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Research Article

Bacterial Infections across the Ants: Frequency and Prevalence of *Wolbachia*, *Spiroplasma*, and *Asaia*

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Bacterial endosymbionts are common across insects, but we often lack a deeper knowledge of their prevalence across most organisms. Next-generation sequencing approaches can characterize bacterial diversity associated with a host and at the same time facilitate the fast and simultaneous screening of infectious bacteria. In this study, we used 16S rRNA tag encoded amplicon pyrosequencing to survey bacterial communities of 310 samples representing 221 individuals, 176 colonies and 95 species of ants. We found three distinct endosymbiont groups—*Wolbachia* (Alphaproteobacteria: Rickettsiales), *Spiroplasma* (Firmicutes: Entomoplasmatales), and relatives of *Asaia* (Alphaproteobacteria: Rhodospirillales)—at different infection frequencies (at the ant species level: 22.1%, 28.4%, and 14.7%, resp.) and relative abundances within bacterial communities (1.0%–99.9%). *Spiroplasma* was particularly enriched in the ant genus *Polyrhachis*, while *Asaia* relatives were most prevalent in arboreal ants of the genus *Pseudomyrmex*. While *Wolbachia* and *Spiroplasma* have been surveyed in ants before, *Asaia*, an acetic acid bacterium capable of fixing atmospheric nitrogen, has received much less attention. Due to sporadic prevalence across all ant taxa investigated, we hypothesize facultative associations for all three bacterial genera. Infection patterns are discussed in relation to potential adaptation of specific bacteria in certain ant groups.

1. Introduction

Recent studies have shown that insects are associated with a broad range of unrelated microbial taxa [1, 2]. These interactions shape the ecology and evolution of hosts and bacterial symbionts and often heavily impact host biology [3, 4]. Congruent evolutionary histories between some symbiotic partners show the likely obligate nature of this relationship [5], while other associations occur sporadically and can vary both spatially and temporally [6]. Bacterial endosymbionts sometimes inhabit specialized host cells or structures [7, 8] and might even share metabolic pathways with their hosts [9], while others occur loosely in unspecific tissues or hemolymph [10].

Microbes associated with insects are extremely diverse and span-wide taxonomic groups, even within individual hosts. One of the best-characterized endosymbiont groups is comprised of insect-associated bacteria that increase the nutritive value of their hosts' diets. These bacteria are often highly specialized and coevolved associates, playing particularly important roles in insects with nutritionally limited or deficient diets. Some well-known examples of such endosymbionts include *Buchnera aphidicola* in aphids, which provide their hosts with essential amino acids lacking in the sugar-rich but nitrogen-poor phloem sap [11]. Other examples are the cospeciated and essential amino acid synthesizing *Blochmannia* endosymbionts of Camponotini ants [12, 13], nitrogen fixing taxa in the fungal gardens of the leaf-cutter ants [14], *Wigglesworthia glossinidia,* which provides vitamins that are lacking in the blood meals of its host, the tsetse fly [15], and the nitrogen-fixing microflora of termites [16, 17]. In ants, several recent studies have highlighted the importance of bacterial symbionts for nutrition, especially in ant taxa feeding low on the trophic scale [18–20].

Symbiotic bacteria can also play other beneficial roles by protecting insects from parasites and pathogens and thus

defending their hosts against natural enemies [4, 7, 21]. For example, *Spiroplasma* can convey increased resistance to nematode infections in *Drosophila* flies [22], and secondary symbionts in aphids can confer resistance to parasitic wasps [23]. Some insect-associated bacteria also contribute to nest hygiene [7]. For example, actinomycetes in the fungal gardens of leaf-cutter ants inhibit the growth of fungal pathogens, but not of mutualistic fungi [24]. Actinomycetes are also found in antennal glands of bee wolves and protect larvae in their nests against infestation by pathogens [25]. Other mutualistic bacteria can increase host tolerance to unfavorable abiotic conditions such as temperature stress [26] or facilitate the use of novel hosts [27].

While the associations described above are typically beneficial to hosts, many bacterial endosymbionts are detrimental reproductive manipulators. *Wolbachia*, for example, can cause cytoplasmic incompatibility, parthenogenesis, male killing, and male feminization [28]. There are also examples of *Wolbachia*, which protect their host against RNA viruses, thus acting as defensive mutualists [29]. An estimated 66% of insect species and about 30% of ant species have been reported to be facultatively infected with *Wolbachia* [30, 31]. Other less prevalent reproductive manipulators in insects include *Cardinium*, *Arsenophonus,* and *Spiroplasma* [32, 33]. *Spiroplasma*, although beneficial to hosts in some cases [22], can have various negative effects on their insect hosts, including manipulation of sex ratios, male killing, and entomopathenogenicity [33–35].

Despite these fascinating findings, our knowledge of bacterial symbionts is based on a relatively small number of organisms. Thus, we still know little about the identities and ecological or physiological functions of bacteria associated with most animal groups [36]. In-depth analyses and extensive surveys of the bacterial communities present in a wide range of eukaryotic taxa are required to understand the diversity and the function of microbial symbionts [37]. Here, we analyzed bacterial communities across the ants (Hymenoptera: Formicidae) using 16S rRNA tag encoded amplicon pyrosequencing (454 pyrosequencing) to survey for infection patterns with potential parasitic microbes. Due to their sporadic prevalence and unknown effects on host ant biology, we refer to these microbes as infections. In total, we screened 310 ant samples of 176 colonies from 95 ant species and encountered high prevalence of three bacterial groups: *Wolbachia*, *Spiroplasma,* and *Asaia*.

2. Materials and Methods

A total of 299 ant samples were subjected to 454 pyrosequencing and combined with data from 11 samples analyzed by Ishak et al. [38], that is for a total of 310 samples. All samples represented 176 different colonies and 95 different ant species belonging to the genera *Camponotus* (Formicinae; 1 species), *Cephalotes* (Myrmicinae; 7 species), *Crematogaster* (Myrmicinae; 6 species), *Myrmecia* (Myrmeciinae; 2 species), *Myrmecocystus* (Formicinae; 1 species), *Oecophylla* (Formicinae; 1 species), *Paraponera* (Paraponerinae; 1 species), *Polyrhachis* (Formicinae; 32 species), *Pseudomyrmex* (Pseudomyrmecinae; 36 species), *Solenopsis* (Myrmicinae; 2 species) and *Tetraponera* (Pseudomyrmecinae; 6 species). DNA extractions were either prepared from entire ants or from dissected ant parts as described in Kautz et al. [39]. A complete list of samples used for this study can be found in Supplementary Table 1 (see Supplementary material available online at http://dx.doi.org/10.1155/2013/936341).

2.1. 454 Pyrosequencing. To screen ant samples for overall bacterial diversity, bacterial tag-encoded FLX amplicon pyrosequencing was performed by the Research and Testing Laboratory (Lubbock, TX, USA) as described by Dowd et al. [40]. The 16S rRNA universal eubacterial primers 28F (5′-GAGTTTGATCITGGCTCAG) and 519R (5′-GWATTACCGCGGCKGCTG) were used to amplify approximately 500 bp of the variable regions V1–V3.

2.2. Bacterial 16S rRNA Data Processing and Analysis. All 16S rRNA pyrosequencing reads were quality controlled and denoised using the QIIME v1.5.0 implementation of AmpliconNoise v1.25 using default parameters [41]. Chimeras were removed by Perseus, a component of the AmpliconNoise pipeline [42]. All the remaining reads were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UCLUST [43]. We used the longest sequence in a cluster as the representative sequence for that OTU. Singletons, that is, OTUs with only one read in the entire dataset, were removed. We used the QIIME implementation of the Ribosomal Database Project [44] classifier trained on the February 4, 2011 release of the greengenes database [45] to classify OTUs at the level of bacterial orders. Default settings were used, including a 0.8 confidence cutoff for classifications.

Our filtering approach recovered infections with *Wolbachia* (Alphaproteobacteria: Rickettsiales), *Spiroplasma* (Firmicutes: Entomoplasmatales), and *Asaia* (Alphaproteobacteria: Rhodospirillales). All OTUs classified as Rickettsiales, Entomoplasmatales, and Rhodospirillales that accounted for more than one percent of reads within a sample were considered as infections by the respective order and included in further analyses. This cutoff also allowed us to control the relatively high error rate of 454 pyrosequencing. We classified the sequences at the genus level using the RDP classifier (see Supplementary Table 2 for results). All OTUs used in further analyses have been deposited in GenBank (accessions KF015767-KF015856; Supplementary Table 2).

We downloaded the closest relatives of each OTU from GenBank. Additionally, we were interested in retrieving any other sequence from GenBank of those three orders that were associated with ants and insects in general. Thus, we searched for sequences using the search keywords "16S" and "symbiont" as well as the name of the respective order. GenBank sequences with 99% identity that were isolated from the same source were considered duplicates and deleted from the dataset.

2.3. Phylogenetic Tree Construction. Sequences were compiled and edited using Geneious v5.3.6 [46]. The alignment

was generated using the infernal secondary-structure-based aligner of the ribosomal database project (RDP) [44]. We inferred a maximum likelihood phylogeny of the most common OTUs and their GenBank relatives using the RAxML 7.2.8 Black Box [47] on the CIPRES web portal [48]. The model GTR+I+G was employed. We then uploaded the most likely tree to the iToL website [49] to facilitate graphical illustration of bacterial source, ant subfamily, and geographic region for each sequence. Trees with branch length and bootstrap support are provided as supplementary material (Figures S1–S3).

3. Results and Discussion

3.1. Wolbachia (Alphaproteobacteria: Rickettsiales). In our study, 21 of 95 ant species had at least one individual infected with *Wolbachia* (Table 1). Across all 304 samples from which we obtained data (Supplementary Table 1), we found 30 *Wolbachia* OTUs. Overall, with 22.1% of infected species this is a lower infection rate of *Wolbachia* across ants than has been reported before. In an extensive compilation of existing data, about 28.6% of ant species carried *Wolbachia* infections [31], while a frequency of up to 50% had been found previously [50]. This discrepancy from our study to general trends could be due to several reasons. Often a species is counted as being infected with *Wolbachia* when just one individual carries this infection. However, not all individuals of a species or individuals from the same colony need to be infected. Thus, discrepancies in infection rate across studies might merely be due to natural variation among individuals. Also, there is a strong bias in infection rate among different ant groups. Species from the genera *Acromyrmex*, *Formica*, *Solenopsis,* and *Tetraponera* are often infected with *Wolbachia,* while *Dolichoderus* and *Leptogenys* mostly lack infection [51]. For example, in a screening of 24 *Polyrhachis* species, 5 (20.8%) were infected with *Wolbachia* [31]. In the present study, we found the genera *Cephalotes* (57%) and *Solenopsis* (50%) to have particularly high infection rates, *Tetraponera* (33.3%) and *Polyrhachis* (25.0%) with intermediate rates, *Crematogaster* (16.7%) and *Pseudomyrmex* (13.9%) with rather low rates, and no infections in the samples of *Camponotus*, *Myrmecia*, *Myrmecocystus*, *Oecophylla,* and *Paraponera* included here.

Most studies that screen for *Wolbachia* use diagnostic approaches by conducting PCR with *Wolbachia*-specific primers. This is the most reliable means of *Wolbachia* detection [51]. However, even when using diagnostic PCR, negative results can occur due to variations in the primer sequence or low titers of the bacterial symbionts [52]. In our study, we found high variability in *Wolbachia* titers, ranging from 1.03% to 97.36% (Supplementary Table 1). We used a 1% relative abundance within a sample as the cutoff to control error rates of 454 pyrosequencing, which might also have led to lower detected infection rates among species.

In addition to the 30 *Wolbachia* sequences obtained in this study, we downloaded sequence data from GenBank and compiled a dataset of 111 taxa including the outgroup *Rhizobium leguminosarum* (Alpha-proteobacteria: Rhizobiales). The total alignment had a length of 1224 characters. Four ant-specific clades of *Wolbachia* were recovered in the inferred tree (Figure 1; Figure S1). Ant clade 1 comprised *Wolbachia* that was isolated from Australian *Polyrhachis* (6 sequences) as well as one sequence detected in *Cephalotes varians* from the Nearctic. Ant clade 2 included mostly Australian *Polyrhachis* (9 sequences) in addition to sequences found in Nearctic *Solenopsis* and Neotropical *Pseudomyrmex*. Ant clade 3 exclusively contained sequences from European *Formica* species, while ant clade 4 was the most diverse. This fourth clade comprised the majority of ant-associated *Wolbachia* sequences from our dataset as well as existing GenBank data and included the ant subfamilies Dolichoderinae, Ecitoninae, Formicinae, Myrmicinae, Ponerinae, and Pseudomyrmecinae from the Afrotropics, Nearctics, Neotropics, and Palearctics. Overall, 68 out of 82 (82.9%) ant-associated *Wolbachia* sequences clustered in ant-specific clades indicating a certain degree of host specialization. Even though neither ant relatedness (subfamily) nor biogeographic region (continent) was a strong determinant for infection with similar *Wolbachia* strains, related *Wolbachia* seemed to infect related hosts from the same geographic region to some extent. A rather low degree of host specificity has previously been reported for *Wolbachia* across ants and butterflies, while strict cospeciation between *Wolbachia* and its hosts has not been found [51, 53].

Wolbachia are reported to be the most prevalent bacterial symbionts across insects and ants [31], although infections with other bacterial groups were often more frequent in our present study. Despite this ubiquity, to date no studies have been able to show the functional role of *Wolbachia* in ants. This is due to the difficulty of breeding most species of ants in the laboratory, and thus, we have to restrict our knowledge to the correlations of *Wolbachia* infections with specific host traits. *Wolbachia* most commonly manipulate host reproduction, but in ants no such phenomena are known [51]. In *Formica truncorum*, *Wolbachia* infection leads to a reduced production of sexuals, although this could be due to physiological costs rather than direct manipulation [54]. However, worker production is not affected and it has been suggested that *Wolbachia* might reduce the ability of workers to provide resources to alate development [51]. Curing of *Wolbachia* infection within individuals has been observed, which seems to be unique to ants, but the mechanisms behind this phenomenon are not understood [54]. Lastly, ants often show exceptionally high levels of coinfection with multiple *Wolbachia* strains adding another layer of complexity to this poorly understood symbiosis [51]. It has been speculated that eusociality or haplodiploidy might have an impact on *Wolbachia* infection [50, 55], but such mechanisms have never been confirmed. Also, there seems to be a weak correlation of *Wolbachia* infection with colony founding mode as species that found new colonies independently are less frequently infected than species relying on dependent colony founding [50]. Speculations on effects of *Wolbachia* on colony-founding behavior and colony structure have often been made as ants can show exceptional variations in these traits ranging from a single queen that mated once to multiple queens and/or multiple matings per queen [56–58].

Table 1: *Wolbachia*, *Spiroplasma*, and *Asaia* detected by 454 amplicon pyrosequencing across 310 ant samples.

Ant genus and subfamily	Species screened	Individuals screened	Colonies screened	Number (and percent) of infected species and number of individuals/colonies		
				Wolbachia	*Spiroplasma*	*Asaia*
Camponotus (Formicinae)	1	1	1	0	1 (100%) 1/1	0
Cephalotes (Myrmicinae)	7	17	12	4 (57.1%) 4/4	2 (28.5%) 6/3	1 (14.3%) 1/1
Crematogaster (Myrmicinae)	6	6	6	1 (16.7%) 1/1	0	0
Myrmecia (Myrmeciinae)	2	3	3	0	0	0
Myrmecocystus (Formicinae)	1	1	1	0	0	0
Oecophylla (Formicinae)	1	1	1	0	0	0
Paraponera (Paraponerinae)	1	23	9	0	1 (100%) 2/2	1 (100%) 1/1
Polyrhachis (Formicinae)	32	64	60	8 (25.0%) 10/10	15 (46.9%) 15/15	0
Pseudomyrmex (Pseudomyrmecinae)	36	88	72	5 (13.9%) 5/5	5 (13.9%) 5/5	12 (33.3%) 15/15
Solenopsis (Myrmicinae)	2	11	5	1 (50%) 1/1	2 (100%) 2/1	0
Tetraponera (Pseudomyrmecinae)	6	6	6	2 (33.3%) 2/2	1 (16.7%) 1/1	0
Total	95	221	176	21 (22.1%)	27 (28.4%)	14 (14.7%)

3.2. Spiroplasma (Tenericutes: Entomoplasmatales). A total of 27 (28.4%) ant species were infected with *Spiroplasma* relatives (Mollicutes: Entomoplasmatales) leading to one of the highest frequency estimates of this bacterial group across the ants to date (Table 1). Previously, an infection rate of 6.2% across ant species had been reported, and the infection rates of approximately 6% were documented for Coleoptera, Diptera, Hymenoptera, and Lepidoptera in general, while 23.1% of spiders (Araneae) carried *Spiroplasma* symbionts [31]. There appears to be a strong bias towards certain groups of ants that are more often associated with this group of bacteria [31, 59]. The ant genus *Polyrhachis* showed a high infection rate of 46.9% (15 of 32 species were infected). The phenomenon of enriched *Spiroplasma* symbionts in this ant genus is in line with a study by Russell et al. [31] and is particularly interesting as ants of the tribe Camponotini, to which *Polyrhachis* belong, carry obligate *Blochmannia* endosymbionts, which are housed in specific bacteriocytes and provide essential amino acids to the ant host [12, 13]. Studying the prevalence of spiroplasmas in more genera of the Camponotini, particularly the hyperdiverse genus *Camponotus*, would reveal whether these bacteria are likely to interact within their hosts. Infections per species were high in *Camponotus* (1/1), *Paraponera* (1/1), and *Solenopsis* (2/2). However, these values are not representative due to the low number of species included. Outside of *Polyrhachis,* infection rates were moderate in the better sampled genera *Cephalotes* (2/7), *Pseudomyrmex* (5/36), and *Tetraponera* (1/6). No infection was detected in *Crematogaster*, *Myrmecia*, *Myrmecocystus*, and *Oecophylla* (Table 1). Again, sampled species numbers were low for these ant genera so infection frequency can only be regarded as preliminary.

An alignment of 175 taxa and 1311 characters was generated including *Selenomonas ruminantium* (Firmicutes: Selenomonadales) as an outgroup. In this molecular phylogeny, three large ant-specific clades of spiroplasmas were identified: ant clade 1 that includes endosymbionts of *Cephalotes*, *Solenopsis*, *Tetraponera*, *Pseudomyrmex* and *Neivamyrmex*; ant clade 2 that comprises spiroplasma-associates of the ant genera *Polyrhachis*, *Camponotus*, *Pseudomyrmex,* and *Cephalotes;* and ant clade 3 which was dominated by army ants (subfamilies Aenictinae, Dorylinae, and Ecitoninae) (Figure 2). Additionally, several small clades containing only ant-associated spiroplasmas were scattered throughout the phylogeny as well as several individual ant-associated OTUs. Overall, bioregion did not seem to be a strong predictor for relatedness among *Spiroplasma* symbionts (Figure 2; Figure S2).

Clade 3, which is dominated by army ants from the New and Old World, has been identified before [60]. In our analysis, GenBank-derived *Spiroplasma* sequences that were isolated from the ant genera *Odontomachus* and *Pachycondyla* also fell into this clade (Figure 2). Army ants are characterized by the "army ant syndrome" of nomadism and group predation [61]. Due to their specialized diet and a weak correlation of Entomoplasmatales infection with trophic position, a nutritive symbiosis between army ants and Entomoplasmatales has been suggested [60]. Even though this clade of Entomoplasmatales is highly dominated by army ants, the association is not obligate as infection rates vary with respect to species and individuals, and the symbionts are not necessary for host development and reproduction [60]. As Entomoplasmatales are generally absent in eggs and larvae, horizontal transmission is assumed.

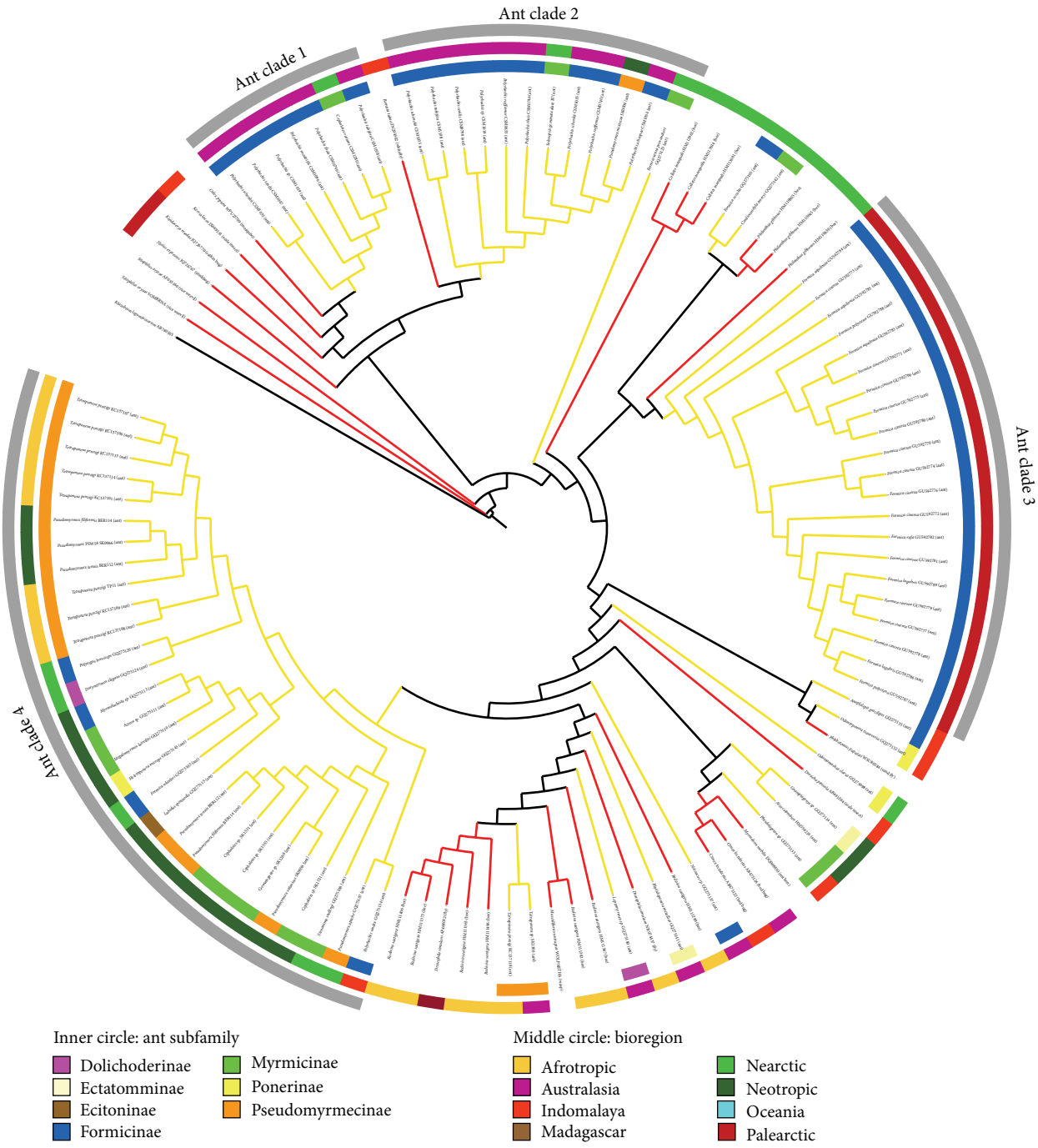

FIGURE 1: Phylogenetic tree of *Wolbachia* symbionts associated with ants and their closest relatives with sequence data available in GenBank. A maximum likelihood phylogeny of the 16S rRNA region of bacterial symbionts is shown. The host name is given together with the GenBank accession number (GenBank sequences) or collection code (sequences generated in the present study). Yellow and red branches represent bacteria isolated from ant hosts and other insect hosts, respectively. The inner circle shows ant subfamily, and the outer circle refers to the continent from which host organisms were collected. Four ant-specific clades of *Wolbachia* symbionts are highlighted (Ant clades 1–4). *Rhizobium leguminosarum* was used as an outgroup.

Even outside the army ants, a certain degree of host specificity of Entomoplasmatales bacteria is evident from our phylogeny and has been described for ants, *Drosophila,* and other arthropod-associated spiroplasmas [60]. In our molecular phylogeny, clades 1 and 2 exclusively contained ant-associated Entomoplasmatales (Figure 2). However, both clades contained symbionts from different ant subfamilies and biogeographic regions indicating that neither phylogeny nor geographic range drives the association with these symbionts, and repeated environmental acquisition is common.

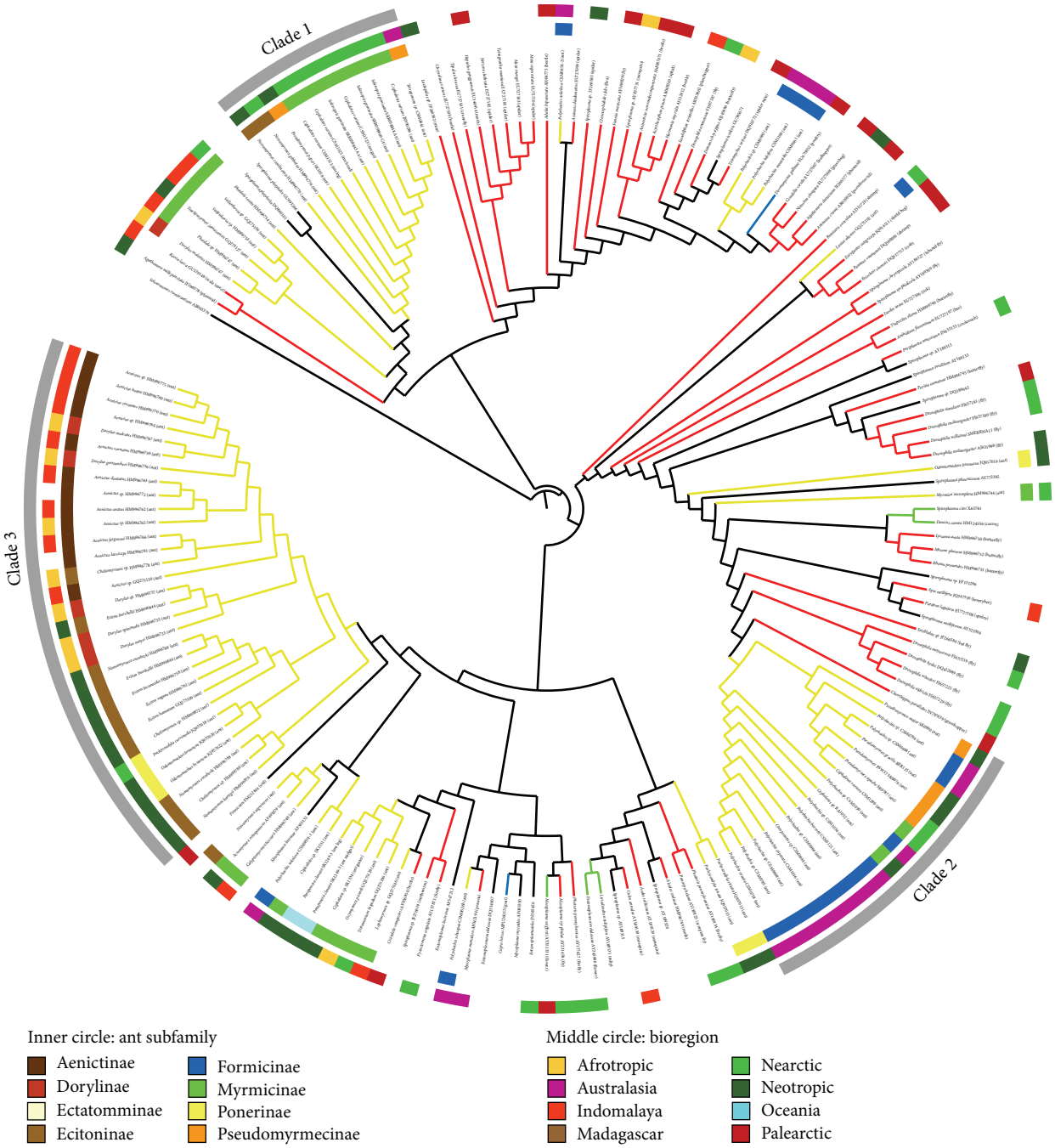

Figure 2: Phylogenetic tree of *Spiroplasma*-related ant symbionts and their closest relatives with sequence data available in GenBank. A maximum likelihood phylogeny of the 16S rRNA region of bacterial symbionts is shown. The host name is given together with the GenBank accession number (GenBank sequences) or collection code (sequences generated in the present study). The branch color refers to the source from which the bacteria were isolated with yellow representing ant hosts, red other insect hosts, blue vertebrates, and green plants. The inner circle refers to the ant subfamily, and the outer circle refers to the continent from which samples were collected. The three largest ant-specific clades of *Spiroplasma* symbionts are indicated (Clades 1–3). *Selenomonas ruminantium* was used as an outgroup.

The infection with *Spiroplasma* seems to be systemic, as we found high titers of this bacterium in association with ant guts, heads, and legs (Supplementary Table 1).

Entomoplasmatales can be pathogenic to plants and vertebrates [59, 62] and have been isolated from various insect taxa including aphids, ants, bees, beetles, butterflies, fruit flies, and horse flies [63–68]. Mutualistic spiroplasmas can grant insects resistance to parasitic nematodes [22] and an increased ability to overwinter [69]. Pathogenic phenotypes usually lead to insect death [34] and reproductive manipulation includes altered sex ratios [33] and male killing [35, 70, 71]. In ants, spiroplasmas have been surveyed, and

biocontrol potential has been hypothesized, but their role remains elusive to date [31, 38, 60]. Functional studies that compare the performance of infected and uninfected individuals would improve our understanding of the role of these facultative symbionts.

3.3. Asaia (Alphaproteobacteria: Rhodospirillales). Of 95 ant species, 14 hosted bacteria related to *Asaia* (Alphaproteobacteria: Rhodospirillales) (Table 1). For these bacteria, no previous surveys on their prevalence across the ants have been conducted. We found a particularly high infection rate of 33.3% (12/36 species) in *Pseudomyrmex*. In contrast, *Asaia*-related symbionts were lacking in *Camponotus*, *Crematogaster*, *Myrmecia*, *Myrmecocystus*, *Polyrhachis*, *Oecophylla,* and *Solenopsis*. Low infection frequency was present in *Cephalotes* (1/7) and *Paraponera* (1/1 species) (Table 1). The enrichment of *Asaia* symbionts in *Pseudomyrmex* is particularly interesting as this ant genus is arboreal and contains several obligate plant ants, which exclusively feed on plant-derived food sources [58, 72]. However, this bacterial group occurred facultatively in arboreal generalists and plant mutualists alike indicating that even if these symbionts are more frequent in arboreal or mutualistic *Pseudomyrmex* ants, the association is not obligate.

In total, we obtained 25 *Asaia*-related OTUs in our dataset. Of these OTUs, 21 were associated with *Pseudomyrmex*, 3 with *Paraponera,* and 1 with *Cephalotes*. We inferred a maximum likelihood phylogeny of these OTUs, their closest GenBank relatives, and other endosymbiotic Rhodospirillales bacteria from GenBank. The total alignment consisted of 91 taxa and had a length of 1313 characters. We used *Wolbachia pipientis* (Alphaproteobacteria: Rickettsiales) as an outgroup. The phylogenetic tree shows three clades in which ant-associated *Asaia* OTUs cluster together (Figure 3): (1) a small clade with two *Pseudomyrmex*-associated OTUs and one *Paraponera*-associated OTU, (2) a clade that appears to be Hymenoptera specific containing the bulk of *Pseudomyrmex*-associated OTUs, a *Formica*-associated sequence from GenBank, and bacteria isolated from several bee species, and (3) a clade comprised of many insect-associated *Asaia* bacteria and five of our OTUs. This last clade is particularly interesting as it comprised several strains that were isolated from different mosquito species as well as three ant-associated *Asaia* sequences from GenBank. One sequence (JF514556), was isolated and cultivated from *Tetraponera rufonigra* in India [73]. The *nifH* gene, a gene associated with the fixation of atmospheric nitrogen, has also been found in this bacterium (GenBank accession JF736510) and it has been experimentally shown that this strain is capable of fixing nitrogen *in vitro* suggesting possible nitrogen fixing attributes in its natural environment, the ant body cavity [73]. The two other sequences are cultivated bacteria from *Cephalotes varians* and were generated in the framework of a previous study from our lab (GenBank accessions JX445137 and JX445138) [39].

Bacteria from the family Acetobacteraceae are commonly known as "acetic acid bacteria" and have the metabolic capacity to oxidize ethanol to acetic acid [74]. *Asaia*, also a member of the Acetobacteraceae, however, only weakly oxidizes ethanol and shows higher rates of sugar oxidation [74]. These bacteria are environmentally ubiquitous, but have also been found in association with insects, such as bees [75, 76], mosquitoes [77], *Drosophila melanogaster* [78], leafhoppers [79], and mealybugs [80]. All these insects rely on sugar-rich and often nitrogen-limited diets, and it has been suggested that the bacteria function as nutritional symbionts. Some acetic acid bacteria have the capacity to fix atmospheric nitrogen [73]. However, it remains entirely speculative whether this function can be retained in the insect gut environment and whether these bacteria actually contribute to insect nitrogen metabolism or recycling [81]. Interestingly, neither acetic acid bacteria nor lactic acid bacteria are commonly found in the core gut microbiota of arboreal Cephalotini ants, an ant group with one of the most thoroughly studied microbiomes [18, 19, 39]. The metabolic capacities of the core gut microbiota of the Cephalotini consisting of Burkholderiales, Opitutales, Pseudomonadales, Rhizobiales, and Xanthomonadales might be redundant with the role that acetic acid bacteria play in other insects.

In *Drosophila,* acetic acid bacteria are part of the normal commensal bacterial gut community and can be involved in the regulation of the innate immune system. In healthy flies, a stable equilibrium of different gut microbes is maintained. Perturbation of the normal gut community, which can be caused by a defective regulation of antimicrobial peptide, leads to the dominance of the pathogenic commensal *Gluconobacter morbifer* and ultimately to gut apoptosis [82]. Potential other mechanisms by which acetic acid bacteria benefit insect immunity are by decreasing the gut pH making it an unfavorable environment for pathogenic microorganisms or by competitive exclusion [81]. However, these acetic acid bacteria are not essential for the fitness and reproduction of most insects as even in the well-studied *Asaia*-mosquito interaction, experimental removal of bacteria had no detectable negative impact on the host [81].

Several studies have been conducted to analyze the microbial diversity associated with ants [18, 19, 39, 60, 83]. However, the symbiotic relationships of ants with Rhodospirillales have rarely been observed. In fact, only two ant-associated Rhodospirillales sequences had been deposited in GenBank (GQ275104 from *Formica occulta* and JF514556 from *Tetraponera rufonigra*) prior to work from our group [39]. Clone libraries generated for the Cephalotini ants [18, 19] as well as tag-encoded amplicon data sets [38, 39] are among the most extensive microbial data collections available for ants to date, and acetic acid bacteria were only sporadically associated with the ant taxa that were investigated. Thus, the interaction of *Asaia* relatives with ants is generally poorly understood, but due to the metabolic capacities of these bacteria to utilize sugar-rich substrates and fix nitrogen, they might play an important nutritional role. Particularly, they might be functionally important in the ant subfamily Pseudomyrmecinae, in which they seem to be enriched as indicated by our present study.

The phylogenetic history of ant-associated Rhodospirillales does not show host specificity and suggests likely acquisition from the environment (Figure 3). These observations

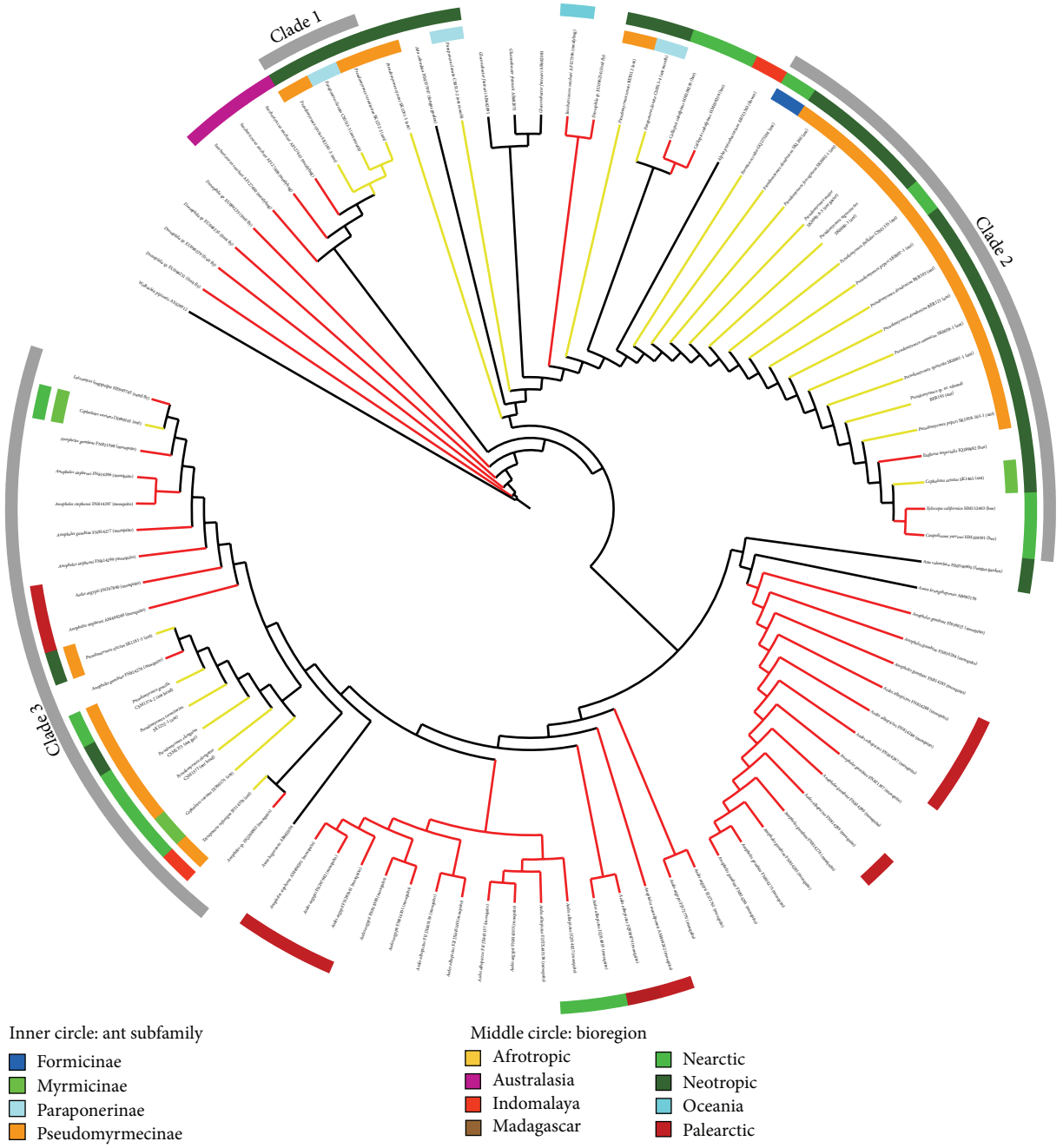

Figure 3: Phylogenetic tree of *Asaia*-related symbionts associated with ants and closest relatives with sequence data available in GenBank. A maximum likelihood phylogeny of the 16S rRNA region of bacterial symbionts is shown. The host name is given together with the GenBank accession number (GenBank sequences) or collection code (sequences generated in the present study). The branch color refers to the source from which the bacteria were isolated with yellow representing ant hosts and red other insect hosts. The inner circle refers to the ant subfamily, and the middle circle refers to the bioregion from which samples were collected. The outer circle indicates three clades (Clades 1–3), which contained several ant-associated symbionts. *Wolbachia pipientis* was used as an outgroup.

indicate that Rhodospirillales are most likely environmentally transmitted and support the hypothesis that they are only facultative associates of ants. One clade of ant-associated Rhodospirillales was closely related to endosymbionts isolated from mosquitos (Figure 3). It has been experimentally shown that mosquito-associated *Asaia* can successfully colonize leafhoppers further emphasizing the low-host specificity of this bacterial group [77].

4. Conclusion

Our broad bacterial screening approach has contributed to our understanding of the prevalence of ant-associated microbes, particularly with regard to their *Wolbachia* and *Spiroplasma* symbionts. Furthermore, we provide the first extensive survey for ant-associated *Asaia*-related symbionts. While these symbionts of the order Rhodospirillales infect

ants only sporadically, some strains are capable of fixing atmospheric nitrogen and might retain this function in ants. Alternatively, these bacteria might have an important functional role for upgrading nitrogen-poor diets of some herbivorous ants, which comprise the majority of all ant taxa [20]. Even though we do not have experimental evidence of the role of most bacterial symbionts in ants, previous studies illustrate a broad variety of effects of these bacteria on insect hosts [4, 7, 9]. Even a single group of microbes can have very different effects on different hosts. Our study shows that despite several extensive bacterial surveys across the ants, the diversity and functional role of ant-associated microbes is far from being fully understood, and broad next generation sequencing approaches will provide a fast and cost-effective tool to deepen our knowledge of the rare (and not so rare) biosphere.

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