OPEN

The ISME Journal (2017) 11, 1019–1028

www.nature.com/ismej

ORIGINAL ARTICLE

Plant–mediated horizontal transmission of *Wolbachia* between whiteflies

Shao-Jian Li[1,2,5], Muhammad Z Ahmed[1,3,5], Ning Lv[1], Pei-Qiong Shi[1], Xing-Min Wang[1], Ji-Lei Huang[4] and Bao-Li Qiu[1]

[1]*Department of Entomology, Key Laboratory of Bio-Pesticide Innovation and Application, South China Agricultural University, Guangzhou, China;* [2]*Institute of Plant Protection, Henan Academy of Agricultural Sciences, Zhengzhou, China;* [3]*Tropical Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Homestead, FL, USA and* [4]*Instrumental Analysis and Research Center, South China Agricultural University, Guangzhou, China*

Maternal transmission is the main transmission pathway of facultative bacterial endosymbionts, but phylogenetically distant insect hosts harbor closely related endosymbionts, suggesting that horizontal transmission occurs in nature. Here we report the first case of plant-mediated horizontal transmission of *Wolbachia* between infected and uninfected *Bemisia tabaci* AsiaII7 whiteflies. After infected whiteflies fed on cotton leaves, *Wolbachia* was visualized, both in the phloem vessels and in some novel 'reservoir' spherules along the phloem by fluorescence *in situ* hybridization using *Wolbachia*-specific 16S rRNA probes and transmission electron microscopy. *Wolbachia* persisted in the plant leaves for at least 50 days. When the *Wolbachia*-free whiteflies fed on the infected plant leaves, the majority of them became infected with the symbiont and vertically transmitted it to their progeny. Multilocus sequence typing and sequencing of the *wsp* (*Wolbachia* surface protein) gene confirmed that the sequence type of *Wolbachia* in the donor whiteflies, cotton phloem and the recipient whiteflies are all identical (sequence type 388). These results were replicated using cowpea and cucumber plants, suggesting that horizontal transmission is also possible through other plant species. Our findings may help explain why *Wolbachia* bacteria are so abundant in arthropods, and suggest that in some species, *Wolbachia* may be maintained in populations by horizontal transmission.

The ISME Journal (2017) **11,** 1019–1028; doi:10.1038/ismej.2016.164; published online 9 December 2016

Introduction

Offspring vertically inherit both nuclear and non-nuclear genetic material from their mothers. Intracellular bacteria are the non-nuclear materials inherited vertically from mother to offspring (Oliver *et al.*, 2010). There has been an increasing interest in intracellular bacteria over the past two decades, because of their widespread distribution in nature and their significance to the ecology, evolution and reproductive biology of their hosts (Gotoh *et al.*, 2007; Himler *et al.*, 2011; Segoli *et al.*, 2013; Baldini *et al.*, 2014). Innumerable species of insects and other arthropods are associated with various intracellular bacteria (Hilgenboecker *et al.*, 2008; Watts *et al.*, 2009; Jaenike and Brekke, 2011). These bacteria often live in symbioses with their hosts (Oliver *et al.*, 2010), and may be obligate (that is, primary endosymbionts essential for host survival) or facultative (that is, secondary endosymbionts that can increase or decrease host fitness; Himler *et al.*, 2011; Jiggins and Hurst, 2011). The obligate symbionts are found within specialized cells and typically share a long evolutionary history with their hosts (Buchner, 1965), whereas the facultative symbionts tend to have more recently formed associations with their hosts. *Wolbachia* (Alphaproteobacteria: Rickettsiales) is a genus of facultative endosymbionts common among arthropods and is estimated to have infected the majority of arthropods and filarial nematodes. In arthropods, *Wolbachia* most commonly interact with their hosts via a parasitic manipulation of the reproductive system (Werren *et al.*, 2008). As with other facultative endosymbionts, *Wolbachia* have been thought to undergo primarily vertical transmission from mother to offspring with high fidelity. The horizontal transmission pathway is thought to be the most likely explanation for closely related symbionts occurring in phylogenetically distant insects (Werren *et al.*, 1995; Vavre *et al.*, 1999; Noda *et al.*,

Correspondence: B-L Qiu, Department of Entomology, South China Agricultural University, No. 483, Wushan Road, Tianhe, Guangzhou 510640, China.
E-mail: baileyqiu@scau.edu.cn
[5]These authors contributed equally to this work.
Received 19 May 2016; revised 19 August 2016; accepted 20 October 2016; published online 9 December 2016

2001; Baldo *et al*., 2008; Ahmed *et al*., 2013). Over the past two decades, there have been multiple phylogenetic and transinfection studies reporting evidence of *Wolbachia* transmission between both phylogenetically close and phylogenetically distant species (Boyle *et al*., 1993; Heath *et al*., 1999; Vavre *et al*., 1999). Thus, it is probable that *Wolbachia* horizontal transmission is occurring between some arthropod taxa (Ahmed *et al*., 2015). Although *Wolbachia* has been shown to undergo extensive horizontal transmission between several host taxa (Werren *et al*., 1995; Baldo *et al*., 2006; Chiel *et al*., 2009; Raychoudhury *et al*., 2009; Oliver *et al*., 2010; Ahmed *et al*., 2015), the mechanisms for this are poorly understood.

There is growing evidence for common horizontal transmission of *Wolbachia* from one species to another (Ahmed *et al*., 2013; Gerth *et al*., 2013; Brown and Lloyd, 2015), and the transmission route is becoming a hotspot of research, given its importance in ecological and evolutionary biology (Vavre *et al*., 1999; Sintupachee *et al*., 2006; Caspi-Fluger *et al*., 2012; Gehrer and Vorburger, 2012). Recently, *Wolbachia* horizontal transmission through invertebrate predators and parasitoids has been revealed (Huigens *et al*., 2000, 2004; Le Clec'h *et al*., 2013; Ahmed *et al*., 2015). However, the presence of identical strains of *Wolbachia* among species that do not share predators and parasitoids but have similar habitats, such as shared host plants or food sources, suggests that plants or food may be involved in *Wolbachia* horizontal transmission (Sintupachee *et al*., 2006; Caspi-Fluger *et al*., 2012; Weinert *et al*., 2015).

The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) is a small hemipterous insect that feeds on phloem sap of numerous host plants; it has a very wide host plant range of over 500 species worldwide (Stansly and Naranjo, 2010). *Bemisia tabaci* is a complex of distinct cryptic species, harboring various bacterial symbionts such as *Wolbachia*, *Arsenophonus*, *Cardinium*, *Hamiltonella* and *Rickettsia*, but endosymbionts vary largely among the different whitefly species (Chiel *et al*., 2007; Ahmed *et al*., 2010; Skaljac *et al*., 2013). Here, we investigated if host plant had a role in the horizontal transmission of *Wolbachia* between whiteflies. We also studied the transfer dynamics of *Wolbachia* during this plant-mediated transmission.

Materials and methods

Plants and insects

Cotton plants (*Gossypium hirsutum* L. var. Lumianyan no. 32) were used in this study. Cotton seeds were sown in 15-cm-diameter plastic pots containing a soil–sand mixture (10% sand, 5% clay and 85% peat) in a greenhouse at ambient temperature and photoperiod. Plants were watered as necessary before being used in experiments at the 6–8 expanded leaf stage.

The whiteflies used in the study were *Bemisia tabaci* AsiaII7 (formerly known as Cv biotype), which is an indigenous cryptic species in South China (De Barro and Ahmed, 2011). Details regarding the collection, rearing and *Wolbachia* infection monitoring of AsiaII7 whiteflies are shown in the Supplementary Method S1.

Wolbachia *transmission from whiteflies to cotton plants*

We investigated the effects of the number and feeding time of *Wolbachia*-positive AsiaII7 on the efficiency of *Wolbachia* transmission from whiteflies to cotton plants. We collected AsiaII7 adults 24–48 h after they emerged from the pupal stage, using a hand aspirator from the *Wolbachia*-positive subcolony. In AsiaII7, *Wolbachia* created the scattered infection pattern described by Ahmed *et al*. (2015). Whiteflies were released into leaf cages (2 cm high, 3 cm diameter) covered on the undersurface of clean, healthy cotton leaves (as shown in Supplementary Figure S5). We studied three treatments, each with a different amount of whiteflies per cage (1 pair, 5 pairs and 10 pairs), and performed 10 replicates per treatment.

Two days after the AsiaII7 *B. tabaci* were released into the leaf cages, we recorded which leaves were being fed upon by the whiteflies (herein referred to as 'fed leaves'). We then began cutting ~0.01 g of leaf material (equivalent to leaf surface area of ~50 mm^2) every 24 h from both the fed leaf and from the leaf immediately below it on the same stem (~2 cm distance between the bases of the two leaves). The presence of *Wolbachia* in the cotton leaves was detected by PCR using the *Wolbachia*-specific primers of *Wolbachia* surface protein (*wsp*) gene and 16S rRNA genes (O'Neill *et al*., 1992; Braig *et al*., 1998); the primers and protocols for PCR detection are shown in Supplementary Table S1 and Supplementary Method S2. The initial time when *Wolbachia* was positively detected in cotton leaves was recorded for each replicate. For negative controls, the same procedure was followed using whiteflies that were collected from the *Wolbachia*-free subcolony.

Visualization of Wolbachia *in plants*

Fluorescence *in situ* hybridization (FISH) was used to identify the location of *Wolbachia* in cotton plants. Ten pairs of AsiaII7 were released onto a single leaf of each plant and were allowed to feed for 25–28 days. Then, a 50 mm^2 leaf section (10 mm × 5 mm) was removed by cutting longitudinally along the leaf vein; an equally sized section was also removed from the leaf immediately below the fed leaf. These leaf samples were placed in Carnoy's fixative. FISH detection was then performed following the method of Sakurai *et al.* (2005; see Supplementary Method S3), using a *Wolbachia*-specific 16S rRNA probe (W2-Cy3:

5′-CTTCTGTGAGTACCGTCATTATC-3′) that had its specificity tested with the Ribosomal Database Project II 'probe match' analysis tool (http://rdp.cme.msu.edu) by Gottlieb *et al.* (2008). The stained leaf samples were mounted and viewed under a Nikon eclipse Ti-U FluoView inverted microscope (Nikon Instruments Inc., Tokyo, Japan). Specificity of *Wolbachia* detection was confirmed using two controls: (1) *Wolbachia*-infected cotton leaves without the *Wolbachia* 16S rRNA probe and (2) *Wolbachia*-free cotton leaves with the *Wolbachia* 16S rRNA probe. FISH visualization experiments were also performed on cowpea and cucumber plants, with the same protocol and primers used in the cotton plant experiment.

Transmission electron microscopy was used to confirm the location of *Wolbachia* in the cotton leaves. Samples of *Wolbachia*-deposited cotton leaves (1.0 mm × 0.5 mm) were fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.4) at 4 °C for 24 h, and then overnight in 1% osmium tetroxide. The fixed leaf samples were dehydrated through an alcohol series and embedded in Spurr's resin. Ultrathin sections were collected on copper grids with a single slot, stained with 1% uranyl acetate and lead citrate, and finally examined under a Transmission electron microscopy (JEOL, Tokyo, Japan).

Persistence of Wolbachia *in cotton plants*

Two experiments, each with three replicates, were performed to study the persistence of *Wolbachia* in the cotton leaves. In the first experiment, 10 two-day-old pairs of *Wolbachia*-positive AsiaII7 adults were released into a leaf cage to feed on the cotton leaves for 24 days (we have already shown that the 24th day is approximately the earliest day in which *Wolbachia* can be detected in plant leaves). On day 25th, the adult whiteflies and their immature progeny were collected and 0.01 g of the fed leaves were cut for DNA extraction and the detection of *Wolbachia* presence using PCR and quantitative real-time PCR (q-PCR) with the *wsp* primers. For the next 50 days, additional 0.01 g leaf segments were cut every 5 days and tested for the presence of *Wolbachia*; a total of 11 sets of leaf samples were tested in this experiment. The second experiment was run with a nearly identical protocol, except the 10 pairs of AsiaII7 whiteflies and their offspring were not collected on day 25th. Instead, they were allowed to feed continuously on the cotton leaves during the experiment. The *wsp* primers used in PCR detection were *wsp* 81F and *wsp* 691R (Supplementary Table S1) and *wsp* primers used in q-PCR were *wsp*-QF and *wsp*-QR (Supplementary File Supplementary Method S2). A *UBQ7* gene of cotton fiber (DQ116441) was used as an internal control for data normalization and quantification (Tu *et al.*, 2007). Detailed procedures for PCR and q-PCR *Wolbachia* detection are shown in Supplementary Method S2.

To test whether the *Wolbachia* detected in the plants was still alive, RNA was extracted from leaf discs (2 cm diameter) of five cotton plants that had each been exposed to *Wolbachia*-positive AsiaII7 whiteflies for a different amount of time (24, 34, 44, 54 or 64 days). RNA was also extracted from a cotton plant that had not been exposed to whiteflies, as a negative control. Extractions were performed using the Trizol RNA Extraction Kit (Omega, Stamford, CT, USA) following the protocol in the instruction manual. The RNA was then reverse transcribed to cDNA using Moloney murine leukemia virus (Invitrogen, Carlsbad, CA, USA) and the specific 16S rRNA primers (Supplementary Table S1).

Wolbachia *transmission from cotton to whitefly and its subsequent vertical transmission*

To detect the horizontal transmission of *Wolbachia* from cotton plants to whiteflies, 10 newly emerged adults collected from the *Wolbachia*-negative colony were released into 10 different leaf cages. They were allowed to feed on the *Wolbachia*-positive cotton leaves for 20 days, and then collected for *Wolbachia* PCR detection. This experiment was repeated 10 times.

To evaluate the *Wolbachia* acquisition efficiency of recipient whiteflies, 100 pairs of AsiaII7 adults from the *Wolbachia*-negative colony were introduced into 20 leaf cages (five pairs per cage) covered on the *Wolbachia*-positive cotton leaves. Additional *Wolbachia*-negative whitefly adults were released into a leaf cage with *Wolbachia*-free leaves, as a negative control. Every 2 days, five randomly selected pairs of adults were collected for *Wolbachia* PCR and q-PCR using the *wsp* primers (see Supplementary Table S1 and Supplementary Method S4, respectively). A *β-actin* gene of *B. tabaci* was used as an internal control for data normalization and quantification in q-PCR (Ghanim and Kontsedalov, 2009); the detailed procedure for q-PCR is described in Supplementary Method S4. This q-PCR experiment was repeated three times.

In the instances where *Wolbachia* was detected in a recipient whitefly, RNA was extracted from the whitefly to test whether the *Wolbachia* was still alive. Extractions were performed using the Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) following the procedure in the instruction manual. DNase was added to remove DNA contamination, and cDNA was synthesized using a Verso cDNA Kit (Thermo Scientific, Waltham, MA, USA). Reactions without the reverse transcriptase enzyme (to exclude DNA contamination) were used as negative controls for the RT-PCR.

To test if the *Wolbachia* acquired by AsiaII7 during feeding can be vertically transferred in subsequent generations of AsiaII7, we randomly selected 10 pairs of adults that had been feeding on *Wolbachia*-positive leaves for 15 days (*Wolbachia* can be initially detected in a recipient whitefly after

10 days). Each pair was then introduced into a separate leaf cage containing new healthy cotton leaves to encourage oviposition. After 24 h, the adult whiteflies were removed from the cages and used for *Wolbachia* PCR detection; all eggs (the F_1 generation) were left in the cages and allowed to fully mature. Twenty randomly selected specimens of the offspring adults were then used to examine the *Wolbachia* distribution pattern, and the others were used to determine the percentage of F_1 whiteflies that retained *Wolbachia*. This experiment was repeated 10 times.

Multilocus sequence typing and phylogenetic analysis of Wolbachia

Multilocus sequence typing (MLST) was used to identify *Wolbachia* strains in (1) donor *Wolbachia*-positive whiteflies, (2) recipient *Wolbachia*-negative whiteflies, (3) the F_1 generation progeny of the newly infected whiteflies and (4) cotton leaves. Five MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) as well as the *wsp* gene were sequenced following the methods of Baldo *et al.* (2006). The five MLST genes were concatenated using *Geneious* (version r8; Kearse *et al*., 2012). MLST loci were blasted against the *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*). Two MLST loci from supergroup A, four from supergroup B, one from supergroup D and our MLST loci were analyzed, using maximum likelihood (ML) in RAxML (Stamatakis, 2006), to construct a phylogenetic tree (Supplementary Table S2 and Supplementary Method S5).

Transmission of Wolbachia *through other plant species*

To examine *Wolbachia* transmission through other plant species, we repeated the *Wolbachia* horizontal transmission and FISH visualization experiments using cucumber, *Cucumis sativus* L. (var. Xiayou168), and cowpea, *Vigna unguiculata* (L.) Walp (var. Kefeng), instead of cotton. *Wolbachia* was transmitted from *Wolbachia*-positive AsiaII7 to cucumber and cowpea plants, and then to the *Wolbachia*-negative AsiaII7 individuals and their F_1 generation offspring. PCR detection of *Wolbachia* in the new host plant species, determinations of the strain genotypes and phylogenetic analyses were all conducted using the same methodology as in the cotton plant experiments.

Results

Wolbachia *can be transmitted to cotton plants by whiteflies*

Results of the *Wolbachia* PCR detection using *wsp* and 16S rRNA gene primers (Supplementary Table S1) revealed that this endosymbiont could be detected in cotton plants after several weeks of *Wolbachia*-positive whitefly feeding. In samples taken from leaves that the whiteflies were directly feeding on (that is, the fed leaves), the time interval from when the whiteflies began feeding to when *Wolbachia* was initially detected in the leaf was inversely correlated with the number of infected whiteflies in the leaf cage (Figure 1, 35.8 ± 0.6, 27.4 ± 0.8 and 23.4 ± 0.7 days for the groups of 1, 5 and 10 pairs of insects, respectively, mean ± s.e.). *Wolbachia* was also detected in samples taken from leaves immediately below the fed leaves; that is, in each treatment, *Wolbachia* was positively detected 1–3 days after *Wolbachia* was detectable on the corresponding fed leaf (Figure 1). In the negative control treatments, which only involved *Wolbachia*-free whiteflies, *Wolbachia* was not detected on any of the cotton leaves.

The localization of Wolbachia *in cotton leaves*

Using a cyanine3-labeled *Wolbachia*-specific 16S rRNA probe (Supplementary Method S3), the FISH visualization of the fed leaves revealed that *Wolbachia* could be found in most parts of the phloem sieve tube, which is usually 5–8 cm from the whitefly feeding site. *Wolbachia* was unexpectedly also found in some novel globular regions along the phloem sieve tube (Figure 2a and Supplementary Figure S1). *Wolbachia* was also found in the phloem of the leaves immediately below the fed leaves (Figure 2b). This presence of *Wolbachia* in cotton leaves that had not been directly fed on by whiteflies suggests that *Wolbachia* can move between leaves after the initial transmission. As with the cotton leaves, spherules of *Wolbachia* were also in the phloem of the cowpea and cucumber leaves that had been exposed to *Wolbachia*-positive whiteflies (Supplementary Figure S2). However, the quantities and sizes of these spherules were different from those found in the cotton leaves (Supplementary Figure S2). *Wolbachia* was not observed in any leaves from the negative controls (Supplementary Figure S3).

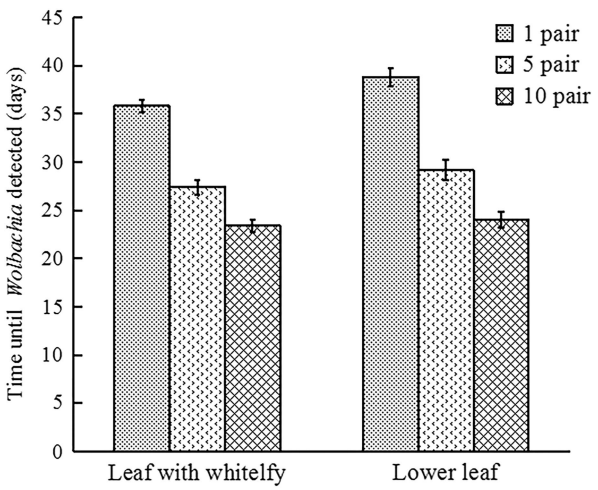

Figure 1 Correlation between the number of *Wolbachia*-infected whiteflies (AsiaII7 *Bemisia tabaci*) feeding on cotton plants and the amount of time (as of the initial whitefly introduction) before *Wolbachia* was initially detected in the cotton leaves. The column and error bars are the mean ± s.e. of time (in days) and 10 replicated were repeated in each column.

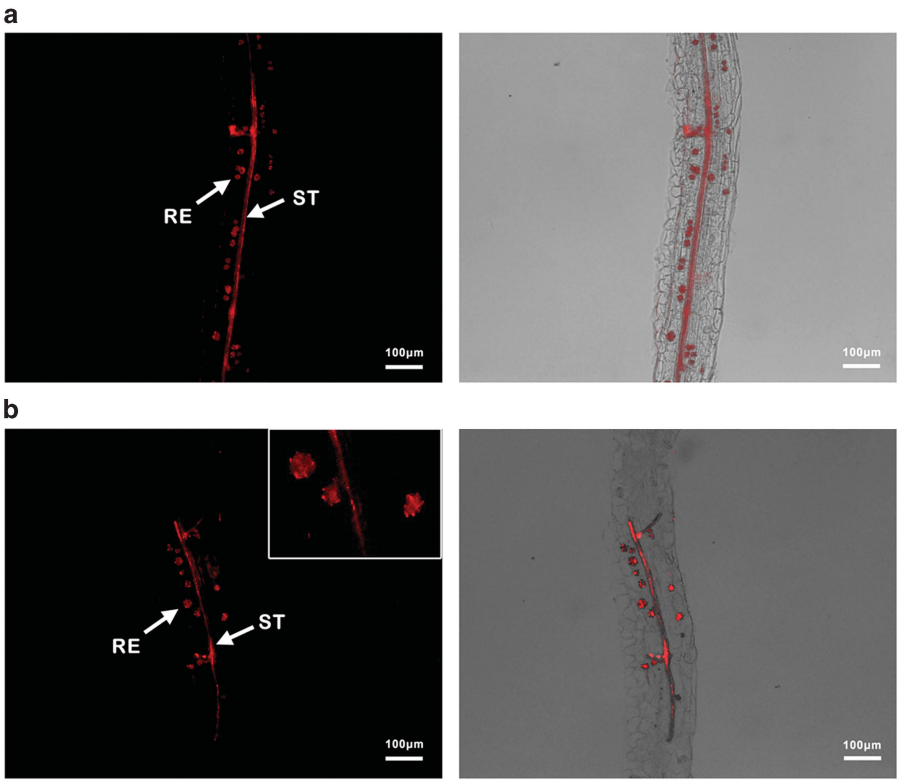

Figure 2 FISH visualization of *Wolbachia* in cotton leaves. Figures show the leaf tissue longitudinally along the leaf phloem. (**a**) *Wolbachia* in a leaf directly fed on by AsiaII7 whiteflies; (**b**) *Wolbachia* in the leaf immediately below the fed leaf; ST, phloem sieve tube; RE, reservoir of *Wolbachia* along the phloem; left panels: fluorescence in the dark field; right panels: fluorescence in the bright field.

Transmission electron microscopy images were used to visualize *Wolbachia* morphology in the bacteriocytes located in the adult whitefly abdomen and in the cotton leaf phloem. In the whitefly bacteriocytes, most individual *Wolbachia* have a small (0.5–1 μm), irregular coccoid form with a double membrane (Figures 3a and b). In the phloem, *Wolbachia* was found in the vacuole of a plant cell and was morphologically similar to those in whitefly adult abdomens (Figures 3c and d).

Changes in the amount of Wolbachia *in cotton leaves over time*

In this study, two plant groups were exposed to *Wolbachia*-infected AsiaII7 whiteflies. In one group, the whiteflies were removed after the first 24 days, whereas in the other group, the whiteflies were left on the plants for the entire duration of the experiment (74 days). *Wolbachia* persisted in cotton leaves for at least 50 days after its first detection (that is, 74 days after the *Wolbachia*-positive whiteflies first fed on the leaf) in both of the plant groups, regardless of whether the whiteflies had been removed (Figure 4). The relative quantity of *Wolbachia* in all treatments increased to its highest amount during the first 5–10 days after it was positively detected (that is, days 25th–34th), and during this period the leaves that were continuously fed on by whiteflies had significantly higher quantities of *Wolbachia* than the leaves that had the whiteflies removed. After reaching this peak quantity, the amount of *Wolbachia* reduced gradually in leaves from both groups.

Wolbachia *transmission from cotton to whiteflies and its subsequent vertical transmission*

When uninfected adult whiteflies continuously fed on the *Wolbachia*-infected cotton leaves, *Wolbachia* was initially detected in the recipient whiteflies after 10.0 ± 0.3 days. After 20 days of feeding, *Wolbachia* was detected in 62.0 ± 5.5% of the recipient female whiteflies. In the more sensitive q-PCR evaluation, *Wolbachia* was initially detected in the recipient whiteflies after 4 days of feeding on the *Wolbachia*-infected cotton leaves, and the relative quantity of *Wolbachia* in female adults greatly increased as the feeding time increased (Figure 5). We also found that 95.0 ± 1.67% of the F_1 whitefly adults tested positive for *Wolbachia*, indicating that *Wolbachia* can be vertically transmitted from newly infected female whiteflies to their offspring (Supplementary Figure S4).

Multilocus sequence typing and phylogenetic analysis of Wolbachia from plants and whiteflies

Our genetic analysis of the *Wolbachia* strain being transmitted between cotton plants and AsiaII7

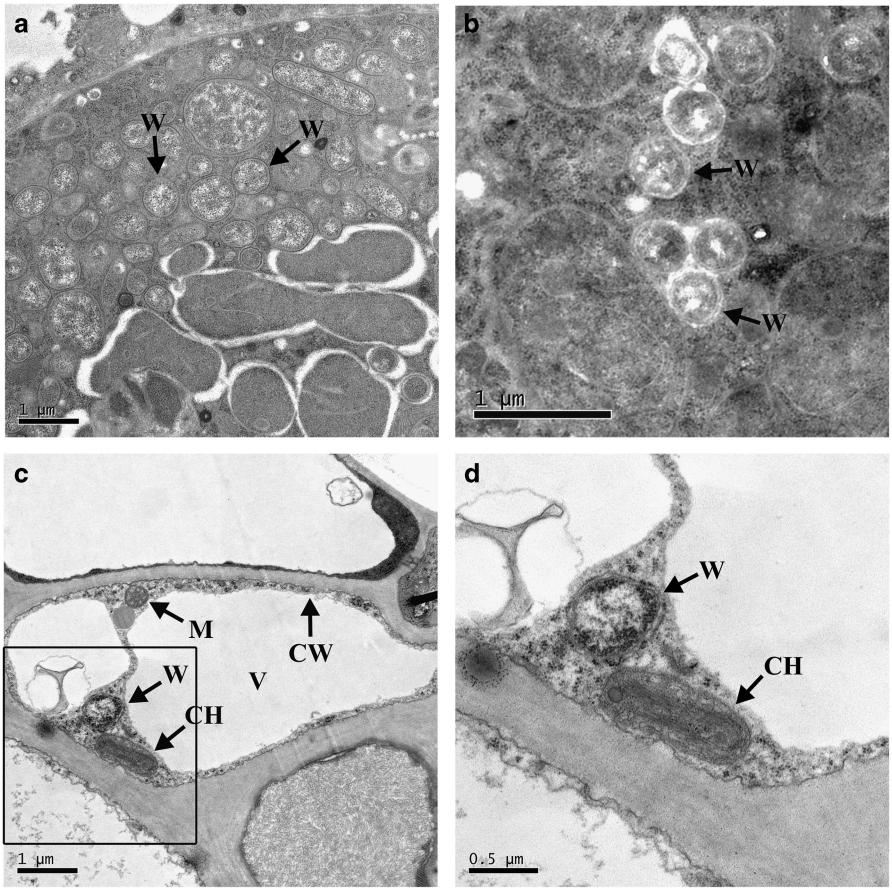

Figure 3 TEM images of *Wolbachia* in the bacteriocyte located in the abdomen of an adult AsiaII7 whitefly (**a**, **b**) and in the phloem sieve tube of a cotton leaf (**c**, **d**). CW, cell wall of the plant phloem; CH, chloroplast; M, mitochondrion; V, vacuole of the plant cell; W, presence of *Wolbachia* in a double membrane cell.

whiteflies revealed that all of the tested *Wolbachia* samples were identical. The *Wolbachia* from the donor whiteflies, the infected cotton leaves, the recipient AsiaII7 whiteflies and the recipient whitefly offspring are all *Wolbachia* ST388 (Figure 6a and Supplementary Table S3). These results were confirmed by DNA sequencing and by our phylogenetic analysis (Figure 6b). Furthermore, all of the tested *Wolbachia* endosymbionts belong to the *Con* group within Supergroup B (Figure 6). No sequence variation was found in the *wsp* gene of these *Wolbachia* endosymbionts.

Transmission of Wolbachia *through different plant species*

After repeating our *Wolbachia* transmission and detection experiments with two additional types of plants, cucumber and cowpea, we found evidence that the whiteflies' choice of host plant does not have any effect on the sequence type or the fidelity of *Wolbachia* during horizontal transmission (Figure 6). This demonstrates that this particular *Wolbachia* strain (ST388) remains highly conservative during its plant-mediated horizontal transmission.

Discussion

There has been an increased interest in studying the horizontal transmission of intracellular bacterial endosymbionts over the past two decades, particularly *Wolbachia* and *Rickettsia* because of their widespread distribution and significant role in the ecology and evolution of their hosts (Vavre *et al*., 1999; Sintupachee *et al*., 2006; Gotoh *et al*., 2007; Himler *et al*., 2011; Caspi-Fluger *et al*., 2012). The vector-mediated interspecific transmission of intracellular bacterial endosymbionts was observed through shared food sources (in *Wolbachia*, *Spiroplasma*, *Hamiltonella defensa*; Rigaud and Juchault, 1995; Oliver *et al*., 2010; Caspi-Fluger *et al*., 2012), ectoparasitic mites (Jaenike *et al*., 2007; Gehrer and Vorburger, 2012), host plants (in *Rickettsia*, *Arsenophonus*; Caspi-Fluger *et al*., 2012; Bressan, 2014) and parasitoids (in *Arsenophonus*, *Wolbachia*; Duron *et al*., 2010; Ahmed *et al*., 2015). The hypothesis that *Wolbachia* can be horizontally

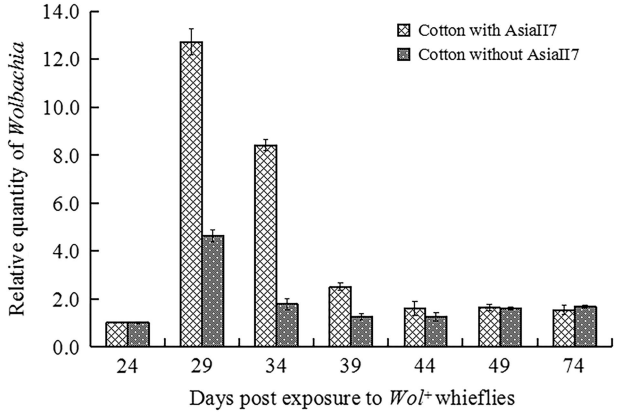

Figure 4 Retention time (days) and relative quantity of *Wolbachia* endosymbiont in cotton leaves since the initial introduction of *Wolbachia*-positive whiteflies to the 50th day after introduction. The relative quantity (rq) of *Wolbachia* was calculated based on its *wsp* gene using the formula $rq = 2^{-\Delta\Delta ct}$. The column and error bars were the mean ± s.e. of time (days) with three replicates for each column.

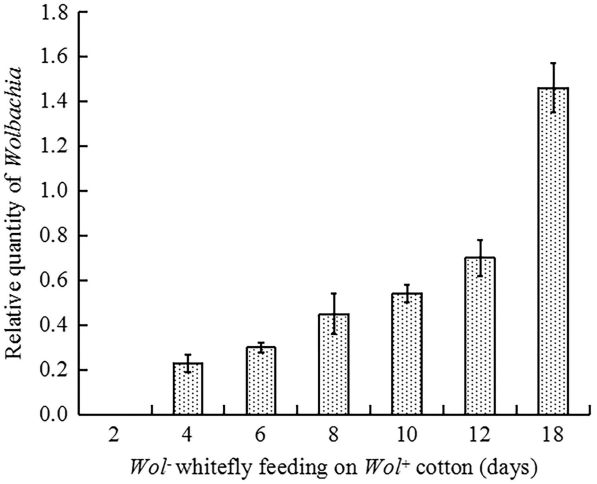

Figure 5 Dynamics of *Wolbachia* in recipient AsiaII7 whiteflies that have fed on *Wolbachia*-positive cotton leaves. The column and error bars are the mean ± s.e. of time (in days) with three replicates repeated in each column.

transmitted between two insect species has been supported by both phylogenetic and experimental analyses (Vavre *et al.*, 1999; Huigens *et al.*, 2000, 2004; Sintupachee *et al.*, 2006; Ahmed *et al.*, 2013; Yang *et al.*, 2013). Ahmed *et al.* (2013) compared the phylogeny of different *Bemisia* species and their endosymbionts, revealing the incongruence of *Wolbachia* with their whitefly hosts and suggesting a host shift of *Wolbachia* through horizontal transmission. Recently, Ahmed *et al.* (2015) revealed that parasitoids can transmit *Wolbachia* by feeding and probing their uninfected hosts. Both the phylogenetic analysis and transmission experiments demonstrated that parasitoids could serve as potential routes for horizontal transmission of *Wolbachia* between different hosts. Sintupachee *et al.* (2006) discovered a potential route for lateral transmission of *Wolbachia* between different insects that share the same leaf substrate in pumpkin plants. Huigens *et al.* (2000, 2004) discovered a frequent horizontal transmission of *Wolbachia* from infected to uninfected wasp larvae (*Trichogramma kaykai*) when they feed on a common food source: eggs of the butterfly *Apodemia mormo deserti*. After the wasps matured, the females then vertically transmitted *Wolbachia* to their offspring. Sintupachee *et al.* (2006) showed that four taxonomically diverse insects feeding on the same host plant contained very closely related *Wolbachia*, suggesting the potential role of host plants in *Wolbachia* horizontal transmission (Sintupachee *et al.*, 2006). Work by Yang *et al.* (2013) also showed that identical strains of *Wolbachia* can be shared by two species that live in the same plant tissue: the gall wasp *Andricus mukaigawae* and its inquiline wasp *Synergus japonicas*. Stahlhut *et al.* (2010) used a multigene approach to provide evidence that ecological associations can facilitate horizontal transmission of *Wolbachia* within mycophagous fly communities. Our current study supplements the findings on *Wolbachia* transmission by providing direct evidence of plant-mediated transmission of *Wolbachia*, including the transmission efficiency, distribution pattern and persistence of *Wolbachia* in both plant tissues and in the progeny of its recipient host.

The distribution patterns of endosymbionts in their hosts may have important influences on their transmission ability. Caspi-Fluger *et al.* (2011) found that another endosymbiont, *Rickettsia*, can have two distribution patterns in its whitefly host: a scattered pattern localized in the whitefly hemocoel and a confined pattern restricted to the bacteriocytes. Moreover, Chiel *et al.* (2009) found that the scattered pattern of *Rickettsia* facilitates its transmission to whitefly parasitoids. Previous work by Ahmed *et al.* (2015) showed that, like *Rickettsia*, *Wolbachia* can also be found in scattered and confined distribution patterns. The scattered *Wolbachia* have the potential to be transmitted horizontally between whiteflies through the feeding or oviposition probing of an *Eretmocerus* parasitoid. In the current study, we found that *Wolbachia*-positive AsiaII7 whiteflies with a scattered *Wolbachia* pattern can transmit *Wolbachia* to cotton leaves, and *Wolbachia*-negative AsiaII7 whiteflies can become infected with scattered-pattern *Wolbachia* after feeding on *Wolbachia*-positive cotton leaves for at least 10 days. This suggests similarities between the plant-mediated transmission routes of *Wolbachia* and *Rickettsia*.

The distribution pattern of *Wolbachia* in plant tissues has not been previously demonstrated. *Wolbachia* could not be detected in plant tissue until the *Wolbachia*-positive whiteflies had been feeding for 24 days, at which point the amount of *Wolbachia* noticeably increased for the ensuing 5 days. We surmise that *Wolbachia* requires 24 days to accumulate of a titer, with the subsequent amplification being a barrier break in the interaction

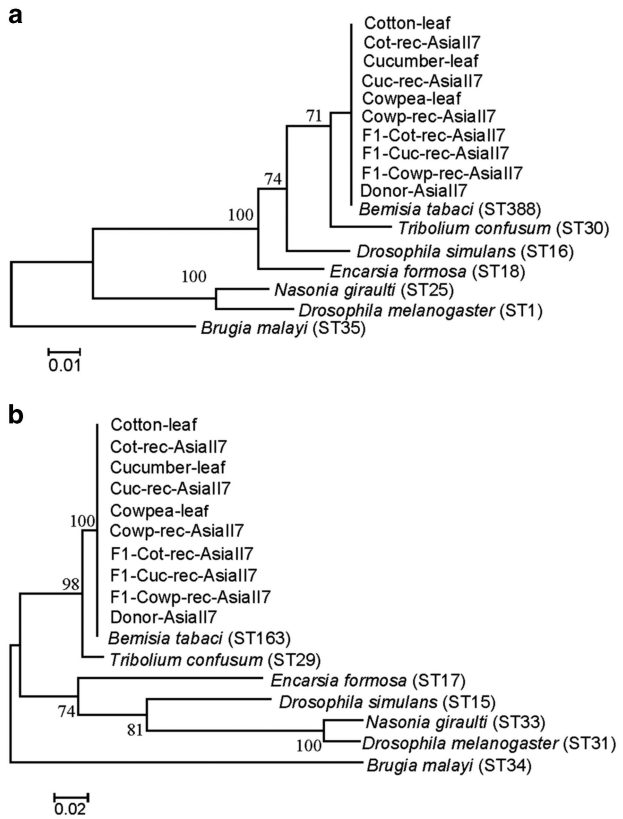

Figure 6 Phylogenetic analysis of *Wolbachia* strains detected from different hosts. (**a**) The ML phylogenetic tree based on five MLST genes, using a GTR model. Support values are based on 1000 bootstrap iterations. The numbers within parentheses represent the sequence types of *Wolbachia* in the *Wolbachia* MLST database. The capital letters represent the supergroups of different *Wolbachia* resources. (**b**) The ML tree based on *wsp* gene sequences using a Tamura 3-parameter model. Support values are based on 1000 bootstrap iterations. *Wolbachia* hosts: 'Donor AsiaII7': *Wolbachia*-positive AsiaII7 *B. tabaci*; 'Cotton/Cucumber/Cowpea leaf': *Wolbachia* in the infected cotton/cucumber/cowpea plants; 'Cot/Cuc/Cowp-rec AsiaII7': recipient AsiaII7 *B. tabaci* that became infected with *Wolbachia* after feeding on *Wolbachia*-positive cotton/cucumber/cowpea leaves; 'F_1 Cot/Cuc/Cowp-rec AsiaII7': progeny of the Cot/Cuc/Cowp-rec AsiaII7 whiteflies.

between invasive *Wolbachia* and the physiological contents of the plant leaf. Here, for the first time, we have shown that *Wolbachia* can persist in cotton leaves for more than 50 days; our RNA RT experiments revealed that all of the *Wolbachia* endosymbionts present in the cotton leaves for 24–64 days were alive. In order for *Wolbachia* to live that long in the plant, there should be some sort of interaction effect or nutritional support. This requires further investigation. Interestingly, we found that some *Wolbachia* cluster in an irregular globular shape along the leaf phloem. The biological role of these globular clusters is unclear, but we suspect that these may be the reservoirs of *Wolbachia* in plant leaves. Purcell *et al.* (1994) found that a bacterial parasite of the leafhopper *Euscelidius variegatus* can be horizontally transmitted between different individuals of *E. variegatus* that feed on the same plant leaves. This particular bacterium did not multiply or move within the plant. In contrast, we found that *Wolbachia* can move within its plant after transmission; FISH visualization showed the presence of *Wolbachia* in leaves that had not been fed on by whiteflies.

Facultative endosymbionts have already been shown to change host fitness or biology for multiple reasons, including host protection against entomopathogenic fungi and parasitic wasps, amelioration of the detrimental effects of heat and influence on host plant suitability (Oliver *et al.*, 2003, 2005, 2010; Scarborough *et al.*, 2005). One main consequence of *Wolbachia* horizontal transmission is the induction of unknown phenotypes in the novel host (Werren *et al.*, 2008; Ahmed *et al.*, 2015). *Wolbachia* can confer positive fitness benefits by increasing the resistance against natural pathogens in fruit flies (Teixeira *et al.*, 2008). Hornett *et al.* (2006) revealed that *Wolbachia*, in some host species, do not currently induce any phenotype but may have done so in the past, implying that more species have had their biology affected by *Wolbachia* than previously estimated. In other situations, after transinfection of *Wolbachia*, the newly induced phenotype can be suppressed by its novel host (Hornett *et al.*, 2008) and can also be changed into a completely different phenotype (Sasaki *et al.*, 2002). It is therefore necessary to investigate each strain's genotype and phenotype in its natural host, as well as other possible hosts in which it may have been transferred through shared host plants.

Plant-mediated transmission might explain the widespread abundance of *Wolbachia* infection in phytophagous arthropods and the presence of its identical strains in evolutionarily distant species. Overall, plant-mediated transmission might be having a crucial unknown role in ecological and evolutionary biology. In this study, some novel 'reservoir' spherules were found along the cotton leaf phloem using FISH, but this kind of spherule was not found when the *Rickettsia*-infected *B. tabaci* MEAM1 species fed upon cotton leaves while contaminating the phloem with *Rickettsia* (An *et al.*, 2015). The biological roles of the *Wolbachia* 'reservoir' in plant leaves, and the consequent plant-mediated transmission, need to be further investigated to fully understand the dynamics of *Wolbachia* infection.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We are very grateful to Francis M Jiggins, John J Welch (University of Cambridge) and David Plotkin (University of Florida) for their constructive responses on an earlier version of this manuscript and review the language. This research was funded by the National Basic Research Program of China (2013CB127600), the National Department Public Benefit Research Foundation (201303019) and

the Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (201419) to BLQ.

Author contributions

BLQ and SJL designed the study; SJL, MZA, NL, PQS and JLH performed the experiments; BLQ, SJL, XMW and MZA analyzed the data; BLQ and MZA wrote the paper; BLQ supported the grants.

References

Ahmed MZ, De Barro PJ, Ren SX, Greeff JM, 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 *et al.* (2015). The intracellular bacterium *Wolbachia* uses parasitoid wasps as phoretic vectors for efficient horizontal transmission. *PLoS Pathog* **10**: e1004672.

Ahmed MZ, Ren SX, Xue X, Li XX, Jin GH, Qiu BL. (2010). Prevalence of endosymbionts in *Bemisia tabaci* populations and their *in vivo* sensitivity to antibiotics. *Curr Microbiol* **61**: 322–328.

An X, Li YH, Li SJ, Guo CF, Ren SX, Qiu BL. (2015). Preliminary research on the distribution and transmission efficiency of *Rickettsia*, an endosymbiont of whitefly *Bemisia tabaci*. *Chinese J Appl Entomol* **52**: 135–142.

Baldini F, Segata N, Pompon J, Marcenac P, Shaw WR, Dabire RK *et al.* (2014). Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. *Nat Commun* **5**: 3985.

Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, Werren JH. (2008). Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. *Mol Ecol* **17**: 557–569.

Baldo L, Hotopp JCD, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR *et al.* (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia* pipientis. *Appl Environ Microb* **72**: 7098–7110.

Boyle L, Oneill SL, Robertson HM, Karr TL. (1993). Interspecific and intraspecific horizontal transfer of *Wolbachia* in Drosophila. *Science* **260**: 1796–1799.

Braig HR, Zhou W, Dobson SL, O'Neill SL. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia* pipientis. *J Bacteriol* **180**: 2373–2378.

Bressan A. (2014). Emergence and evolution of Arsenophonus bacteria as insect-vectored plant pathogens. *Infect Genet Evol* **22**: 81–90.

Brown AN, Lloyd VK. (2015). Evidence for horizontal transfer of *Wolbachia* by a *Drosophila* mite. *Exp Appl Acarol* **66**: 301–311.

Buchner P. (1965). *Endosymbiosis of Animals with Plant Microorganisms*. John Wiley and Sons: New York, NY, USA.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Katzir N, Portnoy V, Belausov E *et al.* (2012). Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proc R Soc Ser B* **279**: 1791–1796.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Mouton L, Hunter MS, 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.

Chiel E, Gottlieb Y, Zchori-Fein E, Mozes-Daube N, Katzir N, Inbar M *et al.* (2007). Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bull Entomol Res* **97**: 407–413.

Chiel E, Zchori-Fein E, Inbar M, Gottlieb Y, Adachi-Hagimori T, Kelly SE *et al.* (2009). Almost there: transmission routes of bacterial symbionts between trophic levels. *PLoS One* **4**: e4767.

De Barro PJ, Ahmed MZ. (2011). Genetic networking of the *Bemisia tabaci* cryptic species complex reveals patterns of biological invasions. *PLoS One* **6**: e25579.

Duron O, Wilkes TE, Hurst GDD. (2010). Interspecific transmission of a male-killing bacterium on an ecological timescale. *Ecol Lett* **13**: 1139–1148.

Gehrer L, Vorburger C. (2012). Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biol Lett* **8**: 613–615.

Gerth M, Roethe J, Bleidorn C. (2013). Tracing horizontal *Wolbachia* movements among bees (Anthophila): a combined approach using multilocus sequence typing data and host phylogeny. *Mol Ecol* **22**: 6149–6162.

Ghanim M, Kontsedalov S. (2009). Susceptibility to insecticides in the Q biotype of *Bemisia tabaci* is correlated with bacterial symbiont densities. *Pest Manag Sci* **65**: 939–942.

Gotoh T, Sugasawa J, Noda H, Kitashima Y. (2007). *Wolbachia*-induced cytoplasmic incompatibility in Japanese populations of *Tetranychus urticae* (Acari: Tetranychidae). *Exp Appl Acarol* **42**: 1–16.

Gottlieb Y, Ghanim M, Gueguen G, Kontsedalov S, Vavre F, Fleury F *et al.* (2008). Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies. *FASEB J* **22**: 2591–2599.

Heath BD, Butcher RDJ, Whitfield WGF, Hubbard SF. (1999). Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. *Curr Biol* **9**: 313–316.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. (2008). How many species are infected with *Wolbachia*?—a statistical analysis of current data. *FEMS Microbiol Lett* **281**: 215–220.

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE *et al.* (2011). Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332**: 254–256.

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N *et al.* (2006). Evolution of male-killer suppression in a natural population. *PLoS Biol* **4**: 1643–1648.

Hornett EA, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD *et al.* (2008). You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. *Evolution* **62**: 1258–1263.

Huigens ME, De Almeida RP, Boons PAH, Luck RF, Stouthamer R. (2004). Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc R Soc Ser B* **271**: 509–515.

Huigens ME, Luck RF, Klaassen RHG, Maas MFPM, Timmermans MJTN, Stouthamer R. (2000). Infectious parthenogenesis. *Nature* **405**: 178–179.

Jaenike J, Polak M, Fiskin A, Helou M, Minhas M. (2007). Interspecific transmission of endosymbiotic Spiroplasma by mites. *Biol Lett* **3**: 23–25.

Jaenike J, Brekke TD. (2011). Defensive endosymbionts: a cryptic trophic level in community ecology. *Ecol Lett* **14**: 150–155.

Jiggins FM, Hurst GDD. (2011). Rapid insect evolution by symbiont transfer. *Science* **332**: 185–186.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S *et al.* (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647–1649.

Le Clec'h W, Chevalier FD, Genty L, Bertaux J, Bouchon D, Sicard M. (2013). Cannibalism and predation as paths for horizontal passage of *Wolbachia* between terrestrial isopods. *PLoS One* **8**: e60232.

Noda H, Miyoshi T, Zhang Q, Watanabe K, Deng K, Hoshizaki S. (2001). *Wolbachia* infection shared among planthoppers (Homoptera: Delphacidae) and their endoparasite (Strepsiptera: Elenchidae): a probable case of interspecies transmission. *Mol Ecol* **10**: 2101–2106.

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* **89**: 2699–2702.

Oliver KM, Degnan PH, Burke GR, Moran NA. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* **55**: 247–266.

Oliver KM, Moran NA, Hunter MS. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc Natl Acad Sci USA* **102**: 12795–12800.

Oliver KM, Russell JA, Moran NA, Hunter MS. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100**: 1803–1807.

Purcell AH, Suslow KG, Klein M. (1994). Transmission via plants of an insect pathogenic bacterium that does not multiply or move in plants. *Microbiol Ecol* **27**: 19–26.

Raychoudhury R, Baldo L, Oliveira DCSG, Werren JH. (2009). Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. *Evolution* **63**: 165–183.

Rigaud T, Juchault P. (1995). Success and failure of horizontal transfers of feminizing *Wolbachia* endosymbionts in woodlice. *J Evol Biol* **8**: 249–255.

Sakurai M, Koga R, Tsuchida T, Meng XY, Fukatsu T. (2005). *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont Buchnera. *Appl Environ Microb* **71**: 4069–4075.

Sasaki T, Kubo T, Ishikawa H. (2002). Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: a *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics* **162**: 1313–1319.

Scarborough CL, Ferrari J, Godfray HCJ. (2005). Aphid protected from pathogen by endosymbiont. *Science* **310**: 1781–1781.

Segoli M, Stouthamer R, Stouthamer CM, Rugman-Jones P, Rosenheim JA. (2013). The effect of *Wolbachia* on the lifetime reproductive success of its insect host in the field. *J Evolution Biol* **26**: 2716–2720.

Sintupachee S, Milne JR, Poonchaisri S, Baimai V, Kittayapong P. (2006). Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. *Microbiol Ecol* **51**: 294–301.

Skaljac M, Zanic K, Hrncic S, Radonjic S, Perovic T, Ghanim M. (2013). Diversity and localization of bacterial symbionts in three whitefly species (Hemiptera: Aleyrodidae) from the east coast of the Adriatic Sea. *Bull Entomol Res* **103**: 48–59.

Stahlhut JK, Desjardins CA, Clark ME, Baldo L, Russell JA, Werren JH *et al.* (2010). The mushroom habitat as an ecological arena for global exchange of *Wolbachia*. *Mol Ecol* **19**: 1940–1952.

Stamatakis A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Stansly PA, Naranjo SE. (2010). *Bemisia: Bionomic and Management of a Global Pest*. Springer: New York, NY, USA.

Teixeira L, Ferreira A, Ashburner M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6**: 2753–2763.

Tu LL, Zhang XL, Liu DQ, Jin SX, Cao JL, Zhu LF *et al.* (2007). Suitable internal control genes for qRT-PCR normalization in cotton fiber development and somatic embryogenesis. *Chinese Sci Bull* **52**: 3110–3117.

Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol Biol Evol* **16**: 1711–1723.

Watts T, Haselkorn TS, Moran NA, Markow TA. (2009). Variable incidence of *Spiroplasma* infections in natural populations of *Drosophila* species. *PLoS One* **4**: e5703.

Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc R Soc Ser B* **282**: 20150249.

Werren JH, Baldo L, Clark ME. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**: 741–751.

Werren JH, Zhang W, Guo LR. (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc R Soc Ser B* **261**: 55–63.

Yang XH, Zhu DH, Liu ZW, Zhao L, Su CY. (2013). High levels of multiple infections, recombination and horizontal transmission of *Wolbachia* in the *Andricus mukaigawae* (Hymenoptera; Cynipidae) communities. *PLoS One* **8**: e78970.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)