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Tripartite associations among bacteriophage WO, *Wolbachia*, and host affected by temperature and age in *Tetranychus urticae*

Ming-Hong Lu · Kai-Jun Zhang · Xiao-Yue Hong

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Abstract A phage density model of cytoplasmic incompatibility (CI), which means lytic phages reduce bacterial density associated with CI, significantly enhances our understanding of the tripartite associations among bacteriophage WO, *Wolbachia* and host. However, WO may alternate between lytic and lysogenic life cycles or change phage production under certain conditions including temperature, host age and host species background. Here, extreme temperatures can induce an alteration in the life cycle of WO and change the tripartite associations among WO, *Wolbachia* and CI. Based on the accumulation of the WO load, WO can transform into the lytic life cycle with increasing age. These findings confirmed that the environment plays an important role in the associations among WO, *Wolbachia* and host.

Keywords Two-spotted spider mite · WO · *Wolbachia* · Temperature · Age

Introduction

Wolbachia are maternally inherited obligatory intracellular symbionts that infect a wide range of arthropods and filarial nematodes (Werren 1997; Bandi et al. 1998; Stouthamer et al. 1999). Current data show that *Wolbachia* are estimated to infect 66 % of insect species (Hilgenboecker et al. 2008), and they are likely the most prevalent obligate intracellular symbionts. *Wolbachia* have been divided into eleven supergroups (A-K) using three protein-coding genes (*ftsZ*, *gltA* and *groEL)* and the 16S rRNA gene. The spider mite harbors B-group *Wolbachia* except for *Bryobia* species V (Ros et al. 2009). In arthropod hosts, *Wolbachia* mainly reside in ovaries and testes (Zabalou et al. 2008) and can induce various reproductive disorders, such as cytoplasmic incompatibility (CI), thelytokous parthenogenesis, feminization and male killing (Werren et al. 1995; Hurst et al. 1999; Sasaki and Ishikawa 1999; Riegler and O'Neill 2006). However, the molecular mechanisms and genes responsible for these phenomena are still unknown, and the absence of

M.-H. Lu · K.-J. Zhang · X.-Y. Hong (✉)
Department of Entomology, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China
e-mail: xyhong@njau.edu.cn

any correlation between *Wolbachia* phylogeny and the effects they induce in hosts have led several authors to speculate on the evolution of *Wolbachia*-induced phenotypes (Stouthamer et al. 1999). One hypothesis that has been posed is that the genes responsible for *Wolbachia* incompatibility are conveyed by extra-chromosomal particles, such as plasmids or phages (Stouthamer et al. 1999; Fujii et al. 2004). No plasmid has been detected in *Wolbachia*, but a bacteriophage-like genetic element of *Wolbachia* has been identified and has been named bacteriophage WO (Masui et al. 2000).

Bacteriophage WO is widespread in the genus, and harbored by 89 % of *Wolbachia* (Gavotte et al. 2007; Bordenstein and Wernegreen 2004), which is due to the vertical transmission and horizontal transfer. Phage vertical transmission appears stable across insect generations (Gavotte et al. 2004). Horizontal transfer of WO could occur either between several *Wolbachia* infections in a single host or, hypothetically, by paternal transmission of phage particles to a fertilized egg that harbors a phage-free *Wolbachia* strain (Kent and Bordenstein 2010). Based on phylogenetic analyses, a lack of congruency between *Wolbachia* and phage WO phylogenies indicates that WO is able to successfully transfer itself horizontally between different insects, with or without its bacterial host (Gavotte et al. 2007; Masui et al. 2000; Bordenstein and Wernegreen 2004; Chafee et al. 2010).

The absence of a relation between phage phylogeny based on *orf7* sequences and the effects induced by *Wolbachia* suggests that WO is not directly involved in these effects (Gavotte et al. 2007). Furthermore, several studies have demonstrated a negative correlation between WO density and *Wolbachia* density (Ahantarig et al. 2008; Bordenstein et al. 2006), and a positive correlation between *Wolbachia* density and CI (Bordenstein et al. 2006). Based on these, the Phage Density Model (Bordenstein et al. 2006) is proposed: (a) when phage WO is lysogenic and titers of *Wolbachia* are high in male reproductive tissues, CI intensity is high after mating with an uninfected female; (b) when phage WO in these *Wolbachia* becomes lytic, *Wolbachia* cell titers decrease as a result of cell lysis and cause the CI intensity also decreased. However, WO can be either lysogenic or lytic and can possibly alternate lytic and lysogenic life cycles, but the genetic mechanisms that drive prophage induction and lytic activity are currently unknown. The hypothesis that has been posed is that WO might change phage production or alternate lytic and lysogenic life cycles under certain conditions including temperature, host age and host species background (Kent and Bordenstein 2010). Except for the indirect effect on CI, WO may also directly code for proteins, such as the proteins involved in antibiotic resistance or toxins, which are a valuable auxiliary for bacteria and host arthropods (Miao and Miller 1999; Duron et al. 2006; Degnan and Moran 2008; Oliver et al. 2009; Tanaka et al. 2009; Kent and Bordenstein 2010).

To better understand the tripartite associations among WO, *Wolbachia* and host, the diversity and evolutionary dynamics of WO—*Wolbachia* associations were studied. The tripartite associations among WO, *Wolbachia*, and CI under different temperature conditions were analyzed using the two-spotted spider mite *Tetranychus urticae* Koch as the subject. The association between WO and *Wolbachia* on the effect of age was also studied.

Materials and methods

Insect strains and rearing

The two-spotted spider mite (red form) was collected from different geographic regions in China in 2008 and 2009 (Table 1). The mites were examined carefully by professional

Table 1 The collection records and infect status of two-spotted spider mites

Location	Abbr.	Host Plant	Collection date	Latitude Longitude	*Wolbachia* strains	WO types
Changchun City, Jilin Province	CC	*Solanum melongena*	August, 2009	43°54′N 125°19′E	*w*UrtOri2	WO*w*Urt1
Beijing	BJ	*Vigna unguiculata*	August, 2009	39°56′N 116°18′E	+	−
Qingtongxia City, Ningxia autonomous region	QTX	*Solanum melongena*	July, 2009	38°01′N 106°04′E	+	−
Handan City, Hebei Province	HD	*Vigna unguiculata*	August, 2008	36°36′N 114°28′E	+	−
Taiyuan City, Shanxi Province	TY	*Solanum melongena*	August, 2009	37°30′N 111°50′E	*w*UrtOri3	WO*w*Urt1
Yuncheng City, Shanxi Province	YC	*Solanum melongena*	August, 2008	35°01′N 110°59′E	*w*UrtOri3	WO*w*Urt1
Tianshui City, Gansu Province	TS	*Solanum melongena*	August, 2008	34°37′N 105°42′E	+	−
Xi'an City, Shănxi Province	XA	*Solanum melongena*	August, 2008	34°39′N 109°14′E	+	−
Zhengzhou City, Henan Province	ZZ	*Vigna unguiculata*	August, 2009	34°46′N 113°40′E	*w*UrtOri3	WO*w*Urt1
Zhenjiang City, Jiangsu Province	ZJ	*Vigna unguiculata*	August, 2008	32°11′N 119°27′E	*w*UrtCon2	WO*w*Urt1
Huai'an City, Jiangsu Province	HA	*Vigna unguiculata*	August, 2008	33°36′N 119°01′E	+	−
Shanghai	SH	*Solanum melongena*	August, 2008	31°03′N 121°45′E	+	−
Huzhou City, Zhejiang Province	HZ	*Vigna unguiculata*	July, 2009	30°52′N 120°06′E	+	−
Cixi City, Zhejiang Province	CX	*Gossypium hirsutum*	August, 2008	30°10′N 121°14′E	+	−
Pengze County, Jiangxi Province	PZ	*Solanum melongena*	August, 2009	29°54′N 116°33′E	*w*UrtOri1	WO*w*Urt2
Yingtan City, Jiangxi Province	YT	*Solanum melongena*	August, 2009	28°14′N 117°03E	+	−
Changsha City, Hunan Province	CS	*Solanum melongena*	August, 2008	28°12′N 112°59′E	+	−
Fuzhou City, Fujian Province	FZ	*Solanum melongena*	August, 2008	26°05′N 119°18′E	+	−
Longyan City, Fujian Province	LY	*Solanum melongena*	August, 2009	25°06′N 117°01′E	+	−
Maoming City, Guangdong Province	MM	*Vigna unguiculata*	August, 2009	21°40′N 110°53′E	+	−

Acari taxonomists (Xiao-Feng Xue and Xiao-Yue Hong) in our lab with the Zeiss Discovery V12 and Zeiss Imager A2 research microscopes (Göttingen, Germany) to ensure that the identification was correct. They were reared separately on leaves of the common bean (*Phaseolus vulgaris*), which were placed on a water-saturated sponge mats in petri dishes at 25 °C under light/dark 16:8 and 60 % RH.

DNA extraction

First, more than 40 individual mites from each geographic population were collected, and all infected mites from every geographic area were checked for the presence of WO phages. DNA was extracted by homogenizing a single female or male adult in a 25 μl (PCR) or 10 μl (real-time qPCR) mixture of STE buffer (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 2 μl of proteinase K (10 mg/ml) in a 1.5 ml Eppendorf tube. The mixture was incubated at 37 °C for 30 min, heated at 95 °C for 5 min, and then centrifuged at 3,000 rpm for 10 s. The mixture was either used immediately for the PCR reactions or deposited at −20 °C (<1 month) for later use.

PCR amplification

Wsp gene coding for an outer membrane protein of *Wolbachia* (Braig et al. 1998) and *orf7* gene coding for a minor capsid protein of WO (Masui et al. 2000) were used to detect *Wolbachia* and WO, respectively. The *wsp* gene from *Wolbachia* was amplified by the primers *wsp* 81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and *wsp* 691R (5′-AAA AAT TAA ACG CTA CTC CA -3′) designed by Braig et al. (1998). PCR was performed in a 25 μl reaction volume consisting of 14.3 μl of H_2O, 2.5 μl of 10 × buffer, 2.0 μl of 25 mM $MgCl_2$, 2.0 μl of dNTP (2.5 mM each), 1 μl of primers (10 μM each), 0.2 μl of Taq DNA polymerase (1 U) and 2.0 μl of sample. Amplification was done under the following thermal profile: 94 °C for 3 min followed by 35 amplification cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min, and 72 °C for 5 min. The *orf7* gene of WO was amplified by the primers *orf7*F (5′-GAA ATG CTT GTT CAG CTA ATA GC-3′) and *orf7*R (5′-CCA GAA AAA ATA GGA GAA TTT AT-3′) designed by Fujii et al. (2004). PCR was conducted in a 25 μl reaction volume consisting of 14.8 μl of H_2O, 2.5 μl of 10 × buffer, 1.5 μl of 25 mM $MgCl_2$, 2.0 μl of dNTP (2.5 mM each), 1 μl of primers (10 μM each), 0.2 μl of Taq DNA polymerase (1 U) and 2.0 μl of sample. The PCR conditions were 94 °C for 3 min followed by 40 amplification cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s, and 72 °C for 5 min.

Cloning and sequence analysis

The *wsp* and *orf7* PCR products were purified by EZgene™ Gel Extraction Kit (BIOMIGA) and were cloned into *Escherichia coli* of TOP10 using the p-GEMT Vector system (Promega). Cells containing the plasmid were selected on LB/Ampicillin/X-Gal/IPTG plates (Trypton 1 %, Yeast Extract 0.5 %, NaCl 1 %, agarose 1.5 %, ampicillin 0.1 mg/ml, X-Gal 0.04 mg/ml, IPTG 0.024 mg/ml), and those containing plasmids with the PCR product were discriminated by a white/blue screening. At least three clones were selected and incubated in Luria–Bertani (LB) liquid medium with ampicillin (100 mg/ml). These clones were sequenced by TAKARA Company (Dalian, Liaoning Province, China). Sequence homology analysis was performed with the BLAST program in NCBI Web (http://www.ncbi.nlm.nih.gov/) and GenDoc software. The DNA sequences of *orf7* genes are available in GenBank under the following accession numbers: HM623911 and HM623912.

Phylogenetic analyses

In addition to our sequences, 45 other ORF7 sequences (amino acid sequences) were retrieved from the databases and subjected to analysis (accession numbers are indicated in

Fig. 1). All sequences were aligned with the Clustal W (Thompson et al. 1994) software and visually modified using BioEdit version 7.0.9.0 (Hall 1999). Analysis of the genetic and phylogenetic relationship was performed using MEGA (http://www.megasoftware.net). A phylogenetic tree was constructed by the neighbor -joining (NJ) method using bootstrap analysis, which was performed with the parameter pairwise deletion method. In total, 1,000 replications were used to estimate the reliability of tree branch nodes.

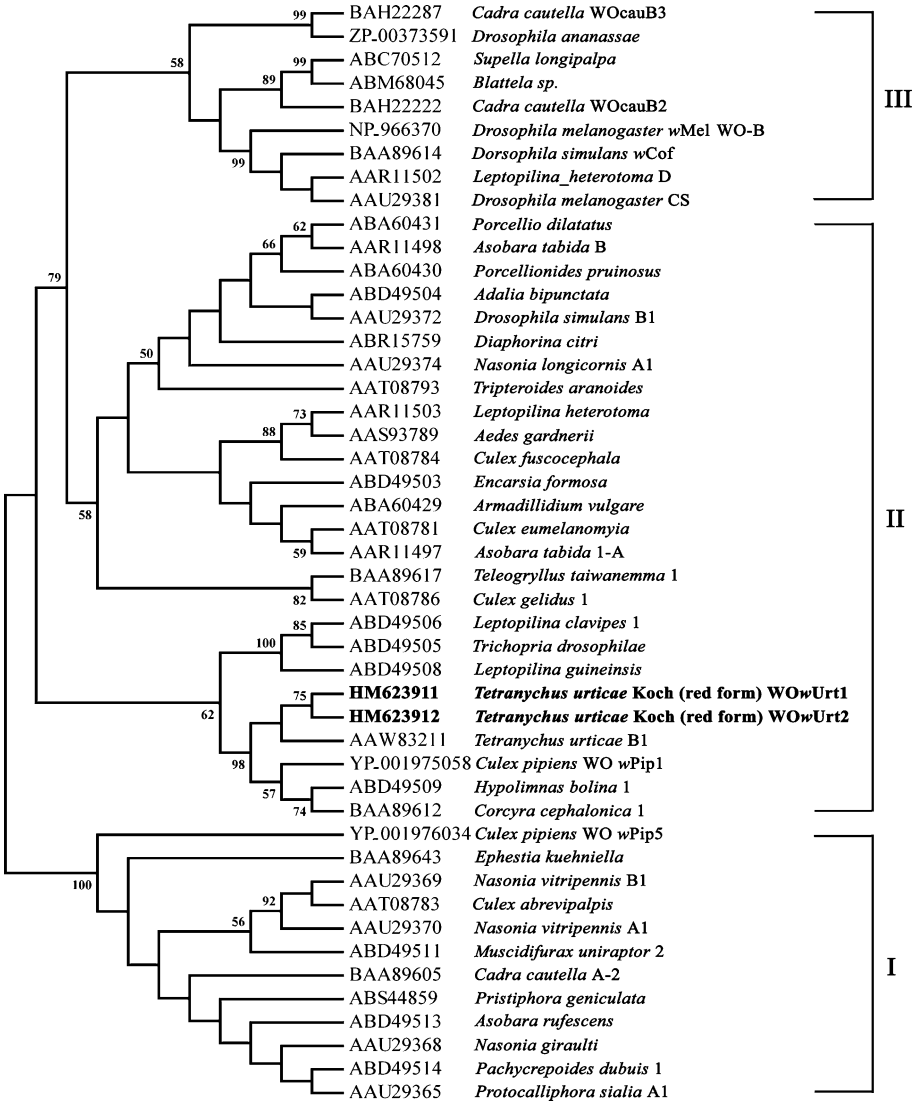

Fig. 1 Molecular phylogenetic analysis of WO*w*Urt1 and WO*w*Urt2 on the basis of ORF7 sequences. In addition to our sequences (in *bold*), 45 other ORF7 sequences were retrieved from the databases and subjected to analysis. A neighbor-joining tree inferred from 125 aligned amino acid sites is shown. Bootstrap values higher than 50 % are shown at nodes I, II, and III represent the ORF7 clusters identified by Bordenstein and Wernegreen (2004)

Screening of WO infection lines

Because not all of the *Wolbachia* found in mites for a geographical population carried WO, the infected *Wolbachia* lines were screened for our experiment. One female was allowed to lay eggs without being crossed with males. When the eggs grew into male adults, a backcross occurred between the female and the males. After the cross, the female adult was transferred to a new leaf disk and was allowed to lay eggs for 3–5 days. A female was checked for WO by PCR amplification. The eggs were separately reared on new leaf disks depending on the infection status of the mother. The above process continued for three to four generations until 100 % bacteriophage-infected populations were obtained.

Real-time quantitative PCR (RTQ-PCR)

To estimate the abundance of *Wolbachia* and WO, the copy numbers of the *wsp* and *orf7* genes were measured by RTQ-PCR in an ABI PRISM 7300 Sequence Detection System (*Applied Biosystems*). Based on the *wsp* sequence variability, the *wsp* primers specific for the Con *Wolbachia* were Qcon*wsp* F (5′-CTC GTT ACT TCG GTT CTT ATG GC-3′) and Qcon*wsp* R (5′-TTA AAC GCT ACT CCA GCT TCT GC-3′); *wsp* primers specific for the Ori *Wolbachia* were Qori*wsp* F (5′-GCA GCG TAT GTA AGC AAT CC-3′) and Qori*wsp* R (5′-ATA ACG AGC ACC AGC ATA AAG-3′), corresponding to a 112 and 138-bp fragment of the *wsp* gene of *Wolbachia,* respectively. For quantification of WO, the primers were employed as previously described (Bordenstein et al. 2006): NvWOF1 (5′-GTC TGG AAA GCT TAC AAA AAG-3′) and NvWOR1 (5′-CTC GCC AAA ATA TAG CCC TGC-3′). These primers amplified a 91-bp product.

The amplification reaction was monitored using SYBR green. The 20 µl volume reaction mixture consisted of 10 µl of 2 × SYBR® *Premix Ex Taq*™ (*Applied Biosystems*), 6.8 µl of sterile water, 0.4 µl each of 10 µM forward and reverse primers, 0.4 µl of 50 × ROX Reference Dye and 2 µl of DNA template. The PCR thermal program was as follows: 1 cycle (10 s 95 °C) followed by 40 cycles (5 s 95 °C, 31 s 60 °C), and finally 1 cycle (15 s 95 °C, 1 min 60 °C, 15 s 95 °C). A melting curve was analyzed to check for any nonspecific amplification or primer dimers.

To prepare standard solutions for RTQ-PCR, PCR products of *wsp* or *orf7* were electrophoresed on TAE 1.0 % (w/v) agarose gels, extracted, and the DNA fragments were measured by an optical density (OD) absorbance at of 260 nm. The copy number of the stock standards was calculated from this, and a tenfold dilution series from 10^{-8} to 10^{-3} copy numbers were prepared. Threshold cycles (Ct), in which the fluorescence began to increase from the background level, were measured. The number of molecules in all samples was determined from the threshold cycles in the PCR based on a standard curve. Negative controls were included in all amplification reactions.

A linear fit with a slope approximately between −3.1 and −3.6, equivalent to 90–110 % reaction efficiency, was typically acceptable for most applications requiring accurate quantification (Echaubard et al. 2010). In our study, the slope of each standard curve was –3.36 (Qcon*wsp*), −3.43 (Qori*wsp*) and −3.12 (Q*orf7*). The reaction efficiencies of the standard curves were all 0.99 to 1.

Effect of temperature on the tripartite associations between WO, *Wolbachia*, and CI

Wolbachia can be removed from two-spotted spider mites at 32 ± 0.5 °C after six generations (Opijne and Breeuwer 1999). As a result, the experiment was carried out at the

following temperatures: 19, 22, 25, 28 and 31 °C. All the mite lines had been kept at these temperatures for at least two generations before the experiment was performed. At each temperature condition, the abundance of WO and *Wolbachia* in ten adult females (virgin) and ten adult males (unmated) were measured. Males and females molting into adults in the previous 24 h were used.

For cross experimentation purposes, two cross combinations were conducted: uninfected females were crossed with infected males to evaluate the intensity of CI, and uninfected females were crossed with uninfected males for controls. Females in the last developmental stage before adult emergence (teliochrysalid) were placed with one virgin male (one-day-old) on the same leaf disk. Males were discarded after the females oviposited for 2 days, and mated females were allowed to oviposit for 5 days. Eggs on leaf disks were checked daily to determine the hatchability, the survival rate in immature stages and the sex ratio. In this experiment, the intensity of CI was evaluated by hatchability. To exclude the effect of temperature on the host itself, the percent CI was calculated as 100 % − (the hatchability of W−♀ × W+♂/the hatchability of W−♀ × W−♂).

Effect of male age on the abundance of WO and *Wolbachia*

This experiment was performed at 25 °C. Females were allowed to lay eggs without being crossed with males. Before the eggs developed into male adults, the females were removed. To reduce the differences in the abundance of *Wolbachia* and WO between individuals during development, the time period from 10:00 a.m to 6:00 p.m. was set as 1 day. The male adults that emerged during this time span were reared on a new leaf disk on specific days (3, 5, 7, 9, 11, 13, 15 and 17 days), and then their DNA were extracted and deposited at −20 °C for later use. Seven adult males were tested from each age class.

Statistical analysis

All statistical analyses were done with SPSS (17.0). The abundance of *Wolbachia* and WO was analyzed with the Independent *t* test to assess the effects of different temperatures and ages. CI intensity was analyzed with one-way ANOVA (analysis of variance), and the Tukey test was used.

Results

No phylogenetic congruence was found beween WO and *Wolbachia*

As shown in Table 1, all of the 20 isolated geographic populations of the two-spotted spider mite were infected with *Wolbachia*, and only six geographic populations were found carried WO. The *Wolbachia* that carried WO belonged to two subgroups of B-*Wolbachia*, Con and Ori (Zhou et al. 1998). The *Wolbachia* infecting ZJ population belonged to Con, while the others belonged to Ori. A sequence homology analysis showed sequences of five geographic populations of Ori that were divided into three *Wolbachia* types: *w*UrtOri1, *w*UrtOri2, and *w*UrtOri3 (HM486515 to HM486517 in GenBank). Sequences of WO in six geographic populations can be divided into two phage types: WO*w*Urt1 (HM623911) and WO*w*Urt2 (HM623912). The two types differed by 16 bp in the *orf7* sequence. Only the WO-infecting *Wolbachia* of the PZ population belonged to WO*w*Urt2 type. To sum up, the

two different subgroups Ori and Con of B supergroup *Wolbachia* were infected with phages that had an identical partial *orf7* sequence, here names the WO*w*Urt1 type (for example, ZJ and YC), whereas the Ori subgroup of *Wolbachia* was infected with two different phage WO types (for example, ZJ and PZ). As is the case in many other studies (Masui et al. 2000; Fujii et al. 2004; Gavotte et al. 2007), no phylogenetic congruence was found between WO and *Wolbachia* in our study.

Based on phylogenetic analysis (Fig. 1), WO in *Wolbachia* that infected the two-spotted spider mite was confirmed to belong to group II (Tanaka et al. 2009, Bordenstein and Wernegreen 2004). They also had a higher similarity with *Tetranychus urticae* B1, whose host belonged to the same genus *Tetranychus*. These results indicate that WO*w*Urt1 and WO*w*Urt2 are related to each other, but they are two distinct phages.

Temperature-induced alterations in life cycles of WO

The ZJ and YC geographic populations, which were infected with two different *Wolbachia* but carried the same WO type (WO*w*Urt1), were studied. In these two populations, temperature affected the abundance of *Wolbachia* in nearly the same way: the abundance was the highest at the moderate rearing temperature (25 °C), and then significantly decreased with temperature increasing or decreasing (Independent *t* test. Fig. 2A, male, 25 vs 22 °C, $P < 0.001$; Fig. 2a, male, 25 vs 28 °C, $P = 0.012$; Fig. 2a, female, 25 vs 22 °C, $P = 0.006$; Fig. 2a, female, 25 vs 28 °C, $P < 0.001$; Fig. 3a, female, 25 °C vs 22 °C, $P = 0.002$; Fig. 3a, female, 25 vs 28 °C, $P = 0.002$.). However, the male mites of the YC (Ori) population were a special case in that the abundance of *Wolbachia* was the highest at 28 °C (Figs. 2, 3). Despite this, the abundance of *Wolbachia* in the YC (Ori) population was lower by at least tenfold when compared to the ZJ (Con) population; otherwise, there was no significant difference in the abundance of *Wolbachia* between males and females in the YC population (one-way ANOVA, $F = 5.350$, $P = 0.023$), whereas the abundance of *Wolbachia* was always significantly higher in males than in females in the ZJ population (one-way ANOVA, $F = 236.823$, $P < 0.001$). These two differences between the YC and

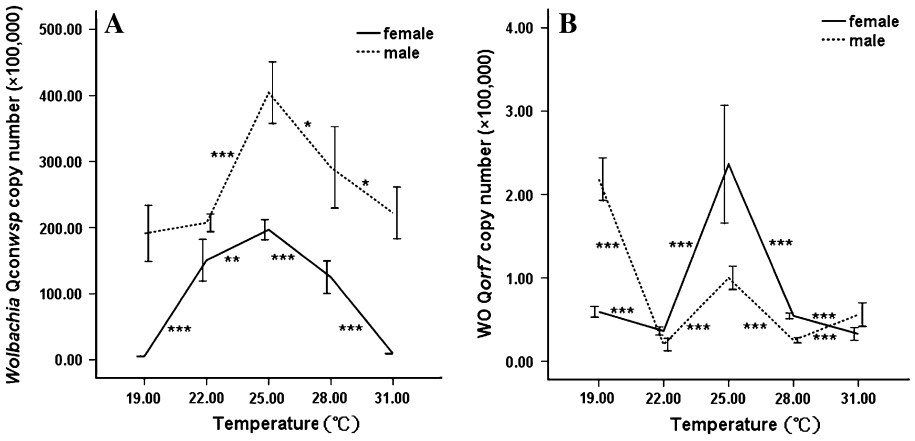

Fig. 2 Effect of temperature on the abundance of *Wolbachia* (Fig. 2a) and WO (Fig. 2b) in the ZJ (Con) population. The Independent *t* test was used. Blank means no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. *Error bar* 95 % confidence interval

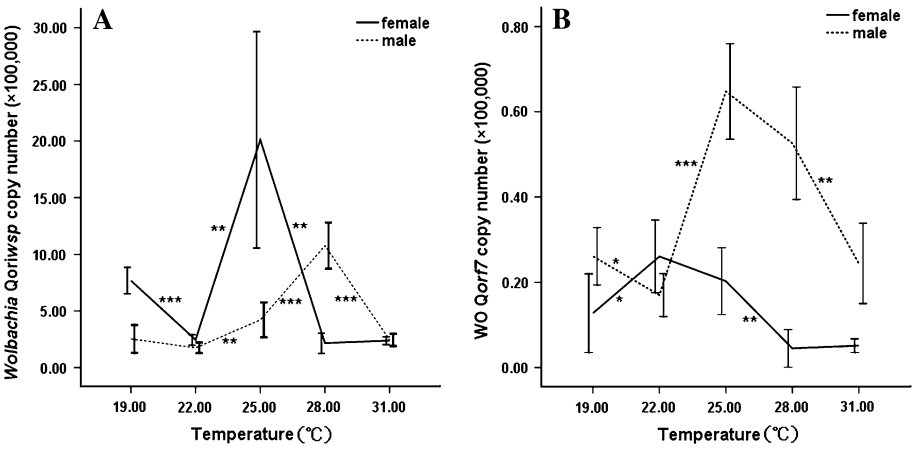

Fig. 3 Effect of temperature on the abundance of *Wolbachia* (Fig. 3a) and WO (Fig. 3b) in the YC (Ori) population. The Independent *t* test was used. Blank means no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. *Error bar* 95 % confidence interval

the ZJ populations provide a reason to further explore the difference between Ori and Con in the two-spotted spider mite. Similar to the abundance of *Wolbachia*, WO abundance also decreased after peaking at the moderate rearing temperature. However, WO abundance increased at extremely high or low temperatures. For instance, in the ZJ (Con) population, the abundance of WO increased in both females and males at 19 °C and 31 °C; it also increased faster in males than in females (Fig. 2b). In general, at the moderate rearing temperature, WO was synchronized while *Wolbachia* changed; whereas at extreme high or low temperatures, WO increased, but *Wolbachia* still decreased.

Accumulation of WO abundance with aging led to transformations to lytic cycles

In the YC and ZJ populations, the abundance of WO and *Wolbachia* generally increased with increasing ages up to 17 days after adult emergence. Both *Wolbachia* and WO*w*Urt1 increased significantly (Fig. 5) when the copy number of WO*w*Urt1 was under ≈70,000 per mite (1–17 days, YC; 1–11 days, ZJ). *Wolbachia* and WO*w*Urt had a positive relationship at that time. *Wolbachia* would change into a stable stage if WO*w*Urt1 went through a significant growth period in which the copy number was between ≈70,000 per mite and ≈140,000 per mite (11–15 days, ZJ) (Fig. 4). Once WO*w*Urt1 was up to ≈140,000 (15 days, ZJ) per mite, *Wolbachia* would begin to decrease significantly (Independent *t* test, $P < 0.05$) (Fig. 4).

The effect of temperature on CI

Crossing experiments were conducted in the ZJ population, and the response to CI was investigated when WO and *Wolbachia* were altered by way of changing temperature. The hatchability of W−♀ × W+♂ was significantly lower than the hatchability of W−♀ × W−♂ at all temperature conditions (Independent *t* text, $P < 0.05$) (Table 2). This finding indicates that the *Wolbachia* of the ZJ population can induce partial CI. The difference in CI intensity was due to the different amounts of *Wolbachia* infecting males

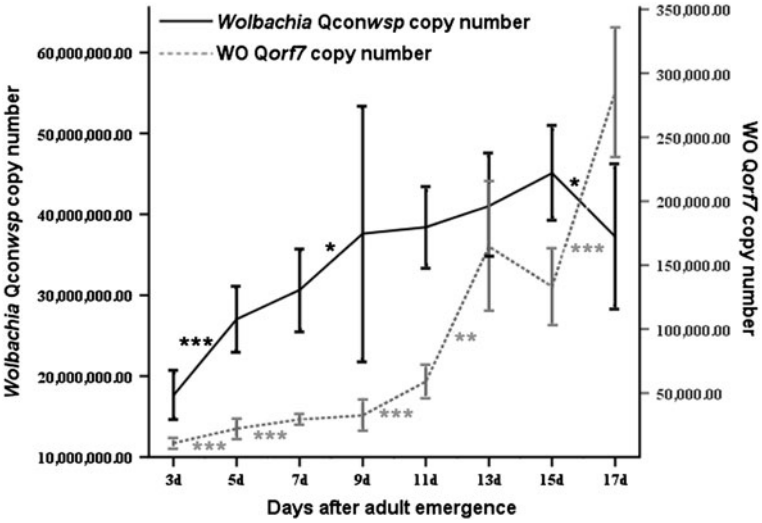

Fig. 4 Effect of age on the abundance of *Wolbachia* and WO in the ZJ (Con) population. The Independent *t* test was used. Blank means no significant difference; * $p < 0.05$; ** $P < 0.01$; *** $P < 0.001$. *Error bar* 95 % confidence interval

Table 2 Percent cytoplasmic incompatability of ZJ population at different temperature conditions

Temperature (°C)	Cross ♀ × ♂	N	Number of eggs[a]	Hatchability[b]	Percent CI[c]
19	W− × W+	20	8.95 ± 1.85	0.91 ± 0.07	6.17 ± 7.63[a]
	W− × W−	23	12.52 ± 3.58	0.97 ± 0.05	
				$P = 0.004$	
22	W− × W+	23	19.43 ± 4.10	0.92 ± 0.07	6.15 ± 6.58[a]
	W− × W−	17	17.00 ± 3.43	0.99 ± 0.02	
				$P < 0.001$	
25	W− × W+	17	20.00 ± 3.50	0.86 ± 0.08	11.60 ± 8.05[ab]
	W− × W−	18	18.67 ± 3.43	0.97 ± 0.03	
				$P < 0.001$	
28	W− × W+	21	26.43 ± 5.00	0.77 ± 0.12	19.02 ± 12.75[b]
	W− × W−	24	28.25 ± 6.10	0.95 ± 0.06	
				$P < 0.001$	
31	W− × W+	19	32.68 ± 6.31	0.83 ± 0.08	11.83 ± 8.60[ab]
	W− × W−	13	48.31 ± 11.75	0.94 ± 0.07	
				$P < 0.001$	
					$F = 7.352$***

[a] Mean number of eggs (± SD)

[b] Hatchability (mean ± SD) was analyzed with Independent *t* test

[c] Percent CI was calculated as 100 %−(the hatchability of W−♀ × W+♂/the hatchability of W−♀ × W−♂), and analyzed with one-way ANOVA (Tukey test)

*** $P < 0.001$

and the host response to different temperature conditions. The temperature 25 °C was the most suitable temperature condition for the two-spotted spider mite to live (Zhou et al. 2003). At this temperature, mites had the strongest capacity to resistant external interferences including the modification of *Wolbachia* in male mites. Compared to the abundance found at 25 °C, the abundance of *Wolbachia* was less at 28 °C, but the resistant capacity was also lower. That is why the CI intensity was stronger at 28 °C.

Discussion

Temperature can influence the tripartite associations

WO can directly code proteins such as the proteins involved in antibiotic resistance or toxins, which were a valuable auxiliary for bacteria and host arthropods (Miao and Miller 1999; Duron et al. 2006; Tanaka et al. 2009; Kent and Bordenstein 2010). For example, WO contains a gene encoding a similar protein with the protein of APSE, the bacteriophage of *Hamiltonella defensa* that conferred pea aphid resistance to parasitoid attack (Degnan and Moran 2008; Oliver et al. 2009). In the ZJ population, WO increased at 19 and 31 °C (Fig. 2b), which indicated that WO may transfer from lysogenic to lytic, and produce a special protein that has a strong effect on rescuing the spider mites or lyse the *Wolbachia* cells to raise the hatchability of spider mites. As the results (Figs. 2, 3) indicate, the relationship of WO-*Wolbachia* was positive at optimal temperature but negative at extreme temperatures. Otherwise, the relationship between *Wolbachia* and CI was always positive (Fig. 2a, Table 2). Therefore, the associations changed according to temperature. However, more studies on gene products should be conducted.

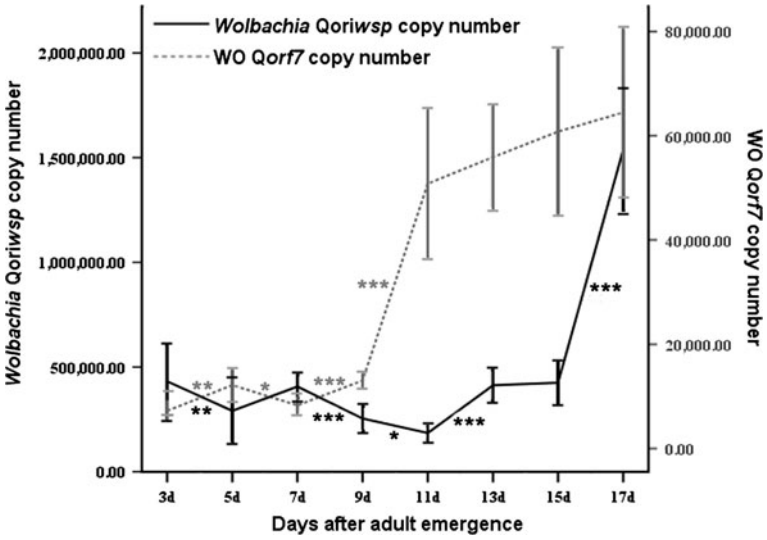

Fig. 5 Effect of age on the abundance of *Wolbachia* and WO in the YC (Ori) population. The Independent *t* test was used. Blank means no significant difference; * $p < 0.05$; ** $P < 0.01$; *** $P < 0.001$. *Error bar* 95 % confidence interval

The tripartite associations on the effect of age

Age has been found to affect the expression of CI in several hosts including the fruit fly (Turelli and Hoffmann 1995; Reynolds and Hoffmann 2002), the planthopper *Laodelphax striatellus* (Noda et al. 2001) and *Armigeres subalbatus* (Jamnongluck et al. 2000). Sometimes, the decrease in CI intensity was attributed to the decrease in *Wolbachia* load with an increase in age. However, age was considered to have no effect on CI intensity even though *Wolbachia* increased with age in *Culex pipiens* (Duron et al. 2007). The influence of *Wolbachia* load on CI intensity above cannot be directly extended to the two-spotted spider mite. In our study, the negative relationship between WO*w*Urt1 and *Wolbachia* demonstrates that WO*w*Urt1 transformed to the lytic cycle when the copy number reached $\approx$140,000 per mite. It showed that a process of basic copy number accumulation was required for changing lysogenic life cycles into lytic life cycles in WO*w*Urt1. While the abundance of *Wolbachia* first increased with age, then keep invariant, and at last decreased when WO changed to the lytic cycle (Fig. 4). Therefore, the complex WO-*Wolbachia* association may influence CI intensity in different ways, which future studies can reveal.

WO was lysogenic at appropriate conditions in the two-spotted spider mite

According to the classic lambda-like models, the phage density should always equal (no lytic activity) or exceed (lytic activity producing multiple phage virions) the *Wolbachia* density (Bordenstein et al. 2006). Most surprisingly, however, was that the abundance of *Wolbachia* was about 10- to 10^2-fold the abundance of WO in the two-spotted spider mite, which is similar to the results of Chauvatcharin et al.'s study (2006). In many different mosquito strains, the average WO-B phage density was about 10^5- to 10^{10}-fold lower than *Wolbachia* densities and was explained that WO was in the lysogenic life cycle and *Wolbachia* free of WO-B phage infection co-present within the same host (in the absence of lytic switch in the infected individuals). In our two-spotted spider mite populations, the infection rate was only 6/20, and not all of the *Wolbachia* found in mites for a geographical population carried WO (Table 1). So on an evolutionary scale, it was probable that the time WO transferred into this mite was not long. In addition, the stable vertical transmission of WO was used for screening, it is very possible that WO was lysogenic and infected just a part of *Wolbachia* in one individual of the two-spotted spider mite at the optimal temperature and in the early development stage. To better understand life cycles of WO, more studies should be carried out by using fluorescence in situ hybridization to evident the situation of lysogenic phage in *Wolbachia*.

Wolbachia load is higher in males than in females

Previous studies have demonstrated that the *Wolbachia* load in females was higher than in males. This was observed in several hosts such as *Culex pipiens* (Berticat et al. 2002; Echaubard et al. 2010), *Aedes albopictus* (Dobson et al. 1999; Wiwatanaratanabutr and Kittayapong 2006, 2009) and two planthopper species (Noda et al. 2001). However, in our study, the *Wolbachia* load in males was higher than in females in the ZJ population of the two-spotted spider mite. This finding is consistent with the density of *w*AlbB in *Aedes albopictus* (Tortosa et al. 2010). This interesting result can be explained by the time of development in females longer than in males. In haplo-diploid organisms, males are more developmentally unstable than females responding to the same environment stimuli

(Clarke 1997; Wiwatanaratanabutr and Kittayapong 2006), which is also observed in the two-spotted spider mite (data unpublished). Moreover, the *Wolbachia* load in females and males increased with age in the ZJ population. As a consequence, the *Wolbachia* load of one-day-old males increased faster than of one-day-old females, and expressed as the *Wolbachia* load in males higher than in females.

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