Diversity of bacteria associated with Hormaphidinae aphids (Hemiptera: Aphididae)

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Abstract
Bacteria are ubiquitous inhabitants of animals. Hormaphidinae is a particular aphid group exhibiting very diverse life history traits. However, the microbiota in this group is poorly known. In the present study, using high-throughput sequencing of bacterial 16S ribosomal RNA gene amplicons, we surveyed the bacterial flora in hormaphidine aphids and explored whether the aphid tribe, host plant and geographical distribution are associated with the distribution of secondary symbionts. The most dominant bacteria detected in hormaphidine species are heritable symbionts. As expected, the primary endosymbiont Buchnera aphidicola is the most abundant symbiont across all species and has coexisted with its host aphids. Six secondary symbionts were detected in Hormaphidinae. Arsenophonus is widespread in Hormaphidinae species, suggesting the possibility of ancient acquisition of this symbiont. Ordination analyses and statistical tests show that the symbiont composition does not seem to relate to any of the aphid tribes, host plants or geographical distributions, which indicate that horizontal transfers might occur for these symbionts in Hormaphidinae. Correlation analysis exhibits negative interference between Buchnera and coexisting secondary symbionts, while the interactions between different secondary symbionts are complicated. These findings display a comprehensive picture of the microbiota in Hormaphidinae and may be helpful in understanding the symbiont diversity within a group of aphids.

Key words Arsenophonus; balance selection; horizontal transfer; symbiont interactions

Introduction
Insects frequently harbor a variety of symbiotic bacteria, and the interactions between them may have important effects on their evolution. Aphids are a group of insects that feed on plant phloem sap and have established a mutualistic relationship with the bacterial symbiont Buchnera aphidicola, which inhabits specialized bacteriocytes and supplies essential nutrients that are lacking in the aphid diet (Buchner, 1965; Douglas, 1993; Sandström & Moran, 1999). Buchnera experiences strictly vertical transmission and diversifies parallel to their host during long-term evolution (Buchner, 1965; Munson et al., 1991; Moran et al., 1993; Baumann et al., 1995; Baumann et al., 1997; Clark et al., 2000; Jousselin et al., 2009; Liu et al., 2013, 2014; Xu et al., 2018).

In addition to the obligate symbiont Buchnera, various secondary symbionts inhabit aphids, namely, Arsenophonus, Fukatsuya symbiotica, Hamiltonella defensa, Regiella insecticola, Rickettsia, Rickettsiella viridis, Seratia symbiotica, Spiroplasma and Wolbachia (Oliver et al., 2010, 2014; Zytynska & Weisser, 2016; Guo et al., 2017). These secondary symbionts distribute erratically in aphids and undergo vertical and some horizontal transmission (Chen & Purcell, 1997; Sandström et al., 2001; Russell et al., 2003; Russell & Moran, 2005; Vorburger, 2009).
et al., 2017; Rock et al., 2018). They play important roles in aphid performance in various environments including protection against parasitic wasps (Oliver et al., 2003, 2005; Vorburger et al., 2010; Hansen et al., 2012; Brandt et al., 2017; Frago et al., 2017), resistance to fungal pathogens (Ferrari et al., 2004; Scarborough et al., 2005; Łukasik et al., 2013), modification of body colors (Tsuchida et al., 2010; Nikoh et al., 2018), interactions with host plants (Leonardo & Müiru, 2003; Tsuchida et al., 2004; Wagner et al., 2015), and thermal tolerance (Chen et al., 2000; Montllor et al., 2002). Furthermore, several facultative symbionts have evolved as co-obligate endosymbionts to supplement Buchnera, such as Erwinia haradaeae, F. symbiotica, H. defensa, S. symbiotica, Sodalis in some Lachninae species (Pérez-Brocal et al., 2006; Lamelas et al., 2011; Manzano-Marin & Latorre, 2014; Manzano-Marin et al., 2016, 2017, 2019; Meseguer et al., 2017) and Wobelia in Pentalonia nigronervosa Coquerel (De Clerck et al., 2015; but see Manzano-Marin, 2019).

The associations between microbial symbionts and aphids varies in different aphid groups. Żytynska and Weisser (2016) reviewed studies about aphid–symbiont associations in 156 aphid species with a strong focus on Western Palearctic samples and mainly Aphidinae and Lachninae groups, 89 and 46 species each, respectively. They revealed that the biological roles of secondary symbionts were dependent on many factors (e.g., aphid species, host plant, genotype); distribution patterns of different symbionts were variable within aphids, which might be contributed by aphid species, host plant species, geography and several environmental factors; and interactions between symbionts were complicated, which might be influenced by both internal (e.g., aphid and symbiont variation) and external factors (e.g., host plant species/abundance, parasitism rate and temperature). Through high-throughput sequencing, new symbiotic associations with Erwinia- and Sodalis-related bacteria and Type-X (later named F. symbiotica) were detected in Cinara species (Jousselin et al., 2016; Meseguer et al., 2017). Using the same method, Fakhour et al. (2018) revealed that the compositions of aphid bacterial flora were not limited to commonly known symbionts, and several other bacteria were also present.

However, few studies have revealed the bacterial community and factors that shape it within a large aphid group. Hormaphidinae is an extraordinary group with complex life cycles. Many species in this subfamily are heteroecious, seasonally obligate alternating between primary and secondary plants. They exhibit strong primary host plant specificity, with each tribe feeding on one generic plant, while the associations with secondary host plants are more relaxed, that is, Cerataphidini on Gramineae, Compositae, and Loranthaceae; Hormaphidini on Betula (Betulaceae) and Picea (Pinaceae); and Nipponaphidini on Fagaceae, Lauraceae and Moraceae (Aoki & Kurosu, 2010; Chen et al., 2014). Hormaphidinae aphid species form morphologically diverse galls on primary hosts, secrete a visible wax coating and produce specialized sterile soldiers (Aoki et al., 1977; Aoki & Miyazaki, 1978; Ghosh, 1985, 1988; Stern & Foster, 1996; Chen & Qiao, 2009; Aoki & Kurosu, 2010; Chen et al., 2014). They are mainly distributed in eastern and southeastern Asia (Heie, 1980; Ghosh, 1985, 1988; von Dohlen et al., 2002).

Almost all aphids harbor B. aphidicola as the primary endosymbiont, but in some hormaphidine species, Buchnera has been lost and replaced by yeast-like symbionts (Fukatsu & Ishikawa, 1992; Fukatsu et al., 1994; Xu et al., 2018). Secondary symbionts in Hormaphidinae have been little studied. Two Hormaphidini species were included in Russell et al. (2003) to explore the distributions of H. defensa, R. insecticola and S. symbiotica, but none of the three symbionts were found in hormaphidine species. Wang et al. (2014) sampled six Hormaphidinae species and detected Wobelia in all of them. Beyond these studies, the bacterial flora in this extraordinary group remains largely unknown to date.

In the present study, based on extensive taxon sampling, we characterize the microbial communities of Hormaphidinae aphids, evaluate the impact of aphid phylogeny, host plant and geographical distribution on the bacterial community and discuss the symbiont infection patterns and interactions using high-throughput sequencing of 16S ribosomal RNA (16S rRNA).

Materials and methods

Sampling and extraction of total DNA

Forty-nine samples representing 23 genera and 49 Hormaphidinae species were collected in this study (Table S1). All specimens were preserved in 95% or 100% and 75% ethanol for molecular experiments and voucher specimens, respectively. All aphid voucher specimens and samples were deposited in the National Zoological Museum of China, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

Each sample analyzed contained three to 10 individuals from the same aphid clone. Aphid specimens were first immersed in 70% ethanol, washed for 5 min (with vortexing and centrifugation) and then rinsed with sterile water four times to remove body surface contaminations. Total DNA was extracted from pure aphids using the DNeasy
Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions, and two negative controls were set. The standard cytochrome oxidase subunit I (COI) barcode of each sample was amplified using the primer pair LCO1490/HCO2198 (Folmer et al., 1994) to verify the aphid species identification and to eliminate parasitized aphids. Three other aphid gene sequences, *Cytb*, *EF-1α* and *LWO*, which were used to reconstruct the phylogeny of Hormaphidinae, were amplified or downloaded from GenBank. All the new sequenced data have been submitted to the GenBank database (Table S1). Phylogenetic congruence between Hormaphidinae species and *Buchnera* was tested. The more detailed methods of these analyses are provided in the Supporting information, Extended methods.

16S rRNA amplicon amplification and sequencing

The 16S rRNA amplicon of the V3–V4 regions was amplified using the primer pair (341F, 5′-CCTAYGGGRBGCASCAG; and 806R, 5′-GGACTACNNGGGTAAAACTTAAT) with a barcode. All polymerase chain reaction (PCR) amplifications were performed in a 30 μL reaction mixture containing 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μmol/L forward and reverse primers and approximately 10 ng template DNA. The PCR conditions were as follows: initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s and final extension at 72 °C for 5 min. Each sample was amplified in duplicate, and one negative and one positive control containing equal amounts of sterile water and DNA of *Escherichia coli* instead of the aphid DNA were prepared for PCR amplification. The PCR assays for the negative controls (two negative controls in the DNA extraction process and one negative control in the 16S rRNA amplification process), *Cerataphis brasiliensis* (Hempel), *Glyphinaphis bambusae* van der Goot and four species in the genus *Tuberaphis* Takahashi were negative; therefore, these samples were not used for library construction. PCR products were mixed in the same volume with 1× loading buffer (containing SYBR green) and subjected to electrophoresis on a 2% agarose gel. Samples with a bright band between 400 and 450 bp were chosen for further experiments. The target bands of the PCR products were excised and mixed in equidensity ratios and then purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Massachusetts, United States). A sequencing library was constructed using the NEB Next Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Waltham, MA, USA) following the manufacturer’s recommendations, and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent Bioanalyzer 2100 system. The library was then sequenced on an Illumina HiSeq2500 platform, and 250 bp paired-end reads were generated. The raw reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) database under BioProject accession number PRJNA553318.

Sequencing data analysis

Paired-end reads were assigned to samples based on their unique barcodes. The reads were then merged using FLASH (V1.2.11) (Magoc & Salzberg, 2011), and low-quality tags and chimeras were filtered by QIIME (Caporaso et al., 2010). Sequences were clustered into operational taxonomic units (OTUs) at 97% identity by pick_de_novo_otus.py in QIIME. The most abundant sequence was picked as a representative sequence for each OTU to annotate taxonomic information with the RDP Classifier based on the SILVA 132 database (Wang et al., 2007; Quast et al., 2013; Yilmaz et al., 2014). The OTUs of singletons and chloroplasts were excluded (Navas-Molina et al., 2013). The OTU abundance of each species was rarefied to the value corresponding to the minimum sum of OTU sequences across all the samples to mitigate the differences in the sequencing effort, and then the relative abundance was calculated based on this rarefied abundance data by dividing the abundance of each OTU by the total abundance of a given species. Subsequent diversity analyses were all performed based on this rarefied abundance or relative abundance data. All OTUs assigned to the reported secondary symbionts of aphids were screened out, and the relative abundance of each secondary symbiont was calculated to better explore the symbiont diversity.

Diversity analysis

To evaluate the alpha diversity of aphid bacterial community, the observed species, the Shannon index and the Simpson index of each species were calculated using the phyloseq package in R 3.5.1 (McMurdie & Holmes, 2013; R Core Team, 2018) based on the OTU abundance table. A rarefaction curve was generated based on the index of observed species.

All samples of Hormaphidinae were grouped according to tribe (including three groups) and host plant family (including four groups with samples ≥3) (Table S2).
Significance tests of the alpha diversity indices (the Shannon and the Simpson indices) for aphid bacteria and secondary symbionts from different groups were performed using the Kruskal-Wallis and Tukey’s honestly significant difference (HSD) tests implemented in the vegan (Oksanen et al., 2018) and agricolae packages (de Mendiburu, 2017), respectively.

The dissimilarities of the bacterial communities and secondary symbiont communities between samples were quantified by calculating the Bray-Curtis dissimilarity using the vegan package (Oksanen et al., 2018). The bacterial communities and secondary symbiont communities among groups were clustered using constrained principal coordinate analysis (CPCoA) and nonmetric multidimensional scaling (NMDS) in the vegan package (Oksanen et al., 2018) based on the relative abundance of each genus and the Bray-Curtis dissimilarity and plots were created in the ggplot2 package (Wickham, 2016). Based on the Bray-Curtis dissimilarity, permutational multivariate analysis of variance (PERMANOVA or ADONIS) was performed in the vegan package (Oksanen et al., 2018) to discern statistically significant differences as a result of grouping factors, and analysis of similarities (ANOSIM) was used to test whether the dissimilarity between groups was significantly greater than those within groups in the pegas package (Paradis, 2010). Bipartite networks between secondary symbionts and their aphid hosts were constructed based on the relative abundance data using the bipartite package (Dormann et al., 2008). The specificity coefficient (d’) for each secondary symbiont was estimated using the function specieslevel in the bipartite package, which compares the relative abundance of interactions of a secondary symbiont with an aphid species with the average relative abundance of interactions of that particular secondary symbiont across all aphid species (Dormann et al., 2008; Dormann, 2011).

To explore the effect of geographic distance among species on structuring the bacterial community, the Spearman correlation coefficient between beta diversity index (Bray-Curtis) and geographic distance matrix was tested. A geographic distance matrix was constructed from geographic points (latitudes and longitudes; Table S2) using the GeoDistanceInMetresMatrix function written by Peter Rosenmai. The Spearman correlation coefficient (\(\rho\)) between the two matrices was calculated, and the significance of the statistic was evaluated by a permutation procedure using the Mantel test in the vegan package (Oksanen et al., 2018).

Spearman’s rank correlation coefficient (\(\rho\)) was calculated to explore the interactions between different symbionts associated with Hormaphidinae based on their relative abundance in the Hmisc package (Harrell & with contributions from Charles Dupont and many others, 2018).

Results

Sequencing data

The sequencing of the 16S rRNA V3–V4 amplicons yielded 9 249 188 raw reads. After quality filtering and removal of chimeric sequences, a total of 8 001 564 effective tags with an average length of 427 nt were obtained. The sequences were classified into 3420 OTUs at 97% sequence identity. The rarefaction curve for each sample tended to saturate (data not shown).

Bacterial diversity across Hormaphidinae aphids

After discarding singletons and chloroplast sequences, 3093 OTUs were obtained and annotated to 23 phyla, 203 families and 469 genera. Overall, 43.62% of these OTUs were attributed to Proteobacteria, 18.76% to Firmicutes and 12.58% to Bacteroidetes. The alpha diversity of bacteria in Hormaphidinae was relatively low (mean Shannon index = 0.53, mean Simpson index = 0.25). The bacterial communities were dominated by B. aphidicola, Serratia, Arsenophonus and Wolbachia (Fig. 1). In addition, Gilliamella (the family Orbaceae) was detected in 29 Hormaphidinae species with high relative abundance (average relative abundance across all samples: 1.32%; Fig. S1). The total relative abundance of the above bacteria was more than 93.00% in most samples, and the other bacterial genera accounted for less than 0.50%.

The primary endosymbiont B. aphidicola was detected in all species with an average relative abundance of 81.73%. Buchnera and the corresponding hormaphidinae species are cospediated. The result of the Jane analysis showed significant phylogenetic concordance between hormaphidinae aphids and Buchnera (\(P < 0.01\)) (Fig. S2). The ParaFit analysis rejected the null hypothesis that the phylogenetic trees of aphids and Buchnera were randomly associated (ParaFitGlobal = 1.1776, \(P = 0.0001\)), and 40 individual host-parasite-associated links contributed to the global trace statistic (\(P < 0.05\)).

A total of six aphid secondary symbionts were detected in hormaphidinae aphids (Fig. 2), and a bipartite network analysis of secondary symbiont interactions with Hormaphidinae species is reported in Figure 3. Arsenophonus inhabited all the species (detection frequency: 43/43; average relative abundance across all samples: 3.79%), followed by Wolbachia (40/43; 2.47%) and...
S. symbiotica (35/43; 4.15%). H. defensa (11/43; 0.11%) and Rickettsia (21/43; 0.15%) detected in hormaphidine species with low abundance, while R. insecticola was only present in two species with extremely low abundance (<0.004%). Every secondary symbiont contained just several OTUs. There were seven OTUs for Arsenophonus, six for S. symbiotica, four for Wolbachia, two for Rickettsia and only one for H. defensa and R. insecticola. The reads belonging to each OTU were not equal, but there were no more than two OTUs dominating in each aphid species. Noticeably, the same phylotypes were present in some distant aphid species. Almost all of the sampled species (42/43) were infected with at least two secondary symbionts except for Hormaphis betulae Osten-Sacken, which was only infected with Arsenophonus (Table S3 and S4). The combination of Arsenophonus, S. symbiotica and Wolbachia (12/43) was the most common type, followed by those of Arsenophonus, Rickettsia, S. symbiotica and Wolbachia (10/43) and Arsenophonus, H. defensa, Rickettsia, S. symbiotica and Wolbachia (10/43). Furthermore, Hybothoracaphis laevigata Chen, Jiang, Chen & Qiao was infected with all six detected secondary symbionts. The specificity (d') of secondary symbionts was inferred (Fig. 3). The specificities of H. defensa, Rickettsia and R. insecticola were 0.57, 0.53 and 0.07, respectively. Arsenophonus, S. symbiotica and Wolbachia had a specificity of zero.

Measurement of within-sample diversity (alpha diversity) of bacteria showed significant differences between Hormaphidini and both Cerataphidini and Nipponaphidini (Kruskal-Wallis test: \( P < 0.05 \) between Hormaphidini and Cerataphidini and between Hormaphidini and Nipponaphidini for the Shannon and the Simpson index; Tukey’s HSD test: \( P < 0.05 \) between Hormaphidini and Cerataphidini for the Shannon and the Simpson index; Fig. S3A and B). The microbiota of Hormaphidini including four sampled species had lower diversity than those of Cerataphidini and Nipponaphidini (Fig. S3A and B). However, there were no significant differences in the diversity of secondary symbionts among the three tribes (Fig. 4A and B). The alpha diversity of neither bacteria (Fig. S3C and D) nor secondary symbionts (Fig. 4C and D) showed significant differences among hormaphidine species exploiting different plant families. We found that compositions of the bacterial microbiota and secondary symbiont community were similar among different groups. CPCoA and NMDS did not form any clusters of either the bacterial community (Fig. S3E–H) or the secondary symbiont community (Fig. 4E–H) for three hormaphidine tribes and for species feeding on different plant families.

**Comparison of bacterial and secondary symbiont communities associated with Hormaphidinae among different grouping sets**

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plants. Statistical analyses showed that there were no significant differences in bacterial community compositions among the three hormaphidine tribes (ADONIS: $F_{2,40} = 0.77, R^2 = 0.04, P = 0.57$; ANOSIM: $R = -0.11, P = 0.97$) and among aphids feeding on different plants (ADONIS: $F_{3,27} = 0.96, R^2 = 0.10, P = 0.42$; ANOSIM: $R = -0.06, P = 0.68$). The composition of the secondary symbiont community was also not different among the three hormaphidine tribes (ADONIS: $F_{2,40} = 1.17, R^2 = 0.06, P = 0.29$; ANOSIM: $R = -0.004, P = 0.50$) and among aphids feeding on different plants (ADONIS: $F_{3,27} = 0.83, R^2 = 0.08, P = 0.54$; ANOSIM: $R = 0.05, P = 0.26$). The correlation between the beta diversity index of both bacteria and secondary symbionts and specimen geographic distance was not significant (Mantel test: $\rho = -0.08–0.01, P = 0.79–0.39$).

**Correlation test between different symbionts associated with Hormaphidinae aphids**

The results of Spearman's correlation coefficient are shown in Figure 5 and Table S5. The relative abundance of *Arsenophonus* and *Rickettsia* ($r = 0.48, P < 0.01$), *Hamiltonella* and *Regiella* ($r = 0.44, P < 0.01$), *Hamiltonella* and *Rickettsia* ($r = 0.43, P < 0.01$), *Hamiltonella* and *Serratia* ($r = 0.62, P < 0.001$) and *Rickettsia* and *Wolbachia* ($r = 0.41, P < 0.01$) were positively correlated.
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Fig. 3 Interaction network structure between aphids and secondary symbionts. The width of the links is proportional to the relative abundance of secondary symbionts associated with a given aphid species. The bottom and top boxes represent the Hormaphidinae species and secondary symbionts, respectively. Colors correspond to different secondary symbionts, as shown in the legend. Specificity values (d’) for secondary symbionts are reported.

B. aphidicola had significantly negative correlations with Serratia (r = −0.56, P < 0.001) and Wolbachia (r = −0.57, P < 0.001).

Discussion

Symbionts inhabiting Hormaphidinae aphids

Our study revealed the bacterial communities of Hormaphidinae. B. aphidicola inhabited all the sampled species with the highest relative abundance. Considering the obligate nutritive role of Buchnera and the long-term endosymbiotic association between aphids and Buchnera (Douglas & Prosser, 1992; Moran et al., 1993; Shigenobu et al., 2000), the ubiquity of the high abundance of Buchnera in the present study seems to be predictable. Buchnera and aphids have been demonstrated to diversify in parallel in several aphid groups (Buchner, 1965; Munson et al., 1991; Moran et al., 1993; Baumann et al., 1995; Baumann et al., 1997; Clark et al., 2000; Jousselin et al., 2009; Liu et al., 2013, 2014; Xu et al., 2018). In the present study, the codiversification of hormaphidine species and the corresponding Buchnera is also confirmed, which presents an instance of parallel evolution between aphids and Buchnera within a subfamily.

Six secondary symbionts were detected in this study; however, the infection patterns of these symbionts varied in Hormaphidinae. Our data showed that Arsenophonus was present in all the sampled Hormaphidinae species with high relative abundance. The ubiquity of Arsenophonus in Hormaphidinae species suggests that this symbiont acquisition could be ancient in this aphid subfamily and followed by vertical transmission. Jousselin et al. (2013) largely surveyed the diversity of Arsenophonus in aphids and revealed a high incidence of Arsenophonus in the Aphis Linnaeus genus; and Zouari et al. (2018) detected Arsenophonus in all studied Aphidini species (two Aphis and three Hyalopterus Koch species). Our results confirm that Arsenophonus is a major secondary symbiont of aphids and is widespread across aphid taxa. Arsenophonus could increase the growth of the soybean aphid population (Wulff & White, 2015), and Aphis craccivora Koch hosting Arsenophonus promoted specialization in locust host plants (Wagner et al., 2015). Similarly, the high prevalence of Arsenophonus in Hormaphidina could not be random, which suggests that this symbiont may play an important role in these species. However, all of these require further investigation.

Wolbachia is a common symbiont of terrestrial arthropods and can manipulate the reproduction of mutualists (Stouthamer et al., 1999; Zug & Hammerstein, 2012). Several studies failed to detect Wolbachia in aphids (West et al., 1998; Tsuchida et al., 2002; Kittayapong et al., 2003; Nirgianaki et al., 2003; Carletto et al., 2008), while Wang et al. (2014) found a widespread infection of Wolbachia in Chinese aphid populations. Consistent with the results of Wang et al. (2014), all but two species in this study hosted Wolbachia. The true figure of Wolbachia diversity might have been underestimated because of its low titer, its high genetic divergence or inappropriate detection methods (Augustinos et al., 2011). However, the effects of Wolbachia in aphids are still unclear, and further detailed studies are needed to illustrate the exact role of Wolbachia.

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Fig. 4 Boxplot comparing the Shannon index of hormaphidine secondary symbiont communities from three tribes (A) and four plant families (C); comparing the Simpson index of hormaphidine secondary symbiont communities from three tribes (B) and four plant families (D). No significant differences across groups on the basis of Kruskal-Wallis and Tukey’s honestly significant difference test. The bottom and top edges of the boxes mark the 25th and 75th percentiles (i.e., first and third quartiles), respectively. Lines within boxes represent the medians, hinges represent the ±25% quartiles, and whiskers represent up to 1.5× the interquartile range. Constrained principal coordinate analysis (CPCoA) plot illustrating the separation of samples based on differences in secondary symbiont community structure among three tribes (5.4% of the total variance, $P = 0.29$) (E) and four plant families (9.36% of the total variance, $P = 0.48$) (G). Nonmetric multidimensional scaling (NMDS) plot illustrating the separation of samples based on differences in bacterial community structure among three tribes (stress = 0.07) (F) and four plant families (stress = 0.07) (H). Colors correspond to different groups, as shown in the legend.

For aphids suffering from heat shock or subjected to constant high temperature, their fitness increased while hosting *S. symbiotica*, possibly by rescuing the primary symbiont *Buchnera* (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006). Field studies showed a higher prevalence of *S. symbiotica* in aphids collected in the summer season than in aphids collected 2–4 months earlier at the same site (Montllor et al., 2002). The main distribution areas of Hormaphidinae aphids are eastern and southeastern Asia with warm or hot climates (Heie, 1980; Ghosh, 1985, 1988; von Dohlen et al., 2002). In the present study, all samples were collected in subtropical or tropical zones in China. Chronic exposure to high temperature may reduce the fitness of aphids; hence, most sampled species (86.05%) host *S. symbiotica*, which may increase their thermal tolerance and suitability under high temperature.

The defensive roles of *H. defensa*, *R. insecticola* and *Rickettsia* have been documented in many studies (Ferrari et al., 2004; Oliver et al., 2005; Scarborough et al., 2005; Vorburger et al., 2010; Hansen et al., 2012; Lukasik et al., 2013; Oliver et al., 2014; Vorburger & Rouchet, 2016). However, in the present study, *Rickettsia* and *H. defensa* were detected in no more than half of the samples with low abundance (<1%), and *R. insecticola* was only screened in two of all sampled species with an extremely low abundance (<0.004%). Most species in Hormaphidinae secrete wax to protect against fungal infection, and the visible wax coating can protect against parasitoids and predators (Smith, 1999; Moss et al., 2006; Pope, 2010; Chen & Qiao, 2012; Su et al., 2016). Alternating between different host plants could also help hormaphidine species hide from parasitoids and predators (Way & Banks, 1968; Eastop, 1998). These intrinsic characteristics of...
Hormaphidinae can reduce the pressures of parasitoids and predators on these aphid groups. Furthermore, carrying defensive symbionts can entail costs in aphids (Chen et al., 2000; Oliver et al., 2008; Polin et al., 2014; Zytynska et al., 2019). Our results revealed negative correlations between the primary symbiont *Buchnera* and most secondary symbionts, which indicates that competition for limited resources and inhabitations within aphids between the primary and secondary symbionts may exist (Zytynska & Weisser, 2016). Therefore, the patchy distributions of these symbionts within Hormaphidinae may be a balance selection (Oliver et al., 2014).

We also found a *Gilliamella* bacterium in more than half of the sampled species in the present study. The relative abundance of this bacterium was high in several species and second only to the primary symbiont *Buchnera*. *Gilliamella* is a gut symbiont of bees and stimulates bees to utilize several toxic sugars; therefore, *Gilliamella* maintains the health of the bee host (Kwong & Moran, 2013; Kwong et al., 2014; Zheng et al., 2016). We propose that this bacterium might also be a gut symbiont in aphids. However, the actual role of *Gilliamella* in aphids remains to be experimentally tested.

**Fig. 5** Heatmap of Spearman correlation coefficients of symbionts. Positive correlations are indicated as red gradients from 0 to 1.0 and negative correlations are indicated as blue gradients from −1.0 to 0, as shown in the legend.

The influence of geographical distribution, host plant and aphid phylogeny on symbiont communities

Geographical distribution and food plants of aphids have been reported to influence symbiont communities, but the samples involved in these studies were mainly different populations from the same species (Najar-Rodriguez et al., 2009; Jones et al., 2011; Ferrari et al., 2012; Russell et al., 2013; Brady et al., 2014; Henry et al., 2015; Zhao et al., 2016; GalloFranco et al., 2019; Guo et al., 2019). Our results showed that there were no correlations between the community of aphid secondary symbionts and aphid phylogeny or aphid distributions. The aphid food plant also appears to have no impact on the community profiles of bacteria and symbiotic microbes. Neither ordination analyses nor statistical tests revealed effects of both the host plant and hormaphidine tribes on bacteria or secondary symbiont communities. These findings are consistent with previous studies that reported similar results in which the geographical distributions or host plants did not structure symbiont communities (Fakhour et al., 2018), and the presence of certain secondary symbionts was not affected by aphid phylogeny (Henry et al., 2015). No obvious specificities of secondary symbionts toward aphid species were found in the network analysis. Overall, secondary symbionts did not form any specific clusters but showed a relatively uniform distribution across hormaphidine taxa. Furthermore, every secondary symbiont contained just a few OTUs, and the same phylotype was shared by distantly related aphid taxa. These results indicate that horizontal transfers of secondary symbionts may occur in Hormaphidinae. Horizontal transmission has been reported to repeatedly occur in several secondary symbionts (Sandström et al., 2001; Russell et al., 2003; Jousselin et al., 2013). Bacterial symbionts can perform horizontal transfer during aphid sexual reproduction via aphid host plants and through sequential stabbing in different aphids by parasitoids (Moran and Dunbar, 2006; Gehrer & Vorburger, 2012; Chrostek et al., 2017; Pons et al., 2019). Many hormaphidine species are heteroecious holocyclic (Ghosh, 1985, 1988). The species in Hormaphidinae with sexual generation, seasonal host alternation between primary and secondary host plants and repeated migrations among different secondary host plants could greatly increase the possibility of horizontal transmission of their secondary symbionts.

**Interactions between secondary symbionts**

Multi-infections with secondary symbionts occurred commonly in Hormaphidinae. Strong positive correlations of various secondary symbiont combinations were revealed by Spearman correlation analysis in this study. The superinfection of secondary symbionts has been documented in several studies (Ferrari et al., 2012; Russell et al., 2013; Smith et al., 2015; Zytynska et al., 2016; Zhang et al., 2019). Multiple infection may be a result of
frequent horizontal transmission of secondary symbionts and alternatively form a horizontal gene pool for recombination or transfer (Moran & Dunbar, 2006; Henry et al., 2013; Russell et al., 2013). Coinfections of Hamiltonella–Serratia and Hamiltonella–Fukatsuia exhibited greater resistance to parasites in Acyrthosiphon pisum (Harris) (Oliver et al., 2006; Guay et al., 2009). However, co-infection with Hamiltonella and Arsenophonus enhanced the self-fitness of Aphis gossypii Glover rather than the resistance against parasitoids (Ayoubi et al., 2020). In contrast, coinfecting Hamiltonella negatively affected the beneficial phenotype provided by Rickettsiella (Leclair et al., 2017). Furthermore, the cost of hosting multiple symbionts may additively combine (Oliver et al., 2006; Leclair et al., 2017). McLean et al. (2018) showed a polymorphic figure of multiple infections with different symbiont combinations. These findings suggest that the interactions between secondary symbionts can be synergistic, additive or antagonistic.

Conclusions

In this study, using high-throughput sequencing of bacterial 16S rRNA gene amplicons, we described the bacterial diversity in the aphid subfamily Hormaphidinae. The primary endosymbiont Buchnera unsurprisingly inhabited all species, in accordance with its obligate mutualist role. Otherwise, we provide a good example of codiversification between aphids and Buchnera at the subfamily level. Arsenophonus was the predominant secondary symbiont in Hormaphidinae species, and its high prevalence might indicate an ancient acquisition of this symbiont. There were no relationships between symbiont diversity and any of the aphid tribes, host plants or geographical distributions. These reveal unspecific clusters of secondary symbionts, which suggest horizontal transmission may occur for these secondary symbionts. Moreover, multiple infections of secondary symbionts were common in Hormaphidinae, but the interactions between them were very complicated. In addition, we first reported the bacterium Gilliamella in Hormaphidinae, and the high abundance of Gilliamella indicated that it may exert biological effects on aphids.

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Disclosure

The authors declare that they have no conflict of interests.

References

Aoki, S. and Kurosu, U. (2010) A review of the biology of Cerataphidini (Hemiptera, Aphididae, Hormaphidinae), focusing mainly on their life cycles, gall formation, and soldiers. Psyche, 2010, 1–34.
Aoki, S. and Miyazaki, M. (1978) Notes on the pseudoscorpion-like larvae of Pseudoregma alexanderi (Homoptera, Aphidoidea). Kontyä, 46, 433–438.
Aoki, S., Yamane, S. and Kiuchi, M. (1977) On the biters of Astegopteryx styracicola (Homoptera, Aphidoidea). Kontyä, 45, 563–570.
Augustinos, A.A., Santos-Garcia, D., Dionyssopeulou, E., Mor-eira, M., Papapanagiotou, A., Scarvelakis, M. et al. (2011) Detection and characterization of Wolbachia infections in natural populations of aphids: is the hidden diversity fully unraveled? PLoS ONE, 6, e28695.
Ayoubi, A., Talebi, A.A., Fathipour, Y. and Mehrabadi, M. (2020) Coinfection of the secondary symbionts, Hamiltonella defensa and Arsenophonus sp. contribute to the performance of the major aphid pest, Aphis gossypii (Homoptera: Aphididae). Insect Science, 27, 86–98.
Baumann, P., Baumann, L., Lai, C.Y., Rouhbakhsh, D., Moran, N.A. and Clark, M.A. (1995) Genetics, physiology, and evolutionary relationships of the genus Buchnera: intracellular symbionts of aphids. Annual Review of Microbiology, 49, 55–94.
Baumann, P., Moran, N.A. and Baumann, L. (1997) The evolution and genetics of aphid endosymbionts. Bioscience, 47, 12–20.
Brady, C.M., Asplen, M.K., Desneux, N., Heimpel, G.E., Hopper, K.R., Linnen, C.R. et al. (2014) Worldwide populations of the aphid Aphis craccivora are infected with diverse facultative bacterial symbionts. Microbial Ecology, 67, 195–204.

© 2019 The Authors. Insect Science published by John Wiley & Sons Australia, Ltd on behalf of Institute of Zoology, Chinese Academy of Sciences, 28, 165–179.
Brandt, J.W., Chevignon, G., Oliver, K.M. and Strand, M.R. (2017) Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. *Proceedings of the Royal Society B: Biological Sciences*, 284, 20171925.

Buchner, P. (1965) *Endosymbiosis of Animals with Plant Microorganisms*. Interscience Publishers, New York.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336.

Carletto, J., Gueguen, G., Fleury, F. and Vanlerberge-Masutti, F. (2008) Screening the bacterial endosymbiotic community of sap-feeding insects by terminal-restriction fragment length polymorphism analysis. *Entomologia Experimentalis et Applicata*, 129, 228–234.

Chen, D.Q., Montllor, C.B. and Purcell, A.H. (2000) Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pismum*, and the blue alfalfa aphid, *A. kondoi*. *Entomologia Experimentalis et Applicata*, 95, 315–323.

Chen, D.Q. and Purcell, A.H. (1997) Occurrence and transmission of facultative endosymbionts in aphids. *Current Microbiology*, 34, 220–225.

Chen, J., Jiang, L.Y. and Qiao, G.X. (2014) A total-evidence phylogenetic analysis of Hormaphidinae (Hemiptera: Aphididae), with comments on the evolution of galls. *Cladistics*, 30, 26–66.

Chen, J. and Qiao, G.X. (2009) A study on diversity of aphid’s galls of Hormaphidinae. *Acta Zootaxonomica Sinica*, 34, 269–276.

Chen, J. and Qiao, G.X. (2012) Wax gland plates in Hormaphidinae (Hemiptera: Aphididae): morphological diversity and evolution. *Entomological News*, 122, 27–45.

Chrostek, E., Pelz-Stelinski, K., Hurst, G.D.D. and Hughes, G.L. (2017) Horizontal transmission of intracellular insect symbionts via plants. *Frontiers in Microbiology*, 8, 2237.

Clark, M.A., Moran, N.A., Baumann, P. and Wernegreen, J.J. (2000) Coalescence between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution*, 54, 517–525.

De Clerck, C., Fujiwara, A., Joncour, P., Leonard, S., Felix, M.L., Francis, F. *et al.* (2015) A metagenomic approach from aphid’s hemolymph sheds light on the potential roles of co-existing endosymbionts. *Microbiome*, 3, 63.

de Mendiburu, F. (2017) *Agricolae: statistical procedures for agricultural research*. R package version 1.2-8. https://CRAN.R-project.org/package=agricolae

Dohlen, C.D. von, Kurosu, U. and Aoki, S. (2002) Phylogenetics and evolution of the eastern Asian-eastern North American disjunct aphid tribe, Hormaphidini (Hemiptera: Aphididae). *Molecular Phylogenetics and Evolution*, 23, 257–267.

Dornmann, C.F. (2011) How to be a specialist? Quantifying specialization in pollination networks. *Network Biology*, 1, 1–20.

Dornmann, C.F., Gruber, B. and Fruend, J. (2008) Introducing the bipartite package: analysing ecological networks. *R News*, 8, 8–11.

Douglas, A.E. (1993) The nutritional quality of phloem sap utilized by natural aphid populations. *Ecological Entomology*, 18, 31–38.

Douglas, A.E. and Prosser, W.A. (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pismum*) symbiosis. *Journal of Insect Physiology*, 38, 565–568.

Eastop, V.F. (1998) Why do aphids do that? *Aphids in Natural and Managed Ecosystems: Proceedings of the Fifth International Symposium on Aphids* (eds J.M. Nieto Nafria & A.F.G. Dixon), pp. 37–47. University of Leon, Leon.

Fakhour, S., Ambroise, J., Renoz, F., Foray, V., Gala, J.L. and Hance, T. (2018) A large-scale field study of bacterial communities in cereal aphid populations across Morocco. *FEMS Microbiology Ecology*, 94, fiy003.

Ferrari, J., Darby, A.C., Daniell, T.J., Godfray, H.C.J. and Douglas, A.E. (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology*, 29, 60–65.

Ferrari, J., West, J.A., Via, S. and Godfray, H.C. (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. *Evolution*, 66, 375–390.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.

Frage, E., Mala, M., Weldegergis, B.T., Yang, C., Mclean, A., Godfray, H.C.J. *et al.* (2017) Symbionts protect aphids from parasitic wasps by attenuating herbivore-induced plant volatiles. *Nature Communications*, 8, 1860.

Fukatsu, T., Aoki, S., Kuros, U. and Ishikawa, H. (1994) Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. *Zoological Science*, 11, 613–623.

Fukatsu, T. and Ishikawa, H. (1992) A novel eukaryotic extracellular symbiont in an aphid, *Astegopteryx styraci* (Homoptera, Aphididae, Hormaphidinae). *Journal of Insect Physiology*, 38, 765–773.

Gallo-Franco, J.J., Duque-Gamboa, D.N. and Toro-Perea, N. (2019) Bacterial communities of *Aphis gossypii* and *Myzus persicae* (Hemiptera: Aphididae) from pepper crops (*Capsicum sp.*). *Scientific Reports*, 9, 5766.

Gehrer, L. and Vorburger, C. (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biological Letters*, 8, 613–615.
Ghosh, A.K. (1985) Hormaphidinae: distribution, phylogeny and systematics. Evolution and Biosystematics of Aphids: Proceedings of the International Aphidological Symposium at Jablonna, 1981 (ed. H. Szelegiewicz), pp. 303–336. Polska Akademia Nauk, Warsaw.

Ghosh, A.K. (1988) The Fauna of India and the Adjacent Countries (Hemiptera: Aplicoidea). Part 4. Subfamilies: Phloeomyzinae, Anoeiinae and Hormaphidinae. Zoological Survey of India, Calcutta, India.

Guay, J.F., Boudreault, S., Michaud, D. and Cloutier, C. (2009) Impact of environmental stress on aphid clonal resistance to parasitoids: Role of Hamiltonella defensa bacterial symbiosis in association with a new facultative symbiont of the pea aphid. Journal of Insect Physiology, 55, 919–926.

Guo, J.Q., Watt, S., He, K., Chen, J., Francis, F. and Wang, Z. (2017) Nine facultative endosymbionts in aphid species. A review. Journal of Asia-Pacific Entomology, 20, 794–801.

Guo, J.Q., Liu, X.W., Poncelet, N., He, K.L., Francis, F. and Wang, Z.Y. (2019) Detection and geographic distribution of seven facultative endosymbionts in two Rhopalosiphum aphid species. Microbiologyopen, 8, e00817.

Hansen, A.K., Vorburger, C. and Moran, N.A. (2012) Genomic basis of endosymbiont-conferred protection against an insect parasitoid. Genome Research, 22, 106–114.

Harrell, F.E. Jr and with contributions from Charles Dupont and many others (2018) Hmisc: Harrell Miscellaneous. R package version 4.2-0. https://CRAN.R-project.org/package=Hmisc

Heie, O.E. (1980) Fauna Entomologica Scandinavica: The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. Volumen I. General Part. The Families Mindaridae, Hormaphididae, Thelaxidae, Anoeiidae, and Pemphigidae. Scandinavian Science Press, Klampenborg.

Henry, L.M., Maiden, M.C., Ferrari, J. and Godfray, H.C. (2015) Insect life history and the evolution of bacterial mutualism. Ecology Letters, 18, 516–525.

Henry, L.M., Peccout, J., Simon, J.C., Hadfield, J.D., Maiden, M.J., Ferrari, J. and et al. (2013) Horizontally transmitted symbionts and host colonization of ecological niches. Current Biology, 23, 1713–1717.

Jones, R.T., Bressan, A., Greenwell, A.M. and Fierer, N. (2011) Bacterial communities of two parthenogenetic aphid species cocolonizing two host plants across the Hawaiian Islands. Applied and Environmental Microbiology, 77, 8345–8349.

Jousselin, E., Clamens, A.L., Galan, M., Bernard, M., Maman, S., Gschloessl, B. et al. (2016) Assessment of a 16S rRNA amplicon Illumina sequencing procedure for studying the microbiome of a symbiont-rich aphid genus. Molecular Ecology Resources, 16, 628–640.

Jousselin, E., Coeur D’acier, A., Vanlerberghae-Masutti, F. and Duron, O. (2013) Evolution and diversity of Arsenophonus endosymbionts in aphids. Molecular Ecology, 22, 260–270.

Jousselin, E., Desceives, Y. and Coeur D’acier, A. (2009) Fine-scale cospeciation between Brachycauda and Buchnera aphidicola: bacterial genome helps define species and evolutionary relationships in aphids. Proceedings of the Royal Society B: Biological Sciences, 276, 187–196.

Kittayapong, P., Jammongluk, W., Thipakorsorn, A., Milne, J.R. and Sindhusake, C. (2003) Wolbachia infection complexity among insects in the tropical rice-field community. Molecular Ecology, 12, 1049–1060.

Kwong, W.K., Engel, P., Koch, H. and Moran, N.A. (2014) Genomics and host specialization of honey bee and bumble bee gut symbions. Proceedings of the National Academy of Sciences USA, 111, 11509–11514.

Kwong, W.K. and Moran, N.A. (2013) Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of Snodgrassella alvi gen. nov., sp. nov., a member of the family Neisseriaceae of the Betaproteobacteria, and Gilliamella apicola gen. nov., sp. nov., a member of Orbaceae fam. nov., Orbales ord. nov., a sister taxon to the order ‘Enterobacteriales’ of the Gammaproteobacteria. International Journal of Systematic and Evolutionary Microbiology, 63, 2008–2018.

Lamelas, A., Gosalbes, M.J., Manzano-Marín, A., Peretó, J., Moya, A. and Latorre, A. (2011) Serratia symbiotica from the aphid Cinara cedri: a missing link from facultative to obligate insect endosymbiont. PLoS Genetics, 7, e1002357.

Leclair, M., Polin, S., Joussevenne, T., Simon, J.C., Sugio, A., Morlière, S. et al. (2017) Consequences of coinfection with protective symbionts on the host phenotype and symbiont titres in the pea aphid system. Insect Science, 24, 798–808.

Leonardo, T.E. and Muiru, G.T. (2003) Facultative symbionts are associated with host plant specialization in pea aphid populations. Proceedings of the Royal Society B: Biological Sciences, 270, S209–S212.

Liu, L., Huang, X.L., Zhang, R.L., Jiang, L.Y. and Qiao, G.X. (2013) Phylogenetic congruence between Mollitrichosiphum (Aphididae: Greeneiniae) and Buchnera indicates insect-bacteria parallel evolution. Systematic Entomology, 38, 81–92.

Liu, L., Li, X.Y., Huang, X.L. and Qiao, G.X. (2014) Evolutionary relationships of Pemphigus and allied genera (Hemiptera: Aphididae: Eriosomatinae) in their modern endosymbiont, Buchnera aphidicola. Insect Science, 21, 301–312.

Łukasik, P., van Asch, M., Guo, H.F., Ferrari, J. and Godfray, H.C.J. (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecology Letters, 16, 214–218.

Magoc, T. and Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics, 27, 2957–2963.

Manzano-Marín, A. (2019) No evidence for Wolbachia as a nutritional co-obligate endosymbiont in the aphid
Pentalonia nigronervosa. bioRxiv, https://doi.org/10.1101/609511.
Manzano-Marín, A., Coeur d’acier, A., Clamens, A.L., Orvain, C., Cruaud, C., Barbe, V. et al. (2019) Serial horizontal transfer of vitamin-biosynthetic genes enables the establishment of new nutritional symbionts in aphids’ di-symbiotic systems. bioRxiv, https://doi.org/10.1101/556274.
Manzano-Marín, A. and Latorre, A. (2014) Settling down: the genome of Serratia symbiotica from the aphid Cinara tujafilina zooms in on the process of accommodation to a cooperative intracellular life. Genome Biology and Evolution, 6, 1683–1698.
Manzano-Marín, A., Simon, J.-C. and Latorre, A. (2016) Re-inventing the wheel and making it round again: evolutionary convergence in Buchnera–Serratia symbiotic consortia between the distantly related Lachninae aphids Tuberochlaus salignus and Cinara cedri. Genome Biology and Evolution, 8, 1440–1458.
Manzano-Marín, A., Szabo, G., Simon, J.-C., Horn, M. and Latorre, A. (2017) Happens in the best of subfamilies: establishment and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environmental Microbiology, 19, 393–408.
McLean, A.H.C., Parker, B.J., Hrecz, J., Kavanagh, J.C., Wellham, P.A.D. and Godfray, H.C.J. (2018) Consequences of symbiont co-infections for insect host phenotypes. Journal of Animal Ecology, 87, 478–488.
McMurdie, P.J. and Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE, 8, e61217.
Meseguer, A.S., Manzano-Marín, A., Coeur d’acier, A., Clamens, A.L., Godefroid, M. and Jousselin, E. (2017) Buchnera has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts. Molecular Ecology, 26, 2363–2378.
Montllor, C.B., Maxmen, A. and Purcell, A.H. (2002) Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. Ecological Entomology, 27, 189–195.
Moran, N.A. and Dunbar, H.E. (2006) Sexual acquisition of beneficial symbionts in aphids. Proceedings of the National Academy of Sciences USA, 103, 12803–12806.
Moran, N.A., Munson, M.A., Baumann, P. and Ishikawa, H. (1993) A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proceedings of the Royal Society B: Biological Sciences, 253, 167–171.
Moss, R., Jackson, R.R. and Pollard, S.D. (2006) Mask of wax: Secretions of wax conceal aphids from detection by spider’s eyes. New Zealand Journal of Zoology, 33, 215–220.
Munson, M.A., Baumann, P., Clark, M.A., Baumann, L., Moran, N.A., Voegtlin, D.J. et al. (1991) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. Journal of Bacteriology, 173, 6321–6324.
Najar-Rodriguez, A.J., McGraw, E.A., Mensah, R.K., Pittman, G.W. and Walter, G.H. (2009) The microbial flora of Aphis gossypii: patterns across host plants and geographical space. Journal of Invertebrate Pathology, 100, 123–126.
Navas-Molina, J.A., Peralta-Sánchez, J.M., González, A., McMurdie, P.J., Vázquez-Baeza, Y., Xu, Z. et al. (2013) Advancing our understanding of the human microbiome using QIIME. Methods in Enzymology, 531, 371–444.
Nikoh, N., Tsuchida, T., Maeda, T., Yamaguchi, K., Shigenobu, S., Koga, R. et al. (2018) Genomic insight into symbiosis-induced insect color change by a facultative bacterial endosymbiont, “Candidatus Rickettsiella viridis”. MBio, 9, e00890–e00818.
Nirgianaki, A., Banks, G.K., Frohlich, D.R., Veneti, Z., Braig, H.R., Miller, T.A. et al. (2003) Wolbachia infections of the whitefly Bemisia tabaci. Current Microbiology, 47, 93–101.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Meglinn, D. et al. (2018) vegan: Community Ecology Package. R package version 2.5-4. https://CRAN.R-project.org/package=vegan
Oliver, K.M., Campos, J., Moran, N.A. and Hunter, M.S. (2008) Population dynamics of defensive symbionts in aphids. Proceedings of the Royal Society B: Biological Sciences, 275, 293–299.
Oliver, K.M., Degnan, P.H., Burke, G.R. and Moran, N.A. (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annual Review of Entomology, 55, 247–266.
Oliver, K.M., Moran, N.A. and Hunter, M.S. (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proceedings of the National Academy of Sciences USA, 102, 12795–12800.
Oliver, K.M., Moran, N.A. and Hunter, M.S. (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. Proceedings of the Royal Society B: Biological Sciences, 273, 1273–1280.
Oliver, K.M., Russell, J.A., Moran, N.A. and Hunter, M.S. (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences USA, 100, 1803–1807.
Oliver, K.M., Smith, A.H. and Russell, J.A. (2014) Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. Functional Ecology, 28, 341–355.
Paradis, E. (2010) pegas: an R package for population genetics with an integrated-modular approach. Bioinformatics, 26, 419–420.
Pérez-Brocal, V., Gil, R., Ramos, S., Lamelas, A., Postigo, M., Michelena, J.M. et al. (2006) A small microbial genome:
the end of a long symbiotic relationship? Science, 314, 312–313.
Polin, S., Simon, J.-C. and Outreman, Y. (2014) An ecological cost associated with protective symbionts of aphids. Ecology and Evolution, 4, 826–830.
Pons, I., Renoz, F., Noël, C. and Hance, T. (2019) Circulation of the cultivable symbiont Serratia symbiotica in aphids is mediated by plants. Frontiers in Microbiology, 10, 764.
Pope, R.D. (2010) Some aphid waxes, their form and function (Homoptera: Aphididae). Journal of Natural History, 17, 489–506.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SLIVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research, 41, D590–D596.
R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. https://www.R-project.org/
Rock, D.L., Smith, A.H., Joffe, J., Albertus, A., Wong, N., O’Connor, M. et al. (2018) Context-dependent vertical transmission shapes strong endosymbiont community structure in the pea aphid. Acyrthosiphon pisum. Molecular Ecology, 27, 2039–2056.
Russell, J.A., Latorre, A., Sabater-Muñoz, B., Moya, A. and Moran, N.A. (2003) Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Molecular Ecology, 12, 1061–1075.
Russell, J.A. and Moran, N.A. (2005) Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host. Applied and Environmental Microbiology, 71, 7987–7994.
Russell, J.A. and Moran, N.A. (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proceedings of the Royal Society B: Biological Sciences, 273, 603–610.
Russell, J.A., Weldon, S., Smith, A.H., Kim, K.L., Hu, Y., Lukasik, P. et al. (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. Molecular Ecology, 22, 2045–2059.
Sandström, J. and Moran, N. (1999) How nutritionally imbalanced is phloem sap for aphids? Entomologia Experimentalis et Applicata, 91, 203–210.
Sandström, J.P., Russell, J.A., White, J.P. and Moran, N.A. (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. Molecular Ecology, 10, 217–228.
Scarborough, C.L., Ferrari, J. and Godfray, H.C. (2005) Aphid protected from pathogen by endosymbiont. Science, 310, 1781.
Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. (2000) Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature, 407, 81–86.
Smith, A.H., Lukasik, P., O’Connor, M.P., Lee, A., Mayo, G., Drott, M.T. et al. (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. Molecular Ecology, 24, 1135–1149.
Smith, R.G. (1999) Wax glands, wax production and the functional significance of wax use in three aphid species (Homoptera: Aphididae). Journal of Natural History, 33, 513–530.
Stern, D.L. and Foster, W.A. (1996) The evolution of soldiers in aphids. Biological Reviews of the Cambridge Philosophical Society, 71, 27–79.
Stouthamer, R., Breeuwer, J.A. and Hurst, G.D. (1999) Wolbachia pipiens: microbial manipulator of arthropod reproduction. Annual Review of Microbiology, 53, 71–102.
Su, M., Tan, X.M., Yang, Q.M., Wang, J.Q., Wan, F.H. and Zhou, H.X. (2016) Distribution of wax gland pores on the body surface and the dynamics of wax secretion of wooly apple aphid Eriosoma lanigerum (Hemiptera: Aphididae). Entomological News, 126, 106–120.
Tsuchida, T., Koga, R. and Fukatsu, T. (2004) Host plant specialization governed by facultative symbiont. Science, 303, 1989.
Tsuchida, T., Koga, R., Horikawa, M., Tsunoda, T., Maoka, T., Matsumoto, S. et al. (2010) Symbiotic bacterium modifies aphid body color. Science, 330, 1102–1104.
Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. and Fukatsu, T. (2002) Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, Acyrthosiphon pisum. Molecular Ecology, 11, 2123–2135.
Vorburger, C., Gehrer, L. and Rodriguez, P. (2010) A strain of the bacterial symbiont Regiella insecticola protects aphids against parasitoids. Biology Letters, 6, 109–111.
Vorburger, C. and Rouchet, R. (2016) Are aphid parasitoids locally adapted to the prevalence of defensive symbionts in their hosts? BMC Evolutionary Biology, 16, 271.
Vorburger, C., Siegrist, G. and Rhyner, N. (2017) Faithful vertical transmission but ineffective horizontal transmission of bacterial endosymbionts during sexual reproduction of the black bean aphid, Aphis fabae. Ecological Entomology, 42, 202–209.
Wagner, S.M., Martinez, A.J., Ruan, Y.M., Kim, K.L., Lenhart, P.A., Dehnel, A.C. et al. (2015) Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. Functional Ecology, 29, 1402–1410.
Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology, 73, 5261–5267.
Wang, Z., Su, X.M., Wen, J., Jiang, L.Y. and Qiao, G.X. (2014) Wide spread infection and diverse infection patterns of Wolbachia in Chinese aphids. Insect Science, 21, 313–325.

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Way, M.J. and Banks, C.J. (1968) Population studies on the active stages of the black bean aphid, *Aphis fabae* Scop., on its winter host *Euonymus europaeus* L. *Annals of Applied Biology*, 62, 177–197.

West, S.A., Cook, I.M., Werren, J.H. and Godfray, H.C.J. (1998) *Wolbachia* in two insect host–parasitoid communities. *Molecular Ecology*, 7, 1457–1465.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York.

Wulff, J.A. and White, J.A. (2015) The endosymbiont *Arsenophonus* provides a general benefit to soybean aphid (Hemiptera: Aphididae) regardless of host plant resistance (*Rag*). *Environmental Entomology*, 44, 574–581.

Xu, T.T., Chen, J., Jiang, L.Y. and Qiao, G.X. (2018) Historical and cospeciating associations between Cerataphidini aphids (Hemiptera: Aphididae: Hormaphidinae) and their primary endosymbiont *Buchnera aphidicola*. *Zoological Journal of the Linnean Society*, 183, 236–236.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C. *et al.* (2014) The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Research*, 42, D643–D648.

Zhang, S., Luo, J.Y., Wang, L., Zhang, L.J., Zhu, X.Z., Jiang, W.L. *et al.* (2019) Bacterial communities in natural versus pesticide-treated *Aphis gossypii* populations in North China. *Microbiologyopen*, 8, e00652.

Zhao, Y., Zhang, S., Luo, J.Y., Wang, C.Y., Lv, L.M. and Cui, J.J. (2016) Bacterial communities of the cotton aphid *Aphis gossypii* associated with *Bt* cotton in northern China. *Scientific Reports*, 6, 22958.

Zheng, H., Nishida, A., Kwong, W.K., Koch, H., Engel, P., Steele, M.I. *et al.* (2016) Metabolism of toxic sugars by strains of the bee gut symbiont *Gilliamella apicola*. *mBio*, 7, e01326–e01316.

Zouari, S., Ben Halima, M.K., Reyes-Prieto, M., Latorre, A. and Gil, R. (2018) Natural occurrence of secondary bacterial symbionts in aphids from Tunisia, with a focus on genus *Hyalonaphus*. *Environmental Entomology*, 47, 325–333.

Zug, R. and Hammerstein, P. (2012) Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE*, 7, e38544.

Zytynska, S.E., Meyer, S.T., Sturm, S., Ullmann, W., Mehrparvar, M. and Weisser, W.W. (2016) Secondary bacterial symbiont community in aphids responds to plant diversity. *Oecologia*, 180, 735–747.

Zytynska, S.E., Thighiouart, K. and Frago, E. (2019) A meta-analysis on the benefits and costs of hosting secondary endosymbionts in sap-sucking insects. *bioRxiv*, https://doi.org/10.1101/563031.

Zytynska, S.E. and Weisser, W.W. (2016) The natural occurrence of secondary bacterial symbionts in aphids. *Ecological Entomology*, 41, 13–26.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Voucher information and GenBank accession numbers for all aphid species used in this study.

**Table S2.** Group information.

**Table S3.** Occurrence of secondary symbionts in Hormaphidinae.

**Table S4.** Infection pattern of secondary symbiont across Hormaphidinae.

**Table S5.** Spearman correlation coefficients of symbionts in Hormaphidinae.

**Fig. S1.** Phylogeny of Hormaphidinae species with the relative abundance of the bacteria *Gilliamella* displayed as triangle at the tips of the phylogeny. Triangle size corresponds to the different relative abundance according to the inset legend.

**Fig. S2.** Cophylogeny of Hormaphidinae and *Buchnera* from Jane 4.0. Blue and black lines indicate the phylogenies of the *Buchnera* and aphids, respectively. The reconciled trees were from the ML aphid tree and the ML *Buchnera* tree. Hollow circles indicate cospeciation events, solid circles indicate duplications, solid circles with arrows indicate host switch events, and dashed lines indicate loss events.

**Fig. S3.** Boxplot comparing the Shannon index of bacterial community among Hormaphidinae samples from three tribes (A) and four plant families (C); comparing the Simpson index of bacterial community among Hormaphidinae samples from three tribes (B) and four plant families (D). Boxes with the same letter are not significantly different, while those without same letters are significantly different on the basis of Kruskal-Wallis and Tukey’s HSD tests. The bottom and top edges of the boxes mark the 25th and 75th percentiles (that is, first and third quartiles), respectively. Lines within boxes represent the medians, hinges represent the +/− 25% quartiles, and whiskers represent up to 1.5x the interquartile range. CPCoA plot illustrating the separation of samples based on differences in bacterial community structure among three tribes (E) and four plant families (G); NMDS plot illustrating the separation of samples based on differences in bacterial community structure among three tribes (F) and four plant families (H). Colors correspond to different groups, as shown in the legend.