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ORIGINAL ARTICLE

Effects of host genotype against the expression of spiroplasma-induced male killing in *Drosophila melanogaster*

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Increasing attention has been paid to the maternally inherited microbes that are capable of manipulating the reproduction of their hosts for their own benefit. Although several studies have revealed that the host genotype can affect the intensity of the manipulation, the underlying genetic basis is poorly understood. Here, we examined the intensity of spiroplasma-induced male killing in various wild-type stocks of *Drosophila melanogaster* to clarify the genetic basis of the host factors responsible for the variation in the male-killing intensity. Among ten lines examined by mating experiments (that is, nuclear introgression), eight lines including Oregon-R and Canton-S were found to have nuclear factors that allowed strong expression of male killing. In contrast, the nuclear factors of the lines Sevelen and Hikone partially suppressed or remarkably retarded the expression of male killing. These results were confirmed by artificial transfer experiments of spiroplasma infection across the fly lines by means of microinjection. A series of mating experiments revealed that the nuclear factors acting against male killing were mainly located on autosomes in Sevelen and on the X chromosome in Hikone. In both lines, the suppressors were inferred to act maternally with a dominant effect. The nuclear factors of Sevelen and Hikone scarcely affected spiroplasma densities in reproductively active young insects, suggesting that the suppressors may act on the male-killing expression directly rather than through suppressing bacterial proliferation.
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Introduction

The reproductive systems of arthropods are often manipulated by endosymbiotic microorganisms, for example by bacteria belonging to the genera *Wolbachia*, *Rickettsia*, *Spiroplasma* or *Cardinium*, or by microsporidian parasites belonging to the genera *Amblyospora*, *Nosema* or *Octosporea*. These microorganisms are maternally inherited and their various effects on host reproduction, such as cytoplasmic incompatibility, parthenogenesis induction, feminization and male killing, are advantageous for their own transmission (Werren, 1997; Stouthamer *et al*., 1999; Dunn *et al*., 2001; Bourtzis and Miller, 2003, 2006).

It is recognized that the intensity of endosymbiont-induced reproductive manipulation can be affected by the host genotype (Sakaguchi and Poulson, 1963; Boyle *et al*., 1993; Poinsot *et al*., 1998; McGraw *et al*., 2001; Riegler *et al*., 2004; Tinsley and Majerus, 2007). A well-known example is that of *Wolbachia* bacteria, which induce cytoplasmic incompatibility in *Drosophila melanogaster* and its close relative *Drosophila simulans* (reviewed by Merçot and Charlat, 2004). The naturally occurring strain of *Wolbachia* in *D. melanogaster* (*w*Mel) induces very weak cytoplasmic incompatibility in *D. melanogaster*. However, when transfected into *D. simulans*, *w*Mel induces strong cytoplasmic incompatibility (Poinsot *et al*., 1998). On the other hand, the naturally occurring strain of *Wolbachia* in *D. simulans* (*w*Ri) induces strong cytoplasmic incompatibility in *D. simulans*, but when transfected into *D. melanogaster* *w*Ri induces weak cytoplasmic incompatibility (Boyle *et al*., 1993). These transfection experiments clearly show that the intensity of *Wolbachia*-induced cytoplasmic incompatibility can be greatly affected by the host genotype. However, the genetic bases underlying the suppression of reproductive manipulations are poorly understood. In this context, intraspecific genetic variation that led to variation in the level of suppression of endosymbiont-induced reproductive manipulations would be an ideal situation for genetic studies because of the ability to perform mating experiments.

Male killing is a phenomenon in which only males die during immature stages due to the effect of maternally transmitted endosymbionts, such as bacteria belonging to genera *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Spiroplasma* and *Flavobacteria*, and microsporidian parasites like *Amblyospora* (Hurst, 1991; Hurst *et al*., 1997, 2003; Hurst and Jiggins, 2000). Among these, the mechanism of male killing has been relatively well studied in spiroplasma

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strains that infect *D. melanogaster*. The population density of the male-killing spiroplasma strain NSRO was found to be consistently higher than that of its non-male-killing derivative NSRO-A (Anbutsu and Fukatsu, 2003), thereby leading to the proposal of a density-dependent hypothesis for the expression of male killing.

In *Drosophila*, dosage differences of genes on X chromosomes between sexes are compensated for by hypertranscription of X-linked genes in males. This process of hypertranscription requires a dosage compensation complex consisting of five protein components, namely MSL-1, MSL-2, MSL-3, MLE and MOF (Bashaw and Baker, 1996; Marin *et al*., 2000; Gilfillan *et al*., 2004). Classical genetic experiments using loss-of-function mutants revealed that spiroplasma failed to kill males lacking any of the five protein components. Therefore, it was concluded that a functional dosage compensation complex is required for the expression of male killing in *D. melanogaster* (Veneti *et al*., 2005). However, the direct target and mechanism of male killing remain to be elucidated.

In this study, we aimed to clarify how the level of reproductive manipulation is affected by the host genotype using male-killing spiroplasma (strain NSRO) and various wild-type stocks of *D. melanogaster*. NSRO was originally found in *D. nebulosa* (reviewed by Williamson and Poulson, 1979), and experimentally transfected into *D. melanogaster* (Oregon-R) in the 1960s. In the nuclear background of Oregon-R, NSRO induces almost complete male killing, although a small number of male progeny from very young mothers can survive (Anbutsu and Fukatsu, 2003; Kageyama *et al*., 2007). Here, we transferred the NSRO infection into various wild-type *D. melanogaster* stocks by two different means; backcrosses of NSRO-infected females with wild-type males (paternal introgression of nuclear factors) or by microinjection of NSRO-laden hemolymph into wild-type females (inoculation of NSRO), which consistently showed that the expression intensity of male killing was affected by the host genotype. A series of classical genetic experiments was performed to elucidate the fundamental genetics affecting the expression intensity of male killing. Furthermore, the population densities of the spiroplasma were examined to clarify their possible relationships with male-killing intensity.

Materials and methods

Fly stocks

Wild-type stocks of *D. melanogaster* (Canton-S, Amherst, Harwich, Samarkand, Swedish-c, Florida, Cremia, Hikone and Sevelen) were provided by Dr Etsuko Matsuura (Ochanomizu University, Tokyo, Japan). Spiroplasma-infected and -uninfected Oregon-R lines were provided by Dr Takao Koana (Railway Technical Research Institute, Tokyo, Japan). A strain with attached-X chromosomes and X chromosome balancer (FM7i/C(1)DX, y^1 f^1:DGRC (*Drosophila* Genetic Resource Center) No. 108132), wherein attached-X chromosomes are inherited exclusively from mothers to daughters, was obtained from the DGRC of Kyoto Institute of Technology, Japan. A female of this strain was injected with spiroplasma-laden hemolymph and mated to Oregon-R males. By backcrossing with Oregon-R males for more than ten generations, an all-female line with attached-X chromosomes was established. This line stably exhibited all-female production. Flies were reared on a standard cornmeal agar medium at 25 °C (optimal temperature for the expression of male killing by NSRO) with a 16L/8D photoperiod. On the basis of the strong expression of male killing and stable maintenance of spiroplasma for >30 years, the Oregon-R strain is considered to be highly susceptible to male killing and suitable for maintenance of spiroplasma infection.

Detection of among-strain variation in susceptibility to male killing

Paternal introgression of the nuclear genome: Backcrosses of the NSRO-infected Oregon-R females with males of a single line allow us to partially replace the Oregon-R genetic background with that of the male line. To detect among-strain variation in susceptibility to male-killing, sex ratios of F_1 and F_2 offspring were examined (Figure 1a).

Transfection of NSRO: To detect among-strain variation in susceptibility to male killing, sex ratios of various lines artificially infected with NSRO were examined. For artificial infection of NSRO, microinjection of NSRO-laden hemolymph was performed as described earlier (Kageyama *et al*., 2006). Briefly, collection and injection of hemolymph was conducted under a dissecting microscope with thin glass capillary tubes made with a microelectrode puller (PN-3; Narishige, Tokyo, Japan). For capillary collection of hemolymph, the tip was inserted into the thorax of adult donor flies (NSRO-infected Oregon-R strain). Approximately 0.1 µl of spiroplasma-laden hemolymph was injected into the thorax of adult recipient flies.

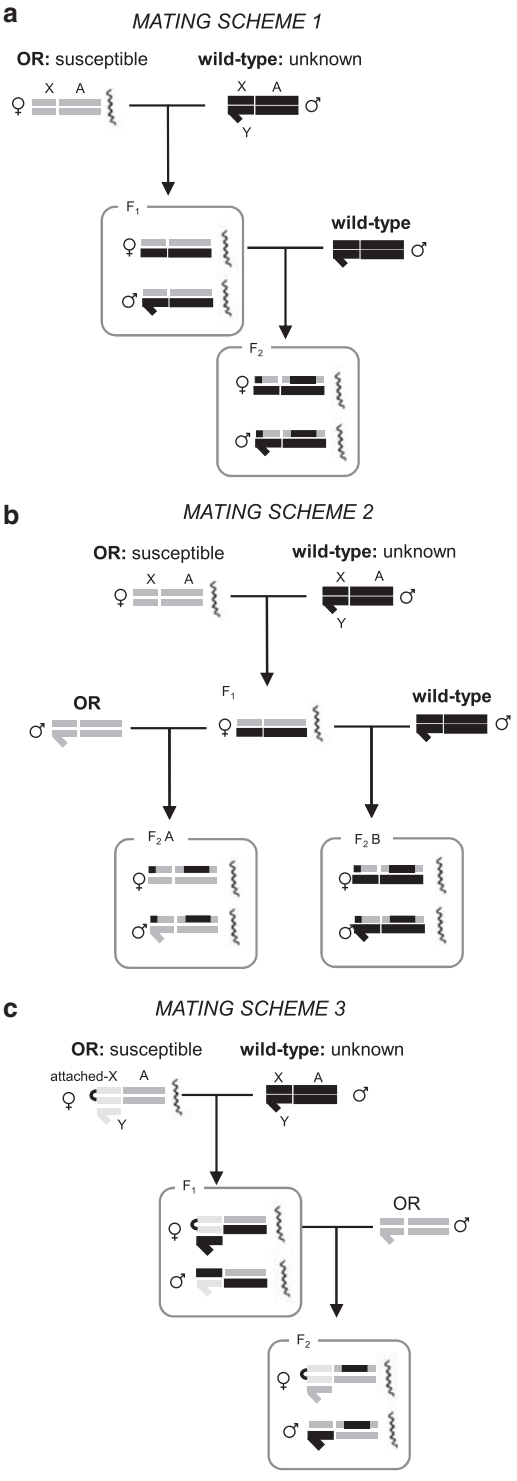
Mating schemes to characterize the suppressor alleles against the expression of male killing

To characterize the genetic factors that underlie the among-strain variation in male-killing susceptibility, we designed three mating schemes (*MATING SCHEME 1*, *2* and *3*). Integration of the results obtained by these experiments allows us to distinguish some of the important genetic features (that is, maternal versus zygotic, dominant versus recessive and X chromosomal versus autosomal) that influence the male-killing susceptibility.

Comparison of sex ratios between F_1 progeny and paternally backcrossed F_2 progeny (*MATING SCHEME 1*): The sex ratios to be compared are those of F_1 offspring produced by mating between NSRO-infected Oregon-R females and respective strain males and those of F_2 offspring produced by backcrossing F_1 females with respective strain males (see Figure 1a for genotypes). Detailed procedures of *MATING SCHEME 1* are as follows. A total of 100 NSRO-infected Oregon-R females were separated into individual vials (one female per vial). Next, sets of 10 of these females were individually mated to two males (two males per vial) derived from one of the following ten lines: Oregon-R, Canton-S, Harwich, Cremia, Florida, Amherst, Samarkand, Swedish-c, Sevelen or Hikone. The flies were transferred to new vials after an interval of 3–4 days to examine the change of offspring sex ratio according to

the maternal age. For each category of mating, five female offspring were picked up from two vials producing all-female broods, and each of these ten females was placed in a single vial and mated to two males of the same strain as their father. The sex ratios of the F_1 progeny and their progeny (F_2) were examined.

Comparison of sex ratios between two different types of F_2 progeny (*MATING SCHEME 2*): In this experiment, the sex ratios to be compared were those of offspring of F_1 females mated to either Oregon-R males or respective strain males (F_2A and F_2B; see Figure 1b for genotypes). These crosses were performed independently of the crosses performed in the *MATING SCHEME 1*. Detailed procedures of *MATING SCHEME 2* are as follows. Five NSRO-infected Oregon-R females were mated to five males derived from one of the following five strains: Hikone, Sevelen, Canton-S, Swedish-c or Cremia. The flies were transferred to new vials after an interval of 3–4 days to examine the change of offspring sex ratio according to the maternal age. For each category of mating, ten female offspring were individually mated to two males of the same strain as their fathers and another ten female offspring were individually mated to two Oregon-R males. The sex ratios of the two groups of progeny were examined and compared.



Comparison of sex ratios between F_1 progeny produced from attached-X mothers and their progeny (*MATING SCHEME 3*): In this experiment, the sex ratios to be compared are of F_1 offspring produced by mating between attached-X females having the Oregon-R background and respective strain males and of F_2 offspring produced by mating between F_1 females and Oregon-R males (see Figure 1c for genotypes). Detailed procedures of *MATING SCHEME 3* are as follows. Females of the NSRO-infected attached-X strain with the Oregon-R background were separated into individual vials and individually mated to one male derived from one of the following three lines: Canton-S, Sevelen or Hikone. Overall, 26, 31 and 27 vials were generated for the mating combinations involving Canton-S, Sevelen and Hikone, respectively. The flies were transferred to new vials after an interval of 3–4 days to examine the change of offspring sex ratio according to the maternal age. Female progeny (F_1) were picked up from the vials producing all-female broods and individually mated to an Oregon-R male. In total, 22, 13 and 32 vials were generated for the mating combinations involving Canton-S, Sevelen and Hikone, respectively. The sex ratios of the F_1 and F_2 progeny were examined.

Measurement of spiroplasma densities to infer a possible relationship with the suppression of male killing

To infer a possible relationship with the suppression of male killing in the F_2 generation of *MATING SCHEME 1*,

Figure 1 (**a**) *MATING SCHEME 1*. The sex ratios of F_1 (offspring produced by mating between NSRO-infected Oregon-R females and respective wild-type stock males) and F_2 (offspring produced by mating between F_1 females and respective wild-type stock males) were compared. Results are shown in Figure 2. (**b**) *MATING SCHEME 2*. The sex ratios of F_2 offspring produced by two different types of backcrosses (mating between F_1 females and wild-type stock males and mating between F_1 females and Oregon-R males) were compared. Results are shown in Figure 4. (**c**) *MATING SCHEME 3*. The sex ratios of F_1 (offspring produced by females of the attached-X strain with the Oregon-R autosomal background mated to respective wild-type stock males) and F_2 (offspring produced by F_1 females and Oregon-R males) were examined. The chromosomes of Oregon-R are shown in gray and the chromosomes of wild-type strains to be tested are shown in black. The spiral structures adjacent to the chromosomes indicate infection with NSRO. X: X chromosome; Y: Y chromosome; A: a set of three autosomes (chromosomes 2, 3 and 4). OR: Oregon-R. Detailed explanations of the experiments are provided in Materials and methods.

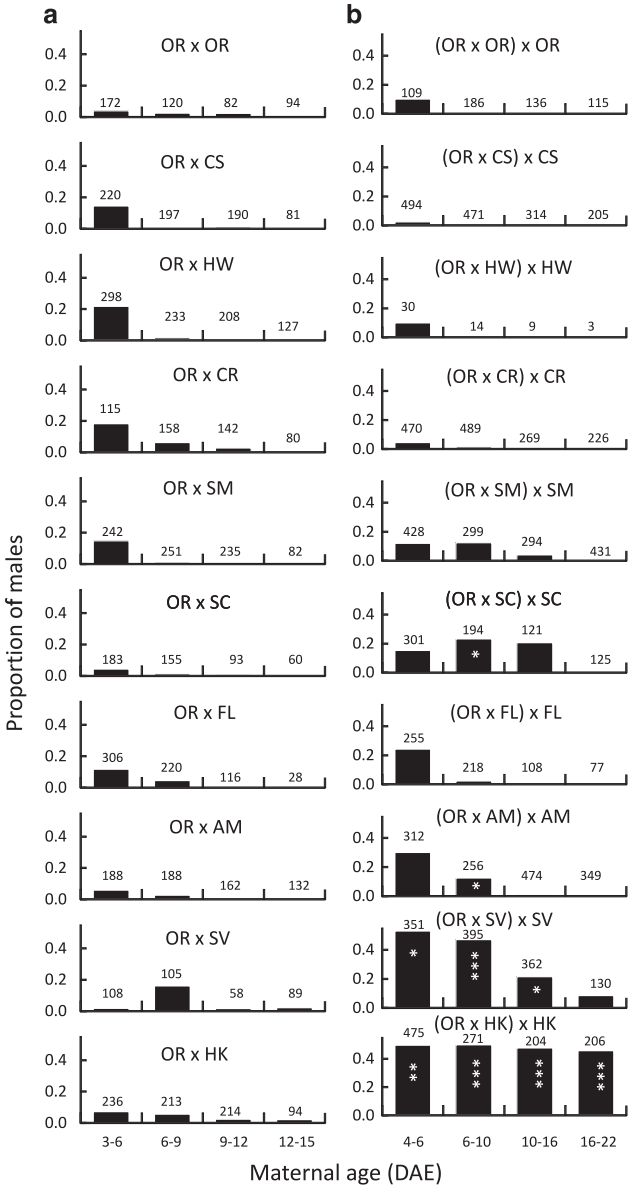


Figure 2 Offspring sex ratio obtained from the *MATING SCHEME 1*. (**a**) Proportion of males among F_1 progeny produced by NSRO-infected OR females mated with respective wild-type males. The crossing formulas are presented as female × male. (**b**) Proportion of males among F_2 progeny produced by backcrossing the F_1 females with respective wild-type males. The crossing formulas are presented as (female × male) × male. Numbers of individual progeny are shown above bars. See Figure 1a for the crossing scheme. OR: Oregon-R, CS: Canton-S, AM: Amherst, HW: Harwich, SM: Samarkand, SC: Swedish-c, FL: Florida, CR: Cremia, HK: Hikone, SV: Sevelen, DAE: days after eclosion.

F_1 adults in *MATING SCHEME 1* were examined for NSRO densities according to adult aging.

Total DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). To monitor the spiroplasma populations during aging of the host insects, real-time fluorescence detection quantitative PCR was performed using a TaqMan PCR and ABI PRISM 7700 sequence detection system (PE Applied Biosystems, CA, USA), essentially as described earlier (Anbutsu and Fukatsu, 2003, 2006). As an index of the spiroplasma titers, the copy numbers of the *dnaA* gene were determined using the probe DnaA180T (5′-FAM-AGCTTCAAATCCACCAAGATCATCAGGA-TAMRA-3′) and primers DnaA109F (5′-TTAAGAGCAGTTTCA AAATCGGG-3′) and DnaA246R (5′-TGAAAAAAACA AACAAATTGTTATTACTTC-3′). To estimate the spiroplasma densities, the copy numbers of the host *elongation factor 1 alpha* (*ef1α*) gene were determined in the same samples using the probe EF157T (5′-FAM-CAAGTCGA CGACCACCGGCCAC-TAMRA-3′) and primers EF23F (5′-TTAACATTGTGGTCATTGGCCA-3′) and EF123R (5′-CTTCTCAATCGTACGCTTGTCG-3′).

Statistical analyses

Sex ratio data: All analyses on binary outcomes (for example, male or female offspring) were performed with generalized linear mixed models (GLMMs) with binomial error distribution to assess the effects of fly stock on sex ratio. Generalized linear mixed models are an extension to the generalized linear models (McCullagh and Nelder, 1989) in which the linear predictor contains random effects in addition to the fixed effects (Schall, 1991). To account for stochastic among-brood variation (that is, repeated observation within single broods), we included the random effects of brood identity in the models. As some of the data sets contain hierarchical error structures (that is, effects of mothers and grandmothers), they were included as the hierarchical random effects in the models. All these statistical analyses were calculated by the function *lmer* of the program package lme4 using the software R version 2.4.0 (R Development Core Team, 2005). Generalized linear mixed models were fitted using Laplace approximation. Multiple comparisons were performed with Bonferroni corrections.

Spiroplasma density data: The spiroplasma density data (either *dnaA* copies per *ef1α* copy or *dnaA* copies per individual insect) were analyzed with generalized linear models (McCullagh and Nelder, 1989) using the software R version 2.4.0. Using the function *glm* originally implemented in R, we adopted a generalized linear model for Gaussian, inverse Gaussian or gamma distributions, which was selected according to the Akaike information criterion. Multiple comparisons were performed with Bonferroni corrections.

Results

Among-strain variation in susceptibility to male killing

Paternal introgression of nuclear genome: In all the mating combinations, small numbers of males appeared in the F_1 progeny produced by young mothers, whereas no or extremely small numbers of males appeared from older mothers. There were no significant differences in the F_1 sex ratios between different mating combinations in any of the maternal ages ($P > 0.05$; Figure 2a).

In the F_2 progeny produced by F_1 mothers, two categories of mating involving Sevelen and Hikone exhibited sex ratios that are significantly different from those involving Oregon-R (Figure 2b). In the mating involving Sevelen, the sex ratio gradually became biased towards female as the mothers got older. In the progeny

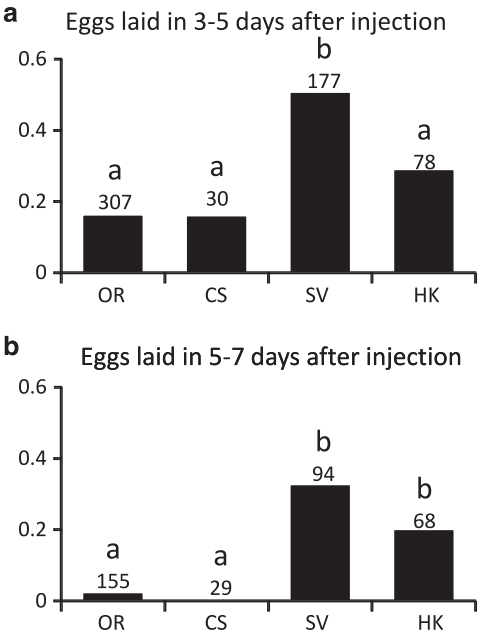


Figure 3 Offspring sex ratio produced by females injected with NSRO-laden hemolymph. Values are proportions of males. (**a**) Progeny laid 3–5 days after injection. (**b**) Progeny laid 5–7 days after injection. Numbers above bars indicate numbers of progeny. The same alphabets above the bars indicate no significant difference by generalized linear mixed model analyses ($P > 0.05$). OR: Oregon-R, CS: Canton-S, SV: Sevelen, HK: Hikone.

produced by mothers of 16–22 *d*ays *a*fter *e*closion (16–22 DAE), sex ratios had not become significantly different from those involving Oregon-R ($P > 0.05$). On the other hand, the mating combination involving Hikone produced sex ratios that were consistently nearly 1:1 irrespective of the maternal age (that is, when compared with crossings involving Oregon-R, $P < 0.01$ for 4–6 DAE and $P < 0.001$ for 6–10 DAE, 10–16 DAE and 16–22 DAE). From other mating, a slight, but significant, difference in sex ratios from those of Oregon-R was detected in the mating involving Swedish-c and Amherst in which maternal age was 6–10 DAE ($P < 0.05$; Figure 2b).

Transfection of NSRO: Adult females of four strains (Oregon-R, Canton-S, Sevelen and Hikone) were microinjected with hemolymph from NSRO-infected adults and the sex ratios of their progeny were examined. 3–5 days after injection, progeny laid by Sevelen females exhibited a significantly higher proportion of males compared with progeny laid by Oregon-R, Canton-S and Hikone females. No significant difference in sex ratio was detected between progeny produced by Oregon-R, Canton-S and Hikone females (Figure 3a). 5–7 days after injection, progeny laid by Sevelen and Hikone females exhibited a significantly higher proportion of males compared with progeny laid by Oregon-R and Canton-S females. No significant difference in sex ratio was detected between progeny produced by Oregon-R and Canton-S females and between progeny produced by Sevelen and Hikone females (Figure 3b).

Genetic basis of the suppression of male killing in Sevelen and Hikone strains

To characterize the genetic feature of the suppression of male killing observed in Sevelen and Hikone strains, the effects of maternal versus zygotic genotype on the expression of male killing were examined by three different experiments, which are supplementary to each other.

In the *MATING SCHEME 1* (Figure 1a), suppression of male killing was absent in F_1, but present in F_2 (Figure 2), indicating that the suppressors of male killing in both Sevelen and Hikone have either (i) maternal and dominant effects or (ii) zygotic and recessive effects. The suppressors could be located on the autosomes or on the X chromosome.

In the *MATING SCHEME 2*, F_1 females produced by crossings between NSRO-infected Oregon-R females and five different lines of males (Hikone, Sevelen, Swedish-c, Cremia or Canton-S) were mated to either males of the same strain as their fathers (F_2B in Figure 1b) or Oregon-R males (F_2A in Figure 1b). All the mating combinations involving Canton-S, Cremia, Swedish-c, Sevelen and Hikone showed no significant differences in the sex ratios between F_2A and F_2B for any of the maternal ages (Figure 4). As shown in Figure 1b, F_2A and F_2B have different genotypes in their autosomes, but the same genotype in their X chromosomes, which is the same as their mothers' genotype. The absence of sex ratio differences in these offspring excludes the possibility that the suppressors of male killing of Hikone and Sevelen are located on autosomes and have zygotic effects. Therefore, the remaining possibilities are that the suppressors are (i) autosomal with maternal and dominant effects, (ii) X-linked with maternal and dominant effects or (iii) X-linked with a zygotic effect.

In *MATING SCHEME 3*, females of an attached-X strain stably expressing NSRO-induced male killing were crossed with males derived from three different strains (Canton-S, Sevelen and Hikone). Subsequently, F_1 females were mated to Oregon-R males and sex ratios were compared between F_1 and F_2 progeny (Figure 1c). In the F_1 generation, male killing was not suppressed in any of the mating combinations (Figure 5). As X chromosomes of males are inherited by their sons in this experiment, the third hypothesis (that is, X-linked with a zygotic effect) can be excluded. Hence, the remaining possibilities for the suppressors would be the first and second hypotheses. Mating involving Canton-S and Sevelen did not exhibit a significant difference in sex ratio between F_1 and F_2 progeny (Figures 5a and b). On the other hand, in mating involving Hikone, proportions of males in F_2 were significantly higher compared with those in F_1 ($P = 1.146 \times 10^{-7}$ for 0–3.5 DAE and $P = 0.027$ for 3.5–7 DAE) (Figure 5c). These results suggest that, in the F_2 generation, suppression of male killing was absent in the progeny of the mating combination involving Sevelen, but unambiguously present in the progeny of the mating combination involving Hikone.

Collectively, these results suggest that the suppressors of male killing in Sevelen are mainly located on the X chromosome and have maternal and dominant effects (that is, the second hypothesis), whereas those in Hikone are mainly located on autosomes and have maternal and dominant effects (that is, the first hypothesis).

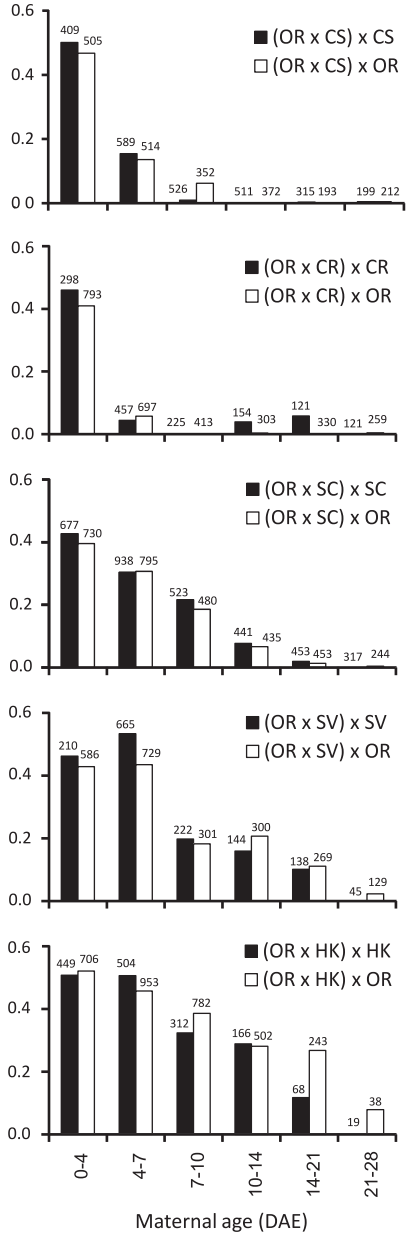


Figure 4 Offspring sex ratio obtained from the *MATING SCHEME 2*. Proportions of males among F_2 progeny produced by two different types of mating. Numbers above the bars indicate the total numbers of progeny. See Figure 1b for the mating scheme and the Materials and methods for detailed explanations. OR: Oregon-R, CS: Canton-S, SC: Swedish-c, CR: Cremia, HK: Hikone, SV: Sevelen.

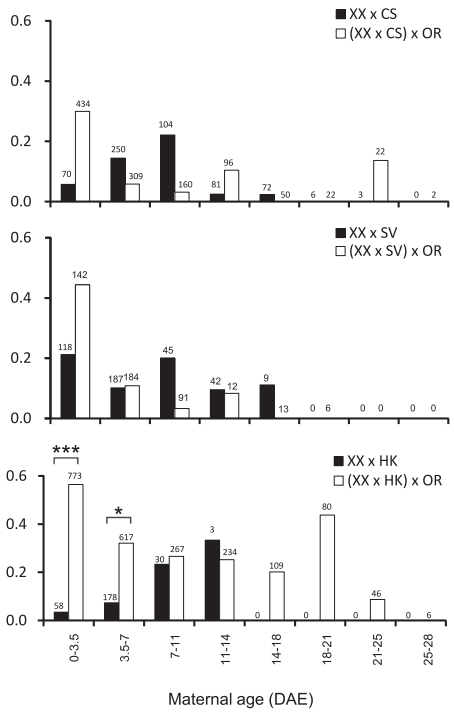


Figure 5 Offspring sex ratio obtained from the *MATING SCHEME 3*. Replacement of the genomic content of an NSRO-infected attached-X strain. Black bars indicate proportions of males among F_1 progeny produced by NSRO-infected attached-X females mated with respective wild-type males. White bars indicate proportions of males among progeny produced by F_1 females mated with respective wild-type males. Numbers above the bars indicate the total numbers of progeny. See Figure 1c for the crossing scheme and the Materials and methods for detailed explanations. XX: attached-X strain with OR genetic background, OR: Oregon-R, CS: Canton-S, HK: Hikone, SV: Sevelen.

Spiroplasma densities are decreased by nuclear factors of Sevelen, but not of Hikone

We suspected that the NSRO density was associated with the observed variations in the intensity of expression of male killing in the F_2 generation of *MATING SCHEME 1* (Figure 2b). Therefore, F_1 adults in *MATING SCHEME 1* were examined for NSRO densities with respect to adult aging. As an estimate of the NSRO density, the spiroplasma *dnaA* copy numbers per host nuclear *ef1α* copy (Figure 6a) or spiroplasma *dnaA* copy numbers per host individual (Figure 6b) were adopted. In 2-DAE adults, there were no significant differences in the NSRO densities between host genotypes. In 10-DAE adults, there were no consistent differences in the NSRO densities between host genotypes, although a slight tendency that the progeny from Canton-S males exhibited higher NSRO density compared with others. On the other hand, among 20-DAE adults, the progeny from Sevelen males showed remarkably lower NSRO densities compared with others (Figure 6).

Discussion

It is recognized that the host genetic background can profoundly affect the intensity of reproductive manipulation induced by endosymbiotic bacteria (Sakaguchi and Poulson, 1963; Boyle *et al*., 1993; Poinsot *et al*., 1998; McGraw *et al*., 2001; Riegler *et al*., 2004; Tinsley and Majerus, 2007). We found that the expression intensity of spiroplasma-induced male killing varies depending on the host genetic background within a single host species by two different experiments. Partial replacement of the nuclear background of spiroplasma-infected *D. melanogaster* by backcrosses revealed that the nuclear factors of eight strains (including Oregon-R and Canton-S) allowed strong expression of male killing, whereas the nuclear factors of two strains (Sevelen and Hikone) considerably retarded or attenuated the expression of male killing. Independently, transfection experiments (that is, micro-

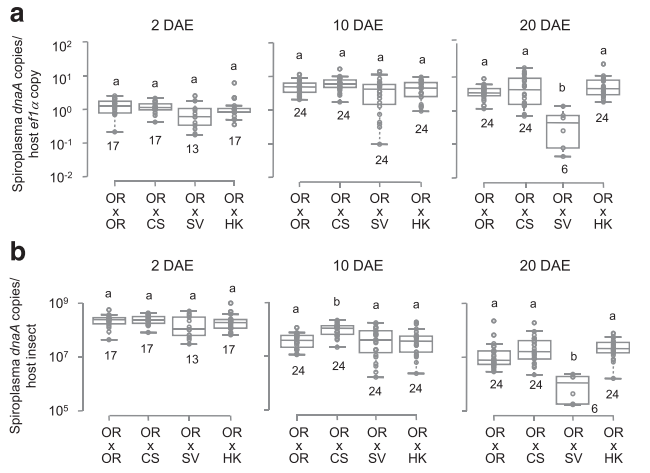


Figure 6 Infection densities of spiroplasma in F_1 females. (**a**) Spiroplasma *dnaA* copies per host nuclear *ef1α* copy. (**b**) Spiroplasma *dnaA* copies per individual host. 2 DAE: Females at the second day after eclosion. 10 DAE: Females at the tenth day after eclosion. 20 DAE: Females at the 20th day after eclosion. In the X-axis, mating combinations are indicated as the mother strains at the top and the father strains at the bottom. Numbers below the plots indicate the sample sizes. Each box represents the first (lower) quartile, second quartile (median) and third (upper) quartile. Bars below and above boxes represent the smallest and largest non-outlier observations. The same letters above the bars indicate no significant difference between the mating combinations. OR: Oregon-R, CS: Canton-S, HK: Hikone, SV: Sevelen.

injection of spiroplasma-laden hemolymph into uninfected strains) gave results that were consistent with the nuclear replacement experiments, namely strong expression of male killing in Oregon-R and Canton-S and attenuated or retarded expression of male killing in Sevelen and Hikone. Although the nuclear factors of Sevelen and Hikone suppressed the expression of male killing to considerable levels, they could not accomplish complete suppression of male killing (that is, when mothers become sufficiently old, suppression of male killing is attenuated and eventually lost). Similar results were obtained in an earlier study using the spiroplasma strain WSRO (male-killing spiroplasma derived from *D. willistoni*), which is closely related to NSRO (Montenegro *et al*., 2005; Kageyama *et al*., 2006). Specifically, microinjection of WSRO-laden hemolymph into *D. melanogaster* revealed that the male-killing intensity was high in Oregon-R, but weak in Sevelen (Sakaguchi and Poulson, 1963). Therefore, the nuclear factors of Sevelen can attenuate or retard the expression of male killing induced by either NSRO or WSRO, which may imply similar mechanisms of male killing induced by NSRO and WSRO.

Yamada *et al*. (1985) surveyed resistance genes against NSRO-induced male killing among 100 sets of autosomes derived from a Japanese natural population of *D. melanogaster*. None of the 100 sets exhibited any suppression of male killing in the heterozygotic state with Oregon-R chromosomes. In the homozygotic state, although complete suppression of male killing was never observed, 42 sets exhibited attenuated or retarded expression of male killing at variable levels. These results suggest that a considerable proportion of individuals in natural populations of *D. melanogaster* harbor nuclear factors that influence the intensity of male killing.

In *Drosophila*, few populations harbor male-killing spiroplasma. Even in populations harboring male-killing spiroplasma, the frequencies of infection are extremely low (typically below 3%) (Williamson and Poulson, 1979; Montenegro *et al*., 2005; Pool *et al*., 2006). These observations may be partly explained by the presence of suppressors like the ones found in this study and in Yamada *et al*. (1985) in various species and strains of *Drosophila*.

A series of mating experiments revealed that the suppressors against spiroplasma-induced male killing found in both Sevelen and Hikone have maternal and dominant effects. It was also found that the suppressors in Sevelen were mainly located on the X chromosome, whereas the suppressors in Hikone were mainly located on autosomes. The different localizations of the major suppressors in these two strains imply independent origins of the male-killing suppressors. It is likely that partial suppression or retardation of male-killing expression in natural populations is accomplished in complex ways (for example, possible additive effects of suppressors). Although a certain effort would be required, identification of the genes responsible for male-killing suppression may greatly contribute to our understanding of the mechanism of male killing and/or the mechanism of stable symbiosis between spiroplasma and their host insects.

To examine whether partial suppression of male killing is due to a reduction in spiroplasma density, the densities of NSRO were compared among adult offspring derived from Oregon-R females mated with either Hikone, Sevelen, Canton-S or Oregon-R males. When the adults were young (2 and 10 DAE), no differences in the NSRO densities were observed in any of the genotypes. As the nuclear factors of Hikone and Sevelen remarkably suppressed the expression of male killing in progeny produced by young mothers (<10 DAE; Figures 2 and 4), the above results suggest that the male-killing suppression in these progeny of Hikone or Sevelen is not due to reductions in the spiroplasma densities in the maternal bodies. Despite the significant decline in the spiroplasma densities in 20-DAE individuals, the nuclear factors of Sevelen allowed strong expression of male killing in progeny produced by mothers of a comparable age. This result strongly suggests that the suppression of male killing caused by the nuclear factors of Sevelen is independent of the NSRO density. Therefore, the nuclear factors of Sevelen and Hikone may have some other mechanisms for suppressing male killing rather than just simple suppression of the NSRO density, and these may involve more direct actions against the expression of male killing. However, care should be taken in concluding that bacterial densities were not associated with the suppression of male killing detected in Sevelen and Hikone, as bacterial densities across whole maternal tissues were measured. The possibility still remains that bacterial density in specific tissues only, such as ovaries or embryos, is repressed in Sevelen and/or Hikone.

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