Microbial Ecology (2019) 77:257–266
https://doi.org/10.1007/s00248-018-1210-4

HOST MICROBE INTERACTIONS

Multiple Infection and Reproductive Manipulations of *Wolbachia* in *Homona magnanima* (Lepidoptera: Tortricidae)

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Received: 9 February 2018 / Accepted: 22 May 2018 / Published online: 21 June 2018

Abstract

Endosymbiotic bacterium *Wolbachia* interacts with host in either a mutualistic or parasitic manner. *Wolbachia* is frequently identified in various arthropod species, and to date, *Wolbachia* infections have been detected in different insects. Here, we found a triple *Wolbachia* infection in *Homona magnanima*, a serious tea pest, and investigated the effects of three infecting *Wolbachia* strains (*w*Hm-a, -b, and -c) on the host. Starting with the triple-infected host line (W^{abc}), which was collected in western Tokyo in 1999 and maintained in laboratory, we established an uninfected line (W^{-}) and three singly infected lines (W^{a}, W^{b}, and W^{c}) using antibiotics. Mating experiments with the host lines revealed that only *w*Hm-b induced cytoplasmic incompatibility (CI) in *H. magnanima*, with the intensities of CI different between the W^{b} and W^{abc} lines. Regarding mutualistic effects, *w*Hm-c shortened larval development time and increased pupal weight in both the W^{c} and W^{abc} lines to the same extent, whereas no distinct phenotype was observed in lines singly infected with *w*Hm-a. Based on quantitative PCR analysis, *Wolbachia* density in the W^{a} line was higher than in the other host lines ($p < 0.01$, $n = 10$). *Wolbachia* density in the W^{b} line was also higher than in the W^{c} and W^{abc} lines, while no difference was observed between the W^{c} and W^{abc} lines. These results indicate that the difference in the CI intensity between a single or multiple infection may be attributed to the difference in *w*Hm-b density. However, no correlation was observed between mutualistic effects and *Wolbachia* density.

Keywords *Wolbachia* density · Reproductive fitness · Cytoplasmic incompatibility · *Homona magnanima*

Introduction

Endosymbionts are frequently detected in a variety of insect species [1, 2]. Some endosymbionts interact with the host in a mutualistic or parasitic manner [2, 3]. As an example, endosymbiotic bacteria [4–8], microsporidia [9], and an RNA virus [10] are known to manipulate host reproduction. The best-studied endosymbiont is the alpha-proteobacterium *Wolbachia*, which generally is responsible for three types of reproductive manipulation, namely, male-killing, feminization, and cytoplasmic incompatibility (CI) [11]. Recent studies revealed that *Wolbachia* infects approximately 40% of terrestrial arthropod species [12]. Some *Wolbachia* strains exert a negative effect on the host, e.g., by distorting the sex ratio, whereas others benefit the host by increasing its fecundity [13]; elongating the host's lifetime [14]; providing nutrition [15]; and resisting viral infection [16]. Conversely, other *Wolbachia* strains do not discernibly affect the host fitness [17, 18]. Hence, interactions between *Wolbachia* and its hosts are complicated.

Wolbachia sometimes coexists with other endosymbiotic bacteria, such as *Spiroplasma* [19] and *Cardinium* [20]. Multiple *Wolbachia* infections have been also reported in many insect hosts, on the order level: Coleoptera [21], Hymenoptera [22, 23], Lepidoptera [24], and Diptera [25]. In some cases, the CI intensity induced by *Wolbachia* decreases as a result of coexistence with *Cardinium* [26] or other *Wolbachia* strains [27]. The phenotype caused by the endosymbionts is appreciably correlated with endosymbiont density [27, 28]. On the other hand, the phenotype and *Spiroplasma* density are not affected by the presence of *Wolbachia* because the bacterial microhabitats are different [29]. Hence, phenotypes caused by the endosymbiotic bacteria may exhibit various types when multiple infections occur in an individual.

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Homona magnanima (Tortricidae, Lepidoptera) is a serious tea pest in East Asia. Two male-killing agents, a presumed RNA virus [10, 30] and *Spiroplasma* [31], have been identified in this species. Although *Wolbachia* has also been detected in the field population of *H. magnanima* [31], its effect on host reproduction or development had not been investigated in detail.

In the current study, we first evaluated the endosymbiont infection status of *H. magnanima* collected from a tea plantation in Tokyo, Japan, in 1999. We then established three lines singly infected with *Wolbachia* and one uninfected line in a laboratory-maintained *H. magnanima*. Finally, we investigated the effect of each *Wolbachia* strain on the development and CI modification of *H. magnanima*.

Materials and Methods

Insects

H. magnanima was originally collected in Akiruno city (Tokyo, Japan) in 1999 and is positive for *Wolbachia* [31]. Insects were continuously reared under laboratory conditions (16L:8D, 25 °C, and 60% relative humidity). All larvae hatched from an egg mass were reared on an artificial diet, SilkMate 2S (Nosan Co., Ltd., Yokohama, Japan) in a plastic container (23 × 16 × 8 cm). For mating, 15 males and 10 females were placed in a plastic box (30 × 20 × 5 cm) [31]. Those rearing and mating treatments were done in each generation.

Detection of Endosymbiotic Bacteria

To detect endosymbiotic bacteria, total DNA was extracted from the abdomen of newly emerged adult females. Endosymbiotic bacteria showed tissue tropism in its host; however, these bacteria are usually located in the host ovary for maternal transmission [29, 32, 33]. As the abdomen of female *H. magnanima* bears the ovaries, we chose this organ for DNA extraction and for further experiments. First, the female adult abdomen was separated using forceps and placed into a 1.5-ml plastic tube. Each sample was homogenized using a sterilized pestle in 900 μl of cell lysis solution (10 mM Tris-HCl, 100 mM EDTA, and 1% SDS, pH 8.0) and incubated with 1.1 μg/μl proteinase K (Merck, Darmstadt, Germany) at 50 °C for 5 h. The samples were then incubated with 10 μg/μl RNase (Nippongene, Tokyo, Japan) at 37 °C for 1 h. Two hundred microliters of Protein precipitation solution (Qiagen, Hilden, Germany) was added to collect the debris. After centrifugation, 600 μl of the supernatant was transferred to a new tube, and 600 μl of 100% isopropanol was added. Precipitated DNA was washed with 500 μl of 70% ethanol [*v*/*v*], and the supernatant was removed by pipetting. After drying, the DNA was suspended in 30 μl of distilled water and stored at 4 or − 35 °C.

To detect three types of endosymbionts (*Wolbachia*, *Spiroplasma*, and *Rickettsia*), DNA samples were amplified using specific primer sets: *wsp*81F and *wsp*691R, for *Wolbachia* [34]; HA-IN and SP-ITS-N, for *Spiroplasma* [35]; and RpCS.877P and RpCS.1258, for *Rickettsia* [36]. PCR was conducted using TaKaRa Ex Taq kit (TaKaRa Bio Inc., Shiga, Japan). The reaction mix (total 10 μl) consisted of 1.0 μl of 10X Ex Taq buffer, 0.8 μl of dNTP mixture (2.5 μM in each), 0.05 μl of TaKaRa Ex Taq (5 U/μl), 6.75 μl of Milli-Q water, 0.2 μl of each forward and reverse primer (10 μM), and 1.0 μl of sample DNA (50–100 ng/μl). The amplification reaction parameters for *Wolbachia wsp* (*wsp*81F and *wsp*691R) were as follows: 3 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C; with a final extension for 7 min at 72 °C. The conditions for *Spiroplasma* (HA-IN and SP-ITS-N) and *Rickettsia* (RpCS.877P and RpCS.1258) were as follows: 3 min at 96 °C; followed by 30 cycles of 30 s at 96 °C, 30 s at 52 °C for *Spiroplasma* and 55 °C for *Rickettsia*, and 30 s at 72 °C; with a final extension for 7 min at 72 °C. To verify the success of DNA extraction, a housekeeping gene of lepidopteran insects encoding β-actin was amplified as a control, using the primers actin-S and actin-AS [31]. The temperature profile of the amplification reaction was as follows: 3 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; with a final extension for 7 min at 72 °C. DNA, which was extracted, as mentioned above, from laboratory colonies of *Laodelphax striatellus* and *Nephotettix cincticeps*, was subjected to PCR as a positive control of each symbiont. *Laodelphax striatellus* was positive for *Wolbachia* and *Spiroplasma* [37, 38], and *Nephotettix cincticeps* was positive for *Rickettsia* [39]. PCR products were resolved by electrophoresis on a 1.5% (*w*/*v*) agarose gel, stained with ethidium bromide, and the bands observed on a transilluminator.

Establishment of Lines Singly Infected with *Wolbachia* and an Uninfected Line

Host line infected with three *Wolbachia* strains (*w*Hm-a, *w*Hm-b, and *w*Hm-c) was designated as "W^{abc}." To establish the uninfected line (W^{-}) and the three singly infected lines (W^{a}, W^{b}, and W^{c}), the W^{abc} line was treated with antibiotics, as follows. The W^{-} line was established from W^{abc} line reared on SilkMate 2S supplemented with tetracycline [0.05% (*w*/*w*)] for one generation. The W^{a} line was established from the W^{abc} line reared on SilkMate 2S supplemented with tetracycline [0.0125% (*w*/*w*)] for one generation. The W^{b} and W^{c} lines were established from the W^{abc} line reared on SilkMate 2S supplemented with rifampicin [0.06% (*w*/*w*)] for two generations. The offspring of each antibiotic-treated line was maintained as mentioned above. To confirm the *Wolbachia*

infection status, specific primer sets for *wsp* of *w*Hm-a, *w*Hm-b, and *w*Hm-c were designed and used for PCR-based detection, as follows: *w*Hm-a_F173 (5′-CCTATAAGAAAGACAATA-3′) and *w*Hm-a_R565 (5′-TTTGATCATTCACAGCGT-3′), for *w*Hm-a; *w*Hm-b_F176 (5′-GGTGCTAAAAAGAAGACTGCGG-3′) and *w*Hm-b_R667 (5′-CCCCCTTGTCTTTGCTTGC-3′), for *w*Hm-b; and *w*Hm-c_F188 (5′-CATATAAATCAGGTAAGGACAAC-3′) and *w*Hm-c_R603 (5′-CACCAGCTTTTGCTTGATA-3′), for *w*Hm-c. The following amplification reaction conditions were employed: 3 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 55 °C, or 60 °C (for *w*Hm-a, *w*Hm-b, and *w*Hm-c, respectively), and 30 s at 72 °C; with a final extension for 7 min at 72 °C.

Sequencing of *Wolbachia* Genes

To determine the status of *Wolbachia* infection, DNA of W^{abc} line was subjected to *Wolbachia wsp* gene amplification as mentioned above. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen). Purified product was ligated with the pGEM-T easy vector (Promega, Madison, WI, USA) using a ligation mix (TaKaRa). Competent cells (*Escherichia coli* JM109, TaKaRa) were then transformed with the plasmid. Plasmid DNA was extracted using the Pure Yield Plasmid Miniprep System (Promega, Madison, WI, USA), according to the manufacturer's protocol, and stored at 4 °C.

DNA extracted from females of each line singly infected with *Wolbachia* was used as a template for *Wolbachia* multilocus sequence typing (MLST). MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) were amplified using specific primer sets, and PCR analysis was conducted as described by Baldo et al. [40]. PCR products were purified using the Qiaquick PCR purification kit and directly sequenced. Sequencing reactions were performed using the BigDye terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Specific primers for T7 and SP6 promoters were used to amplify the cloned *wsp* genes, while purified amplimers generated using the MLST-specific primers were sequenced with the primer sets used for PCR. The sequencing was performed in both, forward and reverse direction. Products of each reaction were pelleted with a mixture of 30 μl of 99% ethanol and 2.5 μl of 125 mM EDTA and washed with 70% ethanol. Purified products were suspended in 10 μl of Hi-Di™ formamide (Applied Biosystems) and incubated for 2 min at 95 °C. The sequencing was performed using the 3100 Genetic Analyzer (Applied Biosystems).

Transmission Rate of the Individual *Wolbachia* Strains

One female from the W^{abc} line and one male from the W^{-} line were mated in a plastic box (15 × 15 × 7 cm). The crossing was replicated six times; ten larvae were chosen randomly from each egg mass and reared individually until eclosion. *Wolbachia* infection status was checked in F_1 adults by PCR, as described above, using strain-specific primer sets. The transmission rate of each *Wolbachia* strain was calculated as the number of F_1 adults infected with *Wolbachia* divided by the number of adults in each crossing experiment.

The Effects of *Wolbachia* on Host Development and Reproduction

To investigate the effects of *Wolbachia* infection on the host, larvae of each host line were individually reared on an artificial diet, INSECTA LF (Nosan Co., Ltd.). The sex ratio, larval development time, and pupal weight were recorded.

Crossing experiments were performed to evaluate the CI modification and rescue of each *Wolbachia* strain. Three males (0 ± 1 day post eclosion) from the *Wolbachia*-infected lines (W^{abc}, W^{a}, W^{b}, and W^{c}) were mated with three virgin females from the W^{-} line in a plastic box (15 × 15 × 7 cm). Each crossing experiment was replicated four times with different generations. The W^{-} males and W^{-} females were also mated, as a control. Females were allowed to oviposit for 7 days. Since low hatchability was observed in the crosses of $W^{abc} \times W^{-}$ and $W^{b} \times W^{-}$ lines, to characterize the phenotypes, males and females of the W^{abc} and W^{b} lines were mated with each other.

The hatchability of F_1 generation from each crossing experiment described above was calculated. It was defined as the number of late-stage embryos per number of eggs in an egg mass, because the number of late-stage embryos was almost the same as the number of larvae that hatched successfully. First, to analyze the correlation between the egg mass area and the number of eggs, 50 egg masses from the control crossing ($W^{-} \times W^{-}$), which showed high hatchability, were chosen randomly. Each egg mass area was determined using Image J (https://imagej.nih.gov/ij/) and the eggs were counted under a microscope. Both parameters were analyzed by JMP v 9 (SAS, Cary, NC, USA) using a general linear model (see below) to obtain a regression line. Using this line, the number of eggs in an egg mass from each crossing experiment was estimated from the egg mass area. In addition, late-stage embryos in each egg mass were counted. Finally, the hatchability of each egg mass was calculated as described above.

Determination of *Wolbachia* Density

To evaluate *Wolbachia* density, five newly emerged males and females from each line were chosen for qPCR determinations. The qPCR primer set was designed based on the *wsp* sequence, to amplify the *w*Hm-*wsp* universal region of about 100 bp: *w*Hm-uni_qpcrF, 5′-TGGTGTTGGTGCAGCGTAT-

3′; and *w*Hm-uni_qpcrR, 5′-AACTAACACCAGCTTTTGCT TGA-3′. The qPCR reaction was performed as described by Iwata et al. [41]. The reaction mixture contained 10 ng of DNA, 30 μM of each primer, and 5 μl of the FastStart Universal SYBR Green master mix (Roche, Basel, Switzerland). The reactions were performed using the StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA, USA). The cycle conditions were as follows: 10 min at 95 °C; followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Dissociation curve analysis of the amplified product was performed after the amplification, as follows: 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. The Ct value of each sample DNA was determined twice as described by Iwata et al. [41]. The quantity of *Wolbachia* in each sample was calculated based on a standard curve generated using 10^{-4}–10 ng of DNA of a plasmid harboring a cloned partial *wsp* sequence. The number of *wsp* copies in 10 ng of sample DNA was estimated from the molecular weight of the *wsp* sequence.

Data Analysis and Statistics

The obtained sequences were aligned with sequences obtained by Baldo et al. [40] using ClustalW [42], except for *Trichogramma deion*, which lacks *wsp* sequences. For phylogenetic tree construction, the maximum likelihood method with bootstrap re-sampling of 1000 replications was performed in MEGA6 [43]. Correlation between the size and number of eggs in an egg mass was determined by regression analysis using a general linear model. χ^2 test was used to confirm whether the sex ratio of each host line was biased. The hatchability, larval development time, pupal weights, and *Wolbachia* densities in each host line were analyzed using the Steel-Dwass test. All statistical analyses were performed using JMP v9. Sequences of *Wolbachia* were deposited in GenBank under accession numbers LC363921 to LC363938.

Results

Detection of Endosymbionts and the Transmission and Phylogeny of *Wolbachia*

Laboratory-maintained *H. magnanima* (Tokyo population; W^{abc}) was positive for *Wolbachia*, but negative for *Spiroplasma* and *Rickettsia*. The 46 clones of *wsp* fragments generated from W^{abc} line could be divided into three sequence types: *wsp*-a (25 clones), *wsp*-b (12 clones), and *wsp*-c (9 clones), indicating a multiple infection of *H. magnanima* with three *Wolbachia* strains (*w*Hm-a, *w*Hm-b, and *w*Hm-c). The transmission rate of *w*Hm-a and *w*Hm-c to F_1 generation was 100.0% ($n = 60/60$), while that of *w*Hm-b was 90.0% ($n = 54/60$, Table 1).

Table 1 Transmission rate of each *Wolbachia* strain

	Sample size	Infection status					
		*w*Hm-a		*w*Hm-b		*w*Hm-c	
Female	31	31	100%	31	100%	31	100%
Male	29	29	100%	23	79%	29	100%
Total	60	60	100%	54	90%	60	100%

MLST sequence data also supported the presence of three distinct strains (Table 2). Both *w*Hm-a and *w*Hm-b belonged to supergroup A, and *w*Hm-c belonged to supergroup B (Fig. 1).

Establishment of the Three Lines Infected with a Single *Wolbachia* Strain

The W^{abc} line was firstly treated with tetracycline [0.0125% (*w*/*w*)]. The randomly chosen eight individuals of the treated line (Gt1) showed only multiple infection, as revealed by diagnostic PCR experiment: triple infection (6/8 in number of individuals) and double infection with *w*Hm-b and -c (2/8). In Gt2 generation, the two Gt1 triply infected lines were reared on SilkMate without antibiotics. The randomly chosen four individuals in each Gt2 line showed *w*Hm-a single infection (2/4 and 3/4, respectively) and no infection (2/4 and 1/4 in each). The offspring of the Gt2 *w*Hm-a singly infected line showed stable infection after the third generation (Gt3); therefore, we chose one line as a *w*Hm-a singly infected line for further experiments (Fig. 2a).

Secondly, the W^{abc} line was treated with rifampicin [0.06% (*w*/*w*)]. The randomly chosen nine individuals of the treated line (Gr1) showed the following infection statuses, as revealed by diagnostic PCR experiments: triple infection (2/9 in number of individuals); double infection with *w*Hm-a and -c (3/9), *w*Hm-a and -b (1/9), and *w*Hm-b and -c (1/9); and single infection with *w*Hm-a (1/9) and *w*Hm-c (1/9). In Gr2 generation, the two lines of Gt1 (doubly infected line with *w*Hm-a and -b and singly infected line with *w*Hm-c) were re-treated with rifampicin [0.06% (*w*/*w*)]. The randomly chosen six individuals of the offspring of doubly infected line with *w*Hm-a and -b showed the following three infection statuses: single infection with *w*Hm-b (1/6) and *w*Hm-a (2/6) and no infection (3/6). The randomly chosen six individuals of the offspring of singly infected line with *w*Hm-c showed stable infection only with *w*Hm-c in Gr2 generation (6/6). In Gr3, the singly infected line with *w*Hm-b and -c was reared on SilkMate without antibiotics and showed stable infection only with both *Wolbachia* strains. Thus, we determined the singly infected line with *w*Hm-b and *w*Hm-c as the W^b and W^c lines, respectively, for further experiments (Fig. 2b).

Table 2 *wsp* and MLST gene typing of *w*Hm-a, *w*Hm-b, and *w*Hm-c strains

Strain	*wsp*	*gatB*	*coxA*	*hcpA*	*fbpA*	*ftsZ*
*w*Hm-a	83	54	59	68	67	Partial match: 164(1)
*w*Hm-b	108	Partial match: 7(2)	6	7	8	Partial match: 164(3)
*w*Hm-c	61	16	14	40	4	Partial match: 73(4)

The numbers below each locus indicate the most similar sequences type number in the *Wolbachia* MLST database. (1) 44 differences found; (2) one difference found; (3) insertions or deletions present; (4) 43 differences found

The Effect of *Wolbachia* on Host Sex Ratio and Development

The percentages of females in the W^{abc}, W^{a}, W^{b}, W^{c}, and W^{-} lines were determined, with no significant difference between the lines ($\chi^2 = 6.704$, $p = 0.152$, Table 3).

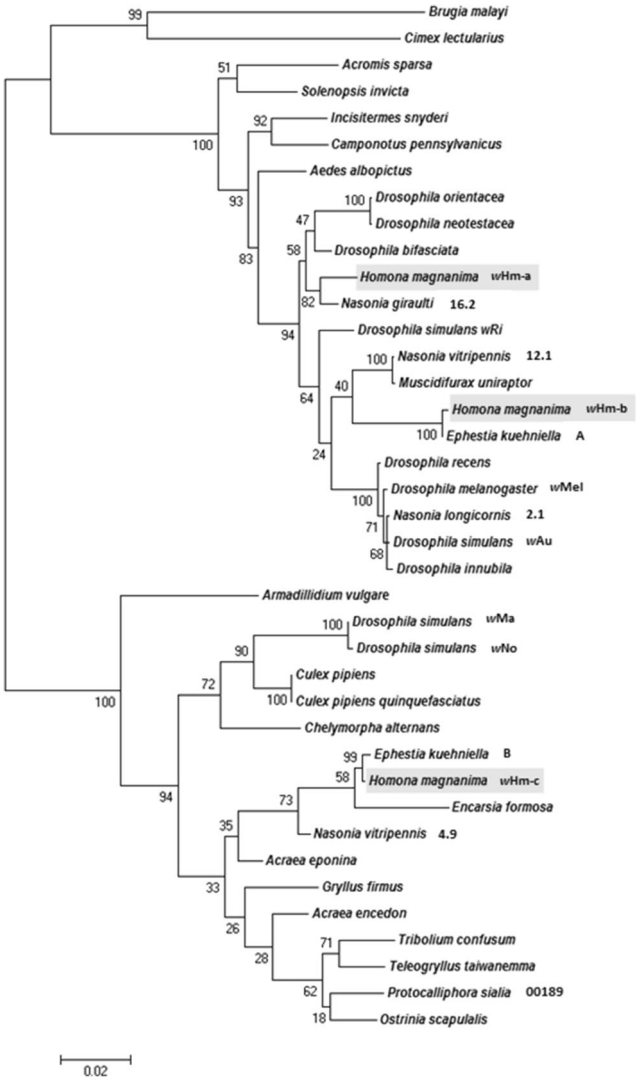

Fig. 1 Phylogeny of *Wolbachia* based on *wsp* and MLST gene sequences. Phylogenetic analysis was conducted using *H. magnanima* infected with *Wolbachia* and 36 strains from Baldo et al. [40], after exclusion of *T. deion*, using maximum likelihood method based on the Tamura-Nei model. Bootstrap values exceeding 50% are shown (1000 replicates)

The mean development time of female larvae from the W^{abc} line (19.5 ± 0.17 days, mean ± SD) was significantly shorter than those from the W^{-} line (21.5 ± 0.67 days, Steel-Dwass test, $Z = 2.79304$, $p < 0.05$), the W^{a} line (21.5 ± 0.57 days, $Z = -3.12190$, $p < 0.05$), and the W^{b} line (22.5 ± 0.62 days, $Z = -4.19888$, $p < 0.01$), but was not significantly different from those from the W^{c} line (19.4 ± 0.19 days, $Z = 0.28314$, $p = 0.9986$). The mean development time of female larvae from the W^{c} line was also significantly shorter than those from the W^{-} line ($Z = 2.76255$, $p < 0.05$), the W^{a} line ($Z = -3.07285$, $p < 0.05$), and the W^{b} line ($Z = -4.19888$, $p < 0.01$). No significant difference in the mean development time of male larvae was observed between the host lines ($p > 0.05$).

The mean female pupal weights in the W^{abc} line (94.5 ± 1.50 mg) and the W^{c} line (94.0 ± 1.9 mg) were significantly higher than those in the W^{-} line (86.1 ± 2.10 mg; Steel-Dwass test: $Z = -2.89617$, $p < 0.05$ for the W^{abc} line comparison; $Z = -3.62427$, $p < 0.01$ for the W^{c} line comparison) but were not significantly different from those in the W^{a} line (85.8 ± 2.76 mg, $Z = -0.23769$, $p = 0.9993$) and the W^{b} line (87.6 ± 3.71 mg, $Z = -0.74415$, $p = 0.9461$). No significant differences in the mean male pupal weights were observed between the host lines ($p > 0.05$).

The Effect of *Wolbachia* Infection on Host Compatibility and the CI Strength

A positive significant relationship between egg mass size (x; area, mm^2) and the number of eggs (y) per egg mass (general linear model, $p < 0.01$, $r^2 = 0.9704$, $y = 4.7974x + 2.1326$) was observed. The mean hatchability for the W^{abc} male × W^{-} female crossing pair (56.8 ± 3.0%) was significantly lower than that for W^{-} male × W^{-} female (77.5 ± 2.3%, $Z = -4.49572$, $p < 0.01$), W^{a} male × W^{-} female (76.0 ± 2.0%, $Z = -0.33743$, $p < 0.01$), and W^{c} male × W^{-} female pairs (76.7 ± 2.5%, $Z = 0.17050$, $p < 0.01$). The mean hatchability for the W^{b} male × W^{-} female crossing pair (31.9 ± 2.7%) was also significantly lower than that for the W^{-} male × W^{-} female ($Z = -9.03885$, $p < 0.01$) and W^{abc} male × W^{-} female pairs ($Z = -5.58632$, $p < 0.01$, Fig. 3).

These results indicated that *w*Hm-b was involved in CI and hence, the hatchability for mating between the W^{abc} male × W^{abc} female and W^{b} male × W^{b} female pairs was examined to confirm whether CI would be rescued. The hatchability for the

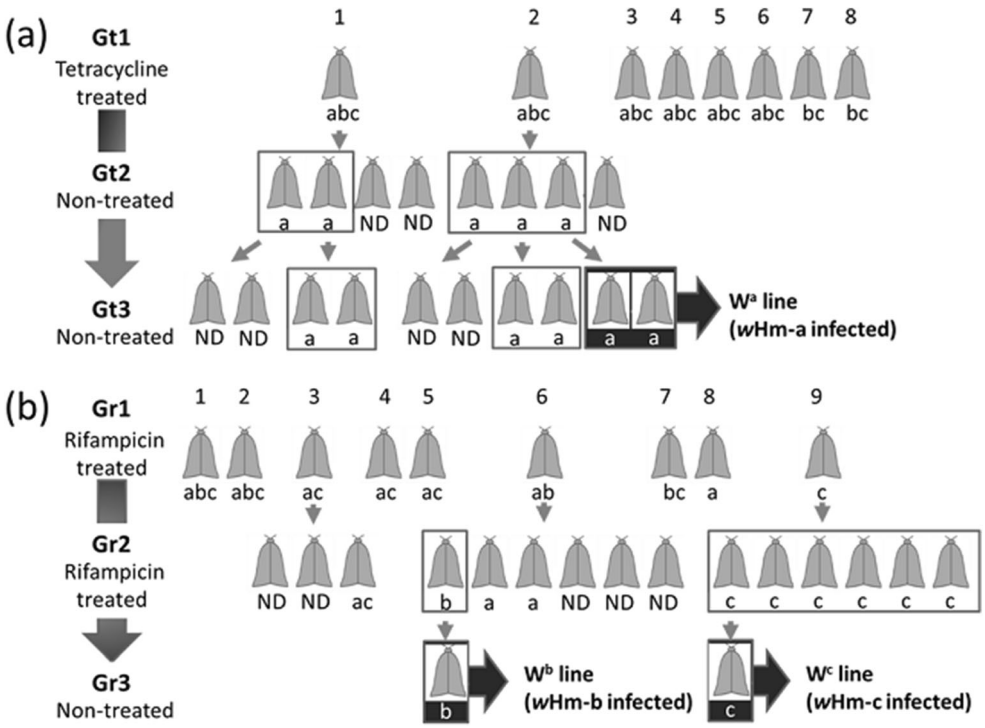

Fig. 2 Processes for segregation and the infection status of *Wolbachia*. *Wolbachia* infection was confirmed by strain-specific PCR. Three alphabets: a, b, and c in the figure indicate *w*Hm-a, -b, and -c, respectively. **a** The process of the establishment of W^a line. **b** The process of the establishment of W^b and W^c lines

W^{abc} male × W^{abc} female pair (72.7 ± 1.6%) was significantly higher than that for the W^{abc} male × W^- female pair ($Z = -4.49572$, $p < 0.01$); that for the W^b male × W^b female pair (70.5 ± 1.6%) was also significantly higher than that for the W^b male × W^- female pair ($Z = -8.62484$, $p < 0.01$). These results indicated that the CI-inducing factor was *w*Hm-b.

Wolbachia Density in Each Host Line

No significant difference in *wsp* copy number per 10 ng of DNA was apparent between males and females from each line ($p > 0.05$, data not shown). The *wsp* copy number in the W^a line was significantly higher than that in the W^b ($Z = -4.79807$, $p < 0.01$), W^c ($Z = -5.48650$, $p < 0.01$), and W^{abc} lines ($Z = -5.54392$, $p < 0.01$). *Wolbachia* density in the W^b line was also significantly higher than that in the W^c ($Z = -4.72656$, $p < 0.01$) and W^{abc} ($Z = -4.60516$, $p < 0.01$) lines, whereas no significant difference in the *wsp* copy number was observed between the W^c and W^{abc} lines ($Z = 1.79579$, $p = 0.2753$, Fig. 4).

Discussion

In the current study, we demonstrated multiple *Wolbachia* infections in *H. magnanima*, their phenotypes, and high transmissibility to the host's offspring. From the three investigated *Wolbachia* strains, *w*Hm-b differently induced CI in the host,

Table 3 Larval development time, pupal weight, and the percentage of females in host lines singly infected (W^a, W^b, and W^c), multiply infected (W^{abc}), and uninfected (W^-) with *Wolbachia*

	Larval development time (d)		Pupal weight (mg)		Percentage of females
	Male	Female	Male	Female	
W^{abc}	19.2 ± 0.33^a (53)	19.5 ± 0.17^a (54)	61.7 ± 1.08^a (48)	94.5 ± 1.50^a (54)	50.4^a
W^a	20.4 ± 0.47^a (47)	21.5 ± 0.57^b (27)	56.7 ± 1.42^a (47)	85.8 ± 2.76^{ab} (22)	36.5^a
W^b	19.6 ± 0.60^a (15)	22.5 ± 0.62^b (22)	58.9 ± 2.96^a (15)	87.6 ± 3.71^{ab} (12)	59.6^a
W^c	20.3 ± 0.46^a (30)	19.4 ± 0.19^a (39)	61.0 ± 1.12^a (30)	94.0 ± 1.89^a (39)	56.5^a
W^-	20.7 ± 0.44^a (45)	21.5 ± 0.67^b (35)	59.3 ± 1.25^a (41)	86.1 ± 2.10^b (36)	44.4^a

For larval development time and pupal weight, the data are presented as the mean ± SD. Sample sizes are given in brackets. The percentage of females was calculated as the number of female pupa divided by the total number of pupa (shown in brackets). Different letters indicate significant differences between the lines, for the larval period and pupal weight (Steel-Dwass test, $p < 0.05$). No significant differences in female ratios were observed between the different host lines (χ^2 test, $p > 0.05$)

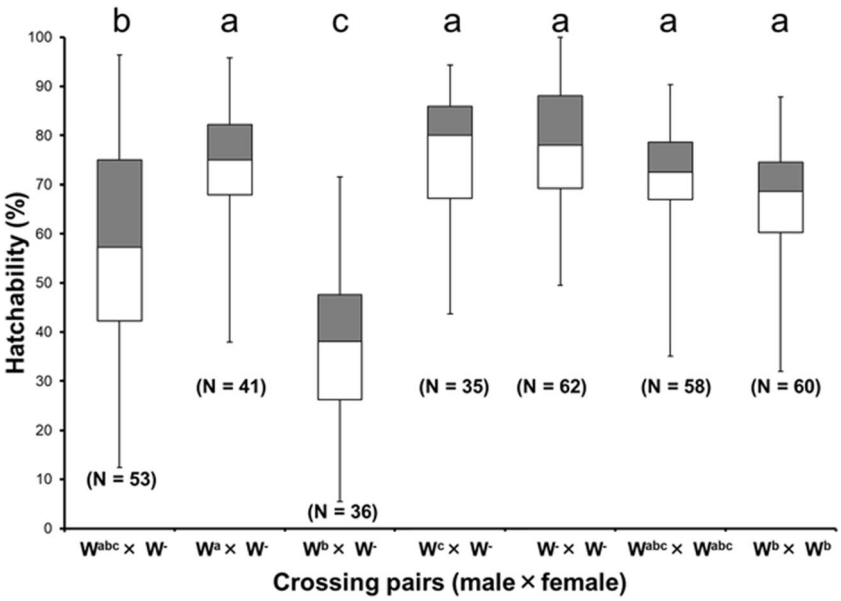

Fig. 3 Mating experiments with *H. magnanima* lines, uninfected or infected with *Wolbachia.* The center line within the box (a border between dark gray and white) represents the median. The upper and lower boundaries of the box indicate upper quartile and lower quartile, respectively. Sample size in each data point is indicated in parentheses below the plots. Different letters indicate significant differences between groups (Steel-Dwass test, $p < 0.05$)

depending on the *Wolbachia* infection status, while *w*Hm-c increased host pupal weight and shortened the larval development time. No distinct phenotype was observed for the *w*Hm-a infection.

Wolbachia is frequently found in various arthropod species, affecting the host in various ways [12]. CI is one of the most representative reproductive manipulations of *Wolbachia* [11]. Since CI-inducing *Wolbachia* kills the host's offspring when an infected male mates with uninfected females, the phenomenon is directly correlated with a rapid increase in the prevalence of CI-inducing *Wolbachia* [44]. Although the reported intensities of CI caused by *Wolbachia* or other endosymbionts are different in different studies, some authors have shown that *Wolbachia* density in the host is positively correlated with the intensity of CI [27, 28]. In the current study, the intensity of CI and *Wolbachia* density in the W^b line were significantly higher than in the W^{abc} line, suggesting that the density of *w*Hm-b indeed determined the intensity of CI.

Wolbachia may also be beneficial for its host [13–16]. For example, one *Wolbachia* strain reportedly contributes to iron metabolism in *Drosophila* [14, 15]. In the current study, *w*Hm-c infection correlated with the host development in terms of host pupal weight and development time. Previous studies of Lepidoptera indicated that the weight of female pupae is positively correlated with the oviposition period, the number of eggs laid, and the longevity of the adult [45–47]. Thus, the increase of pupal weight associated with the *w*Hm-c infection was beneficial for the survival and reproduction of the host. In terms of the host development, host death is a crucial problem for *Wolbachia.* Variety of parasitoids and insect pathogenic viruses has been isolated from *H. magnanima*, characterized, and shown to act as natural enemies [48–50]. Since virus infection is mainly restricted to the host larval stages, short larval development time associated with a *w*Hm-c infection may contribute to a lower risk of viral infection. Unlike *w*Hm-b, no differences in the phenotype caused by *w*Hm-c were apparent when singly and multiply infected lines were compared. The density of *Wolbachia* in the W^{abc} line was significantly lower than in the W^b line but did not differ from the W^c line, indicating that the phenotype of *w*Hm-c was less dependent on bacterial density than that of *w*Hm-b. In *Drosophila*, *Wolbachia* and *Spiroplasma* exhibit their own specific localization patterns or host organ specificity [29, 51], and *Spiroplasma* also restricts *Wolbachia* density in a co-infected tissue [29]. It is likely that the microhabitats of *Wolbachia* in *H. magnanima* or *Wolbachia-Wolbachia* interactions affect bacterial density and the associated phenotypes.

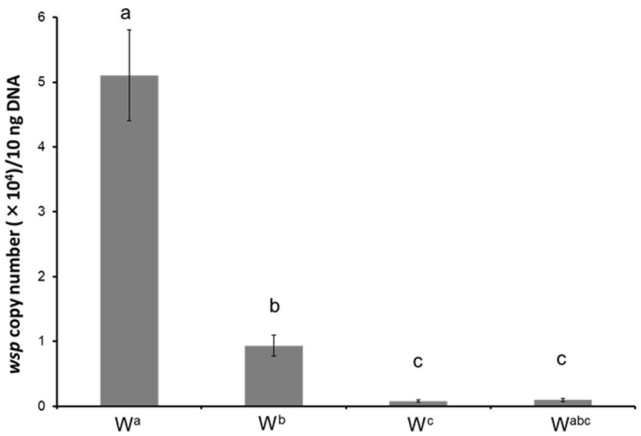

Fig. 4 *Wolbachia* density in each *Wolbachia*-infected host line. Different letters indicate significant differences between groups (Steel-Dwass test, $p < 0.05$)

Infections with some *Wolbachia* strains were shown to be associated with no cost or benefit to the host [17, 18], despite successfully invading the host population [52]. Although *w*Hm-a infection was not associated with any apparent cost or benefit to *H. magnanima* in the current study, this strain was highly transmissible to the host's offspring. Another curious observation for *w*Hm-a was that in the W^a line, *Wolbachia* density was significantly higher than in any other host line. Previous studies demonstrated that *Wolbachia* density depends on the host's genetic background [28, 53, 54]. In the current study, all host lines used in the experiments were established from the W^{abc} line, and their genetic background may be similar to the W^{abc} line. Thus, it is reasonable to assume that the difference in strain densities depends on the characteristics of each *Wolbachia* strain. Although *w*Hm-a did not show any distinct phenotype in the current study, the existence of *w*Hm-a reduced the density of *w*Hm-b during a multiple infection, which could remedy the death of offspring caused by *w*Hm-b. From that perspective, *w*Hm-a may indirectly contribute to the host's fitness. In the current study, we examined the *Wolbachia* density and its phenotype using only laboratory-maintained colonies. As the density of *Wolbachia* can be altered depending on field conditions or seasons [55], it is also worth studying the prevalence, phenotype, and density of *Wolbachia* in field populations of *H. magnanima* for understanding the dynamics of *Wolbachia*-host interactions.

Previous studies revealed that multiple *Wolbachia* infections are highly prevalent in several insects in nature [21–25]. Interestingly, these multiply infected hosts often harbor one or more CI-inducing *Wolbachia* strains [21, 24, 27]. For example, *Eurema hecabe* harbors CI-inducing *Wolbachia* (*w*CI) and feminization-inducing *Wolbachia* (*w*Fem), and *w*Fem is only detected together with *w*CI [24]. Since it is well-known that CI-inducing *Wolbachia* strains are maintained and spread rapidly in wild host populations [44], CI-inducing *Wolbachia* is thought to a play crucial role in the establishment of a multiple infection. Considering the induction of CI by *w*Hm-b and data from studies mentioned above, it is reasonable to propose that the co-infection of *w*Hm-a and *w*Hm-c with *w*Hm-b is highly prevalent. For the host, such multiple infections might be beneficial not only in terms of a decreased offspring mortality compared with a single *w*Hm-b infection, but also in terms of reproductive and developmental benefits associated with *w*Hm-c infection. The three strains of *Wolbachia* cannot co-infect their host with such high density as during a single infection, although they continue to invade the host population on account of their high transmissibility. Hence, infections with multiple endosymbionts are thought to constitute a compromise between selfish microorganisms and their host.

In the current study, we characterized three *Wolbachia* strains isolated from *H. magnanima* found in Tokyo. We also demonstrated that none of the three strains caused sex ratio distortion in *H. magnanima*. Interestingly, a presumed RNA virus and *Spiroplasma* have both been identified as the male-killing agents in Ibaraki and Shizuoka *H. magnanima* populations, respectively [10, 30, 31]. Although *Wolbachia* is also well-known as a sex ratio-distorting factor [56], *w*Hm-a, *w*Hm-b, and *w*Hm-c do not appear to be involved in host sex abnormalities in Tokyo populations of *H. magnanima*. Previous studies indicated that the phenotypes caused by *Wolbachia* strains depend on the genetic background of the host [57, 58]. Hence, further studies of the phenotypes and prevalence of *Wolbachia* in various field populations may contribute to the understanding of *Wolbachia* strain dynamics in fields. In addition, the presented *H. magnanima*-symbiont system will likely provide useful insights to facilitate the understanding of the host-endosymbiont-endosymbiont interactions.

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