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HOST MICROBE INTERACTIONS

Colonization by the Red Imported Fire Ant, *Solenopsis invicta*, Modifies Soil Bacterial Communities

Nicholas V. Travanty[1] · Edward L. Vargo[2] · Charles S. Apperson[1,3] · Loganathan Ponnusamy[1,3]

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Abstract

The long-standing association between insects and microorganisms has been especially crucial to the evolutionary and ecological success of social insect groups. Notably, research on the interaction of the two social forms (monogyne and polygyne) of the red imported fire ant (RIFA), *Solenopsis invicta* Buren, with microbes in its soil habitat is presently limited. In this study, we characterized bacterial microbiomes associated with RIFA nest soils and native (RIFA-negative) soils to better understand the effects of colonization of RIFA on soil microbial communities. Bacterial community fingerprints of 16S rRNA amplicons using denaturing gradient gel electrophoresis revealed significant differences in the structure of the bacterial communities between RIFA-positive and RIFA-negative soils at 0 and 10 cm depths. Illumina sequencing of 16S rRNA amplicons provided fine-scale analysis to test for effects of RIFA colonization, RIFA social form, and soil depth on the composition of the bacterial microbiomes of the soil and RIFA workers. Our results showed the bacterial community structure of RIFA-colonized soils to be significantly different from native soil communities and to evidence elevated abundances of several taxa, including *Actinobacteria*. Colony social form was not found to be a significant factor in nest or RIFA worker microbiome compositions. RIFA workers and nest soils were determined to have markedly different bacterial communities, with RIFA worker microbiomes being characterized by high abundances of a *Bartonella*-like endosymbiont and Entomoplasmataceae. Cloning and sequencing of the 16S rRNA gene revealed the *Bartonella* sp. to be a novel bacterium.

Keywords *Solenopsis invicta* · Bacterial community · 16S rDNA · Soil microbiome

Introduction

It is increasingly recognized that insects have close associations with microorganisms. Microorganisms can vary from beneficial symbionts to pathogens within the diverse microbial community residing in a particular habitat or host [1, 2]. The structure and species composition of host-associated bacterial communities can substantially influence the ecology and evolution of host species populations [3–5]. In turn, the activity of host populations can alter the organization of associated bacterial communities. For example, in termite nest mounds, termite activity alters nutrient availability and soil pH, which affects soil bacterial community structure [6]. Compared to native soils, community structure in termite mounds is characterized by decreased abundances of Firmicutes and increased abundances of Actinobacteria [6–8], and an overall lower community diversity [9]. The relatively stable conditions within termite mounds (temperature and humidity) are also considered factors that affect the bacterial community structure of nest soil [9].

For other social insects, recent studies have also demonstrated that ant colonies can alter soil microbial communities and modify their composition at the scale of individual nests [10, 11]. Little is known, however, about the bacterial microbiome of ant colonies and their nest soils for invasive species, such as the red imported fire ant (RIFA), *Solenopsis invicta* Buren. RIFA is an invasive species that arrived in the USA in the early twentieth century [12]. Because this species can out-compete native ant species for food resources [13], and natural enemies of this species are scarce in North

✉ Loganathan Ponnusamy
lponnus@ncsu.edu

[1] Department of Entomology and Plant Pathology, North Carolina State University, NC 27695 Raleigh, USA

[2] Department of Entomology, Texas A&M University, College Station, TX 77843, USA

[3] Comparative Medicine Institute, North Carolina State University, Raleigh, NC 27695, USA

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America [14], RIFA populations have thrived and become established throughout the southern United States [15]. Colonies of invasive RIFA have two distinct social forms: monogyne colonies each organized around a single queen, and polygyne colonies in which several queens cooperate. Monogyne RIFA are highly territorial and aggressive toward non-nestmates, and polygyne RIFA are defined by sharing resources intercolonially and being less antagonistic with other polygyne RIFA [16]. Polygyne RIFA carry a chromosome with a large inverted region that prevents recombination and therefore accumulates mutations distinct from the monogyne RIFA genome [17]. The presence of this genetic element determines the social genotype. Among the genes encoded in this chromosomal region is the *Gp-9* gene which encodes an odorant-binding protein associated with chemical communication [18]. Monogyne ants are homozygous for the social *B* allele (*BB*) at the *Gp-9* locus, and polygyne ants are heterozygous *Bb* [17].

The process of RIFA nest construction involves physical disruption of the soil in which worker ants establish networks of subterranean tunnels that radiate from a central nest. The excavated soil is deposited on the ground surface creating a characteristic tumulus of the ant nest [19]. Comparatively, monogyne RIFA colonies each occupy a single large mound, while polygyne RIFA colonies regularly consist of several smaller mounds connected by tunnels to one another. Nest soils are exposed to a suite of chemicals that are released by the RIFA occupying the soil, including several compounds that have anti-microbial activity[20]. To inhibit microbial growth, anti-microbial venom is released by workers onto brood and the nest substrate [21]. Metapleural gland secretions deposited by worker ants also inhibit development of microbial fauna [22]. Physical disruption caused by nest building, dissemination of anti-microbial compounds, deposition of waste materials, and introduction of foraging and exogenous resources into the soil environment, taken together, are likely to alter microbial communities of native soils. We expected the diversity of bacterial communities between polygyne nests (that share resources) to be relatively similar compared to the bacterial communities of monogyne RIFA (that do not share resources).

Pyrosequencing of microbial marker genes has been used to characterize bacterial community composition of the RIFA and their colony soils [23, 24], and culture-based approaches have been used to characterize bacteria associated with RIFA colony soils [25]. However, it is likely that previous culture-based studies significantly underrepresented the total bacterial diversity. In fact, an estimated less than 2% of bacteria in environmental samples are culturable, resulting in a significant underreporting of total diversity [26, 27]. Also, more current next-generation sequencing (NGS) methods, such as Illumina technology, generates a greater number of reads than pyrosequencing, which allows for a more robust and representative bacterial profiling [28]. In this study, we use both denaturing gradient gel electrophoresis (DGGE) and Illumina MiSeq NGS to analyze 16S rRNA bacterial community amplicons and investigate bacterial community structure dependencies on RIFA colonization and social form. DGGE was used in preliminary analyses to evaluate the variability of bacterial community structure within and between RIFA colonies of both social forms. Because DGGE is a comparatively coarse scale analytical method, we followed these preliminary experiments with NGS to gain a deeper understanding of bacterial species diversity across some of the same soil samples.

Material and Methods

Soil and Ant Collections

Collection of soils from RIFA nests and adjacent RIFA-negative soils were made in Raleigh, NC. Two primary areas were targeted including North Carolina State University Centennial Campus (CC), and at the Lake Wheeler Agricultural Research Station (LW). Additional collections were made at a third site within the Carl Alwin Schenck Memorial Forest (SF). GPS coordinates of these collection areas are given in **Table S1**. At each active RIFA nest, mound soil and the adjacent RIFA-negative soil were collected separately. The term "collection site," hereafter, refers to the ant nest site from which these paired soil collections were made. All collections were made between August and October 2015 on days in which precipitation did not occur for the previous 72 h. Collections within each area (CC, LW, or SF) were made within 13 days of each other.

A galvanized steel cylindrical soil corer (45 cm length, 3.2 cm diameter, fabricated from electrical conduit) was inserted into the soil, driven vertically to a depth of 30 cm, and then extracted. From the extracted core, samples of ant-free soil (each *ca*. 5 g) were removed through a longitudinal corer window at depths of 0, 10, and 20 cm using a sterilized stainless-steel spatula. Soil samples from active nests (RIFA-positive) were collected with the corer insertion point positioned at one-half of the height of the mound on the side of the tumulus. For RIFA-negative soil collections, corers were inserted directly into the ground surface approximately 1 m from the ant mound. Corer samples of both RIFA-positive and RIFA-negative soil were taken in triplicate from each collection site (Fig. 1). Between each sampling, the corer was brushed to remove soil residue, washed with 2% bleach, and then rinsed several times with sterile distilled water, and the spatula used to remove soil from the corer was sterilized in the same manner. Each soil sample was transferred to a sterile 50 ml Falcon tube (Becton, Franklin Lakes, NJ) and immediately placed on ice. Additionally, RIFA workers were collected from each nest by flicking ants off of the spatula into sterile

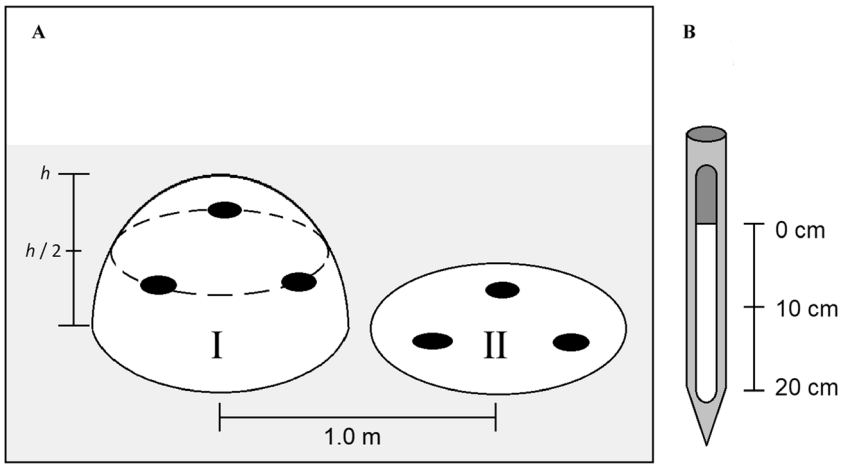


Fig. 1: **A** Diagram of soil collection from (I) RIFA mound and (II) RIFA-negative soil, insertion point of corer shown in triplicate. **B** Corer containing extracted soil sampled at the indicated depths

Falcon tubes. Capped tubes were kept on ice while transferred from the field to the laboratory. Within 2 h of collection, soil and RIFA worker samples were processed in the lab. Soil samples were stored at −80 °C, and RIFA samples were transferred to sterile 1.5 ml centrifuge tubes containing 95% ethanol prior to storage at −80 °C.

DNA Extraction and Identification of the Social Form of Fire Ants

Groups of approximately 15 workers recovered from each RIFA nest were washed with 95% ethanol to remove any residual soil particles, then transferred to sterilized 2 ml conical microcentrifuge tubes (Fisher Scientific, Hampton, NH), each containing 12 sterilized 3 mm diameter glass beads (#11-312A, Fisher Scientific, Hampton, NH). RIFA were surface sterilized by immersion in 500 μl 1% bleach, shaking for 45 s, then rinsing 5 times with 500 μl of sterile 0.85% NaCl. The final rinse was retained for later verification of sterility by a polymerase chain reaction (PCR) assay (described below). Lysis buffer (200 μl) was added to each ant sample, which was homogenized in a FastPrep™ FP120 Cell Disruptor (Thermo Electron Corporation, Carlsbad, CA) (speed = 4.0, 30 s), and then DNA was extracted using methods of Smith et al. [29]. An additional DNA purification step was performed with the Wizard DNA clean-up Kit (Promega, Madison, WI) using the manufacturer's instructions. DNA purity and yield were measured by Nanodrop 1000 (Thermo Scientific, Waltham, MA). Extracted total genomic DNA (gDNA) concentrations were standardized to a range of 75 to 125 ng/μl.

Identification of RIFA social form was based on genotyping of *Gp-9* allele variants using primer pairs that target the $Gp\text{-}9^B$ and the $Gp\text{-}9^b$ alleles with the multiplex PCR assay of Valles and Porter [30]. PCR products were electrophoresed in 1.5% agarose gels followed by ethidium bromide staining. RIFA social form was identified by the presence of gel band (s) of the expected size. To confirm PCR results, a band resulting from an apparent monogyne sample and two bands from an apparent polygyne sample were excised from electrophoresed agarose gels, purified by QIAquick Gel Extraction Kit (Qiagen, Germantown, MD), and Sanger sequenced (Eton Bioscience, Morrisville, NC). The resulting sequences were compared with the GenBank database using the BLASTn program [31] to identify the closest related species. Sequences were deposited into GenBank (Accession nos. MT246862 to MT246864).

DNA Extraction from Soils

The phenol-chloroform DNA extraction method used was adapted from Smith et al. [29], which included alkalization of lysis buffers (to disrupt DNA binding to soil clay particles), removal of lysozyme from cell lysis (due to inactivity of lysozyme in basic conditions), and addition of sodium dodecyl sulfate (SDS) for cell lysis (to facilitate bacterial cell disruption). Approximately 100 mg of each soil sample was transferred to a separate sterilize conical 2 ml microcentrifuge tube, each containing 12 sterilized 3 mm glass beads (#11-312A, Fisher Scientific), 240 μl of cell lysis buffer 1 (modified by addition of 1*N* NaOH to pH = 12.0), 30 μl 20% (W/V) SDS (#L3771, Sigma, St. Louis, MO), and 30 μl (W/V) proteinase K (20 mg/ml). The samples were homogenized in a FastPrep™ FP120 Cell Disruptor (speed = 4.0, 30 s) and then incubated for 1 h at 37 °C. Cell lysis buffer 2 (modified by addition of 1*N* NaOH to pH = 12.0) was then added (300 μl per sample), and the samples were incubated for an additional 1 h at 56 °C. After cell lysis, DNA was extracted and purified using the methods described by Smith et al. [29]. Extracted DNA was further purified using the Wizard DNA clean-up system and OneStep™ PCR Inhibitor Removal Kit (#D6030, ZYMO, Irvine, CA), which is designed to remove humic acid and other compounds that can inhibit polymerase activity. DNA purity and yield were measured by Nanodrop

1000 and gDNA concentrations were then normalized to a range of 25 to 50 ng/μl. To assure that the gDNA extracted from soil samples did not result from contamination, for each round of soil extractions, a 100-mg sample of autoclaved sand was subjected to the same extraction procedures to serve as a negative control.

Testing Bacterial Community Variability Between Replicate Soil Samples and Between Soil Depths Using DGGE

Prior to testing our main hypotheses (effects of RIFA social form and RIFA-colonization on soil bacterial communities), preliminary DGGE assays were performed to (1) measure variation in bacterial community structure between replicate soil samples and (2) determine which soil depths showed significant contrasts in bacterial community structure between RIFA-colonized soils and native soils. Preliminary analyses included soil samples collected from six collection sites (see Table S1 for details) from the CT area (three monogyne and three polygyne nests at 0, 10, and 20 cm depths) for a total of 108 soil samples. Amplification of 16S rRNA gene fragments was performed by PCR using universal bacterial primers 357F-GC and 518R [32] with DNA extracted from soil samples. Touchdown PCR amplification, gradient gel casting, reference ladder composition, separation of amplicons by DGGE, gel image capture, and OTU detection followed the protocols of Ponnusamy et al. [33].

Based on the presence/absence and relative abundance of OTUs, weighted Bray-Curtis distances were calculated between samples using Community Analysis Package Software (CAPS) (ver. 3.1, Pieces Conservation Ltd., UK). A goodness-of-fit test (Shapiro-Wilk) showed that distance values were not normally distributed. Accordingly, nonparametric Kruskal-Wallis tests (α = 0.05) were performed to compare the mean distances. Goodness-of-fit and Kruskal-Wallis tests were performed using JMP® Pro software (ver. 14.1.0, SAS Institute Inc., Cary, NC). In brief, we performed tests (1) to compare distances between replicate samples to distances between samples from different collection sites and (2) to compare distances between replicate samples to distances between RIFA-positive and RIFA-negative soils from a collection site. See **Supplementary Materials and Methods** for details.

Bacterial 16S rRNA Gene Amplicon and Illumina Library Preparations

Soil samples included those from 11 monogyne and 11 polygyne nest collection sites, each including RIFA-positive soil and RIFA-negative soil at depths of 0 and 10 cm. 16S rRNA amplicons (described below) from the collection replicates at each soil depth were pooled for a total of 88 library samples. To characterize and compare the RIFA worker microbiome to soil microbial communities, RIFA workers were collected from one additional monogyne nest and one additional polygyne nest for a total of 24 samples (12 monogyne and 12 polygyne). Worker samples included those obtained from these 22 nests and two additional nests (one monogyne nest and one polygyne nest).

Bacterial communities in soil and RIFA workers were analyzed by Illumina MiSeq 16S rRNA gene sequencing. In each PCR, purified gDNA from soil and RIFA samples and negative control extractions were used as templates. Reaction mixes (25 μl) included 12.5 μl KAPA HiFi HotStart ReadyMix, 5 μl (1 μM) forward primer (Illumina adapter +515F), 5 μl (1 μM) reverse primer (Illumina adapter +806F), and 2.5 μl template DNA. Each PCR assay also included a negative control reaction that was performed using all reagents with DNA-free water serving as the template. PCR products used for Illumina library preparations followed the protocol of the Illumina 16S Metagenomic Sequencing Library Preparation Guide (part number 15044223, revision B). To validate surface sterilization of fire ants used in the *Gp-9* PCR assays, additional PCR were performed using the last rinse water from fire ant surface sterilizations as templates. Gel electrophoresis of the negative control amplification reactions from the last rinsates and the negative controls of the Illumina sequencing verified that fire ants were surface sterilized and PCR reagents were free of contamination.

To detect potential contamination that occurred through the DNA isolation and amplification processes, DNA extractions from autoclaved sand and an empty microcentrifuge tube were included in PCR assays and amplicon library preparations. Also included was a ZymoBIOMICS Microbial Community DNA Standard (positive control) composed of eight known bacterial species (D6305, ZymoBIOMICS™, Irvine, CA). Inclusion of the mock community allowed us to determine if PCR bias, sequencing bias, and taxa classification inaccuracies affected study results. All soil and RIFA DNA samples were quantified using a Quant-iT PicoGreen kit (Invitrogen, Carlsbad, CA, USA) after each were pooled in equimolar amounts. Paired-end sequencing of the Illumina libraries was performed on the Illumina Mi-Seq PE300 platform at the University of North Carolina Core Microbiome Facility (Chapel Hill, NC).

Amplification of fire ant worker samples included non-target host DNA amplicons (described in results). An additional Illumina library was, therefore, prepared to measure bacterial communities of RIFA worker ants using 16S rRNA primers that span the V3–V4 region (approximately 464 bp). The library included 24 samples (12 monogyne, 12 polygyne worker gDNA samples) and was prepared as previously described, differing only by the use of 341F/805R primers [34]. In the library, a negative control and a mock bacterial community were also included, as previously described. Paired-end sequencing of the

library was performed on Illumina Mi-Seq PE300 at the UNC Microbiome Core Facility.

Sequence Processing, Quality Control, and Mock Community Analysis

Bioinformatic processing of samples was done using QIIME 2.0 [35, 36]. Paired-end reads were merged and quality filtered into error-corrected amplicon sequence variants (ASVs) using the DADA2 plugin (ver. 2019.4.0) using the default settings [37], which represent unique bacterial taxa. Rarefaction curves were constructed to compare sampling depth to observed ASV richness for samples from each Illumina run. Following the inspection of saturation plateaus, a rarefaction depth of 9000 sequences was chosen for diversity analyses from each Illumina run.

Because fragment length varied between the libraries prepared with different primer sets, the sequences provided different taxonomic resolutions. To facilitate diversity analyses, sequences covering the V3–V4 region were trimmed (using the QIIME "cutadapt" function [38]) to have homologous coverage (same V4 region) as amplicons generated using the 515F/806R primers. Then, prior to diversity analyses, ASVs tables, representative sequence files, and metadata from Illumina runs were merged. Following quality control and paired-end merging, taxonomic assignments were made to the sequence data from each mock community using the Greengenes 13_8 99% database [39]. Genus-level taxonomic proportions in each sample were compared to the theoretical and measured compositions of the mock community.

Alpha and Beta Diversity Analyses

Alpha diversity (which estimates diversity within samples) was measured using three metrics: (1) "observed ASVs" measured the richness of unique sequence variants in each sample and was an estimate of species richness [37]; (2) "Shannon's diversity" was based on abundances and evenness of individual ASV in each sample [40]; and (3) "Faith's phylogenetic diversity" (Faith's PD) which calculated the sum of the phylogenetic branch distances lengths between all ASVs within each sample [41].

To analyze the differences in the overall bacterial diversity across the samples, beta diversity was measured using unweighted and weighted UniFrac indices [42]. UniFrac is based on the presence and absence of ASVs (taxa) between sample pairs and the phylogenetic distances between sequences to estimate sample similarities. Weighted UniFrac factors in the relative abundances of ASVs, while unweighted UniFrac does not.

A series of tests were performed to evaluate effects of several independent variables (colonization by RIFA, RIFA social form, and soil depth) on the composition of soil and RIFA bacterial communities. For each test, RIFA worker microbiome data were included so that differences in diversity (alpha and beta) of bacterial communities between RIFA workers and soil could be examined. To test for the effects of the RIFA on soil bacterial communities, comparisons were made between RIFA-positive soil, RIFA-negative soil, and RIFA workers. To test for effects of RIFA social form, comparisons were made between soil bacterial communities in monogyne RIFA soil, polygyne RIFA soil, monogyne RIFA workers, and polygyne RIFA workers. Finally, to test for effects of soil depth, comparisons were made between soil at the 0 cm depth and soil at the 10 cm depth of RIFA-positive and RIFA-negative soils.

Using JMP® Pro software (ver. 14.1.0, SAS Institute Inc., Cary, NC), each alpha diversity metric's distributions were tested for normality using Shapiro-Wilk goodness-of-fit tests [43]. As each of the three metrics failed to meet normality requirements, nonparametric Kruskal-Wallis tests were used to evaluate effects of each independent variable on alpha diversity. Following each test, pairwise comparisons were then made using the Wilcoxon signed-rank test at $\alpha = 0.05$.

Testing for effects of colonization by RIFA, RIFA social form, and soil depth on beta diversity was performed within QIIME2 by permutation analysis of variance (PERMANOVA). In each test ($\alpha = 0.05$), the average distances were calculated from the unweighted and weighted UniFrac distance matrices [42]. Average distances were compared by PERMANOVA [44] for their dependence on RIFA colonization (+ vs. −), colony social form (polygyne vs. monogyne), and soil depth (0 vs. 10 cm). Pairwise PERMANOVA tests were carried out to evaluate effects of independent variables on microbiome composition at a significance level of $\alpha = 0.05$.

Taxonomic Assignments

Taxonomic assignments of soil and RIFA samples, performed in QIIME2 using the Greengenes 13_8 99% database [39], were merged and heatmaps of taxonomic abundances were composed using the "feature-table heatmap" function in QIIME2. Heatmaps were designed to focus only on the most abundant taxa (whose total abundance was greater than 1% across study samples), and taxonomic comparisons were made at the class and family levels. Samples were grouped (monogyne RIFA (+) soil, polygyne RIFA (+) soil, RIFA (−) soil, monogyne RIFA, and polygyne RIFA) in which average abundances of taxa were $\log_{10}$ converted and visualized on a heatmap gradient.

Linear Discriminate Analysis of Differential Taxa Abundance

To determine which bacterial taxa contributed to differences in community structure between RIFA-positive and

RIFA-negative soils, taxa that were differentially represented between the two groups were identified by linear discriminate analysis (LDA). LEfSe compares features (taxa) between samples to identify differences between samples based on biological categories [45]. Taxa with logarithmic LDA scores > 2.0 were determined to be differentially represented between RIFA-positive and RIFA-negative soils.

Cloning Library Construction and Analysis of Sequences

Analyses of high-throughput sequencing results identified a bacterial taxon as *Bartonella* sp. Two of the RIFA samples with high abundances of this taxon (SI147 (99.9% *Bartonella*) and SI447 (94.1% *Bartonella*) were further evaluated to identify the bacterium at a higher taxonomic resolution. 16S rRNA genes were amplified from DNA using the universal bacterial primers 27F and 1492R [46] in 50 μl reactions. Cloning, blue-white colony selection, and sequence analyses followed previously described methods [47]. Sequences were trimmed to the first 600 bases and identified by comparison to the NCBI 16S rRNA database. DNA from a colony (sample #SI147) that matched closely to *Bartonella apis* (95.2% similarity, NCBI accession no. KP987884) was again Sanger sequenced using the 520F and 968F primers. Using the MUSCLE sequence alignment algorithm [48] in MEGA (ver. 7.0.18) [49], sequences were aligned and combined to a consensus sequence with at least 200 base overlap of 100% similarity. The consensus sequence (1451 bases) was checked for vector contamination and chimeras and then identified by comparison to the standard NCBI GenBank nucleotide blast database. The sequence was deposited in NCBI GenBank with the accession number MN788445.

Construction of *Bartonella* Phylogeny

To identify the bacteria represented by our consensus sequence more accurately, we constructed a phylogeny based on 16S rRNA gene variation. Using MUSCLE, we aligned our consensus sequence with several type-species of bacteria within the Rhizobiales (including *Bartonella*) that were acquired from Ribosomal Database Project (RDP) and several Rhizobiales sequences from NCBI GenBank. Homologous regions were compared by trimming the aligned sequences to 1347 bases, from which a phylogeny was generated with the maximum likelihood method using the Tamura-Nei model. Bootstrapping was performed at 1000 replications, and bootstrap values above a 50% cutoff were included in the phylogeny [50]. The tree was rooted by *Orientia tsutsugamushi* as the outgroup taxon.

Results

Gp-9 Sequence Analyses

Multiplex PCR primers produced a 517 bp amplicon for monogyne RIFA and 517 and 423 bp amplicons for polygyne RIFA. Both social forms of the red imported fire ant were positively identified from the ant samples by visualization of the amplicons of the expected size on agarose gels. Thus, in our study, 12 nests were identified as monogyne, and 12 nests were identified and as polygyne **(Table S1)**. Our examination of the sequencing results (Table S2) of three *Gp-9* amplicons confirmed the social form assignments. The sequences were 97% and 99% similar to sequences of *S. invicta Gp-9*^{B} and *Gp-9*^{b} allele variants, respectively, deposited in the NCBI database.

Testing Bacterial Community Variability Between Replicate Soil Samples and Between Soil Depths Using DGGE

Preliminary analyses were based on 16S rRNA amplicons from 108 soil samples from six RIFA collection sites that were separated on six DGGE gels **(Fig. S2)**. Bray-Curtis distances between replicate samples (d_r) at each collection site were significantly lower ($p < 0.05$) than distances between samples from different collection sites (d_c) **(Table S3)**, thus showing low variability of bacterial communities within each nest at a given depth for both monogyne and polygyne RIFA nests. Replicate samples were, therefore, pooled in subsequent Illumina analyses.

In soil at the 0 cm depth, distances between replicate samples (d_r) of RIFA-colonized soil were significantly lower than distances between RIFA-negative samples (d_p) **(Table S4)** in monogyne nests (Kruskal-Wallis, $X^2 = 15.44$, $p < 0.001$) and in polygyne nests ($X^2 = 4.18$, $p = 0.041$). Likewise, in soil at 10 cm deep, Bray-Curtis distances between replicate samples of RIFA-colonized soil were lower than distances between RIFA-negative samples **(Table S4)** in both monogyne ($X^2 = 19.73$, $p < 0.001$) and polygyne nests ($X^2 = 18.75$, $p < 0.001$). In contrast, at the 20 cm soil depth, replicate sample distances (d_r) were not significantly different ($p > 0.05$, for each test) between RIFA-colonized and RIFA-negative samples (d_p) **(Table S4)**. Bacterial communities of RIFA-colonized and RIFA-negative soil at the 20 cm depth were similar. Soil samples collected at the 20 cm depth were, therefore, not included in subsequent Illumina analyses.

QIIME2 Analysis of Bacterial Microbiome

A total of 3,642,104 high-quality paired-end 16S rRNA sequences were obtained from the soil samples. Sequences from 83 soil samples were included in the analyses with an average

of 43,881 (range 9343–85,493) sequences per sample prior to rarefaction. A rarefaction depth of 9000 sequences per sample was selected based on the visualization of saturation plateaus in rarefaction curves (Fig. S3). Soil samples included 896 different ASVs (Supplementary file 1).

Bacterial 16S rRNA amplicons generated from RIFA worker samples using the V4 universal primers had high abundances of non-target ASVs. An ASV classified only as "bacteria" was present in all of the samples, in abundances between 0.58 and 96.77%, with an average (± SE) abundance across worker RIFA samples of 44.92% (± 9.96). The identity of the most common ASV in the "bacteria" classification (48.95%) was reevaluated by comparison to the NCBI nucleotide database. This ASV matched closest to *Solenopsis invicta* uncharacterized LOC113004795 mRNA (matching to NCBI accession no. XM 026139155). It was therefore concluded that non-target host amplifications had occurred. Unfortunately, when these non-target amplicons were subtracted from the analysis, the average number of sequences in RIFA worker samples was reduced to 5998 sequences per sample. Rarefaction curves showed that the reduced number of sequences failed to capture bacterial community diversity in most of these samples (data not shown).

Accordingly, PCR was repeated with alternative universal bacterial primers (V3–V4). This approach successfully amplified bacterial 16S rRNA genes in the RIFA worker samples without non-target amplification. A total of 1,623,697 high-quality paired-end reads were generated for these samples with an average of 77,319 reads per sample. To coordinate bacterial alpha diversity comparisons between RIFA worker samples and soil samples, the same rarefaction depth was selected (9000 sequences); this sampling depth fell within the rarefaction curve saturation plateau (Figure S3) and ensured that bacterial diversity was adequately represented in the analysis. The selected depth allowed for the inclusion of 21 of the 24 RIFA samples (11 monogyne and 10 polygyne). RIFA samples included 678 different ASVs (Supplementary file 2).

Negative controls (from autoclaved sand and from a microcentrifuge tube) each had low numbers of sequences: 390 and 25, respectively. The number of ASVs in each was also relatively low (two from the sand and nine ASVs from the vial). Taken together, these results suggest that exogenous contaminations that may have occurred during DNA isolation, PCR, and library preparations were minimal, and the sequences derived from our test samples were valid. It was anticipated that sequencing of the mock DNA would detect the component eight species, based on compositional details from the supplier. Using the V4 primers, one bacterium, *Listeria*, was not detected. The remaining taxa, however, were measured in similar proportions as the theoretical relative abundances. The mock community developed using V3–V4 primers detected all of the expected bacterial taxa, indicating a wider taxonomic sensitivity of V3–V4 primers compared to the V4 primers. The similarity of the measured and theoretical abundances of each taxon and the accuracy of taxa identifications (Table 1) validated the bioinformatics pipeline used for microbiome analysis.

Alpha Diversity

Alpha diversity of bacterial communities was lower in RIFA workers compared to soil samples using all of the alpha diversity metrics that were measured and tested (Fig. 2). In detail, Kruskal-Wallis tests comparing alpha diversity of RIFA (+) soil, RIFA (−) soil, and RIFA workers showed significant differences for all three metrics: ASV richness (X^2 =13.14, p = 0.001), Shannon's diversity (X^2 =41.24, $p < 0.001$), and Faith's PD (X^2 =30.19, $p < 0.001$). Post hoc pairwise tests showed that for each alpha diversity metric, bacterial communities of RIFA workers had significantly lower diversity compared to soil communities, and there were no significant differences between communities of RIFA (+) and RIFA (−) soils ($p > 0.05$, for each metric). Tests comparing monogyne RIFA soil, polygyne RIFA soil, monogyne RIFA, and polygyne RIFA found significant differences between groups: ASV richness (X^2 =14.76, p = 0.002), Shannon's diversity (X^2 =41.45, $p < 0.001$), and Faith's PD (X^2 =31.03, $p < 0.001$). Post hoc testing of ASV richness, Shannon's diversity, and Faith's PD generally resulted in lower diversity in the RIFA workers compared to the soil samples. Bacteria community alpha diversity in colony soil was not dependent on colony social form (p >0.05, for each metric), and RIFA worker bacterial community diversity was not dependent on RIFA social form (p >0.05). ASV richness of monogyne RIFA soil and monogyne RIFA workers were not significantly different from one another (p >0.05, for each metric). Finally, testing for effects of soil depth on bacterial community alpha diversity showed that soil depth did not significantly affect any of the bacterial community alpha diversity metrics ($p > 0.05$, for each metric).

Beta Diversity

RIFA colonization of soil significantly affected weighted (PERMANOVA, F = 18.857, p = 0.001) and unweighted (PERMANOVA, F = 6.751, p = 0.001) UniFrac distances. Pairwise testing showed differences between all independent variables (RIFA (−) soil, RIFA (+) soil, and RIFA workers) for both weighted and unweighted UniFrac indices (Fig. 3**A)** to be significant (PERMANOVA, p <0.05).

Significant differences in weighted UniFrac indices (PERMANOVA, F = 8.949, p = 0.001) and unweighted UniFrac indices (PERMANOVA, F = 3.976, p = 0.001) (Fig. 3B) were found between monogyne RIFA, polygyne RIFA, monogyne RIFA nest soil, and polygyne RIFA nest

Table 1. Bacterial mock community theoretical and measured taxonomic abundances and species identifications from two Illumina 16S rRNA amplicon libraries

Mock community		V4 library mock community		V3–V4 library mock community	
Mock community taxa	Theoretical abundance (%)	Identified taxa	Measured abundance (%)	Identified taxa	Measured abundance (%)
Pseudomonas aeruginosa	4.2	*Pseudomonas* sp.	14.6	Pseudomonadaceae	5.3
Escherichia coli	10.1	*Escherichia coli*	7.1	*Escherichia coli*	8.9
Salmonella enterica	10.4	Enterobacteriaceae	14.4	*Salmonella* sp.	13.8
Lactobacillus fermentum	18.4	*Lactobacillus* sp.	18.3	*Lactobacillus* sp.	21.8
Enterococcus faecalis	9.9	*Enterococcus* sp.	8.6	*Enterococcus* sp.	6.4
Staphylococcus aureus	15.5	*Staphylococcus* sp.	20.4	*Staphylococcus* sp.	15.4
Listeria monocytogenes	14.1	Listeriaceae	0	*Listeria* sp.	11.5
Bacillus subtilis	17.4	*Bacillus* sp.	16.6	*Bacillus* sp.	16.8
				Other taxa	0.06

V4 library composed using 515F/806R primers and V3–V4 library composed using 341F/805R primers

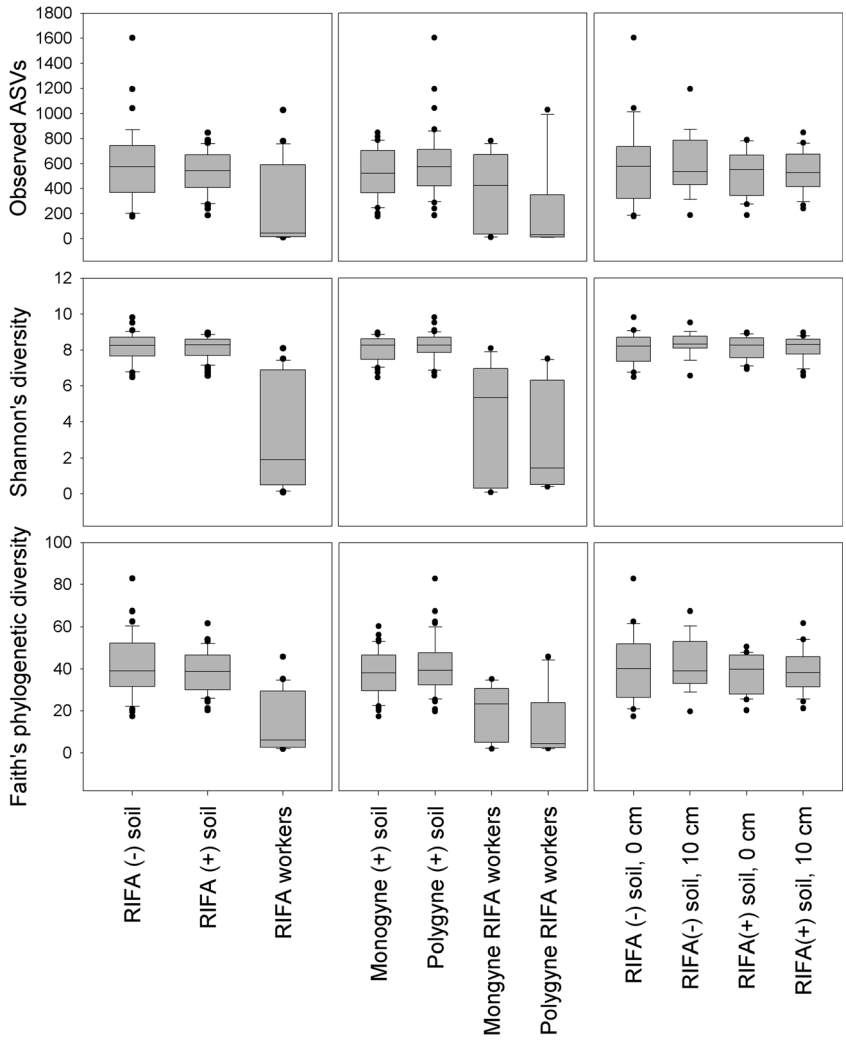


Fig. 2: Boxplots showing higher bacterial community alpha diversity of soil compared to RIFA workers. Average observed ASVs, Shannon's diversity, and Faith's phylogenetic distances were tested for dependencies on RIFA colonization, RIFA social form, and soil depth by Kruskal-Wallis tests ($\alpha = 0.05$). Significantly different Wilcoxon pairwise test groups are indicated by lowercase letters

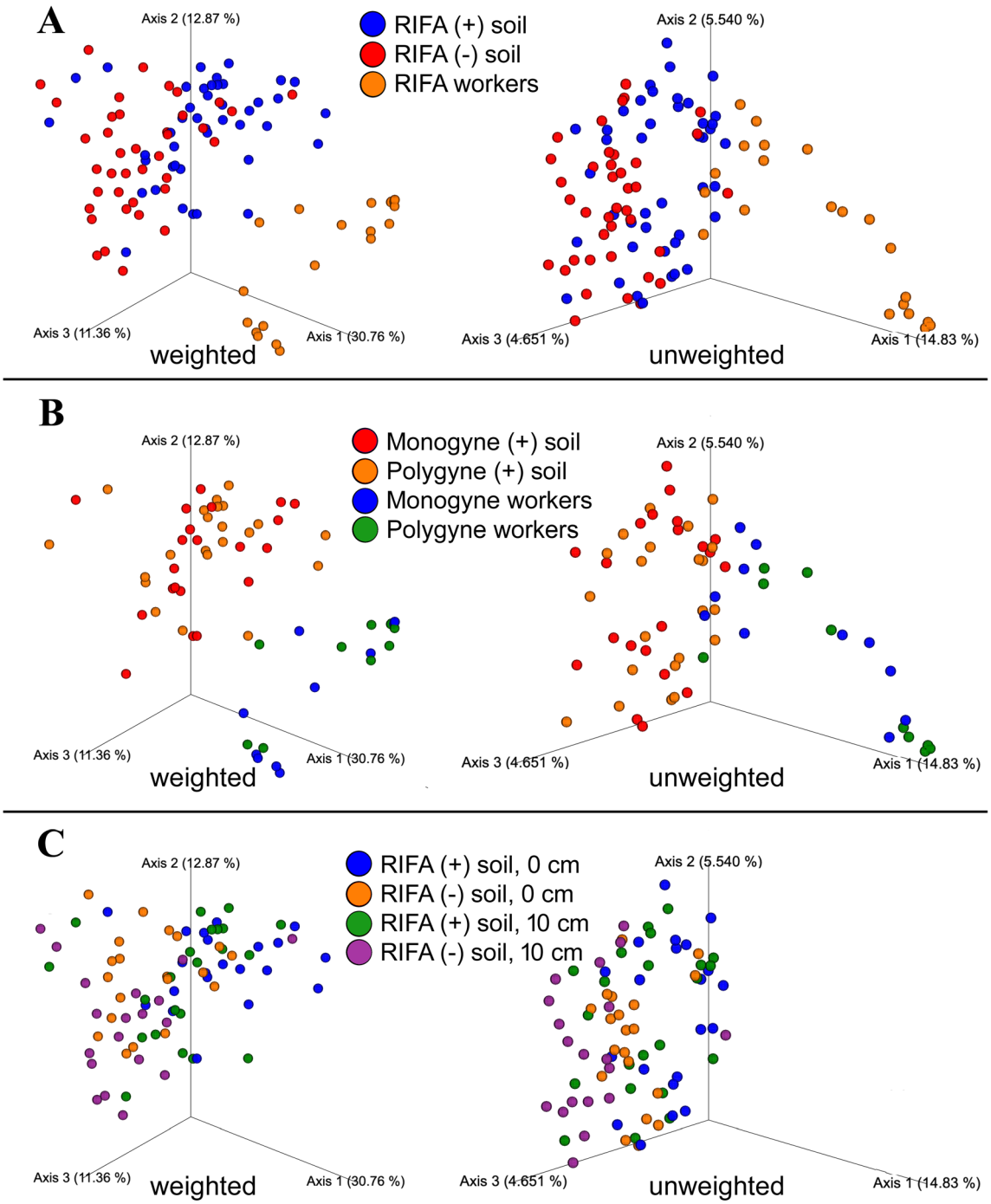


Fig. 3: Principle coordinate analysis of bacterial community composition based on weighted (left) and unweighted (right) UniFrac distances. **A** Comparison of soil (stratified by RIFA colonization) and RIFA workers. **B** Comparison of RIFA colony soil and RIFA workers (each stratified by RIFA social form). **C** Comparison of soils stratified depth and RIFA colonization

soil. Pairwise comparisons, however, revealed no differences in UniFrac distance between monogyne and polygyne soil communities (weighted UniFrac PERMANOVA, $F = 0.799$, $p = 0.593$; unweighted UniFrac PERMANOVA, $F = 1.021$, $p = 0.380$) or between monogyne and polygyne RIFA worker communities (weighted UniFrac PERMANOVA, $F = 1.351$, $p = 0.214$; unweighted UniFrac PERMANOVA, $F = 1.219$, $p = 0.195$).

Site-specific tests (LW and CC) each also showed soil and RIFA bacterial community structure was not dependent on RIFA social form. Among the LW samples, UniFrac distances were not different between monogyne and polygyne nest soils (weighted Unifrac, $F = 0.705$, $p = 0.703$; unweighted Unifrac, $F = 0.968$, $p = 0.500$), and distances were not different between monogyne and polygyne worker RIFA (weighted Unifrac, $F = 0.525$, $p = 0.688$; unweighted Unifrac, $F =$

0.675, $p = 0.892$). Likewise, among the CC samples, nest soils were not dependent on nest social form (weighted Unifrac, $F = 1.089$, $p = 0.297$; unweighted Unifrac, $F = 1.115$, $p = 0.192$), and RIFA worker bacterial communities were not dependent on social form (weighted Unifrac, $F = 2.612$, $p = 0.107$; unweighted Unifrac, $F = 2.146$, $p = 0.055$).

Within RIFA (+) soil, 0 cm deep soil and 10 cm deep soil could not be differentiated using either the weighted (PERMANOVA, $F = 1.653$, $p = 0.083$) or unweighted (PERMANOVA, $F = 0.939$, $p = 0.529$) UniFrac index (Fig. 3C). The bacterial communities of RIFA (−) soil, however, were dependent on soil depth (weighted UniFrac PERMANOVA, $F = 2.534$, $p = 0.012$; unweighted UniFrac PERMANOVA, $F = 1.879$, $p = 0.001$).

Heatmap Analysis and Linear Discriminate Analysis

Common taxa found across the study varied in abundance between RIFA-positive soil (monogyne and polygyne), RIFA-negative soil, and RIFA (monogyne and polygyne) (Fig. 4). Comparisons of RIFA-colonized soil to RIFA-negative soil showed some taxa co-occurring in large abundances (i.e., Solibacteraceae, Chitinophageaceae, Sphingomondaceae) with the abundances of some taxa associated with RIFA colonization (Nocardiaceae, Nocardiodiaceae, Oxalobacteraceae). Pronounced contrasts occurred between RIFA workers and soil. Bartonellaceae and Entomoplasmataceae were rare in soil samples but highly abundant in RIFA workers. Also, RIFA workers had comparatively high abundances of Actinobacteria, including Microsporaceae, Nocardiaceae, and Nocardioidaceae.

Lefse analysis showed that RIFA-colonized soil had significantly higher abundances of 23 genus-level taxa and lower abundances of 9 genus-level taxa compared to RIFA (−) soil. When expanded to the family level, there were 13 significantly more abundant taxa in RIFA (+) soil and 18 families of lower abundance compared to RIFA (−) soil (Fig. 5). Colonization by RIFA was found to be related to decreases in taxa, including several *Elusimicrobia* and *Acidobacteria* compared to adjacent uncolonized soil. Taxa that had increased abundances in RIFA colonized soil were diverse, including TM7-3, Burkholderiales, and Actinobacteria.

Cloning and Phylogenetic Analysis

Six sequences, obtained from two RIFA worker samples (SI147 and SI447), matched closest to an uncultured Bartonellaceae bacterium isolated from the ant *Megalomyrmex modestus* (NCBI accession no. LC027776.1) (see **Table S5** for details on sequence identifications). The six sequences were nearly identical to one another with an average (± SE) Jukes-Cantor distance of 0.004 (±0.002) substitutions per site. One transformed colony (from sample SI147) was further evaluated with additional sequencing from which a 1451 base consensus sequence was created. The closest published match to the sequence was an uncultured Rhizobiales bacterium isolated from *Pheidole* sp. (NCBI accession no. FJ477647).

The consensus sequence of our *Bartonella*-like species was compared to 16S rRNA sequences of several other taxa within the Rhizobiales. Phylogenetic analyses determined that the pathogenic *Bartonella* (*Bartonella henselae* and *Bartonella quintana*) and *Bartonella* associated with *Apis mellifera* (including *Bartonella apis* and an uncultured *Bartonella* sp.) all clustered within the same clade. Our unknown *Bartonella*-like species clustered into a sister clade along with several uncultured Rhizobiales, each of which had been isolated from various ant host species (Fig. 6).

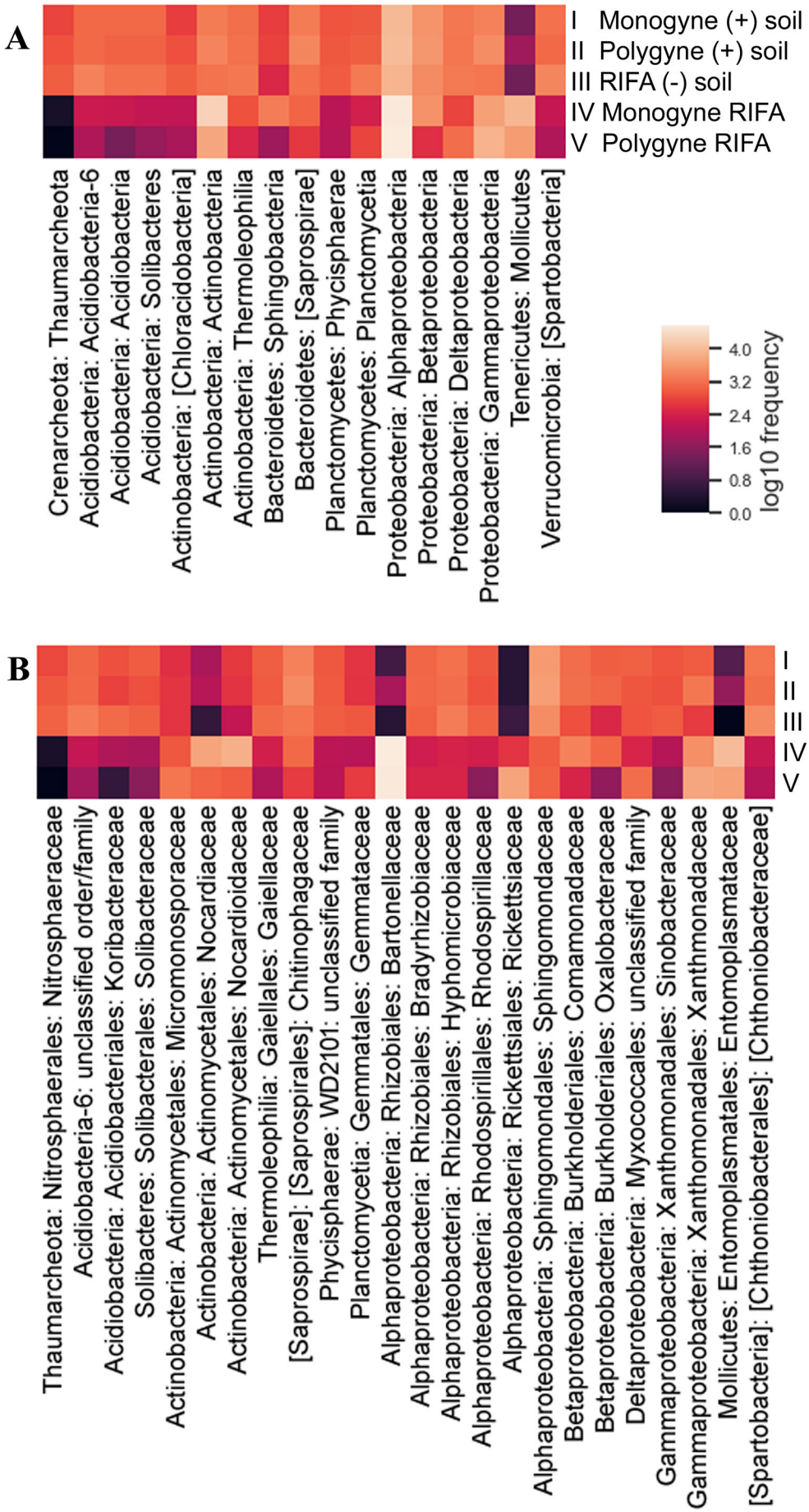
Discussion

Soil Bacterial Community Dependence on RIFA Colonization and RIFA Social Form

Our Illumina Miseq sequencing results showed that there were categorical differences in bacterial community membership and abundance in RIFA-colonized soils compared to native soils, thus supporting our hypothesis that the species composition of soil bacterial communities of RIFA nests are driven by RIFA colonization. Beta diversity analyses described by permutation analysis of variance revealed lower diversity in the bacterial communities of ant-colonized soils compared to native soils. Our results are consistent with previous findings [51] that the process of RIFA colonization reduces bacterial community structure. Linear discriminant and heatmap analyses identified taxa that characterized RIFA nest soil communities and contrasted the bacterial microbiome of the types of soil samples that we analyzed. Changes in the physical and chemical attributes of soil are likely responsible for altering the microbial community structure in our current study. Soil bacterial communities have been shown to be sensitive to pH [52, 53], soil porosity [54], and physical disruption, such as tilling [55].

Interestingly, Actinobacteria (Nocardiaceae and Intrasporangiaceae) were among the bacterial taxa identified in RIFA nest soils. These bacteria are notable for expressing antifungal compounds [56] and have known associations with subterranean Hymenoptera (including many ant species) that use these bacteria to mitigate fungal development in their nests [57, 58]. Elevated abundances of these bacteria in RIFA colonized soil may be indicative of their symbiosis with RIFA.

Various Proteobacteria and TM7-3 bacteria were also in relatively high abundance in RIFA nest soil. Several of these Proteobacteria (Caulobacteraceae, Sphingomondaceae Oxalobacteraceae, Moraxelleraceae, and


A
I Monogyne (+) soil
II Polygyne (+) soil
III RIFA (-) soil
IV Monogyne RIFA
V Polygyne RIFA
Crenarcheota: Thaumarcheota
Acidiobacteria: Acidiobacteria-6
Acidiobacteria: Acidiobacteria
Acidiobacteria: Solibacteres
Actinobacteria: [Chloracidobacteria]
Actinobacteria: Actinobacteria
Actinobacteria: Thermoleophilia
Bacteroidetes: Sphingobacteria
Bacteroidetes: [Saprospirae]
Planctomycetes: Phycisphaerae
Planctomycetes: Planctomycetia
Proteobacteria: Alphaproteobacteria
Proteobacteria: Betaproteobacteria
Proteobacteria: Deltaproteobacteria
Proteobacteria: Gammaproteobacteria
Tenericutes: Mollicutes
Verrucomicrobia: [Spartobacteria]
4.0
3.2
2.4
1.6
0.8
0.0
log10 frequency
B
I
II
III
IV
V
Thaumarcheota: Nitrosphaeraceae
Acidiobacteria-6: unclassified order/family
Acidiobacteria: Acidiobacteriales: Koribacteraceae
Solibacteres: Solibacteraceae
Actinobacteria: Actinomycetales: Micromonosporaceae
Actinobacteria: Actinomycetales: Nocardiaceae
Actinobacteria: Actinomycetales: Nocardioidaceae
Thermoleophilia: Gaiellales: Gaiellaceae
[Saprospirae]: [Saprospirales]: Chitinophagaceae
Phycisphaerae: WD2101: unclassified family
Planctomycetia: Gemmatales: Gemmataceae
Alphaproteobacteria: Rhizobiales: Bartonellaceae
Alphaproteobacteria: Rhizobiales: Bradyrhizobiaceae
Alphaproteobacteria: Rhizobiales: Hyphomicrobiaceae
Alphaproteobacteria: Rhodospirillales: Rhodospirillaceae
Alphaproteobacteria: Rickettsiales: Rickettsiaceae
Alphaproteobacteria: Sphingomondales: Sphingomondaceae
Betaproteobacteria: Burkholderiales: Comamonadaceae
Betaproteobacteria: Burkholderiales: Oxalobacteraceae
Deltaproteobacteria: Myxococcales: unclassified family
Gammaproteobacteria: Xanthomonadales: Sinobacteraceae
Gammaproteobacteria: Xanthomonadales: Xanthomonadaceae
Mollicutes: Entomoplasmatales: Entomoplasmataceae
[Spartobacteria]: [Chthoniobacterales]: [Chthoniobacteraceae]

◀ **Fig. 4:** Heatmaps of most abundant taxa (each represent >1% of total taxa across study samples) at (**A**) class-level of taxonomic classification (phylum: class) and (**B**) family-level of taxonomic classification (class: order: family). The average abundance of each taxon for the five subject groups is $\log_{10}$-converted

Xanthomonadaceae) and bacteria from the TM7 phylum have been associated with diverse soil environments [59–63]. Some members of the Moraxelleraceae [64–67] and some bacteria from the TM7 phylum [68, 69] have previously been reported to have associations with ant microbiomes or ant nests. No specific roles for these bacteria have been proposed. It has not been determined whether their occurrences in ant microbiomes are incidental or whether these bacteria have specialized interactions with ants. Like the Actinobacteria noted above, several species within *Acinetobacter* (Moraxelleraceae) produce antifungal compounds [70, 71], and could potentially function to inhibit fungal development in RIFA nests. Bacteria of the Oxalobacteraceae have been associated with sap-feeding insects with evidence of symbiotic interactions in which the bacteria synthesize amino acids that are utilized by host insects [72].

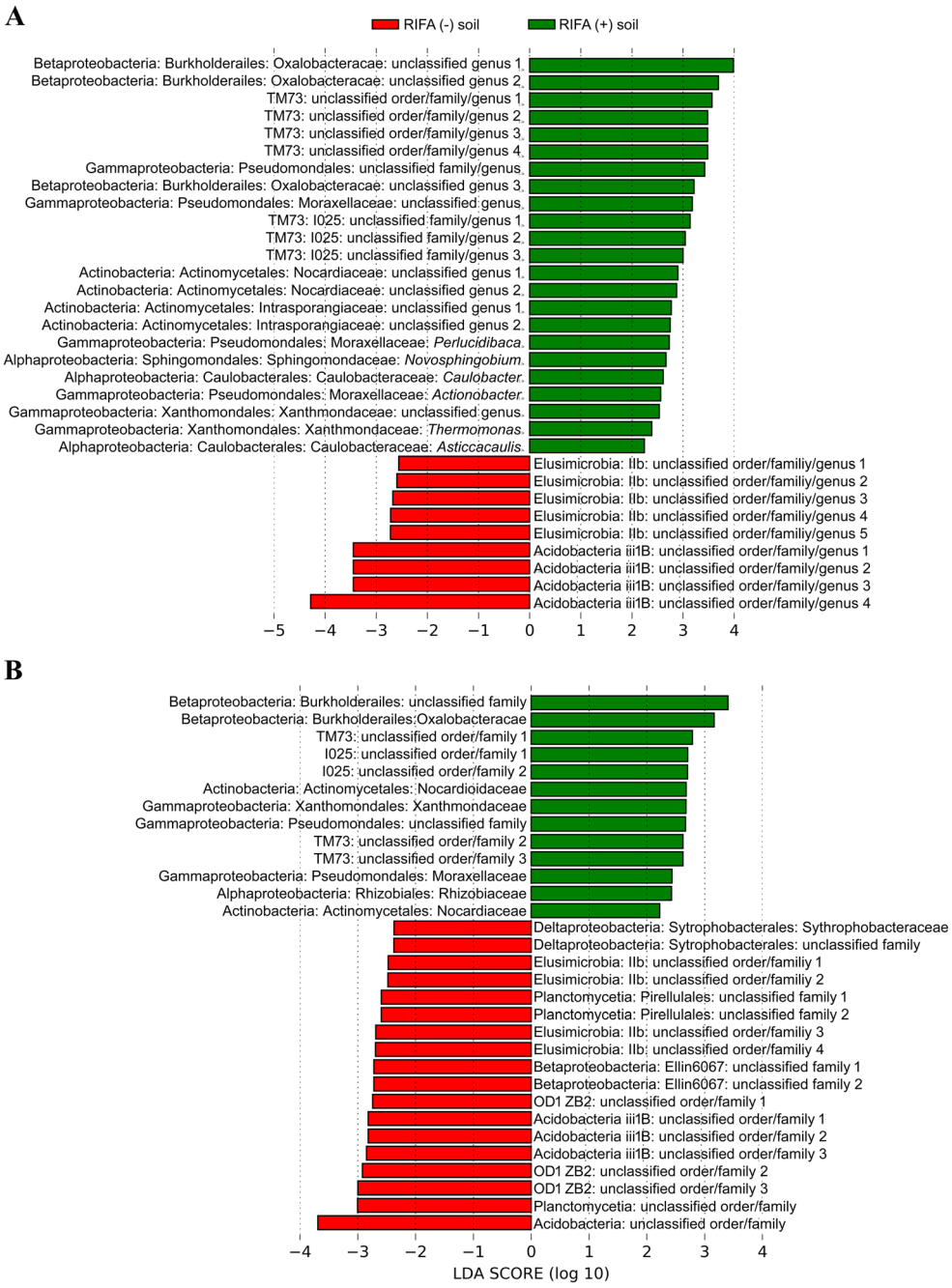


Fig. 5: Linear discriminate analysis (LDA) shows that over-representation of bacterial taxa in soil is dependent on RIFA colonization. **A** LDA scores for genus-level taxa (class: order: family: genus). **B** LDA scores for family-level taxa (class: order: family)

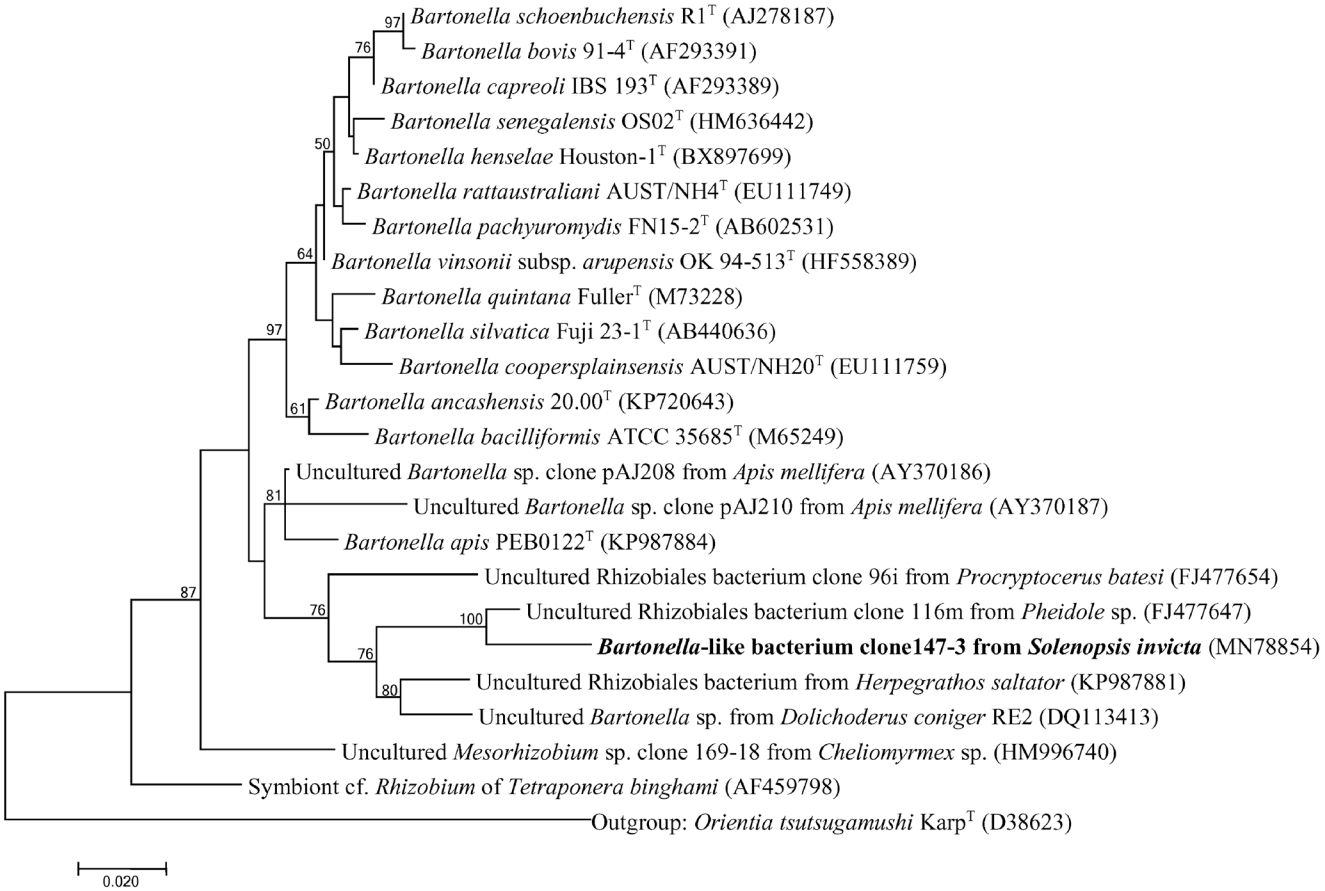


Fig. 6: Phylogenetic tree reconstructed by the maximum likelihood method based on the Tamura-Nei model using 16S rRNA gene sequences showing relationship between unknown *Bartonella*-like cloned sequence (147-3) and related bacterial taxa. Bootstrap percentages (based on 1000 replications) with 50% cutoff values shown at upper left of nodes; GenBank accession numbers for each species are shown in parentheses. The tree is rooted with *Orientia tsutsugamushi* (Type) (D38623) as the outgroup

Native soils were characterized by higher abundances of *Elusimicrobia* and *Acidobacteria*. Based on our analyses, we determined that RIFA colonized soils have reduced abundances of these taxa. While several members of the Elusimicrobia are endosymbionts of insects [73, 74], the lineage identified here (IIB) has been found in soils and aquatic sediments [75]. These bacteria and *Acidobacteria* [76] are likely common inhabitants of soil. Our results showing their occurrence in low abundance in ant nest soil suggest that these bacteria are sensitive to RIFA activity.

We had hypothesized that the food resources by polygyne ants would be shared among adjacent nests leading to relatively similar microbial communities in nest soils. Our results, however, did not show bacterial community membership or structure to be dependent on RIFA social form. It is unknown whether foraged resources are a significant factor contributing to the structure of nest microbial communities. It is also not clear whether food resources are uniformly distributed across polygyne RIFA nests. These questions need to be addressed to understand how RIFA social form potentially affects nest microbial community composition.

RIFA Worker Microbiome

The RIFA worker microbiome was largely characterized by two taxa that have been previously reported in ant microbiomes—*Bartonella*-like Rhizobiales [77–79] and Entomoplasmataceae [65, 80]. By cloning and sequencing nearly full-length fragments of the *Bartonella* sp. 16S rRNA gene, we were able to place the *Bartonella*-like endosymbiont among several other nitrogen-fixing Rhizobiales that have been linked to herbivory in ants [81]. The *Bartonella*-like species that we identified in RIFA workers is possibly a participant in this type of interaction, suggesting that local populations of *S. invicta* include plant resources in their diet. Hemipteran species that commonly contain *Rickettsia* endosymbionts [82–85] have a greater survival rate to adulthood, develop faster, and produce a higher proportion of female offspring [86]. However, *Rickettsia* are not known to be symbionts of ants and were possibly acquired by RIFA in our study through predation. If so, this finding reveals how an interaction between sap feeding hemipterans and RIFA workers shapes aspects of the RIFA microbiome. Members

of *Actinobacteria*, frequently listed as abundant in bacterial communities associated with ants [87–89], also commonly occurred in the RIFA microbiome in our study.

The co-occurrence of taxa in RIFA workers and their nests presents an intriguing question about whether the nest soil drives the RIFA microbiome or whether the RIFA-associated bacteria drive the nest community composition. While bacterial communities of RIFA-colonized soils were found to be distinct compared to those of RIFA workers, many taxa co-occurred between these samples. Of the 21 genus-level bacterial taxa that characterized RIFA-colonized soils (according to our Lefse analysis), 15 of these taxa co-occurred in RIFA worker microbiome samples. Of these co-occurring taxa, only one unclassified genus of Nocardiaceae (referred to as Nocardiaceae: unclassified genus 1) was determined to be a major taxon in RIFA colony soil samples with an average abundance of 3.32%. The remaining 14 taxa were relatively minor in the soil with average abundances ranging from 0.0087 (Xanthomonadaceae, unclassified genus) to 0.7169% (*Acinetobacter* sp.).

The bacterial community membership that we found in *S. invicta* workers is similar to what was reported previously by Ishak et al. [23]. In this study, large abundances of several Actinobacteria, *Acinetobacter* (Moraxellaceae), Nocardioidaceae, *Marmoricola* (Nocardioidaceae), and *Nocardioides* (Nocardioidaceae) were identified by 454 pyrosequencing of 16S rRNA reads. Interestingly, previous studies [23–25] did not report the presence of the *Bartonella*-like endosymbiont in RIFA populations. Occurrences of the *Bartonella* sp. in RIFA workers may be a feature of local populations that we sampled. Additional surveys of *S. invicta* populations would need to be performed to determine if this putative symbiont is geographically widespread in other RIFA populations. Effects of this bacteria on the survival of RIFA also needs to be examined.

Future Studies

Based on 16S rRNA gene variation, the *Bartonella*-like bacteria we found in RIFA are unique, and our phylogenetic tree analysis strongly suggests that it is a novel bacterial species. In order to better characterize these bacteria and more accurately determine their phylogenetic relationship to other Rhizobiales, additional gene targets need to be sequenced, from which a more robust phylogeny can be constructed.

Actinobacteria were found in high abundance in the bacterial communities of RIFA-colonized soils and in RIFA workers. Because these bacteria potentially convey protection against fungal infections, eliminating or reducing the abundance of these bacteria may stress RIFA colonies enabling native ants to successfully compete with fire ants. Accordingly, a more geographically comprehensive survey for these bacteria is needed. Using phylum-specific primers [90], metagenomic surveys could be performed to focus on how RIFA colonization affects these potentially important bacteria while providing identifications of Actinobacteria community members at a higher resolution.

While effects of RIFA colonization on native soils were significant, the time course needed for the effects of colonization should be evaluated. To better understand how RIFA colonization alters soil bacterial communities, it would be informative to determine how bacterial communities change over time following RIFA colonization. Future lab-based or field studies could include a repeated sampling component to determine the effects of colony age on the composition of the nest microbiome.

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Author Contribution N.V.T. collected samples, extracted DNA from samples, and carried out PCR, DGGE, and 16S Illumina library preparations. N.V.T. and L.P. analyzed the data. N.V.T. wrote original draft of the manuscript and N.V.T., E.L.V., C.S.A., and L.P. reviewed and edited final version of the manuscript.

Data Availability Newly determined sequence data were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA690596.

Declarations

Ethics Approval Not applicable.

Consent to Participate All authors approve the final draft of the publication and consent to submission.

Conflict of Interest The authors declare no competing interests.

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