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Host phylogeny and diet structure its bacterial community: a case study of various fig wasps coexisting in *Ficus hispida*

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ABSTRACT

The composition of a microbial community may be driven by geographic isolation, diet, and the phylogenies of host organisms. A null hypothesis was that the evolutionary history of microbial communities reflects the phylogenies of their respective hymenopteran hosts. The objective of this study was to test this hypothesis using four fig wasp species (*Ceratosolen solmsi Mayr, Apocrypta bakeri* Joseph, *Philotrypesis pilosa Mayr*, and *Philotrypesis* sp. Forster), which coexist in the spatially isolated fig cavities of *Ficus hispida* Linnaeus. The bacterial communities of the four fig wasps were investigated using culture-independent methods. The results show that these wasps harbored a total of 53 operational taxonomic units (3% distance cutoff for 16S rDNA sequences), and were dominated by Proteobacteria. Herbivore *C. solmsi* mainly harbored γ-proteobacteria (65.3%) and Actinobacteridae (23.9%). Potential omnivore *P. pilosa* was dominated by α- and γ-proteobacteria (80.5% and 4.9%). β-proteobacteria and Acidobacteria represented the majority of the bacterial communities of carnivores *Philotrypesis* sp. (51.2% and 16.8%) and *A. bakeri* (52.7% and 12.5%). Contrary to our hypothesis, the bacterial communities of the four fig wasps were clustered into three groups, which might be structured by both the diet and phylogenies of the host species. The fig-fig wasp system provides an isolated model for the extensive exploration of the ecological associations between insects and their microbes.

INTRODUCTION

Virtually all macro-organisms have symbiotic microbes. Insects, which benefit greatly from their bacterial symbionts, are the largest and most successful group of terrestrial macro-organisms. Insect-associated bacterial symbionts participate in many of the life processes of their macro-symbionts, such as nutrition [1,2], immune defense [3,4], and reproduction [5,6]. Some bacteria also play roles in the coevolution and speciation of their host insects [7,8].

In contrast, insect hosts may constrain or drive variation in their bacterial communities. Both host phylogeny and diet may influence the structures of bacterial communities associated with insects, with host phylogeny appearing to be especially important in hymenopterans [9,10]. For example, in some hymenopteran lineages, including honey bees and bumble bees, the linkage among bacterial communities associated with hosts exhibits patterns similar to those of their hosts' phylogeny [11,12]. Besides those of

social bees, the bacterial community compositions at the larval, pupal, and adult phases of three closely related species of jewel wasps (*Nasonia*) are also parallel to their hosts' phylogenetic histories significantly [13,14]. Thus, a null hypothesis is that the history of a microbial community reflects the phylogeny of its hymenopteran hosts. However, to date, the above hypothesis has not been tested among different species of hymenopterans with close ecological relationships, existing within a spatially isolated habitat.

The fig-fig wasp mutualism system associated with *Ficus hispida* Linnaeus filius (Moraceae) may provide a valuable model for exploring the relationship between insects and their microbes in a spatially isolated micro-syconium. The fig ovaries of *F. hispida* are inhabited by one pollinating fig wasp species (PFW, *Ceratosolen solmsi* Mayr) and three non-pollinating fig wasp species (NPFWs, *Apocrypta bakeri* Joseph, *Philotrypesis* sp. Forster, and *Philotrypesis pilosa Mayr*). The four wasp species differ in their phylogenetic relationships and diets. Phylogenetically, PFW *C. solmsi* belongs to the family Agaonidae, while the NPFWs belong to the family Pteromalidae subfamily Sycoryctinae. Among NPFWs, the sister species *P. pilosa* and *Philotrypesis* sp. belong to the tribe Sycoryctini, whereas *A. bakeri* is a member of the tribe Apocryptini. With respect to diet, *C. solmsi* is a gall-maker that oviposits during the female phase of the fig syconia. *P. pilosa* appears to be an inquiline of *C. solmsi* [15] (Table 1), and oviposits shortly after C. solmsi. Both *A. bakeri* and *Philotrypesis* sp. parasitize larval *C. solmsi* and oviposit during the male phase of the figs [16-18].

Taken together, these differences among the four fig wasp species facilitate the testing of the null hypothesis that the compositions of bacterial communities associated with fig wasps co-varied with the hosts' phylogeny. Thus, the objective of this study was to test the null hypothesis using the four fig wasp species of *Ficus hispida*. The bacterial communities of the four fig wasp species were investigated using culture-independent methods.

MATERIALS AND METHODS

Samples: The fig wasps that were used for the screening of bacterial communities were collected from *F. hispida* in the Hainan and Yunnan Provinces, China in 2012–2013 (Table 1). Healthy, ripe syconia were taken to the laboratory prior to wasp emergence. The wasps were then collected synchronously upon emergence from the syconium, and immediately identified to the species level according to their morphology. Individual wasps were stored separately in 95% ethanol at -80°C.

DNA extraction: To reduce the potential biases of individuals, each sample contained 20 individual wasps. DNA was separately extracted from 13 samples, including three female *C. solmsi*, one male *C. solmsi*, and three female samples from each of the three species of NPFWs (Table 1). Each sample was washed three times with sterilized phosphate buffer solution (PBS) using an ultrasonic (30 Hz) cleaner, then frozen in liquid nitrogen and crushed with a sterilized pestle. Total genomic DNA was then isolated using a DNeasy Tissue Kit (Qiagen, United Kingdom) according to the manufacturer's instructions. A sample-free extraction using the same kit served as a negative control.

DNA barcoding of fig wasps: Given the difficulty of discriminating between the tiny wasps due to their similar morphologies, molecular identification techniques were employed to confirm the morphological taxonomy of the four wasp species. To the best of our knowledge, both *CO* I and *ITS* sequences are commonly used to investigate the evolution of insect species. As the *ITS2* sequence of *C. solmsi* failed to be amplified, *CO* I was selected for molecular identification of the four fig wasp species.

Partial *COI* fragments of the fig wasps were amplified and sequenced for each DNA extraction via PCR, using the conserved primers 1490f and 2198r [19]. The sequences were deposited in GenBank under the accession number KF778382–KF778394.

Construction of clone libraries: 16S rDNA gene fragments from the 13 fig wasp DNA extractions were amplified via PCR, using the primers 27f and 1492r, as previously described [20]. The PCR product was cleaned up using an EasyPure Quick Gel Extraction Kit (TransGen, Beijing, China) according to manufacturer's protocols. Parallel negative controls were run synchronously containing PCR mixture but no DNA template; these were always negative. The same amplifications were performed with blank extractions; these were also consistently negative. Subsequently, the clone libraries of the complete 16S rDNA gene fragments were constructed as described by Martinson et al. [12].

Amplified ribosomal DNA restriction analysis (ARDRA) and sequencing: For each library, more than 100 clones were used to detect the targeted fragment of 16S rDNA. A small amount of each bacterial colony was diluted in 300 µL of LB broth and grown for 12 h. The target fragments were amplified directly from the bacterial culture medium via a 50 µL PCR mixture. Samples of each PCR product were run on a 1% agarose gel stained using EB.

Successful PCR products (5 µL) with targeted fragments were digested separately with restriction enzymes 1 U *Hae* III and 1 U *Hha* I (TaKaRa, Dalian, China) for 1 h. The digested products were run on 2.5% agarose gel stained using EB. Each gel was analyzed using the Bio Imaging System with GENE SNAP (Syngene, Gene Co.). Band sizes were qualified by comparing them to a 100-bp plusII DNA ladder (TransGen, Beijing, China). Next, one representative fluid culture of the bacterial clone was selected using each type of ARDRA profile from each clone library. The culture was then purified and sequenced in both directions using the primers M13F and M13R. Sequencing was performed as described above. Potential chimeric sequences were identified using BELLEROPHON [21] on the Greengenes web site (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi); these were excluded from further analysis. The remaining sequences were deposited in GenBank under the accession numbers HQ639416–HQ639583 and KC708242–KC708336.

Coverage and richness: Coverage, representing the proportion of OTUs observed out of the estimated total number of OTUs in the sampled population, was calculated for each library using the formula [1- (n/N)], where n was the number of phylotypes represented by only one clone, and N was the total number of clones [22,23]. The Shannon-Wiener diversity index was used to

represent the community diversity of the 16S rDNA clone libraries, Chao1 and ACE, both of which represented the community richness of the 16S rDNA clone libraries.

Taxonomic assignment and phenogram: The taxonomic assignments of 16S rDNA gene sequences were confirmed using RDPCLASSIFIER [24] on the RDP web site (http://rdp.cme.msu.edu/) [25]. We set the confidence level at 80%, stopping the assignment at the last clear taxonomic level and designating successive levels as unclassified (uc). All available sequences were aligned using ClustalW [26] in BIOEDIT 7.0.0 [27], with its default settings. We were not interested in phylogenetic relationships of the bacteria, per se, but rather their taxonomic identity based on similarity. Thus, neighbor-joining phenograms were constructed using MEGA 6.0 [28] with 1000 bootstrap replicates, to visualize similarity.

Phylotype definition: Sequences sharing 97% similarity were clustered into a single operational taxonomic unit (OTU0.97) using FASTGROUP II (http://biome.sdsu.edu/fastgroup/) [29]. These phylotypes were used to study the 'species-level' bacterial composition. A representative sequence from each OTU was used for taxonomic assessment via BLAST searching. All checked clones were assigned to taxonomic groups according to their ARDRA profiles.

Statistical analyses: The similarity of the bacterial communities among samples was analyzed using the principal components analysis (PCA) of a dissimilarity matrix. The matrix consisted of proportioned Bray-Curtis coefficients generated from the 16S rDNA data. PCA was run in PAST 2.03 (http://folk.uio.no/ohammer/past/). We tested the pairwise differences between the bacterial community compositions of different wasp species using a one-way analysis of similarity (ANOSIM) carried out in PAST with 10,000 permutations on the Bray-Curtis dissimilarity matrix [30]. ANOSIM compared the average rank similarity between samples within one wasp species with that of other species, and computed R values ranging from -1 to 1 [30]. A priori, R>0.75 was assumed to indicate strong separation between species, R>0.5 separation with overlap, and R<0.25 barely separable [31]. The difference in the abundance of particular bacterial taxa among samples was tested using one-way ANOVA carried out in SPSS 16.0. Significance was defined as being P<0.05.

Results

The morphological taxonomies of the four fig wasp species were confirmed via DNA barcoding.

Evaluation of clone libraries: Based on the 13 clone libraries from the four fig wasp species, 223 16S rDNA sequences were obtained from 1244 clones, including 200 bacterial sequences, 17 potentially chimeric sequences, and six chloroplast sequences **(Table 1)**.

Table 1. Characteristics of the 16S rDNA clone libraries. (OTUs are defined at a distance cutoff of 3%. Both Chao1 and ACE indices represent the community richness of the 16S rDNA clone libraries. Shannon indices represent the community diversity of the 16S rDNA clone libraries. Coverage for each library represents the proportion of OTUs observed out of the estimated total number of OTUs in the sampled population).

Samples	No. of OTUs	Chao1	Ace	Shannon	Coverage	No. of clones
Wasp	53					1244
Cso	14	16.00	18.4444	1.32077		319
CsoF1	6	6.00	6.0000	0.91064	97.1%	102
CsoF2	2	2.00	2.0000	0.67749	100.0%	17
CsoF3	9	12.00	11.5667	1.36143	95.6%	99
CsoM3	5	5.50	6.7867	0.49073	95.2%	101
Ppi	22	29.00	29.3220	1.18927		299
PpiF1	11	11.33	11.8713	1.62229	94.4%	105
PpiF2	3	3.00	4.1111	0.19195	97.9%	111
PpiF3	6	6.00	6.0000	1.10141	98.8%	83
Psp	26	39.20	44.0220	1.80361		296
PspF1	16	16.60	17.5445	2.25588	90.0%	90
PspF2	6	6.00	6.6667	0.93169	97.4%	117
PspF3	5	5.00	5.0000	1.02903	98.9%	89
Aba	22	25.00	27.9573	1.87792		330
AbaF1	24	26.50	28.2196	2.89579	94.9%	103
AbaF2	4	4.00	4.0000	0.85058	100.0%	99
AbaF3	4	5.00	5.0000	0.89859	99.2%	128

Two lines of evidence support the feasibility of our ARDRA sampling strategy. First, multiple 16S rDNA sequences were sequenced randomly within an ARDRA profile (altogether 15 sequences), all of which were identical or nearly so, belonging to a same OTU (3% distance cutoff). Second, as described below (in the section of "Bacterial communities of fig wasps"), 30 out of 53 OTUs contained at least two ARDRA profile types, indicating that our sampling strategy was sufficiently representative to reveal common bacterial diversity.

Coverage averaged 97.5% and exceeded 94% for each clone library. The Chao1 values of the bacterial communities related to the four wasp species were approximately 27.3 on average. *C. solmsi* displayed the lowest Chao1 value of 16; *A. bakeri* and *P. pilosa* were similar to each other with 25 and 29, and *Philotrypesis* sp. had the highest value, at 39. These values of Chao1, Ace, and the Shannon index also varied in intra-species samples **(Table 1).**

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Bacterial communities of fig wasps: The 200 qualificatory 16S rDNA sequences were grouped into 53 OTUs. The number of OTUs averaged 7.8 OTUs per library of fig wasps, yet varied greatly among libraries within the same species. *A. bakeri* was most variable in richness per library, having from 4 to 24 OTUs. The bacterial OTU richness also varied among the fig wasp species **(Table 1 and Figure1)**. The mean number of OTUs per species was 21. *C. solmsi* hosted only 14 OTUs, while *A. bakeri*, *P. pilosa*, and *Philotrypesis* sp. had 22, 22, and 26 OTUs, respectively. The sister species *P. pilosa* and *Philotrypesis* sp. shared 6 of 39 OTUs (15.4%), yet fig wasps from different families shared more. For example, *C. solmsi* and *P. pilosa* shared 7 of 26 OTUs (26.9%). In total, 18 of 53 OTUs (34%) were shared by at least two species of fig wasps. All fig wasps possessed OTU 9, and the three NPFWs shared OTU 17. The ratios of shared OTUs did not correlate with the phylogenies of the fig wasps.

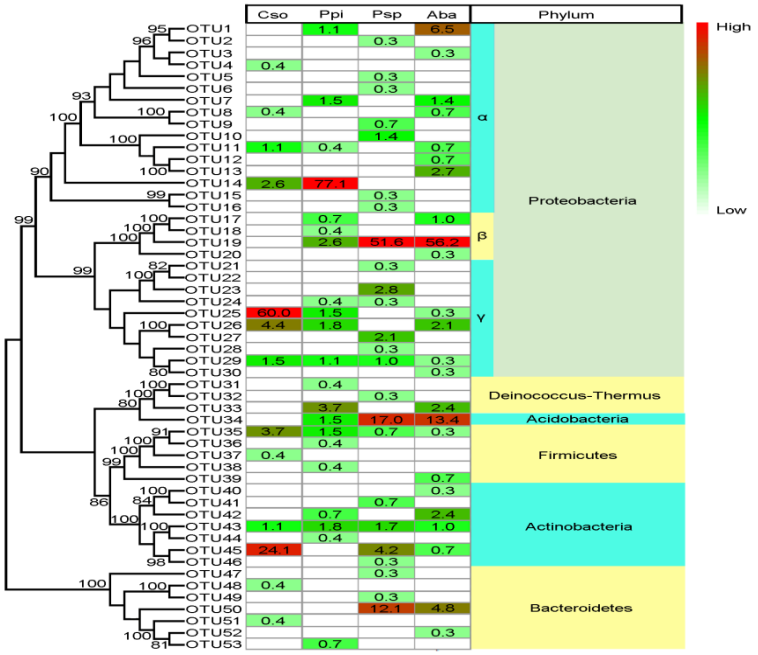

Figure 1. Heatmap of the relative abundances of bacterial communities of the four fig wasp species associated with *Ficus hispida*. *Ceratosolen solmsi, Philotrypesis hispida, Apocrypta bakeri, and Philotrypesis* sp. are abbreviated to Cso, Ppi, Aba, and Psp, respectively. The neighbor-joining phenogram on the left side is constructed from 16S rDNA sequences selected randomly from each OTU. The phyla on the right correspond to the classification of the OTUs on the left. The heatmap in the center is based on the relative abundances of OTUs of each host species, and the numbers in squares are the percentages of corresponding OTUs.

The overall bacterial community of the four fig wasp species included six phyla, 13 classes, 25 families, and 43 genera **(Figure1)**. The bacterial phyla represented were as follows: Proteobacteria 72% (α-proteobacteria 24%, β- 28%, and γ- 20%), Firmicutes 11%, Actinobacteria 11%, Acidobacteria 8%, Bacteroidetes 5%, and Deinococcus-Thermus 2%. The bacterial community composition of each wasp species is detailed in **Figure 2**. The most abundant bacteria in Proteobacteria were assigned to *Wolbachia* (α-, 12.9%), *Tepidimonas* (β-, 27.6.0%), and to the family Enterobacteriaceae (γ-, 9.2%).

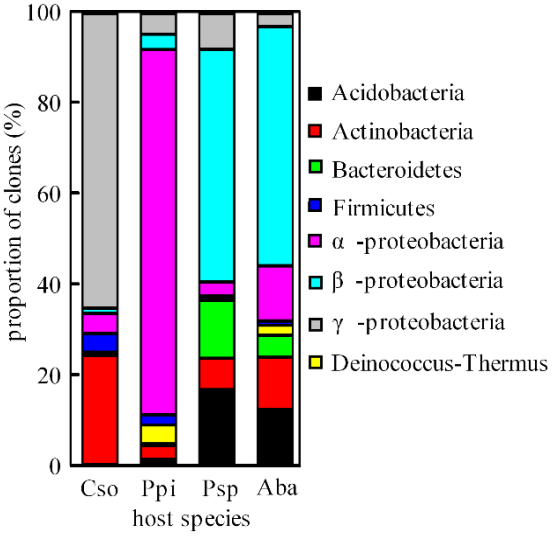

Figure 2. Proportion of bacterial phyla distributed in the four fig wasp species associated with *Ficus hispida. Ceratosolen solmsi, Philotrypesis pilosa, Apocrypta bakeri, and Philotrypesis* sp. are abbreviated to Cso, Ppi, Aba, and Psp, respectively.

Only three (Aba1-73, Ppi1-108, and Psp2-78) of the 200 sequences from fig wasps exhibited <97% similarity to sequences in GenBank. In addition, 37 sequences were highly matched (>97% similarity) to taxonomically undescribed bacterial genera in RDP II and GenBank. The remaining sequences were highly similar to those either from other insects, or uncultured environmental samples in GenBank.

Neighbor-joining trees constructed using the 16S rDNA sequences from fig wasp bacteria depicted curious patterns with respect to the three bacterial genera. Some sequences of *Acinetobacter* from fig wasp species evolved into a particular cluster, with no more than 97% similarity to any GenBank sequences **(Figure 3)**. Sequences from NPFWs exhibited separate clusters on the trees of Acidobacteria and Tepidimonas. Acidobacteria in fig wasps clustered in subdivision 4, which was not observed in the other insect symbionts. *Tepidimonas* in fig wasps clustered with an uncultured environmental bacterium classified as an undescribed species (99% similarity).

Comparisons among the bacterial communities of fig wasps: The bacterial communities of the four wasp species were resolved into three groups by principal components analysis (PCA) performed at the genus level. One group harbored the four samples of *C. solmsi*, the second had the three samples of *P. pilosa*, and the third included the six samples from *A. bakeri* and *Philotrypesis* sp. Fig wasps within the same group possessed shared bacterial communities **(Figure 3A)**. The ANOSIM revealed pairwise differences between the bacterial communities of different wasp species. The bacterial community of *C. solmsi*, the only herbivore, differed substantially from those of the NPFWs *P. pilosa*, *Philotrypesis* sp., and *A. bakeri*, with high R values (0.8519, 0.9259, and 0.8519, respectively). The smallest R value (0.0830), between the communities of *A. bakeri* and *Philotrypesis* sp., indicated the absence of any major differences. The sister species *P. pilosa* and Philotrypesis sp. [32] had an extremely high R value of 1.0000, which far exceeded that between the bacterial communities of *A. bakeri* and *Philotrypesis* sp. Inquiline *P. pilosa* had R values >0.75 with the other three wasps, indicating the uniqueness of the composition of its bacterial community.

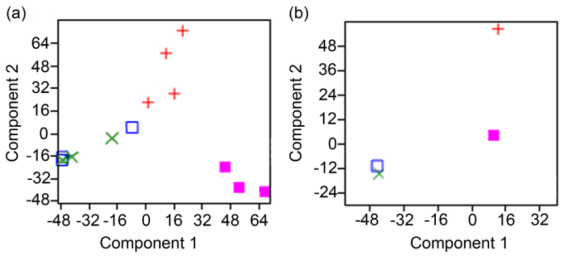

Figure 3. Principal component analysis of bacterial compositions of wasps. (A) Principal component analysis (PCA) of the bacterial communities of four species of fig wasps. (B) PCA for *Wolbachia*-free bacterial communities of four species of fig wasps. Red Cross, plum Solid Square, moss green cross, and blue blank square represent the bacterial community structures of *Ceratosolen solmsi, Philotrypesis pilosa, Philotrypesis* sp., and *Apocrypta bakeri*, respectively.

The greatest difference between the bacterial composition of PFWs and NPFWs involved the family Enterobacteriaceae, mainly from the genera *Enterobacter* and *Pantoea*. The 164 clones of the family Enterobacteriaceae constituted 51.4% of all screened clones in the four libraries from *C. solmsi*. In contrast, only three clones were from the NPFWs *A. bakeri* and *P. pilosa* observed. Sequences from libraries of *C. solmsi* sampled in summer and winter were assigned separately to the genera *Pantoea* and *Enterobacter*, respectively.

Wolbachia, the only known heritable endosymbiotic bacteria in fig wasps, also differed significantly among the four wasp species. *Wolbachia* infected all *P. pilosa* and some *C. solmsi* samples, but never infected *A. bakeri* or *Philotrypesis* sp. The average abundance of *Wolbachia* in *P. pilosa* (77.1%) was much higher than that in *C. solmsi*.

Furthermore, PCA was performed after filtering the *Wolbachia* (OTU 14) data from the libraries of *C. solmsi* and *P. pilosa*. Without *Wolbachia*, the other bacteria from *P. pilosa* were evenly scattered among all phyla **(Figure 2).** Considering that the remaining bacterial data from each *P. pilosa* library are relatively minimal, the data from each wasp species was pooled together as a unit for the PCA, in order to reduce the bias caused by the small size of the dataset. As expected, the *Wolbachia*-free bacterial community of *P. pilosa* still deviated from those of the other three wasp species, which is consistent with the previous pattern **(Figure 3B).**

The third difference among the bacterial communities of the wasp species involved *Tepidimonas* (β-proteobacteria: Burkholderiales) and *Acidobacterium* (Acidobacteria), both of which exhibited similar patterns of distribution among the wasps. The relative abundances of *Tepidimonas* were high in both *A. bakeri* (51.3%) and *Philotrypesis* sp. (50.7%). In contrast, *P. pilosa* exhibited a very low level of infection by *Tepidimonas* (2.6%), and freely infected *C. solmsi*. *Acidobacterium* was similarly abundant in *A. bakeri* (12.2%) and *Philotrypesis* sp. (16.7%), but distinctly low in *P. pilosa* (1.5%).

Finally, Deinococcus-Thermus bacteria occurred only in *A. bakeri* and *P. pilosa* sampled in summer. In terms of similarity, the phyla Bacteroidetes, Firmicutes, and Actinobacteria constituted a common and stable community in all four species of wasp.

DISCUSSION

The complex relationship among the different fig wasp species coexisting in the same fig ovary provides a valuable model with which to test the null hypothesis that the compositions of the bacterial communities associated with fig wasps co-vary with their hosts' phylogenies. In addition, a better understanding of the structures of the system's bacterial communities will

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provide new insights into the mechanism of their coevolution. Thus, this study first investigated the bacterial community structures associated with the four fig wasp species associated with *F. hispida*.

Bacterial community associated with fig wasps: Several authors proposed that fig ovaries offered relatively sterile environments for fig wasps, compared to the habitats of free-living insects [33,34]. The results of this study partially support this conjecture. The four wasp species associated with *F. hispida* harbored relatively simple bacterial communities. In general, of all varieties of insects, bees and wasps harbor relatively small bacterial communities (about 11.0 species/OTUs/samples) [9]. The adult jewel wasp, *Nasonia vitripennis*, averages 20 OTUs, *N. longicornis* averages 17, and *N. girault*i only 9 [13]. In comparison, adult fig wasps average a mere 7.8 OTUs. Thus, fig wasps seem to harbor less bacterial diversity than most hymenopterans that live outside fig fruits, though it was difficult to standardize for sampling depth and methodology. This result might be caused by the following factors. The spatially isolated fig syconium cavity is relatively sterile; thus, the bacterial communities of the fig wasps inhabiting the cavity have little chance of being infected by environmental microbes. Alternatively, some rare or very low-abundance OTUs associated with fig wasps might go undetected by the ARDRA method used in this study.

The bacterial community discovered in fig wasps is commonly found in other insects as well. For instance, order Burkholderiales, the most widespread group of microbes found in herbivorous solitary bees [12], were also highly abundant in the carnivorous parasites *A. bakeri* (51.5%) and *Philotrypesis* sp. (50.6%). Sequences of *Enterobacter* collected from fig wasps were scattered on the neighbor-joining phenogram, and some were associated with either plants or insects living in open environments. Enterobacteriaceae was the most abundant symbiont of *C. solmsi* (51.4%), yet it also dominated the microbial communities of many other hosts, such as red fire ants, *Solenopsis invicta* (>80%), and Nasonia (>70%) [13,35]. The different diets and distributions of these hosts might reflect the broad biological functions of Enterobacteriaceae, including the synthesis of essential amino acids, carbon metabolism, development, reproduction, and immunity to pathogenic bacteria [36-39].

In addition, some special bacterial groups might be formed from fig wasps. A special cluster of *Acinetobacter* from the four fig wasp species was observed. The greatest similarity found by BLASTN search was merely 97%, which was a level that discriminated between bacterial species. *Acinetobacter* occurs in many insects, including human body lice [40], formicid ants [41], honey bees [42], and *Nasonia* [13]. As the most abundant bacteria, *Acinetobacter*, was presumed to help *N. vitripennis* absorb nutrients within its digestive tract, although the precise mechanisms remain elusive [13]. The sequences of *Tepidimonas* from *A. bakeri* and *Philotrypesis* sp. evolved independently on a single cluster, with a maximum similarity to GenBank sequences of 99%. Although *Tepidimonas* is commonly found in hot springs [43], it has been less frequently reported to occur in insects. Thereby, the knowledge of the role of *Tepidimonas* in insect hosts was quite poor. In addition, the *Acidobacterium* from the fig wasps clustered in subgroup 4, distinct from those of other insects, which mainly clustered in subgroup 1 [20]. The different evolutionary histories and habitats of fig wasps and other insects were potential causes of these phenomena.

Host phylogenetic history and diet structure of the bacterial community structures: It is worth noting that, compared to the two *Wolbachia*-free parasites, the *Wolbachia* (77.1%) in *P. pilosa* could possibly swamp other bacteria out of our clone sampling and affect the detection of bacteria from other tissues, such as the gut and blood cavity. However, using the ARDRA method, all the observed *Wolbachia* sequences were assigned to OTU14. The *Wolbachia*-free bacterial diversity of *P. pilosa* (21 OTUs) detected in this study was equal to that of *A. bakeri* (22 OTUs), and much higher than that of *C. solmsi* (14 OTUs), which were infected by only a small population of *Wolbachia*. This high bacterial diversity suggested that the clone-sampling size was large enough to overcome the effect of *Wolbachia* dominance on the investigation of the other prominent bacteria of the *P. pilosa* community. In addition, PCA was performed after filtering the *Wolbachia* data from the wasp libraries. As expected, the *Wolbachia*-free bacterial community of *P. pilosa* continued to deviate from those of the other three wasp species, in a manner consistent with the prior pattern **(Figure 3B)**.

Although it was suggested that hymenopterans and termites typify the pattern in which microbial communities and insect phylogenies are parallel to one another [9,13,23], our discoveries suggest otherwise. The composition of the bacterial communities of the four wasp species appear to be simultaneously driven by the diets and phylogenetic histories of the fig wasps **(Figure 4).**

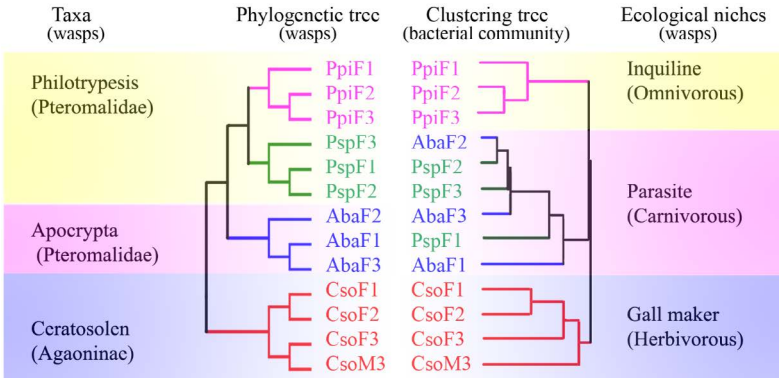

Figure 4. Relationships among the ecological correlation, phylogenesis, and bacterial community structures of four fig wasp species. The phenogram on the left is based on partial sequences of the *COI* gene of fig wasps. The clustergram on the right side is based on the bacterial community structures of fig wasps.

At the fig wasp subfamily level, the bacterial community structure of Agaoninae (*C. solmsi*) was significantly different from that of Sycoryctinae (*A. bakeri*, *Philotrypesis* sp., and *P. pilosa*). The bacterial clustering patterns of the two subfamilies correlated

with the phylogenetic relationships of the fig wasps. This suggests that the significant differences in the bacterial community structures of the two subfamilies are probably a result of the phylogenetic distance between the two subfamilies.

For the three fig wasp species in Sycoryctinae, the bacterial communities appeared to be influenced by diet, rather than the phylogenetic histories of the fig wasps. First, the bacterial community structure of Philotrypesis sp. was similar to that of *Apocrypta bakeri* (*A. bakeri*), but deviated from that of its sister species, *P. pilosa*. This demonstrates that the histories of the bacterial communities do not reflect the phylogenetic relationship of the three NPFWs. This result leads us to reject our hypothesis. Second, both *A. bakeri* and *Philotrypesis* sp. are carnivorous, feeding on *C. solmsi*. In contrast, *P. pilosa* could be considered omnivorous, because it is herbivorous prior to larval phase 2, but later *P. pilosa* may simultaneously consume both fig ovary tissue and *C. solmsi*, which is killed by *P. pilosa* during larval phase 2 [15].

Overall, the bacterial community structures of the four fig wasp species might be shaped by both the feeding habits and the phylogenetic histories of fig wasps **(Figure 4).** Ecological differences between fig wasps and external hymenopterans might account for this discordance. Enclosed fig syconia exert great evolutionary pressure on the fig wasps, which live in darkness, have limited resources, experience fierce competition, and are isolated physically from outside resources [44]. Because of this, fig wasps possess many unique features, such as the degradation of their compound eyes, extreme sexual dimorphism, and male polymorphism [45]. However, no prior data exist which describe how these pressures influence bacterial community composition.

This study investigating the four fig wasp species of *F. hispida* opens the door to further avenues of research. Different bacterial communities likely exist in other fig-fig wasp systems. For example, *Serratia plymuthica*, which was the first microbe cultured and identified from the fig wasp *Blastophaga psenes* L., which pollinates *Calimyrna* figs [46], was not detected in the fig wasps of *F. hispida*. Thus, the conclusions obtained from this study need to be further verified in other fig-fig wasp systems.

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