

Bacterial Diversity in *Solenopsis invicta* and *Solenopsis geminata* Ant Colonies Characterized by 16S amplicon 454 Pyrosequencing

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Abstract Social insects harbor diverse assemblages of bacterial microbes, which may play a crucial role in the success or failure of biological invasions. The invasive fire ant *Solenopsis invicta* (Formicidae, Hymenoptera) is a model system for understanding the dynamics of invasive social insects and their biological control. However, little is known about microbes as biotic factors influencing the success or failure of ant invasions. This pilot study is the first attempt to characterize and compare microbial communities associated with the introduced *S. invicta* and the native *Solenopsis geminata* in the USA. Using 16S amplicon 454 pyrosequencing, bacterial communities of workers, brood, and soil from nest walls were compared between neighboring *S. invicta* and *S. geminata* colonies at

Brackenridge Field Laboratory, Austin, Texas, with the aim of identifying potential pathogenic, commensal, or mutualistic microbial associates. Two samples of *S. geminata* workers showed high counts of *Spiroplasma* bacteria, a known pathogen or mutualist of other insects. A subsequent analysis using PCR and sequencing confirmed the presence of *Spiroplasma* in additional colonies of both *Solenopsis* species. *Wolbachia* was found in one alate sample of *S. geminata*, while one brood sample of *S. invicta* had a high count of *Lactococcus*. As expected, ant samples from both species showed much lower microbial diversity than the surrounding soil. Both ant species had similar overall bacterial diversities, although little overlap in specific microbes. To properly characterize a single bacterial

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community associated with a *Solenopsis* ant sample, rarefaction analyses indicate that it is necessary to obtain 5,000–10,000 sequences. Overall, 16S amplicon 454 pyrosequencing appears to be a cost-effective approach to screen whole microbial diversity associated with invasive ant species.

Introduction

The invasive fire ant *Solenopsis invicta* (Hymenoptera: Formicidae) has become widespread across the southern USA since its introduction to North America in the 1930s. The success of *S. invicta* in reaching high densities may be attributed to a loss of natural enemies such as parasitoids [13, 40], reduced pathogen pressures [6, 24, 59], the ant's strong competitive abilities [14, 34], and its affinity for disturbed habitats [26, 39, 53]. *S. invicta* is considered an important economic and ecological pest due to its aggressive nature and potent sting [60]. Across part of its introduced range, *S. invicta* is sympatric with several native fire ants including *Solenopsis geminata*, which has a more granivorous diet and is seasonally less active in central Texas than *S. invicta* [54].

Microbes play important roles in ant communities where they may act as mutualists, commensals, or pathogens. The need for a deeper understanding of the roles of microbes in fire ant biology, and in particular an interest in identifying potential pathogens of fire ants, motivated several studies of *S. invicta* in both its native (South America) and introduced ranges (USA; summarized by [37]). Pathogens of *S. invicta* that have been described include fungi [44, 51], microsporidia [1, 25, 27], and viruses [56, 57]. The bacterium *Wolbachia* has been found in the native range, but is scarce in North American populations of *S. invicta* [5, 49].

To identify endosymbionts of *S. invicta*, several studies screened for microbes in midgut contents of fourth-instar larvae, the only life cycle stage that can digest solid food [58]. These studies have reported a variety of microbes in larval midguts, such as *Lactococcus*, *Staphylococcus* and *Enterococcus* [38], *Enterococcus*, *Enterobacter*, *Kluyvera*, *Lactococcus*, *Pseudomonas*, *Achromobacter*, *Bacillus*, *Listeria*, and *Serratia* [29]. Lee et al. [28] found mainly *Enterobacteria* strains not considered to be endosymbionts in guts of larvae and little similarity in bacterial communities between colonies. Tufts and Bextine [55] also found *Bacillus* spp. bacteria in *S. invicta* queens, which vertically transmit these bacteria to their progeny. Other studies on bacterial associates of ants have focused more on potentially mutualistic bacteria in species of fungus-growing ants. For example, based on 16S amplicon 454 pyrosequencing of the bacterial communities in fungus-growing ants colonies from central Texas, Sen et al. [48]

documented the occurrence of antibiotic-secreting *Pseudonocardia* and *Amycolatopsis* species on ant workers. Bacterial endosymbionts have been found in the gut of *Camponotus* ants [45], *Tetraponera* ants [4], and a great diversity of other ants [43].

Whereas no study has so far surveyed bacterial associates of *S. geminata*, there have been several culture-dependent surveys of microbes in *S. invicta* gut and hemolymph [18, 28, 29, 38, 55]. These culture-dependent studies undoubtedly underestimated the true bacterial diversity. In contrast, direct field assays of material followed by 454 pyrosequencing of PCR products of the 16S rRNA gene [11] provides a more comprehensive sampling of associated bacteria and an improved measure of relative bacterial abundance, particularly of bacteria that are difficult or impossible to culture or to clone.

Toward developing high-throughput methods to survey bacteria associated with *S. geminata* and *S. invicta* fire ants, we (a) characterized the bacterial assemblages in a small sample of fire ant colonies, (b) compared bacterial associates of workers and brood with those of soils in the nest walls and in the surrounding soil, and (c) compared the bacterial communities of sympatric populations of native (*S. geminata*) and invasive (*S. invicta*) fire ants.

Methods

Study Site and Sample Collection

The study site was the Brackenridge Field Laboratory, University of Texas, Austin (30.285° N, 97.781° W), where populations of native *S. geminata* were displaced by invading *S. invicta* in the early 1980s. A small pocket of *S. geminata* colonies remained in an isolated 0.05-ha grassy patch where only a few *S. invicta* have established. Samples of three colonies each of *S. geminata* and *S. invicta* were collected on 22 November 2009 by three collectors. Each colony was opened with a vertical face approximately 20 cm deep, and material was pried away to reveal uncontaminated colony chambers. Specimens were collected with flame-sterilized forceps and preserved in molecular-grade 100% ethanol (Table 1). Collectors wore fresh sets of latex gloves for each collection. From each colony, we collected where possible 12 larvae (brood), 12 workers, 5 stored seeds, and forceps scrapings (approximately 5 mg) of interior chamber soil and adjacent surface soil (about 10 cm distant, but at the same soil depth as the chamber from which soil was sampled from the chamber wall). For one colony, we also collected three alates (see Table 1 for the list of all collections). Whole ants were pooled together per nest for the DNA extraction; thus, ecto- and endobacteria were sampled in the same assay. We did not attempt to

Table 1 Samples collected for the 454 pyrosequencing bacterial comparisons

Nest	<i>S. geminata</i>			<i>S. invicta</i>		
	1	2	3	1	2	3
Negative control	A1 ^a	A6	B2	C1	D1	E1 ^b
Soil outside	A2	A7	B3	C2	D2	E2 ^b
Soil in chamber	A3	A8	B4	C3	D3	E3 ^b
Worker	A4	A9	B5 ^a	C4	D4	E4 ^b
Brood	A5 ^a		B6	C5 ^a	D5	E5 ^b
Alate			B7			
Stored seed		B1	B8			

^a Less than 1,000 sequences generated

^b Samples excluded from phylogenetic analysis because of failed 454 pyrosequencing reaction for sample E4 and because of poor rarefaction sampling for E5

clean the outside of the ants to remove surface contaminants because interesting bacteria can be found on the ant integument [48]. A negative control sample was taken at each colony by opening the vial and inserting the sterilized forceps. All samples were placed into a -80°C freezer immediately after collection.

Bacterial Tag-Encoded FLX 454-Pyrosequencing

DNA extraction and bacterial tag-encoded FLX 454 pyrosequencing (bTEFAP) were performed by the Research and Testing Laboratory (Lubbock, TX). Samples arrived frozen and were thawed and resuspended with β -mercaptoethanol in 500 μl RLT buffer (Qiagen, Valencia, CA). Bacterial DNA was lysed with 0.1-mm glass beads in a Qiagen Tissue Lyser (Qiagen). One hundred microliters of supernatant and 100 μl of 100% ethanol were added to a DNA spin column and DNA was recovered following standard protocol (starting at step 5) by Qiagen Stool Kit (Qiagen). DNA was eluted with 30 μl of water and samples were diluted to a final concentration of 20 ng μl .

bTEFAP was analyzed on the Genome Sequencer FLX instrument using Titanium protocols and reagents (Roche, Indianapolis, IN) using methods described previously by Bailey et al. [2], Callaway et al. [7], Dowd et al. [11], and Smith et al. [50]. We utilized a one-step PCR with a mixture of Hot Start and HotStar high-fidelity Taq polymerases. The PCR primers for FLX amplicon pyrosequencing were chosen to span the variable regions V1–V3 in the 16S gene: Gray28F 5'GAGTTTGATCNTGGCT CAG and Gray519r 5'GTNTTACNGCGGCKGCTG. All negative controls (A1, A6, B2, C1, D1, E1) and the alate sample (A7) underwent two rounds of pyrosequencing due to the low numbers of sequences generated in the first round. Following sequencing, all failed reads, low-quality

sequence, and sequences <200 bp were removed. Additionally, sequences were depleted of non-bacterial ribosome sequences and chimeras using the black box chimera check software (B2C2) [17]. Pyrosequencing reads are deposited at the GenBank Short Read Archive under accession no. SRA 020746.

BLAST and OTU Designation

The remaining sequences were identified to their closest operational taxonomic units (OTUs) at a minimum of 75% identity using a distributed BLASTn.NET algorithm [12] against a high-quality 16S database derived from the National Center for Biotechnology and curated by the Medical Biofilm Research Institute. All sequences that did not match at the minimum of 75% identity were discarded and were assumed to be either of poor quality or derived from unclassified bacteria. The BLAST hit from each individual sequence was analyzed separately for each sample providing relative abundance information within and among the individual samples based upon relative numbers of reads within each. We have supplied two types of BLAST results presented in this paper (Electronic Supplementary Material (ESM) Table 1 and Table 2). ESM Table 1 presents the percent of bacteria per sample according to its nearest genus, meaning that the percent identity ranges from 75% to 100%. ESM Table 2 presents sequences with identity scores $>97\%$ identity ($<3\%$ divergence) resolved at the species level: between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order levels, and 80% and 85% at the class and 77% and 80% at the phylum levels [2, 7, 11, 50]. The raw BLAST hit identity and unique sequence IDs can be found in ESM Table 3. We also used an OTU rarefaction analyses to test whether the sampling regime adequately represented the bacterial diversity within each sample using 1% and 3% OTU designations and a 5,000 sequence cutoff. Finally, to compare bacterial richness between sample types, we used a Mann–Whitney U test.

Sequence Alignments and Phylogenetic Analysis

We conducted phylogenetic analysis using Fast UniFrac [20]. We excluded from the phylogenetic analysis one of the sampled *S. invicta* nests (samples E1–E5) due to evidence of a failed 454 pyrosequencing reaction for the worker sample E4 and comparatively lower sequences produced for this sample set (see ESM Fig. 1 for rarefaction curves). We further removed all short sequences (<375 bp). Because the numbers of sequences acquired from different samples varied substantially, we randomly selected 1,000 sequences from each sample (exceptions were samples A5

and B5 which yielded only 734 and 676 sequences, respectively). Standardization of sampled sequences in the UniFrac analyses allowed us to compare general patterns of diversity while controlling for the variation in sequence yield per sample. From the 21,423 sequences obtained by random sampling, we clustered redundant sequences together at 97% similarity using the CD-Hit web server [30]. Each cluster was assigned the longest sequence from the cluster as a representative sequence, leaving a total of 4,945 sequences. We used a custom PERL script to produce the Sample Mapping ID file needed for Fast UniFrac analysis, which allowed us to retain the abundance information for all 21,423 sequences but align a smaller dataset. The sequences were aligned using the SILVA bacterial database as a template in Mothur (www.mothur.org) [46]. The alignment was further assembled following the sequence analysis pipeline in Mothur. Specifically, the SILVA database creates a 50,000-bp alignment covering the entire 16S gene, causing an abundance of gaps in the generated alignment. A filter removes the non-informative regions (e.g., gaps across all samples), and a soft filter removed all columns where a dominant base (A,G,C,T) occurred in <10% of the sequences. Additionally, a screen program removes the sequences that were poorly aligned. The final alignment consisted of an average of 351 bp in a 375-bp alignment with 4,897 sequences. An approximate maximum-likelihood phylogenetic tree was generated using the generalized time-reversible (GTR) substitution model in FastTree [41].

To better understand the bacterial composition between *S. geminata* and *S. invicta* ants, we randomly selected a larger subset of 2,000 sequences from each ant sample for workers, brood, and alate (exceptions: samples A5=847 sequences, B5=802, and C5=1,927). The CD-Hit clustering at 97% similarity produced 778 unique clusters from 17,169 total sequences. We performed the same alignment in Mothur and generated an approximate maximum-likelihood phylogenetic tree with the GTR model in FastTree [41].

We calculated a UniFrac significance and principal coordinate analyses (PCoA) using Fast UniFrac analysis to evaluate the degree of similarity of microbial communities associated with the different types of samples (alate, brood, worker, stored seeds, soil chamber, and soil outside) and ant species (ant samples from *S. geminata* nests versus *S. invicta* nests) [20]. UniFrac distances were calculated from phylogenetic distances, particularly the fraction of branch lengths shared between two samples. An unweighted UniFrac analysis only takes into account the branch length distances, whereas a weighted UniFrac calculation weights the branch lengths according to the sequence abundance. Thus, the unweighted UniFrac PCoA is a measure of community membership while the weighted UniFrac PCoA is a measure of community composition because it takes into account the

proportion of bacteria found in each sample [15, 32]. We performed both an unweighted and a weighted PCoA on the entire dataset including all sample types and also ran a PCoA with just the ant samples. We also tested all samples together for a UniFrac significance test without abundance weighting with 1,000 permutations. Finally, to evaluate whether *S. geminata* and *S. invicta* ants had significantly different bacterial communities when analyzed alone, we calculated two abundance pairwise UniFrac significance tests including the alate, brood, and worker samples, each with 1,000 permutations.

Results

Genus and Species Richness

We performed 454 pyrosequencing screens on three *S. geminata* and three *S. invicta* nests (32 samples in total) to characterize their bacterial communities. ESM Table 1 shows the BLAST results for each sample according to the closest genus designation (minimum match stringency at 75% sequence identity), and ESM Table 2 lists results to their closest resolved designation according to more stringent identity scores (species at >97%, genus at 95–97%, family at 95–90%, order at 85–90%, class at 80–85%, and phylum at 77–80%). The rarefaction analysis at a species level (1% sequence difference) indicates that roughly 5,000–10,000 sequences are needed per ant sample to thoroughly cover the species diversity, but more sequences may be necessary to reach an asymptote for soil samples (ESM Fig. 1). However, when analyzed at the genus level (3% sequence differences), we saw that many of the worker, brood, and stored seeds have reached an asymptote and have been adequately sampled at this taxonomic level. Although we undoubtedly undersampled rare bacterial species at our sequencing depth, common genera were adequately covered for the ant samples.

Figure 1 shows the range of microbial generic diversity (OTUs) identified per sample type. Soil from inside the chamber and soil from outside the nest had significantly higher generic richness (mean=196 genera, $n=10$) than ant workers (mean=42 genera, $n=5$; Mann–Whitney U test: $U=50$, $p=0.002$; Fig. 1). In the soil samples, 92% of the bacterial genera were rare (i.e., occurring at <1% abundance among their total sequences; see ESM Table 1).

We observed more bacterial genera from the two *S. invicta* (42 and 86 genera) worker samples than from the corresponding three *S. geminata* samples (22, 26, and 36 genera; Fig. 1). The bacterial genera that were more commonly detected (observed in abundances of >1% of the sequences per sample) are shown in Table 2 for the *S. invicta* and *S. geminata* ants. In two out of the three *S.*

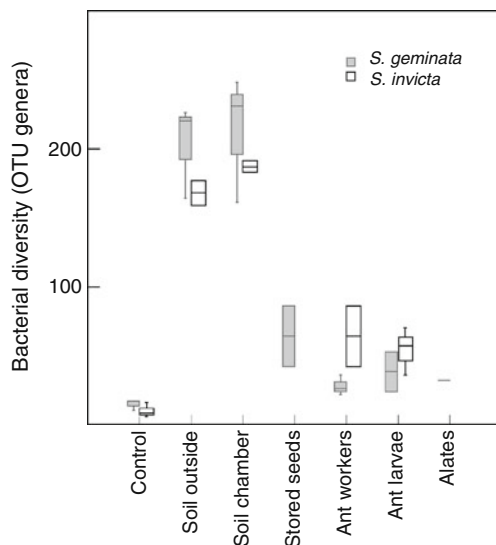


Figure 1 Bacterial diversity (number of OTUs) for ant life stages and soils in *S. invicta* and *S. geminata* colonies. Soils in and outside chambers have the highest bacterial diversity, whereas workers and brood have similar diversities, much lower than soils. *S. invicta* and *S. geminata* have similar diversity counts, although dissimilar community composition (see also Table 2). Box plots show data distributions by quartiles

geminata workers, 99.3% (A4, $N=8,193$) and 98.8% (A9, $N=9,741$) of the total sequences were identified to *Spiroplasma* via BLAST. The majority of these *Spiroplasma* sequences share a 96–97% similarity to *Spiroplasma platyhelix* (GenBank accession no. GU993266). We also detected low levels of *Spiroplasma* (0.03% of all sequences identified by the BLAST) from a *S. geminata* brood sample, as well as possible *Spiroplasma* sequences from a *S. invicta* brood with a BLAST identity match <75% (data not shown in table). When *Spiroplasma* sequences from *S. invicta* were compared to *Spiroplasma* from *S. geminata* sequences, we saw a minimum of 2% sequence divergence (meaning that 11–15 bases were different in the 500-bp fragments). Another noteworthy result is that the bacteria associated with the *S. invicta* brood (C5), which had 90% of its bacterial sequences assigned to *Lactococcus*.

Phylogenetic Analysis with Fast UniFrac

The unweighted UniFrac PCoA is a measure of community membership while the weighted UniFrac PCoA is a measure of community composition because this analysis additionally considers bacterial abundances [15, 32]. Here, unweighted UniFrac was able to distinctly separate all soil samples (outside and chamber) from the ant samples (alate, brood, worker) and the stored seed samples (Fig. 2a). The soil samples (outside and chamber) were tightly clustered, while the ant samples were more loosely clustered (Fig. 2a). In the weighted analysis, all but one of the soil samples remained

tightly clustered (Fig. 2b). However, the ant samples became more dispersed and one brood sample clustered closely with the stored seed samples. The combined PCo1 and PCo2 axes accounted for 29.2% of the total variation in the unweighted PCoA and 57.4% of the variation in the weighted PCoA. UniFrac significance test with all samples together showed that each sample had more unique branch lengths than expected by chance (corrected $p < 0.0001$). Finally, the PCoA for the bacterial communities of *S. geminata* and *S. invicta* ant samples (including alates, brood, and workers) are shown in ESM Fig. 2. This PCoA illustrates that *S. geminata* and *S. invicta* samples do not separate out into two clusters, but have similar bacterial communities.

Analysis of Control Samples

We included a negative control sample for each nest collection. 454 pyrosequencing was performed blind to the identity of the samples, and for every negative control, two rounds of sequencing were performed due to low sequence data in the first round. Thus, the number of bacteria sequences found in the negative controls has been artificially inflated. After undergoing two rounds of sequencing, an average of 1,821 sequences was obtained per control which comprised an average of 14 genera of bacteria. *Acidovorax*, *Acinetobacter*, *Clostridium*, *Comamonas*, *Diaphorobacter*, *Flexibacteraceae*, *Lactococcus*, *Pseudomonas*, *Sphingomonas*, and *Stenotrophomonas* were the most common bacteria found in these control samples. Overall, the negative controls were low in sequence numbers and in bacterial diversity. We saw no need to remove bacteria found in negative controls proportionally from the non-control samples because there was minimal overlap between bacteria in controls and samples. There may have been a collector influence in negative control samples B and D, which were both collected by the same person, because these samples shared bacteria (*Acidovorax*, *Diaphorobacter*, and a genus of *Flavobacteriaceae*) not found in the other controls. However, there was no evidence of collector contamination because these three bacteria were not in any of the non-control B and D nest samples (the only exception being a low percentage found in B7 alate samples).

Discussion

Bacterial Richness Patterns

Using high-throughput sequencing technology, we discovered some potentially important microbes, some observed for the first time in association with *Solenopsis* ants. The native *S. geminata* and the invasive *S. invicta* fire ant species had similar bacterial OTU richness, and

Table 2 Common bacteria genera and their relative abundances (% of total bacteria) identified by BLAST per each *S. geminata* and *S. invicta* sample

Sample	Worker					Brood				Alate
	<i>S. geminata</i>			<i>S. invicta</i>		<i>S. geminata</i>		<i>S. invicta</i>		<i>S. geminata</i>
	A4	A9	B5	C4	D4	A5	B6	C5	D5	B7
No. of raw sequence tags	8,202	9,754	802	12,049	2,197	847	3,214	1,927	5,381	3,914
No. of Blast matches (75–100%)	8,193	9,741	642	11,659	2,071	157	3,101	1,905	5,205	3,802
% Blast identified sequences	99.89	99.87	80.05	96.76	94.26	18.54	96.48	98.86	96.73	97.14
Acidimicrobiaceae genus	–	–	–	1.64	1.26	–	0.19	–	–	–
<i>Acidobacterium</i>	0.01	0.02	2.49	0.77	0.05	–	0.84	–	7.90	–
<i>Acidovorax</i>	0.02	–	–	–	–	–	–	–	–	1.42
<i>Acinetobacter</i>	0.20	0.01	15.42	8.49	2.22	16.56	2.16	0.26	0.15	6.15
<i>Actinomycetospora</i>	–	–	–	–	1.45	–	0.10	–	–	–
<i>Aeromicrobium</i>	–	0.02	0.16	3.10	2.99	–	0.06	–	–	–
<i>Agromyces</i>	–	–	–	–	4.06	–	0.03	–	–	–
<i>Anaeroplasma</i>	–	–	–	4.25	–	–	–	–	–	–
<i>Arthrobacter</i>	0.02	–	–	1.77	–	–	0.55	–	0.21	–
<i>Bacillus</i>	0.01	0.03	10.44	0.23	1.06	–	1.06	0.31	6.88	0.08
<i>Bacteroides</i>	–	–	–	0.01	–	–	2.45	0.05	0.02	4.63
<i>Bradyrhizobium</i>	–	–	–	0.07	5.02	–	0.03	–	–	–
<i>Brevundimonas</i>	–	–	–	–	–	–	–	–	2.04	–
<i>Caldilinea</i>	–	–	–	0.09	11.83	–	–	–	–	–
<i>Candidatus Solibacter</i>	–	–	–	0.01	–	–	–	–	1.19	–
<i>Clostridium</i>	–	0.01	–	0.01	–	–	1.06	–	12.91	–
<i>Comamonas</i>	0.02	–	3.74	0.30	–	–	–	–	–	–
<i>Conexibacter</i>	0.05	0.02	10.90	2.33	3.81	–	2.42	0.16	3.69	0.84
<i>Corynebacterium</i>	–	–	–	–	–	28.66	–	–	–	–
<i>Curtobacterium</i>	–	–	–	–	4.06	–	–	–	–	–
<i>Cystobacter</i>	–	–	–	–	–	–	–	–	9.84	–
<i>Duganella</i>	0.01	–	–	–	–	1.91	–	–	–	–
<i>Enterococcus</i>	–	–	0.93	–	–	–	–	4.36	–	–
<i>Erwinia</i>	–	–	–	–	–	2.55	–	0.05	–	–
<i>Exiguobacterium</i>	–	–	–	–	–	1.91	0.81	–	–	–
<i>Folliculinopsis</i>	–	–	–	–	–	–	–	0.47	5.53	–
<i>Frankia</i>	–	0.01	–	–	–	1.27	–	–	–	–
<i>Gemmatimonas</i>	–	–	–	0.03	0.14	1.27	–	–	–	0.39
<i>Geobacillus</i>	–	–	–	–	–	–	–	–	0.04	20.33
<i>Geopsychrobacter</i>	–	–	–	0.33	–	–	–	–	1.59	–
<i>Geothermobacter</i>	–	–	–	0.67	–	0.64	–	–	1.11	–
<i>Herbaspirillum</i>	–	–	–	–	–	–	0.06	–	–	11.10
<i>Janthinobacterium</i>	–	–	–	–	–	–	–	–	0.02	3.63
<i>Lactobacillus</i>	–	–	–	–	–	–	–	–	11.37	–
<i>Lactococcus</i>	–	–	–	0.29	–	–	0.13	90.60	–	–
<i>Loktanella</i>	–	–	–	–	–	–	–	–	2.23	–
<i>Marmoricola</i>	0.02	–	7.79	9.82	10.00	–	0.29	–	0.02	5.44
<i>Mesorhizobium</i>	–	–	–	–	–	–	–	–	–	16.02
<i>Methylibium</i>	–	–	–	–	–	–	–	–	–	1.79

Table 2 (continued)

Sample	Worker					Brood				Alate
	<i>S. geminata</i>			<i>S. invicta</i>		<i>S. geminata</i>		<i>S. invicta</i>		<i>S. geminata</i>
	A4	A9	B5	C4	D4	A5	B6	C5	D5	B7
<i>Methylobacterium</i>	–	–	1.25	–	–	–	0.06	–	–	–
<i>Methylocystis</i>	–	0.01	–	–	2.27	–	–	–	–	–
<i>Moellerella</i>	–	–	–	–	–	–	29.41	–	–	–
<i>Morganella</i>	–	–	–	–	–	–	2.13	–	–	–
<i>Mycobacterium</i>	0.07	0.01	0.78	3.27	0.34	–	–	–	0.54	1.03
<i>Nitrosovibrio</i>	–	–	–	2.07	–	–	–	–	–	–
Nocardioideae genus	0.02	–	4.83	17.94	26.17	–	–	–	0.69	3.58
<i>Nocardioides</i>	–	0.02	5.45	27.03	4.78	5.10	0.48	0.26	0.04	0.05
Paenibacillaceae genus	–	–	–	–	–	–	1.16	–	–	–
<i>Paenibacillus</i>	0.01	0.01	–	0.03	–	1.27	0.39	–	–	–
<i>Pantoea</i>	–	–	0.16	–	–	6.37	–	0.21	–	–
<i>Patulibacter</i>	–	–	2.02	2.06	–	–	2.35	0.16	0.38	–
<i>Propionibacterium</i>	0.02	–	5.45	–	–	–	–	–	1.36	1.74
<i>Pseudomonas</i>	0.04	–	6.70	1.18	0.05	19.75	0.87	0.10	2.06	–
<i>Pseudonocardia</i>	–	–	–	0.43	3.09	–	0.13	0.10	–	–
<i>Rahnella</i>	–	–	–	–	1.01	–	–	–	–	–
Rhizobiales genus	–	–	–	–	–	–	–	–	1.63	–
Rickettsiaceae genus	–	–	–	–	–	–	–	–	–	14.81
<i>Schlegelella</i>	–	–	–	–	–	–	–	–	2.36	–
<i>Solirubrobacter</i>	0.01	–	1.56	0.02	0.43	–	0.48	–	6.17	–
<i>Sphingomonas</i>	–	–	5.14	0.09	7.34	–	–	–	0.79	0.13
<i>Spiroplasma</i>	99.29	98.83	–	–	–	–	0.03	–	–	–
<i>Staphylococcus</i>	–	–	–	–	–	–	42.37	–	–	–
<i>Stenotrophomonas</i>	–	–	4.98	0.81	0.68	0.64	0.26	0.21	–	–
<i>Streptococcus</i>	–	–	0.78	0.32	–	5.10	0.00	0.26	–	–
<i>Streptomyces</i>	0.01	0.01	–	3.89	2.37	–	–	–	0.02	–
<i>Subaequorebacter</i>	–	–	1.56	–	–	–	–	–	–	–
<i>Sulfurimonas</i>	–	–	–	–	–	–	–	–	3.48	–
<i>Terrabacter</i>	–	–	2.18	–	–	–	–	–	–	–
<i>Thalassobacter</i>	–	–	–	–	–	–	–	–	5.34	–
<i>Vagococcus</i>	–	–	–	–	–	–	3.00	–	–	–
<i>Wolbachia</i>	–	–	–	–	–	–	–	–	–	3.31
<i>Zoogloea</i>	–	–	–	–	0.10	1.27	–	–	–	–
Total	99.9	99.0	94.7	93.3	96.6	94.3	95.4	97.6	91.6	96.5

The BLAST hit for each sequence was analyzed separately for each sample providing relative abundance information within and among the individual samples based upon relative numbers of sequences within each. For better visualization of important bacteria genera, the samples with >1% of their relative abundance per bacterial genus have been indicated in bold. Bold font also identifies the genera that have one or more sample with a relative abundance >10% and perhaps have an important role with *Solenopsis* ants

^a Total BLAST identity matches and abundances including the rare bacteria can be found in ESM Table 1

the sequence BLAST results did not identify any specific microbial genera that were consistently present in one species, but absent in the other. Likewise, UniFrac analysis was unable to detect a distinct separation between *S. geminata* and *S. invicta* ants, suggesting that these

two species do not have a vastly different bacterial community. This result is not surprising given that *S. invicta* has been established in North America since the 1930s and considering that all our ant collections came from the same site.

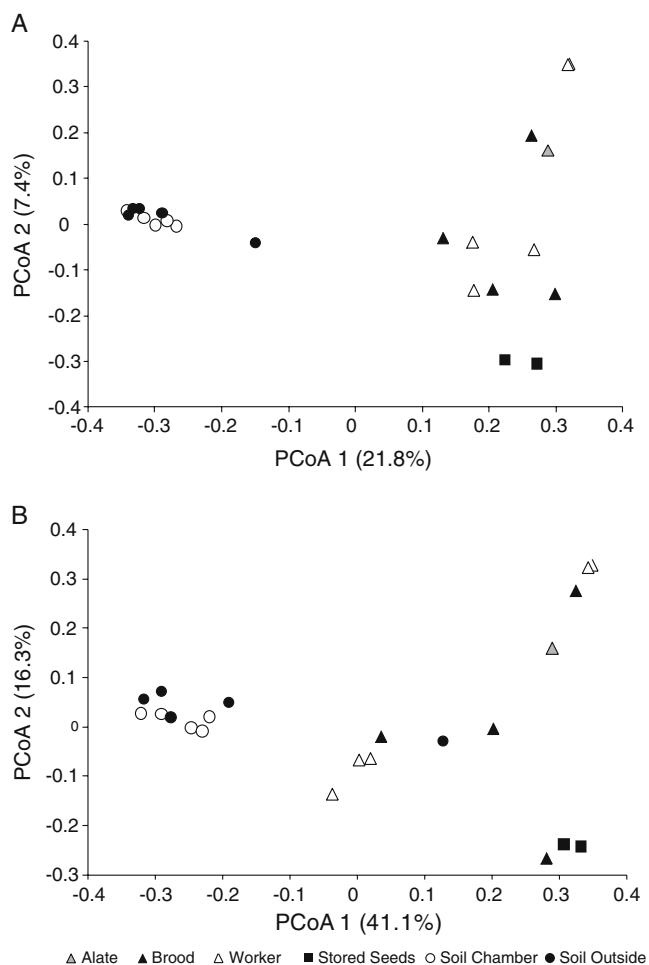


Figure 2 UniFrac unweighted principal coordinate analyses (PCoA) (a) and weighted PCoA (b) for all samples. In the unweighted PCoA (a), the soil, stored seeds, and ant samples cluster with their similar types. In the weighted PCoA (b), the ant samples spread out and become more similar to one outside soil sample and one brood sample clusters closely with the stored seed samples

Although soil samples were undersampled (ESM Fig. 1), ants of all life stages (brood, alates, and workers) contained a significantly lower diversity of bacteria (average of 42 bacterial genera) than either the chamber soil or the surrounding soils (average of 196 bacterial genera; Fig. 1). UniFrac analysis was unable to detect differences between soil samples collected from inside the nest and those collected outside. Both weighted and unweighted UniFrac analyses had soil samples tightly clumped together with the exception of one outside soil in the weighted analysis. This suggests that they shared similar community composition and membership (Fig. 2).

Ecological Functions of Particular Microbes

Ant colonies have evolved diverse defenses and immune responses against pathogenic microbes, such as grooming

behavior, antimicrobial secretions, and relocation of nests [36, 47], all of which reduce the accumulation of and exposure to detrimental microbes. However, beneficial microbial endosymbionts can also contribute to immune and defense responses of insects [10, 23]. A logical starting point for the identification of beneficial microbes is the relative abundance of specific microbial associates. A prime candidate in our survey is *Spiroplasma*, which we found abundantly in two of the *S. geminata* colonies and in low abundance in one colony of *S. invicta* (E4), with a 2% sequence difference between the *Spiroplasma* sequences derived from *S. geminata* and *S. invicta* colonies.

Spiroplasma has been intensively studied in *Drosophila* where its interactions have been variously classified as commensal, mutualistic, or pathogenic [19, 42]. In particular, *Spiroplasma* has gained wide attention as a pathogen or sex ratio distorter in multiple orders of insects, including Hymenoptera [8, 35], but *Spiroplasma* has recently been shown to act as a defensive mutualist in *Drosophila* [23, 61]. *Spiroplasma* has not previously been reported in *Solenopsis* ants, but was detected in *Cyphomyrmex wheeleri* and *Mycocepurus smithii* in a 16S amplicon 454 pyrosequencing screen [48]. The role of *Spiroplasma* in the biology of *Solenopsis* fire ants remains to be determined, but the remarkably high abundance of *Spiroplasma* in several colonies suggests an important relationship. Because *Spiroplasma* are comparatively small bacteria (about 200 nm long \times 3–12 nm wide) [9], they are some of the few microbes capable of passing through the 880-nm pharyngeal filter into the guts of adult workers [16]. Following initial discovery of *Spiroplasma* sequences in the samples, we confirmed the presence of *Spiroplasma* in *S. invicta* and *S. geminata* fire ant colonies from three other sites using the 16STF1 and 16STR1 primers described by Haselkorn et al. [21] (D. Estrada and R. Plowes, unpublished).

The high incidence of *Lactococcus* in the brood of one colony of *S. invicta* is also noteworthy as it confirms similar findings in two previous studies [29, 38]. *Lactococcus* are fermentative bacteria that produce lactic acid from sugars, and may serve an important role in the larval digestive system. In comparison with previous surveys, our assay did not recover any of the common bacteria reported by Baird et al. [3], but we did find *Staphylococcus* in the brood of *S. geminata*, as also reported by Peloquin and Greenberg [38]. *Wolbachia* was also found in the alate sample of *S. geminata*. *Wolbachia* has previously been found in *S. invicta*, with higher prevalence in monogyne colonies than in polygyne colonies. Impacts of *Wolbachia* in *Solenopsis* are unknown [49], and in some insects, this bacterium may function as a defensive symbiont [52]. Almost all *Spiroplasma*-infected *Drosophila* also carry *Wolbachia* [22], but in our study, we did not find any case of co-occurrence of these two microbes.

The occurrence of several bacteria, albeit at low levels, in the negative controls indicated that some collector contamination occurred, and future collections may be improved by use of sterile facemasks. Some of the species found in negative controls (*Pseudomonas* and *Lactococcus*) were also reported by Li et al. [29] as being present in fourth-instar gut contents. We recommend that all 16S amplicons 454 pyrosequencing surveys should include negative controls to factor out possible contamination through storage containers, preservatives, or experimental procedures.

Potential Sources of Variation in Fire Ant Microbial Communities

This study considered colonies from two species in immediate proximity, thus controlling for habitat effects, but the influence of many other factors was not controlled in our sampling scheme. Potential variation in microbial communities between *S. invicta* and *S. geminata* might result from several factors, including (1) colony age, (2) worker age, (3) colony social form (monogyne vs polygyne), (4) species diet differences, or (5) the effects of the invasion process. It is possible that because *S. invicta* is an invasive species, its small founding populations may have carried a different, and possibly less diverse, microbial community when it was established in the 1930s. However, after about 80 years in North America, *S. invicta* carries a broad array of microbes, comparable to the native fire ant *S. geminata*.

The role of microbes in shaping the success of ant invasions may depend on escape from pathogens in the original home range, a lack of virulent pathogens in the introduced range, or the availability of new mutualistic microbes in the invasion zone. While little is yet known of the specific functions of the bacteria found in this survey, such work will contribute to insights in the role of microbes during ant invasions.

Importantly, given that we found significant overlap in the microbiomes of *S. geminata* and *S. invicta*, biological control of invasive fire ants through infection with detrimental bacteria may not be straightforward. Attempts to introduce genetically modified microbes to control invasive ant species [33] must proceed with great caution to prevent spread of biocontrol microbes to other ant species.

Because the high numbers of *Spiroplasma* sequences identified in two out of the three *S. geminata* worker samples may have masked other important microbes in these samples, the observed variation between the two *Solenopsis* ant species may have been underestimated in this study. More refined sampling (e.g., gut versus external microbes; different body parts of individual ants) may overcome the problem of swamping by a

single microbial lineage and thus improve comparisons between host species.

Strengths and Limitations of 454 Pyrosequencing

Traditional methods for studying microbial communities have utilized culture-dependent methods which are known to vastly underestimate the true diversity of microbes present in a sample [48]. 454 pyrosequencing is culture-independent, which enables screening of bacterial lineages that were not captured by traditional culture-dependent methods. Thus, for the amount of information obtained, it appears that pyrosequencing is cost-effective and less biased than traditional bacterial screening methods [31, 48]. Rapid comparisons between life stages and between species are possible using this approach. While we found no large differences between the microbial communities of invasive and native fire ants, the generated sequence information obtained provided novel insights into microbial associations in *S. invicta* and *S. geminata* ants (e.g., *Spiroplasma* in workers; *Lactococcus* in brood). More comprehensive sampling is needed to characterize all the sources of variation between microbial communities associated with *Solenopsis* ants.

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