RESEARCH ARTICLE

Geography-dependent symbiont communities in two oligophagous aphid species

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One sentence summary: The significance of geography and its associated environmental conditions, as an important factor in shaping the symbiont community structure associated with oligophagous aphids.

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ABSTRACT

Aphids and their diverse symbionts have become a good model to study bacteria-arthropod symbiosis. The feeding habits of aphids are usually influenced by a variety of symbionts. Most studies on symbiont diversity have focused on polyphagous aphids, while symbiont community patterns for oligophagous aphids remain unclear. Here, we surveyed the bacterial communities in natural populations of two oligophagous aphids, *Melanaphis sacchari* and *Neophyllaphis podocarpi*, in natural populations. Seven common symbionts were detected, among which *Buchnera aphidicola* and *Wolbachia* were the most prevalent. In addition, an uncommon *Sodalis*-like symbiont was also detected in these two aphids, and *Gilliamella* was found in some samples of *M. sacchari*. We further assessed the significant variation in symbiont communities within the two aphid species, geographical regions and host specialization using statistical and ordination analyses. Geography was an important factor in shaping the symbiont community structure in these oligophagous aphids. Furthermore, the strong geographical influence may be related to specific environmental factors, especially temperature, among different regions. These findings extend our knowledge of the significance of geography and its associated environmental conditions in the symbiont community structure associated with oligophagous aphids.

Keywords: oligophagous aphids; symbiont community; geographical region; environmental factors; biological replicates

INTRODUCTION

Insects are frequently associated with bacterial symbionts that have important effects on their host ecology and evolution. In particular, phloem sap-feeding aphids (Hemiptera: Aphididae) are a good model for the study of bacteria-arthropod symbiosis because they have maintained an obligate mutualistic association with *Buchnera aphidicola* since more than 180 million years ago (Mya) (Buchner 1965; Moran *et al.* 1993). *Buchnera aphidicola*

provides aphids with essential amino acids and vitamins lacking in their diet (Douglas 1998), inhabiting specialized cells called bacteriocytes, and undergoing strict vertical transmission from mother to offspring (Buchner 1965; Rock *et al.* 2018). Aphids may also possess one or more secondary symbionts that can enhance their survival and reproductive abilities (Oliver *et al.* 2010). Most studies have mainly focused on several common secondary symbionts, such as *Arsenophonus*, *Fukatsuia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, *Rickettsiella viridis*, and *Serratia*

symbiotica from the class Gammaproteobacteria, *Rickettsia* and *Wolbachia* from the class Alphaproteobacteria, and *Spiroplasma* from the class Mollicutes (Fukatsu *et al.* 2001; Russell *et al.* 2003; Moran *et al.* 2005; Sakurai *et al.* 2005; Guay *et al.* 2009; Tsuchida *et al.* 2010; Augustinos *et al.* 2011). Secondary symbionts are usually located in secondary bacteriocytes, sheath cells or hemocoels (Fukatsu *et al.* 2000; Oliver *et al.* 2010), and experience vertical transmission and occasional horizontal transfer (Russell *et al.* 2003; Henry *et al.* 2013). Under some conditions, these symbionts can benefit their hosts, conferring resistance to parasitic wasps and fungal pathogens (Oliver *et al.* 2003; Scarborough, Ferrari and Godfray 2005; Frago *et al.* 2017), increasing tolerance to heat stress (Montllor, Maxmen and Purcell 2002; Burke, Fiehn and Moran 2010) and improving performance on particular host plants (Tsuchida, Koga and Fukatsu 2004; Wagner *et al.* 2015).

Aphids are divided into polyphagous, oligophagous and monophagous herbivores according to the range of host plants on which they feed. Polyphagous groups are usually defined as those feeding on species from two or more plant families, but oligophagous insects restricted feeding to two or more genera in a family or closely related families (Cates 1980). Given the diversity of these symbionts, one generality that has emerged from prior studies is that symbiont communities vary strongly among populations of polyphagous aphid species. For example, the bacterial communities of *Acyrthosiphon pisum* vary with host plants (Ferrari *et al.* 2012; Gauthier *et al.* 2015). Other examples include the cowpea aphid *Aphis craccivora* (Brady and White 2013; Brady *et al.* 2014) and the melon aphid *Aphis gossypii* (Xu *et al.* 2020a). In these polyphagous aphid species, host plants are an important factor in shaping the symbiont community (Jones *et al.* 2011; Russell *et al.* 2013; Xu *et al.* 2020a). However, few studies have focused on symbiont communities associated with oligophagous aphids. Compared with polyphagous aphids, oligophagous aphids have a narrow range of host plants; thus, the important factors in the patterns of the symbiont community are unclear. Previous studies have reported that the relative abundance of symbionts in a common oligophagous aphid, *Aphis citricidus*, was different among *Citrus* plants (Guidolin and Cônsoli 2017). Medina, Nachappa and Tamborindeguy (2011) found differences in bacterial diversity between *Phylloxera notabilis* populations feeding on pecan and water hickory. Geography is another important factor in shaping the symbiont community, which has been summarized in a few aphid species (Tsuchida *et al.* 2002; Najar-Rodríguez *et al.* 2009; Zytynska and Weisser 2016; Sepúlveda *et al.* 2017; Guo *et al.* 2019; Xu *et al.* 2020a). Although oligophagous aphids are very restricted by host plants, their geographical distribution is wide. Therefore, it is speculated that geography could be an important factor in shaping the symbiont community structure in oligophagous aphids. Furthermore, some environmental conditions in different geographical regions, such as temperature, altitude, longitude and precipitation, can influence the symbiont community composition (Burke, Fiehn and Moran 2010; Fakhour *et al.* 2018; Duan *et al.* 2020; Heyworth, Smee and Ferrari 2020). Therefore, we speculated that the influence of geographical factors on the symbiont community is likely caused by environmental conditions. More research is needed to estimate the effect of geography and local environmental variables on the bacterial symbiont diversity of oligophagous aphids.

In the present study, we investigated the bacterial symbiont communities of two oligophagous aphid species, *Melanaphis sacchari* and *Neophyllaphis podocarpi*. To date, few studies have reported on the bacterial diversity associated with *M. sacchari* and *N. podocarpi*. Holt *et al.* (2020) detected three symbionts (*Buchnera aphidicola*, *Rickettsiella viridis* and *Serratia symbiotica*) in *M. sacchari* and found differences in bacterial communities between aphid populations collected on sugarcane and sorghum. In a separate study, *Wolbachia* was detected in *Neophyllaphis podocarpi* (Augustinos *et al.* 2011). *Melanaphis sacchari* is an agricultural pest, while *N. podocarpi* is a forestry pest worldwide (Blackman and Eastop 2020). *Melanaphis sacchari* is a vector of the sugarcane yellow leaf virus, and causes leaf disease in *Sorghum* (Fartek *et al.* 2014). *Melanaphis sacchari* feeds on some species of Poaceae, especially *Saccharum* and *Sorghum*, which are extensively cultivated in China, India, Sri Lanka, Australia and the USA (Blackman and Eastop 2020). *Neophyllaphis podocarpi* has been reported to feed on *Podocarpus* spp. and *Nageia nagi* is distributed in China, Japan, Vietnam, Java, Malaya and North America (Blackman and Eastop 2020).

To build on prior studies, we used 16S rRNA Illumina sequencing to explore the microbiota of two oligophagous aphids, *M. sacchari* and *N. podocarpi*, sampled across different geographic regions, to uncover the importance of geography and environmental factors in shaping these aphid symbiont communities.

MATERIALS AND METHODS

Sampling and DNA extraction

Aphid samples consisted of 13 colonies of *Melanaphis sacchari* (MS) mainly feeding on *Sorghum bicolor* from six geographical regions and 22 colonies of *Neophyllaphis podocarpi* (NP) mostly feeding on *Podocarpus macrophyllus* from 10 geographic regions (Fig. 1; Table 1). Specimens were stored in 75% and 100% ethanol for slide mounting and DNA extraction, respectively. All samples were preserved at –20°C. Aphid species identification was performed based on the external morphological characteristics of voucher specimens. All voucher slides and frozen samples were deposited in the National Zoological Museum of China, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

To assess the differences in the symbiont community across different individuals in the same colony or population, three individuals serving as biological replicates (samples were labeled by _1, _2, _3) were prepared to assess the variation in symbiont communities within the same aphid colony. The whole body of an apterous viviparous female per sample was used for extracting DNA. To remove body surface contaminants, each sample was washed with 70% ethanol for 5 min then rinsed with sterile water five times. Total DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Sterile ultrapure water was used as a negative control in the DNA extraction. To verify aphid species identification and eliminate the parasitized samples, the cytochrome oxidase subunit I (COI) barcode was amplified with universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994). The DNA samples were stored at –20°C until 16S rRNA gene amplification.

16S rRNA gene amplification and sequencing

Each DNA sample was amplified in triplicate to serve as technical replicates (samples were labeled by _a, _b, _c) for the estimation of amplification bias. To amplify and sequence the V3–V4 regions of the 16S rRNA gene, two PCR procedures were performed. In the first-step PCR (PCR1), a universal primer pair (338F: 5′-ACTCCTACGGGAGGCAGCA-3′; 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) (Yu *et al.* 2005) was used to
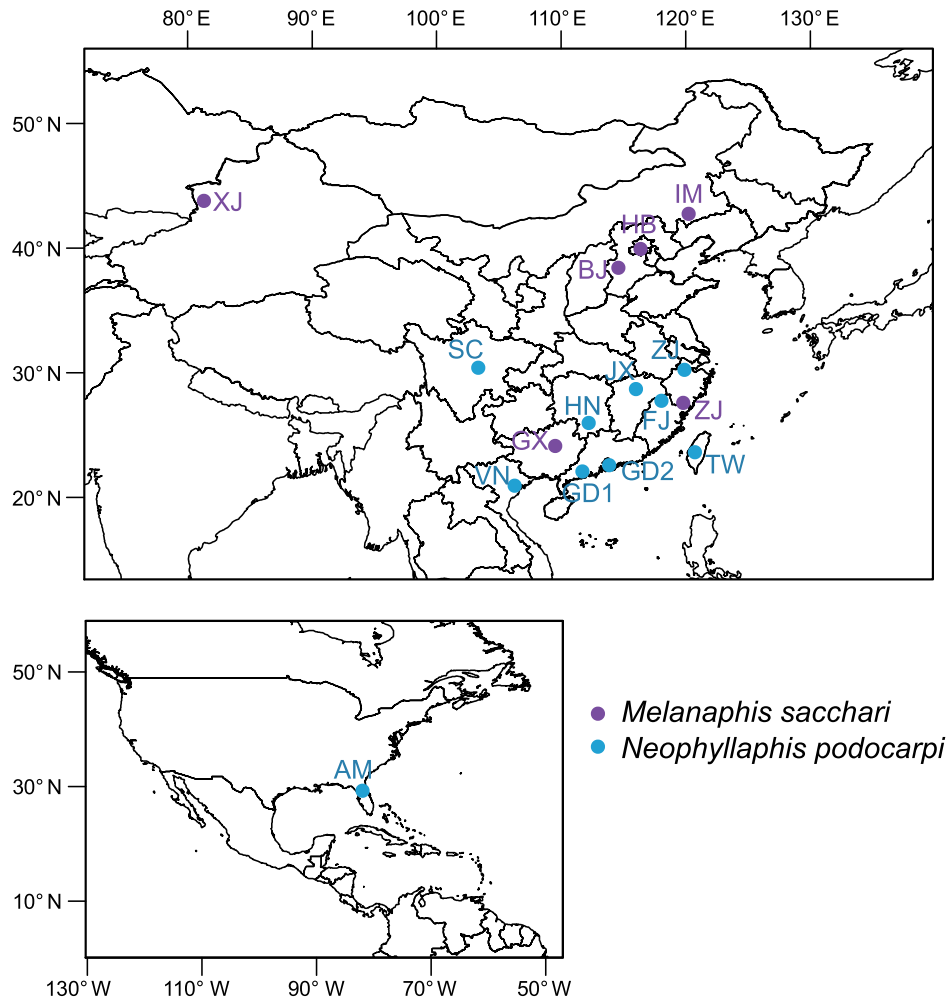
Figure 1. Sampling sites of *Melanaphis sacchari* and *Neophyllaphis podocarpi*. The abbreviations of geographic regions are detailed in Table 1.

amplify the target region. The 50-μL reaction mixture contained 3 μL primers, 10 μL 5 × Q5 reaction buffer (New England Biolabs, Ipswich, MA, USA), 0.4 U Q5 high-fidelity DNA polymerase (New England Biolabs), 1 μL dNTPs (New England Biolabs), 10 μL 5 × Q5 high GC enhancer (New England Biolabs) and 40–60 ng DNA. The PCR1 conditions were as follows: initial denaturation at 95°C for 5 min; 15 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min; and final elongation at 72°C for 7 min. The PCR1 products were purified using VAHTSTM DNA clean beads (Vazyme Biotech, Nanjing, China). To attach indices and adapter sequences, the second-step PCR (PCR2) was performed with the forward primer (5′-AATGATACGGCGACCACCGAGATCTACACNNNNNN NNACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′) and the reverse primer (5′-CAAGCAGAAGACGGCATACGAGATNNNNN NNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′). The 40-μL reaction volume contained 10 μL PCR1 purified product serving as a DNA template, 2 μL primers and 20 μL 2× Phusion high-fidelity PCR master mix (New England Biolabs). The thermocycling program of PCR2 was as follows: initial denaturation at 98°C for 30 s, 10 cycles of 98°C for 10 s, 65°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The final PCR products were checked on a 1.8% agarose gel, and the positive PCR products were purified with VAHTS DNA clean beads. Then the positive samples were quantified with

a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA), mixed at an equal ratio of 1:1 and sequenced on an Illumina HiSeq 2500 PE250 platform (Illumina, San Diego, CA, USA). Blank samples of the negative control were included in the process of amplification and sequencing.

Data analyses

Paired-end reads were assembled using FLASH v. 1.2.11 (Magoč and Salzberg 2011). The raw tags with an average quality score below 20 and length shorter than 300 bp were trimmed using Trimmomatic v. 0.33 (Bolger, Lohse and Usadel 2014). Chimeras were then filtered using UCHIME v. 8.1 (Edgar *et al.* 2011). The clean tags were clustered into operational taxonomic units (OTUs) using the UCLUST module in QIIME (Edgar 2010) with a similarity of ≥97%. The taxonomic annotation of OTUs was performed based on the Silva 16S rRNA gene reference database (Release 128; Quast *et al.* 2013) using the RDP classifier within QIIME (Wang *et al.* 2007). The OTUs with an abundance below 0.005% were then filtered (Bokulich *et al.* 2013) unless they could be classified at the genus level. The bacterial OTUs assigned to the reported symbionts were selected from the OTU table to better investigate the symbiont communities associated with aphids (Table 2). To validate the phylogenetic relationship between these symbionts and reported symbionts, a

Table 1. The sample voucher and grouping information.

Aphid species	Sample ID	Date	Locations	Host plants	Geography	Host plants of MS
Melanaphis sacchari (MS)	16739	6/8/2005	Qapqal, Xinjiang, China	Cyperus rotundus	XJ	NS
	17359	31/7/2005	Taishun, Zhejiang, China		ZJ	NS
	17380	1/8/2005	Taishun, Zhejiang, China	Phragmites australis	ZJ	NS
	17407	4/8/2005	Taishun, Zhejiang, China	Imperata cylindrica var. major	ZJ	NS
	18933	23/5/2006	Guilin, Guangxi, China	Miscanthus floridulus	GX	NS
	24950	25/5/2011	Nanning, Guangxi, China		GX	
	26543	10/10/2011	Shijiazhuang, Hebei, China	Sorghum bicolor	HB	SO
	27276	18/9/2011	Shijiazhuang, Hebei, China	Sorghum bicolor	HB	SO
	27282	19/9/2011	Cangzhou, Hebei, China	Sorghum bicolor	HB	SO
	27284	20/9/2011	Shijiazhuang, Hebei, China	Sorghum bicolor	HB	SO
	37016	14/6/2016	Beijing, China	Sorghum bicolor	BJ	SO
	41939	21/8/2017	Tongliao, Inner Mongolia, China	Sorghum bicolor	IM	SO
	41944	21/8/2017	Chifeng, Inner Mongolia, China	Sorghum bicolor	IM	SO
Neophyllaphis podocarpi (NP)	17278	11/5/2005	Chengdu, Sichuan, China	Podocarpus macrophyllus	SC	
	22542	14/5/2009	Yaan, Sichuan, China	Podocarpus macrophyllus	SC	
	25235	7/11/2010	Pingtung, Taiwan, China	Podocarpus costalis	TW	
	26375	29/5/2011	Kaohsiung, Taiwan, China	Podocarpus costalis	TW	
	26388	2/6/2011	Kaohsiung, Taiwan, China	Podocarpus costalis	TW	
	26493	19/6/2011	Nantou, Taiwan, China	Podocarpus macrophyllus	TW	
	26494	19/6/2011	Nantou, Taiwan, China	Podocarpus macrophyllus	TW	
	26683	26/5/2011	Hangzhou, Zhejiang, China	Podocarpus macrophyllus	ZJ	
	29023	30/11/2012	Vietnam	Podocarpus macrophyllus	VN	
	29533	31/10/2013	Yangjiang, Guangdong, China	Podocarpus macrophyllus	GD1	
	30209	3/5/2014	Shenzhen, Guangdong, China	Podocarpus macrophyllus	GD2	
	30403	22/7/2013	Zhuhai, Guangdong, China	Podocarpus macrophyllus	GD2	
	30429	29/7/2013	Zhuhai, Guangdong, China	Podocarpus macrophyllus	GD2	
	31369	11/6/2014	Dujiangyan, Sichuan, China	Podocarpus macrophyllus	SC	
	34286	12/5/2015	Shenzhen, Guangdong, China	Podocarpus macrophyllus	GD2	
	36480	23/11/2015	Taichung, Taiwan, China	Podocarpus macrophyllus	TW	
	37139	25/4/2016	Nanchang, Jiangxi, China	Podocarpus macrophyllus	JX	
	38914	1/10/2016	Gainesville, Florida, America	Podocarpus macrophyllus	AM	
	39227	9/11/2016	Pingtung, Taiwan, China	Podocarpus macrophyllus	TW	
	39542	27/5/2017	Yongzhou, Hunan, China	Podocarpus macrophyllus	HN	
	39767	13/6/2017	Chenzhou, Hunan, China	Podocarpus macrophyllus	HN	
	39838	21/6/2017	Nanping, Fujian, China	Podocarpus macrophyllus	FJ	

Note: 'Geography' shows the abbreviation of geographical regions. 'Host plants of MS' lists the plant groups of *Melanaphis sacchari* belonging to *Sorghum bicolor* (SO) and Non-*Sorghum* plants (NS). XJ: Xinjiang, ZJ: Zhejiang, GX: Guangxi, HB: Hebei, BJ: Beijing, IM: Inner Mongolia, SC: Sichuan, TW: Taiwan, ZJ: Zhejiang, VN: Vietnan, GD1: Guangdong1, GD2: Guangdong2, JX: Jiangxi, AM: America, HN: Hunan, FJ: Fujian.

Table 2. The infection frequency and average relative abundance of symbionts across all samples of *Neophyllaphis podocarpi* and *Melanaphis sacchari*.

	M. sacchari		N. podocarpi	
	Infection frequency	Relative abundance	Infection frequency	Relative abundance
Buchnera aphidicola	117/117	84.492%	198/198	98.642%
Wolbachia	70/117	3.654%	125/198	0.672%
Rickettsia	48/117	8.220%	69/198	0.128%
Serratia symbiotica	31/117	2.374%	19/198	<0.005%
Arsenophonus	9/117	<0.005%	21/198	<0.005%
Hamiltonella defensa	15/117	0.157%	5/198	<0.005%
Regiella insecticola	1/117	<0.005%	24/198	0.279%
Sodalis-like	8/117	<0.005%	25/198	<0.005%
Gilliamella	7/117	0.215%	0	0

maximum likelihood (ML) phylogenetic tree based on 16S gene fragments was constructed with RAxML software (Stamatakis 2014) by choosing the GTRGAMMA model for bootstrapping and searching for the best ML tree with 1000 bootstrap iterations.

Statistical analyses

The downstream statistical analyses based on the OTU table were conducted in the R v. 3.4.3 software environment (R Core Team 2017). To visualize the relative abundance of each symbiont among different groups (the grouping information is shown in Table 1), an integrated heatmap of symbiont OTUs was constructed using the *pheatmap* package (Kolde 2019).

Alpha diversity indices, including the Shannon and Simpson indices, were calculated using the *diversity* function in *vegan* (Oksanen *et al*. 2018). To assess the variation in alpha diversity with geography, nonparametric Kruskal–Wallis tests were performed to test the differences across all groups, and nonparametric Wilcoxon tests were used to successively detect significant differences between groups. Wilcoxon tests were also conducted to compare the variation in alpha diversity in aphid species and host plants of *M. sacchari*, in which only two groups were included. To examine the variation in alpha diversity among biological and technical replicates, we performed Fisher's least significant difference (LSD) test with Bonferroni correction using the *LSD.test* function in the *agricolae* package (De Mendiburu 2009) when the data were normally distributed.

To balance the heterogeneity of sequencing depth, we normalized the count data of symbiont OTUs using the cumulative sum scaling (CSS) method in the *metagenomeSeq* package (Paulson *et al*. 2013; Weiss *et al*. 2017). Beta diversity based on CSS count data was quantified using the unweighted and weighted UniFrac distances in the *GuniFrac* package (Chen *et al*. 2012). The UniFrac metrics were measured by the phylogenetic distances between OTUs (Lozupone and Knight 2005) and provide more information on community diversity than the commonly used Bray–Curtis distance. Principal coordinates analysis (PCoA) was conducted to display the variation in symbiont and secondary symbiont communities with respect to different groups (i.e. aphid species, geographic regions, host specialization, biological and technical replicates). PCoA was performed based on UniFrac distances using the *pcoa* function in the *ape* package (Paradis and Schliep 2019), and plots were visualized using the *ggplot2* package (Wickham 2016). To further evaluate the significant differences among groups in the symbiont and secondary symbiont communities, analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) based on

UniFrac distances were conducted. Statistical R^2 values in the PERMANOVA were used to evaluate the contribution of these categorical variables. ANOSIM and PERMANOVA were implemented using the *anosim* and *adonis* functions, respectively, in the *vegan* package. To further estimate the impact of geography on the symbiont communities of each aphid species, a Mantel test was performed on geographic distances and weighted UniFrac distances using the Spearman correlation method following Xu *et al*. (2020).

To explore the effect of local climate on structuring symbiont communities associated with *M. sacchari* and *N. podocarpi* separately, redundancy analysis (RDA) and generalized linear models (GLMs) were conducted. First, 19 environmental variables were obtained from the 'WorldClim' dataset using the *getData* function in the *raster* package (Supplementary Table 3). After eliminating the autocorrelation of environmental variables by PCA, altitude (Alt), annual mean temperature (Bio1) and annual precipitation (Bio12) were extracted as predictor variables using the *vif.cca* function in *vegan*. We used the CSS normalized symbiont OTUs as the response variables and three environmental factors as explanatory variables. The RDA was performed using the *rda* function in the *vegan* package. Then partial RDA was conducted to assess the relative contribution of three predictor variables in shaping symbiont community structure (Ter Braak 1988). Prior to GLM analyses based on Shannon and Chao1 indices, the overdispersion in the data was estimated by the *qcc* package (Scrucca 2004). GLM analyses were performed using the Poisson distribution model and *glm* function in the *stats* package (R Core Team 2017).

RESULTS

Microbial communities in two oligophagous aphids

A total of 18 211 874 reads were obtained, with an average sequence number of 57 815 reads per sample. These reads were assigned to 262 OTUs, which were classified into 20 phyla, 59 orders, 43 classes, 98 families and 145 genera. At the phylum level, Proteobacteria represented 99.629% of sequences. OTUs mainly belonged to the class Gammaproteobacteria (94.641%), the order Enterobacteriales (94.522%) and the family Enterobacteriaceae (94.522%). In addition, six aphid symbionts dominated the bacterial community of *M. sacchari* and *N. podocarpi* (Supplementary Fig. 1).

Both *M. sacchari* and *N. podocarpi* harbored *Buchnera aphidicola* and six common secondary symbionts (Table 2). The relative abundance of secondary symbionts in *M. sacchari* was

higher than that in *N. podocarpi* (Table 2, Supplementary Fig. 1). For *M. sacchari*, the top three secondary symbionts were *Rickettsia* (infection frequency: 48/117, average relative abundance: 8.220%), *Wolbachia* (70/117, 3.654%) and *Serratia symbiotica* (31/117, 2.374%). *Gilliamella* (7/117, 0.215%) was found in some *M. sacchari*, which clustered with the strain *Gilliamella apicola* (Supplementary Fig. 2). For *N. podocarpi*, *Rickettsia* (69/198, 0.128%), *Wolbachia* (125/198, 0.672%) and *Regiella insecticola* (24/198, 0.279%) were dominant secondary symbionts with more than 0.1% of the average relative abundance. *Wolbachia* was the most prevalent secondary symbiont associated with the two aphid species (Table 2). A *Sodalis*-like symbiont was also detected in two oligophagous aphids, which was closely related to the *Sodalis* endosymbionts from some heteropteran insects (Supplementary Fig. 2). Although the relative abundance of the *Sodalis*-like symbiont was less than 0.005%, its infection rate (8/117 for *M. sacchari*; 25/198 for *N. podocarpi*) was close to that of other secondary symbionts, such as *Arsenophonus* (9/117, 21/198; Table 2).

Comparison of symbiont communities across replicates and between aphid species

When we assessed different individuals in a colony or population for each aphid species, we did not find any significant difference among biological or technical replicates in alpha diversity (LSD test, $P > 0.05$) (Fig. 2A and B), PCoA (Fig. 2D and E; Supplementary Fig. 3a and b) and ANOSIM or PERMANOVA ($P > 0.05$) (Table 3) based on beta diversity distances. However, there were prevalent differences in some secondary symbionts among biological replicates in the bar plot (Supplementary Fig. 1).

The alpha diversity of symbiont communities across all samples was very low. The alpha diversity of *M. sacchari* symbionts (Shannon: mean = 0.204, SD = 0.264; Simpson: mean = 0.872, SD = 0.178) was slightly higher than that of *N. podocarpi* symbionts (Shannon: mean = 0.052, SD = 0.083; Simpson: mean = 0.977, SD = 0.040; Supplementary Table 1). The results of the Wilcoxon test showed a significant difference between the two species ($P < 0.001$), and the mean values of alpha diversity indices of *M. sacchari* were significantly higher than those of *N. podocarpi* (Shannon: $P < 0.001$, Fig. 2C). Distinct structures of symbiont communities shaped by the aphid species are shown in the PCoA diagrams (Fig. 2F), except for the plot based on unweighted UniFrac distances (Supplementary Fig. 3c). Most of the ANOSIM and PERMANOVA results with the weighted (ANOSIM: R = 0.262, $P < 0.01$; PERMANOVA: $R^2 = 0.122$, $P < 0.01$) and unweighted UniFrac distances (PERMANOVA: $R^2 = 0.012$, $P < 0.01$) showed significant differences in the symbiont communities between the two aphid species (Table 3). Two clusters belonging to different aphid species were displayed in the symbiont OTU distribution heatmap, and samples from the same aphid species were shared with a single highly abundant OTU of *Buchnera aphidicola* (OTU2 of *M. sacchari* and OTU1 of *N. podocarpi*, Fig. 4).

Geography strongly shaped the symbiont communities in *M. sacchari* and *N. podocarpi*

In *M. sacchari*, the Kruskal–Wallis test showed that the alpha diversity was significantly different among geographical regions (Shannon: $P < 0.001$, Fig. 3A), and the Wilcoxon tests found that the alpha diversity of symbiont communities in Guangxi (GX) and Zhejiang (ZJ) was significantly higher than that in other geographical regions (Fig. 3A). In addition to geography, the alpha diversity of symbiont communities in *M. sacchari* feeding on

Sorghum (SO) was significantly lower than that in *M. sacchari* feeding on other non-*Sorghum* plants ($P < 0.001$, Fig. 3B). In *N. podocarpi*, the Kruskal–Wallis test also suggested significant variation in alpha diversity in different geographic regions (Shannon: $P < 0.001$, Fig. 3C). Wilcoxon tests showed that the variation in some groups (AM: America, GD1: Guangdong 1, JX: Jiangxi, ZJ: Zhejiang) was higher than that in the other groups (Fig. 3C).

For *M. sacchari*, the symbiont communities displayed a significant difference associated with geographical regions in the PCoA based on weighted and unweighted UniFrac distances (Fig. 3D, Supplementary Fig. 3d). It is worth noting that the symbiont communities between southern China (MS_GX, MS_ZJ) and northern China (MS_BJ, MS_HB, MS_IM and MS_XJ) were different. ANOSIM and PERMANOVA also found significant variation in symbiont communities using the weighted (ANOSIM: R = 0.363, $P < 0.01$; PERMANOVA: $R^2 = 0.427$, $P < 0.01$) and unweighted UniFrac distances (ANOSIM: R = 0.220, $P < 0.01$; PERMANOVA: $R^2 = 0.274$, $P < 0.01$; Table 3). In addition, the Mantel tests showed a significant positive correlation between geographic distances and symbiont community dissimilarities (MS: r = 0.348, $P = 0.001$; NP: r = 0.069, $P = 0.003$). The relative abundances of *Rickettsia* and OTU4 of *Wolbachia* in *M. sacchari* from Guangxi and Zhejiang were higher than those in other geographic regions (Fig. 4). Apart from geography, we analyzed the effect of host specialization on symbiont communities in *M. sacchari*. The PCoA plots based on all types of beta diversity distances showed a significant separation between *Sorghum* and non-*Sorghum* plants (Fig. 3E, Supplementary Fig. 3e). ANOSIM and PERMANOVA also showed a significant contribution of *Sorghum* to symbiont communities using weighted (ANOSIM: R = 0.432, $P < 0.01$; PERMANOVA $R^2 = 0.224$, $P < 0.01$) and unweighted UniFrac distances (ANOSIM: R = 0.133, $P < 0.01$; PERMANOVA $R^2 = 0.099$, $P < 0.01$; Table 3).

The impact of geography on symbiont communities was also confirmed in another aphid species, *N. podocarpi*. Significant symbiont community patterns structured by geography were found in PCoA (Fig. 3F, Supplementary Fig. 3f). All of the ANOSIM and PERMANOVA results based on weighted UniFrac (ANOSIM: R = 0.231, $P < 0.01$; PERMANOVA $R^2 = 0.354$, $P < 0.01$) and unweighted UniFrac distances (ANOSIM: R = 0.067, $P < 0.05$; PERMANOVA $R^2 = 0.218$, $P < 0.01$) detected significant variation in symbiont communities among different geographic regions (Table 3).

The effect of environmental conditions from different geographic regions on structuring symbiont communities in *M. sacchari* and *N. podocarpi*

We further explored the influence of environmental factors resulting from different geographic regions on symbiont community structures. Redundancy analysis (RDA) reflected a significant correlation between the three environmental variables (altitude, temperature and precipitation) and the symbiont composition (MS: $R^2 = 0.227$, $P = 0.001$; NP: $R^2 = 0.071$, $P = 0.001$; Fig. 5). The partial RDA test suggested a more important effect of temperature (MS: $R^2 = 0.046$, $P = 0.001$; NP: $R^2 = 0.019$, $P = 0.001$) than precipitation (MS: $R^2 = 0.028$, $P = 0.001$; NP: $R^2 = 0.018$, $P = 0.001$) and altitude (MS: $R^2 = 0.031$, $P = 0.001$; NP: $R^2 = 0.017$, $P = 0.001$) in shaping symbiont communities. In addition, GLM analysis suggested a significant impact of temperature and precipitation on the alpha diversity of the symbiont community in *M. sacchari* (Shannon index, Bio1: F = 0.022, $P < 0.001$; Bio12: F = 0.002, $P < 0.001$; Table 4). Temperature was positively correlated
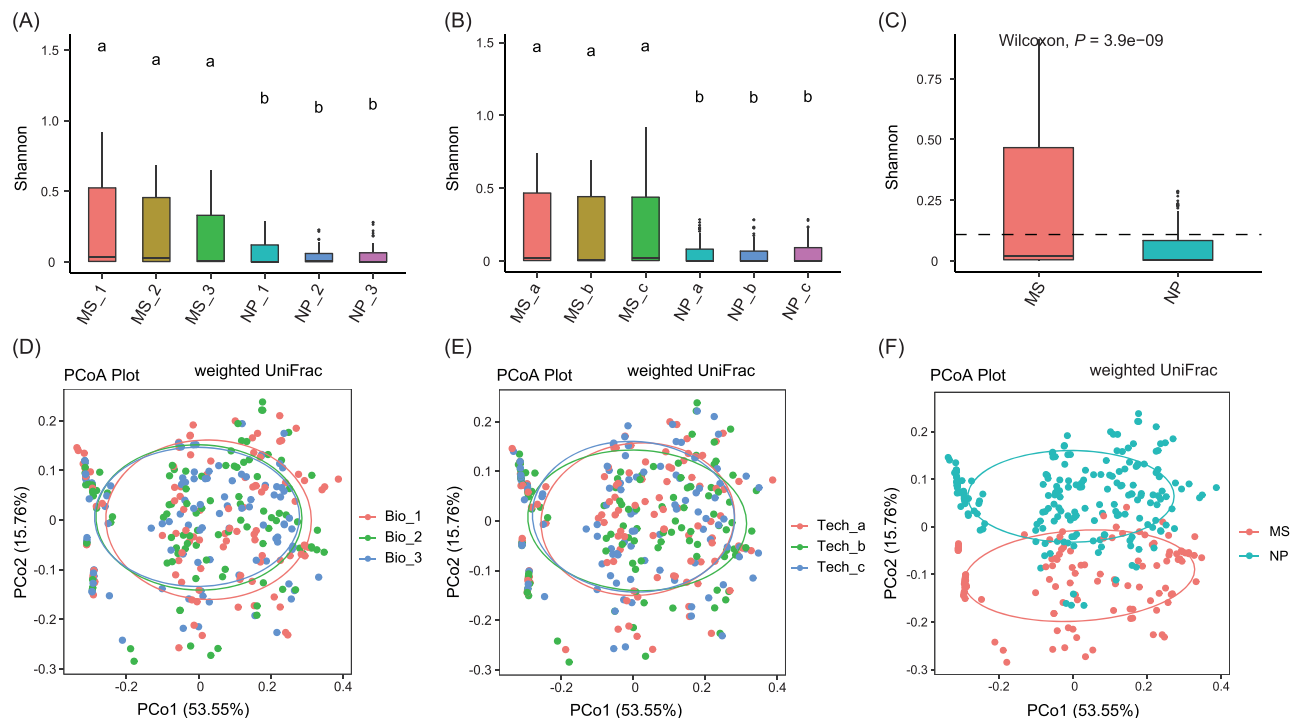
Figure 2. Boxplots of alpha diversity (Shannon index). Different groups included: three biological replicates (_1, _2, _3) grouped by samples of *M. sacchari* (MS) and *N. podocarpi* (NP), respectively, LSD test **(A)**; three technical replicates (_a, _b, _c) grouped by samples of MS and NP, respectively, LSD test **(B)**; two aphid species, Wilcoxon test **(C)**. PCoA plots of weighted UniFrac distances for all samples lumped into three biological replicates (Bio_1, Bio_2 and Bio_3, **D**) and three technical replicates (Tech_1, Tech_2 and Tech_3, **E**), and for two aphid species **(F)** in symbiont communities.

Table 3. Results of ANOSIM and PERMANOVA based on weighted and unweighted UniFrac distances of symbiont communities.

Groups	Weighted UniFrac		Unweighted UniFrac	
	ANOSIM (R, P)	PERMANOVA (R^2, P)	ANOSIM (R, P)	PERMANOVA (R^2, P)
Biological replicates	0, 0.388	0.009, 0.210	0.005, 0.140	0.010, 0.143
Technical replicates	−0.007, 0.991	0.001, 0.998	−0.005, 0.916	0.002, 0.921
Aphid species	0.262, **0.001**	0.122, **0.001**	0.009, 0.196	0.012, **0.009**
Geography_MS	0.363, **0.001**	0.427, **0.001**	0.220, **0.001**	0.274, **0.001**
Geography_NP	0.231, **0.001**	0.354, **0.001**	0.067, **0.018**	0.218, **0.001**
MS_host plants	0.432, **0.001**	0.224, **0.001**	0.133, **0.001**	0.099, **0.001**

Notes: The 'Biological replicates' are grouped by the label of '_1, _2, _3' in samples; the 'Technical replicates' are grouped by the label of '_a, _b, _c' in samples; 'Geography_MS' indicates the aphid groups collected from six geographical regions in *Melanaphis sacchari*, and 'Geography_NP' indicates the aphid groups collected from 10 geographical regions in *Neophyllaphis podocarpi*; 'MS_host plants' includes the group of aphid feeding on *Sorghum* (SO) and non-*Sorghum* (NS) in *Melanaphis sacchari*. $P < 0.05$ and $P < 0.01$ are in bold.

with the symbiont richness of *M. sacchari* (Chao1 index, Bio1: F = 0.002, P = 0.009; Table 4), but altitude negatively affected the symbiont richness of *N. podocarpi* (Chao1 index, Alt: F = −0.0001, P = 0.036; Table 4).

Comparison of secondary symbiont communities across the replicates, aphid species, geographic regions and host plants

After removing the primary endosymbiont *Buchnera*, ANOSIM and PERMANOVA based on UniFrac distances still showed that the secondary symbiont communities significantly differed with respect to geographic populations and host plants (P < 0.05; Supplementary Table 2), except when using weighted UniFrac in ANOSIM for host plants in *M. sacchari* and unweighted UniFrac in ANOSIM for different geographic populations in *N. podocarpi* (P

> 0.05; Supplementary Table 2). No significant difference in secondary symbiont communities was detected among replicates or aphid species (P > 0.05) using UniFrac distances.

DISCUSSION

In our study, the bacterial communities of two oligophagous aphids were dominated by heritable symbionts, most of which commonly inhabit other aphids (Bansal, Mian and Michel 2014; Gauthier *et al.* 2015; Xu *et al.* 2020a). All samples were infected with *Buchnera aphidicola*, which was consistent with its obligate role in aphids (Moran *et al.* 1993; Douglas 1998). We also detected six common secondary symbionts, including *Wolbachia*, *Rickettsia*, *Serratia symbiotica*, *Arsenophonus*, *Hamiltonella defensa* and *Regiella insecticola*. Excluding *Serratia symbiotica*, five other symbionts were detected for the first time in *M. sacchari* from China. The present study first characterized bacterial diversity and
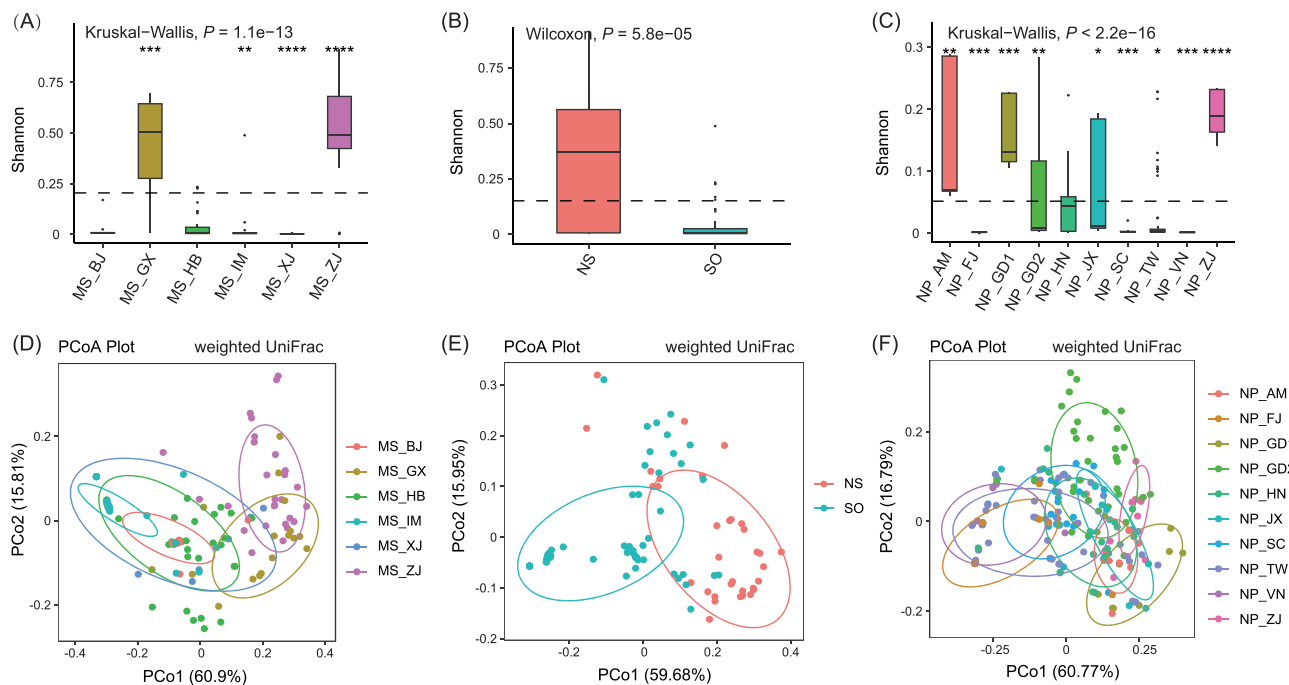
Figure 3. Boxplots of alpha diversity (Shannon index) and PCoA plot based on weighted UniFrac distance for six groups of geographic regions (BJ, GX, HB, IM, XJ, ZJ) in *M. sacchari*, Kruskal–Wallis and Wilcoxon test **(A, D)**; the aphid groups feeding on *Sorghum* (SO) and non-*Sorghum* (NS), Wilcoxon test **(B, E)**; 10 groups of geographic regions (AM, FJ, GD1, GD2, HN, JX, SC, TW, VN, ZJ) in *N. podocarpi*, Kruskal–Wallis and Wilcoxon test **(C, F)**. XJ: Xinjiang, ZJ: Zhejiang, GX: Guangxi, HB: Hebei, BJ: Beijing, IM: Inner Mongolia, SC: Sichuan, TW: Taiwan, ZJ: Zhejiang, VN: Vietnan, GD1: Guangdong1, GD2: Guangdong2, JX: Jiangxi, AM: America, HN: Hunan, FJ: Fujian.

highlighted the importance of geography in shaping the symbiont community in *N. podocarpi*. *Wolbachia* was detected in most samples with high relative abundance, which substantiated its high prevalence in aphids (De Clerck *et al.* 2014; Wang *et al.* 2014; Xu *et al.* 2021). *Wolbachia* are maternally transmitted and mediate host reproduction in many insects, with many supergroups of its phylogenetic relationship (Werren, Baldo and Clark 2008). Wang *et al.* (2009) found two supergroups infected in *Sitobion miscanthi* using the diagnostic PCRs. We also found there are two OTUs of *Wolbachia* in *N. podocarpi* (Fig. 4, OTU4 and OTU6), which suggested that there would be two different clades detected by 16S amplicon sequencing (Supplementary Fig. 2). All analyses proved that the symbiont community diversity was quite different between the two aphid species. Each aphid species harbored a specific OTU of *Buchnera* with the highest relative abundance (Fig. 4). The aphid species dominance of *Buchnera* was consistent with previous studies (Josselin *et al.* 2016; Fakhour *et al.* 2018). Multiple minor OTUs of *Buchnera* were also detected in this study, perhaps in relation to slightly accumulated mutations due to reduced genome and genetic drift (Moran 1996; Moran, McCutcheon and Nakabachi 2008).

In addition, we detected a rare *Sodalis*-like symbiont in these two oligophagous aphids. Previous studies have found *Sodalis*-like symbiont infection in some aphid species of Lachninae, such as *Cinara strobi* and *Eulachnus* spp. feeding on *Pinus* and host-specific *Nippolachnus piri* (Burke *et al.* 2009; Jousselin *et al.* 2016; Manzano-Marín *et al.* 2017; Blackman and Eastop 2020). Manzano-Marín *et al.* (2020) demonstrated that the B-vitamin biosynthesis genes of *Erwinia* were horizontally transmitted from *Sodalis* bacteria to complete the deficiency of *Buchnera*. Thus, the *Sodalis*-like symbiont might also participate in the biosynthesis of nutrients in oligophagous aphids. *Gilliamella*, which was previously found in some aphid species

of Hormaphidinae (Xu *et al.* 2020b), was also detected with high relative abundance in *M. sacchari* (Supplementary Fig. 1, Table 2). Its representative sequence was similar to that of the honeybee gut symbiont *Gilliamella apicola* (homology of 94.04%) when aligned against the NCBI database using BLAST. *Gilliamella apicola* is a common gut symbiont of honeybees and provides benefits such as degradation of pectin in pollen walls and fermentation of sugars (Engel, Martinson and Moran 2012; Zheng *et al.* 2016, 2019). Further work should assess the localization and role of *Gilliamella* associated with *M. sacchari*.

It seems unnecessary to perform technical replicates for aphid microbiome studies in the future because of the non-significant variation among those included in our study. The high-throughput amplicon sequencing should be a mature technique that would cause extremely minimal errors for detecting symbiont community. However, we noticed that a few differences in bacterial communities among biological replicates were related to the secondary symbiont infection pattern in *M. sacchari* (Supplementary Fig. 1). In fact, secondary symbionts are moderately prevalent in aphid populations (Haynes *et al.* 2003; Oliver *et al.* 2010; Zytynska and Weisser 2016). In addition, horizontal transfer plays an important role in shaping the secondary symbiont communities of aphids (Rock *et al.* 2018). Thus, the addition of biological replicates would be more accurate for the description of the aphid symbiont community in the population level.

Most studies have found that host plants are an important factor in structuring the bacterial communities associated with aphids (Medina, Nachappa and Tamborindeguy 2011; Ferrari *et al.* 2012; Gauthier *et al.* 2015; Xu *et al.* 2020a). However, in this study, geography is the main factor in shaping the symbiont community for oligophagous aphids. We found the variation in symbiont communities among different geographical
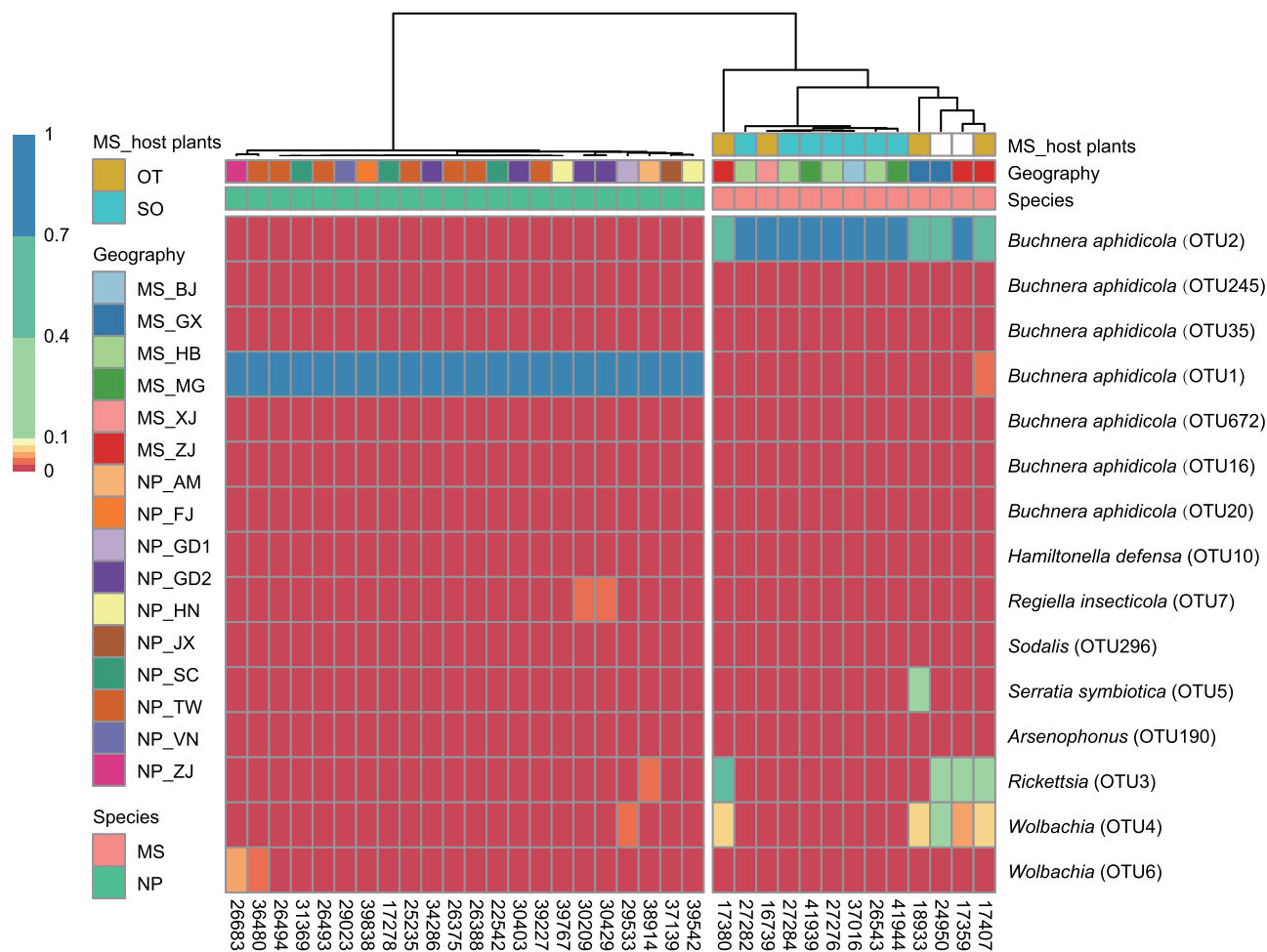
Figure 4. Heatmap showing the distribution and relative abundance of symbiont OTUs in *M. sacchari* and *N. podocarpi*. The hierarchical clustering tree indicating the similarities of symbiont communities is displayed. The abbreviations are shown in Table 1.

regions was shown in these two aphid species (PERMANOVA: $R^2 = 0.218$–0.427; Table 3). In addition, the symbiont communities of *M. sacchari* in southern China (ZJ and GX) differed largely from those in northern China (BJ, HB and IM) (Fig. 3A and D). The result was similar to the groups of *Sorghum* and non-*Sorghum* (Fig. 3B and E; Table 1), which probably due to *Sorghum* is widely cultivated in northern China. Two secondary symbionts, *Rickettsia* and *Wolbachia*, were more abundant in southern China (Fig. 4). The prevalence of both these two secondary symbionts in southern China was consistent with the findings of Tsuchida *et al.* (2002) and Guo *et al.* (2019). Hence, geography is a more important factor than host plants for the symbiont community assemblage of *M. sacchari*. The contribution of geography to symbiont communities was also confirmed in *N. podocarpi* (Table 3). Nibouche *et al.* (2014) revealed low genetic diversity in *M. sacchari* at the worldwide scale and found that its population genetic structure was related to geographical regions instead of host plants. This study also found a significant difference in the symbiont community of *M. sacchari* feeding on *Sorghum* and non-*Sorghum* plants (Table 3), indicating a potential impact of the symbiont community on its host specialization (Nibouche *et al.* 2015). In addition, a massive outbreak of *M. sacchari* occurred on sorghum that might have caused a new invasive genotype to be introduced into America (Nibouche *et al.* 2018). Perhaps

symbiotic bacteria would provide a new argument for the genetic differentiation of *M. sacchari*.

One possible reason for the importance of geography in structuring the symbiont community of two oligophagous aphids is that the aphid symbionts may be affected by environmental conditions from different geographic regions. Mantel tests revealed a significant correlation between geographic distances and symbiont communities in two oligophagous aphids, but the relationship did not occur in the polyphagous aphid *Aphis gossypii* (Xu *et al.* 2020a). On the one hand, the long spatial distance might be challenging for communication among oligophagous aphid populations and finely structure the variation in symbiont composition. On the other hand, the symbiont flora of oligophagous aphids was related to the environmental shift of geographical regions, following the changes in local ecological conditions, such as temperature, precipitation, altitude and natural enemies (Burke, Fiehn and Moran 2010; Frago *et al.* 2017; Fakhour *et al.* 2018; Duan *et al.* 2020). Similar to our results, three environmental variables (altitude, temperature and precipitation) together contributed to 22.7% of the variance in symbiont community structure in *M. sacchari* and 7.1% of the variance in *N. podocarpi* (Fig. 5). Here, we also found that altitude was negatively correlated with the symbiont richness (Chao1) of *N. podocarpi* (Table 4), which was consistent with the findings
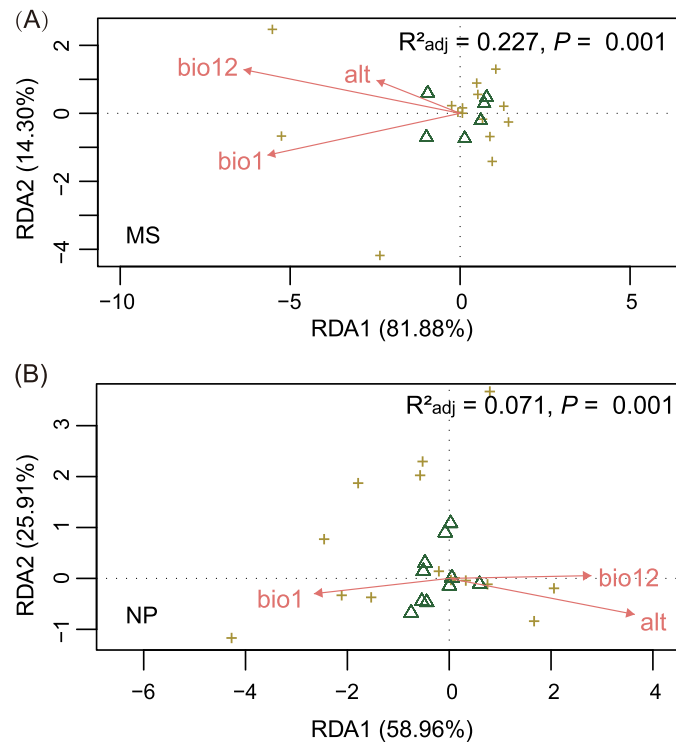
Figure 5. Redundancy analysis (RDA) for the relationship of three environmental factors and symbiont communities of *M. sacchari* (**A**: MS) and *N. podocarpi* (**B**: NP), respectively. The plus symbols represent symbiont OTUs, and the triangular symbols represent different groups for geographical regions. The angles between symbiont OTUs and environmental factor arrows represent the degree of their associations. R^2 measures the total variance of response variables (symbionts) explained by all explanatory variables (environmental factors); alt: altitude; bio1: annual mean temperature; bio12: annual precipitation.

Table 4. Results of GLM and RDA of symbiont communities in *M. sacchari* and *N. podocarpi*.

Environmental factors	*M. sacchari*			*N. podocarpi*		
	Shannon (F, P)	Chao1 (F, P)	Correlation (R^2, P)	Shannon (F, P)	Chao1 (F, P)	Correlation (R^2, P)
Alt	0.002, 0.155	−0.0003, 0.163	**0.031, 0.001**	−0.001, 0.139	**−0.0001, 0.036**	**0.017, 0.001**
Bio1	**0.022, <0.001**	**0.002, 0.009**	**0.046, 0.001**	0.010, 0.409	0.001, 0.357	**0.019, 0.001**
Bio12	**0.002, <0.001**	0.0001, 0.088	**0.028, 0.001**	−0.001, 0.305	−0.0001, 0.152	**0.018, 0.001**

Statistically significant P values ($P < 0.05$) are in bold. Alt: altitude; Bio1: annual mean temperature; Bio12: annual precipitation.

of Fakhour *et al.* (2018) based on the alpha diversity of bacterial symbionts. Sepúlveda *et al.* (2017) uncovered that temperature and precipitation might influence the spatial variation of symbionts in cereal aphids. Duan *et al.* (2020) also showed that the variation in microbial community structure is probably associated with annual mean precipitation and longitude. However, temperature was one of the key factors affecting symbiotic composition in the present study (Fig. 5, Table 4). Some secondary symbionts (i.e. *Serratia*, *Regiella* or *Fukatsuia*) usually protect pea aphids from heat stress by regulating the aphid metabolome or the density of *Buchnera* (Burke, Fiehn and Moran 2010; Heyworth, Smee and Ferrari 2020). Therefore, the similar function of some secondary symbionts indicated that heat stress might also occur in these two oligophagous aphid species, which probably changes the proportion of each symbiont associated with the aphids.

CONCLUSION

We investigated the symbiont diversity of two oligophagous aphids, *Melanaphis sacchari* and *Neophyllaphis podocarpi*. *Buchnera*

aphidicola was unsurprisingly the most dominant symbiont, and six secondary symbionts were found. The symbiont communities of the two oligophagous aphids differed greatly from each other. Geography had the greatest effect on the symbiont community structure, which might be linked to changes in local environmental conditions, such as temperature. Our study unmasks the relationship between the symbiont community and environmental factors associated with geography in oligophagous aphids, which may have been underestimated in previous studies. What is more, this study should have an impact and provide guidance on pest control in the future.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

AVAILABILITY OF SEQUENCING DATA

All sequences were deposited in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA661966.

AUTHOR CONTRIBUTIONS

GQ and JC designed the project. GQ and LJ identified voucher specimens. SX conducted molecular experiments and all analyses. JC and MQ assisted data analyses. SX wrote the manuscript and all authors contributed to revisions.

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