Male-killing *Wolbachia* in the butterfly *Hypolimnas bolina*

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Abstract

Some lines of the butterfly *Hypolimnas bolina* L. (Lepidoptera: Nymphalidae) are characterized by their female-biased sex ratio. In these lines, most males die before reaching the middle larval stage. However, the cause of the bias remains unclear. We detected the proteobacterium *Wolbachia* in all individuals in the female-biased butterfly lines and in some of the lines with a normal sex ratio. Tetracycline treatment of adult females of a female-biased line led to a significant increase in both the hatch rate of their eggs (F1) and the male-to-female ratio of F1 pupae. In addition, certain assays of tetracycline treatment on mother butterflies significantly increased the male to female ratio of F1 adults. Known bacterial sex ratio distorters other than *Wolbachia* were not detected by diagnostic PCR assay, nor by the sequencing of 16S rDNA amplified using general prokaryotic 16S rDNA primers. These results strongly suggest that the distortion of the sex ratio is due to the killing of males by the inherited *Wolbachia*. Sequences of the 16S rDNA amplified using *Wolbachia*-specific primers, the cell division protein gene (*ftsZ*), the molecular chaperone *groE* genes (*groE* operon), and the *Wolbachia* surface protein gene (*wsp*) from *Wolbachia* in lines belonging to three subspecies of the butterfly (*bolina*, *jacintha*, and *philippensis*) revealed no variation among lines nor between female-biased lines and a normal one.

Introduction

Members of the genus *Wolbachia* belong to the α subclass of the Proteobacteria, and are intracellular symbionts that affect the reproduction of their host insects. Four phenotypes of reproductive alterations in hosts have been found to date. The most common is induction of cytoplasmic incompatibility; the others are feminization, parthenogenesis induction, and male-killing. However, some bacteria other than *Wolbachia* also cause some of these reproductive alterations in their hosts, and host effects are important in the expression of the alterations (Weeks et al., 2002). Male-killing is the most recently discovered effect of *Wolbachia*, and it has been found in five insect species: *Adalia bipunctata* (Hurst et al., 1999b), *Acraea encedon* (Hurst et al., 1999b), *Acraea encedana* (Jiggins et al., 2000), *Tribolium madens* (Fialho & Stevens, 2000), and *Drosophila bifasciata* (Hurst et al., 2000). Male-killing of insects is also caused by taxonomically different bacteria, such as *Rickettsia* (Werren et al., 1994), *Spiroplasma* (Williamson & Poulson, 1979), *Arsenophonus nasoniae* (Werren et al., 1986), and an unnamed flavobacterium (Hurst et al., 1999a).

Lines of the great egg-fly, *Hypolimnas bolina* L. (Lepidoptera: Nymphalidae), that show a female-biased sex ratio, were found by Poulton (1923, 1924). Clarke et al. (1975) discovered that the distorted sex ratio was due to the death of larval and embryonic males. More recent reports have shown that lines with a female-biased sex ratio in *H. bolina* harbor *Wolbachia*, whereas lines with a normal sex ratio (hereafter referred to as 'normal lines') do not (Mitsuhashi et al., 1999; Dyson, 2000; Dyson et al., 2002). However, it has not been shown experimentally that the *Wolbachia* causes male-killing in the butterflies; these studies did not include tests showing a normal sex ratio after the antibiotic treatment of the insects, nor searches for male-killing bacteria other than *Wolbachia*.

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In the present study we conducted the above-mentioned essential experiments, and we examined whether or not genetic diversity is present among the *Wolbachia* bacteria in *H. bolina*, which is distributed over a broad range of the Pacific. The results strongly suggested that *Wolbachia* is the causative agent of male-killing in these butterflies.

Materials and methods

Insects

Hypolimnas bolina is not native to Japan, but frequently migrates into southern Japan from tropical or subtropical areas. Adults were collected in the Kagoshima Prefecture, Japan, and the subspecies to which they belonged were identified, based on the color and spot patterns on their wings. Each of the adult females collected in the field laid eggs (F1) without mating with a male after capture. Three female-biased lines were examined in the present study: two were derived from a female of the subspecies *bolina* (referred to as B1 and B2) and the other belonged to the subspecies *jacintha* (referred to as B3). Two normal lines used as controls belonged to the subspecies *bolina* and *philippensis* (referred to as N1 and N2, respectively).

During the experiments, all insects were reared at room temperature (28–18 °C) and host plants – mainly the sweet potato, *Ipomoea batatas*, and *Achyranthes* spp. were used as larval diet and for egg-laying. Whether the sex ratio of the lines (B1, B2, B3, and N1) was normal or female-biased was determined by χ^2 test from the numbers of the male and female adults of the F1 and/or F2. A female of each line was mated with a male in a mosquito net. The N2 was provided by Dr T. Moriyama, Kagoshima, Japan. The F1 larvae of an adult female caught in Kagoshima, Japan were reared separately in two groups by Mr Y. Nakamine, Kagoshima, Japan and Dr T. Moriyama. Eight male and three female F1 adults were obtained by rearing of the larvae by Mr Nakamine, and F1 adults of approximately 1 : 1 sex ratio (total number of adults, ca. 15) were obtained through rearing by Dr Moriyama, thus showing that the N2 was a normal line. Some of the F2 obtained by a cross between the F1 female and male had been donated to us.

DNA extraction

The ovaries of adult females or the Malpighian tubes of last instar larvae were excised. These organs were homogenized with a sterile pestle in a 150 µl extraction buffer (0.1 M Tris-Cl, 10 mM EDTA, 0.5% SDS), and incubated with 54.5 µg proteinase K for 5 h at 37 °C. Subsequent to this, a phenol/chloroform extraction of DNA was performed, although the cetyltrimethylammonium bromide (CTAB) method (Kawakita et al., 2000) was used for some lines (B1 and N1).

Diagnostic PCR for bacteria potentially affecting the reproduction of hosts

We performed diagnostic PCR assays on individuals of each line of *H. bolina* in order to detect bacteria that were able to manipulate the reproduction of their host insects. The number of insects examined was as follows: B1: 5; B2: 3; B3: 5; N1: 5; N2: 6. We used three PCR kits: the Takara LA PCR kit version 2.1 (Takara, Shiga, Japan) for the detection of *Spiroplasma*, the M1861 (Promega, Madison, WI, USA) for *Wolbachia*, and the rTaq DNA Polymerase kit (Toyobo, Tokyo, Japan) for other bacteria. The targeted bacteria and some conditions of the PCR are shown in Table 1A. All reactions in the present study were performed in a 50 µl volume. The composition of each PCR mixture was as follows: Takara LA PCR kit version 2.1: 34–34.5 µl sterile distilled water, 5 µl 10 × buffer, each dNTP at a concentration of 400 µM, each primer at a concentration of 0.5 µM, 1–1.5 µl of genomic DNA, and 2.5 U of polymerase. M1861: 37.75–38.25 µl sterile distilled water, 5 µl 10 × buffer, 1.5 mM Mg^{2+}, each dNTP at a concentration of 400 µM, each primer at a concentration of 1 µM, 1–1.5 µl of genomic DNA, and 1.25 U of polymerase. rTaq DNA Polymerase kit: 35–35.5 µl sterile distilled water, 5 µl 10 × buffer, 1.5 mM Mg^{2+}, each dNTP at a concentration of 160 µM, each primer at a concentration of 0.5 µM, 1–1.5 µl of genomic DNA, and 2.5 U of polymerase. The PCR products were electrophoresed on 1% or 1.2% agarose gels and visualized by ethidium bromide staining. Positive control samples used in the PCR assays were a DNA sample of *Wolbachia* found in the present study for *Wolbachia* detection, a DNA sample of *Spiroplasma* in *Drosophila* spec. for the detection of *Spiroplasma,* and a DNA sample of an unnamed *Rickettsia* for the detection of *Rickettsia*; the last-mentioned two positive control samples were kindly provided by Dr H. Noda, National Institute of Agrobiological Sciences. A DNA sample of the *Wolbachia* found in the present study was used as a substitute for a positive control sample with the *Wolbachia* 16S rDNA specific primers (O'Neill et al., 1992) in trials of the detection of *Arsenophonus nasoniae* and the unnamed flavobacterium. The negative control samples were sterile distilled water. Some of the PCR products (*Wolbachia* 16S rDNA) were used as templates for direct sequencing, as described in the next section.

The quality of the DNA templates (all of N1) with which we had failed to amplify *Wolbachia* 16S rDNA in PCR using was tested by the PCR of both the mitochondrial gene (COI) of hosts and *Wolbachia* 16S rDNA using the rTaq DNA Polymerase kit (Table 1A).

Cloning and sequencing

We sequenced the 16S rDNA, cell division protein gene (*ftsZ*), molecular chaperone genes (*groE* operon; *groES* and

Table 1 Primers and conditions of PCR

Targeted organism	Targeted gene	Primers[a]	Thermal cycle conditions	References
(A) Diagnostic PCR				
Wolbachia	16S rDNA	99F: 5′-TTGTAGCCTGCTATGGTATAACT-3′ 994R: 5′-GAATAGGTATGATTTTCATGT-3′	95 °C for 5 min; 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; 72 °C for 7 min	O'Neill et al. (1992)
Spiroplasma	The spacer region between 16S and 23S rDNA	SP-ITS-JO4: 5′-GCCAGAAGTCAGTGTCCTAACCG-3′ SP-ITS-N55: 5′-ATTCCAAGGCATCCACCATACG-3′	95 °C for 2 min; 30 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min	Majerus et al. (1999) Schulenburg et al. (2001)
Rickettsia	Citrate synthase	5′-GGGGGCCTGCTCACGGCGG-3′ 5′-ATTGCAAAAAGTACAGTGAACA-3′	95 °C for 20 s; 35 cycles of 95 °C for 20 s, 48 °C for 30 s, and 60 °C for 2 min	Regnery et al. (1991)
Arsenophonus nasoniae	16S rDNA	5′-TGTTAAGGTTAATAAG CTTAGCA-3′ 5′-GGAGGCCACAGTTCT TGAC-3′	94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min; 72 °C for 10 min	———
Unnamed flavobacterium	16S rDNA	5′-ATTGTTAAAGTTCCGGCG-3′ 5′-CTGTTTCCAGCTTATTCGTAGTAC-3′	1 cycle of 94 °C for 2 min, 60 °C for 1 min, and 72 °C for 1 min 30 s; 35 cycles of 94 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min 30 s; 1 cycle of 94 °C for 15 s, 59 °C for 1 min, and 72 °C for 10 min	Hurst et al. (1999a)
Insect	COI	C1-J-1751: 5′-GGATCACCTGATATAGCATTCCC-3′ C1-N-2191: 5′-CCCGGTAAAATTAAAATATAAACTTC-3′	95 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; 72 °C for 7 min	Simon et al. (1994)
(B) PCR for sequencing				
General bacteria	Prokaryotic 16S rDNA	fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′ rP2: 5′-ACGGCTACCTTGTTACGACTT-3′	94 °C for 2 min; 31 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; 72 °C for 3 min	Weisburg et al. (1991)
Wolbachia	*ftsZ*	f1: 5′-GTTGTCGCAAATACCGATGC-3′ r1: 5′-CTTAAGTAAGCTGGTATATC-3′	1 cycle of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min; 35 cycles of 94 °C for 15 s, 55 °C for 1 min, and 72 °C for 3 min; 1 cycle of 94 °C for 15 s, 55 °C for 1 min, and 72 °C for 10 min	Werren et al. (1995)
	groE operon (*groES*, *groEL* with an intergenic region)	GroEfl: 5′-TGTATTAGATGATAACGTGC-3′ GroErl: 5′-CCATTTGCAGAAATTATTGCA-3′	94 °C for 2 min; 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min; 72 °C for 7 min	Masui et al. (1997)
	wsp	81F: 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ 691R: 5′-AAAAATTAAACGCTACTCCA-3′	35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min	Zhou et al. (1998)

[a]Table 1A: All primers except for those for insect mt COI are specific to the targeted organism.
Table 1B: all primers except for those for prokaryotic 16S rDNA are specific to the targeted organism.

groEL with an intergenic region), and surface protein gene (*wsp*) of *Wolbachia* amplified from a single host insect in each of B1, B2, B3, and N2 lines. The PCR condition for the 16S rDNA was the same as that described in Table 1A, and those of the other PCRs are shown in Table 1B. A Pyrobest DNA polymerase kit (Takara Shuzo, Shiga) or TAKARA LA PCR kit was used for the PCRs. PCR with the Pyrobest DNA polymerase kit was performed in a 50 μl reaction mixture that consisted of 32.75–33.25 μl sterile distilled water, 5 μl 10 × buffer, each dNTP at a concentration of 200 μM, each primer at a concentration of 1 μM, 1–1.5 μl of genomic DNA, and 1.25 U of polymerase.

PCR products of these genes were sequenced after cloning and/or sequenced directly; four clones of the PCR product were sequenced in case of clone-sequencing alone. On the other hand, at least two clones were sequenced, in addition to direct sequencing, in case the sequences were determined by clone-sequencing with direct sequencing. For determination of the *ftsZ* and the *groE* operon in the four lines and *wsp* sequences in B1 and B2, direct sequencing alone was carried out. For cloning, the PCR products were purified with a SUPREC-02 spin-filter (Takara Shuzo, Shiga) or a QIAEX II Gel Extraction kit (Qiagen GmbH, Hilden, Germany). Then they were cloned into pT7BlueT (Novagen, Madison, WI) or pUC18 (Takara Shuzo, Shiga) vectors and *Escherichia coli* JM 109 competent cells were transformed. The plasmids in the cells were purified with the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison). Clones were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems Inc., Foster City, CA) and an ABI Prism 377XL sequencer (PE Applied Biosystems, Foster City). As sequencing primers, we used the M13 M4 primer and the T7 promoter/M13 RV-M primer. Specific internal primers were also used for the complete sequencing of both strands. The internal primer sequences were as follows: 5′-CGCTAGCCCTCTTCGTTTACAGC-3′ and 5′-TAATACGGAGAGGGCTAGCG-3′ for *Wolbachia* 16S rDNA; 5′-AGATACACTTATTGTCATTCC-3′, 5′-GGAATGACAATAAGTGTATCT-3′, 5′-GTGGTGGAGATATGACTCTA-3′, and 5′-TAGAGTCATATCCACCAC-3′ for *ftsZ*; 5′-TGCAGCAATGGTTGACTCAAC-3′ and 5′-GCTTGCTAATTCCTATTGTATTT-3′ for *gro E*; and 5′-TGAAGATATGCCTATCACTCC-3′and 5′-GAGTGATAGGCATATCTTCAA-3′ for *wsp*. An ABI Prism BigDye Terminator Cycle Sequencing FS Kit or an ABI Prism Dye Terminator Cycle Sequencing FS Kit (PE Applied Biosystems, Foster City) was used for direct sequencing. Sequencing primers were the same as those used for PCR, and the above-mentioned internal primers were used for the complete sequencing of both strands. The sequences were visualized on an ABI Prism 373S or 377XL sequencer.

We also cloned and sequenced PCR products of the bacterial 16S rDNA from an insect of the B2 line in order to detect *A. nasoniae* and the unnamed flavobacterium. PCR conditions using the rTaq DNA Polymerase kit are shown in Table 1B. The procedures for cloning and sequencing followed the above-mentioned cases of 16S rDNA and *wsp* gene amplified with *Wolbachia* specific primers, although specific internal primers were not used as sequencing primers. Both termini (460 bp) of 20 clones were subjected to single-pass sequencing to infer the taxon of bacterium from which each PCR clone originated. The sequences were aligned with those from GenBank using BLAST, and with those of the 16S rDNA of *Wolbachia* in *H. bolina* obtained in the present study.

Phylogenetic analysis

The phylogenetic relationship between *Wolbachia* strains was analyzed using *wsp* sequences. The sequences were aligned with CLUSTAL W version 1.7 (Thompson et al., 1994). The alignment was slightly modified by eye, to correct any obvious mistake by the software. All positions including gaps were then deleted as described by Mitsuhashi et al. (2002), leaving 494 bases for use in the analysis. A phylogenetic tree was constructed with CLUSTAL W on the basis of the Kimura 2-parameter distances by the neighbor-joining method, and a bootstrap analysis with 1000 replications was performed.

Treatment with tetracycline

To determine whether the sex ratio in the butterfly would change after the elimination of the *Wolbachia* infection, five adult females from a line with a female-biased sex ratio (B2) were forced to suck honey containing tetracycline (1 or 2 mg in 1 ml of honey) in a plastic cup by stretching a coiled proboscis with a needle and introducing the tip of proboscis into the diet four times (once a day) before oviposition. Female controls were only fed on honey. Each female was crossed in a mosquito net with a male from a line with a normal sex ratio. The hatch rate of fertilized eggs laid by these females was calculated on the basis of 10 days of observation after oviposition. The sexes of the dead F1 pupae were identified based on the transparent color and spot patterns of the wings of the adults within the cuticles of the pupae, or from the external structures specific to each sex near the end of the pupal abdomens.

Results

Crossing

The numbers of male and female adults, respectively, of F1 and/or F2 were as follows. The number of the insects used

for the DNA extraction was not included in the number of F2 adults. Line B1—F1: 0, 3; F2: 1, 26; B2—F1: 0, ca. 20; F2: 2, 45; B3—F1: 0, 31; F2: 0, 15; N1—F1: 6, 7.

The sex ratios of the F1 and F2 adults in lines B2 and B3 and of the F2 adults in line B1 differed significantly from 1 : 1 (χ^2 test, $P<0.001$), whereas that of the F1 adults in line N1 did not (χ^2 test, $P>0.8$). In the χ^2 test, the number of F1 male adults in line B2 was taken to be 20. Thus the B1, B2, and B3 were regarded as female-biased lines, and N1 as a normal line.

PCR detection of bacteria that potentially affect the reproduction of host insects

In the PCR assays for *Wolbachia*, a PCR product of the expected size (ca. 920 bp) was obtained from all individuals tested of the B1, B2, B3, and N2 lines, whereas no PCR product of this size was detected from any individuals of the N1 line. As for both COI and 16S rDNA of *Wolbachia* using all five DNA samples of the N1 line, we found the expected COI band derived from all DNA samples, and again we did not find a 16S rDNA band in any DNA sample, confirming that the DNA conditions for PCR in all the DNA samples tested for COI DNA amplification were good.

In diagnostic PCR assays for *Rickettsia*, *Spiroplasma*, *A. nasoniae*, and the unnamed flavobacterium, no PCR product representing the targeted genes was obtained in any individual. Positive and negative control samples behaved as expected in all the diagnostic PCR assays.

Sequencing and phylogenetic analysis

The sequences determined (the 16S rDNAs amplified by using the primer set described by O'Neill et al. (1992), the *ftsZ*, the *groE* operon (*groES* and *groEL* with an intergenic region), and the *wsp* sequences) were identical between the four insect lines (B1, B2, B3, and N2). These sequences have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: for the 16S rDNA, AB052745 (subsp. *bolina*), AB085178 (subsp. *jacintha*), and AB085179 (subsp. *philippensis*); for the *ftsZ* gene, AB167352 (subsp. *bolina*), AB167399 (subsp. *jacintha*), and AB167353 (subsp. *philippensis*); for *groE* operon, AB167350 (subsp. *bolina*), AB167398 (subsp. *jacintha*), and AB167351 (subsp. *philippensis*); and for the *wsp* gene, AB085181 (subsp. *bolina*), AB085180 (subsp. *jacintha*), and AB085182 (subsp. *philippensis*). A homology search with BLAST against the sequences in GenBank revealed that both the 16S rDNA and *wsp* sequences in our study were identical to those in the mosquito *Culex pipiens* (AF301012, AF301010, AF216859, AF216860), and that the *ftsZ* and *wsp* sequences were also identical to those in *H. bolina* from Fiji (AJ307075 and AJ307076, respectively). Furthermore, all the compared sequences (*ftsZ*, *groE*, and *wsp*) were identical between *H. bolina* and a nymphalid butterfly *Acraea encedon* (AJ271199, AJ318485, and AJ271198, respectively) from Tanzania, suggesting that these insect species harbor *Wolbachia* strains that are the same as, or closely related to those that we found.

In the sequencing of both termini of 20 clones from PCR using general prokaryotic 16S rDNA primers, the sequences near the 5′ end (460 bp) of 16 of the 20 clones were identical to those of 16S rDNA in both *W. pipientis* (X61768) in GenBank and the *Wolbachia* of *H. bolina* analyzed by using *Wolbachia* 16S rDNA specific primers (O'Neill et al., 1992) in the present study. The sequences of the remaining four clones showed a 99.8% similarity to that in *W. pipientis* (X61768) in GenBank. The sequences (460 bp) near the 3′ end of 12 of the 20 clones were identical to those of 16S rDNA in *W. pipientis* (U23709) in GenBank, and those of the remaining eight clones were quite similar to those in *W. pipientis* (U23709) (99.1–99.8% similarity). However, the minute differences in the nucleotide sequences of the clones from the GenBank data (X61768 or U23709) could be due to polymerase errors in the PCR. Thus, no 16S rDNA from the *A. nasoniae* or flavobacterium was found in the 20 clones sequenced.

A phylogenetic tree for *Wolbachia*, based on the sequences of the *wsp* gene, is shown in Figure 1. Our *Wolbachia* strain was assigned to the *Pip* group (Zhou et al., 1998) in the B-group of *Wolbachia*, and is related to strains such as those from *Drosophila simulans* (AF020074) and *Aedes albopictus* (AF020059).

Treatment with tetracycline

Most of the hatch rates of eggs laid by females treated with tetracycline were close to 100%, whereas those of eggs laid by females not treated with tetracycline were low (mostly below 50%; Table 2). There was a significant difference between the hatch rates of total eggs (fertilized) laid in the tetracycline-treated groups (2 mg ml^{-1} group or 1 mg ml^{-1} group) and that of total eggs (fertilized) laid in the untreated group (Table 2). This suggests that most F1 male embryos of mothers that did not undergo the tetracycline treatment died before hatching, and that treating the mother butterflies with tetracycline enabled the male embryos to survive in the eggs and to hatch.

Many F1 pupae from the tetracycline-treated groups died before emerging as adults, whereas few F1 pupae of the untreated mothers died (Table 2). In the tetracycline-treated groups, most of the dead F1 pupae were males, whereas the few F1 pupae that died in the untreated group were probably all females (Table 2). Some dead pupae could not be sexed. A significant difference was observed between the sex ratio of F1 pupae (including the dead ones) in the two tetracycline-treated groups and that of F1

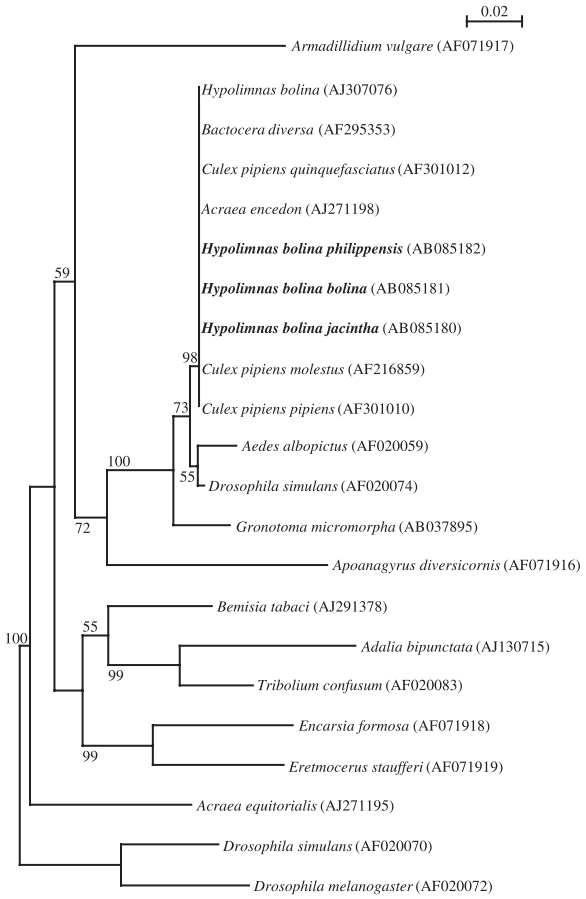

Figure 1 Neighbor-joining tree of *wsp* sequences of B-group *Wolbachia*, with A-group *Wolbachia* of *Drosophila melanogaster* and *D. simulans* as outgroups. The 494 bases within *wsp* were used for the analysis. Accession numbers are shown in parentheses. *Wolbachia* strains are represented by the names of their hosts. Bootstrap values greater than 50 are indicated at the branching points. The bar indicates 0.02 substitutions/site.

pupae (including the dead ones) in the untreated group (Table 2). In addition, the sex ratio of F1 adults was significantly different between the 1 mg ml^{-1} group and the untreated group; however, significant recovery of sex ratio was not observed in the offspring of the 2 mg ml^{-1} group (χ^2 test, P>0.7).

Discussion

The significant improvement in both the hatch rate of eggs and the sex ratio of F1 pupae in the tetracycline treatment groups and the significant increase in the number of F1 adult males of some of tetracycline treated mothers suggest that an inherited bacterium distorts the sex ratio in *H. bolina*. *Wolbachia* was the only kind of bacterium detected by the PCR assays and gene sequencing that is known to affect the reproduction of host insects. These results strongly suggest that the sex ratio distortion in *H. bolina* was caused by the inherited male-killing of *Wolbachia*.

Dyson et al. (2002) failed to change the distorted sex ratio in this species by treating larvae of this species with antibiotics, and speculated that the *Wolbachia* are antibiotic-resistant. Our results indicate that the *Wolbachia* in the female adults (at least in line B2 of this species) were antibiotic-susceptible.

Many F1 pupae from the 2 mg ml^{-1} group died before emerging, and most of the dead pupae were males. In contrast, few pupae died in the untreated group, and they were all females (except one unsexed pupa). *H. bolina* pupates after the 7th instar (sometimes the 8th or 9th) (Fukuda et al., 1972), and most male larvae in lines with sex-ratio distortion die before the 2nd instar (Clarke et al., 1975). Therefore, the most probable explanation is that the *Wolbachia* which survived tetracycline treatment gradually increased in number during the larval development of the F1 progeny and killed their host at the pupal stage. This may imply that this *Wolbachia* strain caused a late male-killing, although the phenomenon occurred under artificial conditions. To date, *Wolbachia* has been reported to cause only early male-killing (Morimoto et al., 2001). If the suppression of *Wolbachia* in the mother butterflies by tetracycline treatment had been more effective, many more F1 male adults would probably have emerged. Some change in the method of tetracycline treatment of these insects may lead to the return to a 1 : 1 sex ratio in F1 adults. In the untreated group, one would have expected almost all the individuals at the pupal stage to have been females, because almost all the males would have died before they grew to the 2nd instar larval stage; the emergence rate was therefore close to 100%.

This butterfly is widely distributed over tropical and subtropical areas of the Pacific. The sequences of five genes in the *Wolbachia*, among lines derived from a wide area were shown to be identical, suggesting that the bacteria in these lines belong to the same strain. This may imply that *Wolbachia* has infected *H. bolina* more recently than it has infected many other species.

As shown in this paper, all genes compared in the *Wolbachia* were identical between *H. bolina* and *A. encedon*, strongly suggesting that the bacteria belong to one strain, or are closely related to each other. This reinforces the suggestion by Dyson et al. (2002), that this *Wolbachia* strain has moved between the two butterfly species. It would be interesting to know how the bacterial transmission between the two species occurred.

Our results indicate that *Wolbachia* is found not only in butterflies (lines B1, B2, and B3 belonging to subsp. *bolina* or *jacintha*) that show sex-ratio distortion, but also in some butterflies (line N2 belonging to subsp. *philippensis*)

Table 2 Effect of tetracycline on growth of a female biased line of *Hypolimnas bolina*

Tetracycline in honey	Female ID number	No. of eggs laid	No. of hatched eggs	No. of F1 pupae			No. of emerged F1 adults		
				Male	Female	Sex unidentified	Male	Female	Sex unidentified
2 mg ml^{-1}	X1	50	49	22	15	0	1	14	0
	X1[a]	50	50	9	12	4	0	11	0
	X2	180	180	37	42	1	1	41	0
	Total	280	279[e]	(68	69)[e]	5	(2	66)	0
1 mg ml^{-1}	y11	20	18	3	8	5	3	8	0
	y12	149	141	19	37	11	7	37	0
	Total	169	159[e]	(22	45)[e]	16	(10	45)[f]	0
1 mg ml^{-1}	y2[b]	34	28[e]	(2	9)	0	(2	9)	0
None	c1	30	18	0	16	0	0	16	0
	c2	312	119	0	26[c]	1	0	23	0
	c3	22	4	2	2	0	2	2	0
	c4	67	25	0	4[c,d]	0	0	4	0
	Total	431	166	(2	48)	1	(2	45)	0

Tetracycline in honey was fed to female adults of a line (B2). They were mated with males, and the number of individuals in the F1 generation was counted at various stages of development.

[a]Female X1 was first mated with a male, laid eggs, and then stopped oviposition. Number of eggs laid was recorded. After that, the female was then mated with another male and number of eggs laid was recorded again. Thus, two series of data were obtained.

[b]This female did not lay until the honey containing the antibiotic was fed 10 times (once a day); the other females appearing in this table were fed four times before laying.

[c]The number of F1 pupae was low, because many larvae had been randomly removed for use in a different study.

[d]The number of dead F1 pupae was not included, because it was not counted.

[e]The hatched to unhatched egg ratios of the total numbers of the fertilized eggs (or the male to female ratios of the total numbers of F1 pupae) are significantly different from ratio in the tetracycline-untreated group (χ^2 test, $P<0.001$).

[f]The male to female ratio of the total number of F1 adults is significantly different from that in the tetracycline-untreated group (χ^2 test, $P<0.05$).

with a normal sex ratio. This fact may be explained in several ways. One explanation is that the sensitivity to sex-ratio distortion associated with *Wolbachia* may be mitigated by some additional factor in the host; that is, the lines with a female-biased sex ratio may differ from line N2 in their genetic susceptibility to male-killing. A second explanation is that very small differences in the genome of *Wolbachia*, such as point mutations, which we did not examine, may contribute to whether or not male-killing occurs. Observation on the phenotypic effects of offspring either from a cross between a female-biased and a normal line having the same strain of *Wolbachia*, or after the implantation of the bacteria to another insect species, will elucidate why some lines of *H. bolina* harboring the *Wolbachia* do not express the male-killing trait.

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References

Clarke C, Sheppard PM & Scali V (1975) All-female broods in the butterfly *Hypolimnas bolina* (L.). Proceedings of the Royal Society of London B 189: 29–37.

Dyson EA (2000) Inherited parasites in the butterfly *Hypolimnas bolina* (Lepidoptera: Nymphalidae). Abstract of First International *Wolbachia* Conference, Crete, Greece, pp. 82–83.

Dyson EA, Kamath MK & Hurst GDD (2002) *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. Heredity 88: 166–171.

Fialho RF & Stevens L (2000) Male-killing *Wolbachia* in a flour beetle. Proceedings of the Royal Society of London B 267: 1469–1474.

Fukuda H, Kubo K, Kuzuya T, Takahashi A, Takahashi M, Tanaka B & Wakabayashi M (1972) Insect Life in Japan, Vol. 3. Butterflies. Hoikusha Publishing, Osaka.

Hurst GDD, Bandi C, Sacchi L, Cochrane AG, Bertrand D, Karaca I & Majerus MEN (1999a) *Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only. Parasitology 118: 125–134.

Hurst GDD, Jiggins FM, Schulenburg JHGVD, Bertrand D, West SA, Goriacheva II, Zakharov IA, Werren JH, Stouthamer R & Majerus MEN (1999b) Male-killing *Wolbachia* in two species of insect. Proceedings of the Royal Society of London B 266: 735–740.

Hurst GDD, Johnson AP, Schulenburg JHGVD & Fuyama Y (2000) Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold in bacterial density. Genetics 156: 699–709.

Jiggins FM, Hurst GDD, Dolman CE & Majerus MEN (2000) High prevalence of male-killing *Wolbachia* in the butterfly *Acraea encedana*. Journal of Evolutionary Biology 13: 495–501.

Kawakita H, Saiki T, Wei W, Mitsuhashi W, Watanabe K & Sato M (2000) Identification of mulberry dwarf-phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. Phytopathology 90: 909–914.

Majerus TMO, Schulenburg JHGVD, Majerus MEN & Hurst GDD (1999) Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Insect Molecular Biology 8: 551–555.

Masui S, Sasaki T & Ishikawa H (1997) *groE*-Homologous operon of *Wolbachia*, an intracellular symbiont of arthropods: a new approach for their phylogeny. Zoological Science 14: 701–706.

Mitsuhashi W, Fukuda H & Sato M (1999) Detection of *Wolbachia* in a line of *Hypolimnas bolina* with female biased sex distortion. Abstract from the 59th Annual meeting of the Entomological Society of Japan, Matsuyama, Japan, p. 75.

Mitsuhashi W, Saiki T, Wei W, Kawakita H & Sato M (2002) Two novel strains of *Wolbachia* coexisting in both species of mulberry leafhoppers. Insect Molecular Biology 11: 577–584.

Morimoto S, Nakai M, Ono A & Kunimi Y (2001) Late male-killing phenomenon found in a Japanese population of the oriental tea tortrix, *Homona magnanima* (Lepidoptera: Tortricidae). Heredity 87: 435–440.

O'Neill SL, Giordano R, Colbert AME, Karr TL & Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proceedings of the National Academy of Science, USA 89: 2699–2702.

Poulton EB (1923) All female families of *Hypolimnas bolina*, bred in Fiji by HW Simmonds. Proceedings of the Royal Entomological Society of London, pp. 9–12.

Poulton EB (1924) Mimicry in the butterflies of Fiji considered in relation to the Euploeine and Danaine invasions of Polynesia and to the female forms of *Hypolimnas bolina* L., in the Pacific. Transactions of the Entomological Society of London, pp. 564–691.

Regnery RL, Spruill CL & Plikaytis BD (1991) Genotypic identification of Rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. Journal of Bacteriology 173: 1576–1589.

Schulenburg JHGVD, Habig M, Sloggett JJ, Webberley KM, Bertrand D, Hurst GDD & Majerus MEN (2001) Incidence of male-killing *Rickettsia* spp. (α-proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L. (Coleoptera: Coccinellidae). Applied Environmental Microbiology 67: 270–277.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H & Flook P (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651–701.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Weeks AR, Reynolds KT & Hoffmann AA (2002) *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? Trends in Ecology and Evolution 17: 257–262.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173: 697–703.

Werren JH, Hurst GDD, Zhang W, Breeuwer JAJ, Stouthamer R & Majerus MEN (1994) Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). Journal of Bacteriology 176: 388–394.

Werren JH, Skinner SW & Huger AM (1986) Male-killing bacteria in a parasitic wasp. Science 231: 990–992.

Werren JH, Zhang W & Guo LR (1995) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society of London B 261: 55–71.

Williamson DL & Poulson DF (1979) Sex ratio organisms (Spiroplasmas) of *Drosophila*. The Micoplasmas (ed. by RF Whitcomb & JF Tully), pp. 175–208. Academic Press, New York.

Zhou W, Rousset F & O'Neill S (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society of London B 265: 509–515.