



Infection by *Wolbachia* bacteria and its influence on the reproduction of the stored-product psocid, *Liposcelis tricolor*

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Abstract

Wolbachia are maternally inherited intracellular bacteria that infect a wide range of arthropods and nematodes and are associated with various reproductive abnormalities in their hosts. The infection by *Wolbachia* of the psocid, *Liposcelis tricolor* (Psocoptera: Liposcelididae), was investigated using long PCR amplification of the *wsp* gene that codes for a *Wolbachia* surface protein. The results showed that *L. tricolor* was positive for *Wolbachia*. Phylogenetic analysis showed that the *Wolbachia* found in *L. tricolor* was related to the B-group. *Wolbachia* infection in *L. tricolor* could be removed through antibiotic treatment. The results of crosses including ♀^{W+} × ♂^{W+}, ♀^{W-} × ♂^{W+}, ♀^{W+} × ♂^{W-}, and ♀^{W-} × ♂^{W-}, suggested that the removal of *Wolbachia* resulted in lower egg production by *L. tricolor*. The mean embryonic mortality of offspring produced by *L. tricolor* without *Wolbachia* was significantly higher than that of control.

Keywords: endosymbionts, molecular detection, antibiotic treatment

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Received: 29.12.2005 | Accepted: 14.3.2006 | Published: 28.9.2006

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ISSN: 1536-2442 | Volume 2006, Number 24

Cite this paper as:

Dong P, Wang J, Zhao Z. 2006. Infection by *Wolbachia* bacteria and its influence on the reproduction of the stored-product psocid, *Liposcelis tricolor*. 7pp. *Journal of Insect Science* 6:24, available online: insectscience.org/6.24

Introduction

Wolbachia, rickettsia-like proteobacteria, are found in the reproductive tissues of a wide range of arthropod species (O'Neill et al. 1997; Werren 1997; Tram and Sullivan 2002). *Wolbachia* are obligate intracellular parasites that are transmitted maternally from infected females to their progeny. *Wolbachia* infections are associated with a variety of reproductive anomalies in the host, including cytoplasmic incompatibility between different populations (Laven 1967) or closely related species (Breeuwer and Werren 1990; Breeuwer et al. 1992), thelytoky or parthenogenesis induction in parasitoid wasps (Stouthamer et al. 1990; 1993), male-killing in coccinellid beetles (Hurst et al. 1999), and feminization of genetic males in isopods (Rousset et al. 1992).

Wolbachia cannot be cultured in defined media and detection within infected gonadal cells may be time consuming. Therefore, detection of *Wolbachia* infection has been based largely on amplification of *Wolbachia* DNA using allele-specific polymerase chain reactions. Primers designed from the 16S rDNA (O'Neill et al. 1992), *ftsZ* (Werren et al. 1995) and *wsp* genes (Zhou et al. 1998; Jeyaprakash and Hoy 2000) have been used to amplify *Wolbachia* DNA from a diverse array of arthropods.

Antibiotic treatment is one established method for producing *Wolbachia*-free individuals (O'Neill and Karr 1990; Yusuf and Turner 2004). A wide range of antibiotics with different modes of action have been used to produce aposymbiotic insects (Stouthamer et al. 1993). The strains of parthenogenetic *Trichogramma* wasps revert to sexual reproduction when rickettsial endosymbionts are eliminated from wasp gonads (Stouthamer et al. 1993).

The psocid, *Liposcelis tricolor*, a bisexual species, is worldwide and commonly found in various processed and unprocessed dry foods in households, granaries, and warehouses (Kalinovi and Rozman 2000). They transmit harmful microorganisms, including fungi and bacteria on their bodies, hair, and in feces. Psocids are found to be harmful pests on seed goods as well by causing damage to seed kernels (Kalinovi and Rozman 2000). This study was initiated to find if *Wolbachia* infest *L. tricolor* and their influence on the reproductive biology of *L. tricolor*.

Materials and Methods

Insects

Stock colonies of *Liposcelis tricolor* originated from larva collected in a wheat warehouse in Shandong, China in 2003. This colony was reared on an artificial diet consisting of whole wheat flour, skim milk and yeast powder (10:1:1) in an air-conditioned room at 27±1 °C and a scotoperiod of 24 h. Cultures were set up in glass bottles (250 ml) with a nylon screen cover and kept in desiccators (5 liter), in which the humidity was controlled with saturated NaCl solution at 75–80% RH. After several generations, insects from the stock colony were used for the tests.

DNA extraction

Twenty adult *L. tricolor* were surface-sterilized in a series of double distilled water and 70% ethanol washes, then frozen under liquid nitrogen and crushed in 300 µl DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine), homogenized using a DNA-free disposable polypropylene pestle and incubated with 2 µl of proteinase-K (20 mg/L) for 2h at 50° C, followed by 5 min at 95 °C for denaturing. One volume of phenol saturated water (pH8.0) and 1 volume of chloroform:isoamyl alcohol (24:1) was added before centrifugation for 10 min at 10000 rpm. The supernatant was collected and gently mixed with 0.2 volume of Na-acetate (3 mM, pH5.2) and 2 volumes of 100% ethanol. After precipitation for 2h at -20 °C, the DNA was washed with 70% ethanol, air dried and finally resuspended in 20 µl of ddH₂O. 3 µl of the DNA sample was used for PCR experiments. DNA quality was determined using insect 12S rDNA primers. The PCR mixtures and cycling conditions were from Yoshizawa and Johnson (2003).

PCR screening procedure for *Wolbachia* infection

Long PCR was performed in a 25 µl containing 50 mM Tris (pH 9.2), 16mM ammonium sulphate, 1.75 mM MgCl₂, 350 µM dNTPs, 800 pM of primers (*wsp*-F, 5'-TGG TCC AAT AAG TGA TGA AAG AAA CTA GCT A and *wsp*-R, 5'-AAA AAT TAA ACG CTA CTC CAG CTT CTG CAC), 1 unit of *Pwo* and 5 units of *Taq* DNA polymerases. The Long PCR was carried out using three linked profiles: (i) one cycle (2min at 94 °C), (ii) 10 cycles (10s at 94 °C, 30s at 65° C, 1min at 68 °C), (iii) 25 cycles (10s at 94 °C, 30s at 65 °C, 1 min at 68 °C, plus an additional 20s added for every consecutive cycle).

To confirm that the PCR products obtained were not due to contamination, an attempt was made to sequence the long PCR products directly from *L. tricolor*. The PCR product was then ligated into a pGEM-T vector. Three clones were produced that were sequenced by Shanghai Sangon Biological Engineering Technology & Services Company.

Phylogenetic analysis

The *wsp* datasets representative sequences for *Wolbachia* groups in different hosts were retrieved from GenBank and included in phylogeny reconstruction. Sequences were aligned using CLUSTAL W (Thompson et al. 1994). Phylogenies for *wsp* were estimated by neighbor-joining analysis of the sequence data. Gaps were coded as missing data. Distances were calculated using maximum likelihood. The model of sequence evolution and the parameter values best fitting the data for each locus were identified using likelihood ratio tests (Huelsenbeck and Pannala 1997), as implemented in PHYLIP 3.57c (Felsenstein 1995) to search for the tree with the highest likelihood. The parameter values suggested by PHYLIP for each dataset were used to specify the distance matrix for neighbor-joining analysis on 1000 bootstrap replicates.

Antibiotic experiments

Rifampicin, a potent inhibitor of DNA-dependent RNA polymerase of bacteria, was used to produce *Wolbachia*-free hosts. Rifampicin (1%) was prepared by mixing 10g of the standard diet with 0.1 g rifampicin (Sigma, www.sigmaaldrich.com). Three grams of this food-antibiotic mixture were further diluted into 10 and 100g of standard diet to make final concentrations of 1%, 0.3%, and 0.03% antibiotic in the standard diet. Dry mixing of the food and antibiotic powder proved unsatisfactory and instead the food-antibiotic mixture was moistened with sterile distilled water, well stirred to create homogeneous slurry, air-dried and ground to a powder (Yusuf and Turner 2004). After 4 weeks treatment with rifampicin, the psocids were checked with the above molecular method. After two generations, the psocids of *Wolbachia*-free strain (W^-) and untreated strain (W^+) were used for the following cross experiment.

Effects of *Wolbachia* removal on fecundity and fertility

Four crosses ($\text{♀}^{W^+} \times \text{♂}^{W^+}$, $\text{♀}^{W^-} \times \text{♂}^{W^+}$, $\text{♀}^{W^+} \times \text{♂}^{W^-}$, and $\text{♀}^{W^-} \times \text{♂}^{W^-}$) were tested in this experiment. The cross of the infected female and male was used as a

control. In each cross experiment, 30–61 maturing females (when the colorless stage begins to change to brown, 12–24 h after the final molt) were removed from culture populations with a fine brush. They were placed individually in a small glass vials containing a piece of filter paper on one side of which was glued the culture medium. The glass vials were covered with fine mesh net to prevent psocids escape. One corresponding matured male was placed into each glass vials. The glass vials were placed in each humidified desiccator (75–80%RH). The desiccators were placed in growth chambers at 27.5 °C with a scotoperiod of 24 h. Eggs produced from the different treatment were counted, and the mortality of eggs from the different treatments was monitored. The total egg numbers within 60 days were counted for each experiment, and the statistical analysis was conducted using SPSS statistical package.

Results

Genomic DNA prepared from *L. tricolor*, was amplified by Long PCR, using the primers (*wsp*-F and *wsp*-R). A 605 bp fragment of the *wsp* gene was amplified (Figure 1) and this sequence was deposited in GenBank with accession number AY639593. The infection frequency of *Wolbachia* in *L. tricolor* was found to be 100% in 5 randomly selected females and 5 males.

The phylogenetic tree based on the *wsp* sequences using neighbor-joining after bootstrapping 1000 times is shown in Figure 2. The topology showed the division of *Wolbachia* into two subgroups, A and B. The A subgroup included fourteen species and the B subgroup included fifteen species. *Wolbachia* in *L. tricolor* belonged to the B subgroup and was close to *Wolbachia Pip* in *Culex quinquefasciatus* and *Aedes albopictus*.

After 4 weeks treatment with 1% rifampicin, no detectable level of the *wsp* gene was amplified, while the same gene fragment remained present in the 0.3% or 0.03% rifampicin treated individuals (Figure 3). A strain of *L. tricolor* without *Wolbachia* infection was obtained.

In cross experiments, all combinations could produce eggs. Total egg production was significantly increased in crosses with *Wolbachia* present, and egg mortality was significantly lower (Table 1, Figure 4).

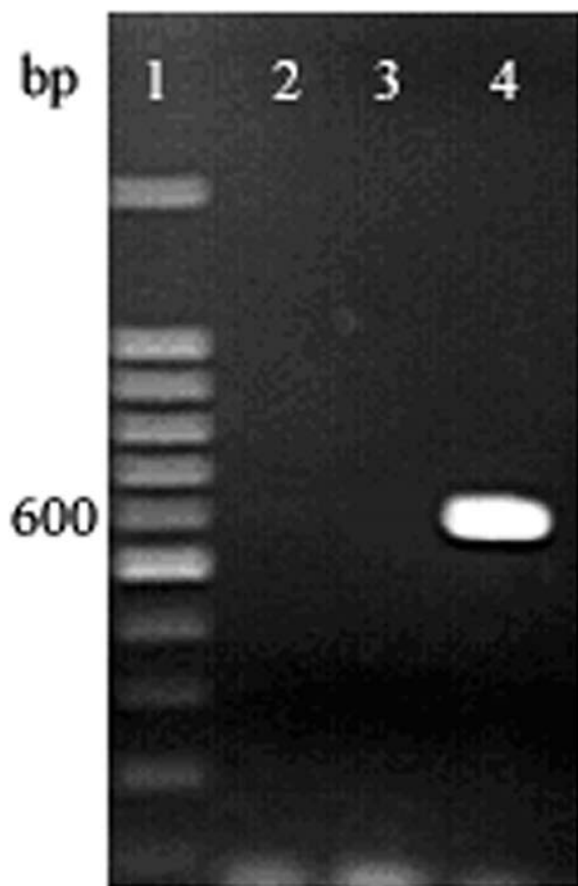


Figure 1. Long PCR amplified *wsp* sequences from *L. tricolor*. Lane 1, 100bp DNA marker; lane 2 and 3 no DNA-control; lane 4, *L. tricolor*

Discussion

In preliminary experiments both regular and long PCR protocols were used to detect the infection of *Wolbachia* in *L. tricolor* in the present study, and the target fragment was scored in both protocols. However, there were some non-specific fragments that appeared using the regular PCR protocol. Therefore, the long PCR protocol was used throughout the experiment.

Phylogenetic evidence has shown that horizontal transfer of *Wolbachia* must have occurred in the course of evolution because closely related bacterial strains can be found in unrelated hosts (Huigens et al. 2004; O'Neill et al. 1992; Werren and Zhang 1995). In this study, phylogenetic analysis showed that *Wolbachia* in *L. tricolor* was close to *wPip* present in *Culex quinquefasciatus* and *Aedes albopictus*.

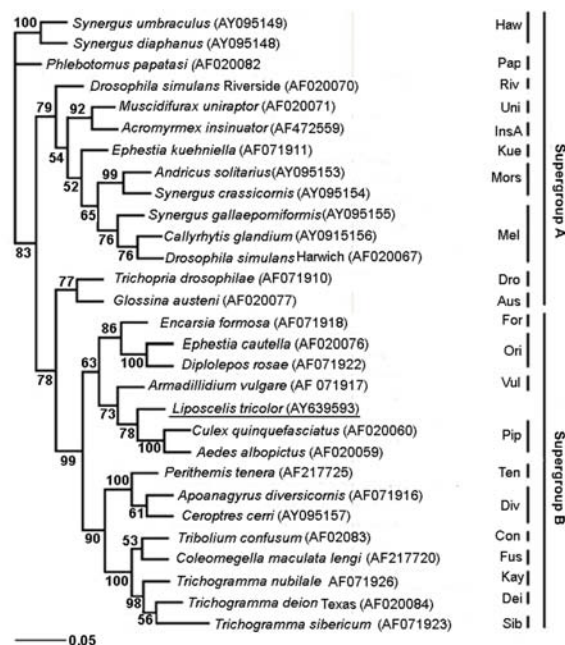


Figure 2. The phylogenetic relationship between the different *Wolbachia* *wsp* sequences as reconstructed using a neighbour-joining algorithm on maximum likelihood estimated distance matrix. Name of the host arthropod species is followed by GenBank Accession no. and clade designation. The *Wolbachia* groups to which sequences belong to are also shown. Bootstrap values of 1000 replicates are presented above the branches. Bootstrap values less than 50 are not shown. *Wolbachia* in *L. tricolor* was shown by underling of group.

In the present study, a strain of *L. tricolor* without *Wolbachia* infection was obtained by treating with 1% rifampicin for 4 weeks. Lower concentrations of rifampicin (0.3% or 0.03%) failed to remove the *Wolbachia* infection completely. Similar results were reported for tetracycline treatment of *Wolbachia* infection in *Aedes albopictus* (Dobson and Rattanadechakul 2001) and rifampicin treatment of *Liposcelis bostrychophila* by Yusuf and Turner (2004).

Wolbachia have a variety of different effects on their hosts' reproduction. Cytoplasmic incompatibility is the most widespread and, perhaps, the most prominent feature that *Wolbachia* endosymbionts impose on their hosts. Cytoplasmic incompatibility resulted in embryonic mortality in crosses between insects with different *Wolbachia* infection status. It can be either unidirectional or bi-directional. Unidirectional cytoplasmic incompatibility is typically expressed when an infected male is mated with an uninfected female (Hoffmann and Turelli 1997; Charlat et al. 2001). However, all cross combinations in this

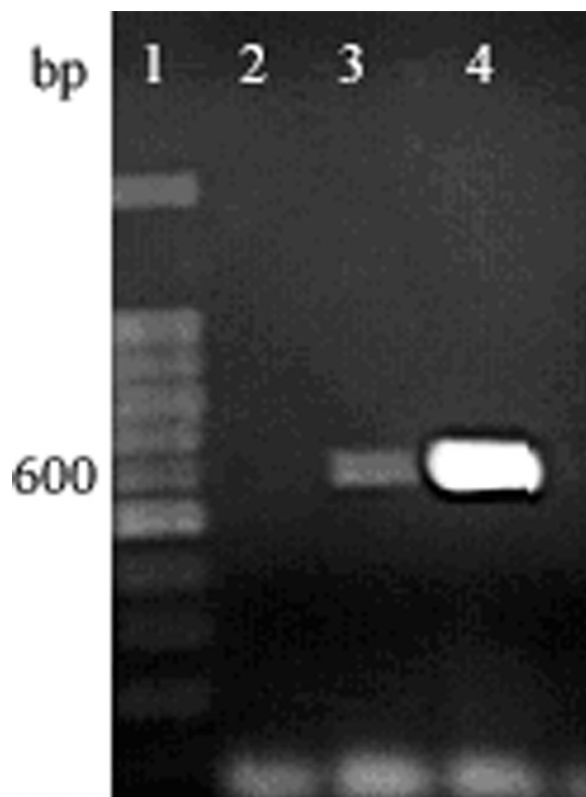


Figure 3. Long PCR amplification of *wsp* sequences from *L. tricolor* treated with antibiotic of different dosages. Lane 1, 100bp DNA marker; Lane 2, 1% rifampicin; lane 3, 0.3% rifampicin; lane 4, 0.03% rifampicin.

study could produce hatching eggs, and the cross $\text{♀}^{W-} \times \text{♂}^{W+}$ did not show higher embryonic mortality compared to the $\text{♀}^{W-} \times \text{♂}^{W-}$ cross. As a result there is not enough evidence to confirm cytoplasmic incompatibility phenomenon caused by *Wolbachia* in *L. tricolor*.

Wolbachia could have positive effects on host fecundity (Girin and Bouletreau 1995; Stolk and Stouthamer 1996) and fertility (Wade and Chang 1995). In this study, egg production of ♀^{W-} mated with ♂^{W-} , ♀^{W+} with ♂^{W-} and ♀^{W-} with ♂^{W+} were all significantly less than the control. The eggs with the highest survival were from the control crosses with both male and female infected, and the other crosses are statistically distinguishable. This suggests that for optimal reproduction, *Wolbachia* must also be present in the male. It implies that *Wolbachia* infection can have positive effects on fecundity and fertility of *L. tricolor*. Similarly, Dedeine et al. (2001) reported that the wasp *Asobara tabida* without *Wolbachia* removed by antibiotics also had lower egg production. By comparison, the mortality of eggs produced by *L.*

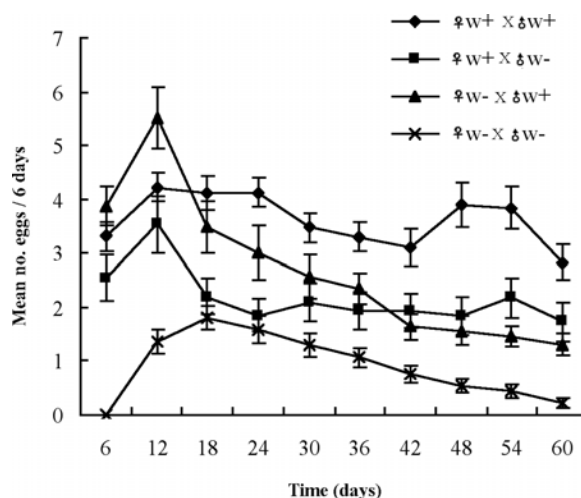


Figure 4. Effects of *Wolbachia* on fecundity and fertility of *L. tricolor*.

tricolor without *Wolbachia* infection was significantly increased. This could be due to cooperative evolution between *Wolbachia* and the psocid host.

In recent years, there has been increasing interest in the biology of *Wolbachia* and in its application as an agent for control or modification of insect population (Zabalou et al. 2004). Therefore, the mode of action of *Wolbachia* manipulation on psocid reproduction warrants further investigation.

Acknowledgements

This research was funded in part by the National Natural Sciences Foundation (30471173), the Program for New Century Excellent Talents in University (NCET-04-0854), and the Specialized Research Fund for the Doctoral Program of Higher Education of China (20040625006). Thanks go to Hong-Bo Jiang, Ji-Zhao Wu, Fu-Xian Jia, Fei Hu, Li-Sha Xiao for technical assistance.

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