

## ***Wolbachia* infection in *Trissolcus* species (Hymenoptera: Scelionidae)\***

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**Abstract.** *Wolbachia* is a maternally transmitted intracellular symbiont which causes reproductive distortions in the arthropods it infects. In recent years there has been an increasing interest in using *Wolbachia* as a potential tool for biological control by genetic manipulation of insect pests. In the present paper we report *Wolbachia* infection in several *Trissolcus* wasps (Hymenoptera: Scelionidae) which are important egg parasitoids of the sunn pest, *Eurygaster integriceps* Puton (Heteroptera: Scutellaridae). We used DNA sequence data for a gene encoding a surface protein of *Wolbachia* (*wsp*) not only to confirm *Wolbachia* infection but also to discriminate *Wolbachia* strains. Phylogenetic analyses indicated that *Wolbachia* strains in *Trissolcus* species were closely related to one another and belonged to supergroup B. Determination of the infection status of various populations, the possible role of *Wolbachia* in causing the incompatibility and knowledge of the reproductive compatibility of *Trissolcus* populations is important for the success of parasitoids in sunn pest management.

### **INTRODUCTION**

The sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) is a major pest of wheat and barley in wide areas of the Near and Middle Eastern, Western and Central Asia, Northern Africa, and Eastern and Southern Europe (Brown, 1962; Critchley, 1998; Parker et al., 2002). The pest feeds on both the vegetative stages of the plant as well as maturing grain. Sunn pest infestations in some areas can lead to 100% crop loss in the absence of control measures. The current management strategy for this pest mainly relies on chemical control. In addition to the high cost, insecticides pollute the environment, as well as killing non-target insects, whilst resistance has developed to various types of insecticides in this species (Critchley, 1998; Bandani et al., 2005; Sukhoruchenko & Dolzhenko, 2008). Thus new control methods are needed to diminish reliance on insecticides for control of this serious pest. Among natural control agents, the most promising of them are hymenopterans egg parasitoids. Of these, the most important belong to the genus *Trissolcus* Ashmead and *Telenomus* Haliday (Hymenoptera: Scelionidae) (Brown, 1962; Kozlov & Kononova, 1983; Orr, 1988; Kocak & Kilincer, 2003; Bouhssini et al., 2004). Common species play a potential role in suppressing sunn pest populations below the economic threshold for control (Barbulescu, 1971; Critchley, 1998). However, the success of the parasitoid in terms of percentage parasitism of the host varies among regions as well as from year to year. Several studies have been conducted in order to improve the efficiency of the parasitoid (Kivan & Kilic, 2002; Trissi et al., 2005; Iranipour et al., 2010, 2011; Fathi et al., 2011). Since the success of a bio-

logical control is dependent on many factors, often including mass rearing of the parasitoid in the laboratory, whilst its release can sometimes be fraught with difficulties, including genetic compatibility between parasitoid and host (Hopper et al., 1993).

Because of its known effects on parasitic Hymenoptera (Zchori-Fein et al., 1992, 2000; Stouthamer, 1997; Plantard et al., 1998; Cook & Butcher, 1999; Hunter 1999; Stary, 1999), we hypothesized that *Wolbachia* infection might affect the establishment of *Trissolcus* species in the field. *Wolbachia* is an intracellular symbiont that causes reproductive alterations including cytoplasmic incompatibility, male killing, feminization and induction of thelytokous parthenogenesis (Breeuwer et al., 1992; O'Neill et al., 1992; Beard et al., 1993; Stouthamer et al., 1993; Sinkins et al., 1995a, b; Wade & Chang, 1995; Werren, 1997; Hurst et al., 1999; Stouthamer et al., 1999). The last effect appears to be most common in the order Hymenoptera, insects that normally reproduce by arrhenotoky where unfertilized haploid eggs develop into males and fertilized diploid eggs develop into females. These effects on individual hosts can also have impacts on host population biology (Jiggins, 2003), mating systems (Jiggins et al., 2000) and even speciation (Bordenstein et al., 2001).

In this study we detected *Wolbachia* as an endosymbiont of *Trissolcus* species, for the first time. We scanned *Trissolcus* species including *T. festivae* Viktorov, *T. grandis* Thompson, *T. simoni* Mayr, *T. semistriatus* Nees, *T. vassillievi* Mayr, *T. rufiventris* Mayr and *T. flavipes* Thompson in terms of *Wolbachia* infection. *Wolbachia* surface protein gene (*wsp*) sequences of different parasitoids have been previously reported. As a result of this

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TABLE 1. The *Wolbachia* infection status of parasitoid species collected from various sites. The values (+ve and -ve) refer to the number of parasitoids sampled by PCR analysis and found positive and negative for the presence of *Wolbachia*, respectively. Percentage (%) infected shows the *Wolbachia* infection observed in the total sampled population.

Parasitoids	Collection sites	Co-ordinates	Hosts	<i>Wolbachia</i> infection status		
				+ve	-ve	% infected
<i>Trissolcus festiva</i> Viktorov	Adana, Turkey	37°14'52"N, 35°17'41"E	<i>Eurygaster integriceps</i> Puton	36	1	97
	Konya, Turkey	37°36'39"N, 33°08'13"E 37°35'21"N, 32°45'35"E	<i>E. maura</i> (Linnaeus, 1758)			
<i>T. grandis</i> Thompson	Ankara, Turkey	39°45'08"N, 32°45'25"E 39°47'48"N, 32°52'42"E	<i>E. maura</i> (Linnaeus, 1758)	42	4	91
	Adana, Turkey	37°06'19"N, 35°06'35"E	<i>E. integriceps</i> Puton			
<i>T. simoni</i> Mayr	Adana, Turkey	37°06'11"N, 35°07'48"E	<i>E. integriceps</i> Puton	14	7	67
	Adana, Turkey	37°05'29"N, 35°37'33"E 37°13'20"N, 35°05'26"E	<i>E. integriceps</i> Puton			
<i>T. semistriatus</i> Nees	Konya, Turkey	37°31'17"N, 33°17'24"E 39°33'38"N, 33°06'36"E	<i>E. maura</i> (Linnaeus, 1758)	0	70	0
	Ankara, Turkey	39°47'06"N, 32°44'04"E	<i>E. maura</i> (Linnaeus, 1758)			
<i>T. vassilievi</i> Mayr	Konya, Turkey	37°35'00"N, 33°06'01"E	<i>E. maura</i> (Linnaeus, 1758)	0	12	0
	Yozgat, Turkey	39°29'11"N, 35°24'25"E	<i>E. maura</i> (Linnaeus, 1758)			
	Ankara, Turkey	39°26'23"N, 32°31'09"E 39°30'05"N, 32°26'31"E	<i>Aelia rostrata</i> Boheman	48	0	100
<i>T. rufiventris</i> Mayr	Konya, Turkey	38°15'33"N, 32°25'08"E 38°10'34"N, 32°24'46"E	<i>A. rostrata</i> Boheman			
<i>T. flavipes</i> Thompson	Ankara, Turkey	39°33'38"N, 33°15'14"E 39°41'12"N, 32°54'56"E	<i>Carpocoris pudicus</i> Poda	35	3	92

study *Wolbachia* infection status of various *Trissolcus* species was empirically demonstrated.

## MATERIAL AND METHODS

The egg batches containing parasitoids of the sunn pest were kept at  $25 \pm 1^\circ\text{C}$  with  $65 \pm 5\%$  relative humidity. The winged adult parasitoids emerging from egg batches were divided into two groups, some of which were kept at  $-80^\circ\text{C}$  for later molecular analysis whilst the rest were placed in tubes with 70% alcohol for taxonomic identification. The *Wolbachia* infection status of parasitoid species collected from various sites are presented in Table 1. The values (+ve and -ve) refer to the number of parasitoids sampled by PCR analysis and found positive and negative for the presence of *Wolbachia*, respectively. Percentage infected shows the *Wolbachia* infection observed in the total sampled population.

Adult parasitoids were homogenized in lysis buffer (50 mM Tris, 50 mM EDTA, 0.4 M LiCl, 1% CTAB, 2% PVPP and 0.5% Tween 20) with a sterile pestle in order to isolate genomic DNA. The homogenate was incubated at  $65^\circ\text{C}$  for 15 min and extracted with chloroform-isoamyl alcohol (24 : 1) followed by isopropanol precipitation. The pellet was washed with 70% ethanol and resuspended in sterile water.

Polymerase chain reactions (PCRs) were performed in a total volume of 50  $\mu\text{l}$  using 1  $\mu\text{l}$  of DNA template and GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions: 5 $\times$  buffer, 10 mM each dNTP, 10 mM each primer and 1.25 u/ $\mu\text{l}$  DNA polymerase. We used universal primers of *wsp* gene in PCR amplifications (Braig et al., 1998), *wsp* 81 F (5'TGG TCC AAT AAG TGA TGA AGA AAC 3') and *wsp* 691 R (5' AAA AAT TAA ACG CTA CTC CA 3'). The sequences obtained with these general primers allowed us to design species specific primers: *Tris wsp* F (5' TGG TCC AAT AAG TGA TGA AGA AAC T 3') and *Tris wsp* R (5' CAC CAT AAG AGC CGA AAT AAC G 3'). A third primer set (12 SAI: 5' CTA GGA TTA GAT ACC CTA

TT 3'and 12 SBI: 5' AAG AGC GAC GGG CGA TG 3') was used to assess the quality of the template DNA. 12S primers are considered universal for insect mtDNA amplifying an approximately 400 bp DNA fragment (Simon et al., 1991). PCR products were visualized by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide. In the case of negative amplification, PCRs were repeated with higher DNA template concentrations to ensure that the dilutions used were not below the sensitivity of the PCR technique. Data were excluded in cases in which the 12S control primers were unable to amplify a product from a given DNA extraction. *Wolbachia* specific PCR products were purified with Wizard SV Gel and PCR Clean up System (Promega). The purified PCR fragments were cloned into PGEM T vector systems (Promega). Sequencing reactions were performed with DTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA), cleaned with Agencourt CleanSeq Kit (Agencourt Bioscience, Brea, CA, USA) and analyzed with CEQ 8800 Genetic Analysis System (Beckman Coulter).

Sequence analysis was performed using blast at NCBI ([www.ncbi.nih.gov/BLAST](http://www.ncbi.nih.gov/BLAST)) and DNA sequences were aligned with ClustalW. The amplified fragments were confirmed to encode the WSP protein through conceptual translational of their DNA sequences and comparison of the translation to closely related WSP protein sequences. The obtained sequences were used to generate phylogenetic tree of *Wolbachia* harbored within *Trissolcus* species. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Pairwise distances were calculated using the Maximum Composite Likelihood method in MEGA4 (Tamura et al., 2004, 2007). The number of base substitutions per site between sequences was calculated. All results were based on the pairwise analysis of five sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). A bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than

50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

Alignments were checked in terms of intragenic recombination using the software package (Martin et al., 2005). Programs used in the RDP2 software package were RDP, GeneConv, Bootscan, MaxChi, Chimaera, and Sister Scanning. Analyses were run with default settings except for window and step sizes.

## RESULTS AND DISCUSSION

Seven *Trissolcus* species including *T. festiva* Viktorov, *T. grandis* Thompson, *T. simoni* Mayr, *T. semistriatus* Nees, *T. vassillievi* Mayr, *T. rufiventris* Mayr, and *T. flavipes* Thompson were screened for *Wolbachia* infection by PCR analysis. A 500 bp fragment of the *wsp* gene was amplified and sequenced from the endosymbionts of five *Trissolcus* species with species specific primers. Results showed that infections could be detected in *T. festiva*, *T. grandis*, *T. simoni*, *T. rufiventris*, and *T. flavipes*, which are the most common species attacking eggs of *Eurygaster integriceps* (Kocak & Kilincer, 2002; Trissi et al., 2004). This finding represents the first record of *Wolbachia* infection in *Trissolcus* species.

Cloning and sequencing of PCR bands revealed a gene encoding WSP protein isolated from diverse arthropods according to BLAST analysis (www.ncbi.nih.gov/BLAST). The sequences have been submitted to GenBank with the accession numbers for *wsp* sequences as follows: HQ447075 for *T. grandis*, HQ447076 for *T. simoni*, HQ447077 for *T. festiva*, HQ447078 for *T. flavipes*, and HQ447079 for *T. rufiventris*. According to pairwise analysis, all *Trissolcus* *wsp* sequences belong to the same bacterial strain. GenBank homology searches of *Trissolcus* *wsp* sequences indicated that the symbionts of all *Trissolcus* species were most closely related to the *Wolbachia* symbiont of other parasitic wasps. A phylogenetic tree of all the *Wolbachia* strains found in infected *Trissolcus* species was constructed. All procedures used for phylogenetic reconstruction (maximum parsimony, maximum likelihood, and neighbor-joining methods) place all *Trissolcus* *Wolbachia* strains in a monophyletic group with those of B group *Wolbachia* with bootstrap values of 100 (maximum likelihood and neighbor-joining analysis). Fig. 1 shows the phylogenetic tree obtained by neighbor-joining method with 1000 bootstrap replicates. The trees based on maximum likelihood and parsimony

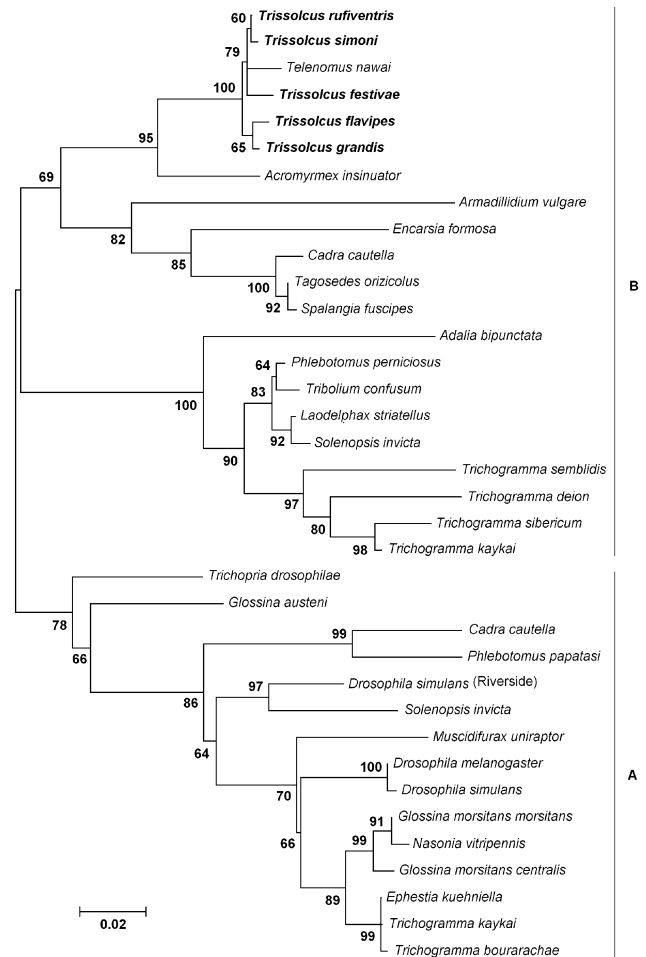


Fig. 1. Phylogenetic analysis of *Wolbachia* infections in *Trissolcus* species in relation to other insect species. Phylogenetic tree was constructed with MEGA 4 Software (Tamura et al., 2007) using neighbor-joining method.

analyses are not shown, but do not significantly deviate from these results. Furthermore, intragenic recombination within the *wsp* gene was analyzed using RDP, GeneConv, Bootscan, MaxChi, Chimaera, and Siscan which are implemented in the RDP2 program. According to these analyses, four recombination events were detected involving three species; *T. rufiventris*, *T. grandis*, and *T. flavipes* (Table 2). The major and minor parents were identified as *Acromyrmex insinuator* (Schultz, Bekkevold & Boomsma 1998) (Hymenoptera: Formicidae) and *Glossina austeni* Newstead (Diptera: Glossinidae), respectively.

Earlier work on *Wolbachia* phylogeny showed that *Wolbachia* strains are clustered into eight divergent clades that are described as supergroups A–H. (O'Neill et

TABLE 2. Summary of recombination events identified by the Recombination Detection Program (RDP2).

Daughter	Major parent	Minor parent	Method	P value
<i>T. rufiventris</i>	<i>Acromyrmex insinuato</i>	<i>Glossina austeni</i>	Bootscan	2.08E–3
<i>T. grandis</i>	<i>A. insinuato</i>	<i>G. austeni</i>	Bootscan	1.626E–3
<i>T. flavipes</i>	<i>A. insinuato</i>	<i>G. austeni</i>	Siscan	6.130E–3
<i>T. grandis</i>	<i>A. insinuato</i>	<i>G. austeni</i>	Siscan	5.677E–3

al., 1992; Werren et al., 1995; Bandi et al., 1998; Zhou et al., 1998; Schulenburg et al., 2000; Lo et al., 2002; Casiraghi et al., 2003, 2005; Bordenstein & Rosengaus, 2005). Among arthropods, supergroup A and B are the most common types (Werren & Windsor, 2000), whereas supergroup E, F, G, and H occur less frequently (Czartnietzki & Tebbe, 2004; Rowley et al., 2004; Bordenstein & Rosengaus, 2005; Casiraghi et al., 2005; Panaram & Marshall, 2006). Supergroups C and D are commonly found in filarial nematodes (Bandi et al., 1998). To date, the 16S rDNA, *ftsZ*, *wsp*, *groEL*, *dnaA*, and *gltA* genes have been characterized and used for phylogenetic analysis of *Wolbachia* species (O'Neill et al., 1992; Werren et al., 1995; Bandi et al., 1998; Zhou et al., 1998; Schulenburg et al., 2000; Lo et al., 2002; Casiraghi et al., 2003, 2005; Bordenstein & Rosengaus, 2005). Among them, the *wsp* gene has been routinely used for supergroup designation (Zhou et al., 1998; Shoemaker et al., 2002; Kikuchi & Fukatsu, 2003; Haine & Cook, 2005; Kyei-Poku et al., 2005; Malloch & Fenton, 2005; Zeh et al., 2005; Sintupachee et al., 2006). However, a Multilocus Sequence Typing (MLST) scheme for *Wolbachia* using five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) has been developed and has been shown to be consistent in terms of supergroup classification of a strain (Baldo et al., 2006). The present study is based on a *wsp* gene based classification system for *Wolbachia* strains. Phylogenetic analysis of *Wolbachia* from five *Trissolcus* species appears to belong to the B supergroup in the "Nawai" subgroup. Further MLST analysis is required in order to discriminate closely related *Wolbachia* strains infecting *Trissolcus* species.

Previous studies have reported that prokaryotic endosymbionts other than *Wolbachia* can cause reproductive alterations in insects (Lawson et al., 2001; Zchori-Fein et al., 2001; Hunter et al., 2003; Hagimori et al., 2006; Perlman et al., 2006). In the present work, we have reported only *Wolbachia* infection in *Trissolcus* species, since other potential secondary endosymbionts of *Trissolcus* species remain as yet unknown. It will be interesting to know how these multiple symbionts interact with their hosts and each other.

It has been suggested that mixing *Wolbachia* infected and uninfected populations may cause reproductive failure in biocontrol programs of parasitoids (Mochiah et al., 2002; Perlman et al., 2006). If parasitoid strains with *Wolbachia* infection are released in an area where a *Wolbachia* free population occurs, sterile mating will diminish the reproductive potential of the introduced parasitoid. Mating between males from populations that harbor *Wolbachia* and females free from *Wolbachia* infection could theoretically contribute to failure in biological control programmes to eradicate pest insects. Differences in distribution and *Wolbachia* infection types in parasitoids may give clues which enable parasitoid strains to be used more effectively. It is particularly important to determine the infection status of different populations when they are mixed either in the laboratory for mass production or when they are released in the field where a

population is already established (Mochiah et al., 2002). On the other hand, *Wolbachia* has also been shown to induce parthenogenesis in parasitic Hymenoptera which can be considered as an advantage in biological control (Stouthamer et al., 1990; Zchori-Fein et al., 1992; Stouthamer, 1997).

Biological control programmers should be aware of *Wolbachia* infections and their effects on parasitoid populations which are important for the establishment of laboratory cultures. This is because mixed populations of differing *Wolbachia* infectivity could result in a severe reduction of population growth in the laboratory. Further studies need to be done to determine the distribution and prevalence of *Wolbachia* infection in *Trissolcus* species. In addition, further crossing experiments should be performed between the same strains or between an infected female and an uninfected male or a treated male in order to determine the influence of *Wolbachia* infection on *Trissolcus* reproduction.

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