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RESEARCH ARTICLE

Plant-mediated horizontal transmission of *Rickettsia* endosymbiont between different whitefly species

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One sentence summary: Cotton plants can mediate the horizontal transmission of *Rickettsia* between whitefly species, and the transmission efficiencies vary with different whitefly species
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ABSTRACT

A growing number of studies have revealed the presence of closely related endosymbionts in phylogenetically distant arthropods, indicating horizontal transmission of these bacteria. Here we investigated the interspecific horizontal transmission of *Rickettsia* between two globally invasive whitefly species, *Bemisia tabaci* MEAM1 and *B. tabaci* MED, via cotton plants. We found both scattered and confined distribution patterns of *Rickettsia* in these whiteflies. After entering cotton leaves, *Rickettsia* was restricted to the leaf phloem vessels and could be taken up by both species of the *Rickettsia*-free whitefly adults, but only the scattered pattern was observed in the recipient whiteflies. Both the relative quantity of *Rickettsia* and the efficiency of transmitting *Rickettsia* into cotton leaves were significantly higher in MEAM1 females than in MED females. The retention time of *Rickettsia* transmitted from MEAM1 into cotton leaves was at least 5 days longer than that of MED. Phylogenetic analysis based on 16S rRNA and *gltA* genes confirmed that the *Rickettsia* extracted from the donor MEAM1, the cotton leaves, the recipient MEAM1 and the recipient MED were all identical. We conclude that cotton plants can mediate horizontal transmission of *Rickettsia* between different insect species, and that the transmission dynamics of *Rickettsia* vary with different host whitefly species.

Keywords: *Bemisia tabaci*; endosymbiont; *Rickettsia*; host plant; interspecies transmission

INTRODUCTION

Numerous arthropods are infected with various intracellular bacterial endosymbionts (Weinert, Araujo-Jnr and Ahmed 2015). These endosymbionts are maternally transmitted and can be classified as obligate (primary) endosymbionts and facultative (secondary) endosymbionts (Moran 2001; Baumann 2005; Chiel *et al.* 2007). Primary endosymbionts, such as *Buchnera* in aphids, *Portiera* in whiteflies and *Carsonella* in psyllids, are necessary for

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host survival and development because they provide essential nutrients that the host cannot obtain from its diet (Douglas 1998; Baumann 2005). In contrast, secondary endosymbionts are not essential for host survival and can have both parasitic and mutualistic effects on host biology (Kontsedalov *et al.* 2008; Giorgini *et al.* 2010). Normally, secondary endosymbionts are vertically transmitted from mother to offspring, but phylogenetic studies have revealed closely related endosymbionts in evolutionarily distant hosts, suggesting that horizontal transmission of endosymbionts may be occurring between different species of arthropods (Baldo *et al.* 2008; Ahmed *et al.* 2013; Gonella *et al.* 2015).

Among the secondary endosymbionts, *Rickettsia* bacteria, usually non-pathogenic in nature, are associated with about one-fourth of arthropod species (Weinert, Araujo-Jnr and Ahmed 2015). Recent data have suggested that *Rickettsia* can play a significant role in the biology of their hosts by influencing host reproduction (Hurst *et al.* 1994; Lawson *et al.* 2001; von der Schulenburg *et al.* 2001; Giorgini *et al.* 2010), increasing host tolerance to high temperature (Brumin, Kontsedalov and Ghanim 2011), protecting their hosts against fungal pathogens (Oliver *et al.* 2003; Ahmed *et al.* 2015b), increasing host fitness (Chiel *et al.* 2009a; Himler *et al.* 2011), increasing viral transmission efficacy (Kliot *et al.* 2014) and increasing their hosts' susceptibility to insecticides (Kontsedalov *et al.* 2008). The substantial effects of *Rickettsia* on host biology make its interspecific transmission routes a matter of great interest (Perlman, Hunter and Zchori-Fein 2006; Weinert *et al.* 2009). Over the past two decades, insect-associated bacteria, with their potential as biological control agents, have been suggested as novel strategies to manage insect pests. Studying the mechanism and probability of *Rickettsia* transmission between different arthropod species via host plants will help us to understand novel, unexpected dynamics of this remarkable and influential microorganism.

Transovarial maternal transmission in arthropod hosts has been suggested as a general transmission mechanism for *Rickettsia* and other secondary endosymbionts. However, recent surveys suggest that they can be transmitted horizontally between different individuals (Ahmed *et al.* 2013, 2015b). Caspi-Fluger *et al.* (2012) provided the first evidence of intraspecific horizontal transmission of *Rickettsia* in *Bemisia tabaci* populations via plants, demonstrating that plants can serve as a reservoir for horizontal transmission of *Rickettsia* in whiteflies. Brumin, Levy and Ghanim (2012) found that *Rickettsia* was not only distributed in the digestive and salivary organs, but also in the testicles and sperm thecae, suggesting the possibility of horizontal transmission of *Rickettsia* between different *B. tabaci* individuals via plant feeding and copulation. Interspecific horizontal transmission of a secondary endosymbiont was recently discovered by Gonella *et al.* (2015), who reported transmission of *Cardinium* between two species of leafhoppers via their host plant. In this study, we provide the first experimental evidence of interspecific horizontal transmission of *Rickettsia*.

The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) is a cosmopolitan polyphagous insect that feeds on phloem sap of numerous host plants (Stansly & Naranjo 2010). Recent studies have identified this pest as actually a species complex containing at least 34 cryptic species (Dinsdale *et al.* 2010; Hu *et al.* 2011; Lee *et al.* 2013) that differed genetically and biologically (Horowitz *et al.* 2005; Elbaz, Lahav and Morin 2010; Qiu *et al.* 2011; Jiao *et al.* 2012; Pan *et al.* 2012a). Previous studies have revealed that *B. tabaci* harbors a diverse array of endosymbionts, including the primary symbiont *Portiera aleyrodidarum* and several other facultative secondary symbionts such as *Arsenophonus*, *Cardinium*, *Hamiltonella*, *Rickettsia* and *Wolbachia*. These secondary endosymbionts are strongly correlated with whitefly species (Ahmed *et al.* 2010a; Pan *et al.* 2012b; Shan *et al.* 2014). Two members of the *B. tabaci* complex, putatively called Middle East Asia Minor 1 (MEAM1, formerly B biotype) and Mediterranean (MED, formerly Q biotype), which are biologically differentiated and reproductively isolated from each other (Horowitz *et al.* 2005; De Barro *et al.* 2011), have invaded globally over the past two decades and have become the two most widespread and damaging whiteflies. In China, these two invasive species are commonly found together in the field (Hu *et al.* 2011). Here, we use standard and real-time PCR and fluorescence *in situ* hybridization (FISH) to demonstrate that interspecific horizontal transmission of *Rickettsia* occurs between these two species of whitefly, via cotton plants.

MATERIALS AND METHODS

Plant and whitefly culture

The cotton plant *Gossypium hirsutum* L. (var. Lumianyan no. 32) was 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) and their seedlings were cultured at ambient temperature and photoperiod in a greenhouse. The plants were watered as needed and were used for experiments at the six- to eight-expanded-leaf stage.

The MEAM1 *B. tabaci* used in this study was originally collected from eggplant (*Solanum melongena*) in 2006 at the training farm of South China Agricultural University (SCAU) in Guangzhou, while the MED *B. tabaci* was collected from pepper plants (*Capsicum frutescens*) in 2006 in Nantong, Jiangsu. Both populations were then reared on hibiscus (*Hibiscus rosa-sinensis*) plants in separate greenhouses at SCAU with ambient temperature, photoperiod and humidity. Their colonies were monitored monthly by sequencing the mitochondrial COI gene, following the protocol used in Qiu *et al.* (2009).

Detection of *Rickettsia* in the MEAM1 and MED whiteflies

To detect the presence of *Rickettsia* in MEAM1 and MED *B. tabaci* greenhouse populations, whitefly adults were individually homogenized in lysis buffer and DNA was extracted and amplified using the protocol described in Ahmed *et al.* (2010b). The primers used for *Rickettsia* detection were *Rb-F* and *Rb-R* for the 16S rRNA gene (Supplementary Table S1). The PCR procedure entailed predenaturation at 94°C for 2 min followed by 32 cycles of 94°C (1 min), 58°C (45 s) and 72°C (45 s). All PCRs were performed in a 25 μl reaction volume that included 2.5 mM $MgCl_2$, 200 mM of each dNTP, 1 μM of each primer and 1 unit DNA Taq polymerase (Invitrogen, Guangzhou, China). PCR amplified products were visualized on a 1% agarose gel containing GoldView colorant. When bands with the expected size were visible on the gels, 20 μl volumes of PCR products were sent to Beijing Genomics Institute (BGI) for sequencing. Thirty female adults were sampled for each of the two whitefly species (three replicates of 10 specimens) for a total of 60 specimens. *Portiera aleyrodidarum* DNA was used as a positive control and ddH_2O was used as a negative control to eliminate potential confounding variables.

Subcolonies of *Rickettsia*-positive and *Rickettsia*-negative whitefly populations

After completion of the *Rickettsia* detection experiment, a *Rickettsia*-positive (R^+) MEAM1 subcolony and *Rickettsia*-negative (R^-) MEAM1 and MED subcolonies were set up for further experiments, following the protocol of Ahmed *et al.* (2015b). An R^+ MED subcolony was not set up because all observed MED individuals were found to be *Rickettsia* negative. The infection status of *Rickettsia* in these subcolonies was checked monthly using PCR with the *Rickettsia* 16S rRNA gene.

Rickettsia transmission from R^+ whiteflies to cotton plant and its visualization

Thirty pairs of 24–48 h-old MEAM1 adults were collected from the R^+ subcolony using an aspirator. Adults were released into a leaf cage (4 cm diameter × 2 cm height) covering abaxially the blade of clean cotton leaves, with the entry hole subsequently blocked with a small cotton ball to prevent whiteflies from escaping (Supplementary Fig. S1). After 2 weeks of R^+ MEAM1 whitefly feeding, pieces of cotton leaves (10 mm × 5 mm, ~0.01 g) inside the leaf cage were cut longitudinally, starting from the leaf edge and along the leaf vein, and analyzed for *Rickettsia* presence and localization using FISH. FISH was also performed on 50–60 third instar nymphs of R^+ MEAM1 whitefly that were randomly collected from the donor subcolonies. The leaves and nymphs were placed in Carnoy's fixative, and FISH was performed with the symbiont-specific 16S rRNA for *Rickettsia* (Rb1-Cy5: 5′-TCCACGTCGCCGTCTTGC-3′) using the method described by Gottlieb *et al.* (2006). Stained whitefly and cotton leaf samples were mounted and viewed under a Nikon eclipse Ti-U inverted microscope. Healthy cotton leaves without whitefly infestation and R^- MED whitefly specimens were used as negative controls.

Rickettsia acquisition of R^- whiteflies from plants

Fifty pairs of 24–48 h-old R^- MED and 50 pairs of R^- MEAM1 *B. tabaci* adults (one male and one female per pair) were randomly collected and introduced into leaf cages (10 pairs per leaf cage) containing R^+ cotton leaves. These leaves had been fed upon continuously by 15–20 pairs of R^+ MEAM1 whitefly adults for at least 20 days to ensure the high deposition of *Rickettsia* into leaf tissues. All the leaves were cleaned and carefully examined under a microscope to ensure that all R^+ whitefly nymphs and eggs had been removed before R^- MED or MEAM1 were introduced. Once the R^- whiteflies were introduced, they were given 24 h to mate and oviposit before being removed. Once the offspring developed into third-instar nymphs, 10 of them were randomly selected from each cage for *Rickettsia* PCR detection, using the method described in Ahmed *et al.* (2010b), and 10 more were randomly selected for *Rickettsia* FISH visualization.

Vertical transmission of *Rickettsia*

Twenty pairs of R^- MED adults were introduced into 20 leaf cages (one pair per cage) containing R^+ cotton leaves, and were given 24 h to mate and oviposit before removal. Once the offspring developed into third-instar nymphs, all but 10 of the nymphs were removed from each leaf cage. Then, five nymphs from each cage were randomly selected for *Rickettsia* PCR detection; if all of them were *Rickettsia* positive, the remaining five nymphs were recorded as *Rickettsia* positive and allowed to mature (deemed as R^+ recipient MED *B. tabaci* F0 adults, 24–48 h old). After eclosion, the F0 adults were mixed and 10 pairs of them were collected randomly and introduced into 10 leaf cages (one pair per cage) that contained new healthy cotton leaves. The adults were given 24 h to oviposit before removal, and the F1 progeny were selected for *Rickettsia* detection by PCR. The R^- MEAM1 individuals were used as controls in these experiments. Each PCR and FISH detection experiment was repeated five times.

Quantitative detection of *Rickettsia* in MEAM1 and MED whiteflies

Quantitative real-time PCR was used to detect the titer of *Rickettsia* in the donor MEAM1, recipient MEAM1 and MED *B. tabaci* adults, following the protocol of Ghanim and Kontsedalov (2009). The DNA of R^+ adult was extracted from 10 females of each species (Ahmed *et al.* 2010b). The primers used for *Rickettsia* qRT-PCR detection were gltA-QF and gltA-QR, and a β-actin whitefly gene was selected as an internal control for data normalization and quantification (Ghanim & Kontsedalov 2009). The variance of the relative quantity of *Rickettsia* across the donor and recipient whiteflies was analyzed by running an ANOVA and Tukey's test, using SAS (9.02).

Rickettsia transmission from R^+ whiteflies to cotton and its persistence in cotton

Two different experimental set-ups were used. In the first, five pairs of 24–48 h-old *B. tabaci* adults were collected from the R^+ subcolonies using a hand aspirator and released into separate leaf cages (4 cm diameter × 2 cm height) containing new clean cotton leaves. In the second set-up, 10 pairs of whitefly adults were used. After 24 h, approximately 0.01 g of leaf material was cut from the leaves every day until *Rickettsia* was positively detected in plant leaves, and the cotton leaf DNA was extracted using the cetyl trimethylammonium bromide method. The initial time that *Rickettsia* was transmitted from R^+ whiteflies to cotton plants was detected using PCR, as described previously. The DNA of the primary endosymbiont *Portiera aleyrodidarum* and ddH_2O were used as positive and negative controls, respectively, in the PCR reactions. Ten replicates were performed for each of the four set-ups (MEAM1 and MED whiteflies, five- and 10-pair set-ups). The overall experiment was repeated three times.

In order to detect the persistence of *Rickettsia* in cotton plants after it was transferred from MEAM1 and MED whiteflies, 30 pairs of 24–48 h-old R^+ adults from each species were released into a leaf cage to feed on the cotton leaves for 15 days (primary detection revealed that *Rickettsia* is detectable after 30 pairs of R^+ MEAM1 or MED adults continuously feed on cotton leaves for 12 days). Afterwards, all the whiteflies were removed. Approximately 0.01g of cotton leaf was cut from one edge of these R^+ cotton plants longitudinally every 5 days, and DNA was extracted and used for *Rickettsia* qRT-PCR detection with the aforementioned *gltA* primers. The detections continued for 30 days from the first sampling day. Healthy cotton leaves without whitefly or other arthropod infestation were used as negative controls in these qRT-PCR detections. Experiments were repeated three times.

Phylogenetic analysis of *Rickettsia* in whiteflies and cotton plants

To assess the similarity of the *Rickettsia* strains in R^+ donor MEAM1 *B. tabaci*, R^- recipient MEAM1 and MED *B. tabaci*, and R^+ cotton plant leaves, two *Rickettsia* genes, the 16S rRNA and

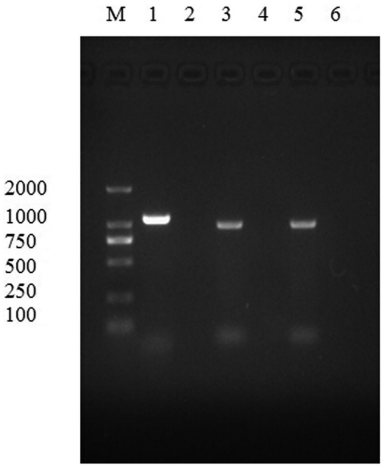

Figure 1. The detection of *Rickettsia* in whitefly populations. M, DNA marker; lane 1, positive control (16S rRNA gene of *Portiera*); lane 2, negative control (ddH_2O); lane 3–4, *Rickettsia* positive and negative subcolonies of MEAM1; lane 5–6, *Rickettsia* positive and negative subcolonies of MED.

citrate synthase (*gltA*) (Supplementary Table S1), were extracted following the procedure described in Caspi-Fluger *et al.* (2012). All DNA fragments were PCR amplified in a 25 μl reaction volume that contained 2.5 mM $MgCl_2$, 200 mM of each dNTP, 1 μM of each primer and 1 unit DNA Taq polymerase (Invitrogen), and PCR products were sent to BGI for sequencing after the expected bands became visible on 1% agarose gels.

All the DNA sequences were edited and aligned manually using Clustal X1.83 (Thompson *et al.* 1997) in Mega 5 (Tamura *et al.* 2011), then concatenated using SequenceMatrix (Windows 1.7.8). Genetic distances were calculated using Mega 5. The best model and partitioning scheme were chosen using the Bayesian information criterion in PartitionFinder v. 1.0.1 (Lanfear *et al.* 2012). Phylogenetic analysis was undertaken using maximum likelihood (ML) in RAxML (Stamatakis 2006). To estimate the best ML tree in RAxML, we used the '-f a' option to estimate 1000 bootstraps and perform a likelihood search, as well as 200 '-f d' searches that started from randomly generated parsimony trees. We selected the tree with the best likelihood score as the best tree and used RAxML to calculate bootstrap values. The tree was viewed and edited in FigTree v. 1.4.0. Six different strains of *Rickettsia* from eight arthropod species were included in the phylogenetic analysis, and *Orientia* was used as an outgroup (Supplementary Table S2). All the 16S rRNA, *gltA* and *Pgt* gene sequences were submitted to GenBank (Supplementary Table S3).

RESULTS

Rickettsia detection in MEAM1 and MED whiteflies

PCR detection showed that the MEAM1 *B. tabaci* population is *Rickettsia* positive. The screening of 30 individuals of MEAM1 adults showed that 58.7 ± 3.6% of them were infected with *Rickettsia*, whereas all the detected MED individuals were *Rickettsia* free. The PCR results indicated that we successfully set up a *Rickettsia*-negative MEAM1 subcolony for our transmission experiment (Fig. 1).

FISH detection of *Rickettsia* in R^+ whitefly and cotton plant

When R^+ MEAM1 *B. tabaci* fed upon cotton leaves, *Rickettsia* was successfully transmitted from the whiteflies into the cotton leaves. FISH visualization showed that this bacterium was located inside the phloem vessels of plant leaves; no *Rickettsia* was found outside of the phloem (Fig. 2a and b). No fluorescence of *Rickettsia* was visualized in the new cotton leaves (which were never exposed to whiteflies) or in the cotton leaves exposed to R^- whiteflies (Supplementary Fig. S2).

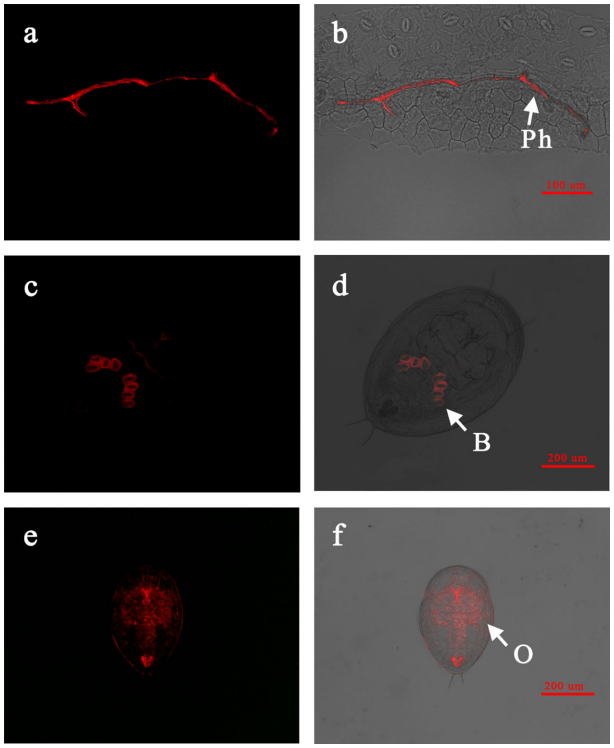

Figure 2. FISH visualization of *Rickettsia* in cotton leaf (**a**, **b**) and donor whitefly nymphs (**c–f**). Ph: phloem vessel of plant leaf; B: bacteriocyte in which *Rickettsia* is localized; O: other tissue of whitefly nymph. (c, d) *Rickettsia* confined in MEAM1 whitefly nymphs; (e, f) scattered *Rickettsia* in MEAM1 whitefly nymphs. Left panels: fluorescence in dark field; right panels: fluorescence in bright field.

The R^+ whitefly nymphs exhibited two different distribution patterns of *Rickettsia*: scattered and confined patterns. In the confined pattern, *Rickettsia* was restricted to the bacteriocytes in the abdomen of the host (Fig. 2c and d); this was observed only in MEAM1 whiteflies. In the scattered pattern, *Rickettsia* was localized throughout the hemocoel and other organs of *B. tabaci* simultaneously, but excluding the bacteriocytes that were found in the abdomen of the host (Fig. 2e and f). The scattered pattern was observed in both MEAM1 and MED whiteflies.

Rickettsia acquisition for R^- whiteflies from plants and its vertical transmission

After R^- MED and R^- MEAM1 adults (i.e. the recipients) fed on the R^+ cotton leaves (i.e. the leaves that had previously been fed on by R^+ MEAM1 whitefly adults), *Rickettsia* was detected in third-instar nymphs of both species of recipients, with an infection frequency of 78 ± 5.8% in MED (39/50 in total) and 86 ± 5.1% in MEAM1 (43/50 in total). Interestingly, only the scattered pattern of *Rickettsia* was found in both recipient species. Unexpectedly, PCR results revealed that all the F1 progeny of both species of recipient whiteflies were *Rickettsia* negative (Table 1).

Table 1. The infection percentage of *Rickettsia* in the recipient whiteflies and their F1 progeny. Data were analyzed by *t* test; there is no significant difference between the infection rates of *Rickettsia* in recipient MEAM1 and MED *B. tabaci* populations.

Whitefly species	Infection in recipient whitefly adults (%)	Infection in the F1 nymphs of recipient (%)
MEAM1	86 ± 5.10	0
MED	78 ± 5.80	0
t	1.21	
P	0.294	

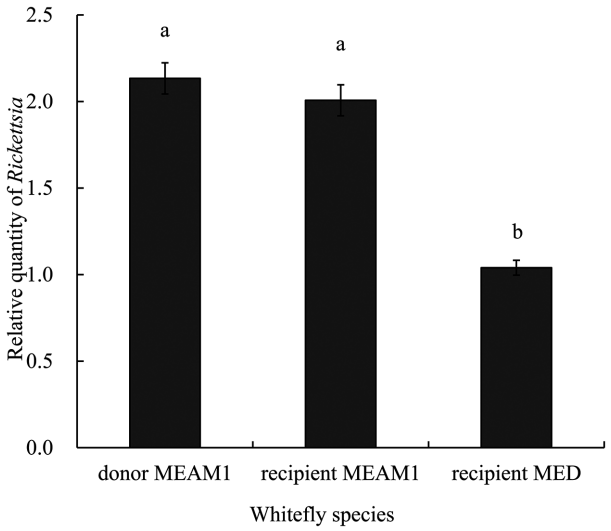

Figure 3. The quantity of *Rickettsia* in R^+ donor MEAM1 and recipient MEAM1 and MED whiteflies. Taking a β-actin gene as house-keeping gene, qRT-PCR results revealed that the relative quantity of *Rickettsia* in donor MEAM1 is similar to recipient MEAM1 adults, but approximately two times that of recipient MED adults. The relative quantity of *Rickettsia* was calculated using the method of $2^{-\Delta\Delta Ct}$. The column and error bars represent the fold change (titer) as mean ± SE, and three replicates of each qRT-PCR were used in each column. There was a significant difference among these three ($F_{2,27} = 67.13$, $P < 0.0001$).

Quantitative detection of *Rickettsia* in whiteflies

Taking a β-actin gene from *B. tabaci* as a house-keeping gene, the qRT-PCR results revealed that the titer of *Rickettsia* between the donor and recipient whiteflies varied significantly ($F_{2,27} = 63.13$, $P < 0.0001$). The relative quantity of *Rickettsia* in donor MEAM1 is similar to that of recipient MEAM1 adults, but approximately two times greater than in recipient MED adults (Fig. 3).

Rickettsia transmission from R^+ whiteflies to cotton plant and its persistence

The R^+ MEAM1 and MED *B. tabaci* showed different horizontal transmission efficiencies. In the five-pair experimental set-up, *Rickettsia* was initially detected in cotton leaves after 21.8 days in the MEAM1 cages and 32.8 days in the MED cages. In the 10-pair set-up, the times for initial detection decreased to 17.2 and 28.2 days, respectively (Fig. 4). Our results suggest that the transmission efficacies of *Rickettsia* may be related to the species being fed upon. However, in the case of the same species, a higher number of *B. tabaci* adults feeding on the plant may lead to a higher titer of *Rickettsia* in the host plant in a given time period.

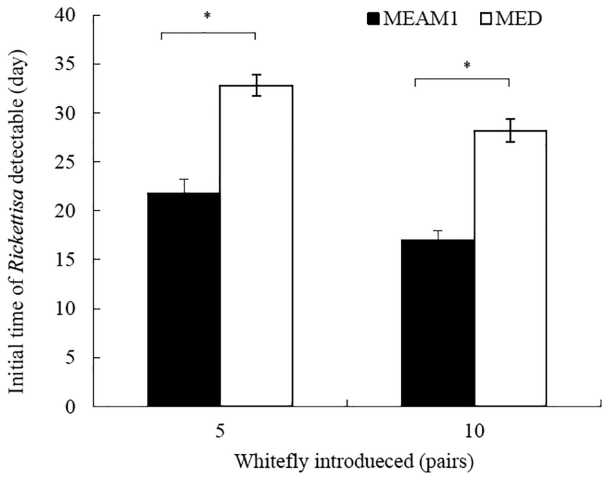

Figure 4. The time *Rickettsia* was first detected in cotton plants. PCR detection results reveal that the transmission efficacies of *Rickettsia* were correlated with the number of whitefly adults fed on plant leaves. The asterisks on the bars indicate a significant difference between the initial detectable time of *Rickettsia* in the MEAM1 and MED whitefly ($t = -52.18$, $P < 0.0001$ for five-pair experiment and $t = -38.54$, $P < 0.0001$ for 10-pair experiment on different cotton plants).

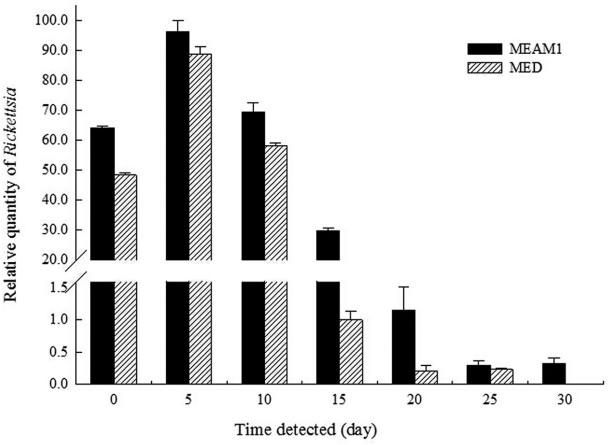

Figure 5. The retention time of *Rickettsia* in cotton leaves infested by MEAM1 and MED whiteflies. *Rickettsia* transmitted from MEAM1 can persist at higher quantity in the cotton leaves for first 15 days and is eventually lost on the 30th day; *Rickettsia* from MED has the same retention dynamics but was lost 5 days earlier than that from MEAM1 whiteflies.

Once *Rickettsia* was transmitted into the cotton leaves, the titer of *Rickettsia* increased during the first 5 days, then gradually decreased, but the retention time in the host plant varied between species of donor whitefly. Compared with the titer quantity of cotton housekeeping gene *UBQ7*, the relative quantity of *Rickettsia* transmitted from MEAM1 that persisted in cotton leaves was 29.86–96.14 times higher during the first 15 days, followed by a sharp reduction and a complete absence from cotton by the 30th day. In contrast, the *Rickettsia* transmitted from MED persisted in cotton leaves for 25 days, with a relative titer quantity ranging from 57.98 to 88.80 times higher before a sharp reduction at day 15 (Fig. 5). This shows that the MEAM1 recipients from donor R^+ MEAM1 may have a higher relative *Rickettsia* retention than their MED counterparts, which received the same *Rickettsia* from R^+ MEAM1. The intraspecific transmission of *Rickettsia* (from R^+ MEAM1 to R^- MEAM1) might be for the *Rickettsia* a lesser change in cellular environment than transmission to a

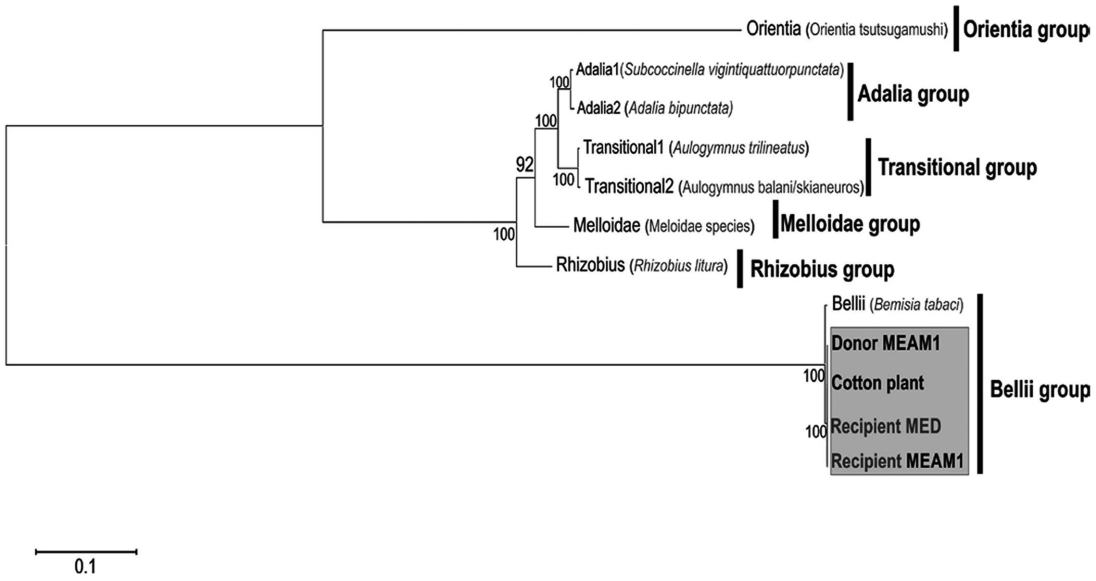

Figure 6. Maximum likelihood phylogenetic analysis of *Rickettsia* from different hosts using concatenated sequences of 16S rRNA, *gltA* and *Pgt* gene. *Rickettsia* from donor MEAM1, R^+ cotton leaf, recipient MEAM1 and MED whiteflies are clustered into one branch within *Bellii* group.

different species (from R^+ MEAM1 to R^- MED). Our results reveal that the retention time of *Rickettsia* in cotton differs depending on the species of whitefly used for transmission, but the reason for this variation is currently unclear.

Phylogenetic analysis of *Rickettsia* in whiteflies and cotton plant

All the *Rickettsia* sequences of 16S rRNA, *gltA* and *Pgt* genes are shown in Supplementary Table S3. Genetic analysis indicated that the *Rickettsia* in the R^+ donor MEAM1, the R^+ cotton leaves, and the recipient MEAM1 and MED were all identical. In the phylogenetic tree, all these *Rickettsia* were clustered into one branch with the *Rickettsia* from *B. tabaci* belonging to the *Bellii* group, while the *Rickettsia* symbionts from other arthropod hosts were clustered into branches belonging to separate *Rickettsia* groups (Fig. 6). Our genetic analysis revealed that the *Rickettsia* symbionts in our study were highly conserved during the plant-mediated horizontal transmission.

DISCUSSION

The routes and mechanisms of endosymbionts' horizontal transmission have become the hotspots of symbiont research over the last two decades (Purcell, Suslow and Klein 1994; Sintupachee *et al.* 2006; Jaenike *et al.* 2007; Chiel *et al.* 2009b; Hirunkanokpun *et al.* 2011; Caspi-Fluger *et al.* 2012; Woodbury, Moore and Gries 2013; Yang *et al.* 2013; Zhang, Han and Hong 2013). Recently, the plant-mediated horizontal transmission between conspecific individuals was investigated (Caspi-Fluger *et al.* 2012; Li *et al.* 2016), but until now, it has been unclear as to whether *Rickettsia* can also be transmitted between different species feeding on the same host plant. Our study provides novel findings about the horizontal transmission of *Rickettsia*. We report the horizontal transmission of *Rickettsia* between two different cryptic species, MEAM1 and MED, via cotton plants; previous studies have revealed that these two species are biologically differentiated and reproductively isolated (Horowitz *et al.* 2005; Elbaz, Lahav and Morin 2010; De Barro *et al.* 2011; Jiao *et al.* 2012; Pan *et al.* 2012a). We also compared the transmission efficiency of *Rickettsia* from a host plant to two recipient *B. tabaci* species, and we find that the ability of MEAM1 to pick up *Rickettsia* from cotton plants was much higher than that of MED. Finally, we demonstrate a certain variation in retention time of *Rickettsia* in cotton plants, depending on the recipient *B. tabaci* species; the retention time in cotton leaves of *Rickettsia* that were input by MEAM1 is at least 5 days longer than for those input by MED. This might be due to higher pick-up ability or higher retention capacity of MEAM1 as compared with MED. This could be clarified by testing the *Rickettsia* titer load in salivary glands of these two species in the future.

The invasive MEAM1 and MED *B. tabaci* were first recorded in China in the mid-1990s and the early 2000s, respectively. Both of them have spread throughout most provinces of China, but the endosymbiont infection status in these two *B. tabaci* species varied significantly in different geographical populations (Pan *et al.* 2012b). For example, in the case of *Rickettsia*, infection rates in MEAM1 and MED *B. tabaci* were 70.2% and 4.1%, respectively, in Shandong province (Chu *et al.* 2011), and 100% and 0%, respectively, in Zhejiang province (Shan *et al.* 2014). The infection rate of *Rickettsia* in MEAM1 in our study was 58.7%, which is much lower than the rates in Shandong and Zhejiang provinces. In a study from the USA, *Rickettsia* infection rates in MEAM1 increased from 1% in 2000 to 51% in 2003 and to 97% in 2006 (Himler *et al.* 2011). The parasitoid-vectored or plant-mediated interspecies horizontal transmission is considered responsible for this increase in infection frequencies (Vavre *et al.* 1999; Chiel *et al.* 2009b; Caspi-Fluger *et al.* 2012; Ahmed *et al.* 2015b). Caspi-Fluger *et al.* (2012) reported plant-mediated *Rickettsia* horizontal transmission within one whitefly species (MEAM1), and before this Purcell, Suslow and Klein (1994) revealed plant-mediated transmission of another bacterial endosymbiont between leafhoppers via feeding on the same host plant. Moreover, the phylogenetic study conducted by Sintupachee *et al.* (2006) proposed that plant-mediated horizontal transmission is responsible for a shared *Wolbachia* strain among taxonomically distant species

through feeding on the same host plant at the same time. However, the biology of plant-mediated horizontal transmission was poorly understood before this study.

Our FISH visualization results showed that *Rickettsia* was restricted in the phloem vessels of cotton leaves after input by its whitefly host. *Rickettsia* did not cause any visible pathogenic symptoms in the cotton plant; these results were similar to those of Caspi-Fluger *et al.* (2012). Purcell, Suslow and Klein (1994) reported that the bacterial endosymbiont cannot move within plants. However, Caspi-Fluger *et al.* (2012) assumed that *Rickettsia* can move within the phloem and can be found near the feeding sites in whitefly-free plant parts. In this study, *Rickettsia* could also be detected by PCR and FISH in the lower adjacent leaf, which was never fed on by whiteflies (S-J. Li, PhD dissertation, unpublished data), and thus we speculate that *Rickettsia* can move along the phloem vessel in plants. It would be interesting to investigate the speed of *Rickettsia* propagation after initial infection in plant tissues in a future study.

Our current study suggested that the relative quantity of *Rickettsia* endosymbiont in cotton leaves increased in the first 5 days, and reduced gradually thereafter. Caspi-Fluger *et al.* (2012) found that *Rickettsia* transmitted from whiteflies to the cotton-leaf phloem remained alive, and our study also indicated that *Rickettsia* was alive and might have self-multiplied in plant tissues. After *Rickettsia* has been introduced into the plant leaves, host plants may need a few days to produce, accumulate and transport some chemical compounds to defend or even eliminate the invading endosymbiont (P-Q. Shi and B-L. Qiu, unpublished data). Inactivation through defensive chemicals reducing the multiplication speed of *Rickettsia* might be the reason for the decrease in the quantity of *Rickettsia* in plant leaves (P-Q. Shi and B-L. Qiu, unpublished data). However, this deduction, together with the absence of *Rickettsia* in the F1 progeny of recipient whiteflies, needs to be further investigated.

The distribution patterns of *Rickettsia* in insects may vary between host species (Sokolova, Zinkevich and Zakharov 2002; Sakurai *et al.* 2005; Gottlieb *et al.* 2006; Gottlieb *et al.* 2008; Caspi-Fluger *et al.* 2011), and there are two distribution patterns of *Rickettsia* reported in MEAM1: the scattered pattern, in which *Rickettsia* is located throughout the whitefly hemocoel (apart from bacteriocytes), and the confined pattern, in which *Rickettsia* is restricted to the bacteriocytes (Caspi-Fluger *et al.* 2011). In this study, both of these patterns were found in the MEAM1 *B. tabaci*, among which the confined pattern is around 70%, and the scattered pattern is approximately 30%, but the MED *B. tabaci* only showed the confined pattern. We suspect that the scattered pattern is perhaps the result of acquisition of *Rickettsia* infection via plant-mediated horizontal transmission from other whitefly species like MEAM1. This is further reflected by field surveys in which *Rickettsia* infection frequency in MED remains between 0% and 4.1% in China (Chu *et al.* 2011; Shan *et al.* 2014).

The different localization patterns of *Rickettsia* may give us some clues to its possible transmission routes. For example, by being localized in the reproductive system of its whitefly host, the confined *Rickettsia* symbiont may remain stable and maternally transmit to the whitefly offspring. In the scattered localization pattern, the absence of infection in ovarian tissues of recipient adult whiteflies may explain the inability of *Rickettsia* to be transmitted vertically from mother to offspring. However, if *Rickettsia* is scatter-localized in the salivary glands, it has a higher opportunity to be delivered to plant phloem during feeding and can then be picked up by a recipient whitefly feeding on the same plant leaves (Caspi-Fluger *et al.* 2012, and our current study). Moreover, when localizing in the hemolymph of its whitefly host, *Rickettsia* may have a high probability of being transported by a parasitoid wasp during probing; similar examples of phoresy were previously demonstrated with the endosymbiont *Wolbachia* (Ahmed *et al.* 2015b). In addition, Ahmed *et al.* (2015b) found that a *Wolbachia* strain remained unchanged after horizontal transmission between two insect hosts, even when transmission occurs between two different species. Similarly, our phylogenetic analysis of *Rickettsia* strains in the donor R^+ MEAM1, the R^+ cotton plant, and the recipient MEAM1 and MED revealed that symbionts remained identical during horizontal transmission, underlying the fidelity of symbionts. Although *Rickettsia* is unlikely to evolve in such a short duration, it was important to make sure that the *Rickettsia* strain that recipient whiteflies picked up was identical to the one delivered by donor whiteflies, and to assess whether it remained conserved without becoming mutated due to intercellular interaction with the defensive chemicals released by plants when responding to invading bacteria.

To summarize, we explored the distribution patterns and infection biology of the endosymbiont *Rickettsia* in two members of the *B. tabaci* complex (MEAM1 and MED) and provided novel evidence for the plant-mediated horizontal transmission of a bacterial endosymbiont between two different host species. The ecological dynamics of an endosymbiont in its interspecific horizontal transmission and the interactions of a host plant, insect and endosymbiont could be the focus of future bacterial endosymbiont research. This will help elucidate why the endosymbionts are so abundant in arthropod communities and why phylogenetically distinct arthropods often harbor closely related endosymbionts in nature (Ahmed *et al.* 2013; Weinert, Araujo-Jnr and Ahmed 2015; Ahmed *et al.* 2015a,b). Novel strategies of symbiont-based pest control technology require a deeper understanding of the symbiont dynamics during insect–plant interactions.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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AUTHOR CONTRIBUTIONS

YHL, MZA, SJL, XSC, NL and PQS carried out the experiments, participated in data analysis and carried out sequence

alignments; YHL, SJL and BLQ carried out the statistical analyses; BLQ and MZA designed the study, and wrote the manuscript. All authors gave final approval for publication.

Conflict of interest. None declared.

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