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Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*

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Abstract

Wolbachia, a bacterial endosymbiote of arthropods, causes cytoplasmic incompatibility (CI) in many insect species. CI traits were studied in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*, and *Wolbachia* densities in these planthopper species were calculated by quantitative PCR methods. The CI level of *L. striatellus* was quite high and even aged males strongly caused CI. In contrast, *S. furcifera* showed partial CI, and males lost their ability to cause CI with age. *Wolbachia* infecting these two planthopper species were the same with respect to the nucleotide sequences of *Wolbachia* genes, 16S rDNA, *ftsZ* gene, *groE* genes, and *wsp* gene. Two methods for quantitative PCR, one using a DNA sequencer and the other a real-time sequence detection system, were established to calculate the amount of *Wolbachia* in the planthoppers. The density of *Wolbachia* in *S. furcifera* males was quite low. The difference in CI levels between the two planthopper species seems to be due to different amounts of *Wolbachia* infecting males.

Keywords: *Wolbachia*; *Laodelphax striatellus*; *Sogatella furcifera*; Planthopper; Cytoplasmic incompatibility; Quantitative PCR

1. Introduction

Cytoplasmic incompatibility (CI) is found in many arthropod species of Coleoptera, Diptera, Homoptera, Hymenoptera, Lepidoptera (Werren and O'Neill, 1997) and Acarina (Breeuwer, 1997). Rickettsial symbiote, *Wolbachia*, is a causative agent of CI. Embryonic mortality is caused in crosses between *Wolbachia*-infected male hosts and females that are uninfected or infected with a different *Wolbachia* strain. The CI mechanism is generally explained by two separate actions of *Wolbachia*, "modification" of sperm and "rescue" in eggs (Bourtzis and O'Neill, 1998; Werren, 1997). Sperm or male chromosomes are modified by *Wolbachia* in the testis and become unable to normally participate in the first mitotic division in the eggs. This incompatibility is restored by the same kind of *Wolbachia* in the females, and a normal zygote is formed in the *Wolbachia*-infected eggs.

CI and the CI level are affected by various factors, such as *Wolbachia* strains (Bourtzis et al., 1998; Giordano et al., 1995; Hoffmann et al. 1996, 1994; Merçot and Poinsot, 1998), superinfection of *Wolbachia* (Bordenstein and Werren, 1998; Rousset and Solignac, 1995; Sinkins et al., 1995), host genetic background (Bordenstein and Werren, 1998; Breeuwer and Werren, 1993a), host age (Hoffmann et al., 1986; Singh et al., 1976; Subbarao et al., 1977), temperature (Clancy and Hoffmann, 1998; Hoffmann et al. 1990, 1986), food quality (Hoffmann et al., 1990; Sinkins et al., 1995), and rearing density of host insect (Sinkins et al., 1995). Except for the *Wolbachia* strain, these factors more or less appear to affect bacterial density in the host insects. The importance of *Wolbachia* density in host insects for CI level has been pointed out by many authors (Boyle et al., 1993; Bressac and Rousset, 1993; Clancy and Hoffmann, 1998; Poinsot et al., 1998; Sinkins et al., 1995). A specific threshold level of bacterial infection density is believed to be required for the modification and rescue (Boyle et al., 1993; Breeuwer and Werren,

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1993b). Bacterial density has been examined by microscopic observation of fluorescent staining of *Wolbachia* (Bressac and Rousset, 1993), dot blots of nucleic acids, and western blots of proteins (Bourtzis et al., 1998; Rousset et al., 1999). More accurate detection methods of *Wolbachia* would be useful for precisely analyzing the relation between bacterial density and CI level.

CI of the small brown planthopper *Laodelphax striatellus* was found through crossing experiments between two laboratory colonies of red-eyed mutant and black-eyed wild type (Noda, 1984a). This species shows unidirectional incompatibility among field populations; the crosses between females of northeastern populations and males of southwestern populations in Japan are incompatible (Noda 1984b, 1987). Hoshizaki and Shimada (1995) reported that the southwestern populations are infected with *Wolbachia* and the distribution of southwestern populations have spread to the northeast since previous investigation (Noda, 1984b). This spreading of *Wolbachia*-infected populations appears to be assisted by CI. Laven (1959, 1967) proposed that a *Wolbachia*-infected population expels the uninfected population from the co-existing area and expands its distribution. A similar phenomenon is also recognized in *Drosophila simulans* populations in California (Turelli and Hoffmann 1991, 1995)

Wolbachia is well known to be sensitive to tetracycline and is eliminated from host arthropods by antibiotic treatment. When insects are infected with CI-inducing *Wolbachia*, the crosses between antibiotic-treated females and untreated males are incompatible, and most eggs deposited by the pairs do not develop. The reciprocal cross (between antibiotic-treated males and untreated females) is compatible. In *L. striatellus*, tetracycline and rifampicin effectively altered the crossing cytotype, and the expected results of test crosses were obtained after the antibiotic treatment (unpublished). The antibiotic treatment also revealed that the white-backed rice planthopper, *Sogatella furcifera*, is infected with CI-inducing *Wolbachia*. Preliminary crossing experiments, however, indicated that *S. furcifera* showed partial incompatibility in the crosses between tetracycline-treated females and untreated males, i.e. some eggs developed normally from the incompatible pairs.

In this report, we conducted crossing experiments to compare the CI level between *L. striatellus* and *S. furcifera*. The nucleotide sequences of several genes of *Wolbachia* from the two planthopper species were compared to examine the relation between these two *Wolbachia*. We then compared the density of *Wolbachia* in the two planthopper species by quantitative PCR methods. A possible relation between the partial incompatibility and low *Wolbachia* density in males of *S. furcifera* is shown.

2. Materials and methods

2.1. Insects

L. striatellus and *S. furcifera* (Homoptera, Delphacidae) were collected at Izumo in 1987 and were reared on rice seedlings at 25 to 26°C and 16 h daily illumination.

2.2. Antibiotic treatment

Tetracycline hydrochloride (Wako Pure Chemical) and rifampicin (Sigma) were administered to planthoppers orally through rice seedlings. A small piece of cotton was stuffed into the bottom of a glass test tube (135 mm in length and 12 mm in diameter), and about 2 ml of water was added to the cotton. Four to five water-soaked rice seeds were put on the cotton and allowed to germinate in the tube. After three to four days, newly hatched first instar nymphs were individually introduced into the tubes. Preliminary testing showed that the volume of the water in the cotton was reduced from 2 ml to about 1.5 ml by evaporation and absorption into the seeds. Therefore, 0.5 ml of 0.4% antibiotic solution or suspension was added to the cotton. The concentration of the antibiotics in the tube should thus become about 0.1% [0.4×0.5/(1.5+0.5)]. The test tubes were plugged with cotton and held at 25 to 26°C without additional water supply. Seven or eight days later, the planthoppers were transferred to new antibiotic-treated (0.1%) rice seedlings, and were reared to adults.

Initially, the effective concentration of tetracycline was examined in *L. striatellus*. From 0.01 to 0.25% of tetracycline was provided to the rice seedlings to rear nymphs, and 0.05 to 0.1% antibiotics was sufficient to alter cytotype in crossing experiments. Too high a concentration (0.25%) deteriorated rice seedlings and caused much delay in adult emergence.

To establish *Wolbachia*-free planthopper colonies, nymphal stages of planthoppers were treated with the antibiotics (0.1%) for two successive generations. *L. striatellus* and *S. furcifera* were treated with rifampicin and tetracycline, respectively. *Wolbachia* was not detected by diagnostic PCR in the colonies of either species after the antibiotic treatments.

2.3. Test crosses

Planthoppers were individually reared in the test tube containing rice seedlings. A male and a female of a few days old were introduced into the test tube that contained rice seedlings. The test pair was allowed to lay eggs into the rice seedlings at 25 to 26°C. The planthoppers were removed from the tube after one week. After another week, newly hatched nymphs by this time were counted, and the remaining deposited eggs in the seedlings were

dissected to microscopically observe the development. Eye pigmentation (red in color) was examined as an indicator for egg development.

2.4. PCR amplification

Wolbachia specific primers, 99F/994R that amplify about 890 bp of rDNA of *Wolbachia* (O'Neill et al., 1992) and Holden's primers that amplify about 770 bp of *ftsZ* gene of *Wolbachia* (Holden et al., 1993), were used. Two other primer pairs for *ftsZ*, Adf–Adr and Bf–Br (Werren et al., 1995), were also used for determining the type of *Wolbachia*, A or B. Genomic DNA was usually prepared according to the method of O'Neill et al. (1992) using proteinase K. Template DNA for examining *Wolbachia* infection rate was obtained by boiling adult abdomen with 40 μl of STE (100 mM NaCl, 1 mM EDTA [pH 8.0], 10 mM Tris–HCl [pH 8.0]) or the abdomen of each fourth instar nymph with 20 μl of STE. Amplifications were performed in 25 μl of buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$) with 0.15 mM each dNTP, 10 pmol primers and 1.5 U *Taq* DNA polymerase (TAKARA). Two microliters of template was added to the reaction mixture. The PCR thermal program was 95°C for 30 s; followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 2 min, and 72°C for 5 min as a final extension after the last cycle.

2.5. Nucleotide sequencing

16S rDNA (O'Neill et al., 1992), *ftsZ* gene (*ftsZ* f1 primer by Werren et al., 1995 and reverse primer by Holden et al., 1993), *groE* genes (*groES* and *groEL*) (Masui et al., 1997), and *wsp* gene (Braig et al., 1998) of *Wolbachia* were amplified from planthoppers by the specific primers. The PCR products were inserted into the *Sma*I or *Eco*RV site of pBluescript II (Stratagen) or were cloned into the T-plasmid vector of pBluescript II produced by the method of Marchuk et al. (1991). Isolated plasmid DNA or PCR-amplified DNA was used for sequencing templates. The sequences were determined by the *Taq* dye primer cycle sequencing method with a DNA Sequence System (model 373S or 377, PE Applied Biosystems) or by the Thermo Sequenase fluorescent labeled primer cycle sequencing method with DNA sequencer ALF Express (Amersham Pharmacia Biotech). The sequences were determined for at least three clones having opposite orientations.

2.6. Quantitative PCR (Q–PCR)

Wolbachia in the planthoppers were quantitated by amplifying 16S rDNA. The amplified products during exponential phase in PCR were detected with an automatic DNA sequencer. PCR performance in each sample was evaluated by internal standard template DNA (Wang et al., 1989; Porcher et al., 1992). The target DNA was 370 bp of 16S rDNA of *Wolbachia*, which was amplified by a primer pair, 99F, 5′-TTG TAG CCT GCT ATG GTA TAA CT-3′ (O'Neill et al., 1992) and 468R, 5′-CCG TCA TTA TCT CCC TCA CTA-3′. This 370-bp DNA was cloned into the t-vector of pBluescript II as a control target DNA. The internal standard was constructed by deletion from this 370-bp DNA (Fig. 1). Two PCR-amplified DNA were ligated to make a 354-bp internal standard. The first DNA fragment is amplified by the 99F and 326R, 5′-CAG *CTG CAG* TGT GGC TGA TCA TCC TCT-3′, and the second one by 343F, 5′-ACG *CTG CAG* ACT CCT ACG GGA GGC AG-3′, and the 468R. The primers 326R and 343F included *Pst* I site (italic), and these two PCR products were ligated after *Pst* I endonuclease digestion, producing a 354-bp DNA including slight difference from original 370-bp DNA at the ligated site. The sequences of the constructed control target DNA (370 bp) and internal standard DNA (354 bp) were confirmed by DNA sequencing as mentioned above. The templates of the 370-bp and 354-bp DNAs for Q–PCR were prepared by PCR amplification using M13–20 and reverse primers. The amplified DNAs include extra flanking sequences of 227 bp corresponding to a cloning site of pBluescript II. The DNAs were purified with a spin column of Sephacryl S-300 HR (Pharmacia Biotech), and their concentration was measured by OD absorbance at 260 nm.

Adult planthoppers were crushed in 100 μl of STE buffer and incubated at 37°C for 1 h after adding 4 μl of proteinase K (10 mg/ml). One to three microliters of template samples were used for each PCR, and the reaction mixture (15 μl) included the above-mentioned

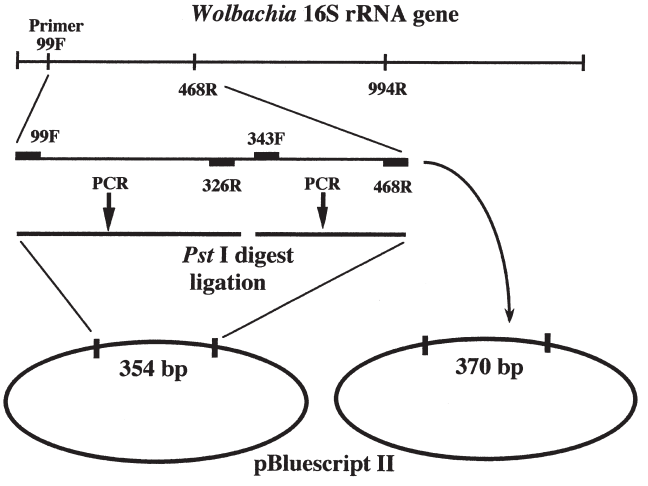

Fig. 1. Control target DNA (370 bp) and internal standard DNA (354 bp) constructed from 16S rDNA of *Wolbachia* for quantitative PCR. The internal standard DNA was made by ligation of two PCR-amplified DNA from the 370-bp DNA. The PCR products were cloned into the t-vector of pBluescript II. The target and internal standard DNA share an identical sequence for primer annealing.

buffer for diagnostic PCR. The 99F primer whose 5′ end was labeled with Cy5 and 468R primer were used for PCR. Fifty to 200 fg of 354-bp internal standard DNA was added to each tube. The PCR thermal program was 95°C for 2 min; followed by 17 to 20 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min, and 72°C for 5 min as a final extension after the last cycle. Co-amplified target sample DNA (370 bp) and internal standard DNA (354 bp) were separated by the DNA sequencer ALF Express (Amersham Pharmacia Biotech) using 7.5% acrylamide gel and analyzed with the software "AlleleLinks" (Amersham Pharmacia Biotech). The relative quantity of *Wolbachia* calculated from the peak area of 370-bp DNA was compensated based on the quantitated values of the amplified internal standard DNA. The measured amount of the target DNA was calculated into the number of target molecules (see Appendix A). We assure the number of target molecules equals the number of *Wolbachia* because Bensaadi-Merchermek et al. (1995) observed that *Wolbachia* isolated from *Culex pipiens* and *Ephestia cautella* has a single copy of rDNA.

2.7. Real-time quantitative PCR (RTQ–PCR)

Another quantitation method of *Wolbachia* was developed to compare and evaluate the two methods. RTQ–PCR was performed with an ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems) (Heid et al., 1996). RTQ–PCR does not detect PCR products but measures fluorescence that is released from a reporter dye attached to the probe. In this system, threshold cycles (C_T), in which the fluorescence begins to increase from the background level, are measured. The number of molecules in the samples is determined from the threshold cycles (C_T) in the PCR based on a standard curve.

PCR primers and probes were designed using software Primer EXPRESS™ 1.0. PCR conditions were initially optimized using a part of DNA of pBluescript II as a target DNA. A primer pair, forward 5′-ACAAAAGCTGGGTACCGGG (Tm 61°C at 300 nM, GC 58%) and reverse 5′-TAGGGCGAATTGGAGCTCC (Tm 62°C at 300 nM, GC 58%) that amplifies a part of the cloning site of pBluescript II, was designed together with a probe 5′-ATCGAATTCCTGCAGCCCGGG (Tm 68°C at 50 nM, GC 62%) that was labeled with a reporter dye (FAM [6-carboxyfluorescein]) and a quencher (TAMRA [6-carboxy–tetramethyl–rhodamine]) at the 5′ and 3′ ends, respectively.

Primers and probe used for the real-time quantitation of *Wolbachia* were designed from the *ftsZ* gene of *Wolbachia*. A pair of primers, which amplified 111-bp DNA, were ftsZ77 Bf1, 5′-TTATCACAGCAGGGATGGGT (Tm 60°C at 300 nM, GC 50%) and ftsZ77 Br1, 5′-TTTTTTCTTTTGCTCCTTTATCTTTAACTA (Tm 60°C at 300 nM, GC 23%). A fluorogenic probe, which possesses a reporter dye FAM and a quencher TAMRA at the 5′ and 3′ ends, respectively, was designed as ftsZ77 BPro1, 5′-ACTGGAACAGGTGCTGCACCG (Tm 64°C at 50 nM, GC 62%). These primers and probe were designed to specifically amplify and detect the *ftsZ* gene of B-group *Wolbachia*. Standard DNA for quantitation was prepared from a planthopper homogenate by PCR with ftsZ77 Bf1 and ftsZ77 Br1 primers. The standard DNA was purified with a spin column of Sephacryl S-300 HR (Pharmacia Biotech).

A TaqMan™ PCR Reagent Kit was used for the PCR reaction. The amplification solution (20 μl) contained a DNA sample, a pair of 300 nM of primers, 50 nM probe, 200 μM of ATP, CTP and GTP, 400 mM of UTP, 0.2 U of AmpEraseUNG, 0.6 U of Ampli *Taq* Gold, 8% glycerol and 5 mM $MgCl_2$. Two microliters of template DNA samples, which were used for the Q–PCR mentioned above, were also used. The standard DNA was simultaneously amplified with the following amount in each of two replications: 0, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 molecules. The number of molecules was calculated from the weight data by OD at 260 nm. Cycle parameters were 50°C for 2 min to work AmpErase UNG to prevent amplicon carryover contamination and 95°C for 10 min to activate Ampli*Taq* Gold, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Results

3.1. Effect of tetracycline on progeny production and egg development in planthoppers

Tetracycline (0.1%) was applied in nymphal stages, and the resultant female adults were crossed with untreated *Wolbachia*-bearing males. The proportion of egg development deposited by pairs (n=8) of the tetracycline-treated females and untreated males of *L. striatellus* was 0% (0/171), whereas that between untreated female and male (n=9) was 73.0% (176/241). In contrast, the proportion of developed eggs from the crosses between antibiotic-treated females and untreated males of *S. furcifera* (n=9) was 18.6% (74/397), and that by untreated male and female (n=9) was 66.4% (298/449).

Wolbachia-free colonies were established by antibiotic treatment through two successive generations in both *L. striatellus* and *S. furcifera*, and crossing experiments were done between a *Wolbachia*-free colony and the *Wolbachia*-infected original colony. Fig. 2 shows the four combinations of crosses in both planthoppers. The cross between *Wolbachia*-free females and *Wolbachia*-infected males of *L. striatellus* did not produce any viable eggs, whereas that of *S. furcifera* produced 2.7% viable eggs. This difference was more marked in the crosses with aged males. Young (three days old or less)

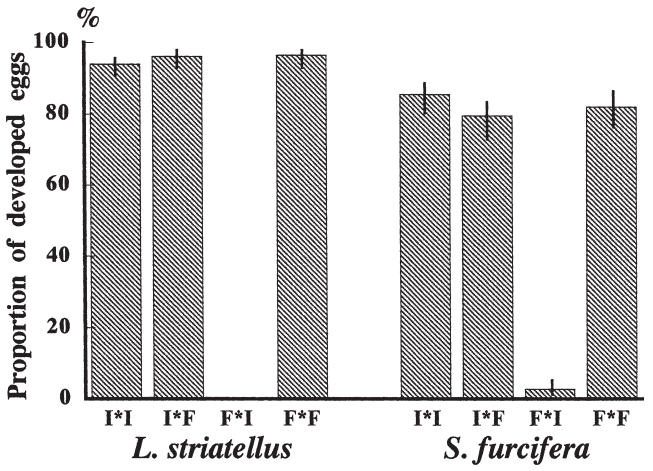

Fig. 2. CI in the crosses between *Wolbachia*-free (F) and *Wolbachia*-infected (I) males (right) and females (left) in two planthopper species. Young females (less than three days old) and males (less than one day old) were crossed individually for one week, and deposited eggs and emerged nymphs were observed one week later. F*I means the cross between *Wolbachia*-free female and *Wolbachia*-infected male. Bars indicate the 95% confidence interval.

Wolbachia-free females were crossed with *Wolbachia*-infected aged males in both *L. striatellus* and *S. furcifera*. In *L. striatellus*, incompatibility was quite complete even if the males were aged (Fig. 3). In contrast, the rate of developed eggs increased as the male aged in *S. furcifera* and 14 to 15 day-old males produced 18.0% of developed eggs. This result indicates that *L. striatellus* shows strong CI and *S. furcifera*, partial CI.

3.2. Nucleotide sequences of genes of Wolbachia from two planthopper species

Since the incompatibility level differed between *L. striatellus* and *S. furcifera*, it was considered possible

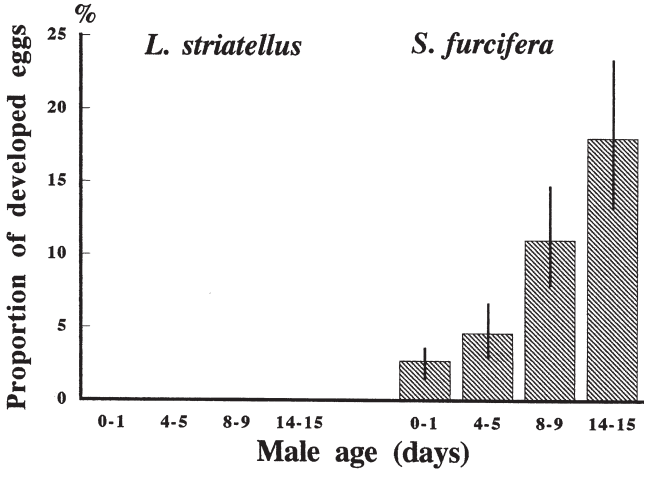

Fig. 3. The effect of male age on CI of two planthopper species. Young females from a *Wolbachia*-free colony were crossed with the males of 0–1 to 14–15 day-old from a *Wolbachia*-infected colony. Bars indicate the 95% confidence interval.

that the *Wolbachia* strains infecting *L. striatellus* and *S. furcifera* were different from each other. PCR with Adf–Adr and Bf–Br primers for *ftsZ* gene (Werren et al., 1995) showed both *Wolbachia* were type B. We then determined the sequences of *Wolbachia* genes to discriminate between the two *Wolbachia* from the different planthopper species. However, the nucleotide sequences of 16S rDNA (853 bp), *ftsZ* gene (1060 bp), *groE* genes (783 bp) and *wsp* gene (555 bp) were identical between two *Wolbachia* from *L. striatellus* and *S. furcifera* (DNA database accession numbers for 16S rDNA, AB039036 and AB039037; for *ftsZ* gene, AB039038 and AB039039; for *groE* genes, AB039040 and AB 039041; and for *wsp* gene, AB039042 and AB039043).

3.3. PCR detection of Wolbachia in two planthopper species

During PCR experiments, we noticed that PCR failed to amplify distinct products in some adult males of *S. furcifera*. The presence of *Wolbachia* in the nymphs and adults in the two planthopper species was then examined by PCR using the 99F/994R primer pair for 16S rDNA. Templates were simply prepared from boiled samples. *Wolbachia* were detected in all stages of *L. striatellus* examined with only a few exceptions in males (Table 1). In *S. furcifera*, however, PCR-positive rates of *Wolbachia* were clearly different between male and female. Most adult females possessed *Wolbachia*, whereas the PCR products were not observed in many of the newly emerged adult males (19/34). The males seemed to gradually lose *Wolbachia* with age, and only 15% of nine-day-old males were positive for *Wolbachia*. Even in nymphal stages, some individuals did not show the positive products of PCR under the present conditions of template preparation and PCR amplification.

3.4. PCR quantitation of Wolbachia

The infection rate of *Wolbachia* was low in *S. furcifera*. The infection density, however, cannot be measured by conventional PCR. Therefore, quantitative PCR (Q–PCR) was performed using a DNA sequencer to compare the density of infection between two planthopper species and between male and female.

Conditions for PCR in the thermal cycler and electrophoresis in the DNA sequencer were initially established using two cloned DNAs; a 370-bp control target DNA and 354-bp internal standard DNA (Fig. 1). First, amplified 370-bp DNA was run in all 40 lanes of the gel in the DNA sequencer (ALF Express, Pharmacia Biotech). The detection level of fluorescence in the sequencer fluctuated among lanes (probably due to the non-uniformity of the glass plate used for gel electrophoresis and operator handling errors), resulting in the highest and lowest levels differing by a factor of two. We con-

Table 1
PCR detection of *Wolbachia* in different stages of two planthoppers

Species	Sex	4th instar[a]	Adult: days after adult emergence		
			0	4	9
L. striatellus	female	30/30[b] (100.0)[c]	25/25 (100.0)	24/24 (100.0)	16/16 (100.0)
	male		24/25 (96.0)	27/27 (100.0)	27/31 (87.1)
S. furcifera	female	21/30 (70.0)	30/30 (100.0)	31/31 (100.0)	43/46 (93.5)
	male		19/34 (55.9)	12/39 (30.8)	6/40 (15.0)

[a] Including both male and female.
[b] The number of *Wolbachia*-detected planthoppers/the number of planthoppers tested.
[c] Parentheses show percentage.

sidered that this difference is not a serious problem for quantitating PCR products because an internal standard revises the value and three replications compensate for the fluctuation. Second, as a control the two template DNAs (370 bp and 354 bp) were amplified in separate tubes and loaded on the sequencer. A linear increase was observed at least from 10 to 1000 fg (17 PCR cycles), from 10 to 600 fg (18 PCR cycles), and from 1 to 100 fg (20 PCR cycles) of template DNA. Using these cycles, DNA could be detected quantitatively (data not shown). Third, the two template DNAs were amplified in the same tubes. Two hundred fg of the internal standard DNA was added to each PCR tube, and from 10 to 640 fg target DNA was added. After 18 cycles of amplification, the two products were separated by electrophoresis on the DNA sequencer, and the areas of the each fluorescent peak were measured. Amplification of internal standards was stable, and a linear increase in the target DNA was obtained under the present conditions. Template DNA was successfully calculated at the range of 10–320 fg (Fig. 4).

Adult males and females of *L. striatellus* and *S. furcifera* were subjected to this quantitative PCR. Three stages of adult females, 0, 4, and 9 days old, and four stages of adult males, 0, 4, 9, and 14 days old were examined in five samples for each stage (Fig. 5). There were more *Wolbachia* in females than in males. Four-day-old females of *L. striatellus* possessed 3.1×10^7 *Wolbachia*, about 2.3 times higher than the males of the same age. The quantity of *Wolbachia* in an adult female increased after adult emergence in both species. In the males of *L. striatellus*, *Wolbachia* decreased in later stages of adults. The number of *Wolbachia* in the males of *S. furcifera* was less than 5.4×10^5 and much fewer (about 1/20) than in the males of *L. striatellus*.

3.5. Real-time quantitative PCR

Another quantitative PCR method, real-time quantitative PCR (RTQ–PCR), was applied to quantitate *Wolbachia*. We preliminarily tested this method by quantitating the DNA molecules of a part of a plasmid DNA, pBluescript II, and obtained highly reproducible results (data not shown). The present procedure did not include an internal standard, therefore we cannot disregard possible PCR inhibitors in the templates of planthopper homogenates. To investigate possible PCR inhibitors a planthopper homogenate was then examined by amplifying a part of the plasmid DNA. Two microliters of the homogenate of nine-day-old females, which was used for the above-mentioned Q–PCR, was diluted and added to the PCR solution to compare the PCR performance at various dilution rates. The dilution did not affect the PCR results. C_T values at ten-amol template were 16.846, 16.819, 16.903, and 16.835 at the dilution rates of 1, 0.3, 0.1, and 0.03, respectively. Similar results were also observed in the experiments for 30- and 100-amol template concentrations. Therefore, the effect of possible PCR inhibitors in the planthopper samples is negligible in this procedure, and we used the planthopper template solution without further purification.

The planthopper samples that were used for Q–PCR were also used here. Five individuals of each stage of male and female adults were examined twice. We calculated the number of *Wolbachia* on the assumption that the *ftsZ* gene is a single copy gene per genome. The overall trend of the fluctuation in the number of *Wolbachia* in the adults of two planthopper species in RTQ–PCR (Fig. 6) was similar to that measured by Q–PCR (Fig. 5). The relatively low density of *Wolbachia* in the males of *S. furcifera* was also confirmed in this RTQ–PCR; the number of *Wolbachia* decreased from 6.2×10^5 at 0 days old to 3.3×10^5 at 14 days old and was less than one tenth of that in the males of *L. striatellus*. There was, however, a dissimilarity between the two quantitation methods. The number of *Wolbachia* in four-day-old females was relatively higher in Q–PCR.

The numbers of *Wolbachia* in the testis and ovary were measured independently using four-day-old adults (Table 2). The numbers of *Wolbachia* infecting four-day-old adults were in rather good accordance with the results in Fig. 6. The numbers of *Wolbachia* in the ovary or testis were lower than those of the whole body. The number of the *Wolbachia* infecting the testes and whole body of *S. furcifera* males varied, shown by relatively large values of standard error. The calculated number in

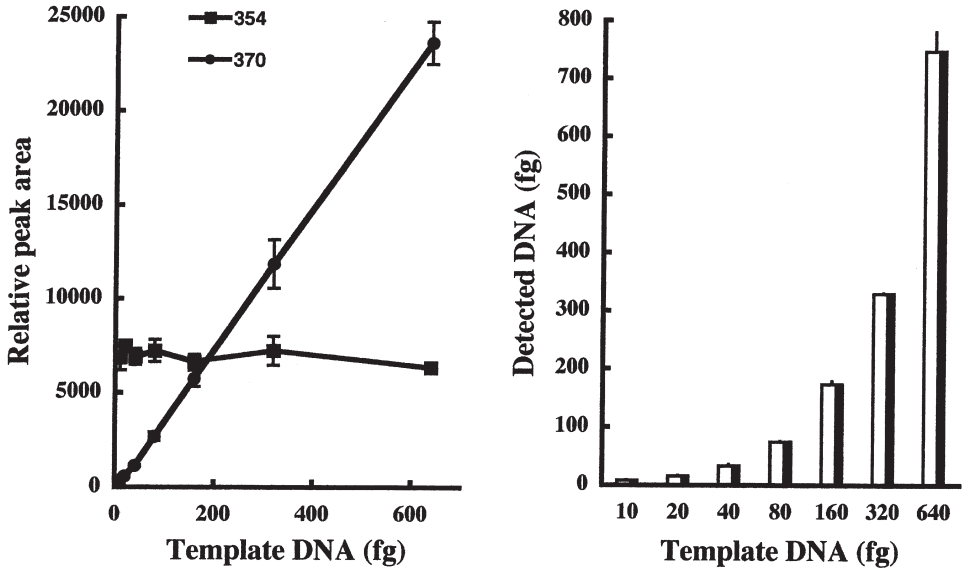

Fig. 4. Evaluation of quantitation of PCR products. Template DNAs of 354- and 370-bp rDNA sequences of *Wolbachia* were co-amplified in the same tubes. Each tube contained appropriate amounts (10 to 640 fg) of 370-bp DNA and 200 fg of 354-bp DNA as templates. Three replicates were prepared for each amount of 370-bp target DNA. (a) Relation between the amount of template DNAs and relative peak area in data analyzed by the AlleleLinks (Amersham Pharmacia Biotech). (b) Calculated amount of 370-bp DNA after correction based on the amount of the internal standard DNA (354-bp DNA). Bars indicate standard errors.

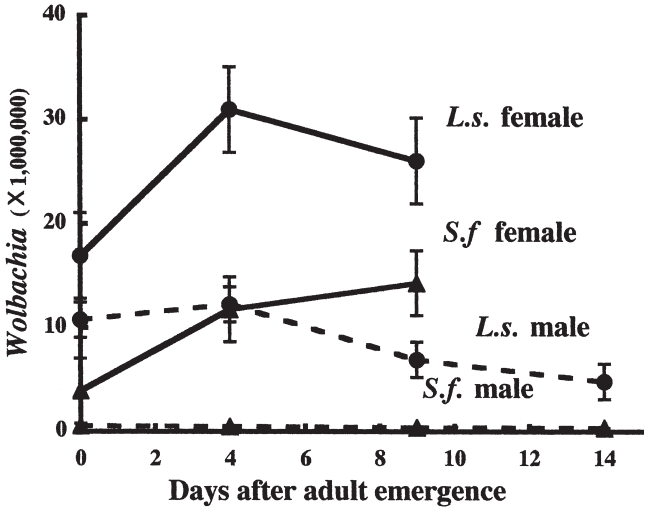

Fig. 5. Quantitative PCR of 16S rDNA of *Wolbachia* in male and female adults of two planthopper species. The samples of *L. striatellus* and *S. furcifera* were amplified for 18 and 20 cycles, respectively. The number of *Wolbachia* is shown as the average of five samples. Bars indicate standard errors.

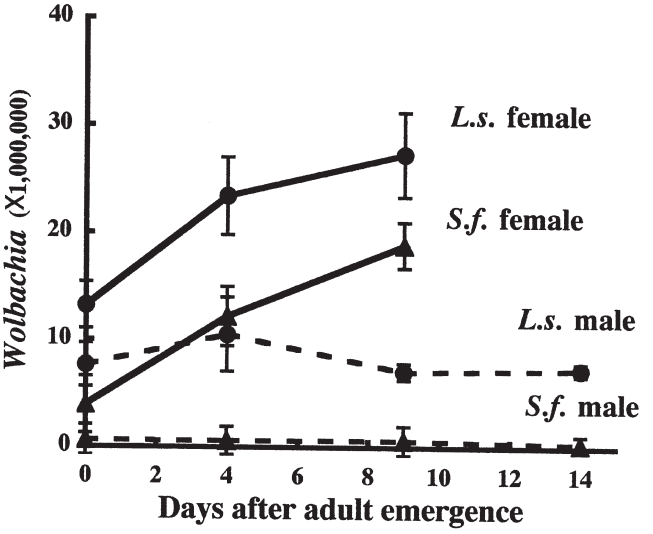

Fig. 6. Real-time quantitative PCR of *ftsZ* gene of *Wolbachia* in male and female adults of two planthopper species. Each point is the average of two measurements of five samples. Bars indicate standard errors.

the testes of five samples, for example, were 0.3, 0.3, 0.7, 15, and 319×10^3. These numbers were quite small in comparison with the number in the testes of *L. striatellus*, 176, 319, 328, 353, and 395×10^3.

The decrease of *Wolbachia* with age of males was also confirmed in another samples of the testes of *S. furcifera*. The numbers of *Wolbachia* in the testes of zero-, six-, and twelve-day-old males (n=6–7) were 4620±2610, 1530±1050, and 1770±1000, respectively.

4. Discussion

CI in laboratory-reared *L. striatellus* was strong and almost complete (Figs. 2 and 3). When field collected *L. striatellus* were tested for incompatibility after rearing for two or three generations, only a small number of offspring was obtained from incompatible crosses (Noda 1984b, 1987). In general, the incompatibility level of this species is one of the highest. In contrast, *S. furcifera* showed weaker incompatibility than *L. striatellus* (Figs.

Table 2
The number of *Wolbachia* infecting testis and ovary of 4-day-old adults

Sample	*L. striatellus* ×1,000,000[a]	*S. furcifera*
Female whole body	28.9±3.2	7.6±2.1
Ovary	3.5±0.5	2.3±0.6
Male whole body	9.1±1.0	0.6±0.3
Testis	0.3±0.04	0.06±0.06

[a] average of five samples±standard error.

2 and 3). The difference in incompatibility level between two planthopper species were then examined in the present study from the viewpoint of quality and quantity of *Wolbachia*, which are important factors for characterizing CI in each insect species.

Quality was judged by nucleotide sequences of four genes of *Wolbachia* from *L. striatellus* and *S. furcifera* to learn how closely or distantly these two *Wolbachia* are related to each other. Generally, *Wolbachia* from different hosts show different nucleotide sequences in some genes (Rousset et al., 1992; Werren et al., 1995; Zhou et al., 1998), except in some host–parasitoid associations (van Meer et al., 1999; Huigens et al., 2000). Nucleotide sequences of 16S rDNA, *ftsZ* gene, *groE* genes and *wsp* gene, in total 3250 nt, were identical in *Wolbachia* of *L. striatellus* and *S. furcifera*. The present results strongly suggest that *Wolbachia* from the two planthopper species are identical and the CI ability of the two *Wolbachia* should be the same.

The density of *Wolbachia* was first precisely detected by Sinkins et al. (1995) using a PCR method. They quantitated *Wolbachia* by competitive PCR. In the present study, two less-laborious methods were established to quantitate *Wolbachia*. First, Q–PCR using an automated DNA sequencer is based on the principle of comparative PCR; the PCR products, which included a fluorescent primer, were quantitated in the exponential phase of reaction (cycles 17 to 20) before the reaction plateau (Kellog et al., 1990; Porcher et al., 1992). Employing an internal standard made it possible to test the unpurified insect homogenates. A more convenient method, RTQ–PCR with a sequence detection system, successfully estimated the number of *Wolbachia*. This method has a very wide dynamic range of starting target molecule determination (Heid et al., 1996). In the present study, we did not set an internal standard. We did not further purify the proteinase K-treated template solutions because we may have lost a part of the template DNA during the purification process. Fortunately, initial tests for the effect of PCR suppressors in the template solution of the homogenates (nine-day-old females of *L. striatellus*) indicated that adding the homogenates did not retard PCR amplification.

In these methods, 16S rDNA or *ftsZ* gene were used for calculation of the number of *Wolbachia*. 16S rDNA is present in a single copy per genome (Bensaadi-Merchermek et al., 1995) and the copy number of *ftsZ* gene is also presumably one because all more than 20 prokaryotes, whose whole genomes were fully sequenced and deposited in the Entrez Genome in the National Center for Biotechnology Information, have only one copy of the locus. In this respect, *Buchnera*, endosymbiote of aphids, have multiple copies of a genome in a single bacterium (Komaki and Ishikawa, 1999). Such phenomenon is rare and is usually not known in other bacteria. *Wolbachia*, therefore, seems likely to have a single copy of a genome, and the measured number of target DNA molecules estimates the number of *Wolbachia* in a planthopper. However, we are not assure that the aforementioned DNA extraction method using proteinase K releases all of the *Wolbachia* chromosomes from the samples and makes them accessible to PCR amplification. We should keep it in mind that the measured number of *Wolbachia* may not be an absolute value but an "index" of relative density.

The results of the two quantitation methods were similar (Figs. 5 and 6), but an incongruent point was evident between the two quantitation methods. The *Wolbachia* population in four-day-old females of *L. striatellus* is relatively larger in Q–PCR than in RTQ–PCR; gradual increase of *Wolbachia* in *L. striatellus* female was observed in RTQ–PCR, whereas the number of *Wolbachia* increased in the first four days and slightly decreased later in Q–PCR. The measurable range of target template DNA in Q–PCR is narrower than in RTQ–PCR, and there may easily be a shortage of resources, for example primer, dNTP, and enzyme in Q–PCR for quantitating a large amount of target DNA. We then reduced the amount of template and standard DNA to ease the resource competition in the PCR tube. However, a similar result was obtained, and the incongruence remained (data not shown). The reason is unclear at this moment. Nevertheless, overall results are quite similar and these methods are useful for comparing the relative and absolute densities in host insects. The authors have the impression that RTQ–PCR is more accurate because it is based on a unique PCR and detection system in comparison with conventional PCR, and they seem to

suffer less interference by various factors involved in PCR reaction. Handling is also easier in RTQ–PCR.

Bacterial density, was first fully discussed by Breeuwer and Werren (1993b) and Boyle et al. (1993). Chromosome modification, which is believed to be a key factor for CI, apparently requires a certain threshold of bacterial density. Aged males have less ability to cause CI in *Cx. pipiens* (Singh et al., 1976; Subbarao et al., 1977) and *D. simulans* (Hoffmann et al., 1986). This phenomenon is explained by the fact that aged males decrease the proportion of *Wolbachia*-infected sperm cysts in *Drosophila* (Bressac and Rousset, 1993). Decreased density in aged males does not presumably modify the sperms any more. The partial CI in *S. furcifera* seems to be explained by this bacterial density model of CI. Males of *S. furcifera* possessed fewer *Wolbachia* in comparison with males of *L. striatellus*, and some testes of *S. furcifera* were almost free of *Wolbachia*. Some sperms in *S. furcifera* males appear to be hardly modified because of the low *Wolbachia* density. The present results suggest that partial CI is caused by low *Wolbachia* density in males.

RTQ–PCR showed that there were much fewer *Wolbachia* in the ovary or testis than in the whole body, indicating that *Wolbachia* infects tissues outside the gonad. In this respect, Dobson et al. (1999) reported that *Wolbachia* infect various tissues other than the gonad in *D. simulans* and some other species. They presented clear results against the unchallenged idea that *Wolbachia* is restricted to the gonads of insects. The present results support their observation that *Wolbachia* infect organs other than the gonad.

As Werren (1997) points out, exploration of regulation mechanism of bacterial density is an interesting and important question. In this respect, the yeastlike symbiotes (YLS) of planthoppers are well regulated in the host planthoppers (Noda, 1974). The number of YLS increases after adult emergence in females and decreases in males. This is comparable with the present data on *Wolbachia*. Growth regulation of *Wolbachia* will be an important study area for clarifying the mechanism of CI expression and host-endosymbiote interactions. The outgrowth of the popcorn strain of *Wolbachia* found in *Drosophila* (Min and Benzer, 1997) is also apparently related to the mechanism of growth regulation of the bacteria.

S. furcifera is distributed in Southeast Asia and expands its distribution every summer by long distance flight (Kisimoto, 1987). Actually, this species cannot overwinter in the main islands of Japan and immigrates from the continent in June and July every year. *L. striatellus* has a wider distribution and exhibits nymphal diapause (Kisimoto, 1958; Noda, 1992). The present results indicate that the same strain of *Wolbachia* infects *L. striatellus* and *S. furcifera*. This raises the possibility that *Wolbachia* in the two planthopper species have been horizontally transmitted to each other. *Wolbachia* are now being detected in planthoppers from different locations, and the vehicle of *Wolbachia* distribution among planthoppers is now being explored.

Acknowledgements

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Appendix A

Wolbachia amount (the number of *Wolbachia*) was calculated based on the following equations. Two hundred fg of internal standard DNA (354 bp) was usually used. The number of molecules of target DNA (370 bp) was calculated as follows.

* Size of the amplified internal standard DNA:

354 bp (insert DNA)+227 bp (cloning site)=581 bp

* Average molecular weight of dNMP is assumed to be 330.

Therefore,

Molecular weight of the double standard DNA of 581 bp is:

$330\times581(bp)\times2=383460$

* Weight of internal standard DNA, which was amplified by PCR, was calculated by OD (OD 1=45 μg DNA in 1 ml water). The solution was then diluted to the order of fg (usually 200 fg).

1 fg of the standard DNA is:

$1/383460$ fmol$=2.6\times10^{-6}$ fmol$=2.6\times10^{-3}$ amol

* One amol (1×10^{-18} mol) corresponds to:

1×10^{-18} (mol)$\times6.02\times10^{23}$ (Avogadro number)$=6.02\times10^{5}$ molecules (copies)

Therefore,

One fg corresponds to:

2.6×10^{-3} (amol)$\times6.02\times10^{5}$ (copies)$=1.57\times10^{3}$ copies

Therefore,

200 fg of standard DNA should include 3.14×10^{5} copies ($1.57\times10^{3}\times200$).

When 2 μl of PCR template was used and 200 fg of internal standard DNA was added,

A=104/2=52 (104 μl=100 μl buffer+4 μl proteinase K solution)

B=peak area of the target DNA/peak area of the standard DNA

Number of copies of the target DNA$=3.14\times10^{5}\times A\times B$

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