor	Emigration	February, 2014
EB16_Minor3	*E. burchellii*	Minor	Emigration	February, 2014
EB16_Submajor1	*E. burchellii*	Submajor	Emigration	February, 2014
EB16_Submajor2	*E. burchellii*	Submajor	Emigration	February, 2014
EB16_Submajor3	*E. burchellii*	Submajor	Emigration	February, 2014
EB16_Submajor4	*E. burchellii*	Submajor	Emigration	February, 2014
EB4_Major1	*E. burchellii*	Major	Bivouac collection	February, 2013
EB4_Major2	*E. burchellii*	Major	Bivouac collection	February, 2013
EB4_Major3	*E. burchellii*	Major	Bivouac collection	February, 2013
EB4_Male1	*E. burchellii*	Male	Bivouac collection	February, 2013
EB4_Male2	*E. burchellii*	Male	Bivouac collection	February, 2013
EB4_Male3	*E. burchellii*	Male	Bivouac collection	February, 2013
EB4_Media1	*E. burchellii*	Media	Bivouac collection	February, 2013
EB4_Media2	*E. burchellii*	Media	Bivouac collection	February, 2013
EB4_Media3	*E. burchellii*	Media	Bivouac collection	February, 2013
EB4_Minor1	*E. burchellii*	Minor	Bivouac collection	February, 2013
EB4_Minor2	*E. burchellii*	Minor	Bivouac collection	February, 2013
EB4_Minor3	*E. burchellii*	Minor	Bivouac collection	February, 2013
EB4_Submajor1	*E. burchellii*	Submajor	Bivouac collection	February, 2013
EB4_Submajor2	*E. burchellii*	Submajor	Bivouac collection	February, 2013
EB4_Submajor3	*E. burchellii*	Submajor	Bivouac collection	February, 2013

906

907 Tab. S2:

Species	Number of samples	Total number of sequences	Median of sequences	Minimum of sequences	Maximum of sequences
E. burchellii	12	1,507,858	44,457	44,202	541,285
E. dulcium	9	742,620	65,332	35,887	244,025
E. hamatum	11	1,118,479	84,414	3,134	185,609
E. lucanoides	2	163,007	81,504	47,861	115,146
E. mexicanum	9	1,559,332	104,191	26,328	474,935
E. vagans	6	1,701,889	99,020	33,762	1,117,978

908

909 Tab. S3:

Castes	Number of samples	Number of reads	Median of reads	Minimum of reads	Maximum of reads
Minor	6	228,230	30,511	15,962	70,093
Media	5	260,917	43,621	12,344	102,894
Submajor	7	513,360	62,666	12,719	146,628
Major	5	477,851	74,836	40,937	185,448
Male	3	141,471	20,596	14,490	106,385

910

911 Tab. S4:

Species	**Incidences**	***S***	***S* rare ± SD**	e^H	e^H **rare ± SD**
E. burchellii	123	16	10.25±1.94	13.48	9.14±1.19
E. dulcium	67	11	8,35±0.91	9.46	7.35±0.91
E. hamatum	88	13	9.51±1.06	11.40	8.48±1.06
E. lucanoides	18	11	11.00±0.00	10.50	10.50±0.000
E. mexicanum	75	14	9.65±1.20	8.48	8.48±1.20
E. vagans	48	13	9.88±1.05	8.79	8.79±1.05

912

913 Tab. S5:

Castes	**Incidences**	***S***	***S* rare ± SD**	e^H	e^H **rare ± SD**
Male	16	7	7.00±0.00	6.44	6.44±0.00
Major	33	8	7.09±0.61	7.32	6.38±0.61
Submajor	48	10	7.54±0.79	8.50	6.73±0.79
Media	33	8	7.06±0.69	7.32	6.36±0.69
Minor	30	6	5.91±0.32	6.00	5.47±0.32

914

915 "Appendix, as digital supplementary material to this article, at the journal's web pages"

916 Tab. S6: and Tab. S7 are in the attached excel file (Mendoza-Guido Tab S6-Tab S7).