INSECT-SYMBIONT INTERACTIONS

Population Dynamics of Noncytoplasmic Incompatibility-Inducing Wolbachia in Nilaparvata lugens and Its Effects on Host Adult Life Span and Female Fitness

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Environ. Entomol. 39(6): 1801–1809 (2010); DOI: 10.1603/EN10051

ABSTRACT Wolbachia are bacteria that live intracellularly in a wide variety of arthropods. They are maternally inherited and can affect both reproduction and fitness of its host. When infected males mate with uninfected females or females infected by a different Wolbachia strain, there is often a failure of karyogamy, which is usually attributed to cytoplasmic incompatibility (CI). We measured the strength of CI induced by Wolbachia and the fitness effects in three Chinese populations of the brown planthopper Nilaparvata lugens from Hainan, Yunnan, and Guangxi provinces, respectively. No evidence for CI was found in any of the populations, whereas an enhanced fecundity and shortened longevity were observed only in the Hainan population. The infection density was significantly higher in the Hainan population than in the Guangxi population. The Wolbachia strain infecting the three populations appeared to be the same based on the nucleotide sequence of the wsp gene. Therefore, the variable effects of Wolbachia on host fitness seem to be the result of differences in the host genetic background and Wolbachia infection density. The ability of the non-CI-inducing Wolbachia to maintain themselves in their hosts may be attributed to their positive effects on host fecundity and efficient maternal transmission.

KEY WORDS Wolbachia, Nilaparvata lugens, cytoplasmic incompatibility, infection density, quantitative polymerase chain reaction

Wolbachia are intracellular, maternally inherited bacteria that infect a broad range of arthropods and nematodes (Stouthamer et al. 1999, Stevens et al. 2001), and that have strikingly diverse effects on their hosts (Stouthamer et al. 1999, Goryacheva 2004, Markov and Zakharov 2005). In different arthropod species, Wolbachia infection can result in various alterations of sexuality and reproduction, including cytoplasmic incompatibility (CI) (Bourtzis et al. 1996, Hoffmann and Turelli 1997), thelytokous parthenogenesis (Stouthamer et al. 1993), male killing (nonviability of male offspring) (Hurst et al. 1999), and feminization (transformation of genetic males into females) (Rousset et al. 1992, Hiroki et al. 2002, Negri et al. 2006). Each of these phenomena is considered to provide a reproductive advantage to infected females, thereby allowing Wolbachia to persist and spread into host populations (Hoffmann and Turelli 1997).

Based on current data, CI occurs when infected males mate with uninfected females or with females infected by a different, incompatible Wolbachia strain. In such crosses, fertilization is apparently normal, but subsequent mitoses are disrupted, leading to the death of the zygote (Reed and Werren 1995; Callaini et al. 1996). The expression of CI in different hosts varies widely, from no noticeable CI to very strong CI. The basis of this variability is not well understood, but is often considered to be the result of the Wolbachia strains, infection density (Bourtzis and O’Neill 1998), and host genetic background differences (Bordenstein and Werren 1998). Expression of CI is also affected by other factors, such as host age (Hoffmann et al. 1986), temperature (Clancy and Hoffmann 1998, Hoffmann et al. 1990), food quality (Hoffmann et al. 1990, Sinkins et al. 1995), and rearing density of host insect (Sinkins et al. 1995).

Reduced infection density may result in imperfect vertical transmission and consequent loss of infection, whereas excessive infection density may lead to pathological and negative effects on host fitness. It is expected that there are mechanisms for controlling infection density within an appropriate range, and these may involve the genotypes of both symbiont and host (Mouton et al. 2003).

The brown planthopper Nilaparvata lugens (Stål) is a largely long-distance migratory insect (Hemiptera: Delphacidae) that sucks juices from the stems of rice plants. Migrating from southeastern Asian countries (Viet Nam, Myanmar, Thailand) into mainland China in early summer, N. lugens reproduces and feeds on rice and then returns in late autumn (Hong and Ding...
N. lugens seasonal northward and southward migrations has caused it to become the major pest of rice in China and various other countries and regions in Asia. Its landing and endangering also abruptly result in serious damage to rice production. Wolbachia-induced CI could be used as a novel environmentally friendly tool for the control of insect pests. The Thai population of the brown planthopper was found to be infected with Wolbachia (uLug) (Kittayapong et al. 2003). Preliminary studies by our laboratory showed that Wolbachia was also widely distributed in the Chinese populations of N. lugens, but little is known about the effects of Wolbachia on the Chinese populations. In this study, we attempted to determine how the infection density of Wolbachia affects the reproduction and fitness of two migratory populations and one indigenous population of N. lugens in China.

Materials and Methods

Insect Materials. Brown planthoppers were collected from rice paddy fields in three provinces in south and southwestern China in July 2007. The locations were Sanya city in Hainan province (108°30′E, 18°12′N), Ning'er county in Yunnan Province (100°48′E, 27°12′N), and the city of Nanning in Guangxi Zhuang Autonomous Region (108°18′E, 22°48′N). The three populations, designated HN, YN, and GX, respectively, were reared on typical susceptible rice variety seedlings in a climate-controlled room (25°C, 60% RH, 16L:8D photoperiod).

The population on Hainan Island, from which the present HN population was collected, is an indigenous nonmigratory population. The YN and GX populations from mainland China are migratory populations that spend the summers in China and the winters in Laos and Viet Nam, respectively (Bao-Ping Zhai, Nanjing Agricultural University, personal communication).

Polymerase Chain Reaction (PCR) Amplification and Sequencing. Samples were prepared, as described previously (O’Neill et al. 1992), with minor modifications. Individual planthoppers were homogenized in 40 μl of STE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) in a 1.5-ml Eppendorf tube and incubated with proteinase K (10 mg/ml, 2.5 μl) at 37°C for 30 min, followed by 5 min at 95°C. The samples were centrifuged briefly, and used immediately for the PCR reactions or stored at −20°C for later use.

PCR was performed in a 25 μl reaction volume: 14.3 μl of double-distilled water (ddH2O), 2.5 μl of 10× buffer, 2.5 μl of 2.5 mM dNTP, 2.5 μl of 25 mM MgCl2, 0.2 μl of Taq (1 U) (Takara Shuzo, Otsu, Japan), 2 μl of template DNA, and 1 μl of primers (20 μmol each). The primers used in this study were for the wsp gene, which encodes a surface protein of Wolbachia (Zhou et al. 1998): 5′-TGTTGCCAATATAAGTAGTTGAAGAAGAC-3′ and 5′-AAAAATTTAAAGCCATCCTCA-3′. Thermal cycles were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1 min, and 72°C for 7 min as a final extension after the last cycle. A volume of 10 μl of PCR product was separated on 1% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

The resulting PCR products were cloned into a pGEM-T vector (Promega, Madison, WI). Their sequences were obtained twice with each primer, making a total of four sequences obtained independently from one DNA extract, from which a consensus was derived. Each base from the final consensus sequence was present in at least three of the four sequences for every site. The sequence was determined by the Dye Terminator Sequencing method with a DNA Sequencer (model 377 and 3700, PE Applied Biosystems, Foster City, CA).

Preparation of Infected and Uninfected Lines. To obtain 100% Wolbachia-infected and 100% Wolbachia-free lines, we placed pairs of male and female adults of natural populations in individual plastic cups (120 mm in height and 80 mm in diameter) that contained rice seedlings. These pairs were allowed to lay eggs into the rice seedlings, and then removed from the cup after 1 wk. We checked the infection status of these pairs by PCR amplification. Only offspring from parents that tested either positive or negative for Wolbachia by PCR screening were continually reared and used as parental stock. This selection regime was maintained for several successive generations until 100% Wolbachia-infected populations and 100% Wolbachia-free populations were obtained.

Cross Experiments. To determine the capability for CI expression, four crosses were designed in each population, as follows: infected female with infected male (I × I), infected female with infection-free male (I × F), infection-free female with infected male (32 I), and infection-free female with infection-free male (F × F). Wolbachia-infected colonies were designated as ‘I’ and Wolbachia-free colonies were designated as ‘F’.

For each cross, nymphal planthoppers were individually reared in a tube containing rice seedlings until emergence (to ensure they were virgins). A female and a male of 1 d old were introduced into the test cup for 1 wk (to allow for mating and oviposition), and then removed from the cup. After 2 wk, newly hatched nymphs by this time were counted, and the remaining deposited eggs in the seedlings were dissected to microscopically observe their development. Eye pigment (red in color) was examined as an indicator of egg development (Noda et al. 2001). Fecundity of these four crosses was estimated as the total number of eggs laid in the first week.

After the eclosion of all nymphs, the sex statuses of the offspring were recorded to determine the sex ratio (percentage of daughters). Data were analyzed with one-way analysis of variance after arcsine transformation, and means were compared using a Tukey’s honestly significant difference test (SPSS 13.0). To normalize the data, a natural log transformation was used for the number of eggs laid per female, and an arcsine square-root transformation was used for egg-hatching rate and sex ratio.
Host Longevity Assays. To determine longevity, newly emerged planthoppers from both the Wolbachia-infected and Wolbachia-free lines (n = 50 for each line) were placed in individual cups containing rice seedlings. Each day, the number of new deaths was recorded. Surviving planthoppers were transferred to cups with fresh rice seedlings every 5 d until all had died. Survivor curves for individual hosts were compared using the Kaplan–Meier method and log-rank test (SPSS 13.0).

Estimating Wolbachia Density. To estimate the abundance of Wolbachia, the copy numbers of the wsp gene were measured using an Applied Biosystems 7300 real-time PCR system. Based on the sequences of wsp gene of Wolbachia strain (vLug) we sequenced, new specific primers (WSP/F 5'-TGAAGTAAGACCG-GAGATGTGGC-3' and WSP/R 5'-TTTTGTCGTA-AAGGGTTGC-3') were designed (Primer Premier 5.0) for quantitative PCR, which amplified a 170-bp DNA fragment.

To estimate the copy numbers of the wsp gene, we plotted a standard curve using dilutions of a pGEM-T vector containing part of the wsp sequence. A SYBR Premix Ex Taq PCR Reagent Kit (Takara Shuzo) was used to measure the copy number of Wolbachia genes. Under the assumption that each genome has one copy of the wsp gene, the number of wsp copies was taken as an estimate of the number of Wolbachia bacteria. PCR conditions were as follows: 30 s at 95°C, then 40 cycles of 95°C for 5 s, 60°C for 31 s, and a dissociation stage (95°C for 15 s, 60°C for 1 min, and 95°C for 5 s) at last. The PCR included SYBR Premix Ex Taq 10 µl, 0.2 µM concentration of each primer, 7.2 µl of double-distilled water (ddH2O), and 2 µl of template DNA made up to a 20-µl total volume. Each DNA template was analyzed in triplicate.

Five developmental stages of nymphs (first to fifth instars) and adult females and males of 12 stages (1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 d old) in the HN and GX populations were examined (n = 10 for each stage). Because N. lugens of the YN population was also long-distance migratory population as the GX population, the real-time PCR was not performed in the YN population. The instars were collected at the second day of each instar, each instar usually lasting for 3 d.

Results

Sequences of Wolbachia Strains. Wolbachia strains infected in the HN, YN, and GX populations of N. lugens had an identical wsp gene sequence. The referred wsp gene sequences were identical to the wsp sequences of Wolbachia, which originated from these three corresponding natural populations of N. lugens (GenBank accession numbers: FJ713760, GU289763, and GU289769).

CI Expression Not Found in N. lugens. In the HN population (Fig. 1A), we found that crosses between uninfected females and infected males (F × I) produced 92.95 ± 1.82% egg-hatching rate, which was not significantly lower (F = 0.858; df = 3, 50; P = 0.469) than the other crosses (I × I: 93.28 ± 2.10%; F × F: 94.52 ± 1.10%; I × F: 95.87 ± 0.84%). The same situation was found in the YN population (Fig. 1B) (I × I: 88.43 ± 2.69%; F × F: 90.24 ± 4.05%; I × F: 93.65 ± 1.69%; F × I: 92.31 ± 1.70%) (F = 0.968; df = 3, 69; P = 0.418) and the GX population (Fig. 1C) (I × I: 92.97 ± 1.08%; F × F: 95.29 ± 0.96%; I × F: 93.81 ± 0.98%; F × I: 95.08 ± 0.99%) (F = 1.022; df = 3, 77; P = 0.39).

In each population, the hatching rates of eggs for each type of cross were not significantly different. This indicates that Wolbachia did not cause CI in the three populations.

Fitness of N. lugens. The fitness of N. lugens was measured by fecundity of females, sex ratio (percent-age of daughter offspring), and longevity of adults. In the HN population, a significant effect of female infection status was found, with infected females (94.60 ± 7.76; 97.57 ± 10.57) producing more eggs in average than uninfected ones (56.55 ± 3.5; 61.86 ± 8.56) regardless of the infection status of males (F = 10.516; df = 3, 80; P < 0.001). No difference in fecundity was observed between infected (196.00 ± 13.41; 205.09 ± 12.77) and uninfected females (191.70 ± 11.43; 175.11 ± 9.34) in the YN population and the GX population (infected: 118.47 ± 4.53, 112.00 ± 7.29; uninfected: 115.17 ± 4.93, 128.00 ± 6.32). In summary, Wolbachia in the HN population was the only strain that could promote the fecundity of infected females. The sex ratios in the crosses are shown in Table 1. Except for a small difference between F × F and I × F combinations of the YN population (F = 2.969; df = 3, 69; P = 0.048), no significant difference was found in other crosses.

The effects of Wolbachia on host longevity are shown in Fig. 2. In the HN population, infected adults (19.76 ± 0.40) were significantly shorter lived than uninfected adults (27.02 ± 0.60) (χ² = 28.323; df = 1, P < 0.001) (Fig. 2A). No difference of longevity was observed in comparisons of the infected (22.79 ± 1.26) and uninfected adults (25.07 ± 0.86) in the YN population (χ² = 0.85, df = 1, P = 0.357) (Fig. 2B). Like the YN population, the GX population also showed no difference of longevity between the infected (25.27 ± 0.94) and uninfected planthoppers (26.35 ± 0.85) (χ² = 1.542, df = 1, P = 0.214) (Fig. 2C).

Wolbachia Density in Host Nymph and Adults. In nymphs, Wolbachia copy numbers significantly increased along with the developmental stage in the GX and HN populations (Fig. 3). In adults, the Wolbachia infection density varied according to the developmental stage of the GX and HN populations. The quantity of Wolbachia decreased with age after adult emergence in the GX population (Fig. 3A), although a slight increase was observed in 16-d-old females and males. Remarkably, there were more Wolbachia in males than in females for the GX population, the opposite of some reports (Bourtzis and O’Neill 1998, Rousset et al. 1992, Dobson and Rattanadechakul 2001, Noda et al. 2001). In the HN population (Fig. 3B), the Wolbachia density fluctuated randomly throughout development. In addition, we found that the Wolbachia...
Fig. 1. Mean percentages of hatching eggs in single-pair crosses (female × male) of Wolbachia-infected (I) and Wolbachia-free (F) strains in the HN (A), YN (B), and GX (C) populations of *N. lugens*. Bars indicate standard errors; values above bars represent the number of replicate crosses.
density in nymphs and adults was significantly higher in the HN population than in the GX population \((P < 0.001)\).

### Discussion

CI allows *Wolbachia* to invade host populations by specifically inducing sterility in crosses between infected males and uninfected females. The spread of CI-inducing *Wolbachia* in uninfected populations, as well as its maintenance after invasion, depend on three factors, as follows: the level of CI, maternal transmission efficiency, and fitness effects (Turelli and Hoffmann 1995, Hoffmann and Turelli 1997). However, several *Wolbachia* variants that are widely distributed in the field do not seem to induce CI (Bourtzis et al. 1996, 1998; Hoffmann et al. 1996; Reynolds and Hoffmann 2002; Charlat et al. 2003). In the current work, to avoid negative effects of tetracycline (Bandi et al. 1999) in this study, we used *N. lugens* field populations that were naturally free of *Wolbachia* to measure the strength of CI induced by *Wolbachia*. According to the results, we reported another unambiguous case of non-CI-inducing *Wolbachia* harbored in the brown planthoppers *N. lugens*. And, incidentally, similar with some other cases of non-CI-inducing *Wolbachia* strain, *Wolbachia* infection rate of *N. lugens* is lower than 20% (our unpublished data). All of these works will enrich understanding of the mechanism of CI caused by *Wolbachia*.

Because some *Wolbachia* strains cannot induce CI, what maintains these endosymbionts in their hosts? Infection dynamics models predict that in the absence of CI expression or sex ratio distortion, *Wolbachia* infections should be lost from natural populations unless they are beneficial to the host or are perfectly transmitted from infected mothers to their offspring (Hoffmann and Turelli 1997). Our observations fit the latter case: we did not find any evidence for CI in the three *N. lugens* populations, but we did find transmission from infected females to their offspring was nearly perfect in the three populations, as we found hardly any uninfected individuals in the tested samples (data not shown). In addition, we also found a possible positive effect on host fecundity and shortened longevity in the HN population, but not in the other two populations. Accordingly, it has been suggested that the mechanism of how these non-CI-inducing *Wolbachia* strains could maintain in the population of its host in the long-term evolutionary history is still not very clear.

The *Wolbachia* strains in the YN, GX, and HN populations appeared to be the same. However, the effects of *Wolbachia* on the fitness of the three populations were not the same. *Wolbachia* infection had a positive effect on fecundity in the *H*3 population, but not on the other two populations. This difference might be related in some way to the different life histories of these populations, as follows: the *H*3 population are year-round residents, whereas the YN and GX populations are migratory populations. Further studies are needed to determine whether the effect of *Wolbachia* on fecundity in the HN population is related to its nonmigratory behavior.

*Wolbachia* density has been shown to strongly affect the strength of CI in insects (Breeuwer and Werren 1993, Bressac and Rouset 1993, Bourtzis et al. 1996, Bordenstein et al. 2006). It shows that bacterial density is correlated with compatibility differences between male and female parasitoid wasp *Nasonia vitripennis*. Males from strains with high bacterial numbers are incompatible with females from strains with lower numbers (Breeuwer and Werren 1993). In *Drosophila* species, *Drosophila simulans* Hawaii, *Drosophila sechellia*, and *Drosophila auraria* exhibit high levels of cytoplasmic incompatibility relative to levels of infection; all the other species and *D. simulans* Riverside exhibit significantly lower levels of cytoplasmic incompatibility relative to levels of infection (Bourtzis et al. 1996). However, this was not the case in the populations studied in this work. Similarly, no link between CI intensity and *Wolbachia* density was found in *Culex pipiens* mosquitoes (Duron et al. 2006). Moreover, embryos of *D. simulans* and *Drosophila melanogaster* have similar loads of non-CI-inducing and CI-inducing *Wolbachia*, respectively, suggesting that the inability to cause CI is probably a function of the *Wolbachia* strain rather than bacterial density (Hoffman et al. 1996).

### Table 1. Fecundity and sex ratio of offspring in the crosses between *Wolbachia*-infected and *Wolbachia*-free lines in Yunnan, Guangxi, and Hainan populations of *Nilaparvata lugens*

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<th>Cross (female × male)</th>
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<tr>
<td>(F \times F)</td>
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<tr>
<td>21</td>
<td>196.00 ± 13.41</td>
<td>40.96 ± 0.02</td>
<td>12.77 ± 0.03</td>
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<tr>
<td>(F \times I)</td>
<td>205.09 ± 12.73</td>
<td>43.59 ± 0.03</td>
<td>10.48 ± 0.03</td>
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<tr>
<td>(I \times F)</td>
<td>191.70 ± 11.43</td>
<td>51.16 ± 0.03</td>
<td>14.27 ± 0.02</td>
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<tr>
<td>(I \times I)</td>
<td>175.11 ± 9.34</td>
<td>41.58 ± 0.02</td>
<td>14.79 ± 0.02</td>
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<tr>
<td>(F^b)</td>
<td>0.84NS</td>
<td>2.96*</td>
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\(F\), *Wolbachia* free; GX, Guangxi; HN, Hainan; I, *Wolbachia* infected; YN, Yunnan.

* Number of pairs tested.

\(^b\) Means (±SE) differ significantly at \(P < 0.05\) (*) and \(P < 0.001\) (**) (analysis of variance); NS, not significant at the 5% level. Values in a column followed by different letters are significantly different at \(P < 0.05\) (Tukey’s honestly significant difference test). The number of eggs per female was ln transformed before ANOVA. The female ratios were arcsine square root transformed before analysis of variance.
Symbiont density is a major factor in host-symbiont relationships, because it can influence both the efficiency of transmission and expression of symbiont functions. When symbionts are vertically transmitted, host and symbionts may act on the regulation of symbiotic bacterium density to optimize the trade-off between these two parameters (Ebert and Bull 2003). In Wolbachia symbiosis, both bacterial and host genotypes are involved in density regulation. Obviously, within a given multiply infected host, the density of each strain can differ, demonstrating that the Wolbachia genotype plays a role in the regulation process (Mouton et al. 2003). The involvement of the host genotype on the regulation of Wolbachia density has also been demonstrated (Ikeda et al. 2003, Clark et al. 2003). As mentioned above, environmental factors also affect Wolbachia density (Mouton et al. 2007). Further

**Fig. 2.** Comparison of Wolbachia effect on adult longevity in the HN (A), YN (B), and GX (C) populations. ‘I’ indicates Wolbachia-infected strains, and ‘F’ indicates Wolbachia-free strains. Survivor curves for individual hosts were compared using the Kaplan-Meier method and log-rank test.

**Fig. 3.** Wolbachia density in the developmental stages of nymphae and adults in the GX (A) and HN (B) populations. Nymphs were collected at the second day of each instar. For each population, 10 samples were analyzed at each stage. Bars indicate standard errors.
Acknowledgments

We thank Li-Li Zhou, Dong-Xiao Zhao, and Ming-Zhi Yu for help in collecting the brown planthopper and discussing the experiments. This work was supported by grants from the National Key Basic Research Program (973 Program, 2006CB102001) from the Ministry of Science and Technology of China and the Science and Technology Research Program of the National Agricultural Public Welfare Fund (nyhyzx-200803003, nyhyzx-200803051) from the Ministry of Agriculture of China. We are very grateful to Rong-Rong Xie for critical comments and suggestions on the early draft.

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Received 22 February 2010; accepted 1 July 2010.