Diversity and evolution of the *Wolbachia* endosymbionts of *Bemisia* (Hemiptera: Aleyrodidae) whiteflies

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**Abstract**

*Wolbachia* is the most prevalent symbiont described in arthropods to date. *Wolbachia* can manipulate host reproduction, provide nutrition to insect hosts and protect insect hosts from pathogenic viruses. So far, 13 supergroups of *Wolbachia* have been identified. The whitely *Bemisia tabaci* is a complex containing more than 28 morphologically indistinguishable cryptic species. Some cryptic species of this complex are invasive. In this study, we report a comprehensive survey of *Wolbachia* in *B. tabaci* and its relative *B. afer* from 1658 insects representing 54 populations across 13 provinces of China and one state of Australia. Based on the results of PCR or sequencing of the 16S rRNA gene, the overall rates of *Wolbachia* infection were 79.6% and 9.6% in the indigenous and invasive *Bemisia* whiteflies, respectively. We detected a new *Wolbachia* supergroup by sequencing five molecular marker genes including 16S rRNA, groEL, gltA, hcpA, and fbpA genes. Data showed that many protein-coding genes have limitations in detecting and classifying newly identified *Wolbachia* supergroups and thus raise a challenge to the known *Wolbachia* MLST standard analysis system. Besides, the other *Wolbachia* strains detected from whiteflies were clustered into supergroup B. Phylogenetic trees of whitefly mitochondrial cytochrome oxidase subunit I and *Wolbachia* multiple sequencing typing genes were not congruent. In addition, *Wolbachia* was also detected outside the special bacteriocytes in two cryptic species by fluorescence in situ hybridization, indicating the horizontal transmission of *Wolbachia*. Our results indicate that members of *Wolbachia* are far from well explored.

**Introduction**

*Wolbachia* are rickettsial endosymbiotic bacteria in the class Alphaproteobacteria. *Wolbachia* bacteria are considered the most widespread endosymbionts in animals as they are found in all major classes of arthropods and some nematodes (Jayaprakash and Hoy 2000; Werren and Windsor 2000; Duron et al. 2008; Russell et al. 2012). A meta-analysis suggests that the proportion of *Wolbachia* infection in insect species in the terrestrial world is about 40% (Zug and Hammerstein 2012).

In some host species, the successful maintenance and spread of *Wolbachia* is mainly achieved by the induction of cytoplasmic incompatibility to produce more female offspring, thus enhancing its maternal transmission (Stouthamer et al. 1999). In addition, manipulation of reproduction by *Wolbachia* includes feminizing genetic males, causing parthenogenesis, and killing male progenies (Stouthamer et al. 1999; Werren et al. 2008). Recent studies found that *Wolbachia* benefits insect hosts by providing essential nutrition (Hosokawa et al. 2010), enhancing host stem cell’s proliferation (Fast et al. 2011), and protecting insect from pathogenic RNA viruses (Hedges et al. 2008).

The genus *Wolbachia* is highly divergent and has so far been divided into 13 supergroups (A-N, except for G which is a combination of A and B) (Lo et al. 2002, 2007; Baldo and Werren 2007; Haegeman et al. 2009; Ros et al. 2009; Augustinos et al. 2011). *Wolbachia* supergroups are characterized mainly with molecular markers such as *rrs* (16S rRNA), *fssZ* (cell division protein), *gltA* (Citrate synthase), *groEL* (Chaperonin GroEL) and *wsp* (*Wolbachia* surface protein) genes (O’Neill et al. 1992; Zhou et al. 1998; Werren and Windsor 2000; Casiraghi et al. 2005). *Wolbachia* genotyping is inferred mainly from multi locus sequence typing (MLST) genes (*gatB*, *coxA*, *hcpA*, *fbpA*, and *fssZ* genes) and amino acid sequences of the four

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**Keywords**

*Bemisia tabaci*, FIISH, horizontal transmission, multilocus sequence typing, vertical transmission, whiteflies, *Wolbachia*.

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**Funding Information**

The work was supported by the National Natural Science Foundation of China (Project number 31321063 and 31390421).

Received: 24 February 2014; Revised: 30 April 2014; Accepted: 1 May 2014

Ecology and Evolution 2014; 4(13): 2714–2737
doi: 10.1002/ece3.1126

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hypervariable regions (HVRs) of WSP protein (Baldo et al. 2005, 2006).

*Bemisia tabaci* (Hemiptera: Aleyrodidae) is a complex containing more than 28 morphologically indistinguishable cryptic species (De Barro et al. 2011; Hu et al. 2011). Through millions of years of evolution, the various cryptic species of this complex show a clear geographic pattern of distribution around the globe (Boykin et al. 2007, 2013; De Barro et al. 2011). However, with the development of modern transport, whiteflies have been transferred frequently among different continents (Naranjo et al. 2010). During the last twenty years, two cryptic species of the *B. tabaci* complex, Middle East-Asia Minor 1 (formerly known as the B “biotype,” hereafter MEAM1) and Mediterranean (formerly known as the Q “biotype,” hereafter MED) have invaded many regions of the world (Dalton et al. 2011). They have caused serious damages to local agriculture through direct plant sap sucking and transmission of plant pathogenic viruses (Oliveira et al. 2001). What is more, the rapid invasion of MEAM1 and MED has caused the replacement of many indigenous whitefly species complex (Liu et al. 2007; Muñiz et al. 2011; Rao et al. 2011). These events provide us a unique opportunity for studying the evolution and transmission of *Wolbachia* among different *B. tabaci* cryptic species, which were geographically isolated in history but have become sympatric recently.

Previous studies have investigated the diversity of *Wolbachia* in the *B. tabaci* species complex (Nirgianaki et al. 2003; Chiel et al. 2007; Gueguen et al. 2010; Chu et al. 2011; Pan et al. 2012; Singh et al. 2012; Bing et al. 2013a). However, most of these reports focused on the two invasive cryptic species MEAM1 and MED and only used one to three marker genes in the investigation, and the distribution of *Wolbachia* in most indigenous *B. tabaci* cryptic species remains largely unknown. In this study, we examined the distribution of *Wolbachia* in *B. afer* and 10 cryptic species of the *B. tabaci* species complex collected from 13 provinces of China and one state of Australia. We report: (1) the prevalence of *Wolbachia* in *B. afer* and *B. tabaci*; (2) the discovery of a probably new *Wolbachia* (supergroup O) in whiteflies by sequencing of *rrs* gene and four protein-coding genes (*fbpA, hcpA, gltA*, and *groEL*); (3) the diversity and phylogenetic status of *Wolbachia* strains within these whiteflies; and (4) evidence for horizontal transfer of *Wolbachia* among *B. tabaci* cryptic species.

**Materials and Methods**

**Whitefly collection and DNA extraction**

*Bemisia* specimens were collected from 13 provinces of China and one state of Australia. Details for collection (geographical locations, host plants, and dates) of those populations are summarized in Fig. 1 and Table A1. Whiteflies collected from the same locality and host plant were considered as one population. Whitefly samples were initially immersed in 95% ethanol after collection and subsequently kept at -20°C until DNA extraction. Total whitefly DNA was extracted from individual adult specimens according to the method of DeBarro and Driver (1997). The quality of the DNA samples was confirmed by PCR amplification of a 0.8 kb fragment of whitefly mitochondrial cytochrome oxidase I (*mtCOI*) gene using the primers C1-J-2195 and L2-N-3014 (Table A2). Cryptic species of *B. tabaci* were first identified based on the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method described by Qin et al. (2013), and the sex of whiteflies was identified through genital morphology. A total of 1658 whitefly DNA samples were positive for PCR amplification using the *mtCOI* primers, indicating satisfactory quality of the DNA templates.

**Diagnostic screening of Wolbachia**

The presence of *Wolbachia* was screened based on the amplification of a 0.6 kb fragment with the *Wolbachia rrs* primers (Table A2). Standard PCR analyses were performed using 2×EasyTaq PCR SuperMix (TransGen, Beijing, China) in a PTC-200 Thermocycler (Bio-Rad, Hercules, CA). PCR procedures were an initial step of 94°C for 3 min, followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final step of 72°C for 10 min. Amplified DNA products were electrophoresed on agarose gels and stained with GelRed (Biotium, San Francisco, CA). To verify PCR results, amplified bands (especially uncertain ones) were purified by AxyPrep DNA gel extraction kit (Axygen, Silicon Valley, CA) and cloned into the pGEM-T vector (Promega, Madison, WI). Plasmids containing the DNA inserts of expected sizes were confirmed by PCR and sequenced in an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequencing results were then checked by Blast in NCBI nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only those individuals, which were blasted to expected products of the specific primers, were considered to be infected. All PCRs included a negative control (sterile water instead of DNA) and a positive control (DNA of China 1 whitefly). For *Wolbachia*-positive populations, *Bemisia* species were further identified by phylogenetic analysis of the *mtCOI* gene (Fig. A3). *Wolbachia* infection rates between whitefly sexes were statistically tested using the Fisher’s exact test. Statistical significance of the infection rates among different *B. afer* and *B. tabaci* cryptic species was calculated using the *χ*² test and corrected by the Bonferroni procedure. All
statistical analyses were performed using the Data Processing System (DPS) software (Tang and Zhang 2013).

**Sequencing and typing of Wolbachia**

As the phylogenetic analysis of the 0.6 kb rrs sequences indicated a possible new supergroup of Wolbachia, we amplified the rrs gene from whitefly populations and introduced an endonuclease VspI (AT/TAAT) (Fisher Scientific, Pittsburgh, PA) to digest the target bands, to investigate the infection prevalence of the new supergroup of Wolbachia. Several whitefly individuals were then randomly selected for sequencing confirmation of PCR-RFLP results. The groEL, wsp, and MLST (gatB, coxA, hcpA, fbpA, and ftsZ) genes from every combination of host species and rrs genotype were amplified by TransTaq-T DNA Polymerase (TransGen), cloned into pEASY-T1 vectors (TransGen) and sequenced on ABI 3730 DNA analyzer (Applied Biosystems).

In addition, to confirm the finding of the new Wolbachia supergroup, nearly a complete rrs sequence (1417 bp) and a partial gltA sequence (659 bp) were amplified from one Wolbachia new supergroup singular-infected population. PCR amplifications, DNA cloning and sequencing procedures were accomplished as described previously. The cycling procedures were the same as described earlier with changes on the annealing temperature for different primers. Primer sequences and annealing temperatures of rrs, gltA, groEL, wsp, and the MLST genes were listed in Table A2.

Sequences of MLST and wsp genes were manually trimmed in line with the template provided in Wolbachia MLST website and compared with sequences in the Wolbachia MLST database (http://pubmlst.org/wolbachia/). Novel sequences were submitted to the database curators as new alleles. Each unique combination of five MLST sequences was designated a strain type (ST) number in the Wolbachia MLST database (Baldo et al. 2006). Previously published sequences from other whiteflies were added to the data set to increase the power of statistical comparisons. All newly obtained allele numbers and ST numbers in this study are summarized in Table 1.

**Molecular phylogenetic analysis**

Phylogenetic analyses were constructed using (1) Wolbachia sequences of rrs, gltA, groEL, MLST, and wsp genes of different supergroups from various hosts; and (2) whitefly mtCOI sequences. All sequences used in this study were edited and aligned manually using Clustal W (ver. 1.6) (Thompson et al. 1994) in MEGA (ver. 5.10) (Tamura et al. 2011). The Gblocks program (ver. 0.91b) (Castresana 2000) was used to remove poorly aligned positions and to obtain nonambiguous sequence alignments. The best-fit evolutionary model for the sequence data was determined using hierarchical likelihood ratio tests and Akaike information criterion with the program jModelTest (ver. 0.1.1) (Posada 2008). Phylogenetic trees were constructed with the Bayesian inference using MrBayes (ver 3.1.2) (Ronquist and Huelsenbeck 2003).
Table 1. GenBank accession numbers of Wolbachia sequences and allele profiles of Wolbachia-positive whitefly populations.

| Pop. | Strain       | Species | rs      | groEL   | ST     | gatB   | coxB   | hcpA   | ftsZ   | ftpA   | wsp    | HRV1 | HRV2 | HRV3 | HRV4 |
|------|--------------|---------|---------|---------|--------|--------|--------|--------|--------|--------|--------|------|------|------|------|
| 1    | wBt_a1       | Bemisia afer | KF454753 | KF452533 | 382    | KF452586 (105) | KF452567 (11) | KF454725 (220) | KF452573 (11) | KF454737 (162) | KF465800 (670) | (231) | (265) | (143) | (23) |
| 2    | wBt_2        | MED     | KF454762 | –       | –      | –      | –      | –      | –      | KF454744 (386) | KF465817 (668) | (230) | (264) | (3)   | (23) |
| 10   | wBt_10       | Asia II | KF454771 | KF452543 | 391    | KF452588 (207) | KF452561 (88) | KF454726 (234) | KF452576 (170) | KF454746 (390) | –      | –    | –    | –    |
| 11   | wBt_11       | China I | JF795502 | KF452544 | 379    | KF452591 (105) | KF452563 (88) | KF454731 (13) | KF452578 (170) | KF454752 (9)  | KF465816 (669) | (2)  | (17) | (3)  | (2)  |
| 13   | wBt_13       | MED     | KF454764 | –       | –      | –      | –      | –      | –      | KF454719 (226) | KF452575 (181) | –    | –    | –    | –    |
| 26   | wBt_26       | Asia II | JF795503 | KF452536 | 378    | KF452590 (105) | KF452562 (88) | KF454730 (106) | KF452577 (7)  | KF454747 (387) | KF465815 (665) | (78) | (88) | (90) | (2)  |
| 27   | wBt_27       | Asia I  | KF454755 | KF452540 | 385    | KF452594 (210) | KF452565 (88) | KF454721 (106) | KF452580 (7)  | KF454751 (387) | KF465810 (163) | (78) | (88) | (90) | (23) |
| 28-1 | wBt_28-1     | Asia II | KF454768 | KF452534 | 392    | KF452596 (207) | KF452560 (88) | KF454727 (230) | KF452581 (170) | KF454734 (392) | KF465806 (669) | (2)  | (17) | (3)  | (2)  |
| 28-2 | wBt_28-2     | Asia II | KF454769 | KF456814 | –      | –      | –      | –      | –      | KF454728 (231) | –      | –    | –    | –    |
| 29-1 | wBt_29-1     | Asia II | KF454758 | KF452535 | 390    | KF452595 (207) | KF452559 (88) | KF454729 (232) | KF452582 (170) | KF454738 (9)  | KF465807 (669) | (2)  | (17) | (3)  | (2)  |
| 29-2 | wBt_29-2     | Asia II | KF454757 | KF452547 | –      | –      | –      | –      | –      | –      | –      | –    | –    | –    | –    |
| 30-1 | wBt_30-1     | Asia II | KF454759 | KF452538 | 389    | KF452600 (208) | KF452551 (88) | KF454715 (106) | KF452584 (170) | KF454735 (393) | KF465808 (669) | (2)  | (17) | (3)  | (2)  |
| 30-2 | wBt_30-2     | Asia II | KF454760 | KF452539 | –      | KF452552 (88) | KF454716 (13) | KF454736 (391) | KF465809 (669) | (2)  | (17) | (3)  | (2)  |
| 35   | wBt_35       | Asia II | KF454761 | KF452601 | 393    | KF452599 (216) | KF452553 (88) | KF454722 (106) | KF452570 (7)  | KF454741 (387) | KF465818 (163) | (78) | (88) | (90) | (23) |
| 38   | wBt_38       | Asia II | KF454767 | KF452537 | 394    | KF452589 (207) | KF452550 (88) | KF471409 (235) | KF452569 (170) | KF454740 (9)  | KF465812 (669) | (2)  | (17) | (3)  | (2)  |
| 39   | wBt_39       | Asia I  | KF454766 | KF452541 | 395    | KF452587 (207) | KF452556 (88) | KF454718 (106) | KF452568 (182) | KF454750 (387) | KF465811 (672) | (2)  | (17) | (261) | (2)  |
| 41   | wBt_41       | China I | KF454765 | KF452548 | 377    | KF452597 (207) | KF452557 (88) | KF454724 (13) | KF452572 (170) | KF454743 (9)  | KF465803 (669) | (2)  | (17) | (3)  | (2)  |
| 42   | wBt_42       | China I | KF454770 | KF452546 | 383    | KF452598 (209) | KF452554 (88) | KF454723 (13) | KF452571 (170) | KF454742 (9)  | KF465802 (669) | (2)  | (17) | (3)  | (2)  |
| 43   | wBt_43       | Asia II | KF454756 | KF452542 | 384    | KF452593 (207) | KF452566 (88) | KF454720 (13) | KF452583 (170) | KF454748 (386) | KF465813 (160) | (2)  | (17) | (3)  | (23) |
| 49-1 | wBt_49-1     | Asia II | KF454763 | KF452549 | 396    | KF452585 (207) | KF452555 (88) | KF454717 (228) | KF452574 (180) | KF454739 (386) | KF465804 (160) | (2)  | (17) | (3)  | (23) |
| 54   | wBt_54       | Australia KF454754 | KF452545 | 380    | KF452592 (207) | KF452564 (88) | KF454732 (221) | KF452579 (170) | KF454749 (9)  | KF465801 (160) | (2)  | (17) | (3)  | (23) |

ST numbers and allele profile numbers in bold represent new sequence data generated from this study.
For these gene data, 5 million generations were run; 50,000 trees were obtained, and the first 25% trees were discarded as burn-in. The resulting phylogenetic trees were visualized in TreeView (ver. 1.6.6) (Page 1996). The comparison between the phylogeny of whitefly mtCOI and Wolbachia concatenate MLST data sets was constructed with Dendroscope (ver. 3.2.8) (Huson and Scornavacca 2012). The new Wolbachia rrs, gltA, groEL, wsp, and MLST gene sequences and whitefly mtCOI gene sequences have been deposited in the GenBank database (Table 1, Fig. A3 and Table A4).

The pairwise genetic divergence of different Wolbachia supergroups was calculated using the Kimura 2-parameter method (Kimura 1980) in MEGA (ver. 5.10) (Tamura et al. 2011). Because recombination of sequences has potentially disruptive influences on phylogenetic-based molecular evolution analyses (Martin et al. 2011), alignments of individual and concatenated genes were checked for significant levels of recombination using the Phi test (Bruen et al. 2006) in SplitsTree4 under default conditions (Huson and Bryant 2006). When recombination was tested to be significant, a phylogenetic network framework was constructed based on uncorrected P distances using the Neighbor-net method (Bryant and Moulton 2004) implemented in SplitsTree4 (ver. 4.13.1) (Huson and Bryant 2006).

FISH

Localization of Portiera and Wolbachia was studied in nymphs and adults of B. tabaci Asia II 1 (Pop. 10) and Asia II 9 (Pop. 43) using fluorescence labeled probes specifically targeting the rrs genes of these bacteria. We followed the previous protocols for the FISH experiments (Bing et al. 2013b). Briefly, specimens were collected directly into Carnoy’s fixative and fixed overnight. After fixation, the samples were hybridized overnight in hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% deionized formamide) containing 10 pmol of fluorescent probes. The probe, BTP1-Cy3 (5’-Cy3- TGTCAGTGTACGCCCAGAG-3’), was used to target rrs gene of Portiera (Gottlieb et al. 2006). A new probe, Wolb-1-488 (5’- Alexa Fluor 488- TAATATTAGGCATGTACTAGCAA -3’), was designed to target rrs gene of Wolbachia. The specificity of the detection was first checked by “probe match” in RDP 10 (update to May 14, 2013) (Cole et al. 2009) and BLAST in nr database of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then confirmed using the following controls: a no probe control and Wolbachia-free whiteflies (samples of the B. tabaci MED species and MEA1 species). Stained samples were wholly mounted and viewed under a Zeiss LSM780 confocal microscope.

Results

Prevalence of Wolbachia in Bemisia species

Bemisia tabaci has a wide distribution in China. In this study, samples of Bemisia were obtained from 24 localities of 13 provinces of this country (Fig. 1). In all, these samples represent one population of B. afer and 52 populations of 9 cryptic species of the B. tabaci complex from China (Table A1). In addition, one sample of an indigenous species of the B. tabaci complex was obtained from Australia (Table A1).

Of the 1658 individuals examined, rrs PCR assays indicated that the infection rates of Wolbachia varied among species, and even among populations of a given species, ranging from 0% to 100% (Fig. 1; Table 2, Chi-square test, P < 0.0001), but did not differ between sexes in each of the populations that were tested statistically (Table A1). The incidence of Wolbachia infection in indigenous whiteflies (79.61%, n = 618) was significantly higher than that in invasive whiteflies (MEAM1 and MED, 0.96%, n = 1040; Fisher’s exact 2-tailed test, P < 0.0001). The infection rate of Wolbachia also varied among different indigenous species of the B. tabaci complex. For instance, 88.4% of China 1 whiteflies were positive for Wolbachia while so were only 2.1% of Asia II 3 whiteflies (Table 2). The rate of Wolbachia infection in B. afer was 77.5% (Table 2).

Diversity of Wolbachia infections

For those Wolbachia-positive populations, 2–3 individuals were further analyzed by sequencing Wolbachia rrs gene (592 bp) and performing Bayesian phylogenetic analysis. Most of whitefly Wolbachia rrs sequences were clustered

Table 2. Rates of Wolbachia infection in Bemisia afer and cryptic species of the B. tabaci complex.

| Whitefly species | n  | No. of localities | Infection rate (%)1 |
|-----------------|----|-----------------|-------------------|
| B. afer         | 40 | 1               | 77.5 a            |
| Asia I          | 123| 2               | 99.2 b            |
| Asia II 1       | 151| 4               | 96.7 bc           |
| Asia II 3       | 48 | 2               | 23.3 d            |
| Asia II 6       | 60 | 2               | 97.4 ab           |
| Asia II 7       | 38 | 1               | 88.6 ab           |
| China I         | 112| 3               | 88.4 ac           |
| Australia       | 11 | 1               | 100.0 ab          |
| MEAM1           | 334| 12              | 0.0 e             |
| MED             | 706| 26              | 1.4 e             |

1Figures followed by different letters differ significantly (adjusted \( P < 0.05 \), using Bonferroni corrections).
Figure 2. Phylogeny of the Wolbachia identified from Bemisia afer and cryptic species of the B. tabaci complex based on bacterial rrs gene sequences (592 sites). Wolbachia strains are characterized by the names of their host species. The tree was constructed using a TPM1uf + G substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. Sequences obtained in this study are shown in bold. The bar indicates a branch length of 0.1 substitutions/site. The sequence names and GenBank accession numbers are listed in Tables A4 and A6.

Identification of Wolbachia supergroup O

Further Bayesian phylogenetic analysis on a nearly complete rrs sequence (1317 bp) of the strange Wolbachia (wBt_10) revealed that this Wolbachia differed widely from other known Wolbachia (Fig. A5). The divergence of rrs between wBt_10 and the supergroup M is 2.52%, which is the smallest of that between wBt_10 and all previously reported Wolbachia supergroups (A to N) (Table A6). In view of these apparent differences, we proposed to name these Wolbachia as supergroup O temporarily.

Our results thus showed the presence of two genetically distant Wolbachia supergroups in whiteflies. To clarify the detailed infection prevalence of Wolbachia O in Bemisia whiteflies, PCR-RFLP was introduced to digest all positive rrs PCR products by the restriction enzymes VspI. No VspI restriction site was found in rrs sequences of Wolbachia supergroup O, whereas rrs amplicons from Wolbachia supergroup B could be digested into multiple bands by VspI (Fig. A7). Whiteflies in populations 2 (MED species) and 10 (Asia II 1 species) were infected singly by Wolbachia O, whereas populations 28, 29, and 30 (all three are Asia II 1 species) were infected by both Wolbachia O and Wolbachia B (Table 3).

At least one of the eight tested protein-coding genes (gltA, groEL, MLST (gatB, coxA, hcpA, fbpA, fssZ), and wsp genes) was successfully amplified and sequenced for all Wolbachia-infected populations in this study. Both neighbor-net analysis of fbpA, gltA, hcpA gene and Bayesian interference of groEL gene supported the existence of the Wolbachia supergroup O (wBt_10) (Figs. 3 and 4, and Figs. A10 and A12). However, it should be noted that the results of phylogenetic analyses with different genes were not always consistent. In particular, analysis of fbpA, groEL, and hcpA gene clustered some strange Wolbachia, such as wBt_29-2 and wBt_30-2, which were identified as
Sixteen STs were identified in whiteflies from this study, and all of them are new to the MLST database (Table 1). Though efforts were made, some PCRs failed when amplifying the MLST and wsp genes from supergroup O-infected whiteflies (Table 1). As a result, sequences from supergroup O-infected whiteflies were excluded from phylogenetic analysis of the concatenated MLST sequences. Respective Bayesian interference of separate gatB, coxA, ftsZ, and wsp genes showed that all Wolbachia detected in whiteflies belonged to supergroup B (Fig. 5, and Figs. A8, A9 and A11). Neighbor-net analysis clustered the majority of Wolbachia into supergroup B.

Table 3. Infection frequencies of the Wolbachia O in five populations of the Bemisia tabaci complex.

| Pop. no. | Cryptic species | n\(^1\) | % without Wolbachia infection | Single infection (%) |
|----------|----------------|--------|-----------------------------|---------------------|
| 2        | MED            | 29     | 93.1                        | 6.9                 |
| 10       | Asia II 1      | 44     |                             | 100                 |
| 28       | Asia II 1      | 43     | 6.9                         | 14.0                |
| 29       | Asia II 1      | 35     | 2.9                         | 5.7                 |
| 30       | Asia II 1      | 29     | 3.4                         | 24.1                |

\(^1\)Number of whitefly individuals collected from the five populations shown with asterisks in Fig. 1 and Table A1.

Figure 3. Phylogenetic position of the Wolbachia identified from the putative species Asia II 1 of the Bemisia tabaci complex based on bacterial gatB gene sequences (636 sites) using the Neighbor-net method. Each edge (or a set of parallel edges) corresponds to a split in the data set and has a length equal to the weight of the split. The sequence obtained in this study is shown in bold. The bar indicates a branch length of 0.1 substitutions/site. The names and sequence GenBank accession numbers are listed in Table A4.

Figure 4. Phylogenetic position of the Wolbachia identified from Bemisia afer and B. tabaci putative species based on bacterial groEL gene sequences (491 sites). Wolbachia strains are characterized by the names of their host species. The tree was constructed using a GTR + G substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. The sequence obtained in this study is shown in bold. The bar indicates a branch length of 0.1 substitutions/site. The names and sequence GenBank accession numbers are listed in Table 1 and Table A4.
except for wBt_10 (Figs. A10 and A12). The hcpA genes from wBt_10 and fbpA from wBt_10 and wBt_28-2 formed a separate branch that differs distinctly from all known reference sequences.

**Co-divergence between the divergence of Wolbachia supergroup B and whitefly species**

The codivergence of *Bemisia* and Wolbachia supergroup B was assessed by studying the sequences of partial mtCOI gene and Wolbachia MLST genes. For those Wolbachia identified from *B. tabaci*, very poor congruence was found between the phylogenies of mtCOI and concatenated MLST genes (Fig. 6). The topology of MLST tree differs obviously from that of mtCOI. Whiteflies belonging to the same cryptic species harbored distant Wolbachia strains. For instance, two populations (wBt_35 and wBt_38) of *Wolbachia* identified from Asia II 6 are clustered in different phylogenetic groups.

**Localization of Wolbachia in Bemisia tabaci**

The FISH of bacteria revealed that *Portiera* was seen exclusively in the bacteriocytes of whiteflies. In the tested nymphs, *Wolbachia* was strictly located in the bacteriocytes among the abundant *Portiera* (Fig. 7). Nevertheless, in the adults, *Wolbachia* was detected both outside and inside the bacteriocytes (Fig. 7). Signals of *Wolbachia* shown at the anterior pole of the oocytes of female adults indicate its vertical transmission (arrows marked in Fig. 7 D & H).

**Discussion**

*Wolbachia* is widely distributed among invertebrates and is considered as the most prevalent symbiont identified so...
far. Though several research groups have investigated the prevalence of Wolbachia in some cryptic species of the B. tabaci complex, our study represents the first comprehensive analysis of Wolbachia infection among both invasive and indigenous cryptic species of the B. tabaci complex in Asia. In addition, compared with previous investigations, we used five more molecular markers in our analyses.

**Prevalence of Wolbachia varies between invasive and indigenous whiteflies**

In this study, Wolbachia infection rates in five (Asia I, Asia II 1, Asia II 7, Asia II 9, and China I) of the seven Chinese indigenous species reached over 70%. In contrast, Wolbachia infection rate in the MED populations from China was only 1.4% (10/706), and no infection (0/334) was detected in all MEAM1 populations from this country. The low rates of Wolbachia infection in MEAM1 and MED agree with those observed in most previous studies. For example, in populations of MEAM1 and MED from Europe and Western Africa, infection rates of Wolbachia varied from 0–8.3% and 0–33% (Nirgianaki et al. 2003; Chiel et al. 2007; Gueguen et al. 2010; Skaljac et al. 2010; Chu et al. 2011; Thierry et al. 2011; GnankinÉ et al. 2013). And in populations of MEAM1 and MED from China, the rates of Wolbachia infection were 0.2% (1/456) and 0% (0/1149), respectively (Pan et al. 2012). As a whole, our data indicate a high variability of prevalence of Wolbachia between cryptic species of the B. tabaci complex. In our sampling, we obtained adequate numbers of whitefly individuals for five (Asia II 1, Asia II 6, China 1, MEAM1, and MED) of the 11 whitefly species from both laboratory and field. The data indicate that the frequencies of Wolbachia infection between laboratory and field populations in each of the five species appeared similar (Table A1). Thus, the laboratory rearing seemed to have exerted little effects on the frequencies of Wolbachia infection in these whitefly species. Until now, factors underlying the high variability of Wolbachia infection between the whitefly species are virtually unknown but certainly warrant future investigations.

In contrast to a previous study that reports absence of Wolbachia infection in B. afer populations from China (Chu et al. 2010), the rate of Wolbachia infection in the B. afer population examined in the current study reached 77.5%. Phylogenetic analysis of rrs, groEL, MLST, and wsp genes showed that the Wolbachia detected from B. afer belongs to supergroup B, which agrees with the report of Nirgianaki et al. (2003).

**Identification of a novel Wolbachia supergroup O**

Preliminary Bayesian phylogenetic analysis based on rrs gene sequences strongly supports the existence of one strange monophyletic group compared with the other Wolbachia identified in whiteflies. The rrs sequences from five of the whitefly populations (wBt_2, wBt_10, wBt_28-2, wBt_29-2, and wBt_30-2) were clustered into group O. Average distance among those strange rrs sequences (592 bp) are 0.48%. The divergence of rrs between wBt_10 and all previously described Wolbachia superfamilies (A to N) is higher than the 2% distance, a level of divergence that may merit the establishment of a new supergroup (Stouthamer et al. 1993; Augustinos et al. 2011). What is more, independent Bayesian analysis of rrs and groEL gene sequences and Neighbor-net analysis of gltA, hcpA, and fbpA gene sequences confirmed the distinct phylogenetic position of wBt_10 from the other superfamilies. Based on the evidence, we propose the strange Wolbachia group as a new supergroup – Supergroup O.
Figure 7. Whole-mount FISH of *Bemisia tabaci* nymphs and female adults using a *Portiera*-specific probe (red) and a *Wolbachia*-specific probe (green). Upper column, Asia II 1 nymph and female adult; lower column, Asia II 9 nymph and female adult. A, C, E, G: *Wolbachia* channel on a dark-field channel. B, D, F, H: Overlay of *Portiera* and *Wolbachia* channels on a bright-field channel. White triangles in D and H indicate anterior poles of the oocytes. Signals on legs, joints, and wings are chitin autofluorescence.
All previously known Wolbachia in Bemisia tabaci belong to supergroup B

Except for the five supergroup O strains, phylogenetic analysis of eight molecular markers (rrs, groEL, gatB, coxA, hcpA, fbpA, ftsZ, and wsp genes) showed that all the Wolbachia strains detected from Chinese whiteflies as well as one strain from the Australia species belong to supergroup B. This is consistent with previous diversity studies on Bemisia and Trialeurodes whiteflies (Nirgianaki et al. 2003; Sintupachee et al. 2006; Gueguen et al. 2010; Singh et al. 2012; Tsagkarakou et al. 2012).

The protein-coding genes are limited in Wolbachia diversity investigation

At the early stage of Wolbachia research, the identification of Wolbachia strains was inferred based on the rrs gene (O’Neill et al. 1992; Stouthamer et al. 1993; Dumler and Walker 2005). As the research progressed, the rrs gene was found too conserved for further analysis of the Wolbachia genus. Subsequently, additional protein-coding genes (gltA, groEL, ftsZ, and wsp genes) were developed for infection and evolutionary analysis of Wolbachia (Werren et al. 1995b; Zhou et al. 1998; Lo et al. 2002, 2007; Casiraghi et al. 2005). Baldo et al. (2006) developed a standard MLST-based system (gatB, coxA, hcpA, ftsZ, and fbpA) for genotyping and strain classification of Wolbachia infections. However, with more exploration of Wolbachia diversity, conflict results occurred among these different markers (Augustinos et al. 2011). In this study, the presence of supergroup O was confirmed by rrs and four protein-coding genes (fbpA, gltA, groEL, and hcp genes) (Figs. 2–4, and Figs. A10 and A12). Whereas phylogenetic analysis of several protein-coding genes (coxA, groEL, gatB, ftsZ, and wsp) clustered many Wolbachia O strains into supergroup B (Figs. 4 and 5, and Figs. A9, A8 and A11). Similar phenomena have been noticed in previous studies. For example, even though the supergroup M and N have been identified as new groups of Wolbachia by rrs gene clustering, Augustinos et al. (2011) found that several popular protein-coding sequences such as gltA, groEL, and MLST genes clustered some individuals of those new groups into the old supergroup B. Besides, failures of amplifying MLST and wsp genes in many Wolbachia O-infected whiteflies (Table 1) indicated protein-coding genes may not be sufficient for investigating the diversity of Wolbachia in B. tabaci. The failure of amplification of ftsZ and wsp genes were also observed in Wolbachia-infected aphids (Augustinos et al. 2011). Consequently, it seems clear that phylogenetic analysis merely using protein-coding genes may underestimate the diversity of Wolbachia.

That inadequacy of protein-coding genes for analyzing the diversity of Wolbachia may be explained by: (1) primers of protein-coding genes are designed based on the earliest known Wolbachia (mostly A and B); and (2) different protein-coding genes suffer different selective pressure and thus have different evolutionary patterns. The rrs gene sequence is more conserved than wsp gene and the results of amplification are more stable compared with that of wsp or ftsZ genes which often produces unexpected bands (not target-size bands or not the gene of Wolbachia). In fact, no single pair of primers can ensure detection of all Wolbachia specifically among various samples (SimÖEs et al. 2011). In view of the limitation of the various primers, we suggest that infection data obtained by any of these genes should be confirmed by vector cloning and sequencing of all representative bands.

Wolbachia in Bemisia tabaci are transmitted horizontally

Our FISH data indicate that Wolbachia can be vertically transmitted in whiteflies (Fig. 7), a result in agreement with that of a previous report (Gottlieb et al. 2008). In addition, our FISH show the distribution of Wolbachia outside of bacteriocytes of the whitefly adults and thus also indicate potential horizontal transmission of Wolbachia. Not surprisingly, incongruence was found between the phylogeny of Bemisia mtCOI sequences and that of Wolbachia supergroup B based on concatenated MLST sequences (Fig. 6). In addition, in several cases, a population of a given whitefly species harbored divergent Wolbachia strains (e.g., Population of 28 in Table A1). As speculated by a rate of rrs gene divergence of 1–2% per 50 million years in bacterial endosymbionts (Moran et al. 1993; Ochman et al. 1999), the divergence between supergroup B and supergroup O probably started more than 120 million years ago. While the divergence date of B. tabaci complex was speculated to start about 50 million years ago, much more recent than that of supergroup B and O Wolbachia (Boykin et al. 2013). The double infection of Wolbachia supergroups B and O in the same population indicates horizontal transmission of Wolbachia. Horizontal transmission of Wolbachia has often been speculated based on phylogenetic analysis (Werren et al. 1995a; Sintupachee et al. 2006; Stahlhut et al. 2010; Schuler et al. 2013; Zhang et al. 2013). Wolbachia has also been reported from other whitefly genera such as Trialeurodes and some parasitoids (Raychoudhury et al. 2009; Cass et al. 2014), and this diversity of distribution may also hint horizontal transmission. Sintupachee et al. (2006) hypothesized that the horizontal transmission of Wolbachia from whiteflies to other arthropods may occur through plants, because whiteflies could feed on plants without ruining plant cells.
Carsi-Fluger et al. (2011) presented a case study of horizontal transmission of \textit{Rickettsia} in whiteflies via plants. Though we are yet unable to speculate on the origin of \textit{Wolbachia} in whiteflies, we suggest that horizontal transmission of \textit{Wolbachia} in whiteflies via plants warrants investigation especially as this bacterium has been detected outside of the bacteriocytes in the insect hosts.

\textbf{Conclusion}

We conducted a comprehensive screening for \textit{Wolbachia} in whiteflies, and the findings have broadened substantially the host spectrum of \textit{Wolbachia} and revealed a new supergroup of \textit{Wolbachia} in whiteflies. Our study also shows the limitations of protein-coding genes as molecular markers for \textit{Wolbachia} investigation. Both specific and efficient molecular markers are needed for intensive surveys of \textit{Wolbachia}. \textit{Wolbachia} are transmitted vertically and horizontally in whiteflies. Clarifying the \textit{Wolbachia} strains of whiteflies and their biological functions may provide novel clues for the development of efficient control technologies against invasive whiteflies and whitefly-transmitted plant viruses.

\textbf{Acknowledgments}

The study was supported by the National Natural Science Foundation of China (Project number 31321063 and 31390421). We thank Qiong Rao and Yun-Lin Su for comments on the manuscript, Qi-Yi Tang for help in doing the statistical analysis, Amanda Avery for assistance in submitting \textit{Wolbachia} sequences to the \textit{Wolbachia} MLST Databases, and Paul De Barro for providing whitefly samples of the “Australia” species.

\textbf{Conflict of Interest}

None declared.

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### Appendix

#### Table A1. Details of screen of *Bemisia afer* and *B. tabaci* cryptic species for *Wolbachia*. Rates of infections do not differ between sexes in each of the populations analyzed with Fisher’s exact test (Populations with fewer than 10 individuals were excluded from the analysis).

| No. | Whitewolf species | Location | Latitude | Longitude | Collection date | Host plant (family) | Sample size | Infection rate (%)<sup>2</sup> |
|-----|-------------------|----------|----------|-----------|-----------------|---------------------|-------------|-------------------------------|
|     |                   |          |          |           |                 |                     | Females | Males | Overall |
| 1   | *Bemisia afer*    | Linyi, Shandong, China | 35°47’N | 118°37’E | July 2012 | *Broussonetia papyrifera* (1) | 24 | 16 | – | 87.50 | 62.50 | 77.50 |
| 2<sup>3</sup> | *B. tabaci* | Hefei, Anhui, China | 31°95’N | 117°48’E | October 2009 | *Solanum melongena* (2) | 21 | 8 | – | 9.52 | 0.00 | 6.90 |
| 3   |                   | Hefei, Anhui, China | 31°95’N | 117°48’E | October 2009 | *Solanum lycopersicum* (2) | 17 | 13 | – | 0.00 | 0.00 | 0.00 |
| 4   |                   | Nanjing, Jiangsu, China | 31°92’N | 117°14’E | October 2009 | *Salvia splendens* (3) | 21 | 7 | – | 0.00 | 0.00 | 0.00 |
| 5   |                   | Nanjing, Jiangsu, China | 32°02’N | 118°54’E | September 2009 | *Solanum melongena* (2) | 25 | 5 | – | 0.00 | 0.00 | 0.00 |
| 6   |                   | Nanjing, Jiangsu, China | 32°02’N | 118°54’E | September 2009 | *Brassica oleracea* var. capitata (4) | 15 | 15 | – | 0.00 | 0.00 | 0.00 |
| 7   | *MEAM1* | Chongmingdao, Shanghai, China | 31°50’N | 121°80’E | November 2009 | *Brassica oleracea* var. capitata (4) | 16 | 11 | – | 0.00 | 0.00 | 0.00 |
| 8   | *MED* | Chongmingdao, Shanghai, China | 31°50’N | 121°80’E | November 2009 | *Capsicum annuum* (2) | 21 | 10 | – | 0.00 | 0.00 | 0.00 |
| 9<sup>4</sup> | *Asia II 3* | Hangzhou, Zhejiang, China | 30°23’N | 120°18’E | April 2009 | *Glycine max* (5) | 26 | 21 | – | 0.00 | 0.00 | 0.00 |
| 10<sup>1,4</sup> | *Asia II 1* | Hangzhou, Zhejiang, China | 29°27’N | 119°18’E | October 2010 | *Gossypium hirsutum* (6) | 29 | 15 | – | 100.00 | 100.00 | 100.00 |
| 11<sup>4</sup> | *China 1* | Hangzhou, Zhejiang, China | 30°23’N | 120°18’E | November 2009 | *Solanum lycopersicum* (2) | 23 | 29 | – | 100.00 | 82.76 | 90.38 |
| 12<sup>4</sup> | *MED* | Ningbo, Zhejiang, China | 29°48’N | 121°35’E | June 2009 | *Capsicum annuum* (2) | 22 | 16 | – | 0.00 | 0.00 | 0.00 |
| 13 | *MED* | Taizhou, Zhejiang, China | 28°30’N | 121°34’E | October 2012 | *Cucurbita moschata* (7) | 22 | 11 | – | 31.82 | 9.09 | 24.24 |
| 14<sup>4</sup> | *MEAM1* | Wenzhou, Zhejiang, China | 27°47’N | 120°39’E | September 2008 | *Solanum melongena* (2) | 20 | 20 | – | 0.00 | 0.00 | 0.00 |
| 15 | *MED* | Nanchang, Jiangxi, China | 28°72’N | 115°91’E | October 2009 | *Cucurbita moschata* (7) | 16 | 11 | – | 0.00 | 0.00 | 0.00 |
| 16 | *MED* | Nanchang, Jiangxi, China | 28°72’N | 115°91’E | October 2009 | *Ipomoea batatas* (8) | 16 | 14 | – | 0.00 | 0.00 | 0.00 |
| 17 | *MED* | Nanchang, Jiangxi, China | 28°72’N | 115°91’E | October 2009 | *Brassica campestris ssp. Pekinensis* (4) | 21 | 8 | – | 0.00 | 0.00 | 0.00 |
| No. | Whitefly species | Location | Latitude | Longitude | Collection date | Host plant (family)¹ | Sample size | Infection rate (%)² |
|-----|-----------------|----------|----------|-----------|----------------|----------------------|-------------|---------------------|
|     |                 |          |          |           |                |                      | Females     | Males               | Overall            |
| 18  | MED             | Nanchang, Jiangxi, China | 28°72’N 115°91’E | October 2009 | *Humulus scandens* (9) | 16 12 – | – | 0.00 0.00 0.00 |
| 19  | MED             | Nanchang, Jiangxi, China | 28°29’N 116°01’E | October 2009 | *Citrus lanatus* (7) | 13 16 – | – | 0.00 0.00 0.00 |
| 20  | MED             | Nanchang, Jiangxi, China | 28°29’N 116°01’E | October 2009 | *Ipomoea batatas* (8) | 20 7 – | – | 0.00 0.00 0.00 |
| 21  | MED             | Jiujiang, Jiangxi, China | 29°76’N 115°79’E | October 2009 | *Capsicum annum* (2) | 16 11 – | – | 0.00 0.00 0.00 |
| 22  | MED             | Jiujiang, Jiangxi, China | 29°76’N 115°79’E | October 2009 | *Ipomoea batatas* (8) | 24 6 – | – | 0.00 0.00 0.00 |
| 23  | MED             | Jiujiang, Jiangxi, China | 29°76’N 115°79’E | October 2009 | *Cucumis sativus* (7) | 20 8 – | – | 0.00 0.00 0.00 |
| 24  | MED             | Jiujiang, Jiangxi, China | 29°76’N 115°79’E | October 2009 | *Solanum melongena* (2) | 13 2 – | – | 0.00 0.00 0.00 |
| 25  | MED             | Jiujiang, Jiangxi, China | 29°76’N 115°79’E | October 2009 | *Phaseolus vulgaris* (5) | 16 11 – | – | 0.00 0.00 0.00 |
| 26  | Asia II 7      | Guangzhou, Guangdong, China | 23°09’N 113°21’E | October 2007 | *Gossypium hirsutum* (6) | 30 8 – | 100.00 | 87.50 97.37 |
| 27  | Asia I          | Zhaoqing, Guangdong, China | 23°56’N 112°1’E | November 2010 | *Ipomoea batatas* (8) | 20 20 – | 100.00 100.00 100.00 |
| 28  | Asia II 1       | Zhaoqing, Guangdong, China | 23°56’N 112°1’E | August 2012 | *Arachis hypogaea* (5) | 31 12 – | 96.77 | 83.33 93.02 |
| 29  | Asia II 1       | Zhaoqing, Guangdong, China | 23°56’N 112°1’E | August 2012 | *Ipomoea batatas* (8) | 32 3 – | 96.88 | 100.00 97.14 |
| 30  | Asia II 1       | Sanya, Hainan, China | 18°24’N 109°42’E | August 2009 | *Ipomoea batatas* (8) | 24 5 – | 100.00 | 80.00 96.55 |
| 31  | MEAM1           | Nanning, Guangxi, China | 22°38’N 108°23’E | August 2009 | *Vigna unguiculata* (5) | 17 13 – | – | 0.00 0.00 0.00 |
| 32  | MEAM1           | Nanning, Guangxi, China | 22°38’N 108°23’E | August 2009 | *Gossypium hirsutum* (6) | 22 0 – | – | 0.00 0.00 0.00 |
| 33  | MEAM1           | Nanning, Guangxi, China | 22°38’N 108°23’E | August 2009 | *Cucumis sativus* (7) | 17 13 – | – | 0.00 0.00 0.00 |
| 34  | MED             | Beihai, Guangxi, China | 21°29’N 109°09’E | August 2009 | *Ipomoea batatas* (8) | 10 12 – | – | 0.00 0.00 0.00 |
| 35  | Asia II 6       | Baise, Guangxi, China | 22°94’N 108°54’E | August 2009 | *Luffa cylindrica* (7) | 15 3 – | 53.33 | 0.00 44.44 |
| 36  | MEAM1           | Baise, Guangxi, China | 23°52’N 106°37’E | August 2009 | *Vigna unguiculata* (5) | 4 9 – | – | 0.00 0.00 0.00 |
| 37  | MEAM1           | Baise, Guangxi, China | 23°45’N 106°47’E | August 2009 | *Benincasa hispida* (7) | 15 12 – | – | 0.00 0.00 0.00 |
| 38  | Asia II 6       | Baise, Guangxi, China | 22°94’N 108°54’E | November 2011 | *Ipomoea batatas* (8) | 36 6 – | 13.89 | 16.67 14.29 |
| 39  | Asia I          | Honghe, Yunnan, China | 24°38’N 103°46’E | November 2011 | *Ipomoea batatas* (8) | 46 37 – | 97.83 | 100.00 98.80 |
| 40  | MED             | Guiyang, Guizhou, China | 26°40’N 106°67’E | July 2009 | *Glechoma longituba* (3) | 9 21 – | – | 0.00 0.00 0.00 |
| 41  | China 1         | Zunyi, Guizhou, China | 27°39’N 107°70’E | July 2009 | *Solanum melongena* (2) | 24 6 – | 75.00 | 83.33 76.67 |
| 42  | China 1         | Zunyi, Guizhou, China | 27°39’N 107°70’E | July 2009 | *Ipomoea batatas* (8) | 23 7 – | 100.00 | 85.71 96.67 |
**Table A1.** Continued.

| No. | Whitefly species | Location | Latitude | Longitude | Collection date | Host plant (family) | Sample size | Infection rate (%) |
|-----|------------------|----------|----------|-----------|-----------------|---------------------|-------------|-------------------|
| 43[^4] | Asia II 9 | Shaoyang, Hunan, China | 26°59’N 111°16’E | October 2011 | (8) | 19  | 87.50 | 88.57 |
| 44 | MED | Jishou, Hunan, China | 28°18’N 109°38’E | September 2009 | Ipomoea batatas | 7  | 0.00 | 0.00 |
| 45 | MED | Luoyang, Henan, China | 34°46’N 112°46’E | September 2009 | Ipomoea batatas | 16  | 0.00 | 0.00 |
| 46 | MEAM1 | Luoyang, Henan, China | 34°66’N 112°51’E | September 2009 | Gossypium hirsutum | 15  | 0.00 | 0.00 |
| 47 | MEAM1 | Luoyang, Henan, China | 34°59’N 112°58’E | September 2009 | Solanum melongena | 20  | 0.00 | 0.00 |
| 48 | MEAM1 | Luoyang, Henan, China | 34°59’N 112°58’E | September 2009 | Cucumis sativus | 26  | 0.00 | 0.00 |
| 49-1 | Asia II 3 | Zhengzhou, Henan, China | 34°78’N 113°66’E | September 2009 | Solanum melongena | 0  | 100.00 | 100.00 |
| 49-2 | MED | Zhengzhou, Henan, China | 34°78’N 113°66’E | September 2009 | Solanum melongena | 18  | 0.00 | 0.00 |
| 50 | MEAM1 | Zhengzhou, Henan, China | 34°78’N 113°66’E | September 2009 | Cucumis sativus | 27  | 0.00 | 0.00 |
| 51 | MED | Xinxiang, Henan, China | 35°47’N 113°75’E | September 2009 | Phaseolus vulgaris | 12  | 0.00 | 0.00 |
| 52 | MED | Xinxiang, Henan, China | 35°47’N 113°75’E | September 2009 | Raphanus sativus | 26  | 0.00 | 0.00 |
| 53 | MED | Xinxiang, Henan, China | 35°47’N 113°75’E | September 2009 | Brassica campestris ssp. Pekinensis | 20  | 0.00 | 0.00 |
| 54 | Australia | Bundaberg, Queensland, Australia | 24°48’S 152°27’E | – | Euphorbia cyathophora | –  | 11  | 100.00 |

F, female adult; M, male adult; UN, unknown sex; –, not ascertained.

[^4]: In all 22 species of host plants from 10 families, figures in parentheses indicate the names of the families: (1), Moraceae; (2), Solanaceae, (3), Lamiaceae, (4), Cruciferae, (5), Fabaceae, (6), Malvaceae, (7), Cucurbitaceae, (8), Convolvulaceae, (9), Cannabaceae, (10), Euphorbiaceae.

[^2]: Infection rates of *Wolbachia* detected by diagnostic PCR of *rrs* gene.

[^3]: Populations for the detection of *Wolbachia* supergroup O (Table 3).

[^4]: Populations maintained in the laboratory on cotton since collection.

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**Table A2.** List of the primers used for screening and sequencing.

| Gene | Hypothetical product | Primer name | Primer sequences (5’-3’) | Tm | Product size | Reference |
|------|----------------------|-------------|-------------------------|----|--------------|-----------|
| *Bemisia* spp. | mtCOI Mitochondrial cytochrome oxidase subunit I | COI-F-C1-I-2195: COI-R-TL2-N-3014: | TTGATTTTTTGTCTCCACAGAATATCCACGACATCGCTCCATGTTA | 54°C | 759 bp | Frohlich et al. (1999) |
| Universal bacteria | *rrs* Ribosomal RNA 16S | 27F: | AGAGTTTGATCMTTGTGCTCACAG | 50°C | 1417 bp | Weisburg et al. (1991) |
| *Wolbachia* spp. | *rrs* Ribosomal RNA 16S | Wol-16S-F: | CCGGGGAAAAAATTAGTATTACGTGTTAAACAAAGAGAGAAGAAAGACAAAGAAACACG | 55°C | 589 bp | Heddi et al. (1999) |
| | | Wol-16S-R: | AGCTGTTAACTACGAAAGAAAGAGAAGAAAGACAAAGAAACACG | 54°C | 471 bp | Baldo et al. (2006) |
| | | gatB | TGGYAAATCGGGAAYAAGATGA | 54°C | 487 bp | Baldo et al. (2006) |
| | | coxA | TTGGRGCRAYAATCTTATAGCTAAAGCTTCTATGGGAGCTGTCGAATGGTAAATCAG | 54°C | 515 bp | Baldo et al. (2006) |
| | Conserved hypothetical protein | hcpA | GAAATACAGTGTTGCAGAAGTAATGCAGATTAGTATTACGTGTTAAACAAAGAGAGAAGAAAGACAAAGAAACACG | 54°C | 515 bp | Baldo et al. (2006) |
Table A2. Continued.

| Gene | Hypothetical product | Primer name | Primer sequences (5'-3') | Tm | Product size | Reference |
|------|----------------------|-------------|--------------------------|----|--------------|-----------|
| *ftsZ* | Cell division protein | *ftsZ*_F1: | ATYATGGARCATATAAARGATAG | 54°C | 524 bp | Baldo et al. (2006) |
|       |                      | *ftsZ*_R1: | TCRAYAGATGGATAGATGATAG |    |              |           |
| *fbpA* | Fructose-bisphosphate aldolase | *fbpA*_F1: | GCTGCACCTGGTGGYWGTAT | 59°C | 509 bp | Baldo et al. (2006) |
|       |                      | *fbpA*_R1: | CRRCCAGARAAAAYACTATTG |    |              |           |
| *wsp* | Outer surface protein | *wsp*_F1: | GTCGCAATATGATGAARAAAG | 59°C | 546 bp | Baldo et al. (2006) |
|       |                      | *wsp*_R1: | CAYGACCAAYAYRTRTAAAC |    |              |           |
| *groEL* | Chaperonin GroEL | *groEL*_F1: | CAACRGRGRRYAACTGCDDG | 54°C | 491 bp | Ros et al. (2009) |
|       |                      | *groEL*_R1: | GATADCCGRTCTAAAYGTC |    |              |           |
| *gibA* | Citrate synthase | *gibA*_F1: | TACGATCCAGGGTTGTTTCTAC | 54°C | 659 bp | Casiraghi et al. (2005) |
|       |                      | *gibA*_R1: | CATTTCATTACACTGGGC |    |              |           |

Figure A3. Phylogenetic analysis of the *Bemisia* spp. based on whitefly mtCOI gene sequences (657 sites). *Trialeurodes vaporariorum* is used as out group. Reference sequences are obtained from the CSIRO data access portal (De Barro and Boykin 2013). The tree was constructed using a TIM3 + I + G substitution model for Bayesian analysis. Bayesian posterior probabilities are indicated at nodes. The sequences obtained in this study are shown in bold. The bar indicates a branch length of 0.1 substitutions/site. The sequence GenBank accession numbers are shown in parentheses.
Table A4. Taxonomic details of *Wolbachia* hosts and the GenBank accession numbers of sequences included in the analysis.

| Phylum         | Class          | Order          | Host species               | 16S rRNA gene | gltA   | groEL | Supergroup |
|----------------|----------------|----------------|----------------------------|----------------|--------|--------|------------|
| Arthropoda     | Insecta        | Hymenoptera    | Muscidifurax uniraptor     | L02882         | –      | –      | A          |
| Arthropoda     | Insecta        | Hymenoptera    | Nasonia vitripennis        | M84688         | AY714795 | –      | A          |
| Arthropoda     | Prostigmata    | Acarina        | Bryobia sarothamnini       | EU499315       | –      | EU499330| B          |
| Arthropoda     | Prostigmata    | Acarina        | Bryobia praetiosa          | EU499317       | EU499327 | EU499332| B          |
| Arthropoda     | Insecta        | Hymenoptera    | Nasonia vitripennis        | M84686         | AY714782 | AY714796| B          |
| Arthropoda     | Insecta        | Hemiptera      | Bemisia tabaci             | JN204507       | –      | –      | B          |
| Nematoda       | Secernentea    | Spirurida      | Onchocerca ochengi          | AJ010276       | AJ609640 | –      | C          |
| Nematoda       | Secernentea    | Spirurida      | Onchocerca gibson          | AJ276499       | AJ609639 | AJ609652| C          |
| Nematoda       | Secernentea    | Spirurida      | Dirofilaria repens         | AJ276500       | –      | AJ609653| C          |
| Nematoda       | Secernentea    | Spirurida      | Dirofilaria immitis        | Z49261         | A       | –      | C          |
| Nematoda       | Chromadorea    | Spirurida      | Brugia malayi              | AF051145       | AJ609643 | AE017321| D          |
| Nematoda       | Secernentea    | Spirurida      | Litomosoides sigmodontis   | AF069068       | A       | –      | D          |
| Nematoda       | Secernentea    | Spirurida      | Litomosoides sigmodontis   | AF069065       | A       | –      | D          |
| Arthropoda     | Collembola     | Collembola     | Folsomia candida           | AJ422184       | –      | –      | E          |
| Arthropoda     | Ellipura       | Collembola     | Mesaphorura macrocheta     | –              | –      | –      | E          |
| Arthropoda     | Insecta        | Neuroptera     | Myrmeleon mobilis          | DQ068882       | –      | –      | F          |
| Arthropoda     | Insecta        | Isoptera       | Kalotermes flavicollis     | Y11377         | AJ609651 | AJ609660| F          |
| Nematoda       | Secernentea    | Spirurida      | Mansoniella ozzardi        | AJ279034       | –      | AJ609657| F          |
| Arthropoda     | Insecta        | Isoptera       | Zootermopsis nevadensis    | AY764280       | AY764282 | AY764277| H          |
| Arthropoda     | Insecta        | Siphonaptera   | Ctenocephalides felis      | AY335923       | A       | –      | I          |
| Arthropoda     | Insecta        | Siphonaptera   | Orchopeas leucopus         | AY335924       | –      | –      | I          |
| Nematoda       | Secernentea    | Spirurida      | Dipetalonema gracile       | AJ548802       | A       | –      | J          |
| Arthropoda     | Arachnida      | Prosphormia    | Bryobia sp.                | EU499316       | EU499326 | EU499331| K          |
| Nematoda       | Phasmida       | Tylennichida   | Radopholus similis         | EU833482       | –      | EU834384| L          |
| Arthropoda     | Insecta        | Hemiptera      | Tuberochlanus salignu      | JN384085       | –      | –      | M          |
| Arthropoda     | Insecta        | Hemiptera      | Aphis sp.                  | JN384091       | –      | –      | M          |
| Arthropoda     | Insecta        | Hemiptera      | Cinara cedri               | –              | –      | JN384053| M          |
| Arthropoda     | Insecta        | Hemiptera      | Toxoptera aurantii         | JN384094       | –      | –      | N          |
| Arthropoda     | Insecta        | Hemiptera      | Toxoptera aurantii         | JN384095       | –      | –      | N          |
| Arthropoda     | Insecta        | Hemiptera      | Bemisia tabaci             | KF454771       | KF587270 | KF452543| O          |

Figure A5. Phylogenetic position of the *Wolbachia* identified from *Bemisia tabaci* putative species Asia II 1 (wBt_10) based on bacterial *rrs* gene sequences (1317 sites). Wolbachia strains are characterized by the names of their host species. The tree was constructed using a HKY + G substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. The sequence obtained in this study is shown in bold. The bar indicates a branch length of 0.01 substitutions/site. The names and sequence GenBank accession numbers are listed in Tables A4 and A6.
Table A6. Divergence between the Wolbachia detected in the putative species Asia II 1 of the B. tabaci complex (wBt_10) and other supergroups based on 16S rRNA gene sequences.

| Host species                  | Supergroup | Divergence (%) | GenBank accession no. |
|-------------------------------|------------|----------------|-----------------------|
| Muscidifurax uniraptor       | A          | 3.73           | L02882                |
| Nasonia vitripennis          | A          | 3.64           | M84688                |
| Bryobia sarothamni           | B          | 4.98           | EU499315              |
| Bryobia praeliosa            | B          | 4.98           | EU499317              |
| Bemisia tabaci               | B          | 5.18           | JN204507              |
| Nasonia vitripennis          | B          | 4.83           | M84686                |
| Onchocerca ochengi           | C          | 5.61           | AJ010276              |
| Onchocerca gibsoni           | C          | 5.44           | AJ276499              |
| Dirofilaria repens           | C          | 5.99           | AJ276500              |
| Dirofilaria immittis         | C          | 5.63           | Z49261                |
| Brugia malayi                | D          | 4.59           | AF051145              |
| Litomosoides sigmodontis     | D          | 5.15           | AF069068              |
| Folsomia candida             | E          | 3.65           | AF179630              |
| Mesaphorura macrocherta      | E          | 4.04           | AJ422184              |
| Mansonella ozzardi           | F          | 4.95           | AJ279034              |
| Myrmeleon mobilis            | F          | 3.90           | DQ068882              |
| Kalotermes flavicollis       | F          | 3.70           | Y11377                |
| Zootermopsis nevadensis      | H          | 4.41           | AY764280              |
| Ctenocephalides felis        | I          | 7.22           | AY335923              |
| Orchopeas leucopus           | I          | 7.48           | AY335924              |
| Dipetalonema gracile         | J          | 5.59           | AJ548802              |
| Bryobia sp.                  | K          | 3.25           | EU499316              |
| Radopholus similis           | L          | 4.79           | EU833482              |
| Tuberolachnus salignus       | M          | 2.52           | JN384085              |
| Aphid sp.                    | M          | 2.59           | JN384091              |
| Toxoptera aurantii           | N          | 4.50           | JN384094              |
| Toxoptera aurantii           | N          | 4.80           | JN384095              |

Figure A7. RFLP pattern of PCR products of rrs gene of the Wolbachia supergroup O and B in Bemisia tabaci corresponding to VspI (AseI) digestion. The different profiles were obtained from individuals representing different Wolbachia in B. tabaci. The bands shown on the bottom are primer dimers. Lane 1, undigested Wolbachia O obtained by PCR from Asia II 1; lane 2–4, undigested Wolbachia B obtained by PCR from Asia II 7, Asia I and China 1, respectively; lane 5–6, Wolbachia PCR amplified production from MEAM1 and MED as controls; lane 8, digested Wolbachia O obtained by PCR from Asia II 1; lane 9–11, Wolbachia B obtained by PCR from Asia II 7, Asia I and China 1, respectively; lane 12–13, Wolbachia PCR amplified production from MEAM1 and MED as controls; lane M, DNA size markers (100, 250, 500, 750, 1000, and 2000 bp from bottom to top).
Figure A9. Phylogenetic position of the *Wolbachia* identified from *Bemisia afer* and *B. tabaci* putative species based on bacterial *coxA* gene sequences (402 sites). *Wolbachia* strains are characterized by the names of their host species and allele numbers from the MLST database. The tree was constructed using a TIM1 + G substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. The sequences obtained in this study are shown in bold. The bar indicates a branch length of 0.1 substitutions/site.

Figure A8. Phylogenetic position of the *Wolbachia* identified from *Bemisia afer* and *B. tabaci* putative species based on bacterial *gatB* gene sequences (369 sites). *Wolbachia* strains are characterized by the names of their host species and allele numbers from the MLST database. The tree was constructed using a TPM2uf + G substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. The sequences obtained in this study are shown in bold. The bar indicates a branch length of 0.1 substitutions/site.
Figure A10. Phylogenetic position of the Wolbachia identified from Bemisia afer and B. tabaci putative species based on bacterial hcpA gene sequences (444 sites) using the Neighbor-net method. Each edge (or a set of parallel edges) corresponds to a split in the data set and has length equal to the weight of the split. The sequence obtained in this study is shown in bold. MLST Database allele numbers of hcpA sequences are shown in parenthesis. The bar indicates a branch length of 0.01 substitutions/site.

Figure A11. Phylogenetic position of the Wolbachia identified from Bemisia afer and B. tabaci putative species based on bacterial ftsZ gene sequences (435 sites). Wolbachia strains are characterized by the names of their host species and allele numbers from the MLST database. The tree was constructed using a TrN + I substitution model for Bayesian analysis. Bayesian posterior probabilities are shown on the branches. The sequences obtained in this study are shown in bold. The bar indicates a branch length of 0.1 substitutions/site.
Figure A12. Phylogenetic position of the Wolbachia identified from *Bemisia afer* and *B. tabaci* putative species based on bacterial *fbpA* gene sequences (429 sites) using the Neighbor-net method. Each edge (or a set of parallel edges) corresponds to a split in the data set and has length equal to the weight of the split. The sequences obtained in this study are shown in bold. MLST Database allele numbers of *fbpA* sequences are shown in parenthesis. The bar indicates a branch length of 0.01 substitutions/site.
Figure A13. FISH controls. No probe control: Asia II 1; No Wolbachia probe control: Asia II 1; Wolbachia-free whiteflies control: Mediterranean (harboring Portiera and Hamiltonella) and Middle East-Asia Minor 1 (harboring Portiera, Hamiltonella and Rickettsia; Bing et al. 2013a). A–C, G–I: Overlay of channels of Portiera (red) and Wolbachia (green); D–F, J–L: Overlay of channels of Portiera (red), Wolbachia (green) and white light.