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Bacterial Communities in Bacteriomes, Ovaries and Testes of three Geographical Populations of a Sap-Feeding Insect, *Platypleura kaempferi* (Hemiptera: Cicadidae)

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Abstract

Mutualistic associations between symbiotic bacteria and their insect hosts are widespread. The bacterial diversity and community composition within hosts may play an important role in shaping insect biology, ecology, and evolution. Here, we focused on the bacterial communities in bacteriomes, ovaries and testes of three representative populations of the cicada *Platypleura kaempferi* (Fabricius) using high-throughput 16S rRNA amplicon sequencing approach combined with light microscopy and confocal imaging approach. The obligate symbiont *Sulcia* was detected in all the examined samples, which showed a relatively high abundance in most bacteriomes and ovaries. The unclassified OTUs formerly identified as an unclassified Rhizobiales bacterium was demonstrated to be the co-obligate symbiont *Hodgkinia*, which showed 100% infection rate in all the examined samples and had an especially high abundance in most bacteriomes and ovaries. *Hodgkinia* and *Sulcia* occupy the central and peripheral bacteriocytes of each bacteriome unit, respectively. Cluster analysis revealed that the bacterial communities in bacteriomes, ovaries and testes of Zhouzhi and Ningshan populations separated strongly from each other. Significant difference was also detected between the Yangling and Ningshan populations, but no significant difference was detected between the Yangling and Zhouzhi populations. This may be related to the difference of host plants and genetic differentiation of these populations. Our findings show that the bacterial communities can be influenced by the population differentiation of the host cicadas and/or the host plants of cicadas, which improve our understanding of the associations between the bacterial community and population differentiation of sap-feeding insects.

Introduction

Cicadas are well-known for their multiple-year life cycles and the loud, complex, and usually species-specific acoustic signals produced by the males [1]. Adult and nymphal cicadas feed exclusively on the xylem fluid from branches and roots of their host plants, respectively [2]. Although cicadas are usually not considered to be major agricultural pests, they are a significant nuisance to many fruit trees because they can reduce the growth of branches, leaves and fruits and may finally lead to branch wilt and death due to their feeding and oviposition [3].

The diet of cicadas and other hemipteran insects is unbalanced, as they live exclusively on plant xylem or phloem. Plant sap may contain some levels of carbohydrates (mainly in the form of sucrose), but is generally poor in nitrogenous compounds such as amino acids, vitamins and cofactors [4]. Therefore, most sap-sucking hemipterans have evolved symbiotic relationships with mutualistic microorganisms, which can aid in the biosynthesis of essential amino acids and vitamins that are limited or lacking in their diet [5]. It has been reported that two obligate endosymbionts, *Candidatus* Sulcia muelleri (hereafter referred to as *Sulcia*) and *Candidatus* Hodgkinia cicadicola (hereafter referred to as *Hodgkinia*), may coexist in the specialized organ called bacteriomes in cicadas [6, 7]. *Sulcia* is an ancient endosymbiont which has developed intimate symbiosis with the common ancestor of Auchenorrhynchan insects, including cicadas, spittlebugs, leafhoppers, treehoppers, froghoppers, and planthoppers [6]. The genome of this symbiont is highly conserved and always synthesizes a set of seven or eight essential amino acids for their hosts [6–8]. In contrast, the genome of *Hodgkinia* is

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drastically reduced and exhibits substantial structural instability, which can synthesize the two remaining essential amino acids (histidine and methionine) for the host cicadas [7, 9–11]. Additionally, some cicada species lost *Hodgkinia* and instead harbor yeast-like fungal associates which can presumably synthesize almost all amino acids, vitamins and other metabolites for the host cicadas [12].

Besides the primary symbionts, some cicada species have been reported to harbor a series of secondary symbionts in the bacteriomes or other organs, e.g., *Arsenophonus*, *Mycobacterium*, *Phyllobacterium*, *Rhodobacter*, *Rhodococcus*, *Meiothermus*, *Rickettsia*, *Sphingomonas*, *Spiroplasma*, *Sodalis,* and *Wolbachia* [12–14]. The diversity and prevalence of secondary symbionts often varies between host species and populations [15]. This has been well studied particularly in aphids, in which a wide range of defensive, heritable secondary symbionts are harbored, and variations in the bacterial community were observed among populations within a species or in a single population of different seasons [16]. However, impacts of secondary symbionts on host biology are difficult to assess [17].

Many factors vary across the geographic range of a species, including natural enemies, food availability, and abiotic factors (e.g., temperature), which may influence the symbiont diversity and abundance of species [18]. In addition, the genetic divergence of hosts appears to influence the bacterial communities through immune-related genes that shape host–microbiota interactions and through vertical transmission of microorganisms [19]. Some studies focused on the microbial diversity across populations of a single species of some insect groups, e.g., in tsetse flies [20], aphids [21], and whiteflies [22]. It has been revealed that different populations within a species may harbor different microbiomes in terms of diversity and relative abundance, for example, in the aphid *Aphis* (*Toxoptera*) *citricidus* the symbiont diversity and relative abundance can be affected by the host plants [23]. Recently, Wang et al. [13, 24] investigated the bacterial composition associated with internal organs (including bacteriomes, testes, ovaries, different parts of guts and Malpighian tubules) of different populations of the cicada *Subpsaltria yangi* using the high-throughput sequencing method. The results revealed that the bacterial communities in various organs of this cicada species clustered moderately by populations, which possibly reflect adaptive changes in the microbiota of related populations. However, whether the bacterial communities of different populations are commonly different in a cicada species remains unknown.

The cicada *Platypleura kaempferi* (Fabricius) is one of the most dominant species in Guanzhong Plain and areas of low altitude of Qinling Mountains, China. Here, the genetic differences among the representative geographical populations of *P. kaempferi* were quantified based on *COI* sequence data. Then, we investigated the bacterial diversity in bacteriomes, ovaries and testes of three representative populations of *P. kaempferi* feeding on different host plants, using 16S rRNA high-throughput amplicons sequencing and microscopy methods. We hypothesized that the microbiota of *P. kaempferi* from different populations may have adaptive changes. The objectives of this research were (1) to classify the symbionts harbored in the bacteriomes and reproductive organs of *P. kaempferi* from different niches, and (2) to uncover whether the bacterial communities in the bacteriomes and reproductive organs are related to the different geographic populations of this sap-feeding insect.

Materials and Methods

Insects

Adults of *P. kaempferi* were netted from Zhouzhi County (34°06′N, 108°02′E; altitude: 500–600 m), Ningshan County (33°03′N, 108°45′E; altitude: 1500–1550 m), and Yangling District (34°28′N, 108°08′E; altitude: 450–540 m), Shaanxi Province, China (Fig. S1), in July and August since 2016 to 2018. They were transferred alive to voile-cages and brought to the laboratory for dissection as soon as possible.

The three representative populations occurred in Yangling (located at the central part of Guanzhong Plain), Zhouzhi (located at the north of piedmont zone of Qinling Mountains) and Ningshan (located in the Qinling Mountains), respectively. The host plant of Zhouzhi population is the apricot tree *Armeniaca vulgaris* Lam. var. *vulgaris*; the host plant of Ningshan population is the pine tree *Pinus tabuliformis* Carrière; while the host plants of Yangling population are complex, mainly including *A. vulgaris* Lam. var. *vulgaris*, *P. tabuliformis*, and the fir tree *Metasequoia glyptostroboides* Hu & Cheng (Table S1). In total, three individuals of both Ningshan and Zhouzhi populations and two individuals of Yangling population were used for the molecular identification and genetic differentiation analysis of *P. kaempferi*; at least 10 individuals for both sexes of each population were used for light, histological and fluorescent microscopy observations; three males and three females were processed per population for 16S rRNA amplicon sequencing. In addition, one population of *P. kaempferi* (four individuals) collected from Fuzhou, Fujian Province and two congeneric species, *P. hilpa* and *P. assamensis*, were chosen as outgroup taxa for phylogenetic analyses to quantify the genetic differentiation among the Ningshan, Yangling and Zhouzhi populations of *P. kaempferi*.

Genetic Divergence of *P. kaempferi*

Total genomic DNA was extracted from leg muscle using a Biospin Insect Genomic DNA Extraction Kit (Bioer

Technology Co., Ltd, Hangzhou, China) following the manufacturer's instructions. Standard polymerase chain reaction (PCR) methods were used to amplify partial sequences of cytochrome c oxidase subunit I (*COI*). The primer pairs used for *COI* gene (~0.7 kb) are LCO1490 and HCO2198 [25]. PCR programs consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 51.4 °C for 30 s, and 72 °C for 1 min, and ending with an extension at 72 °C for 5 min. The amplification was performed in 25 µl reaction volumes with 2.5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (25 mM), 1 µl of dNTP (2.5 mM each), 0.5 µl of each primer (25 µM), approximately 200 ng of template DNA and 1 U of Taq DNA polymerase (TIANGEN, China). PCR products were then purified and sequenced in both directions by Yangling Bai'ao Biological Engineering Technology and Service Co., Ltd. All sequences have been submitted to GenBank (Accession Nos.: MT672251–MT672255, MT762257–MT672264, MW590200). The Bayesian analysis (BI) based on *COI* sequence was conducted using MRBAYES v3.1.2. Mean sequence divergences among populations of *P. kaempferi* were calculated using MEGA v6 and the pairwise Kimura two-parameter (K2P) model.

Light Microscopy

Live male and female adults of *P. kaempferi* (at least 5 individuals for both sexes of each population) were used in dissection. Male and female adults were anaesthetized by chilling at 0 °C, and then dissected in phosphate buffered saline (PBS, 0.1 M, pH 7.2) under a Motic SMZ168 stereoscopic zoom microscope (Motic, Xiamen, China). After the fat bodies covering on bacteriomes were removed, photographs of bacteriomes were taken using a scientific digital micrography system equipped with an Auto-Montage imaging system and a Qimaging Retiga 2000R (Qimaging Corp, Canada) digital camera (CCD).

Histological Microscopy and Fluorescence In Situ Hybridization

Under the Motic SMZ168, bacteriomes of females and males (at least 5 individuals for both sexes of each population) were dissected in PBS and transferred immediately to a tube filled with cold fixative (2.5% glutaraldehyde and 4% paraformaldehyde). After fixed for 12 h at 4 °C, the samples were rinsed five times (each time 10 min) in PBS (0.1 M, pH 7.2). Then, they were dehydrated through 1 h incubations in 70%, 80%, 90% and 100% ethanol series, and then cleared in methylscylate for 2 × 1 h. Paraffin embedding was done under vacuum for 2 × 1 h. Paraffin blocks were thin-sectioned to 4 µm. Some thin tissue sections for histological microscopy were stained with hematoxylin and eosin, and observed under a light microscope Leica DM6 B (Leica, Germany).

To localize related endosymbionts in bacteriomes of *P. kaempferi*, dual hybridization was performed with symbiont-specific 16S rRNA probes: *Sulcia* (Cy3-CCAATGTGGGGGWACGC) and *Hodgkinia* (Cy5-CCAATGTGGCTGACCGT) [26]. Initially, thin sections of bacteriomes were de-paraffinized in xylene for 2 × 10 min and then hydrated though a 2 × 100%, 90%, 80%, 70% ethanol series. After washed with ddH_2O for 10 min, the slides were incubated in prehybridization solution (20 mM Tris–HCl [pH 8.0], 0.9 M NaCl, 0.01% [w/v] sodium dodecyl sulfate, 30% [v/v] formamide) at 37 °C for 1 h in a humidity chamber. Then, the slides were incubated with the hybridization buffer containing 100 nM each of the probes overnight at 37 °C. After that, the slides were washed in PBSTw (0.1 M PBS with 0.1% [w/v] Tween 20) at 37 °C for 1 h and counterstained with Hoechst33258 for three minutes. Then, the slides were washed in PBSTw for 3 × 5 min and preserved with antifade solution and observed under a fluorescence microscope (Olympus FV 3000, Japan). Negative control was done to check the specificity of hybridization using only one symbiont-targeted probe and no probe staining.

Cicada Dissection and DNA Extraction for High-Throughput Sequencing

Prior to dissection, the surface of each cicada was sterilized with 75% ethanol for 2 min and rinsed in sterile water twice to remove body surface contaminations. After that, bacteriomes and testes/ovaries were aseptically extracted with sterile forceps and scissors, and briefly washed three times with sterile water, and then separately placed in different centrifuge tubes. To prevent cross-contamination between samples, forceps and scissors were flame-sterilized when shifting to another organ during dissection. Samples were stored at − 80 °C until DNA extraction performed. Three male and female individuals were processed per population for biological replication (Table S1).

The dissected samples, including bacteriomes of female and male, ovaries and testes, were hand-homogenized in extraction buffer containing 200 µl lysozyme (50 mg/ml), and then incubated at 37 °C for 1 h to achieve DNA extraction from both Gram-positive and Gram-negative bacteria. The DNA in the samples was extracted using the TIANamp Genomic DNA Kit (TIANGEN, China) following the manufacturer's instructions. The quantity and quality of DNA were measured with a Nanodrop 8000 (Thermo Fisher Scientific, USA).

16S rRNA Gene Amplification and Sequencing

According to the concentration, DNA was diluted to 1 ng/μl for use as PCR template. The V4 region of the 16S rRNA gene was amplified by PCR using specific primers 515F (5′-GTG CCA GCM GCC GCG G-3′) and 806R (5′-GGA CTA CVS GGG TAT CTA AT-3′), which have been shown to be effective markers for assessing bacterial diversity [27]. PCR products were mixed in equidensity ratios, and then purified with Qiagen Gel Extraction Kit (Qiagen, Germany). One ovary sample and two testis samples from Yangling were not included in the next analysis due to an error occurred during DNA extraction, resulting 33 samples remained for further sequenced. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and Agilent Bioanalyzer 2100 System. At last, the libraries were sequenced using 2 × 250 bp paired-end sequencing on the HiSeq PE250 platform. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA640760.

16S rRNA-Based Bacterial Community Analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequences, and then these reads were merged to raw tags using FLASH (v1.2.11, http://ccb.jhu.edu/software/FLASH/) quality controlled process. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags by using QIIME (v1.9.1; http://qiime.org/). The tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed.

Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs) using the UPARSE pipeline (v7.0.1001, http://drive5.com/uparse/). Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Greengenes database was used based on RDP classifier (v2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm to annotate taxonomic information. The taxonomic information was exhibited by the "circlize" package in R v3.5.2 [28]. To gain additional information about bacterial taxonomic identity, the sequences of all unassigned OTUs were blasted against the GenBank database. Based on maximum likelihood (ML) with the program RAxML v8.1.5, a ML tree was constructed based on sequences of the 25 OTUs which are related to *Hodgkinia* and other related sequences from GenBank as well.

The α and β diversity indices were calculated on the basis of the rarefied OTU counts by using the QIIME program. The α diversity index represents the diversity in a single sample reflected by parameters, including the richness indices Chao1 and ACE (abundance-based coverage estimator) and the diversity indices Simpson and Shannon. To evaluate significance level ($P < 0.05$) among the α diversity of different tissue samples from the same population and the same tissue sample from different populations, independent sample T-test was applied to normally distributed data sets, as determined by the Anderson–Darling test. Nonparametric tests (Mann–Whitney *U* test) were used for nonnormal data sets. The "vioplot" package in R v3.5.2 was used to display α diversity indices. The β diversity, microbial community variation, was assessed using UniFrac distances within QIIME. Both weighted and unweighted UniFrac were calculated and visualized with Nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA). NMDS analysis was performed in R v3.5.2 with the "vegan" package. PCoA was displayed by "WGCNA" package, "stat" packages and "ggplot2" package in R v3.5.2. Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was also conducted in QIIME. Statistical testing among variation in microbial community composition was carried out using the analysis of molecular variance (AMOVA) and analysis of similarity (ANOSIM). The test of AMOVA was performed as implemented in MOTHUR v.1.33.3 [29]. To account for the number of analyses undertaken, statistical significance was set a priori at $P < 0.05$ (Bonferroni adjustment). In addition, ANOSIM was performed using the "vegan" package in R v3.5.2 with the default permutation number of 999 (based on Bray–Curtis distances) [30], and the level of significance was set at $P < 0.05$.

Results

Genetic Variation of *P. kaempferi* Populations

The pairwise corrected genetic distances based on *COI* sequences of *P. kaempferi* and other *Platypleura* species show that intraspecific genetic distances of *P. kaempferi* (0.000–0.022) are distinctly lower than those between *Platypleura* species (0.041–0.103), without overlap (Table S2). This indicates that the variations of populations of *P. kaempferi* have not reached species level. The intraspecific genetic distances between Ningshan population and Yangling/Zhouzhi populations are higher than that between Yangling and Zhouzhi populations. According to the BI tree constructed

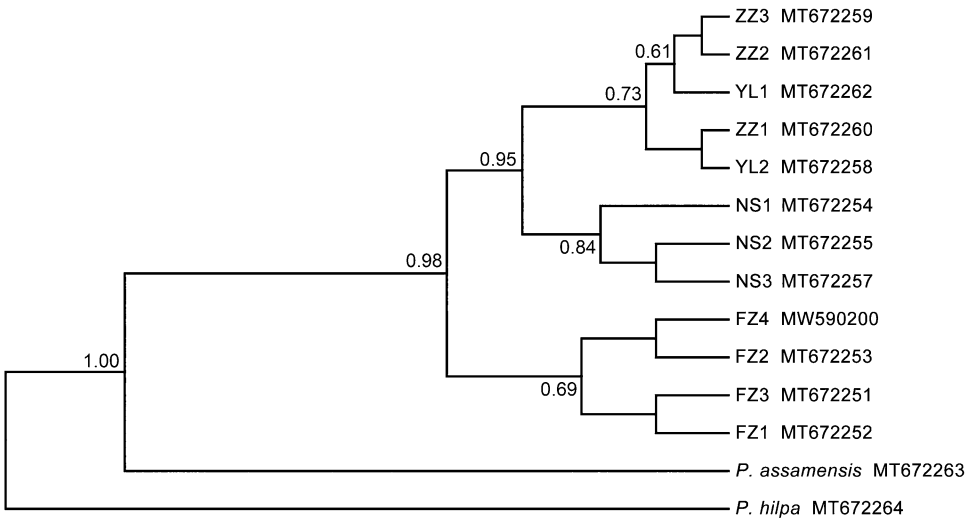


Fig. 1 Phylogram reconstructed based *COI* gene of *P. kaempferi* and allies. Bayesian posterior probabilities are indicated near tree branches. NS, YL, ZZ and FZ represent the population occurring in Ningshan, Yangling, Zhouzhi, and Fuzhou, respectively

based on the *COI* gene, *P. kaempferi* populations were separated into three major clades, i.e., Clade YL + ZZ, Clade NS and Clade FZ (Fig. 1), which also indicates that the Yangling population is more closely related to the Zhouzhi population.

Morphology and Structure of Bacteriomes

For *P. kaempferi*, every individual has two pairs of bacteriomes in the abdomen. The bacteriomes are slightly cyan, and are distinctly recognized as bunches of grapes. Each bacteriome consists of about 12–18 spherical units which are relatively large in size, about 0.6–1.2 mm in diameter. The shape of female bacteriome unit was more round than that of male's (Fig. 2a, b). For each bacteriome unit, two distinct types of bacteriocyte cells were composed underneath the bacteriome sheath (Fig. 2c, d). Results of fluorescence in situ hybridization showed that in each bacteriome unit the cells densely packed within the large syncytium occupying the central part are *Hodgkinia*, and those in the cytoplasm of peripheral bacteriocytes are *Sulcia*. The distribution of these two obligate symbionts in males and females are the same (Fig. 2e, f).

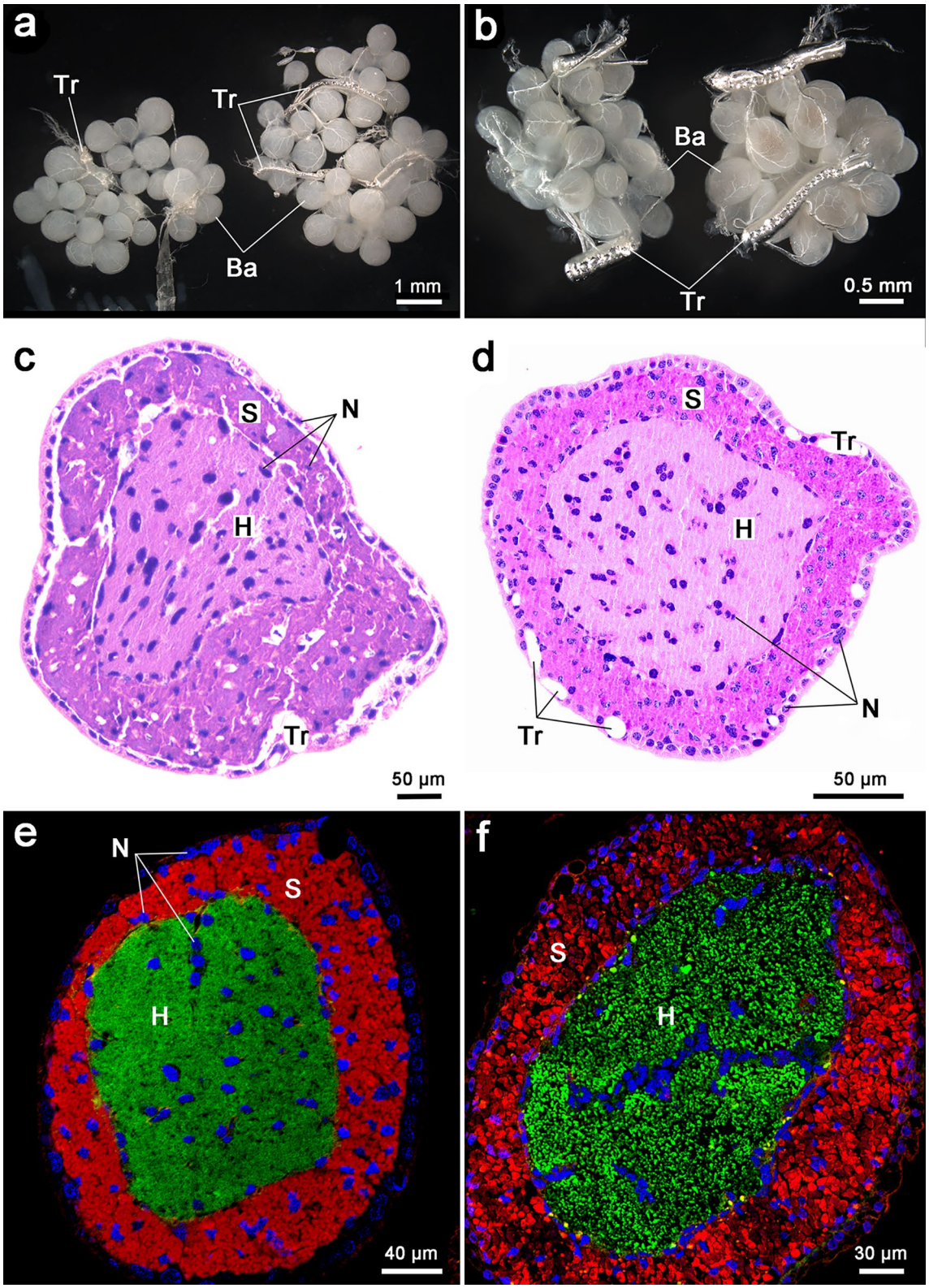
Sequencing Data

An average length of 274 bp region of the 16S rRNA gene was amplified from 33 samples of *P. kaempferi* (Table S3). In total, 2,347,492 sequences were obtained, of which 2,320,805 passed quality checks, resulting in an average of 294 OTUs per sample at 97% similarity. For each sample, the clean sequences and the number of OTUs ranged from 31,463 to 66,263, and 32 to 1563, respectively (Table S3). The rarefaction analysis revealed that the sequencing analysis was comprehensive in covering the bacterial alpha diversity (Fig. S2).

Bacterial Composition of Bacteriomes, Ovaries and Testes

The taxonomic assignment results revealed that the top ten phyla (> 0.1%) at the 97% sequence identity level in *P. kaempferi* were Proteobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Chloroflexi, Actinobacteria, Aminicenantes, Fusobacteria, Nitrospirae and Spirochaetes (Fig. 3a). Among them, the predominant phylum was Bacteroidetes, accounting for 37.70 ± 6.39%, 28.37 ± 7.71% and 21.54 ± 4.07% of the bacterial sequences in Zhouzhi, Yangling and Ningshan populations, respectively. Proteobacteria was the subdominant phylum, which contributed to 6.94 ± 2.92%, 22.12 ± 14.88% and 16.93 ± 5.29% of the bacterial sequences in Zhouzhi, Yangling and Ningshan populations, respectively. Additional phyla with relatively low abundance were mainly enriched in some samples of Zhouzhi and Ningshan populations.

The top ten bacterial genera (> 0.1%) in *P. kaempferi* mainly belong to the phyla Bacteroidetes (*Sulcia*, average relative abundance across all samples: 29.48%), Firmicutes (*Oxalophagus*, *Bacillus* and *Anoxybacillus* of 1.03%, 0.24% and 0.13%, respectively), Proteobacteria (*Liberibacter*, *Rickettsia*, *Pseudomonas* and *Thiobacillus* of 2.40%, 4.22%, 0.83% and 0.21%, respectively), Deinococcus-Thermus (*Meiothermus*, 4.95%) and Actinobacteria (*Rhodococcus*, 0.55%) (Fig. 3b; Table S4). Among the top ten genera, seven genera were shared by the three populations, viz., *Sulcia*, *Meiothermus*, *Oxalophagus*, *Pseudomonas*, *Rhodococcus*, *Thiobacillus*, and *Bacillus*. Only the endosymbiont *Sulcia* was detected in every examined sample, which showed a high relative abundance in most bacteriomes and ovaries (19.30−62.80%). *Rickettsia* and *Anoxybacillus* were both detected in bacteriomes of both sexes and ovaries and testes of the Ningshan population



(*Rickettsia*: 0.29–61.73%; *Anoxybacillus*: < 3.61%), and bacteriomes of both sexes of the Yangling population (*Rickettsia*: < 0.02%; *Anoxybacillus*: < 0.28%), but they were absent in the Zhouzhi population. *Meiothermus* was mainly present in testes and bacteriomes of the three populations, and *Pseudomonas* was mainly present in one bacteriome sample of Yangling population (Ymb3) (21.75%). *Oxalophagus*, *Rhodococcus*, *Thiobacillus* and

◂**Fig. 2** Gross morphology of bacteriomes and distribution of obligate symbionts in bacteriomes of *P. kaempferi*. **a** Gross morphology of bacteriomes in *P. kaempferi* females. **b** Gross morphology of bacteriomes in *P. kaempferi* males. **c** The distribution of symbionts in bacteriomes of *P. kaempferi* females. **d** The distribution of symbionts in bacteriomes of *P. kaempferi* males. **e** Fragment of the bacteriomes of females filled with *Sulcia* and *Hodgkinia*. **f** Fragment of the bacteriomes of males filled with *Sulcia* and *Hodgkinia*. **a**, **b** Light microscopic images. **c**, **d** Histological paraffin sections stained with hematoxylin and eosin. **e**, **f** Fluorescence in situ hybridization images. Blue, red and green visualize DNA, *Sulcia* and *Hodgkinia*, respectively. N, nucleus; Tr, tracheoles, Ba, bacteriome, S, *Sulcia*, H, *Hodgkinia.* (Left) female images and (Right) male images

Bacillus were mainly present in testes of the three populations. Exceptionally, the plant pathogen *Liberibacter* was detected in most samples of Yangling population, and exhibited an extremely high relative abundance (78.76%) in one ovary sample (Yo1).

The total relative abundance of the above bacteria was less than 70% in most samples, while the other bacterial genera accounted for more than 40% (Fig. 3a, b; Table S4). To gain additional information about the bacterial taxonomic identity, we further identified the unclassified OTUs by blasting their sequences with the GenBank database. In total, 860,730 sequence reads (36.67%) of 25 OTUs (Table S5) showed 100% similarity to the previously reported unclassified Rhizobiales bacterium (KR911840−KR911843) which had been detected in *P. kaempferi* [31] and *S. yangi* [13] based on 16S rRNA sequencing. The sequences of these 25 OTUs also showed a high similarity (> 98%) to partial sequence of *Hodgkinia* (LC370455−LC370471) from the *P. kaempferi* detected by Matsuura et al. [18] using genome sequencing. According to the ML phylogenetic tree, all the 25 unclassified OTUs of *P. kaempferi* and the previously reported unclassified Rhizobiales bacterium all clustered in the *Hodgkinia* clade, and formed a monophyletic group with the *Hodgkinia* that harbored in other *Platypleura* species, i.e., *P. yaeyamana* and *P. kuroiwae* (Fig. 4). All these analyses indicated that these 25 unassigned OTUs and the previously reported unclassified Rhizobiales bacterium all belong to *Hodgkinia*. The *Hodgkinia* showed 100% infection rate in all the examined samples of *P. kaempferi*, and especially had a high relative abundance in most bacteriome and ovary samples (Fig. 3c, d).

Bacterial Diversity Across Bacteriomes, Ovaries and Testes

The alpha diversity indices of bacterial assemblages concerned both taxon richness and evenness (Fig. S3 and Table S3). For the Ningshan population, the ACE richness index suggested that the bacterial community of ovaries and testes was richer than that of the bacteriomes of corresponding sex ($P < 0.05$) (Fig. S3a). The Shannon index suggested that the bacterial diversity of bacteriomes of females was significantly higher than that of the males ($P < 0.01$) (Fig. S3b). For the Yangling population, both indices showed no significant differences among different tissues (Fig. S3a, b). For the Zhouzhi population, both alpha diversity indices showed that the bacterial diversity in testes was significantly higher than that in other tissues ($P < 0.05$) (Fig. S3a, b).

The ACE richness index showed that the male bacteriomes, ovaries and testes of the Ningshan population had a more diverse microbial community than the corresponding tissue of the Zhouzhi population ($P < 0.05$) (Fig. S3a). The Shannon index suggested that the bacteriomes of both sexes and ovaries of Ningshan population had a higher bacterial diversity than the corresponding tissue of the Zhouzhi population ($P < 0.05$) (Fig. S3b). The male bacteriomes of the Ningshan population also had a higher bacterial diversity than that of the Yangling population ($P < 0.05$). The male bacteriomes of the Yangling population had a higher bacterial diversity than the male bacteriomes of the Zhouzhi population ($P < 0.05$) (Fig. S3b). After all identified *Sulcia* and *Hodgkinia* OTUs removed, the ACE richness index of all samples decreased, but the Shannon index did not change significantly (Table S3).

Comparisons of Bacterial Community Among Three Populations

The unweighted NMDS and unweighted PCoA showed that the separation between the samples of Ningshan and Zhouzhi populations was robust. The samples of Yangling population grouped moderately with that of Zhouzhi population, but separated moderately from the Ningshan population (Fig. 5a, c). The weighted NMDS and weighted PCoA showed that the separation between the samples of Ningshan and Zhouzhi populations was also robust. But the samples of Yangling population cannot be distinctly separated from the samples of Ningshan and Zhouzhi populations (Fig. 5b, d). Based on the weighted UniFrac UPGMA tree, all samples of the three populations could be divided into two major clades (Fig. 5e): five samples of Yangling population (Yt1, Ymb2, Yo2, Yfb1, Yo1) and all the samples of Zhouzhi population clustered into one clade, and the remaining samples of Yangling population and all samples of Ningshan population clustered into the other clade.

AMOVA results revealed highly significant difference in bacterial community structure between the Ningshan and Zhouzhi populations (AMOVA, unweighted UniFrac, $F_{1, 22} = 9.92$, $P < 0.001$; weighted UniFrac, $F_{1, 22} = 35.69$, $P < 0.001$). The results also revealed highly significant difference in the bacterial community structure between the Yangling and Ningshan populations (unweighted UniFrac, $F_{1, 19} = 7.87$, $P < 0.001$; weighted UniFrac, $F_{1, 19} = 8.83$, $P < 0.001$). The difference of bacterial community structure

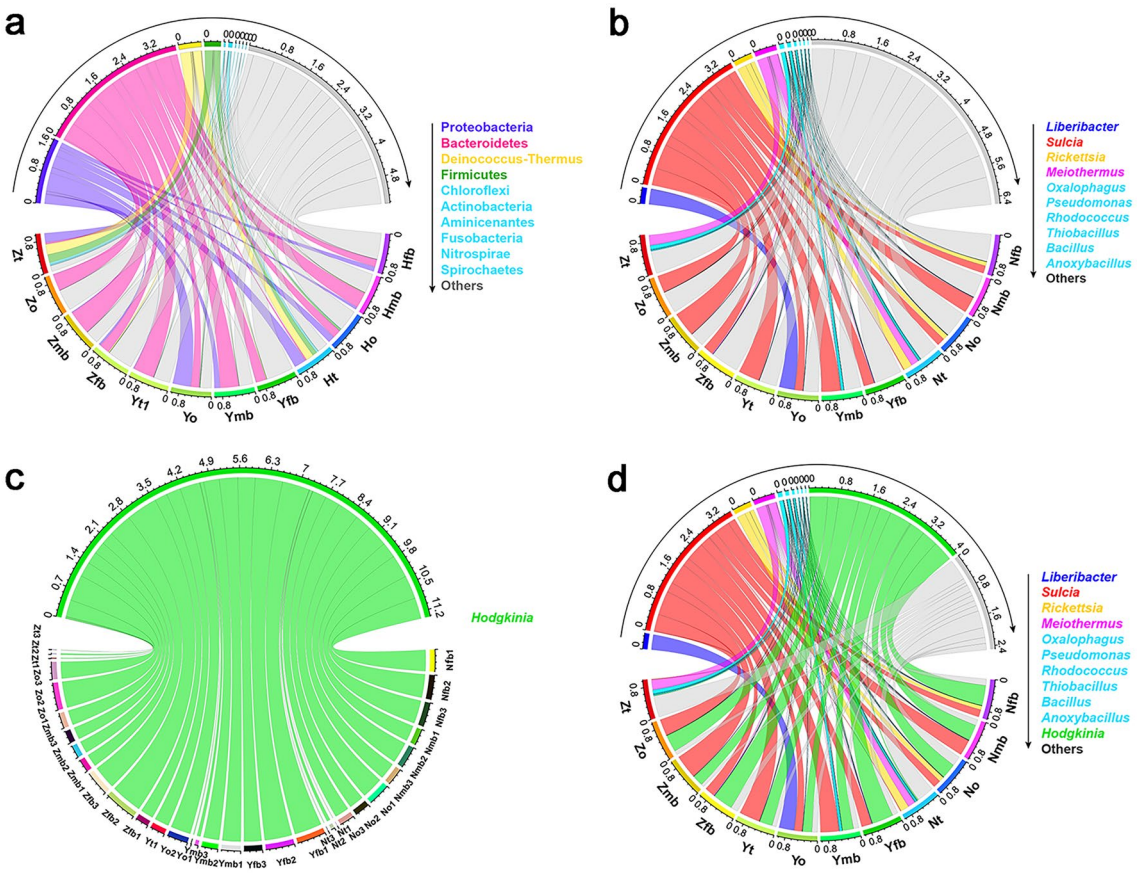


Fig. 3 The relative abundance of main bacterial taxa in *P. kaempferi* based on 16S rRNA gene-targeted amplicon sequencing. **a, b** The relative abundance of main bacterial taxa in *P. kaempferi* at the phylum and genus level, respectively. **c** The relative abundance of *Hodgkinia* in each sample of three populations, based on blasted against with GenBank. **d** Relative abundance of all genus including (**b**) and (**c**). The lower and upper half of each circular plot represents samples and bacterial taxa, respectively. The strings connecting the upper and lower sections represent corresponding bacterial abundance in each sample. Zt, Zo, Zmb, and Zfb, respectively, represent the testes, ovaries, male's bacteriomes, and female's bacteriomes of *P. kaempferi* occurring in Zhouzhi; Yt, Yo, Ymb, and Yfb, respectively, represent the testes, ovaries, male's bacteriomes, and female's bacteriomes of *P. kaempferi* occurring in Yangling; Nt, No, Nmb, and Nfb, respectively, represent the testes, ovaries, male's bacteriomes, and female's bacteriomes of *P. kaempferi* occurring in Ningshan

between the Yangling and Zhouzhi populations was also significant based on weighted UniFrac distance (AMOVA, $F_{1, 19} = 3.63$, $P = 0.027$), but it was not significant based on unweighted UniFrac distance (AMOVA, $F_{1, 19} = 1.57$, $P = 0.107$). ANOSIM revealed highly significant difference in the bacterial community structure between the Ningshan and Zhouzhi populations (ANOSIM, $r = 0.36$, $P = 0.001$). Significant difference was also detected between the Yangling and Ningshan populations (ANOSIM, $r = 0.17$, $P = 0.024$). But no significant difference was found between the Yangling and Zhouzhi populations (ANOSIM, $r = 0.06$, $P = 0.171$).

Discussion

Primary Symbionts of *P. kaempferi*

Morphologically, the bacteriomes consist of a cluster of spherical bacteriome units (Fig. 1a, b), which is similar to that in the previous described cicadas [12]. Although the shape of bacteriome unit of females is more round than that of male's, the internal structure of each bacteriome unit and the distribution of endosymbionts are the same (Fig. 2c, d). The in situ hybridization of oligonucleotides matching the sequence of 16S rRNA clearly clarify that *Sulcia* was harbored in the peripheral bacteriocytes of each bacteriome unit of *P. kaempferi*, and *Hodgkinia* occupied the central syncytial cytoplasm of each bacteriome unit (Fig. 2e, f). *Sulcia* is a highly conserved symbiont, hypothesized to be present in a shared ancestor of the

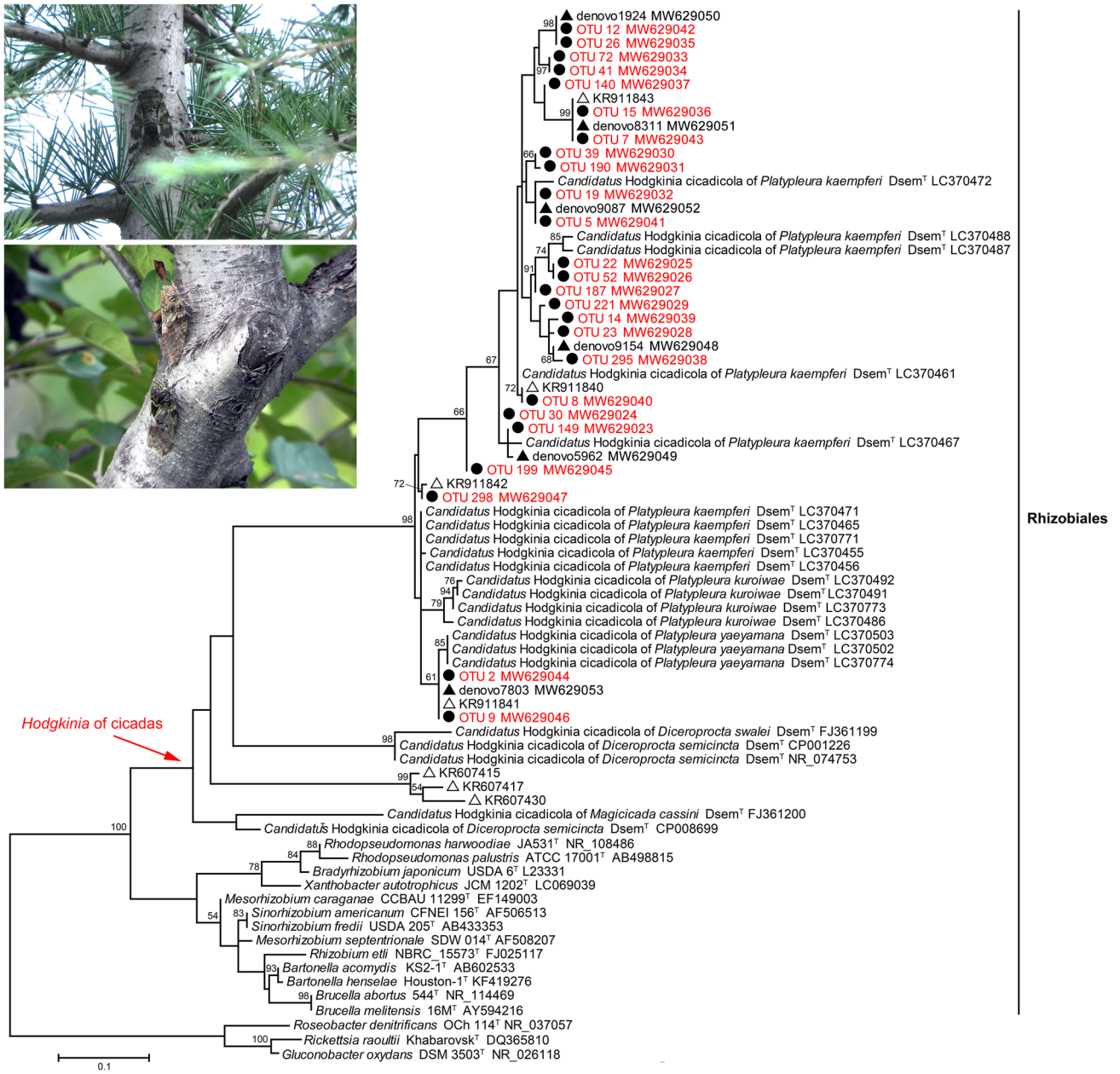
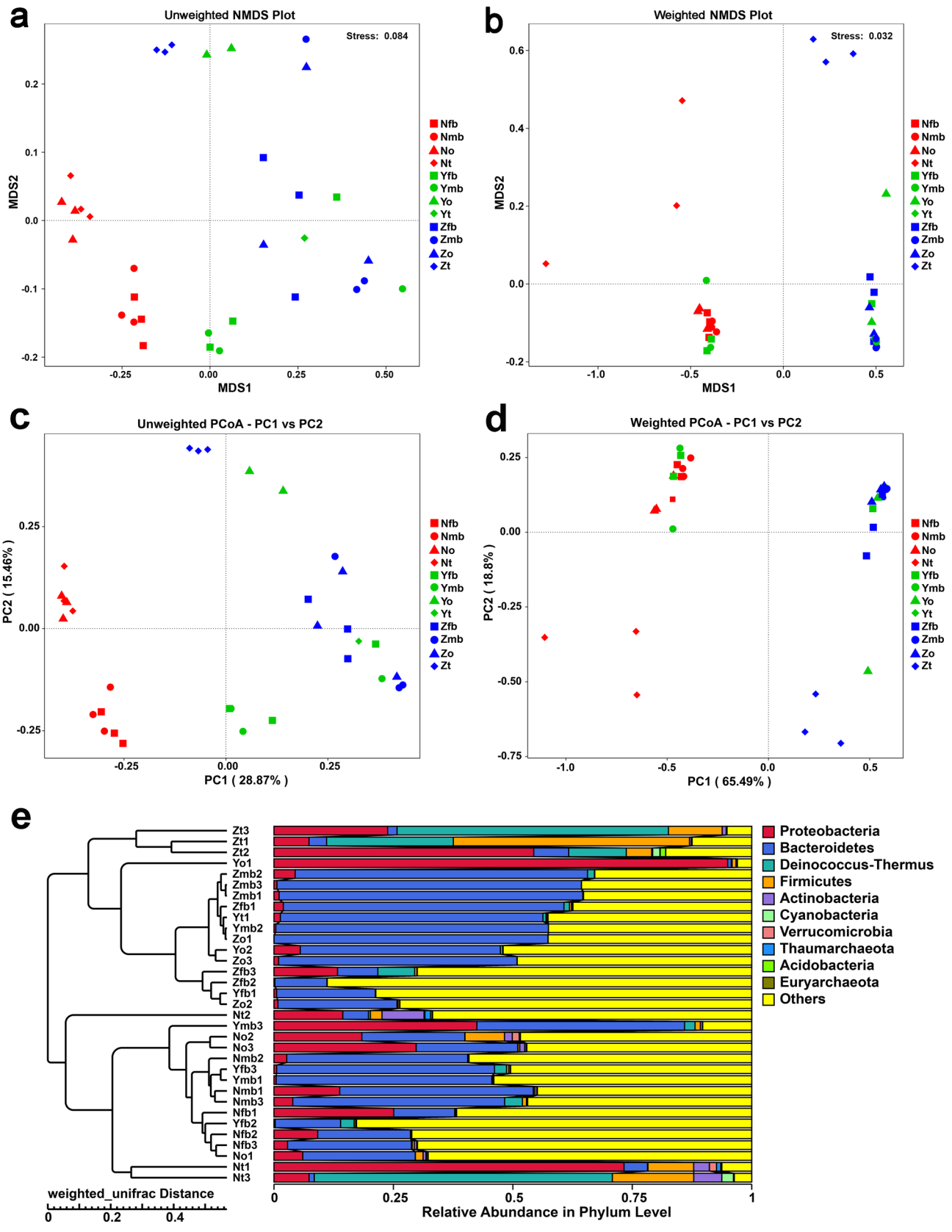


Fig. 4 The ML phylogenetic tree based on 16S rRNA gene sequences of the unclassified OTUs in *P. kaempferi* and other sequences of α-Proteobacteria from GenBank. This tree was generated using the Maximum Likelihood with 1000 bootstrap replicates and Kimura 2-parameter model in MEGA (v5.0) software. The unclassified OTUs of *P. kaempferi* are represented with a red spot. The unclassified Rhizobiales bacterium in *P. kaempferi* from Zheng et al. [29] and in *S. yangi* from Wang et al. [13] are represented with a dark triangle and hollow triangle, respectively. Values less than 50% are not shown

Auchenorrhyncha and retained in most descendant lineages [6]. During the diversification of the Auchenorrhyncha, many lineages have acquired an additional symbiont coexisting with *Sulcia*, such as *Baumannia* in sharpshooters [32], *Zinderia* in spittlebugs [8], and *Vidania* in Cixiidae [33]. In many cicada species, *Hodgkinia* is known as the endosymbiont coexisting with *Sulcia* [6, 7]. The distribution of *Sulcia* and *Hodgkinia* in the bacteriome unit of *P. kaempferi* agrees with the observations of *Sulcia* and *Hodgkinia* by previous authors [9, 10, 12].

Bacteroidetes was the most common phylum identified in the present study, and as expected the cicada primary endosymbiont *Sulcia* was predominant in all bacteriome samples (Fig. 3b), which is consistent with the results of fluorescence in situ hybridization. In addition, *Sulcia* was predominant in all ovaries, but with a low relative abundance in most testes


a
Unweighted NMDS Plot
Stress: 0.084
MDS1
MDS2
b
Weighted NMDS Plot
Stress: 0.032
MDS1
MDS2
c
Unweighted PCoA - PC1 vs PC2
PC1 (28.87%)
PC2 (15.46%)
d
Weighted PCoA - PC1 vs PC2
PC1 (65.49%)
PC2 (18.8%)
Nfb
Nmb
No
Nt
Yfb
Ymb
Yo
Yt
Zfb
Zmb
Zo
Zt
e
Zt3
Zt1
Zt2
Yo1
Zmb2
Zmb3
Zmb1
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Nmb1
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Yfb2
Nfb2
Nfb3
No1
Nt1
Nt3
weighted_unifrac Distance
Relative Abundance in Phylum Level
Proteobacteria
Bacteroidetes
Deinococcus-Thermus
Firmicutes
Actinobacteria
Cyanobacteria
Verrucomicrobia
Thaumarchaeota
Acidobacteria
Euryarchaeota
Others

◂**Fig. 5** Differences in the composition of bacterial community. **a** Unweighted NMDS plots. **b** Weighted NMDS plots. **c** Unweighted PCoA graph. **d** Weighted PCoA graph. **e** UPGMA clustering on weighted UniFrac distances. In (**a**–**d**) each point corresponds to one sample, each sample type is represented by a different shape, and each population is represented by a different color. Abbreviations are listed in the legend of Fig. 3

(Fig. 3b). This distribution pattern is likely related to the vertical transmission of *Sulcia* between insect generations via ovaries. The paucity of paternal symbiont transmission is likely relevant to the extremely streamlined sperm structure: the head consisting of condensed nucleus and the tail made of microtubule bundles, without the symbiont-harboring cytoplasm that is discarded in the process of spermatogenesis [34]. To date, the cellular mechanism for transovarial transmission of *Sulcia* has been studies in some cicada species [12, 14, 35], but more studies are required to clarify the vertical transmission mechanism of related endosymbionts in this Auchenorrhynchan lineage.

In addition, amplicon sequencing detected 25 unclassified OTUs which were found to be identical to the previously reported unclassified Rhizobiales bacterium detected from the bacteriomes of *P. kaempferi* [31] and *S. yangi* [13]. According to the ML phylogenetic tree, these unclassified OTUs clustered with the unclassified Rhizobiales bacterium and *Hodgkinia* in the same clade (Fig. 4), suggesting these 25 unassigned OTUs and the previously reported unclassified Rhizobiales bacterium all belong to *Hodgkinia*. Matsuura et al. [12] revealed that the genome of *Hodgkinia* in bacteriomes of *P. kaempferi* has evolved into complexes of distinct lineages, which may lead to some critical stage of genomic instability and variation. The amplicon sequencing may have impacted the resolution of OTU identification and taxonomic assignment because it analyzed only the V4 hypervariable regions of the 16S rRNA gene [14]. We found *Hodgkinia* was predominant in bacteriomes and ovaries, but with a relatively low abundance in testes (Fig. 3c, d). This is most possibly related to that *Hodgkinia* is vertically transmitted in host generations via the ovaries [14, 15, 35].

Secondary Symbionts of *P. kaempferi*

Liberibacter is a phloem-restricted, nonculturable bacterium which can cause Huanglongbing (HLB) (greening disease) in citrus, even in solanaceous plants such as tomato and potato, and can be vectored by psyllids and leafhoppers [36, 37]. Unexpectedly, we found *Liberibacter* in most samples, especially in ovaries of the Yangling population (Fig. 3b; Table S4). In addition, *Liberibacter* was also described from cicada *M. mongolica* [38] and other phloem-feeding insects, e.g., whiteflies [22]. It seems cicadas and other phloem-feeding insects may also have the opportunity to vector *Liberibacter* to their host plants. But whether *Liberibacter* in the host plants of *P. kaempferi* of Yangling population can cause HLB needs further confirmation.

Rickettsia as symbiont is transmitted vertically in invertebrates, and has been identified from a wide variety of invertebrates such as aphids [39], leafhoppers [40], whiteflies [41], and wasps [42], etc. *Rickettsia* has been reported as a reproductive manipulator of arthropods, similar to the closely related *Wolbachia*, which can induce female bias, increase survival and growth rates, and affect fertility [43]. Moreover, *Rickettsia* can lead to almost complete immunity against the pathogen in aphids [44]. *Rickettsia* was detected in all the samples of the Ningshan population, rare in the Yangling population (only in Yfb3 and Ymb3, $<0.1\%$), and absent in the Zhouzhi population (Fig. 3b; Table S4), which suggest that each population has unique infection patterns by secondary symbionts.

Meiothermus is ubiquitous in alkaline geothermal areas and artificial hot water systems around the world, which can produce restriction enzymes to resist extreme conditions of temperature and pH [45]. Notably, *Meiothermus* was not only detected in all three populations of *P. kaempferi* in this study (Fig. 3b; Table S4), but also detected in other cicada species, including *S. yangi* and *M. mongolica* [13, 24, 38]. Therefore, the function of *Meiothermus* in cicada species needs investigation.

The remaining bacterial taxa such as *Oxalophagus*, *Pseudomonas*, *Rhodococcus*, *Thiobacillus*, *Bacillus*, and *Anoxybacillus* were mainly present in testes and at low frequencies in other tissues (Fig. 3b; Table S4), which may be associated with special functions of related bacteria in testes.

Factors Affecting the Bacterial Community Among Populations

The unweighted and weighted NMDS and PCoA results revealed that the samples of Zhouzhi and Ningshan populations separated strongly from each other (Figs. 5a; S3a), indicating the bacterial communities between these two populations are discrepant. The samples of Yangling population were not separated from the two populations based on weighted NMDS and PCoA results, but were grouped moderately with the samples of Zhouzhi population and separated moderately from that of Ningshan population based on unweighted NMDS and PCoA results (Figs. 5b; S3b). ANOSIM and unweighted AMOVA analyses also revealed significant difference between the Yangling and Ningshan populations, but no significant difference was detected between the Yangling and Zhouzhi populations. These results indicate that the bacterial community in Yangling population is not significantly different from that of Zhouzhi population, but significantly different from that of Ningshan population. The bacterial community across populations of a single insect species can be influenced by many

factors, such as food availability and abiotic factors (e.g., temperature) [18, 46]. Among these factors, diet of the host may be closely related with host phylogeny/taxonomy due to the diet-driven evolution of hosts, and may has a stronger influence on the bacterial community than others do [47]. The population of *P. kaempferi* occurring in Zhouzhi utilizes the apricot tree *A. vulgaris*. The population of *P. kaempferi* occurring in Ningshan feeds mainly on the pine tree *P. tabuliformis*. The different host plants of Zhouzhi and Ningshan populations likely promoted the significantly differentiation of bacterial communities in bacteriomes, ovaries and testes. But no significant difference was detected between the Yangling and Zhouzhi populations, which may be related with the host plants of Yangling population are complex and including that of other two populations, i.e., the fir tree *M. glyptostroboides*, the apricot tree *A. vulgaris* and the pine tree *P. tabuliformis.* Additionally, the intraspecific genetic distances between Ningshan population and Yangling/Zhouzhi populations are higher than that between Yangling and Zhouzhi populations (Fig. 1; Table S2). Therefore, the bacterial community may be influenced by not only the host plants of cicadas but also the population differentiation of the host cicadas, which reflect adaptive changes in microbiota to the host cicada speciation and different diets and habitats.

In addition, many insects are simultaneously infected with more than one symbiont, which lead to the community of secondary symbionts can be shaped by many factors, including horizontal transmission and interactions among symbionts [16, 48]. Zytynska and Weisser [49] have shown that the occurrence of secondary bacterial symbionts in aphids are multiple, which probably depend on both internal (e.g., aphid and symbiont variation) and external factors (e.g., parasitism rate, temperature, and host plant species/abundance). In this study, *Liberibacter* detected in the Yangling population of *P. kaempferi* is a phloem-restricted, non-culturable bacterium, which probably can be acquired and influenced by horizontal transmission. Future work needs to examine more individuals on a broader time scale to get multiple symbiont species of cicadas. It is further important also to consider the surrounding community, e.g., host plants or the natural conditions, to understand how these factors have the potential to mediate cicada-symbiont interactions.

Conclusion

Collectively, the present study revealed that the obligate symbionts *Sulcia* and *Hodgkinia* were harbored in all the examined samples of *P. kaempferi*, which showed a relatively high abundance in most bacteriomes and ovaries. Although the shape of bacteriome unit in females was more round than that of male's, the internal structure of each bacteriome unit and the distribution of endosymbionts are the same. The difference of bacterial communities in bacteriomes, ovaries and testes between the Zhouzhi and Ningshan populations was extremely significant. Significant difference was also detected between the Yangling and Ningshan populations, but not detected between the Yangling and Zhouzhi populations. This may be related to the difference of host plants of these populations. The bacterial communities of different populations can be influenced by the population differentiation of the host cicadas and/or the host plants of cicadas, which reflect adaptive changes in the microbiota of related populations. Our results are informative for further studies of the associations between the bacterial community and population differentiation of sap-feeding insects.

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Author Contributions DW, YL and CW compiled and designed the experiment. All the authors contributed to the interpretation of results and made a significant contribution to the preparation of the manuscript. All the authors have read, revised, and approved the final version of the manuscript.

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Data Availability All sequences have been submitted to GenBank (Accession Nos.: MT672251–MT672255, MT762257–MT672264, MW590200 and MW629023–MW629053). The raw reads of 16S rRNA amplicon sequencing were deposited in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA640760.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This study was carried out in full compliance with the laws of the People's Republic of China. No specific permits were required for our field investigation. All studied species are not included in the 'List of Protected Animals in China'.

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