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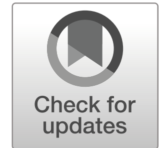
HOST MICROBE INTERACTIONS



Closely Related Male-Killing and Nonmale-Killing *Wolbachia* Strains in the Oriental Tea Tortrix *Homona magnanima*

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Abstract

Wolbachia are inherited intracellular bacteria that cause male-specific death in some arthropods, called male-killing. To date, three *Wolbachia* strains have been identified in the oriental tea tortrix *Homona magnanima* (Tortricidae, Lepidoptera); however, none of these caused male-killing in the Japanese population. Here, we describe a male-killing *Wolbachia* strain in Taiwanese *H. magnanima*. From field-collected *H. magnanima*, two female-biased host lines were established, and antibiotic treatments revealed *Wolbachia* (*w*Hm-t) as the causative agent of male-killing. The *wsp* and MLST genes in *w*Hm-t are identical to corresponding genes in the nonmale-killing strain *w*Hm-c from the Japanese population, implying a close relationship of the two strains. Crossing the Japanese and Taiwanese *H. magnanima* revealed that *Wolbachia* genotype rather than the host genetic background was responsible for the presence of the male-killing phenotype. Quantitative PCR analyses revealed that the density of *w*Hm-t was higher than that of other *Wolbachia* strains in *H. magnanima*, including *w*Hm-c. The densities of *w*Hm-t were also heterogeneous between host lines. Notably, *w*Hm-t in the low-density and high-density lines carried identical *wsp* and MLST genes but had distinct lethal patterns. Furthermore, over 90% of field-collected lines of *H. magnanima* in Taiwan were infected with *w*Hm-t, although not all host lines harboring *w*Hm-t showed male-killing. The host lines that showed male-killing harbored a high density of *Wolbachia* compared to the host lines that did not show male-killing. Thus, the differences in the phenotypes appear to be dependent on biological and genetic characteristics of closely related *Wolbachia* strains.

Keywords Sex ratio distortion · Insect pest · Bacterial density · Symbiont

Introduction

Various microbes, including viruses, fungi, and bacteria, are symbiont of insects [1–3]. Some of these microbes are inherited from mothers to offspring via transovarian transmission [4]. *Wolbachia pipientis*, an alpha-proteobacteria hereafter referred to as *Wolbachia*, is one such intracellular bacteria found in various arthropods [5, 6]. Since its survival mostly relies on female hosts, *Wolbachia* is considered to have unique adaptive strategies to manipulate the host, especially host reproduction [5, 7]. These strategies include male-killing, which directly produces female-biased sex ratios to promote *Wolbachia* inheritance [5, 8]. Other intracellular bacteria, microsporidia, and viruses have also been reported to induce similar male-killing at early embryonic stages (early male-killing) or at larval and pupal stages (late male-killing) [5, 8–10].

Diverse species of intracellular microbes are known to induce male-killing, and the phenomenon has been investigated for many decades in terms of ecological and evolutionary significance and molecular mechanism [5, 11]. The mechanism of male-killing has attracted a great deal of attention and has been well studied in two intracellular bacteria, *Spiroplasma* and *Wolbachia*. Both bacteria cause defects in dosage compensation and sex determination cascade in host insects [12–14]. Recently, a gene encoding the male-killing toxin was identified in *Spiroplasma poulsonii* [12]. A candidate gene has also been postulated for *Wolbachia* male-killing [15]. It has been reported that the density of *Wolbachia* in the host line affects the strength

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of both the male-killing and cytoplasmic incompatibility phenotypes [16–19]. Furthermore, identical *Wolbachia* strains have been observed to exhibit different phenotypes after interspecific transfer to a new host [20–22] or intraspecific introgression to a new host population [23], indicating that host genetic background interacts with the bacteria to generate the different outcomes observed in *Wolbachia* hosts.

Homona magnanima (Tortricidae, Lepidoptera) is a serious pest of tea plants in East Asia. In the Japanese populations of the insect, *Spiroplasma* [24] and a presumed RNA virus [9, 25, 26] were shown to kill males at the embryonic and larval stages, respectively. In addition, three *Wolbachia* strains have also been identified and characterized in the Japanese host population—*w*Hm-a had no effect on the host, *w*Hm-b caused cytoplasmic incompatibility in the host, and *w*Hm-c compensated host reproduction; however, none of them distorted the sex ratio [19]. In this study, we identified a male-killing *Wolbachia* strain from *H. magnanima* in Taiwan. To characterize this male-killing strain, phylogenetic analyses were conducted and the effects of the strain on host development were investigated. A series of crossing experiments were conducted using the Taiwanese and Japanese *H. magnanima* lines to determine whether the male-killing phenotype was dependent on host genetic background. The effects of *Wolbachia* density on host mortality or fecundity were also investigated, along with the prevalence and density of *Wolbachia* in Taiwanese field populations.

Materials and Methods

Insects and Establishment of *H. magnanima* Lines

In 2015, 72 egg-masses of *H. magnanima* were collected from tea plantations at Tea Research and Extension Station (Taoyuan City, Taiwan) and imported with permission from the Ministry of Agriculture, Forestry and Fisheries (No. 27 - Yokohama Shokubou 891). Each field-collected egg-mass was placed on a plastic box (23 × 16 × 8 cm) containing artificial diet INSECTA LF (Nosan, Yokohama, Japan). Larvae hatching from each egg-mass were reared collectively in the box until adult eclosion. The adults that emerged were mated, and offspring maintained in the laboratory as described previously by Tsugeno et al. [24] and Arai et al. [19].

From the 72 egg-masses, three host lines were successfully established and maintained—two female-biased lines named W^{T12} and W^{T24} along with one line with normal sex ratio (NSR). One female adult from each line was subjected to microbe detection assays as mentioned below. Every generation, *Wolbachia* infection was checked using three to five adult females. The female-biased lines were mated with males from the NSR line and maintained in the laboratory as mentioned above. Three lines of Japanese *H. magnanima* W^a, W^b, and W^c lines were also maintained in the same manner. These lines are singly infected with the *Wolbachia* strains *w*Hm-a, *w*Hm-b, and *w*Hm-c [19].

In 2017, a survey of the prevalence of *Wolbachia*, *Spiroplasma*, and RNA virus was completed using 139 egg-masses and 82 larvae of *H. magnanima* collected and imported from the same Taiwanese tea field with permission from the Ministry of Agriculture, Forestry and Fisheries (No. 29 - Yokohama Shokubou 1326). Egg-masses were reared as mentioned above until adult eclosion. Field-collected larvae were reared individually on INSECTA LF in a 1/2-oz cup until adult eclosion. After eclosion, the sex of the individual was confirmed by morphology, and the sex ratio of adults from each egg-mass was calculated. These adults were subjected to microbe detection assays as described below.

Detection of Microbes and Molecular Typing of *Wolbachia*

Total DNA was extracted from individual *H. magnanima* abdomen, as described in Arai et al. [19]. Total RNA was extracted from the abdomen, using ISOGEN (Nippon Gene, Tokyo, Japan) and following the manufacturer's protocol. Briefly, samples were individually placed in a new plastic tube and briefly homogenized in 1000 μL ISOGEN using a sterilized pestle, mixed with 200 μL chloroform, and centrifuged. The resulting supernatant (500 μL) was transferred to a new tube and precipitated with 500 μL isopropanol. The precipitated RNA was then washed with 1000 μL 70% ethanol [v/v], dissolved in 50 μL distilled water, treated with 2 μL DNase I (Nippon Gene), re-extracted with ISOGEN as described in the manufacturer's protocol, and stored at − 80 °C.

To detect *Wolbachia* and *Spiroplasma*, DNA extracted from adults were PCR-amplified as previously reported [19], using TaKaRa Ex Taq or EmeraldAmp MAX PCR Master Mix (TaKaRa Bio, Shiga, Japan) and primer combinations listed in Table S1. DNA extracted from W^a [19] and IN12 [24] lines were used as positive controls for *Wolbachia* and *Spiroplasma*, respectively. To detect male-killing virus, RNA samples were diluted with Milli-Q water to 50–100 ng/μL, reverse transcribed with AMV Reverse Transcriptase XL (TaKaRa), and amplified using primers C3-F and C3-R against MK 1241 [25]. RNA extracted from the LMK line [25] was used as the positive control for male-killing RNA virus. β-Actin of the host insect was amplified as control [19, 24]. PCR products were separated electrophoretically on 1.5% w/v agarose, stained with ethidium bromide, and visualized on a transilluminator. Infection with each microbe was checked once per sample.

For molecular typing of *Wolbachia* in W^{T12} and W^{T24} lines, *wsp* genes and *Wolbachia* multilocus sequence typing (MLST) genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) were

amplified using primer sets designed by Baldo et al. [27]. The PCR products of *wsp* and MLST genes from W^{T12} and W^{T24} lines were cloned and sequenced as described previously [19].

Antibiotic Treatments

Each egg-mass from W^{T12}, W^{T24}, and NSR lines was reared for one generation on the artificial diet SilkMate 2S (Nosan) supplemented with 0.1% w/w tetracycline, with each line treatment in three replicates. The sex ratio at treated generation (G_0) was calculated based on the number of males and females in adult stage, and *Wolbachia* infection was confirmed by PCR amplification of *wsp* gene using one individual from each treatment. G_0 females from each line were mated with NSR males, and G_1 offspring were reared on SilkMate 2S without tetracycline to assess *Wolbachia* infection and the sex ratio.

Crossing Between Taiwanese and Japanese Host Lines

Five females from W^{T12} and W^{T24} lines were mated with five males from the Japanese W^c line, and similarly, five W^c females were mated with five NSR males from Taiwan. Crossing was replicated five times each, and five F_1 egg-masses were selected from each cross. Hatched larvae were individually reared on SilkMate 2S in 1/2-oz cups. After eclosion, the sex ratio was calculated. F_1 females were also backcrossed with W^c or NSR males, respectively, over five generations, and the sex ratio in the offspring was determined.

Determination of *Wolbachia* Density in *Wolbachia*-Infected Host Lines

To estimate *Wolbachia* density, five or six newly emerged females from each *Wolbachia*-infected line (W^{T12}, W^{T24}, W^a, W^b, and W^c) were randomly sampled and individually subjected to DNA extraction as mentioned above. Each DNA template was diluted to 10 ng/μL. A series of qPCR assays were performed using StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA, USA). *Wolbachia wsp* gene copy number in each sample was quantified using a reaction mixture containing 10 ng DNA, 30 μM primers *w*Hm-uni_qpcrF and *w*Hm-uni_qpcrR (Table S1), and 5 μL FastStart Universal SYBR Green master mix (Roche, Basel, Switzerland) [19]. A standard curve was generated using 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 ng plasmid DNA harboring a *wsp* fragment, and the number of *wsp* copies in the plasmid DNA was estimated from the molecular weight of the plasmid and *wsp* sequence. Finally, the total amount of *Wolbachia wsp* copies per 10 ng DNA was calculated.

Effects of *Wolbachia* on Host Survival and Fitness

To survey the effects of *Wolbachia* on host development, egg-masses from W^{T12}, W^{T24}, and NSR lines were obtained as mentioned previously [19]. Survival rate throughout the embryonic stage was quantified as hatchability (number of hatched neonate/number of eggs). The number of eggs per egg-mass was estimated based on a regression line between the area of egg-masses and the number of eggs derived from 50 egg-masses from the NSR line. We note that in *H. magnanima*, matured embryos (pharate) form black head capsules after 4 days post oviposition (dpo), indicating successful development to late embryonic stage (Fig. S1). Healthy pharate hatch within 1 day, while damaged pharate larvae will not. To verify the lethal stage during embryogenesis of *H. magnanima*, both survival rate until late embryonic stage (number of pharate larvae/number of eggs) and survival rate of pharate larvae (number of hatched neonate/number of pharate larvae) were calculated. Hatched neonates and remaining pharate larvae were counted under a microscope.

To determine sex in pharate and neonates, the W chromosome was stained using lactic acetic orcein as previously reported [28]. Briefly, pharates and neonates were dissected on slide glass with forceps, followed by fixation with methanol and acetic acid and staining with lactic acetic orcein. Finally, mortality in female and male pharate larvae was calculated as the number of remaining male or female pharate larvae 7 days post oviposition per total number of pharate larvae in the egg-mass.

Larvae from the W^{T12}, W^{T24}, and NSR lines were individually reared in 1/2-oz cups, using SilkMate 2S as described above. The larval development time and pupal weight (within 1 day post pupation) was measured as mentioned previously [19]. The longevity and number of egg-mass of adult females were measured in a 120-cc plastic cup with cotton supplied with water. Ten adult females per line were individually subjected to the experiment.

Transmission of *Wolbachia* Strains in Female-Biased Lines

One female each from the W^{T12} and W^{T24} lines was mated with males from the NSR line. After oviposition and hatching, 50 neonate larvae were chosen randomly and reared in 1/2-oz cups until adult eclosion, using the artificial diet INSECTA LF. *Wolbachia* infection was tested in F_1 adults by PCR assay as described above. Transmission rate was calculated as the number of infected F_1 adults divided by the total number of F_1 adults tested.

H. magnanima Sex Ratio in the Field and Prevalence and Density of *Wolbachia*

Larvae ($n = 82$) and egg-masses ($n = 96$) collected from the field in 2017 were reared until adult eclosion as outlined above. Total DNA and RNA were separately extracted from abdomen of the 82 samples collected at the larval stage, after dividing the abdomen into two pieces using a pair of forceps, and the template was used to detect *Wolbachia*, *Spiroplasma*, and RNA virus simultaneously. The *Wolbachia*-infected samples were then genotyped using diagnostic primer sets for *w*Hm-a, *w*Hm-b, and *w*Hm-c [19] (Table S1). Three newly emerged females from each egg-mass were individually subjected to DNA extraction, detection of *Wolbachia*, and determination of *Wolbachia* density as mentioned above.

Data Analysis and Statistics

A χ^2 test was used to assess bias in the sex ratio of the host lines. The correlation between egg-mass size and number of eggs per egg-mass was investigated by regression analysis using a general linear model. Hatchability, larval developmental time, pupal weight, and *Wolbachia* density in each host line were analyzed by Steel–Dwass test. *Wolbachia* densities in male-killing egg-masses and nonmale-killing egg-masses were compared using Wilcoxon test. All statistical analyses were performed using JMP v9 (SAS, Cary, NC, USA). The MLST and *wsp* sequences of *w*Hm-t were deposited in GenBank under accession numbers LC427375 to LC427380. The sequences were aligned with known *Wolbachia* sequences obtained from the *Wolbachia* MLST database [27] (Table S2) and *w*Hm-a, *w*Hm-b, and *w*Hm-c (GenBank accession numbers LC363921 to LC363938) using ClustalW [29]. A phylogenetic tree was estimated using MEGA6 [30] by maximum likelihood with bootstrap resampling of 1000 replicates.

Results

Wolbachia Strain *w*Hm-t Is a Causative Agent for the Female-Biased Sex Ratio in Taiwanese *H. magnanima*

Two female-biased lines (W^{T12} and W^{T24}) were established from 72 egg-masses collected in 2015, alongside one line with a NSR (sex ratios 98.6 ± 0.5% female (χ^2 test, $\chi^2 = 886.5$, $p < 0.001$), 99.6 ± 0.2% ($\chi^2 = 927.1$, $p < 0.001$), and 50.4 ± 1.2% ($\chi^2 = 1.837$, $p > 0.05$), respectively (Fig. 1). In the two female-biased lines, *Wolbachia* was detected by diagnostic PCR, and *Spiroplasma* or the male-killing virus was not detected. NSR adults were free of intracellular bacteria and male-killing virus (Fig. 2). To survey multiple *Wolbachia* infections, 16 *wsp* fragments cloned from DNA derived from an adult female from the W^{T12} and W^{T24} lines were sequenced and were found to be identical. Furthermore, sequences of *wsp* and MLST genes were completely identical between *w*Hm-c from Japanese W^c line (normal sex ratio) and *Wolbachia* derived from both W^{T12} and W^{T24} lines (female-biased sex ratio) (Fig. 3). From now on, the Taiwanese *w*Hm-c type *Wolbachia* will be referred as *w*Hm-t.

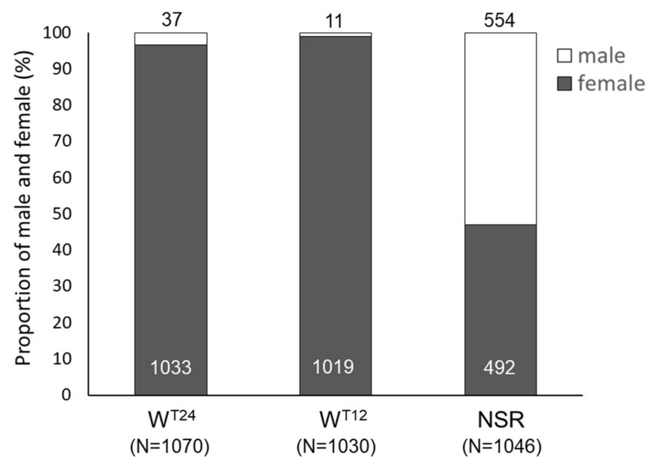

Fig. 1 Proportion of males and females in W^{T24}, W^{T12}, and NSR lines of *Homona magnanima*. The total number of individuals until the 25th generation in each host line is shown on the bar

To investigate whether *w*Hm-t is responsible for the female-biased sex ratio in Taiwanese *H. magnanima* lines, *w*Hm-t was eliminated from these lines by antibiotic treatment. In tetracycline-treated G_0, the sex ratio was still female-biased in W^{T12} and W^{T24} (Fig. 4). The sex ratio at G_1 was not female-biased in any antibiotic-treated lines, implying that a tetracycline-sensitive microbe caused the sex ratio distortion in Taiwanese *H. magnanima*. Furthermore, *Wolbachia* was absent from tetracycline-treated G_0 and G_1 adults of W^{T12} and W^{T24} lines (Fig. 4). Taken together, these results confirm that *w*Hm-t infection distorts the sex ratio in Taiwanese *H. magnanima*.

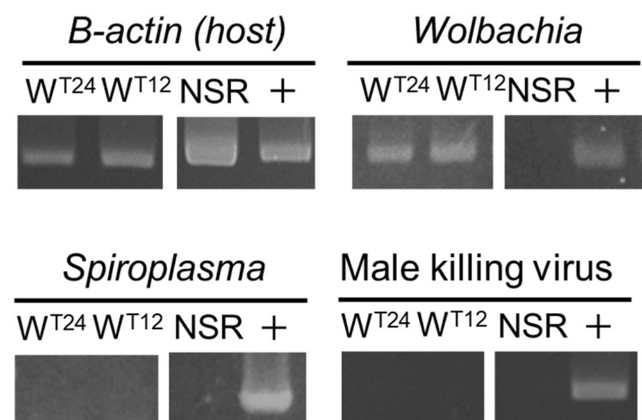

Fig. 2 Infection status of microbes in W^{T24}, W^{T12}, and NSR lines of *Homona magnanima*. Plus indicates positive control for each microbe

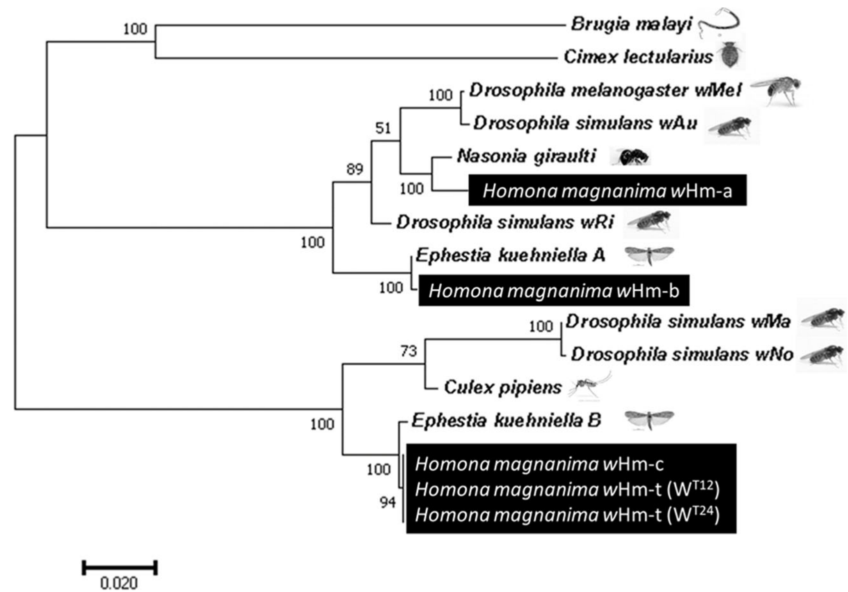

Fig. 3 Phylogenetic analysis of *wsp* and MLST genes in *Wolbachia* infecting *Homona magnanima*. The *Wolbachia* strains quoted are listed in Table S2. The tree was constructed by maximum likelihood based on the Tamura–Nei model. Bootstrap values exceeding 50% in 1000 replicates are indicated

Effect of Host Genetic Background on the Sex Ratio in Japanese and Taiwanese *H. magnanima* Infected with Closely Related *Wolbachia* Strains

Taiwanese (W^{T12}, W^{T24}, and NSR) and Japanese (W^c) *H. magnanima* adults were successfully mated, generating viable, fertile offspring. The sex ratio was near 1:1 (48.5% females, $n = 297$) in the F_1 generation of W^c (female) × NSR (male), but female-biased in the F_1 generation of $W^{T12} \times W^c$ (100% females, $n = 205$) and F_1 generation of $W^{T24} \times W^c$ (100% females, $n = 186$). After backcrossing, the sex ratio was normal in the offspring of F_1 (W^c × NSR) × NSR (back cross: BC1) (48.6% females, $n = 251$), but female-biased in the offspring of F_1 ($W^{T12} \times W^c$) × W^c (100% females, $n = 140$) and F_1 ($W^{T24} \times W^c$) × W^c (100% females, $n = 152$). *H. magnanima* females infected with *w*Hm-t produced only females over five generations of backcrossing with W^c males (Fig. S2). On the other hand, *H. magnanima* infected with *w*Hm-c showed normal sex ratio over the same generations of backcrossing with NSR males (Fig. S2). These results indicate that the host genotype is not a key driver of sex ratio distortion.

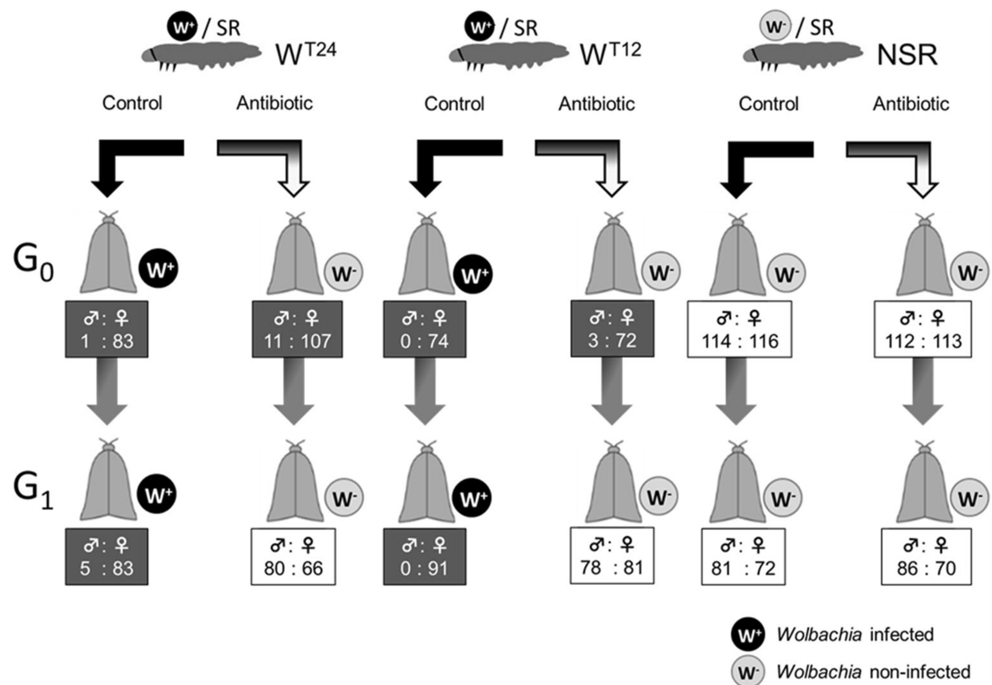

Fig. 4 Effect of tetracycline on *Wolbachia* infection and host sex ratio at G_0 and G_1. *Wolbachia* infection was tested by diagnostic PCR. Black circles marked W^+ indicate lines infected with *Wolbachia*, while gray circles marked W^- are uninfected. Black and white boxes indicate lines with distorted and normal sex ratio, respectively

Wolbachia Density Within Strains, Effects for Host Survival and Fitness, and Transmission of *w*Hm-T Within Taiwanese Host

Wolbachia density was significantly higher in W^{T12} than in any other lines tested, including W^{T24} (Steel–Dwass test, $Z = -4.128$, $p < 0.001$), W^a ($Z = 3.594$, $p < 0.001$), W^b ($Z = 3.923$, $p < 0.001$), and W^c ($Z = 3.923$, $p < 0.001$) (Fig. 5). *Wolbachia* density was also significantly higher in W^{T24} than in W^b ($Z = 3.923$, $p < 0.001$) and W^c ($Z = 3.923$, $p < 0.001$), but was comparable between W^{T24} and W^a ($Z = 0.692$, $p = 0.958$).

The area of an egg-mass (x, mm^2) was significantly correlated with the number of eggs in an egg-mass (y) (general linear model, $y = 2.9273x + 3.9248$ with $R^2 = 0.8556$ and $p < 0.01$). This regression line was then used to estimate survival rate and hatchability. The mean hatchability was significantly higher in NSR than in W^{T12} (Steel–Dwass test, $Z = -5.287$, $p < 0.01$, Table 1) and in W^{T24} ($Z = -3.146$, $p < 0.01$), and higher in W^{T24} than in W^{T12} ($Z = 4.273$, $p < 0.01$), indicating that *w*Hm-t reduced the viability of embryos. The survival rate until late embryonic stage was significantly lower in W^{T12} than in NSR ($Z = -4.249$, $p < 0.01$) and W^{T24} ($Z = 4.610$, $p < 0.01$), but did not differ between NSR and W^{T24} ($Z = 0.929$, $p = 0.62$). The survival rate of pharate larvae was significantly higher in NSR than in W^{T12} ($Z = -6.057$, $p < 0.01$) and in W^{T24} ($Z = -5.047$, $p < 0.01$) and significantly higher in W^{T12} than in W^{T24} ($Z = 5.002$, $p < 0.01$). These results indicate that *w*Hm-t caused male-killing at different stages in the W^{T12} and W^{T24} lines.

Pharate larvae were female-biased in W^{T12} (χ^2 test, $\chi^2 = 23.6$, $p < 0.001$), but not in NSR ($\chi^2 = 0.011$, $p > 0.05$) and W^{T24} ($\chi^2 = 0.137$, $p > 0.05$) based on acetic lactic orcein staining (Fig. S3). On the other hand, neonates were female-biased both in W^{T12} ($\chi^2 = 69.1$, $p < 0.001$) and W^{T24} ($\chi^2 = 45.0$, $p < 0.001$) but not in NSR ($\chi^2 = 0.011$, $p > 0.05$, Fig. S4). Furthermore, mortality in female pharate larvae was significantly higher in W^{T12} than in W^{T24} (Steel–Dwass test, $Z = -4.594$, $p < 0.01$) and NSR ($Z = -4.635$, $p < 0.01$, Table S4). These results indicate that *w*Hm-t caused male-killing in W^{T12} during early embryogenesis and in W^{T24} lines at late embryogenesis and also caused defects in females in the W^{T12} line.

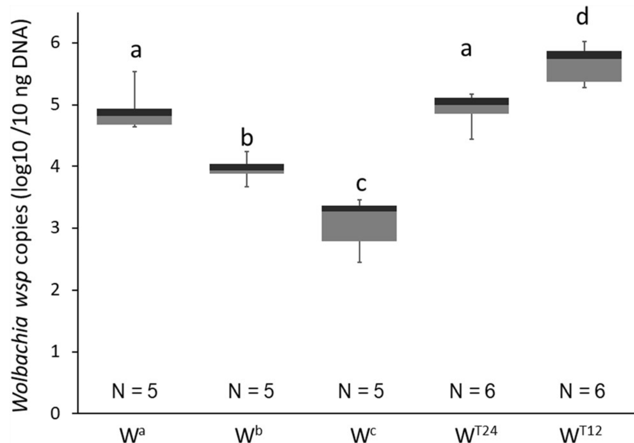

Fig. 5 *Wolbachia* density in infected host lines. Different letters indicate statistically significant difference ($p < 0.05$) by Steel–Dwass test

Mean mortality of larvae and pupae were significantly higher in W^{T24} than in W^{T12} (Steel–Dwass test, $Z = -2.653$, $p < 0.05$) and NSR ($Z = 2.647$, $p < 0.05$) (Table S5), but no significance was detected between W^{T12} and NSR ($Z = 2.207$, $p > 0.05$). Mortality at the first and second instar was higher in W^{T24} than in NSR (first instar: $Z = -2.653$, $p < 0.05$; second instar: $Z = -2.389$, $p < 0.05$) (Table S5).

There were no significant differences in larval development time, adult longevity, pupal weight, number of egg-mass per female, and egg-mass size between lines with or without *Wolbachia* (Table 2). Transmission rates of *w*Hm-t to the next generation were 97.9% (49/50) in W^{T12} and 100% (50/50) in W^{T24}, respectively. These results suggested that *w*Hm-t does not compensate host development and reproduction but can be successfully inherited to host offspring with high transmissibility.

Wolbachia Prevalence and Correlation Between Male-Killing Phenotype and *Wolbachia* Density

A total of 82 larvae collected from the field in 2017 were reared until adult eclosion, producing 57 female and 25 male adults. Larvae hatched from 96 of the 139 egg-masses collected in field were also successfully reared until adult eclosion. Of these, 46 egg-masses were female-biased, while the sex ratio was normal in the other 50 egg-masses.

In adults reared from field-collected larvae, the prevalence of *Wolbachia*, *Spiroplasma*, and male-killing virus was 89.0% ($n = 73/82$), 2.4% ($n = 2/82$), and 14.6% ($n = 12/82$), respectively (Table S3). For *Wolbachia*, *w*Hm-a and *w*Hm-b were not detected (based on strain-specific PCR), but *w*Hm-c was detected at the rate of 89.0% ($n = 73/82$). Similarly, the prevalence of *Wolbachia* in adults reared from field-collected egg-masses was 90.6% ($n = 87/96$).

Although *Wolbachia* prevalence was around 90% in the field, only 46 of 96 egg-masses were female-biased. Since 9 of the 96 egg-masses were not infected with *Wolbachia* as determined by diagnostic PCR, *Wolbachia* densities were quantified by qPCR in newly ecloded adult females derived from the remaining 87 egg-masses. Figure 6 shows the relationship between the proportion of female and *Wolbachia* density. The mean *Wolbachia* density (*wsp* copies/10 ng total DNA) was significantly higher in the female-biased lines ($402{,}955 \pm 50{,}288$, $n = 46$) than that in the NSR lines (511 ± 64, $n = 41$) (Wilcoxon test, $\chi^2 = 194.4$, degree of freedom = 1, $p < 0.001$). Collectively, the data suggest that not only

Table 1 Survival (mean ± SD) of W^{T12}, W^{T24}, and NSR lines of *Homona magnanima* at each developmental stage

Line	Hatchability (%)	Survival rate until late embryonic stage (%)	Survival rate of pharate larvae (%)
W^{T24}	46.5 ± 3.6c (39)	78.8 ± 2.7a (49)	54.7 ± 1.6b (39)
W^{T12}	21.5 ± 2.9b (22)	53.3 ± 3.9b (26)	30.9 ± 2.0c (22)
NSR	69.3 ± 4.8a (29)	75.7 ± 2.2a (76)	78.1 ± 2.7a (29)

Means followed by the same letter are comparable by Steel–Dwass test, $p > 0.05$. Numbers in parenthesis are sample size; the number of egg-mass

Wolbachia infection but also its density is correlated to the occurrence of male-killing.

Discussion

In this study, we observed that a *Wolbachia* strain, *w*Hm-t, causes male-killing in Taiwanese *H. magnanima*. Remarkably, the male-killing strain *w*Hm-t and the nonmale-killing strain *w*Hm-c, isolated from Japanese *H. magnanima* [19], are identical based on phylogenetic analysis of *wsp* and MLST genes. Host-switching experiments confirmed that host genotype did not modify *Wolbachia* phenotypes. However, lethality patterns and mortality in *H. magnanima* differed in response to density of *w*Hm-t—mortality was higher in the host lines with high *w*Hm-t titers (W^{T12}), than in the host line with low *w*Hm-t titers (W^{T24}) (Table 1). Furthermore, pharate mortality in females of W^{T12} line was higher than that in W^{T24} line. Lastly, *w*Hm-t prevalence in the field exceeds 90%, and only host lines infected at high titers undergo male-killing.

The phenotypes of *Wolbachia* are thought to be outcomes of interactions between host and *Wolbachia*. In this study, we found that *w*Hm-t and *w*Hm-c are of the same MLST genotype [27], but the corresponding phenotypes are completely different. The strain *w*Hm-c was previously shown to have no effect on the sex ratio in *H. magnanima*, to shorten larval development time, and to increase pupal weight [19], whereas *w*Hm-t induces female-biased sex ratio but does not affect larval and pupal development (Table 2). *Wolbachia* phenotypes are well known to be modified by host genetic factors. For instance, the *Wolbachia* strain *w*Bol1 caused male-killing in *Hypolimnas bolina* in Polynesia [31], but not in Southeast Asia [23]. Crossing experiments between Polynesian and Southeast Asian populations revealed that the host genetic background influenced the occurrence of male-killing caused by *w*Bol1 in *H. bolina* [23] and was dependent on a single region of the butterfly chromosome 25 [32, 33]. In *H. magnanima*, Japanese and Taiwanese populations were confirmed to be the same biological species based on morphology (U Jinbo, personal communication), and the offspring of the two populations were fertile. The results of crossing experiments showed that *w*Hm-t distorted the sex ratio in the Taiwanese population and in hybrids between Taiwanese and Japanese populations, whereas *w*Hm-c did not distort the sex ratio in the Japanese population nor in the hybrids. This result indicates that *w*Hm-t phenotype did not differ between geographically distinct host populations. Likewise, W^{T12} and W^{T24} lines showed different lethality patterns and *w*Hm-t titers; however, these two host lines appear to have identical genetic backgrounds as they were collected from the same tea field and also mated to the NSR line for over 25 generations in the laboratory. Thus, the male-killing phenotype in *w*Hm-t is due exclusively to its own biological and genetic factors, independent of host genetic background.

Other than the variations in *wsp* and MLST genes, the differences in phenotypes between *w*Hm-c- and *w*Hm-t-related strains are likely due to variations in *Wolbachia* genes that are related to density and/or male-killing. It is well known that closely related bacteria strains may exhibit different pathogenicity due to mutations in specific genomic regions [12, 34, 35]. For example, the pathogenic bacterium *Salmonella typhimurium* harbors various virulence factors in its genome, and mutations in these genes alter pathogenicity and antibiotic resistance [35]. Since *wsp* and MLST genes only represent a tiny fraction of the *Wolbachia* genome, it is necessary to compare genomes of those *Wolbachia* to identify the underlying genomic regions for male-killing and density (as seen in Ellegaard et al. [36] and LePage et al. [37]).

Table 2 Host development and fecundity, with data as mean ± SD

Line	Larval development time (days)	Pupal weight (mg)	Adult longevity (day)	Number of egg-masses per female	Egg-mass size (mm^2)
W^{T24}	20.1 ± 0.1a (186)	139.5 ± 1.4a (206)	8.70 ± 0.4a (10)	3.30 ± 0.3a (10)	60.4 ± 3.4a (26)
W^{T12}	20.3 ± 0.1a (147)	139.9 ± 1.6a (150)	9.50 ± 0.4a (10)	3.30 ± 0.3a (10)	74.7 ± 4.6a (47)
NSR	19.7 ± 0.2a (79)	139.7 ± 2.9a (87)	8.90 ± 0.4a (10)	3.20 ± 0.3a (10)	64.4 ± 2.8a (73)

Means followed by the same letter are comparable by Steel–Dwass test, $p > 0.05$. Numbers in parenthesis show sample size

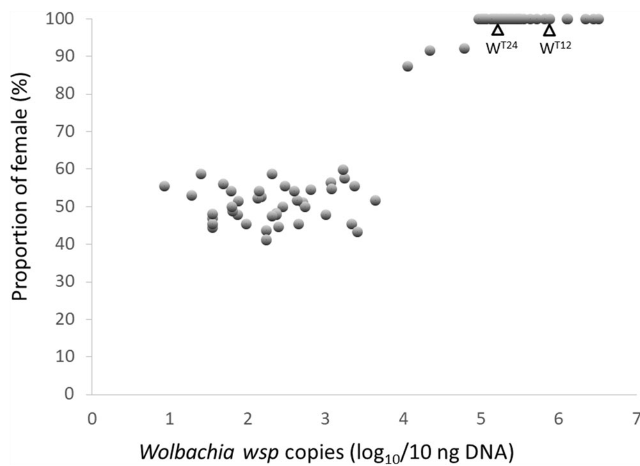

Fig. 6 Relationship between proportion of females and *Wolbachia* density in field-collected *Homona magnanima*. The *Wolbachia* density in established male-killing host lines W^{T12} and W^{T24} are marked with arrows. *Wolbachia* density (*x*-axis) is plotted in Fig. 1 against the proportion of females (*y*-axis, %)

Wolbachia density was previously demonstrated to be crucial in phenotype development [16–19]. For example, in *Drosophila melanogaster,* a *Wolbachia* strain *w*Melpop proliferates to higher densities and exhibit higher virulence than its related strain *w*Mel [38, 39]. In *H. bolina*, a series of antibiotic treatments can artificially modify the timing of male-killing caused by *Wolbachia*, and it was hypothesized that *Wolbachia* represents the ability to kill males during embryogenesis and larval stages based on *Wolbachia* amount and virulence [40]. This is in line with our findings that low-density *w*Hm-t (in W^{T24}) killed insects in late developing stages, but high-density *w*Hm-t (in W^{T12}) killed early developing stages. Furthermore, male-killing by *Wolbachia* in *Drosophila bifasciata* requires a threshold *Wolbachia* density in eggs [16]. In the current study, male-killing of *H. magnanima* is always accompanied by high titer of *w*Hm-t infection, but the nonmale-killing strain *w*Hm-c shows low titer in its host (Figs. 1 and 5). Some insect intracellular bacteria are known to alter transcriptional levels of pathogenic genes depending on the bacterial titer, resulting in changes in pathogenicity, called "phenotypic plasticity" [41–43]. If *w*Hm-c- and *w*Hm-t-related strains have an identical genome, phenotypic plasticity based on bacterial density could be another explanation for differences in phenotypes among *Wolbachia* strains in *H. magnanima.*

Male-killing has been defined as male-specific death [5, 7]. In this study, mortality was also high in female as well as in male of W^{T12} pharate larvae (Table S4). We call this phenomenon "host lethal phenotype" caused by *w*Hm-t infection. Some male-killing bacteria are known to enhance female fitness in terms of fertility or resistance to natural enemies [44, 45] or degrade female activity and longevity [46]; however, "female-killing" as host lethal phenotype would seem counterproductive for an endosymbiont that is mainly transmitted from female hosts to offspring. In fact, there are not many insects infected with *w*Hm-t at high titers, as in W^{T12}, in the field. Possibly, the host lethal phenotype caused by *w*Hm-t in *H. magnanima* may be driven either by (1) male-killing through a molecule that targets only males, with female-killing achieved via other factors; or by (2) differences between males and females in "tolerance" to a specific substance, so that males are killed at lower titer while females are killed at higher titer. So far, a symbiont-encoded male-killing gene has been determined only in *Spiroplasma* infecting *D. melanogaster*, and expression of a single *Spiroplasma* gene reproduces male-killing phenotypes [12], although *Spiroplasma* additionally harbor genes encoding toxins that modulate host biology [47, 48]. *Wolbachia* also have multiple virulence factors in their genomes [49–51]; however, whether a single molecule or multiple molecules cause male-killing phenotype is yet to be elucidated. The virulence factors might affect only males or both males and females, which eventually lead to host lethal phenotype of *w*Hm-t in *H. magnanima*. Further genomic and mechanistic studies may facilitate the understanding of the mechanism of male-killing and interactions between *Wolbachia* and their host.

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Data Archiving The sequence data of *wsp* and MLST genes of *w*Hm-t were deposited in GenBank under accession numbers LC427375 to LC427380.

Authors' Contributions In this work, HA conducted field surveys, all experiments, and data analysis. SRL organized to collect insects in Taiwan and contributed to the discussion. MN supported the entire experiments and contributed to the discussion. YK sampled insects in Taiwanese tea field and contributed to the entire discussions of this study. Lastly, MNI had full access to all data and had responsibility for the decision to submit for publication.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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