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Article

Effects of *Wolbachia* on rDNA-ITS2 variation and evolution in natural populations of *Tetranychus urticae* Koch

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Abstract

In this study, rDNA-ITS2 was sequenced and analyzed to investigate the effect of *Wolbachia* on the rDNA of four populations of *Tetranychus urticae* Koch. The result showed that all four populations of *T. urticae* were infected with two strains of *Wolbachia*. They are *w*UrtOri1 (HM486515–HM486517) and *w*UrtCon1 (HM486518), belonging to the Ori group and Con group of B supergroup, respectively. There was one mutation site among 645 sites for the ITS2 fragments. All the sequenced ITS2 were classified into two haplotypes. The diversity analysis of ITS2 sequences of individuals showed that infection by *Wolbachia* did not significantly change the diversity of rDNA. A neutrality test using ITS2 indicated that the population of *T. urticae* has remained stable during evolution and is not off the Hardy-Weinberg equilibrium.

Key words: *Tetranychus urticae* Koch, *Wolbachia*, rDNA diversity, neutrality test

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is an important agricultural pest that can feed on about 1200 host plant species, among which more than 150 are economically important (Badawy *et al.* 2010). It mainly damages vegetables, fruit trees, cotton, soybean, maize and ornamental crops worldwide (Miao *et al.* 2006; Badawy *et al.* 2010). Its high reproductive potential and short life cycle allow it to quickly develop resistance to many acaricides (Nicastro *et al.* 2010). It is estimated that *T. urticae* has developed resistance to more than 80 acaricides in 60 countries (Badawy *et al.* 2010).It is the most damaging mite species on agricultural crops and fruit trees (Miao *et al.* 2006).

Wolbachia is a very common cytoplasmic symbiont and is maternally inherited (Zhou *et al.* 1998; Hurst *et al.* 1999). It can infect insects, crustaceans, filarial nematodes and mites at rates of 16% to 76% (Miao *et al.* 2006). *Wolbachia* has evolved a large scale of host manipulations such as parthenogenesis induction (Stouthamer *et al.* 1993; Arakaki *et al.* 2001), feminization (Bouchon *et al.* 1998; Hiroki *et al.* 2002), male killing (Hurst *et al.* 1999) and crossing incompatibility which is the most common effect between infected males and uninfected females (Perrot-Minnot *et al.* 1996; Breeuwer 1997; Dobson *et al.* 2001). *Wolbachia* can affect the development and propagation of arthropod hosts (Wang *et al.* 2010).

Both mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) are widely used for population genetic studies. Hillis & Dixon (1991) were the first to use rDNA for phylogenetic analysis, subsequently rDNA was widely used in many studies on evolution and taxonomy for

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insects. Several studies showed that *Wolbachia* have an indirect impact on the DNA diversity of mtDNA of their hosts due to a selective sweep of the mitotype associated with the infection and (or) natural selection acting on *Wolbachia* (Jiggins 2003; Shoemaker *et al.* 2003; Yu *et al.* 2011). But few studies focused on the impacts of *Wolbachia* on the rDNA markers. With the accumulation of mtDNA sequences, it's important to obtain information about rDNA to more precisely learn the evolution together with mtDNA information.

In this study, rDNA -ITS2 (internal transcribed spacer 2) was sequenced from 80 female spider mites across 4 populations to examine the rDNA-ITS2 diversity and investigate the association between *Wolbachia* and rDNA of *T. urticae* Koch.

Materials and methods

Animals and Tissues

T. urticae were collected from *Solanum melongena* L. in 4 regions of 3 provinces, China. Locations, host plants, collection dates and abbreviations are summarized in Table 1. DNA was isolated immediately once mites were taken to the laboratory or the samples were kept at -70°C until use.

Forty female adults were randomly selected from each geographical population to conduct the PCR amplification.

TABLE 1. Collection location, host plant and collection date of *Tetranychus urticae* from China.

Collection location	Abbreviation	Host plant	Collection date	Latitude	Longitude
Yantai, Shandong	YT	*Solanum melongena* L.	Aug., 2008	37.52°N	121.39°E
Taian, Shandong	TA	*S. melongena* L.	Aug., 2008	36.18°N	117.13°E
Kunming, Yunnan	KM	*S. melongena* L.	Aug., 2009	25.05°N	102.70°E
Huhhot, Inner Mongolia	HHHT	*S. melongena* L.	Aug., 2009	40.48°N	111.41°E

DNA extraction

Total DNA was extracted from individual adult females using protocols described in Xie *et al.* (2008). In every sampled locality, three individuals were sequenced.

Amplification of *wsp* gene in *Wolbachia* and ITS2 in *T. urticae*

A fragment in the *wsp* gene of about 596bp was amplified from the DNA with the specific primers (81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'; 691R: 5'-AAAAATTAAACGCTACTCCA-3') (Zhou *et al.* 1998). The amplification reactions were performed in a 50μL volume containing 4μL DNA template solution, 28.6μL ddH_2O, 5μL 10×buffer, 5μL $MgCl_2$ (25 mmol/L), 4μL dNTPs (10mmol/L each), 0.4μL Taq DNA polymerase (5U/μ, TaKaRa, Dalian), 1.5μL each primer (20 μmol/L each) according to the following schedule: 4 minutes at 94°C followed by 30 cycles at 94°C (1 minute), 53°C (1 minute) , 72°C (1 minute).

A 645bp fragment of ITS2 was amplified from the DNA with the specific primers (ITS2-S:5'-ATATGCTTAAATTCAGCGGG-3';ITS2-A: 5'-GGGTCGATGAAGAACGCAGC-3'.) The primers were defined in the highly conserved 5.8S and 28S flanking regions as previously published in Navajas *et al.* (1994). The amplification reactions were performed in a 50μL volume containing

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2μL DNA template solution, 30.6μL ddH_2O, 5μL 10×buffer, 5μL $MgCl_2$ (25 mmol/L), 4μL dNTPs (10mmol/L each), 0.4μL Taq DNA polymerase (5U/μ, TaKaRa, Dalian), 1.5μL each primer (20 μmol/L each) according to the following schedule: 4 minutes at 94? followed by 30 cycles at 94°C (1 minute), 53°C (1 minute), 72°C (1 minute).

The PCR products of the appropriate sizes were gel-purified with the DNA Gel Extraction Kit (Tiangen Biotech, Beijing), cloned into T-easy vector (Promega USA), transformed into *Escherichia coli DH5α* and sequenced in the gene-sequencing company (Bioasia company).

Gene sequencing and sequence analysis

Three samples from each geographical population infected with *Wolbachia* were randomly chosen to sequence. DNA sequences were submitted to the GenBank database and compared online with the published sequences by similarity search engines such as BLAST in NCBI Web, and the identical sequences were used as the standard. Clustal X computer program (Thompson *et al.* 1997) was used to align the *wsp* gene of *T. urticae* infected with *Wolbachia* sequenced in our research with the published *wsp* genes of other mites in Tetranychidae. Analysis of genetic and phylogenetic relationships was performed using MEGA2.1 (Kumar *et al.* 2001). Genetic relationships among every geographical population were estimated based on the distance using the Kimura-2 Parameter method. Phylogenetic trees were constructed by the Neighbor-Joining (NJ) method. Confidence levels for NJ tree were assessed by bootstrapping from 1000 pseudo-replications.

According to detection results of the infection with *Wolbachia*, ten female adults infected with *Wolbachia* and ten which were not infected were chosen from each geographical population. And the DNA isolated from each individual was used as the template to amplify ITS2 fragment with the designed primers (ITS2-S and ITS2-A). Thus, eighty ITS2 sequences were obtained. Haplotype analysis was conducted using TCS1.21 software (Clement *et al.* 2000).

ITS2 sequence diversity and evolution analysis

Haplotype diversity (Hd), nucleotide diversity (π) and average difference of nucleic acid (k) were chosen to determine the differences of ITS2 sequences between *T. urticae* infected with *Wolbachia* and those uninfected. A neutrality test was conducted in order to learn the evolution of ITS2 sequences. All the calculations were performed in the program DnaSP5.10 (Pablo & Rozas, 2009).

Results

Infection with *Wolbachia*

PCR results showed that all four geographical populations of *T. urticae* were infected with *Wolbachia* at an average intraspecific infection rate of 50.4%. Among the four populations, the HHHT and TA populations were infected with the highest rate of 60%, and the infection rate of the YT population was the lowest at 27.5% (Table 2). The four populations were infected with two strains of *Wolbachia* belonging to the Ori group and Con group of B supergroup, respectively, that is, *w*UrtOri1 (HM486515- HM486517) and *w*UrtCon1(HM486518) (Figure 1).

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TABLE 2. Infection rates of four geographical populations of *T. urticae* with *Wolbachia*. Forty female adults were checked for each population.

Geographical Population	Abbreviation	No. infected	Infection Rate (%)	GenBank Accession number
Huhhot, Inner Mongolia	HHHT	24	60	HM486515
Yantai, Shandong	YT	11	27.5	HM486516
Tai'an, Shandong	TA	24	60	HM486517
Kunming, Yunnan	KM	19	47.5	HM486518

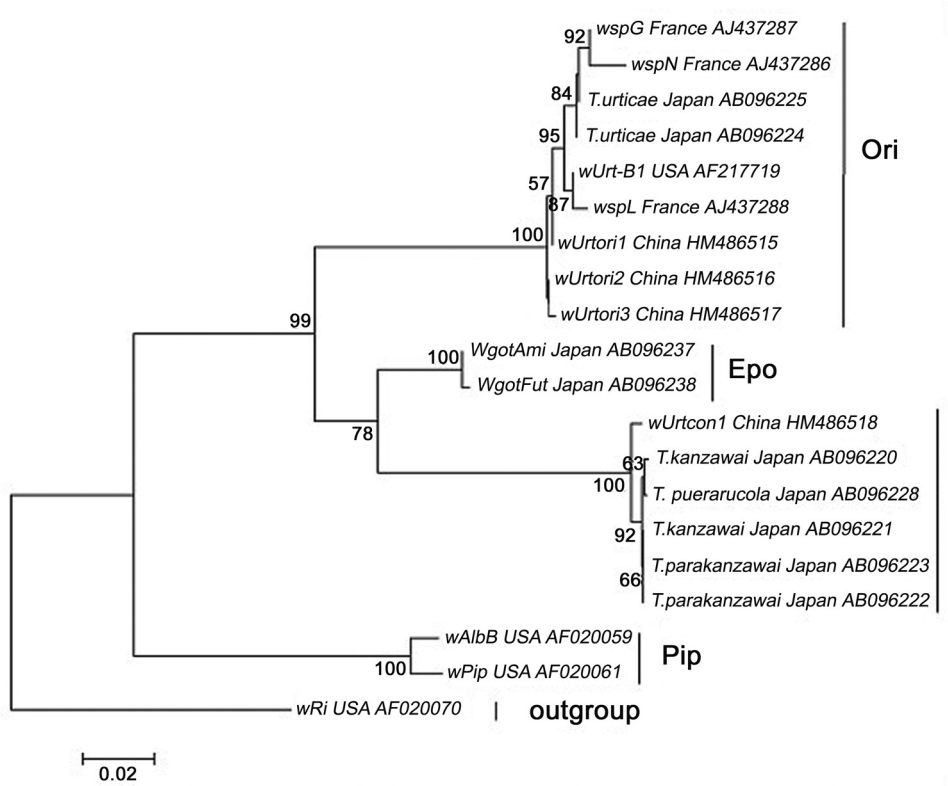

FIGURE 1. Phylogenetic tree of *wsp* genes of four geographical populations of *T. urticae* with other reported Tetranychidae

Alignment of 80 ITS2 gene sequences showed that there were no base insertions or deletions, and that there was just one mutable site (Fig. 2). The 80 ITS2 sequences were classified into 2 haplotypes (Fig. 3).

ITS2 sequence diversity and its evolution analysis

Hd, π and k of ITS2 from *T. urticae* individuals infected with *Wolbachia* were all lower than those of ITS2 from *T. urticae* individuals uninfected with *Wolbachia*, and the differences were not significant. Neutrality tests showed that Tajima'D and Fu&Li'F values fluctuated around 0.

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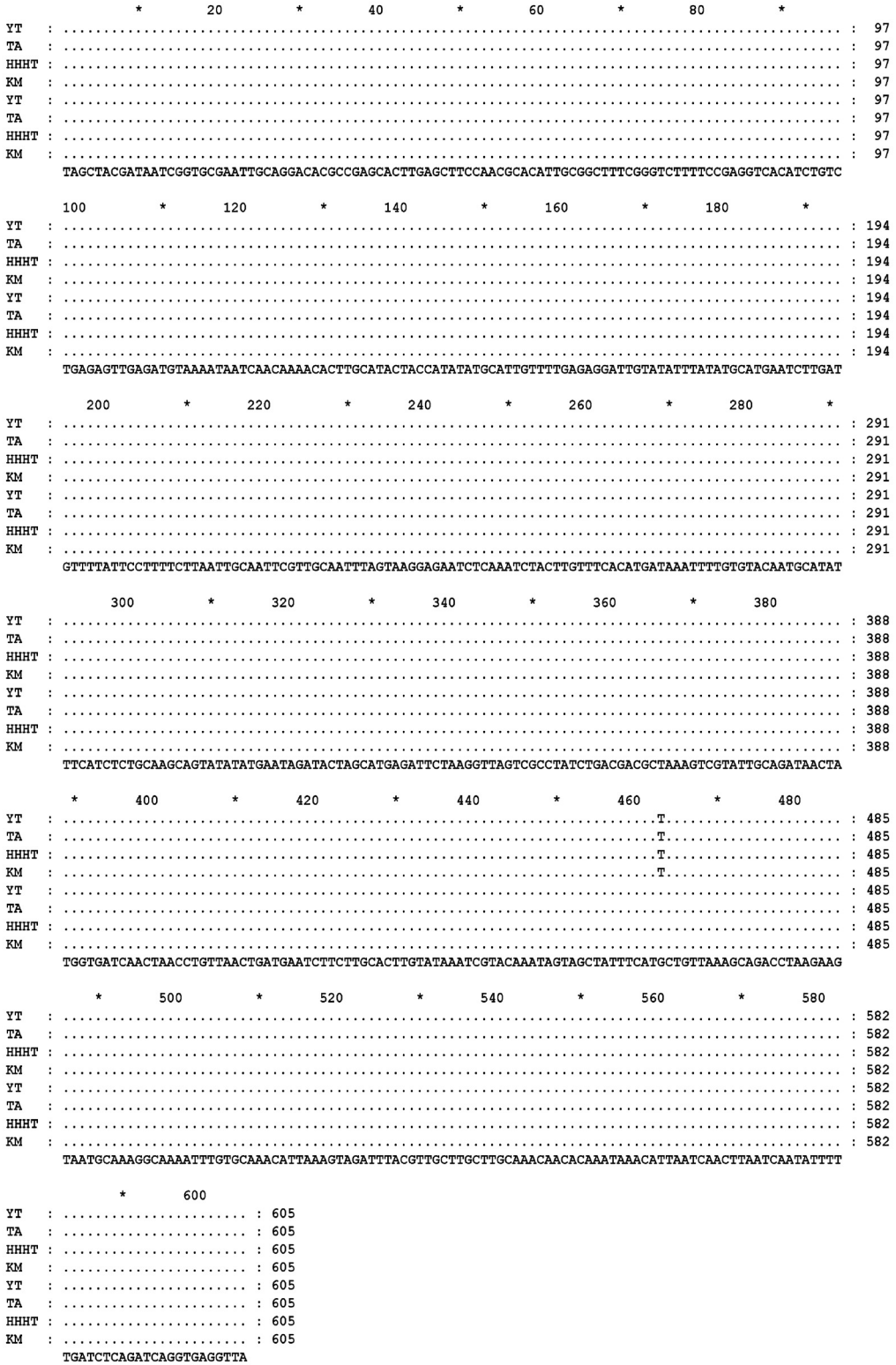

FIGURE 2. Mutation sites of ITS2 in *T. urticae.*

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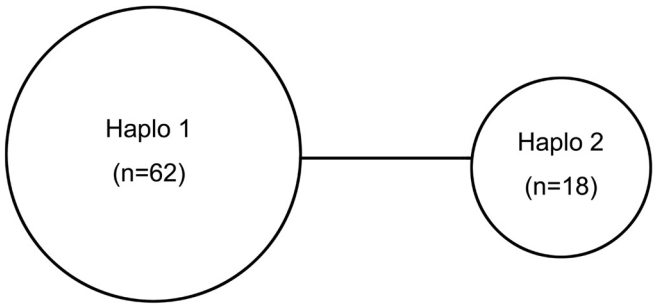

FIGURE 3. Haplotype network of ITS2 sequences in *T. urticae*
*Cycle and square frame represent haplotypes. Area size indicated the number of haplotypes, and the connecting line presented one mutation step.

TABLE 3. ITS2 diversity analysis of four geographic populations of *T. urticae.*

Population	*Wolbachia* strain	Infection status[a]	Molecular diversity			Neutrality test	
			Hd	π	k	Tajima'D	Fu & Li'F
HHHT YT	*w*UrtOri1/	W	0.328	0.00047	0.328	0.56544 P>0.10	0.65111 P>0.10
TA KM	*w*UrtCon1	U	0.364	0.00545	0.364	0.77738 P>0.10	0.56618 P>0.10

a: W and U indicate infected and uninfected with *Wolbachia*

Discussion

Infection rate with *Wolbachia* changed

In California, USA, the rate of spread of *Wolbachia* in populations of *Drosophila simulans* was greater than 100 km/year and populations with lower infected rates were commonly infected within 3 years (Turelli & Hoffmann 1991). Similarly, Gotoh *et al*. (2003, 2007) found the CI-*Wolbachia* strains were widespread in Japan and no geographical trend was observed in the CI-*Wolbachia*. We found that all four populations of *T. urticae* were infected with *Wolbachia* at an average infection rate of 50.4%, while Miao *et al.* (2006) found that the average infection rate of *Wolbachia* in *T. urticae* in a Chinese population was 36.5%, suggesting that *Wolbachia* infections are increasing.

ITS2 sequence analysis

The ITS2 sequence analysis showed that Haplo1 was the most common haplotype and Haplo2 was mutated from Haplo1 (Fig. 3). The fact that there were just two haplotypes in the 80 individuals of *T. urticae* suggests that ITS2 is a poor marker for studies of population genetics. An analogous situation was observed in *Tetranychus evansi* (Boubou *et al*. 2011).

ITS2 sequence diversity and an analysis of ITS2 evolution

Mitochondrial types were different in different *Drosophila* species. Seven major haplotypes have been described, one (*me*) in *D. melanogaster*, three in *D. simulans* (*si* I, *si*II, *si*III), two in *D. mauritiana* (*ma*I, *ma*II), and *D. sechellia* (*se*). Different *Wolbachia* strains infect cytoplasms harboring different mtDNA types (Solignac 2004). However, in this study, no significant differences between Hd, π and k of ITS2 were observed between infected and uninfected individuals, indicating

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that infection by *Wolbachia* does not significantly affect population rDNA. This may be because both *Wolbachia* and mitochondria are maternally inherited, which might lead to a direct influence of *Wolbachia* on mitochondria. But rDNA is not maternally inherited. So the influence of *Wolbachia* on rDNA is different. Or mtDNA is different from rDNA with respect to adaptation to *Wolbachia*.

Deleterious mutations are thought to remain in a population at a rather low rate due to the negative selection or purifying selection, and therefore the value of D and Fu is negative. When some allelic genes are under positive selection in the population, the ratio of neutral or deleterious mutations for those genes closely linked to the allelic gene will also increase in the population with the increase of the allele proportion, a process called hitchhiking or selective sweep (Barton 2000). The D test and Fu test are both significantly negative, which might result from negative selection, hitchhiking, a bottleneck undergone by the population or extensive base insertion or loss (Tajima 1989). When the population is under balancing selection, two or several alleles with high frequency will occur in the population, and the values of D and Fu tests will be positive. In this study, the Tajima' D and Fu & Li' F values both fluctuated around 0, suggesting that *T. urticae* populations remain stable during the evolution process without the influence of selective sweep from *Wolbachia*, suggesting that the population is not off balance.

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