Diversity and evolution of the endosymbionts of *Bemisia tabaci* in China

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The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex, including members that are pests of global importance. This study presents a screening of *B. tabaci* species in China for infection by the primary endosymbiont, *Portiera aleyrodidarum*, and two secondary endosymbionts, *Arsenophonus* and *Cardinium*. The results showed that *P. aleyrodidarum* was detected in all *B. tabaci* individuals, while *Arsenophonus* was abundant in indigenous species of *B. tabaci* Asia II 1, Asia II 3 and China 1 but absent in the invasive species, Middle East-Asia Minor 1 (MEAM1); *Cardinium* presented in the Mediterranean (MED), Asia II 1 and Asia II 3 species but was rarely detected in the MEAM1 and China 1 species. Moreover, phylogenetic analyses revealed that the *P. aleyrodidarum* and *mtCO1* (mitochondrial cytochrome oxidase 1) phylogenies were similar and corresponding with the five distinct cryptic species clades to some extent, probably indicating an ancient infection followed by vertical transmission and subsequent co-evolutionary diversification. In contrast, the phylogenetic trees of *Arsenophonus* and *Cardinium* were incongruent with the *mtCO1* phylogram, potentially indicating horizontal transmission in *B. tabaci* cryptic species complex. Taken together, our study showed the distinct infection status of endosymbionts in invasive and indigenous whiteflies; we also probably indicated the co-evolution of primary endosymbiont and its host as well as potential horizontal transfer of secondary endosymbionts.
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Abstract

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex, including members that are pests of global importance. This study presents a screening of *B. tabaci* species in China for infection by the primary endosymbiont, *Portiera aleyrodidarum*, and two secondary endosymbionts, *Arsenophonus* and *Cardinium*. The results showed that *P. aleyrodidarum* was detected in all *B. tabaci* individuals, while *Arsenophonus* was abundant in indigenous species of *B. tabaci* Asia II 1, Asia II 3 and China 1 but absent in the invasive species, Middle East-Asia Minor 1 (MEAM1); *Cardinium* presented in the Mediterranean (MED), Asia II 1 and Asia II 3 species but was rarely detected in the MEAM1 and China 1 species. Moreover, phylogenetic analyses revealed that the *P. aleyrodidarum* and *mtCO1* (mitochondrial cytochrome oxidase 1) phylograms were similar and corresponding with the five distinct cryptic species clades to some extent, probably indicating an ancient infection followed by vertical transmission and subsequent co-evolutionary diversification. In contrast, the phylogenetic trees of *Arsenophonus* and *Cardinium* were incongruent with the *mtCO1* phylogram, potentially indicating horizontal transmission in *B. tabaci* cryptic species complex. Taken together, our study showed the distinct infection status of endosymbionts in invasive and indigenous whiteflies; we also probably indicated the co-evolution of primary endosymbiont and its host as well as potential horizontal transfer of secondary endosymbionts.
Introduction

*Bemisia tabaci* is a cryptic species complex comprising a minimum of 40 morphologically similar species (De Barro et al. 2011; Dinsdale et al. 2010; Hu et al. 2018; Wang et al. 2017). Among members of the complex, the Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) groups (commonly known as the B and Q biotypes, respectively) have drawn much attention due to their global invasion and vectoring important plant pathogens (e.g. Tomato yellow leaf curl virus, TYLCV) (Brown 1994; Cohen & Nitzany 1966). In China, *B. tabaci* was not considered as a serious pest until the arrival of the MEAM1 group in the mid-1990s (Qiu et al. 2007). In 2004, the MED group was detected, and rapidly became widely distributed in China, causing considerable damage to a wide range of vegetables, fibers, and ornamental crops (Chu et al. 2005). It is interesting that MED has been replacing the earlier invader MEAM1 as well as several indigenous species of whiteflies (e.g. Asia II and China 1) in many regions (Liu et al. 2007; Sun et al. 2013).

Associations between insects and endosymbionts are quite common in nature. It has been estimated that at least 15–20% of all insect species live in symbiotic relationships with bacteria (Douglas 1998; Gosalbes et al. 2010). Endosymbionts associated with insects can be classified into primary endosymbionts (P-endosymbionts) and secondary endosymbionts (S-endosymbionts) (Baumann 2005). The P-endosymbionts are obligate and usually have mutualist relationships with their hosts. Besides, P-endosymbionts are generally localized in bacteriocytes of bacteriome (Caspi-Fluger et al. 2011) and transmitted vertically from mother to progeny (Werren & O’Neill 1997). In contrast, the S-endosymbionts are usually facultative symbionts, and they could reside in several host tissues such as gut, hemolymph, Malpighian tubules, salivary glands or ovarian cells (Cicero et al. 2016; Cooper et al. 2014; Dobson et al. 1999; Zchori-Fein et al. 1998). Infection of secondary endosymbionts can be either maternally inherited or horizontally-transmitted (Moran
It has been discovered that the P-endosymbionts *Portiera aleyrodidarum* and S-endosymbionts such as *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia*, and *Wolbachia* were infected in whiteflies (Bing et al. 2013; Chiel et al. 2007; Chu et al. 2011; Everett et al. 2005; Karut et al. 2017; Thao & Baumann 2004; Zchori-Fein et al. 2014). Previous studies have investigated the prevalence, diversity and evolution of endosymbionts in the *B. tabaci* species complex from different countries or regions (e.g. Turkey, China, Brazil and Africa) (Ahmed et al. 2010; Bing et al. 2013; Bing et al. 2014; Ghosh et al. 2015; Hashmi et al. 2018; Jahan et al. 2015; Karut et al. 2017; Marubayashi et al. 2014; Santos-Garcia et al. 2015; Sseruwagi et al. 2018; Thierry et al. 2011; Thierry et al. 2015). However, most of these reports only focused on the two invasive cryptic species MEAM1 and MED; furthermore, for studies from China, the sample size and distribution range were limited. For example, regarding prevalence of S-endosymbionts in *B. tabaci*, only the laboratory samples from two provinces were investigated (Bing et al. 2013); only one S-endosymbiont (*Wolbachia*) was explored from a slightly larger geographical scale (Bing et al. 2014).

Therefore, although substantial datasets regarding endosymbionts prevalence of *B. tabaci* species complex are present, their current situation in whole China is still not very clear and further investigation is essential. In the present study, our first goal is to investigate the prevalence and diversity of the P-endosymbionts *P. aleyrodidarum* and two common S-endosymbionts *Arsenophonus* and *Cardinium* (as representatives of S-endosymbionts) within *B. tabaci* in wider range of China. We also aim to explore the evolutionary relationships between these three endosymbionts and their host based on phylogenetic analyses of 16S and 23S ribosomal DNA (*rDNA*) (from endosymbionts) as well as mitochondrial cytochrome oxidase 1 (*mtCO1*) gene (from *B. tabaci*). Our study will not only uncover the current status of endosymbionts infection within *B. tabaci* in China, but also greatly provide a supplement for studies of *B. tabaci* endosymbionts worldwide.

**Materials and Methods**
Sample collection

Totally 1,510 B. tabaci individuals were collected from 71 geographical locations, including 19 provinces and four municipalities in China (Fig. 1). At each location, B. tabaci was collected from different leaves of separate plants. The collection details, geographical sites and host plants were described in Table S1.

DNA extraction and gene amplification

Total DNA was extracted from individual whitefly as described in Luo et al. (2002). The primers of mtCO1 gene was used for whitefly species identification. 16S rDNA primers were used to detect P. aleyrodidarum and Cardinium, and for Arsenophonus, 23S rDNA primers were utilized. The primers, annealing temperature, and predicted PCR products size were shown in Table 1. The PCR reaction mixture contained 1 U Taq DNA polymerase, 5 µl (10×) reaction buffer, 3 µl MgCl₂ (final concentration of 25 mmol/L), 2 µl dNTPs (10 mmol/L), 2 µl of forward and reverse primers (20 µmol/L each) and 2 µl of template DNA (Simon et al. 1994). PCR products were visualized by 1.5% agarose gels and sequenced by IGE Biotechnology Co., Ltd (Guangzhou, China). The sequences were deposited in Genbank under accession numbers KP137471 to KP137491 for B. tabaci mtCO1 gene, KP201110 to KP201126 for P. aleyrodidarum 16S rDNA, KP201103 to KP201109 for Arsenophonus 23S rDNA, and KP201127 to KP201134 for Cardinium 16S rDNA (Table 2).

Sequence alignment and phylogenetic analysis

Sequence fragments were assembled using ContigExpress and aligned using the Clustal X 1.83 program (Chenna et al. 2003). The GenBank database was searched for homologous sequences of mtCO1, 16S rDNA and 23S rDNA using the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were constructed using MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003). The best-fit substitution model for each of the aligned sequences was selected with the program Modeltest 3.7 (Posada & Crandall 1998). All the trees were constructed using the GTR+I+G.
model. The Metropolis-coupled Markov chain Monte Carlo algorithm was conducted using four chains. Analyses were initiated with random starting trees, processed for \(3\times10^6\) generations, and sampled every 1,000 generations. For the burn-in period, we discarded 100,000 generations. Posterior clade probabilities obtained from the analysis were used to assess nodal support. Tree information was visualized and edited using FigTree ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

**Results**

**Molecular identification of *B. tabaci* individuals**

Analyses of *mtCO1* sequences indicated that our 1,510 *B. tabaci* samples comprised of two invasive species (MEAM1 and MED) and three indigenous species (Asia II 1, Asia II 3 and China 1). Among the individuals tested, 36.4% (550/1,510) and 39.7% (600/1,510) were identified as MEAM1 and MED, respectively. The remained 4.6%, 9.3% and 9.9% insects were identified as Asia II 1 (70/1,510), Asia II 3 (140/1,510) and China 1 (150/1,510), respectively (Fig. 1A; Table S1). Moreover, both the MEAM1 and MED whiteflies were widely-distributed across China, whereas the three indigenous species (Asia II 1, Asia II 3 and China 1) were relatively less detected and mainly distributed in southeastern part of China (Fig. 1B).

**Prevalence of endosymbionts among five species of *B. tabaci***

As expected, *P. aleyrodidarum* was detected in all whitefly individuals and species (Fig. 2A); 21.7% (327/1,510) individuals harbored S-endosymbiont (*Arsenophonus* or *Cardinium*); 5.6% (84/1,510) whiteflies were co-infected with both, and the remained majority (72.8%; 1,099/1,510) lacked an infection with either of the two S-endosymbionts. Infection frequencies with S-endosymbionts varied across *B. tabaci* species. In detail, *Arsenophonus* was abundant in the indigenous species (Asia II 1, Asia II 3 and China 1), with infection rates ranging from 50.7-55.7%; however, it was infrequent in MED (11.5%) and MEAM1 (0.0%) (Fig. 2B). *Cardinium* was moderately common in MED, Asia II 1 and Asia II 3 populations with frequencies of 20.3-40.7%,
but rarely detected in MEAM1 (0.5%) and not found in China 1 (Fig. 2C).

**Genetic diversity of B. tabaci and its endosymbionts**

Aligned sequences from *B. tabaci* (813 bp, *mtCO1* gene), *P. aleyrodidarum* (886 bp, 16s rDNA), *Arsenophonus* (551 bp, 23S rDNA), and *Cardinium* (460 bp, 16S rDNA) were used to analyze the genetic variation of whitefly and its endosymbionts. The results showed that 21 haplotypes were identified in whiteflies based on *mtCO1* sequences, while 17, 7 and 8 symbiont haplotypes were defined based on analysis of *P. aleyrodidarum, Arsenophonus* and *Cardinium* sequences, respectively (Table 2). These haplotypes sequences were used to construct the phylogenetic trees.

**Phylogenetic analysis of B. tabaci and endosymbionts**

Phylogenetic trees were constructed for *B. tabaci, P. aleyrodidarum, Arsenophonus* and *Cardinium* based on *mtCO1* gene, 16s rDNA, 23S rDNA and 16s rDNA sequences of haplotypes, respectively. We also pulled the related sequences from NCBI to explore the phylogenetic status of our haplotypes. The GenBank number of those related sequences could be found in phylogenetic trees. Based on the *mtCO1* gene, we found five distinct genetic groups of *B. tabaci*, which was corresponding with the five cryptic species including the MEAM1, MED, Asia II 1, Asia II 3 and China 1 (Fig. 3). We can also find that all of our MED individuals belonged to Q1 subclade (Chu et al. 2011). For phylogenetic trees of endosymbionts, several distinct bacterial strains existed within individual bacterium. It is interesting that the phylogenetic tree of *P. aleyrodidarum* were similar to the *mtCO1* tree and exhibited four groups corresponding to MEAM1+MED, Asia II 1, Asia II 3 and China 1 clades to some extent (Fig. 4). While there are still several differences. For example, the sequences of *P. aleyrodidarum* of China 1 are not well clustered and *P. aleyrodidarum* from *B. tabaci* species MED (KP201113) was also present in that China 1 clade. In addition, the MEAM1+MED clade (Fig. 4) contained sequences of *P. aleyrodidarum* from both MEAM1 and MED; however, it was separated into two distinct clades in the *B. tabaci* tree (Fig.
3). In contrast, the phylogenetic trees for *Arsenophonus* and *Cardinium* were totally incongruent with the mtCO1 tree, exhibiting four (A1-A4) and three groups (C1-C3), respectively (Fig 5 and 6).

**Discussion**

In this study, we conducted an extensive screening of *B. tabaci* for the presence one P-endosymbiont of two common S-endosymbionts, along with phylogenetic analyses of these symbionts to compare with host species from the cryptic *B. tabaci* complex. The reason we chose *Arsenophonus* and *Cardinium* as representatives of S-endosymbionts because they are the very common S-endosymbionts in whiteflies, and *Wolbachia* has been thoroughly investigated in the study of Bing et al. (2014). The results showed that P-endosymbiont *P. aleyrodidarum* was detected in all whitefly individuals while S-endosymbionts infection were varied in species. The variation in the prevalence of endosymbionts could be influenced by numerous factors such as host, environmental conditions, geographical location or even climate (Chu et al. 2011; Karut & Tok 2014; Morag et al. 2012; Skaljac et al. 2010). In our study, *Arsenophonus* was abundant in Asia II 1, Asia II 3 and China 1 species but absent in the invasive species MEAM1, which is exactly consistent with previous studies (Bing et al. 2013; Karut et al. 2017); *Cardinium* was present in the MED, Asia II 1 and Asia II 3 species (20.3-40.7%) but was rarely detected in MEAM1 and not detected in China 1. Taken together, it seemed that these two S-endosymbionts had high prevalence in native species rather than invasive species, which is consistent with another S-endosymbiont *Wolbachia* but in contrast to *Hamiltonella*; *Hamiltonella* was found abundant in invasive species rather than native species (Bing et al. 2013).

Previous studies showed that *B. tabaci* could be co-infected with particular pairs of S-endosymbionts, including *Rickettsia* and *Hamiltonella*, *Hamiltonella* and *Cardinium*, or *Rickettsia* and *Arsenophonus*; others were less common, such as *Cardinium* and *Rickettsia*, *Hamiltonella* and *Arsenophonus*, *Cardinium* and *Wolbachia*, and even three or four endosymbionts together (Gueguen et al. 2010; Karut & Tok 2014; Marubayashi et al. 2014; Pan et al. 2012; Skaljac et al. 2010).
In our study, we found evidence for a low rate (5.6%) of co-infection with *Arsenophonus* and *Cardinium* in four *B. tabaci* species (MEAM1 was the exception since *Arsenophonus* was not detected in this species), which has also been reported before (Bing et al. 2013; Chu et al. 2011; Parrella et al. 2014; Zchori-Fein et al. 2014). However, the reason of so few rate of co-infection by *Arsenophonus* and *Cardinium* is that *Arsenophonus* and *Cardinium* are potential reproductive manipulators that compete for resources inside the bacteriocytes, thus compromising the fitness of host (Gottlieb et al. 2008). We have one plausible explanation for the co-infection status, that is *Cardinium* is not restricted to the bacteriocytes (Skaljac et al. 2010), and perhaps the non-overlapping niche makes co-infection of *Arsenophonus* and *Cardinium* possible. In addition, the co-infection symbiont system in whiteflies may indicate the roles of dual endosymbionts: work as important mutually dependents to provide full complement of nutrients to their host (Rao et al. 2015) or affect the fitness and biology of the *B. tabaci* (Ghosh et al. 2018).

Our phylogenetic analyses indicated that *B. tabacia* mtCO1 sequences could be assigned to five distinct clades, which conformed to existing MEAM1, MED, Asia II 1, Asia II 3, and China 1 clades. Similarly, the sequences of the P-endosymbionts *P. aleyrodidarum* were assigned to their own clade and the phylogeny was similar with that of *B. tabaci* genetic groups to some extent. This may potentially indicate an ancient infection followed by vertical transmission and subsequent co-evolutionary diversification (Baumann 2005). Meanwhile, it is important to note that the correlation was not perfect: sequences of *P. aleyrodidarum* from MEAM1 and MED were assigned to the same clade instead of the two distinct clades presented in the mtCO1 tree. The reason could be the dissemination of the MEAM1 and MED species; furthermore, the spread of these two invasive species in China has been frequently associated with founder effects that fix specific mtDNA variation(s) along with particular endosymbionts (Chu et al. 2011; Gueguen et al. 2010). Taken together, although there was similarity between the two trees of *P. aleyrodidarum* and *B. tabaci*, the genetic variation of primary symbiont might not be an ideal reflecting the genetic variation of the cryptic *B. tabaci*.

The S-endosymbionts, *Arsenophonus* and *Cardinium*, both showed a lack of congruence with
the *B. tabaci* *mtCO1* phylogram. This is consistent with the finding from Ahmed et al. (2013), who provided evidence for horizontal transmission of S-endosymbionts in the *B. tabaci* cryptic species complex based on phylogenies studies. There are substantial phylogenetic evidences showing that S-endosymbionts such as *Wolbachia* and *Arsenophonus*, undergoing horizontal transfer among host arthropod species (Ahmed et al. 2013; Chrostek et al. 2017; Kolasa et al. 2017; Li et al. 2017; Russell et al. 2003; Vavre et al. 1999). In some cases, the mechanisms for horizontal transmission of S-endosymbionts are already known, including transferring through parasitoid wasps (Ahmed et al. 2015; Gehrer & Vorburger 2012), plants (Caspi-Fluger et al. 2012; Li et al. 2017) or even sexual transmission (Moran & Dunbar 2006). Therefore, the potential horizontal transfer of S-endosymbionts in our samples could be one or combination of the above ways.

In summary, this study reported the varied prevalence of three endosymbionts within five *B. tabaci* cryptic species. The P-endosymbiont *P. aleyrodidarum* was detected in all whitefly individuals; the S-endosymbionts *Arsenophonus* was abundant in native species while *Cardinium* was common in the invasive species. In addition, the phylogenetic relationships between endosymbionts and their hosts *B. tabaci* probably indicated the vertical transmission and co-evolution of *P. aleyrodidarum* and *B. tabaci*; meanwhile, horizontal transfer of *Arsenophonus* and *Cardinium* may happen in our collecting samples. Our study not only reported current infection status of endosymbionts within *B. tabaci* populations in China, but also demonstrated that S-endosymbionts genetic variation may not reflect host genetic variation and should not be used to infer taxonomic relationships within the host species complex. If funding allows, more endosymbionts should be investigated and future investigations could be the contribution of endosymbionts to invasiveness, population expansion, or even competitiveness of whitefly species.

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References

Ahmed MZ, De Barro PJ, Ren SX, Greeff JM, and Qiu BL. 2013. Evidence for horizontal transmission of secondary endosymbionts in the Bemisia tabaci cryptic species complex. *PLoS one* 8:e53084.

Ahmed MZ, Li SJ, Xue X, Yin XJ, Ren SX, Jiggins FM, Greeff JM, and Qiu BL. 2015. The intracellular bacterium *Wolbachia* uses parasitoid wasps as phoretic vectors for efficient horizontal transmission. *PLoS Pathog* 11:e1004672.

Ahmed MZ, Ren SX, Mandour NS, Greeff JM, and Qiu BL. 2010. Prevalence of *Wolbachia* supergroups A and B in *Bemisia tabaci* (Hemiptera: Aleyrodidae) and some of its natural enemies. *Journal of economic entomology* 103:1848-1859.

Baumann P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* 59:155-189.

Bing XL, Ruan YM, Rao Q, Wang XW, and Liu SS. 2013. Diversity of secondary endosymbionts among different putative species of the whitefly *Bemisia tabaci*. *Insect Science* 20:194-206.

Bing XL, Xia WQ, Gui JD, Yan GH, Wang XW, and Liu SS. 2014. Diversity and evolution of the *Wolbachia* endosymbionts of *Bemisia* (Hemiptera: Aleyrodidae) whiteflies. *Ecology and evolution* 4:2714-2737.

Brown J. 1994. Current status of *Bemisia tabaci* as a plant pest and virus vector in agroecosystems worldwide. *FAO Plant Protection Bulletin* 42:3-32.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Katzir N, Portnoy V, Belausov E, Hunter MS, and Zchori-Fein E. 2012. Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proc R Soc B* 279:1791-1796.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Mouton L, Hunter MS, and Zchori-Fein E. 2011. *Rickettsia* ‘in’ and ‘out’: two different localization patterns of a bacterial symbiont in the same insect species. *PLoS one* 6:e21096.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, and Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic acids research* 31:3497-3500.

Chiel E, Gottlieb Y, Zchori-Fein E, Mozes-Daube N, Katzir N, Inbar M, and Ghanim M. 2007. Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of entomological research* 97:407-413.

Chrostek E, Pelz-Stelinski K, Hurst GD, and Hughes GL. 2017. Horizontal transmission of intracellular insect symbionts via plants. *Frontiers in Microbiology* 8.

Chu D, Gao C, De Barro P, Zhang Y, Wan F, and Khan I. 2011. Further insights into the strange role of bacterial endosymbionts in whitefly, *Bemisia tabaci*: Comparison of secondary
symbionts from biotypes B and Q in China. *Bulletin of entomological research* 101:477-486.

Chu D, Zhang Y, Cong B, Xu B, and Wu Q. 2005. Identification for Yunnan Q-biotype *Bemisia tabaci* population. *Entomological Knowledge* 42:59-62.

Cicero J, Fisher T, and Brown JK. 2016. Localization of ‘*Candidatus Liberibacter solanacearum*’and Evidence for Surface Appendages in the Potato Psyllid Vector. *Phytopathology* 106:142-154.

Cohen S, and Nitzany F. 1966. Transmission and host range of the tomato yellow leaf curl virus. *Phytopathology* 56.

Cooper WR, Sengoda VG, and Munyaneza JE. 2014. Localization of ‘*Candidatus Liberibacter solanacearum*’ (Rhizobiales: Rhizobiaceae) in *Bactericera cockerelli* (Hemiptera: Triozidae). *Annals of the Entomological Society of America* 107:204-210.

De Barro PJ, Liu SS, Boykin LM, and Dinsdale AB. 2011. *Bemisia tabaci*: a statement of species status. *Annual review of entomology* 56:1-19.

Dinsdale A, Cook L, Riggins C, Buckley Y, and De Barro P. 2010. Refined global analysis of *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) mitochondrial cytochrome oxidase 1 to identify species level genetic boundaries. *Annals of the Entomological Society of America* 103:196-208.

Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, and O’Neill SL. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect biochemistry and molecular biology* 29:153-160.

Douglas A. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual review of entomology* 43:17-37.

Everett KD, Thao M, Horn M, Dyzsynski GE, and Baumann P. 2005. Novel chlamydiae in whiteflies and scale insects: endosymbionts ‘*Candidatus Fritschea bemisiae*’strain Falk and ‘*Candidatus Fritschea eriococci*’strain Elm. *International journal of systematic and evolutionary microbiology* 55:1581-1587.

Gehrer L, and Vorburger C. 2012. Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biology letters*:rsbl20120144.

Ghosh S, Bouvaine S, and Maruthi M. 2015. Prevalence and genetic diversity of endosymbiotic bacteria infecting cassava whiteflies in Africa. *BMC microbiology* 15:93.

Ghosh S, Bouvaine S, Richardson SC, Ghanim M, and Maruthi M. 2018. Fitness costs associated with infections of secondary endosymbionts in the cassava whitefly species *Bemisia tabaci*. *Journal of pest science* 91:17-28.

Gosalbes MJ, Latorre A, Llamas A, and Moya A. 2010. Genomics of intracellular symbionts in insects. *International Journal of Medical Microbiology* 300:271-278.

Gottlieb Y, Ghanim M, Gueguen G, Kontsedalov S, Vavre F, Fleury F, and Zchori-Fein E. 2008. Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies. *The FASEB Journal* 22:2591-2599.

Guéguen G, Vavre F, Gnankine O, Peterschmitt M, Charif D, Chiel E, Gottlieb Y, Ghanim M, ZCHORI - FEIN E, and Fleury F. 2010. Endosymbiont metacommunities, mtDNA...
diversity and the evolution of the *Bemisia tabaci* (Hemiptera: Aleyrodidae) species complex. *Molecular Ecology* 19:4365-4376.

Hashmi TR, Devi SR, Meshram NM, and Prasad R. 2018. Assessment of bacterial endosymbionts and the host, *Bemisia tabaci* (Hemiptera: Aleyrodidae), using rRNA and mitochondrial cytochrome oxidase I gene sequences. *Communicative & Integrative Biology* 11:e1433442.

Hu J, Zhang X, Jiang Z, Zhang F, Liu Y, Li Z, and Zhang Z. 2018. New putative cryptic species detection and genetic network analysis of *Bemisia tabaci* (Hemiptera: Aleyrodidae) in China based on mitochondrial COI sequences. *Mitochondrial DNA Part A* 29:474-484.

Jahan S, Lee K, Howlader M, Bashar H, and Hasan G. 2015. Molecular divergence of secondary endosymbiont, *Cardinium* in *Bemisia tabaci* (Gennadius) and associates. *Bangladesh Journal of Agricultural Research* 40:121-135.

Karut K, Mete Karaca M, Döker İ, and Kazak C. 2017. Analysis of Species, Subgroups, and Endosymbionts of *Bemisia tabaci* (Hemiptera: Aleyrodidae) From Southwestern Cotton Fields in Turkey. *Environmental entomology*.

Karut K, and Tok B. 2014. Secondary endosymbionts of Turkish *Bemisia tabaci* (Gennadius) populations. *Phytoparasitica* 42:413-419.

Kolasa M, Montagna M, Mereghetti V, Kubisz D, Mazur MA, and Kajtoch Ł. 2017. Preliminary evidence of the horizontal transmission of *Wolbachia* between *Crioceris* leaf beetles (Coleoptera: Chrysomelidae) and their Asparagus host plants. *European Journal of Entomology* 114:446-454.

Li SJ, Ahmed MZ, Lv N, Shi PQ, Wang XM, Huang JL, and Qiu BL. 2017. Plantmediated horizontal transmission of *Wolbachia* between whiteflies. *The ISME journal* 11:1019.

Liu S-S, De Barro P, Xu J, Luan J-B, Zang LS, Ruan YM, and Wan FH. 2007. Asymmetric mating interactions drive widespread invasion and displacement in a whitefly. *Science* 318:1769-1772.

Luo C, Yao Y, Wang R, Yan F, Hu D, and Zhang Z. 2002. The use of mitochondrial cytochrome oxidase I (mt CO I) gene sequences for the identification of biotypes of *Bemisia tabaci* (Gennadius) in China. *Kun chong xue bao Acta entomologica Sinica* 45:757-763.

Marubayashi JM, Kliot A, Yuki VA, Rezende JAM, Krause-Sakate R, Pavan MA, and Ghanim M. 2014. Diversity and localization of bacterial endosymbionts from whitefly species collected in Brazil. *PloS one* 9:e108363.

Morag N, Klement E, Saroya Y, Lensky I, and Gottlieb Y. 2012. Prevalence of the symbiont *Cardinium* in *Culicoides* (Diptera: Ceratopogonidae) vector species is associated with land surface temperature. *The FASEB Journal* 26:4025-4034.

Moran NA, and Baumann P. 2000. Bacterial endosymbionts in animals. *Current opinion in microbiology* 3:270-275.

Moran NA, and Dunbar HE. 2006. Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences* 103:12803-12806.

Pan H, Li X, Ge D, Wang S, Wu Q, Xie W, Jiao X, Chu D, Liu B, and Xu B. 2012. Factors affecting population dynamics of maternally transmitted endosymbionts in *Bemisia tabaci*. 
Parrella G, Nappo AG, Manco E, Greco B, and Giorgini M. 2014. Invasion of the Q2 mitochondrial variant of Mediterranean *Bemisia tabaci* in southern Italy: possible role of bacterial endosymbionts. *Pest management science* 70:1514-1523.

Posada D, and Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics (Oxford, England)* 14:817-818.

Qiu B, Coats SA, Shunxiang R, Idris AM, Caixia X, and Brown JK. 2007. Phylogenetic relationship of native and introduced *Bemisia tabaci* (Homoptera: Aleyrodidae) from China and India based on mtCOI DNA sequencing and host plant comparisons. *Progress in Natural Science* 17:645-654.

Rao Q, Rollat-Farnier PA, Zhu DT, Santos-Garcia D, Silva FJ, Moya A, Latorre A, Klein CC, Vavre F, and Sagot MF. 2015. Genome reduction and potential metabolic complementation of the dual endosymbionts in the whitefly *Bemisia tabaci*. *BMC genomics* 16:226.

Ronquist F, and Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.

Russell J, Latorre A, Sabater - Muñoz B, Moya A, and Moran N. 2003. Side - stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molecular Ecology* 12:1061-1075.

Santos-Garcia D, Vargas-Chavez C, Moya A, Latorre A, and Silva FJ. 2015. Genome evolution in the primary endosymbiont of whiteflies sheds light on their divergence. *Genome biology and evolution* 7:873-888.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, and Flock P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87:651-701.

Skaljac M, Zanic K, Ban SG, Kontsedalov S, and Ghanim M. 2010. Co-infection and localization of secondary symbionts in two whitefly species. *BMC microbiology* 10:142.

Sseruwagi P, Wainaina J, Ndunguru J, Tumuhimbise R, Taipo F, Guo J-Y, Vrielink A, Blythe A, Kinene T, and De Marchi B. 2018. The first transcriptomes from field-collected individual whiteflies (*Bemisia tabaci*, Hemiptera: Aleyrodidae): a case study of the endosymbiont composition. *Gates open research* 1.

Sun DB, Liu YQ, Qin L, Xu J, Li FF, and Liu SS. 2013. Competitive displacement between two invasive whiteflies: insecticide application and host plant effects. *Bulletin of entomological research* 103:344-353.

Thao ML, and Baumann P. 2004. Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Applied and environmental microbiology* 70:3401-3406.

Thierry M, Becker N, Hajri A, Reynaud B, LETT JM, and Delatte H. 2011. Symbiont diversity and non - random hybridization among indigenous (Ms) and invasive (B) biotypes of
Bemisia tabaci. Molecular Ecology 20:2172-2187.

Thierry M, Bile A, Grondin M, Reynaud B, Becker N, and Delatte H. 2015. Mitochondrial, nuclear, and endosymbiotic diversity of two recently introduced populations of the invasive Bemisia tabaci MED species in La Réunion. Insect Conservation and Diversity 8:71-80.

Vavre F, Fleury F, Lepetit D, Fouillet P, and Bouletreau M. 1999. Phylogenetic evidence for horizontal transmission of Wolbachia in host-parasitoid associations. Molecular biology and evolution 16:1711-1723.

Wang XW, Li P, and Liu SS. 2017. Whitefly interactions with plants. Curr Opin Insect Sci 19:70-75. 10.1016/j.cois.2017.02.001

Werren JH, and O’Neill SL. 1997. The evolution of heritable symbionts. Influential passengers: inherited microorganisms and arthropod reproduction:1-41.

Zchori-Fein E, Lahav T, and Freilich S. 2014. Variations in the identity and complexity of endosymbiont combinations in whitefly hosts. Frontiers in Microbiology 5:310.

Zchori-Fein E, Roush RT, and Rosen D. 1998. Distribution of parthenogenesis-inducing symbionts in ovaries and eggs of Aphytis (Hymenoptera: Aphelinidae). Current microbiology 36:1-8.
Figure Captions

Figure 1. The quantity and distribution of *B. tabaci* cryptic species in China. (A) The quantity of each *B. tabaci* cryptic species based on molecular identification. (B) The locations of the *B. tabaci* cryptic species populations in China. Names of locations are given in Table S1. Maps were created using Esri’s ArcGIS platform (http://www.esri.com/software/arcgis).

Figure 2. Infection frequency of endosymbionts in five *B. tabaci* cryptic species. (A) *P. aleyrodidae*um; (B) *Arsenophonus*; (C) *Cardinium*. Number above bars indicate the number of infection.

Figure 3. The Bayesian phylogenetic tree of *B. tabaci* cryptic species based on mtCOI sequences. The value beside the nodes are posterior probabilities. *Trialeurodes vaporariorum* (AF418672) is used as outgroup. Accession numbers for mtCOI sequences submitted to GenBank are KP137471-KP137491. All mtCOI sequences of *B. tabaci* cryptic species used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. Bold dots indicate the sequences from the present study.

Figure 4. The Bayesian phylogenetic tree of *P. aleyrodidae*um based on 16S rDNA sequences. The value beside the nodes are posterior probabilities. *Trialeurodes vaporariorum* (AY266113) is
used as outgroup. Accession numbers for 16S rDNA sequences submitted to GenBank are KP201110-KP201125. All 16S rDNA sequences of P. aleyrodidarum used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. Bold dots indicate the sequences from the present study. Dotted boxes indicate imperfect cluster of each B. tabaci cryptic species.

**Figure 5. The Bayesian phylogenetic tree of Arsenophonus based on 23S rDNA sequences.**
The value beside the nodes are posterior probabilities. Aleurodicus dispersus (AY264664) is used as outgroup. Accession numbers for 23S rDNA sequences submitted to GenBank are KP201103-KP201109. All 23S rDNA sequences of Arsenophonus used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. A1-A4 indicate the four clusters.

**Figure 6. The Bayesian phylogenetic tree of Cardinium based on 16S rDNA sequences.** The value beside the nodes are posterior probabilities. Paralvinella palmiformis (AJ441237) is used as outgroup. Accession numbers for 16S rDNA sequences submitted to GenBank are KP201127-KP201134. All 16S rDNA sequences of Cardinium used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. C1-C3 indicate the three clusters.
Figure 1

The quantity and distribution of *B. tabaci* cryptic species in China.

(A) The quantity of each *B. tabaci* cryptic species based on molecular identification. (B) The locations of the *B. tabaci* cryptic species populations in China. Names of locations are given in Table S1. Maps were created using Esri’s ArcGIS platform (http://www.esri.com/software/arcgis).
Figure 2

Infection frequency of endosymbionts in five *B. tabaci* cryptic species.

(A) *P. aleyrodidarum*; (B) *Arsenophonus*; (C) *Cardinium*. Number above bars indicate the number of infection.
Figure 3

The Bayesian phylogenetic tree of *B. tabaci* cryptic species based on *mtCOI* sequences.

The value beside the nodes are posterior probabilities. *Trialeurodes vaporariorum* (AF418672) is used as outgroup. Accession numbers for *mtCOI* sequences submitted to GenBank are KP137471-KP137491. All *mtCOI* sequences of *B. tabaci* cryptic species used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. Bold dots indicate the sequences from the present study.
Figure 4

The Bayesian phylogenetic tree of *P. aleyrodidarum* based on 16S *rDNA* sequences.

The value beside the nodes are posterior probabilities. *Trialeurodes vaporariorum* (AY266113) is used as outgroup. Accession numbers for 16S *rDNA* sequences submitted to GenBank are KP201110-KP201125. All 16S *rDNA* sequences of *P. aleyrodidarum* used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. Bold dots indicate the sequences from the present study. Dotted boxes indicate imperfect cluster of each *B. tabaci* cryptic species.
Figure 5

The Bayesian phylogenetic tree of *Arsenophonus* based on 23S *rDNA* sequences.

The value beside the nodes are posterior probabilities. *Aleurodicus dispersus* (AY264664) is used as outgroup. Accession numbers for 23S *rDNA* sequences submitted to GenBank are KP201103-KP201109. All 23S *rDNA* sequences of *Arsenophonus* used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. A1-A4 indicate the four clusters.
Figure 6

The Bayesian phylogenetic tree of *Cardinium* based on 16S *rDNA* sequences.

The value beside the nodes are posterior probabilities. *Paralvinella palmiformis* (AJ441237) is used as outgroup. Accession numbers for 16S *rDNA* sequences submitted to GenBank are KP201127-KP201134. All 16S *rDNA* sequences of *Cardinium* used in this study were clustered with other related references sequences from GenBank and their accession numbers are also indicated in the tree. C1-C3 indicate the three clusters.
Table 1 (on next page)

The sequences, annealing temperature, product size and references of primers used in this study.
### Table 1. The sequences, annealing temperature, product size and references of primers used in this study.

| Gene                          | Primer sequence(5'→3')                                      | Annealing temperature | Product size(bp) | References                  |
|-------------------------------|-------------------------------------------------------------|-----------------------|------------------|-----------------------------|
| *B. tabaci* mtCOI             | C1-J-2195: TTGATTTTTTGGTCATCCAGAAGT                        | 50°C                  | 800              | Frohlich et al., 1999      |
|                               | L2-N-3014: TCCAATGCACTAATCTGCCCATTAA                       |                       |                  |                             |
| *P. aleyrodidarum* 16S rDNA   | Pro-F: TGCAAGTCGAGGCCATCAT                                  | 59°C                  | 1000             | Zghori-Fein and Brown, 2002 |
|                               | Pro-R: AAAGTTCCCGCCTTATGCGT                                |                       |                  |                             |
| Cardinium 16S rDNA            | Ch-F: TACTGTAAGAAATAAGCACCACGAC                            | 57°C                  | 400              | Zchori-Fein et al., 2004   |
|                               | Ch-R: GTGAGATCCTAAAGCTTACGTTT                             |                       |                  |                             |
| Arsenophonus 23S rDNA         | Ars-F: CGTTGATGATTTCTAGTCAAA                                | 60.5°C                | 900              | Thao and Baumann, 2004     |
|                               | Ars-R: GGTCCTCCAGTTAGTTACCCAAAC                             |                       |                  |                             |
Table 2 (on next page)

Haplotype information of whitefly, *P. aleyrodidarum*, *Arsenophonus* and *Cardinium*.
Table 2 Haplotype information of whitefly, *P. aleyrodidarum*, *Arsenophonus* and *Cardinium*.

| Species | n. | Re. Seq. | Acc. no. | Per. (%) | n. | Re. Seq. | Acc. no. | Per. (%) | n. | Re. Seq. | Acc. no. | Per. (%) |
|---------|----|----------|----------|----------|----|----------|----------|----------|----|----------|----------|----------|
| MEAM1/B | 4  | BHZ-1    | KP137471 | 85.1     | 2  | BST-CA1  | KP201110 | 89.1     | 3  | BGD-CR1  | KP201127 | 92.5     |
|         |    | BST-2    | KP137472 | 8.2      |    | BGW-CA2  | KP201111 | 10.9     |    | BHC-CR2  | KP201128 | 2.5      |
|         |    | BSH-3    | KP137473 | 4.9      |    |         |          |          |    | BSRF-CR3 | KP201129 | 5        |
|         |    | BJC-4    | KP137474 | 1.8      |    |         |          |          |    |          |          |          |
| MED/Q   | 4  | QSQ-1    | KP137475 | 90.3     | 6  | QBJ-CA1  | KP201112 | 88.8     | 2  | QHS-AR1  | KP201103 | 89.7     |
|         |    | QST-2    | KP137476 | 0.5      |    | QAH-CA2  | KP201113 | 5.8      |    | QHL-AR2  | KP201104 | 10.3     |
|         |    | QHW-3    | KP137477 | 5.8      |    | QSL-CA3  | KP201114 | 1.0      |    |          |          |          |
|         |    | QSZ-4    | KP137478 | 3.3      |    | QSI-CA4  | KP201115 | 2.0      |    |          |          |          |
|         |    |          |          |          |    | QSH-CA5  | KP201116 | 1.8      |    |          |          |          |
|         |    |          |          |          |    | QAH-CA6  | KP201117 | 0.5      |    |          |          |          |
| Aisa II | 3  | A3AJ-1   | KP137479 | 86.9     | 3  | A3ZW-CA1 | KP201118 | 86.2     | 1  | A3ZL-AR1 | KP201105 | 100.0    |
|         |    | A3HW-2   | KP137480 | 1.5      |    | A3ZL-CA2 | KP201119 | 9.2      |    |          |          |          |
|         |    | A3JN-3   | KP137481 | 0.8      |    | A3GQ-CA3 | KP201120 | 4.6      |    |          |          |          |
|         |    | A3GQ-4   | KP137482 | 6.2      |    |          |          |          |    |          |          |          |
|         |    | A3ZL-5   | KP137483 | 4.6      |    |          |          |          |    |          |          |          |
| Aisa II | 3  | A1ZQ-1   | KP137484 | 81.4     | 2  | A1GQ-CA1 | KP201121 | 94.3     | 2  | A1ZL-AR1 | KP201106 | 92.3     |
|         |    | A1GH-2   | KP137485 | 12.9     |    | A1JS-CA2 | KP201122 | 5.7      |    | A1GS-AR2 | KP201107 | 7.7      |
|         |    | A1GH-3   | KP137486 | 5.7      |    |          |          |          |    | A1GQ-AR1 | KP201133 | 92.0     |
| China 1 | 5  | CAJ-1    | KP137487 | 94.7     | 4  | CCY-CA1  | KP201123 | 2.7      | 2  | CCY-AR1  | KP201108 | 97.6     |
|         |    | CCY-2    | KP137488 | 2.0      |    | CZS-CA2  | KP201124 | 90.7     |    | CAJ-AR2  | KP201109 | 2.4      |
|         |    | CFW-3    | KP137489 | 0.7      |    | CZH-CA3  | KP201125 | 5.3      |    |          |          |          |
|         |    | CJG-4    | KP137490 | 1.3      |    | CZJ-CA4  | KP201126 | 1.3      |    |          |          |          |
|         |    | CGM-5    | KP137491 | 1.3      |    |          |          |          |    |          |          |          |

**TOTAL** 21 17 7 8

2 n.: Haplotype number. Re. Seq.: Representative Sequence. Acc. no.: Accession number. Per.: Percentage in each cryptic species.

3