THE DETECTION AND MIDGUT INTRACELLULAR LOCATION OF *RICKETTSIA* SYMBIONT IN THE CAMELLIA APHID (*APHIS AURANTII*)

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Abstract. The *Rickettsia*, secondary symbiotic bacteria prevalent in arthropods, has been gradually recognized to play a significant role in the biology of their hosts in the past 20 years. A few previous studies have reported the occurrence of *Rickettsia* in several aphid species, located in the secondary bacteriocyte, sheath cell, or aphid hemolymph extracellularly. In this study, we found that *Rickettsia* symbiont infected camellia aphid, *Aphis aurantii*, with a relatively high infection rate (22%). Moreover, based on the next-generation sequencing of 16S rRNA genes, the relative abundance of *Rickettsia* from an *A. aurantii* sample could even reach 59.1%, which exceeded that of *Buchnera*, the primary endosymbiont of aphids. By using transmission electron microscopy, we also observed dense populations of rod-shaped *Rickettsia* symbionts in midgut epithelial cells of *A. aurantii*. Our study is the first to confirm that the *Rickettsia* can invade into midgut epithelial cells of *A. aurantii*, offering new insights into the location and possible infection route of *Rickettsia* in aphids.
Keywords: *bacteria, arthropods, Next Generation Sequencing, electron microscopy, endosymbiont*

Introduction

Aphids (Hemiptera, Aphididea) are considered notorious agricultural pests that cause severely damage to host plants via sucking on the phloem sap and transmitting phytopathogenic viruses (Eastop, 1977; Huang and Qiao, 2014; Skaljac, 2016). Due to the lack of various essential amino acid in the plant phloem sap, primary/obligate symbionts play an important role in providing essential nutrition to complete the diet of sap-feeding insects (Brumin et al., 2012; Hansen and Moran, 2013). For aphids, *Buchnera aphidicola* supplying nutrients as a primary endosymbiont has been reported in numerous studies (Baumann et al., 1995; Shigenobu et al., 2000; Wilson et al., 2010). Besides primary symbionts, insect secondary/facultative symbionts have generally been considered not necessary for host development and reproduction in the past. However, increasing evidence shows that multiple facultative symbionts might have significant influence on aphid fitness (Oliver et al., 2010), including *Hamiltonella defensa* for protection against parasitoids and predators (Schmid et al., 2012; Costopoulos et al., 2014), *Regiella insecticola* for protection against entomopathogen (ŁUkasik et al., 2013), *Wolbachia* for possible nutritional function (Augustinos et al., 2011). Furthermore, the endosymbiont *Serratia* symbiotica seems to undergo an evolutionary scenario from facultative stage to obligate stage, and establish a co-obligate endosymbiotic consortium with *B. aphidicola* in *Cinara cedri* (Lamelas et al., 2011) and *Cinara tujafilina* (Manzano-Marín and Latorre, 2014).

The *Rickettsia* are intracellular symbionts of eukaryotes and ubiquitous in arthropods (Weinert et al., 2009; Merhej and Raoult, 2011), some of which are best known as

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human and animal pathogens vectored by blood-sucking arthropods, being responsible for epidemic typhus, murine typhus or spotted fever (Raoult and Roux, 1997; Sakurai et al., 2005). *Rickettsia* bacteria are thought to be maternally (Weinert et al., 2009) and also plant-mediated horizontally transmitted (Caspi-Fluger et al., 2011). In general, *Rickettsia* bacteria are facultative symbionts in most arthropods, with influences on host fitness such as fitness benefits, female bias and sensitivity to insecticides in whitefly (Kontsedalov et al., 2008; Himler et al., 2011), male killing in ladybird beetle (Von Der Schulenburg et al., 2001), and thelytokous parthenogenesis induction in a parasitoid wasp (Hagimori et al., 2006). However, considering such wide occurrence in arthropods, more distinct and specific functions of *Rickettsia* symbionts await to be discovered in the vast majority of hosts.

For aphids, some studies have shown that *Rickettsia* can live in secondary bacteriocytes, sheath cells (Fukatsu et al., 2000; Skaljac, 2016), as well as in the hemolymph of hosts with extracellular stage (Tsuchida et al., 2005). A recent study using 454 pyrosequencing of 16S rRNA genes indicated the presence of *Rickettsia* in insect guts of 19 taxonomic orders (Yun et al., 2014). It seems to indicate that *Rickettsia* might be distributed more widely across the digestive system of insect taxa than previously thought. However, further studies are needed to investigate the real location of *Rickettsia* in insect digestive system, for example, free living in the gut or within the midgut epithelial cells. The camellia aphid *Aphis Aurantii* is a common pest species in the subtropical and tropical regions. In our study, we found that *Rickettsia* existed in wild *A. Aurantii* populations feeding on the plant *Loropetalum chinense*. We further investigate three questions: 1) whether the *Rickettsia* is common in *A. aurantii* populations; 2) how much can the relative abundance of *Rickettsia* in the symbiotic microbiome of *A. aurantii*; 3) whether the gut cells of *A. aurantii* contain *Rickettsia* symbiont.

Materials and Methods

The samples of *A. aurantii* were collected from seven different localities distributed in Yunnan, Guangxi, Fujian and Zhejiang provinces of southern China (*Fig. 1*). All samples were collected from different locations and plant individuals, making sure these samples were from different clones. All samples were directly preserved in 95%-ethanol in the wild and frozen at −80°C for further experiment in laboratory. For eliminating possible surface contaminants, all 27 individuals from 19 different clones were washed thrice in 70% ethanol, and then rinsed in sterile deionized water. DNA was isolated with DNeasy Blood & Tissue Kit (QIAGEN) as following the manufacturer manual. The specific primer pair (Ric16SF1: 5'-TGGCTGTCGTCAGCTCGT-3' and 5'-Ric16SR1: TCCACGTCACCGTCTTGC-3') (Sakurai et al., 2005) was used to detect and classify the *Rickettsia* infection. All PCRs were carried out in a 50 µl volume with 4 µl DNA template (2-20 ng/µl), 5 µl 10×Buffer, 2.5 mM dNTP mixture, 10 pmol of each primer and 1 unit of ExTaq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). The PCR conditions were an initial step of 3 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C followed in turn by final extension of 10 min at 72°C. PCR products were visualized on 1% agarose. To assure the accuracy of results, sterile deionized water was used as a negative control and some positive products (Individual ID: 17, 22 and 23) were sequenced (*Table 1*).

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To investigate relative abundance of *Rickettsia* in the symbiotic microbiome of *A. aurantii*, three aphid clones (HCX25 from *Mangifera indica*, HCX30 and HCX31 from *L. chinense*) were selected for NGS of bacterial 16S rRNA genes. Ten individuals of each clone followed the above steps of DNA extraction. The primer set (338F: 5'-ACTCCTACGGGAGGCAGCA-3' and 806R: 5'-GGACTACHVGGGTWT CTAAT-3') (Srinivasan et al., 2012) was used to amplify the V3 and V4 region of the bacterial 16S rRNA genes with 95°C for 5 min (1 cycle), 95°C for 30 s, 50°C for 30 s, 72°C for 40 s (25 cycles), followed by 72°C for 7 min. The PCR products was purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, United States). Finally, paired-end sequencing of the 16S rRNA was conducted on Illumina HiSeq 2500 with 2×250bp reads (Illumina, Inc., SanDiego, CA, United States) at Biomarker Bioinformatics Technology, Co., Ltd. (Beijing, China).

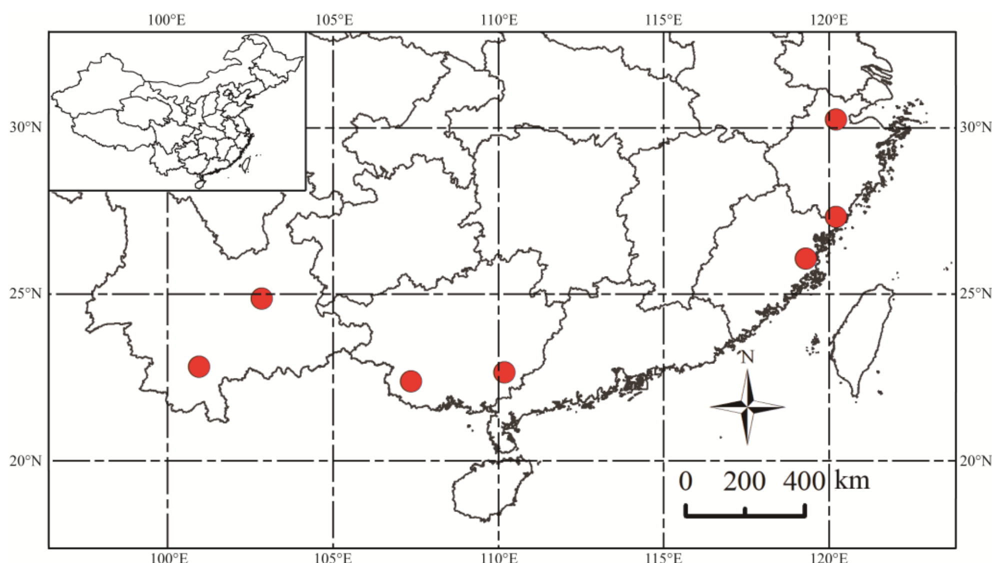

Figure 1. *Overview of geographic distribution of Aphis Aurantii clones collected in this study. Collection sites are labeled with red circle*

The paired-end reads were merged using FLASH v1.2.11 (Magoč and Salzberg, 2011), sequences were further quality trimmed using Trimmomatic v0.33 (Bolger et al., 2014), ensuring >20 quality scores on a sliding window of 50 bp. The chimera sequences were identified and removed using the UCHIME algorithm (Edgar et al., 2011). Sequences with ≥ 97% similarity were clustered into the same operational taxonomic units (OTUs) using UCLUST v10.0 (Edgar, 2010). All OTUs were identified with the RDP classifier (Version 2.2) (Wang et al., 2007) to obtain taxonomic information. The raw sequences have been submitted to the NCBI Sequence Read Archive (accession number SRR8668504, SRR8668505 and SRR8668506).

The 16S sequence of *Rickettsia* from HCX31 was extracted from the NGS sequences of bacterial 16S rRNA genes. The 16S sequences of other *Rickettsia* species was selected and download from GenBank. The data was aligned using MAFFT v 7.409 (Katoh and Standley, 2013), and some sequences not in the same regions were removed using BioEdit 7.0.5 (Hall, 1999). *Orientia tsutsugamushi* (AF062074.1) was chosen as outgroup. Finally, maximum likelihood (ML) tree was conducted using IQTree v1.5.4 and the HKY+F+I model with 1000 ultrafast bootstraps (Nguyen et al., 2014).

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The aphid guts of HCX31 individuals were dissected in 1×PBS using fine forceps. The dissected guts were prefixed in 2.5% glutaraldehyde at room temperature for 2h, and postfixed in 1% osmium tetroxide at room temperature for 60 min. After dehydration through an ethanol series and acetone, the guts were embedded through an SPURR embedding medium series. Ultrathin sections were made on an ultramicrotome (EM UC7; Leica), stained with uranyl acetate and lead citrate, and observed under an H-7650 Hitachi transmission electron microscopy at 80KV.

Table 1. *The detail information of samples from our study*

Clone	SD	NGS	Individual ID	Data	Location collected	Accession number
C1	+		1	04/03/2017	Fuding, Fujian	
C2	+		2	08/06/2017	Kunming, Yunnan	
C3	+		3	04/15/2017	Fuzhou, Fujian	
C4	+		4	10/16/2016	Fuzhou, Fujian	
C5	+		5	09/07/2015	Fuzhou, Fujian	
C6	+		6	10/23/2015	Fuzhou, Fujian	
C7(HCX30)	+	+	7	05/29/2017	Fuzhou, Fujian	SRR8668504
C8(HCX31)	+R	+	8	09/22/2017	Fuzhou, Fujian	SRR8668506
C9	+		9	11/04/2017	Fuzhou, Fujian	
C10	+		10	04/01/2018	Fuzhou, Fujian	
C11	+		11	09/24/2018	Fuzhou, Fujian	
C12	+		12	10/17/2018	Fuzhou, Fujian	
C13	+		13	11/18/2017	Pu'er, Yunnan	
C14	+		14	11/17/2017	Pu'er, Yunnan	
C14	+R		15	11/17/2017	Pu'er, Yunnan	
C15	+		16	11/01/2017	Chongzuo, Guangxi	
C16	+R		17	10/31/2017	Chongzuo, Guangxi	MK577785
C16	+R		18	10/31/2017	Chongzuo, Guangxi	
C2	+		19	08/06/2017	Kunming, Yunnan	
C2	+		20	08/06/2017	Kunming, Yunnan	
C2	+		21	08/06/2017	Kunming, Yunnan	
C17	+		22	10/29/2017	Yulin, Guangxi	MK577784
C18	+R		23	10/27/2017	Yulin, Guangxi	MK577783
C18	+R		24	10/27/2017	Yulin, Guangxi	
C19	+		25	06/17/2018	Hangzhou, Zhejiang	
C19	+		26	06/17/2018	Hangzhou, Zhejiang	
C19	+		27	06/17/2018	Hangzhou, Zhejiang	
C20(HCX25)		+		04/25/2016	Fuzhou, Fujian	SRR8668505

All Clones were collected from *Loropetalum chinense* except for C20 (HCX25) from *Mangifera indica*. NGS=NGS of bacterial 16S rRNA genes; SD=Specific detection using specific primers for *Rickettsia*. + represents using this method. +R represents a fragment of 300 bp obtained from specific primers, indicating the present of *Rickettsia* species

Results

The *Fig. 2* showed the rapid identification result of *Rickettsia* symbiont using the specific primers. A fragment of 300 bp was observed in 6 of 27 individuals. The result suggested a *Rickettsia* occurrence rate of 22.2% in our study. A total of 19 *Rickettsia* species with 403 bp length were selected to construct a ML phylogenetic tree. In the phylogenetic tree (*Fig. 3*), *Rickettsia* were classified into three major clades with high support values: one including *Rickettsia* from leeches, arthropods and our sample; a second clade including *Rickettsia* endosymbiont from *Bemisia tabaci* and some vertebrate pathogen *Rickettsia* such as *R. typhi* and *R. japonica*; a third clade containing sequences obtained from environmental samples.

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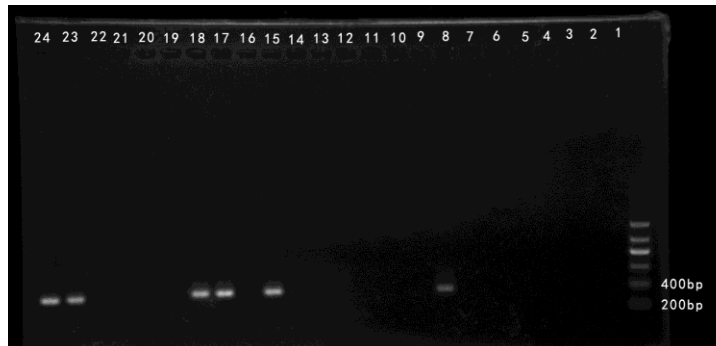

Figure 2. *Specific detection of Rickettsia sp. with primers Ric16SF1 and Ric16SR1 in 19 clones and 27 individuals of A. aurantii. Lanes 1-24: 1-24 individuals, Marker: marker F (200bp 400bp 700bp 1000bp 1500bp 2000bp). The remaining three are not shown due to observation in a small gel, and negative control does not show a fragment of 300 bp. The products in lanes 15, 17 and 23 have been sequenced and submitted to GenBank (Table 1)*

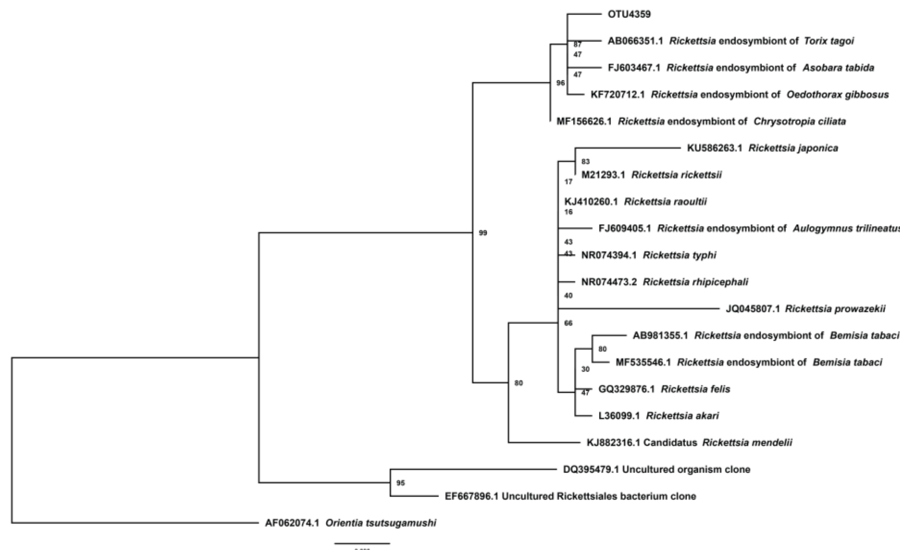

Figure 3. *ML phylogenic tree of Rickettsia based on 16S rDNA sequences. OUT4359 is the sequence from our sample HCX 31. The node values represent the bootstrap values*

The NGS of bacterial 16S rRNA gene yielded 47,579, 54,211 and 54,337 effective tags from HCX25, HCX30 and HCX31, respectively. The average length of effective tags was about 420bp. The OTUs number of the rarefaction curves had very slow growth with the increase of sequencing depth, indicating that the majority of bacterial OTUs had been observed (*Fig. 4A*). A total of 42 OTUs were obtained at 97% sequence-similarity level (31 OTUs for HCX25; 34 OTUs for HCX30 and 34 OUTs for HCX31) (*Fig. 4B*). The top 10 genera based on relative abundance in three samples were illustrated in *Fig. 4C*. For HCX25 and HCX30, the genus of dominant bacteria was *Buchnera*, the primary endosymbiont of aphids, accounting for 98.3% and 98.8%, respectively. For HCX31, the dominant bacteria were *Rickettsia* with 59.1% relative abundance, followed by *Buchnera* with 39.2%. Electron microscopic observation of the gut of HCX31 with dense populations of rod-shaped bacteria in the gut cells (*Fig. 5*). The *Rickettsia* cells ranged from 0.35-0.5 μm in width to 0.5-1 μm in length.

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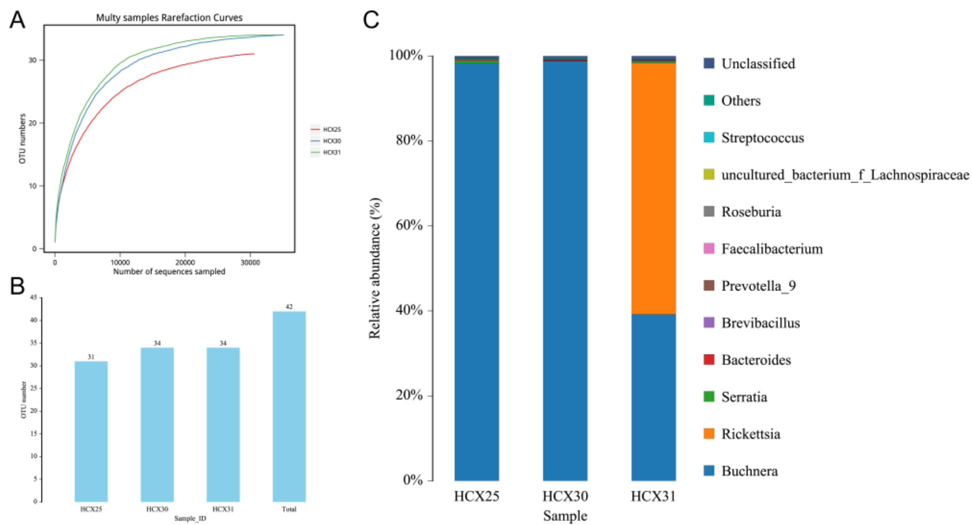

Figure 4. *The NGS analysis results of bacterial 16S rRNA genes. (A) The rarefaction curves. (B) The histogram of the OTUs numbers. (C) Taxonomic composition of bacteria for three A. aurantii samples*

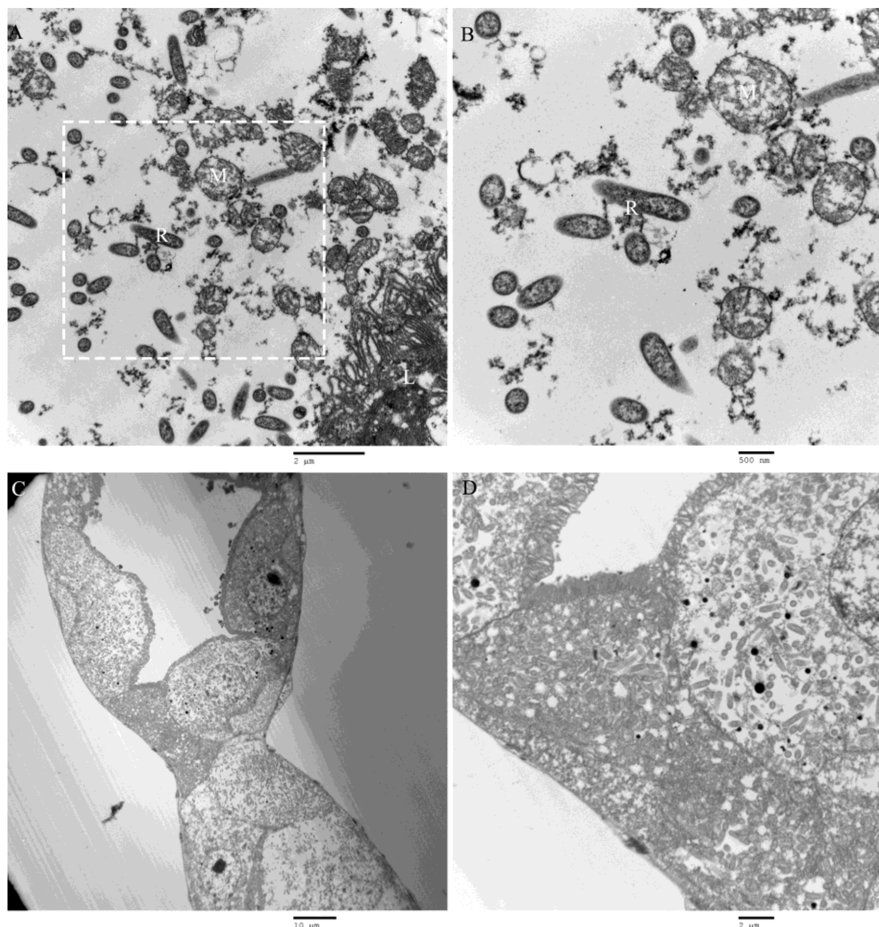

Figure 5. *(A) Electron microscopic observation of the midgut epithelial cells of A. aurantii. (B) Details of the box in A. L, lumen; M, mitochondria; R, Rickettsia. (C) and (D) High density Rickettsia symbionts with rod-shape in the gut cells*

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Discussion

Although all 27 individuals in our study were collected from *L. chinense*, the *Rickettsia* occurrence rate was similar with 17% of Guidolin and Cônsoli (2018) in *Aphis aurantii*. Cautiously, some bacteria occur at low proportion within a host, making limits of detection of specific primers due to low bacterial DNA concentration. The *Rickettsia* occurrence rate of 22.2% might be underestimated. Like other pathogens, some evidence have shown that the plant can be a media and provide a place for horizontal transmission of *Rickettsia* (Perlman et al., 2006; Caspi-Fluger et al., 2011). This may explain the phylogenetically similar symbionts occur in unrelated phytophagous insect species (Caspi-Fluger et al., 2011). The *Rickettsia* sequence from our sample is clustered together with *Rickettsia* from other arthropods in ML tree. *Rickettsia* are popular in many arthropod groups also including some other sap-feeders, but it is rare in aphids (Oliver et al., 2010). All records only include *Acyrthosiphon pisum* (Chen et al., 1996; Sakurai et al., 2005; Sepúlveda et al., 2017), *Amphorophora rubi* (Haynes et al., 2003), *Aphis citricidus* (Guidolin and Cônsoli, 2017, 2018). Haynes et al. (2003) also reported that the *Rickettsia* was very rarely detected in just 1% and 2% occurrence rates in natural populations of *Am. rubi* and *Ac. pisum*, respectively. A relatively high infection rate (22%) of *A. aurantii* was reported in our study, showing a significant difference compared to *Am. rubi* and *Ac. pisum*. Interestingly, the range of host plants of *A. aurantii* is also wider with 104 plant families compared to three families of *Am. rubi* and 23 families of *Ac. Pisum* (host plant information from http://www.aphidsonwor ldsplants.info), which is concordant with the comparative result of *Rickettsia* infection rates among the three aphid species. Guidolin and Cônsoli (2018) also consider that the polyphagous species may tend to more frequently infected with secondary symbionts than the oligophagous one.

Little is known about the influences of *Rickettsia* on their aphid hosts. A few studies show that *Rickettsia* symbionts can enhance aphid host’s resistance to natural enemies (ŁUkasik et al., 2013) and cause reductions in body weight and fecundity in the pea aphid (Chen et al., 2000). The invasive order of the most *Rickettsia* is considered from the gut to the haemocoel and then to the salivary glands (Perlman et al., 2006). Brumin et al. (2012) investigated the subcellular localization of *Rickettsia* endosymbiont in the whitefly, showing that *Rickettsia* infected the digestive salivary and had the possible digestive purposes. For aphids, several previous works of *Rickettsia* location only have shown the distribution of *Rickettsia* endosymbiont in secondary bacteriocytes, sheath cells and hemolymph (Fukatsu et al., 2000; Tsuchida et al., 2005; Skaljac, 2016) with the exception of the gut.

Conclusion

Our research confirms the *Rickettsia* location in gut cells, providing a necessary supplement to reveal a complete and clear *Rickettsia* localization in aphids, contributing to understanding its evolution and function in the future research. To understand *Rickettsia* evolution and pathogenesis, more works such as a comprehensive analysis of the entire genome need be done. Meanwhile, it will be interesting to test whether wider host plant range increases the infection possibility of *Rickettsia* in other aphids in the future.

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