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Transfection of *Wolbachia* in Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*

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Two species of Lepidoptera, *Ostrinia scapulalis* and *Ephestia kuehniella*, harbour *Wolbachia*, which are maternally transmitted intracellular bacteria that often cause reproductive abnormalities in arthropods. While the infection in *O. scapulalis* causes conversion of genetic males into functional females (feminization), that in *E. kuehniella* induces cytoplasmic incompatibility. In the present study, we investigated the relative importance of host and *Wolbachia* factors in the differential expression of reproductive alterations in these insects. We transferred the *Wolbachia* harboured by *O. scapulalis* to *E. kuehniella* in which the original infection had been cured by tetracycline treatment. The transfected strain of *E. kuehniella* expressed a maternally inherited, female-biased sex ratio. Unexpectedly, two lines of evidence suggested that the sex ratio distortion was due to male killing. First, higher mortality of young larvae was observed. Second, the removal of the transferred *Wolbachia* resulted in the recovery of a 1:1 sex ratio, whereas the removal of a feminizer should result in a male-biased sex ratio among offspring. To the authors' knowledge, this is the first report that a single *Wolbachia* strain can cause two distinct sexual abnormalities in different hosts. Our observations highlighted the importance of host–*Wolbachia* interactions in determining the phenotype of reproductive alterations.

Keywords: *Wolbachia*; transfection; cytoplasmic incompatibility; feminization; male killing

1. INTRODUCTION

Many insects and other arthropods harbour *Wolbachia*, which are maternally transmitted rickettsia-like bacteria. These infections are often associated with reproductive alterations in their hosts. The phenotypes induced by *Wolbachia* include thelytokous parthenogenesis in parasitic wasps, feminization in terrestrial isopods, male killing in a ladybird and a butterfly and cytoplasmic incompatibility in diverse insect taxa and other arthropods (reviewed by Stouthamer *et al.* 1999). The widespread distribution of *Wolbachia* (Werren *et al.* 1995) and their abilities to manipulate host sexuality in a variety of ways prompt several questions related to the interaction between host and bacterium. For example, is a *Wolbachia* strain capable of infecting a wide range of hosts or is each *Wolbachia* strain specialized to its own host? If a *Wolbachia* strain can infect different hosts, does the *Wolbachia* have the same effect on the various hosts? Is the type of *Wolbachia*-mediated reproductive alteration dependent on the bacterial strain or is it host dependent?

One method that can be used to answer these questions is experimentally transferring *Wolbachia* between hosts. Intraspecific transfers of cytoplasmic incompatibility-inducing *Wolbachia* have been performed using *Drosophila simulans* (Rousset & De Stordeur 1994; Sinkins *et al.* 1995), the flour beetle *Tribolium confusum* (Chang & Wade 1994) and the Mediterranean flour moth *Ephestia kuehniella* (Sasaki & Ishikawa 2000). In these transfers, the *Wolbachia* induced cytoplasmic incompatibility in the recipients as well as in the donors. The *Wolbachia* of *D. simulans* was also shown to cause cytoplasmic incompatibility in *Drosophila serrata*, a novel host species (Clancy & Hoffmann 1997). Furthermore, an interfamily transfer demonstrated that the *Wolbachia* causing cytoplasmic incompatibility in the mosquito *Aedes albopictus* was able to induce cytoplasmic incompatibility in *D. simulans* (Braig *et al.* 1994). When *Wolbachia* having no reproductive effect on the host was transferred from *Drosophila mauritiana* to *D. simulans*, the *Wolbachia* also failed to induce any effect in the recipient (Giordano *et al.* 1995). While these transfers have supported the view that the expression of cytoplasmic incompatibility mainly depends on the bacterium, transfers between *D. simulans* and *Drosophila melanogaster* have shown that host factors affect the intensity of cytoplasmic incompatibility (Boyle *et al.* 1993; Poinsot *et al.* 1998). Bouchon *et al.* (1998) performed transfers of feminizing *Wolbachia* among isopods and reported that the transfers resulted in four situations: no reproductive effect, expression of feminization, death of recipients and failure of transfection, depending on the combination of the donor and recipient. Thelytoky-inducing *Wolbachia* have been successfully transferred from *Trichogramma pretiosum* to *Trichogramma dendrolimi*, though only partial induction of thelytoky was observed in the transfected lines, probably because of a low density of *Wolbachia* (Grenier *et al.* 1998). Thus, previous transfer experiments have shown first that a *Wolbachia* strain is capable of infecting different hosts,

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second that its ability to manipulate host reproduction may be limited and third that this manipulation depends not only on the bacterial strain but also on the host genetic background.

The basis for the differential expression of reproductive phenotypes in different host–*Wolbachia* systems has not been greatly studied. A useful approach is performing artificial transfers of bacterial strains between hosts in which the natural infections express different phenotypes. To date, only two transfers of such combinations of hosts have been reported. Bouchon *et al.* (1998) reported that *Wolbachia* from *Porcellionides pruinosus* feminized *Porcellio dilatatus petiti*, the natural infection of which is associated with cytoplasmic incompatibility, demonstrating that a single host species can express different phenotypes with different *Wolbachia* strains. Van Meer & Stouthamer (1999) attempted transfer of thelytoky-inducing *Wolbachia* from *Muscidifurax uniraptor* to *D. simulans*. However, no effect on *D. simulans* was detected and the transfection was not stably maintained.

The Lepidoptera are an insect group that is suitable for performing transfers of *Wolbachia* that cause different reproductive alterations because three phenotypes have been observed among this group. Cytoplasmic incompatibility expression has been reported in *Ephestia cautella* (Brower 1976; Kellen *et al.* 1981) and *E. kuehniella* (Sasaki & Ishikawa 1999) and *Wolbachia*-mediated male killing has been found in *Acraea encedon* (Hurst *et al.* 1999). Kageyama *et al.* (1998) reported the occurrence of feminization caused by a microorganism in the Asian corn borer *Ostrinia furnacalis*. The causative agent was recently identified as *Wolbachia*. Feminization induced by *Wolbachia* has also been found in the adzuki bean borer *Ostrinia scapulalis*, a species closely related to *O. furnacalis* (*Wolbachia*-mediated feminization in these lepidopteran insects will be reported elsewhere (D. Kageyama, G. Nishimura, S. Hoshizaki and Y. Ishikawa, unpublished data)).

In the present study, we transferred the *Wolbachia* of feminized *O. scapulalis* to *E. kuehniella* with the following predictions. If the bacterial strains are responsible for the two distinctive phenotypes, the *Wolbachia* transferred from *O. scapulalis* should feminize *E. kuehniella*. On the other hand, if the hosts play major roles in the determination of the phenotypes, the transfected *E. kuehniella* should express cytoplasmic incompatibility. Unexpectedly, however, the transfer resulted in the expression of male killing.

2. MATERIAL AND METHODS

(a) *Insects*

In the present study, the feminizing *Wolbachia* carried by *O. scapulalis* (Pyralidae: Pyraustinae) was transferred to *E. kuehniella* (Pyralidae: Phycitinae).

A matriline of *O. scapulalis* designated as KI-30, which was originally collected at Kuroishi, Japan, was used as the donor strain. KI-30 was infected with a *Wolbachia* strain (*w*Sca) that belonged to the B-group defined by Werren *et al.* (1995) and showed a maternally inherited thelygenous trait. The field-collected mother of KI-30 produced 11 females, out of which five females were crossed with uninfected males for egg collection. In the five broods, we observed exclusively female emergence (108 individuals in total). In the subsequent generation, we examined three broods in which 266 females emerged. KI-30 has continued to produce strongly female-biased offspring. At present, this matriline is at generation 21. Tetracycline treatment of KI-30 resulted in the production of all-male offspring (192 males and no females in a total of three replications). This sex ratio reversal after tetracycline treatment is evidence of feminization caused by a micro-organism sensitive to the antibiotic (Kageyama *et al.* 1998). In brief, sex chromosomes of lepidopteran insects are generally ZZ in males (Traut & Marec 1996) and, therefore, feminized individuals possessing male genotype produce solely ZZ eggs that develop into males after elimination of the feminizer. The expression of feminization in *O. scapulalis* was well associated with *Wolbachia* infection. Besides KI-30, all of the 15 *Wolbachia*-infected lines examined expressed feminization, although this trait has never been observed in uninfected lines. The occurrence of feminization in *O. scapulalis*, together with the identification of the causative *Wolbachia*, will be described in full elsewhere.

Ephestia kuehniella, which was collected in Tsuchiura, Japan, was reared on a diet consisting of wheat bran, dried yeast and glycerol (20:1:2 w/w) at 25 °C under a 16 L:8 D photoperiod. This strain, which was infected with *Wolbachia* (*w*Kue) that belonged to the A group, expressed cytoplasmic incompatibility (Sasaki & Ishikawa 1999). A *Wolbachia*-uninfected strain was established by rearing the insects for two generations on the same diet supplemented with tetracycline at a final concentration of 0.04% (w/w).

(b) *Microinjection*

Injections were performed as described previously (Sasaki & Ishikawa 2000). Freshly laid eggs (< 1 h old) of the uninfected strain of *E. kuehniella*, which was the recipient, were placed on pieces of double-sided tape on a slide. The ovaries of *O. scapulalis*, which was the donor, were collected by dissection from the adult females and also placed on a slide. The ooplasm taken out of the donor ovary was injected into the recipient eggs under a microscope equipped with a three-dimensional micromanipulator and a microinjector.

The injected eggs on the slide were kept at 25 °C in a plastic dish (9 cm in diameter) containing a piece of moist filter paper. Five days after injection, the sticky surface of the tape was covered with flour and the slide was transferred to the diet mixture in a plastic container. The eggs hatched on the sixth or seventh day after injection. The rate of egg hatching was checked by counting the cast-off shells left on the tape.

Adults emerged approximately one month after the eggs hatched. The females were then individually transferred into 30 ml plastic cups (30 mm in diameter × 50 mm in height) where they were allowed to mate with males. The males used were either those developed from the injected eggs or those collected from the stock culture of the uninfected strain. A sample of eggs laid by each female was diagnosed for infection status by a polymerase chain reaction (PCR). The sex ratio was examined upon the emergence of adults.

(c) *PCR for detection of* Wolbachia

The presence or absence of *Wolbachia* was tested for by diagnostic PCR assays using *Wolbachia*-specific primers for the *groE* gene (Masui *et al.* 1998). The template DNA was prepared from eggs or adult insects according to O'Neill *et al.* (1992). Ten to 20 eggs were homogenized in 50 µl of STE buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, pH 8.0) containing

Table 1. *The survival rates of* E. kuehniella

(Fifty eggs were collected from each single-pair cross for examination of the rates of hatching and adult emergence. Females from G_2 post-injection were used as the transfected strain.)

female × male	number of crosses	% egg hatching mean ± s.d. (range)	% adult emergence mean ± s.d. (range)
transfected × uninfected	7	80.3 ± 3.1 (76–86)	39.4 ± 12.1 (18–52)
uninfected × uninfected	6	87.0 ± 12.0 (70–98)	72.3 ± 17.3 (46–94)

Table 2. *The effect of tetracycline treatment on the sex ratio of the transfected strain of* E. kuehniella

(The females from G_7 of the transfected strain were crossed with uninfected males. More than 100 eggs were collected from each single-pair cross and some of them were diagnosed by a PCR. The *Wolbachia*-positive brood was divided into two groups of 50 eggs each; one group was kept on the control diet and the other was given the diet containing tetracycline at 0.04% (w/w). The G_9 eggs were also collected from single-pair crosses and 50 eggs were reared from each cross. Eggs from non-treated G_8 females were tested for infection in order to avoid naturally cured broods. Significantly different from 50% of females by the χ^2-test, $^{*}p < 0.05$, $^{**}p < 0.01$.)

		number of adults (female:male)	
brood	tetracycline treatment	G_8	G_9
1	–	18:0**	23:0**
	+	15:0**	13:20
2	–	21:0**	26:0**
	+	20:0**	13:12
3	–	22:1**	3:0
	+	22:4**	12:20
4	–	19:0**	13:0**
	+	21:0**	24:16
5	–	5:0*	14:1**
	+	6:0*	17:14
6	–	19:5**	16:0**
	+	24:5**	1:2
7	–	2:0	—
	+	0:0	—
8	–	13:0**	13:4*
	+	14:0**	8:10
9	–	18:0**	22:0**
	+	23:0**	14:25
10	–	27:0**	13:0**
	+	22:2**	5:2

0.4 mg ml^{-1} proteinase K and incubated for 90 min at 55 °C and then for 15 min at 95 °C. An adult moth was first homogenized in 100 µl of STE without proteinase K and then 10 µl of the homogenate was added to 90 µl of STE containing proteinase K.

A PCR was performed in a 20 µl reaction mixture using Takara EX Taq. The primers used were groE29f (5′-GTTGCAAGAAGCCTTTCGTG-3′) and groE851r (5′-CCAAAACCTGGAGCTTTTACTG-3′). The PCR cycling conditions were 94 °C for 2 min followed by 35 amplification cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and, finally, 72 °C for 5 min. When necessary, PCR assays were also performed using primers specific for *Wolbachia* 16S rDNA and for the *ftsZ* gene of B-group *Wolbachia* according to O'Neill *et al.* (1992) and Werren *et al.* (1995), respectively.

(d) *Survival rates of* E. kuehniella

Crossing was performed using single pairs of virgin individuals. A female and a male were kept in a 30 ml plastic cup for three or four days. During this period, most females deposited more than 100 eggs onto the wall of the cup. Fifty eggs were collected from each pair and placed onto 1% agarose in a plastic dish (35 mm in diameter). The dish was then placed on the diet mixture in a plastic container (90 mm in diameter 50 mm in height). The eggs were incubated at 25 °C for at least ten days before the hatching rate was scored. The containers were left at 25 °C for at least two months in order to score the rate of adult emergence.

3. RESULTS

Out of 423 eggs of *E. kuehniella* injected with the ooplasm of *Wolbachia*-infected *O. scapulalis*, 127 hatched. The insects that developed from the injected eggs were assigned as generation 0 (G_0). G_1 eggs were collected from each of 66 G_0 females. When testing some (10–20 eggs) of each brood of the G_1 eggs by the PCR assay using the *groE* primers, we detected *Wolbachia* in two broods (lines 1 and 2) of which the remaining eggs were maintained. The sex ratios (% female) of lines 1 and 2, which were checked upon the emergence of G_1 adults, were 70% ($n = 43$) and 67% ($n = 78$), respectively. G_2 eggs collected from each of the G_1 females were diagnosed by PCR as performed on the G_1 eggs. Eight *Wolbachia*-positive broods, four from each line, were selected and maintained further. When the eight lines of G_2 insects attained adulthood, only females emerged. The females from the eight lines were pooled and crossed with uninfected males in order to establish a transfected strain.

The transfected strain continued to produce female-biased progeny. We also observed male emergence, particularly after G_4, although the frequency was low. PCR assays of such rare males showed that transmission failure occasionally occurred.

One possible explanation for the female-biased sex ratio in the transfected strain is that *w*Sca caused feminization in *E. kuehniella* as it did in *O. scapulalis*. However, a female-biased sex ratio can also result from thelytokous parthenogenesis and male killing. The possibility of thelytoky was ruled out because none of the unfertilized eggs collected from the virgin females hatched.

In order to determine whether the mechanism of the sex ratio distortion was feminization or male killing, the survival rate of the transfected strain was examined. As

Table 3. *Infection status and sex ratio of* E. kuehniella *at* G_2 *in the second run of the* Wolbachia *transfer from* O. scapulalis

(More than 50 eggs were collected from each of the G_1 females of lines 3 and 4, some tested for infection by PCR, and then eight infected broods and eight uninfected broods were selected. Fifty eggs of each brood were maintained in order to examine the sex ratio. Significantly different from 50% of females by the χ^2-test, $^{*}p<0.05$, $^{**}p<0.01$.)

line	brood	infection	no. of adults	% female
line 3	3-1	+	14	86**
	3-2	+	12	100**
	3-3	+	4	100*
	3-4	+	21	100**
	3-5	−	24	46
	3-6	−	25	32
	3-7	−	46	42
	3-8	−	10	30
line 4	4-1	+	14	100**
	4-2	+	36	72**
	4-3	+	16	100**
	4-4	+	30	77**
	4-5	−	38	55
	4-6	−	43	63
	4-7	−	38	50
	4-8	−	12	33

shown in table 1, the rate of adult emergence of the transfected strain was significantly lower than that of the uninfected strain ($p < 0.01$ in the *t*-test following arcsine square-root transformation). The reduced adult emergence was mainly because of high mortality of the larvae just after hatching: we observed many dead larvae beside cast-off shells. The survival rate data suggested that the sex ratio distortion in the transfected strain was due to male killing rather than feminization.

In order to distinguish between the two possible mechanisms of thelygeny further, we examined the effect of removal of the *Wolbachia* from the transfected strain on the sex ratio. The sex chromosomes in *E. kuehniella* are ZZ in males and ZW in females (Traut & Rathjens 1973). If feminization occurs, the transfected strain will be dominated by genetically male individuals carrying ZZ chromosomes because ZW females produce both ZZ and ZW offspring and the infected ZZ females (feminized individuals) produce solely ZZ offspring because they are crossed with uninfected ZZ males. In this situation, the removal of the feminizer should result in a male-biased sex ratio. If *w*Sca kills ZZ males and only ZW females survive, all females in the transfected strain should deposit ZW eggs and ZZ eggs at a ratio of *ca.* 1:1. Therefore, once the male killer is removed, the strain will recover a normal sex ratio.

Ten broods of G_8 eggs were treated with tetracycline. All the families showed a normal (1:1) sex ratio after tetracycline treatment for two generations (table 2). Accordingly, it was concluded that the female-biased sex ratio in the transfected *E. kuehniella* was due to male killing.

In order to test whether the relationship between transfection with *w*Sca and the appearance of the male-killing trait in *E. kuehniella* was causal, we performed the transfer again. In this second transfer, both successfully and unsuccessfully transfected lines were maintained in order to examine the sex ratio. Out of 357 eggs injected, we obtained two *Wolbachia*-positive broods that were designated as lines 3 and 4. Since both lines consisted of infected and uninfected individuals at G_1, we were able to obtain infected and uninfected G_2 broods from each line. As shown in table 3, all of the infected broods, but none of the uninfected broods, showed the female-biased sex ratio, indicating that the male killing in the injected lines was caused by *w*Sca and not another unknown factor introduced from *O. scapulalis*. The presence or absence of the *Wolbachia* in the adult insects of each brood was also confirmed by PCR assays using primers specific for *Wolbachia* 16S rDNA and for the *ftsZ* gene of B-group *Wolbachia*.

4. DISCUSSION

In the present study, it was demonstrated that the *Wolbachia* harboured by *O. scapulalis* induced male killing in *E. kuehniella*. Since naturally infected *E. kuehniella* express cytoplasmic incompatibility, the occurrence of the male-killing trait in the transfected strain implies that the two *Wolbachia* strains, *w*Kue and *w*Sca, differ in their ability to induce reproductive alterations. In addition, the observation that the *w*Sca strain induced different effects in *O. scapulalis* and *E. kuehniella* indicated the involvement of host factors in the determination of phenotypes.

At least two explanations can be made for the observation that *w*Sca induced distinct reproductive phenotypes in the two hosts. One is that *w*Sca killed *E. kuehniella* males by a molecular mechanism different from that causing feminization in *O. scapulalis*, possibly by expressing different genes in different intracellular environments. Another explanation is that the two hosts responded differently to the same action of *w*Sca: the bacterial action that induces feminization in *O. scapulalis* might cause the death of *E. kuehniella* males. This could occur if the sex determination and/or differentiation process differs between the two insects. Such a difference may be a consequence of an evolutionary change in *O. scapulalis*. It is possible that *w*Sca had originally been a male killer in *O. scapulalis* but host evolution altered the phenotype from male killing to feminization. Alternatively, the difference may predate the infection event of *w*Sca in *O. scapulalis* and the infection originally expressed feminization.

While rearing the transfected strain, we occasionally observed the emergence of individuals that had a male outer genital apparatus. Although all of these individuals were counted as males, they were of two types. First, some of them were found not to possess testes when dissected. Males without testes were positive for *Wolbachia* infection in the PCR assays. These males might have harboured a low level of *w*Sca, so that they were not killed but normal testis development was prevented. Functional males with well-developed testes also emerged in the transfected strain. These individuals were usually uninfected. Since the maternal transmission of *Wolbachia* occurs at a rate close to 100% in naturally infected *O. scapulalis* and *E. kuehniella* (D. Kageyama and T. Sasaki, unpublished observations), the inefficient vertical

transmission was peculiar to the transfected strain. Reduced maternal transmission was also observed in the transfer from *D. simulans* to *D. serrata* (Clancy & Hoffmann 1997) and in the transfer from *M. uniraptor* to *D. simulans* (Van Meer & Stouthamer 1999). The decrease in transmission rate in the transfected hosts suggests some specialization of *Wolbachia* to its natural host, notwithstanding the phylogenetic evidence that *Wolbachia* has undergone horizontal transmission between phylogenetically distant hosts (e.g. Breeuwer *et al*. 1992; O'Neill *et al*. 1992).

Phylogenetic analyses have shown that closely related *Wolbachia* can cause distinct reproductive alterations in different hosts and that phylogenetically distant *Wolbachia* strains can induce similar phenotypes (e.g. Moran & Baumann 1994; Van Meer *et al*. 1999). In the present study, it was demonstrated that a single *Wolbachia* strain can have distinct effects on different hosts. The importance of host–*Wolbachia* interactions in determining the phenotype of reproductive alterations provides an additional explanation for the lack of congruence between the phylogenetic status of the bacteria and their effects on hosts.

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REFERENCES

Bouchon, D., Rigaud, T. & Juchault, P. 1998 Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond.* B **265**, 1081–1090.

Boyle, L., O'Neill, S. L., Robertson, H. M. & Karr, T. L. 1993 Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* **260**, 1796–1799.

Braig, H. R., Guzman, H., Tesh, R. B. & O'Neill, S. L. 1994 Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. *Nature* **367**, 453–455.

Breeuwer, J. A. J., Stouthamer, R., Barns, S. M., Pelletier, D. A., Weisburg, W. G. & Werren, J. H. 1992 Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Mol. Biol*. **1**, 25–36.

Brower, J. H. 1976 Cytoplasmic incompatibility: occurrence in a stored-product pest *Ephestia cautella*. *A. Entomol. Soc. Am.* **69**, 1011–1015.

Chang, N. W. & Wade, M. J. 1994 The transfer of *Wolbachia pipientis* and reproductive incompatibility between infected and uninfected strains of the flour beetle, *Tribolium confusum*, by microinjection. *Can. J. Microbiol*. **40**, 978–981.

Clancy, D. & Hoffmann, A. A. 1997 Behavior of *Wolbachia* endosymbionts from *Drosophila simulans* in *Drosophila serrata*, a novel host. *Am. Nat.* **149**, 975–988.

Giordano, R., O'Neill, S. L. & Robertson, H. 1995 *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. *Genetics* **140**, 1307–1317.

Grenier, S., Pintureau, B., Heddi, A., Lassabliere, F., Jager, C., Louis, C. & Khatchadourian, C. 1998 Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. Lond.* B **265**, 1441–1445.

Hurst, G. D. D., Jiggins, F. M., Schulenburg, H. G. J., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R. & Majerus, M. E. N. 1999 Male-killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond.* B **266**, 735–740.

Kageyama, D., Hoshizaki, S. & Ishikawa, Y. 1998 Female-biased sex ratio in the Asian corn borer, *Ostrinia furnacalis*: evidence for the occurrence of feminizing bacteria in an insect. *Heredity* **81**, 311–316.

Kellen, W. R., Hoffmann, D. F. & Kwock, R. A. 1981 *Wolbachia* sp. (Rickettsiales: Rickettsiaceae) a symbiont of the almond moth, *Ephestia cautella*: ultrastructure and influence on host fertility. *J. Invertebr. Pathol*. **37**, 273–283.

Masui, S., Sasaki, T. & Ishikawa, H. 1998 *groE*-homologous operon of *Wolbachia*, an intracellular symbiont of arthropods: a new approach for their phylogeny. *Zool. Sci*. **14**, 701–706.

Moran, N. & Baumann, P. 1994 Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *Trends Ecol. Evol*. **9**, 15–20.

O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, T. L. & Robertson, H. M. 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl Acad. Sci. USA* **89**, 2699–2702.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C. & Merçot, H. 1998 *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. *Genetics* **150**, 227–237.

Rousset, F. & De Stordeur, E. 1994 Properties of *Drosophila simulans* strains experimentally infected by different clones of the bacterium *Wolbachia*. *Heredity* **72**, 325–331.

Sasaki, T. & Ishikawa, H. 1999 *Wolbachia* infection and cytoplasmic incompatibility in the almond moth and the Mediterranean flour moth. *Zool. Sci*. **16**, 739–744.

Sasaki, T. & Ishikawa, H. 2000 Transinfection of *Wolbachia* in the Mediterranean flour moth, *Ephestia kuehniella*, by embryonic microinjection. *Heredity* **85**, 130–135.

Sinkins, S. P., Braig, H. R. & O'Neill, S. L. 1995 *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. *Proc. R. Soc. Lond.* B **261**, 325–330.

Stouthamer, R., Breeuwer, J. A. J. & Hurst, G. D. D. 1999 *Wolbachia pipientis*: microbial manipulation of arthropod reproduction. *A. Rev. Microbiol*. **53**, 71–102.

Traut, W. & Marec, F. 1996 Sex chromatin in Lepidoptera. *Q. Rev. Biol*. **71**, 239–256.

Traut, W. & Rathjens, B. 1973 Das W-Chromosom von *Ephestia küehniella* (Lepidoptera) und die Ableitung des Geschlechtschromatins. *Chromosoma* **41**, 437–446.

Van Meer, M. M. M. & Stouthamer, R. 1999 Cross-order transfer of *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Diptera: Drosophilidae). *Heredity* **82**, 163–169.

Van Meer, M. M. M., Witteveldt, J. & Stouthamer, R. 1999 Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Mol. Biol*. **8**, 399–408.

Werren, J. H., Windsor, D. & Guo, L. 1995 Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond.* B **262**, 197–204.