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Research Article

Detection and characterization of *Wolbachia* infection in silkworm

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Abstract

Wolbachia naturally infects a wide variety of arthropods, where it plays important roles in host reproduction. It was previously reported that *Wolbachia* did not infect silkworm. By means of PCR and sequencing we found in this study that *Wolbachia* is indeed present in silkworm. Phylogenetic analysis indicates that *Wolbachia* infection in silkworm may have occurred via transfer from parasitic wasps. Furthermore, Southern blotting results suggest a lateral transfer of the *wsp* gene into the genomes of some wild silkworms. By antibiotic treatments, we found that tetracycline and ciprofloxacin can eliminate *Wolbachia* in the silkworm and *Wolbachia* is important to ovary development of silkworm. These results provide clues towards a more comprehensive understanding of the interaction between *Wolbachia* and silkworm and possibly other lepidopteran insects.

Keywords: *Wolbachia*, silkworm, *wsp*, antibiotics.

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Introduction

Wolbachia is a cytoplasmically inherited rickettsia that was found in a wide range of arthropods (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Hilgenboecker *et al.*, 2008). Infections were detected in all of the major insect orders, including Coleoptera, Diptera, Hemiptera/Homoptera, Hymenoptera, Lepidoptera, and Orthoptera (Werren and Windsor, 2000). Wolbachia can regulate host reproduction via cytoplasmic incompatibility (CI), feminization, parthenogenesis and male killing (Werren *et al.*, 2008; Blagrove *et al.*, 2012).

Zhou *et al.* (1998) established a general naming system basing on the sequence of the *wsp* gene, a single copy gene coding for an outer membrane protein of *Wolbachia*. They classified *Wolbachia* into two supergroups, supergroup A and B, and within these a total of eight potential groups could be recognized within the A group and four within group B. As *wsp* shows relatively high genetic divergence among these strains, the gene has been used extensively in phylogenetic analyses and for microtaxonomic subdivision (Van Meer *et al.*, 1999; Baldo *et al.*, 2005). Several recent studies using FISH or Southern blotting methods reported that *Wolbachia* genes have been horizontally transferred to host chromosomes (Kondo *et al.*, 2002; Nikoh *et al.*, 2008). Such events are present in a variety of insects, including the fruit fly *Drosophila ananassae*, a parasitoid wasp of the genus *Nasonia*, the mosquito *Aedes aegypti*, the pea aphid *Acyrthosiphon pisum*, the longicorn beetle *Monochamus alternates,* and the adzuki bean beetle *Callosobruchus chinensis* (Dunning Hottop *et al.*, 2007; Klasson *et al.*, 2009; Nikoh and Nakabachi, 2009).

In order to identify the function of *Wolbachia* in its host, a frequently used method is the elimination of *Wolbachia* through the use of selective antibiotics (Kuriwada *et al.*, 2010; Voronin *et al.*, 2012). Fytrou *et al.* (2006) found that *Wolbachia*-infected *Drosophila simulans* showed a reduced ability to encapsulate parasitoid eggs compared to a tetracycline-treated, bacterium-free line. Chen *et al.* (2012) used tetracycline to eliminate *Wolbachia* in the rice water weevil, *Lissorhoptrus oryzophilus*, showing that *Wolbachia* is necessary for its host's oocyte production.

The silkworm, *Bombyx mori*, is an economically important lepidopteran insect, domesticated from the wild silkworm *Bombyx mandarina,* which occurs in a range from India to China, Korea, Japan and far into the eastern regions of Russia (Goldsmith *et al.*, 2005). Despite its economic importance, surprisingly few studies focused on *Wolbachia* in the silkworm. One reason may be that *Wolbachia* was not detected in the silkworm strains used in previous studies (Puttaraju and Madhu, 2002; Prakash and Puttaraju, 2007). In this study, we amplified the *Wolbachia*-specific *wsp* gene in silkworm by PCR and cloned the gene. Our results indicate that *Wolbachia* is present in several strains of silkworm and antibiotics treatment revealed that *Wolbachia* plays an important role in silkworm ovary development.

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Materials and Methods

Silkworm samples

The silkworms examined in this study, information about strain and collection locations are listed in Supplementary material Table S1. Samples of adult silkworm moths were collected in 2008 and stored in absolute ethanol at -80 °C. Total DNA of was extracted from each single strain (one individual per sample). DNA extraction was performed by using the QIAamp DNA Mini kit (Qiagen) for PCR amplification or by a standard proteinase K/SDS/phenol/chloroform extraction procedure for Southern blotting (Green and Sambrook, 2012).

PCR and cloning of the *wsp* gene

Wolbachia infection was detected by polymerase chain reaction (PCR) using the following wsp-specifc primers: wsp-81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3'), wsp-691R (5'-AAAAATTAAACGCTACTCCA-3') (Braig *et al.*, 1998; Zhou *et al.*, 1998), resulting in an amplified DNA fragment of about 600 bp. PCR amplications were performed in 25 μL reactions containing 1 μL of DNA, 2.5 μL 10x reaction buffer, 2.0 μL $MgCl_2$ (25 mM), 1 μL dNTPs (25 uM), 0.2 μL of *Taq* polymerase (Takara) and 18.3 μL water. PCRs were run under the following cycling conditions: 94 °C for 4 min, followed by 30 cycles of 40 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C and a final extension step of 10 min at 72 °C. The PCR products were electrophoresed on a 1% agarose gel to determine the presence and general size of the amplified DNA. Considering the possibility of false positive PCR results, all the amplified PCR fragments were sequenced. For this, PCR products were purified by a Gel Extraction Kit (Omega) and purified DNA was ligated into a pMD18-T vector (Takara) for transformation of Escherichia coli DH5a competent cells. Positive clones were picked and sequenced. An additional strategy to detect *Wolbachia* infection was to design specific primers for the *Wolbachia ftsZ* gene: ftsZ-F: TACTGACTGTTGGAGTTGTAACTAAGCCGT and ftsZ-R: TGCCAGTTGCAAGAACAGAAACTCTAACTC. The resulting PCR fragments were cloned and sequenced in the same way.

Phylogenetic analysis

All *Wolbachia wsp* gene sequences obtained in this study were aligned by using the program package ClustalW (Thompson *et al.*, 1994) or MEGA3.1 (Kumar *et al.*, 2008). Sequences downloaded from GenBank representing all currently known supergroups of *Wolbachia* were included in the analysis (Table S2). Phylogenetic analyses were conducted by the Neighbour-Joining algorithm. Bootstrap probabilities were assessed by generating 1000 bootstrap replicates.

Southern blotting analysis

DNA probes for Southern blotting were synthesized by PCR amplification from recombinant *wsp* plasmid DNA as template using the same *wsp* gene specific primers. The amplification products were electrophoresed in 1% agarose gels and purified. Digoxigenin labeling was done by using the DIG Random Primed DNA Labeling Kit (Roche). Genomic DNA preparations of *Bombyx mori Dazao* and wild silkworms were digested with *Hind*III restriction enzyme. The digested DNA samples of 25-30 μg were electrophoresed in 0.8% agarose gels. Plasmid DNA of recombinant *wsp* gene vector was used as positive control. The separated DNA fragments were transferred to nylon membranes and fixed by UV cross-linking. Hybridization, stringency washes and detection were performed by using the DIG Detection Kit (Roche) following the manufacturer's manual.

Antibiotic treatment

The *Bombyx mori* strain *Dazao* used in this experiment was provided by the Silkworm Gene Resource of Southwest University. Silkworm larvae were reared under standard conditions. Antibiotics used included tetracycline, rifampicin, ciprofloxacin and penicillin (Table S3). Antibiotics were dissolved in double-distilled water at a concentration of 100 mg/mL. After the first day of pupation, each silkworm was injected with a volume of 10 μL (1 mg of the antibiotic) into the eighth abdominal segment by using needles pulled from glass capillaries. Negative-control silkworms were injected with the same volume of water. Individual silkworms were collected 10 days after injection and DNA was extracted as per the above described protocol. Quantitative PCR was used to determine *Wolbachia* abundance in silkworms. In order to understand the effect of antibiotic treatment, *Wolbachia* density of untreated samples (Infection free) was also detected by Quantitative PCR.

Given a probable side effect of a high dose of antibiotic, injections were also done with a lower dose of tetracycline (10 mg/mL). Injection volume, time and location were the same as in the former experiment. Furthermore, tetracycline-injected female and male silkworms were mated and the F1 generation was reared to assess the ovary phenotype adult F1 females.

Quantitative PCR

The abundance of *Wolbachia* after antibiotic treatments was determined by quantitative PCR. Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) was used according to the manufacturer's protocol. Real-time quantitative PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primer sequences corresponded to regions of the *Bombyx mori* reference gene *sw22934* (Wang *et al.*, 2008) and the

Wolbachia wsp gene. The *sw22934* primer sequences used were 5'-TTCGTACTGGCTCTTCTCGT-3' and 5'-CAAAGTTGATAGCAATTCCCT-3', while the *wsp* primer sequences were 5'-AGATAGTGTAACAGCGTT TTCAGGAT-3' and 5'-CACCATAAGAACCAAAAT AACGAG-3'. Template-free qPCRs were included as negative controls. Positive controls for the reference gene and *Wolbachia* were also included in each qPCR run. The instrument was programmed to provide initial enzyme activation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Amplification efficiency was calculated according to the method of Tichopad *et al.* (2003).

Results

Screening for *Wolbachia* infections in silkworm populations

By means of a PCR approach using the general *wsp*-specific primers we screened a total of 21 samples of silkworms for the presence of *Wolbachia*. The samples were collected in seven provinces of China (Chongqing, Yunnan, Sichuan, Shandong, Zhejiang, Jiangsu and Guangdong) and represented two types of silkworms (domesticated and wild silkworms). The results showed that PCR amplification products with the expected size of about 600 bp were observed (Figure 1). In order to confirm PCR products, we cloned and sequenced the fragments, showing that the 21 silkworm samples share high sequence similarity with differences in only a few sites (Figures S1 and S2). Taken together, these results demonstrate that *wsp* gene fragments could be amplified in all 21 silkworm samples. The sequence of the silkworm *wsp* gene was submitted to GenBank (Acc. No. KJ659909). Homology searches with BLASTn indicated that the sequence in the Dali wild silkworm sample were the best hit to the *wsp* gene of *Wolbachia* in Orius minutus (Tsukuba), with 98% identity. Additionally, PCR assays were carried out by using ftsZ-specific primers in wild silkworm Dali and Dazao (Figure 1C), and the sequencing results showed that the PCR bands were indeed the *ftsZ* gene of *Wolbachia*. The sequence of *ftsZ* was also submitted to GenBank (Acc. No. KJ659910). In conclusion, the PCR results for the *wsp* and *ftsZ* genes are evidence that there are *Wolbachia* infections in silkworm.

Phylogenetic analysis

Based on previous reports (Zhou *et al.*, 1998; Van Meer *et al.*, 1999), we downloaded 41 *wsp* sequences of *Wolbachia* as a data set (Table S2). First, we merged our *wsp* sequence from the sample of Dali wild silkworm with the data set and performed a phylogenetic analysis. The topology confirmed the division of *Wolbachia* into the two supergroups A and B, showing that our *Wolbachia* belongs to supergroup B (Figure 2). *Wolbachia* strains from Lepidoptera E. kuehniella, E. cautella and silkworm appeared to cluster in different groups. Interestingly, *Wolbachia* from silkworm was closely related to that of the parasitic wasp *T. bedeguaris*. Considering the possibility of a horizontal transmission of *Wolbachia* (Perlman *et al.*, 2006) we hypothesize that *Wolbachia* detected in wild silkworm may have come from Hymenoptera, such as *T. confusum*, *T. bedeguaris* or others. Further experiments are, of course, required to test this hypothesis of horizontal transmission. In a next step we constructed a phylogenetic tree based on our 21 *wsp* sequences, showing that *Wolbachia* identified in domesticated and wild silkworms are quite similar to each other (Figure 3), indicating that *Wolbachia* had infected certain silkworms before strains of this species were domesticated.

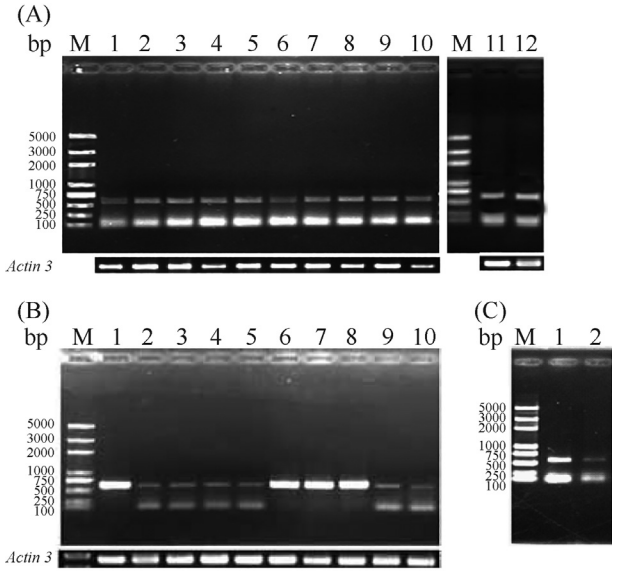

Figure 1 - PCR screening for the presence of *Wolbachia* in silkworms. (A) PCR screening for *wsp* gene in the samples of domesticated silkworms. Lane M represents DL2000 plus DNA marker. Lanes 1-10 represent domesticated silkworm samples from the following strains: lane 1, *871*; lane 2, *932*; lane 3, *7532*; lane 4, *Xianghui*; lane 5, *Yue*; lane 6, *Hang7*; lane 7, *Shi7*; lane 8, *Feng9*; lane 9, *Furong*; lane 10, *Yan7*. Lanes 11 and 12 represent female and male samples from the strain *Dazao*, respectively. (B) PCR screening for the *wsp* gene in samples of wild silkworms. Lane M represents DL2000 plus DNA marker. Lanes 1-10 represent wild silkworm samples from the following areas: lane 1, Dali; 2, Nanchong; 3, Peng'an; 4, Pingdu; 5, Qingmuguang; 6, Haining; 7, Xichang; 8, Huzhou; 9, Zhenjiang; 10, Rongchang. (C) PCR screening for the *ftsZ* gene in samples of silkworm. Lane M represents DL2000 plus DNA marker. Lane 1, Dali; 2, Dazao. All PCR fragments were cloned and sequenced. Sequencing results showed that the bigger bands in lanes 1-12 are fragments of the *wsp* (A, B) or *ftsZ* genes (C), whereas the second, smaller band in lanes was non-specifical PCR product.

Lateral transfer

It was previously reported that *Wolbachia* genes could laterally transfer into host chromosomes (Kondo *et al.*, 2002; Fenn *et al.*, 2006; Nikoh *et al.*, 2008). The genome sequence of the silkworm, *Bombyx mori*, has been completed, and the strain for sequencing was *Dazao* (Xia *et al.*, 2004; International Silkworm Genome Consortium,

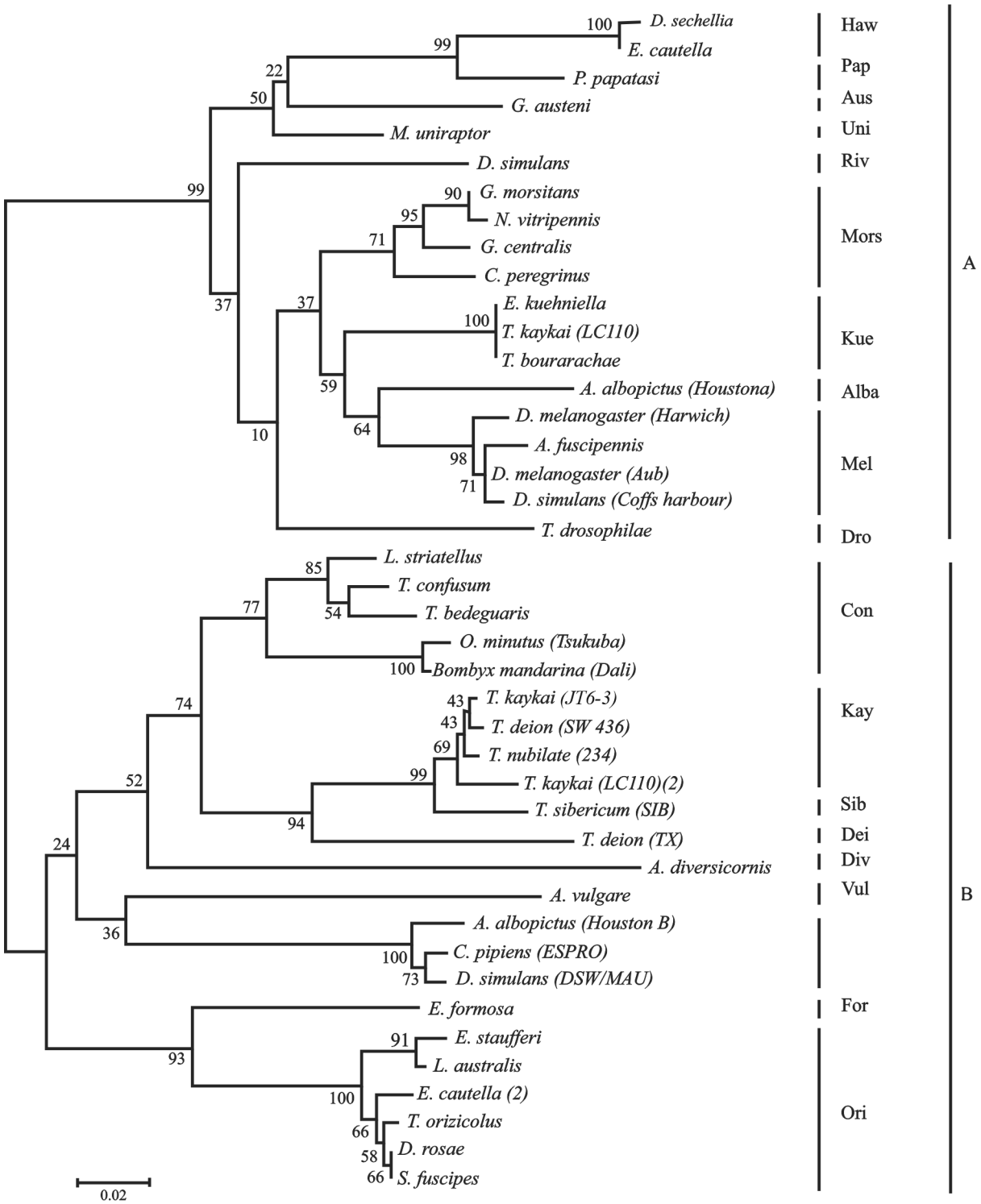

Figure 2 - Phylogenetic tree of *Wolbachia* based on *wsp* gene sequences. The name of the host arthropod species was followed by the group designation. The name `Bombyx mandarina(Dali)' represents *Wolbachia* in wild silkworm from Dali in Yunnan. The tree was constructed by the Neighbour-Joining algorithm. Numbers on the nodes indicated percentages of 1,000 bootstrap replicates.

2008). In order to confirm whether *Wolbachia* genes may have undergone a transfer into the silkworm genome, we performed a BLAST search by using *Wolbachia* gene sequences to query a database of silkworm genomic sequences. The results indicated that the complete genome of the silkworm *Dazao* contained no *Wolbachia* sequence (data not shown). To further confirm this result, we performed Southern blotting by using the *wsp* gene sequence

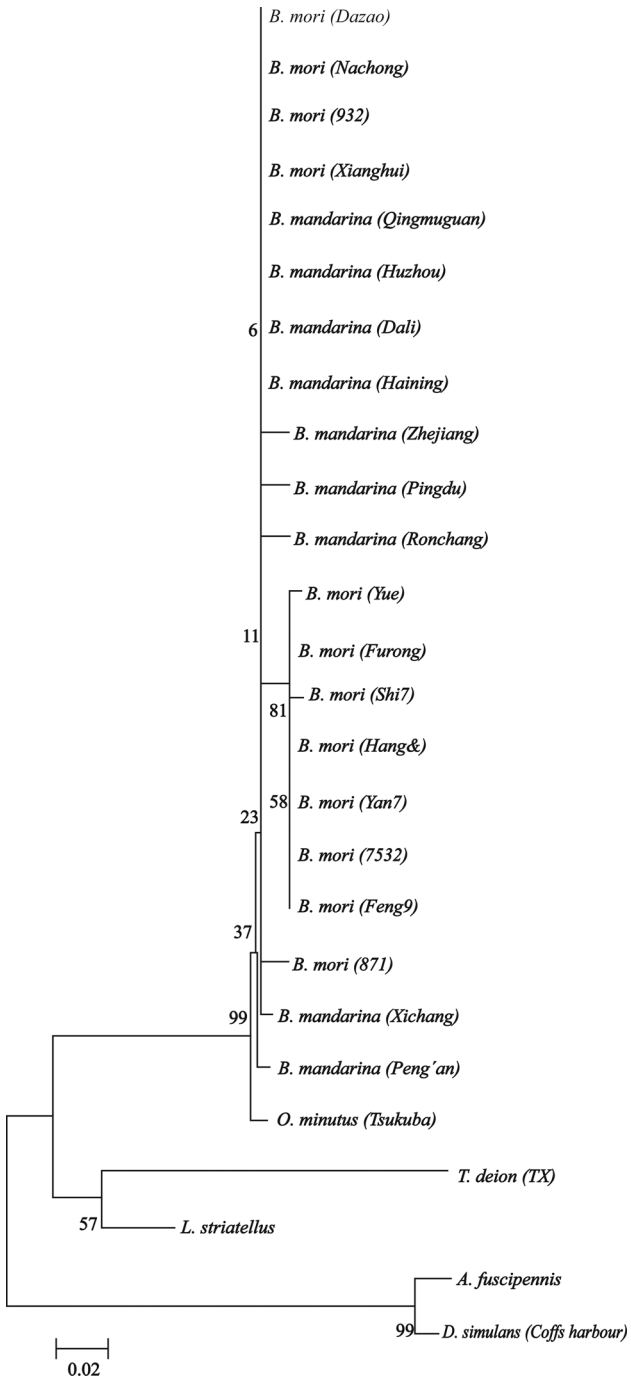

Figure 3 - Phylogenetic tree of *Wolbachia* in silkworms. All 21 *wsp* sequences from samples of silkworms were used to construct the tree.

as probe. This analysis showed that no *wsp* hybridization signal was detected in the *Dazao* genome, but was clearly apparent in the wild type silkworms of Huzhou, Xichan and Pingdu (Figure 4). Since Southern blotting had also previously been used to identify horizontal transfer of *Wolbachia* (Nikoh *et al.*, 2008), we speculate that *Wolbachia* did not transfer into the *Dazao* genome, but that a *wsp* gene had done so in the wild silkworm genomes of Huzhou, Xichan and Pingdu.

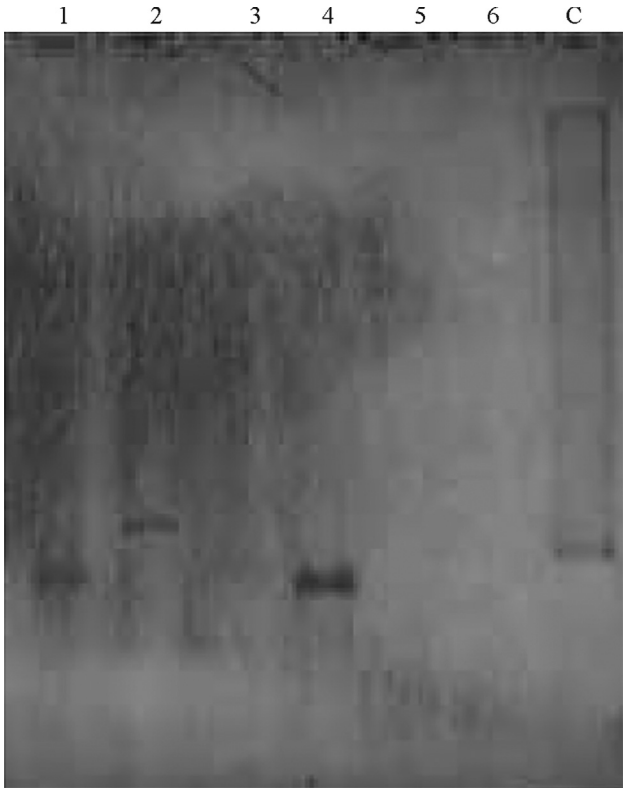

Figure 4 - Southern blotting analysis of the *wsp* gene using genomic DNA from different *Wolbachia* infected silkworms. The blot was hybridized with a probe corresponding to the *wsp* gene. Lane C represents a positive control from plasmid DNA of a recombinant *wsp* gene. Lanes 1-3 represent wild silkworm samples from Huzhou, Xichan and Pingdu, respectively. Lane 4 represents F1 silkworms from a $Male^{Dali}$ x $Female^{Dazao}$ cross. Lanes 5 and 6 represent female and male samples of the *Dazao* strain, respectively.

Effects of *Wolbachia* on silkworm ovary development

In this study, we used four kinds of antibiotics, tetracycline, penicillin, rifampin and ciprofloxacin (Table S3). These were respectively injected into the body of 30 silkworms (half male, half female silkworms). The injected dose was 1 mg per individual. After injection, the survival rate was 100% in all experimental groups. But while in four groups (rifampicin, penicillin, autoclaved H_2O and untreated controls) the silkworms could all normally emerge as adult moths, those injected with tetracycline or ciprofloxacin did not. By dissecting silkworms injected with tetracycline or ciprofloxacin we noted an abnormal ovary phenotype in female moths with a frequency of 93% and more (Table S3). Oviducts lacked the typical structure and were clearly shorter. The number of developing eggs was also lower than in the control groups (Figure 5). In order to verify the elimination of *Wolbachia* by the antibiotics, we performed quantitative PCR assays using *wsp* primers, this showing showed that the density of *Wolbachia* in the group injected with tetracycline or ciprofloxacin was strongly de-

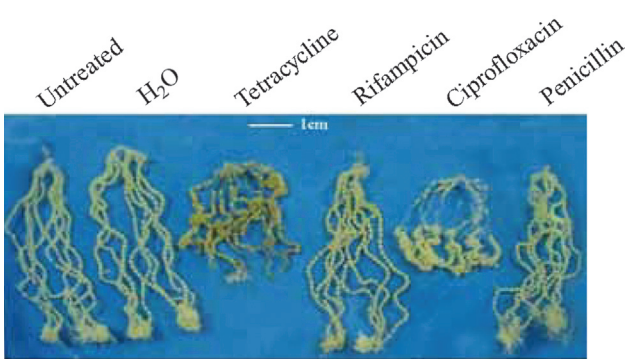

Figure 5 - Phenotype of ovaries after antibiotic treatments. The two groups of H_2O and untreated were used as control. The groups of tetracycline and ciprofloxacin treatment showed an abnormal ovary phenotype compared with that in the control groups.

creased (Figure 6). These results indicated that tetracycline and ciprofloxacin could dramatically reduce the density of *Wolbachia* in silkworm, and that the presence of *Wolbachia* is important for normal development of the ovary.

In view of a probable side effect of a high dose of antibiotic treatment, we repeated the experiment injecting a lower dose of tetracycline (0.1 mg per individual). In this experiment, silkworms were furthermore mated to obtain an F1 generation. Compared with the control group, nearly 29% of the F1 females still showed an abnormal ovary morphology (Figure 7), putting in evidence that a lower dose of tetracycline, for which no or less of a side effect was expected, lead to a similar effect.

Discussion

Wolbachia infects a wide variety of insects and plays important roles in host reproduction. As it was previously reported that *Wolbachia* was not detected in silkworm (Puttaraju and Madhu, 2002; Prakash and Puttaraju, 2007), we conducted a study on both wild type and domesticated silkworm strains using PCR assays for the *Wolbachia*-specific *wsp* gene and subsequent sequencing. This is the first study to detect the presence of *Wolbachia* in silkworm. Interestingly, in the PCR screening of the *wsp* gene we found that the band of a PCR fragment in lanes 1 and 6-8 in Figure 1B showed a stronger signal, suggesting that the *wsp* gene could easily be amplified and detected by PCR in these four samples. It has previously been reported that *Wolbachia* infections in some species could be detected by common PCR, while in others long PCR assays under strict conditions were necessary (Jeyaprakash and Hoy, 2000). These results may imply a difference in the density of *Wolbachia* infecting the hosts.

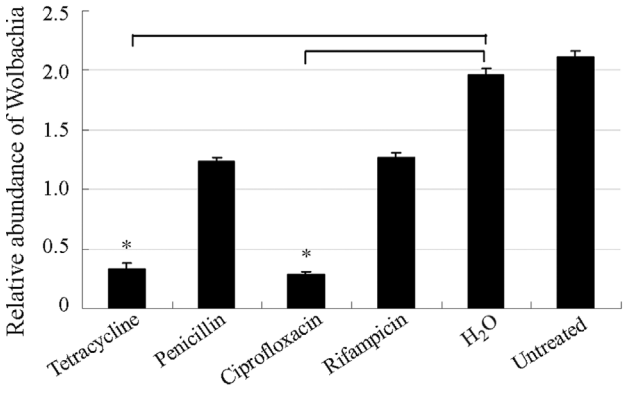

Figure 6 - Relative *Wolbachia* abundance after antibiotic treatments. The graphs show *Wolbachia* abundance relative to the host silkworm reference gene (*sw22934*) as determined by quantitative PCR. The error bars represent standard deviations.

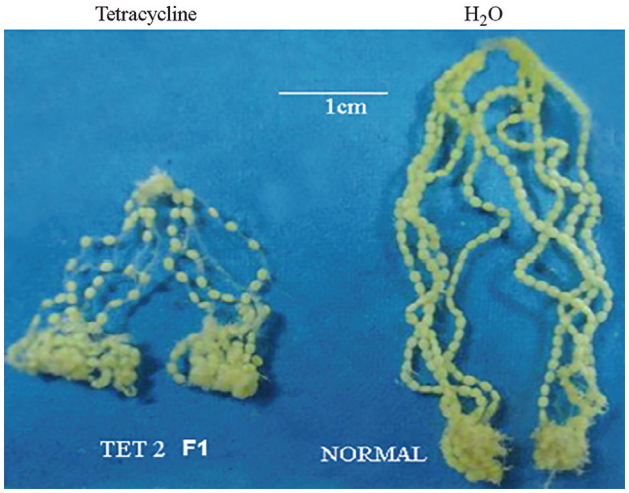

Figure 7 - Ovary phenotype of F1 generation females after tetracycline treatment. The injected H_2O group was used as control. Tetracycline was injected with a lower dose of 0.1 mg per silkworm. After antibiotic treatments, tetracycline-injected females were crossed with tetracycline-injected males. The F1 generation was reared and used to identify the ovary phenotype. Nearly 29% of the F1 female still showed an abnormal ovary morphology.

As reported, *Wolbachia* can play important roles in lepidopteran insects. In the butterfly *Eurema hecabe*, a female-biased sex-ratio distortion was observed due to feminization of genetic males by *Wolbachia* (Hiroki *et al.*, 2002). In the Mediterranean flour moth, *Ephestia kuehniella* Lewis *et al.* (2011) showed that *Wolbachia*-infected males transfer fewer fertile sperm at mating than uninfected ones in their heteromorphic, sperm, and that Wolbachia may affect fertile sperm production. In the adzuki bean borer, *Ostrinia scapulalis* (Walker), *Wolbachia* selectively kills male progeny. This *Wolbachia* strain appears to have a feminizing effect since antibiotic treatment of infected female moths gave rise to male progeny with sexually mosaic phenotypes (Kageyama *et al.*, 2003). In *Wolbachia*-induced sexual mosaics of *O. scapulalis*, which are genetically male, the female-specific isoform of the sex determining gene *Osdsx* was shown to be expressed in addition to the male-specific isoform (Sugimoto *et al.*, 2010). This finding indicated that *Wolbachia* manipulates the sex of its host by interfering either with the

sex-specific splicing of *Osdsx* itself or with another upstream sex determination process.. By injection of different antibiotics for elimination of *Wolbachia* in the silkworm, we could show that the elimination of large amounts of *Wolbachia* by tetracycline and ciprofloxacin can, cause abnormal development of the silkworm ovary. Apparently, *Wolbachia* thus plays an important role in silkworm ovary development and that the biological role of *Wolbachia* in silkworm is different from that in other lepidopteran insects. These results provide some first clues to address the role of *Wolbachia* silkworm biology and possibly also in other lepidopteran insects.

Acknowledgments

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Supplementary Material

The following online material is available for this article:

Figure S1 - Alignment of *wsp* sequences from *Wolbachia* infecting wild silkworms.

Figure S2 - Alignment of *wsp* sequences from *Wolbachia* infecting domesticated silkworms.

Table S1 - Strains of the silkworms used in this study.

Table S2 - Sequences of *wsp* genes downloaded from GenBank.

Table S3 - Statistics of experimental treatment with four antibiotics.

This material is available as part of the online article from http://www.scielo.br/gmb.

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