Prevalence of Inherited Male-Killing Microorganisms in Japanese Population of Ladybird Beetle *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Ann. Entomol. Soc. Am. 98(1): 96–99 (2005)

ABSTRACT Maternally inherited bacteria that kill male but not female hosts during embryogenesis occur in *Harmonia axyridis* (Pallas). In two populations in Japan, Fukuyama, Hiroshima, and Muikamachi, Niigata, *H. axyridis* were infected with male-killing bacteria. According to the sequence analysis of the gene fragment for 16S rDNA, these bacteria belong to the genus *Spiroplasma*. Tetracycline-treated infected females produced female and male progeny, although untreated females produced only female progeny, demonstrating that *H. axyridis* is male-killed by the *Spiroplasma*. The proportion of females infected in Muikamachi and Fukuyama was 0.039 and 0.135, respectively, indicating that prevalence of male-killer in *H. axyridis* is low in Japan.

KEY WORDS ladybird beetle, *Harmonia axyridis*, male-killing, *Spiroplasma*, prevalence

MALE-KILLING, THE KILLING OF male embryos by intracellular, maternally inherited microorganisms, has been reported in a variety of insect taxa (Hurst 1991, Hurst and Majerus 1993, Hurst et al. 1997). Male-killers have been found in a number of bacterial taxa, most notably *Spiroplasma*, *Rickettsia*, *Flavobacteria*, and *Wolbachia* (Williamson and Poulson 1979; Werren et al. 1994; Hurst et al. 1999a, b). Male-killing is a mechanism for the bacteria to enhance their spread through host populations. Infected female hosts avoid inbreeding and antagonistic sibling interactions and may have increased resource availability due to the death of their brothers (Hurst et al. 1997, Hurst and Jiggins 2000).

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) is a common predator of aphids (Dixon 2000). *H. axyridis* has been reported to be infected only by *Spiroplasma* male-killing bacterium (Majerus et al. 1998). The prevalence of *H. axyridis* varies throughout Asia (Majerus et al. 1998, Majerus 2003). The prevalence of male-killers in ladybirds is not inordinately high, usually ranging from 2.2% for *H. axyridis* in Altai, Mongolia (Majerus et al. 1999), to 23.1% for *Coleomegilla maculata* (DeGeer) in Ames, IA (Hurst et al. 1996). However, in Sapporo (43° N, 141° E) Hokkaido Prefecture, Japan, the infection frequency with male-killing *Spiroplasma* of 49% has been recorded in *H. axyridis* (Majerus et al. 1998). If the male-killer were spread more commonly, the host population would be driven extinct as a result of lack of males (Groenenboom and Hogeweg 2002).

Here, we characterize a bacterium found in *H. axyridis* by its 16S rDNA sequence and its relatedness to other bacteria. Furthermore, we describe the prevalence of male-killing in two Japanese populations of *H. axyridis* to understand whether the prevalence in Japan is unusually high compared with the prevalence mentioned above, expect for Sapporo.

Materials and Methods

Ladybird Samples. We collected pupae of *H. axyridis* from Muikamachi (37° N, 139° E) Niigata Prefecture, in May and June 2001. The pupae were transferred to Niigata University where each pupa was placed individually in a petri dish (6.0 cm in diameter, 1.5 cm in height). When the adults emerged, the ladybirds were sexed. Adult samples also were collected from Fukuyama (34° N, 133° E), Hiroshima Prefecture, in April 2003, which were then reared in the National Agricultural Research Center for Western Region. All ladybirds were kept at 20°C, 70–80% RH, and a photoperiod of 16:8 (L:D) h, and fed on pea aphid, *Acyrthosiphon pisum* (Harris). Matrilines from two Japanese populations were designated as the female biased line (SR) or normal (N) (defined by Majerus et al. 1998). One hundred and twenty-seven females from Niigata (5SR and 122N) and 111 females from Fukuyama (15SR and 96N) were assayed.

Polymerase Chain Reaction (PCR) Amplification and Sequencing. DNA was extracted from a single adult ovarium that was crushed with a needle in 30 μl of a Tris-EDTA buffer (5 N NaCl, 500 nM EDTA, pH 8.0, and 1 M Tris-HCl, pH 8.0) and 5% Chelex (Bio-

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Rad, Hercules, CA) and incubated with proteinase K (20 mg/ml) at 56°C for 3 h. Each DNA sample was analyzed for bacterial presence through a PCR reaction by using general bacterial 16S rDNA primers (Weisburg et al. 1991). Primers 27f (5′-GAG AGT T TG ATC CTG GCT CAG-3′) and 1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) were used in 33-μl reactions containing 1.5 U of *Taq* polymerase (Applied Biosystems, Foster City, CA), 3.3 μl of 2 nM dNTPmix, 400 nM forward and reverse primer, 3.3 μl of 10× PCR buffer, 3.3 μl of 125 nM $MgCl_2$, 1 μl of DNA template, and 20.3 μl of sterile water. Temperature cycling was carried out in the ABI thermal cycler (Applied Biosystems PCR System 9700 and 2400) as follows: 10 min at 95°C, 35 cycles for 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, and 2 min at 72°C. Successful amplification was determined by subjecting 3 μl of PCR reaction to electrophoresis on 2% agarose gel (1× TAE), staining with ethidium bromide, and observation under an UV transilluminator. Samples of PCR products with the 16S rDNA primers that yielded amplicons of the expected size were cloned by using p-GEMT Easy Vector system (Promega, Madison, WI). Plasmids were amplified, purified, and sequenced. To determine whether *Spiroplasma* was present, we used *Spiroplasma*-specific primers. The primers used were SP-ITS-JO4 (5′-GCC AGA AGT CAG TGT CCT AAC CG-3′) and SP-ITS-N55 (5′-ATT CCA AGG CAT CCA CCA TAC GC-3′) that were designed to span the spacer region between the 16S and 23S rRNA genes (for methods, see Majerus et al. 1999). Temperature cycling was carried out in the ABI thermal cycler as follows: 10 min at 95°C, 35 cycles for 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, and 2 min at 72°C. Successful amplification was determined by electrophoresing 3 μl of PCR reaction on 2% agarose gel (1× TAE), staining with ethidium bromide, and observation under an UV transilluminator.

Phylogenetic Analysis. To identify the bacteria involved in male-killing in *H. axyridis*, molecular characterization of the 16S rDNA gene was carried out on SR lines. The 16S rDNA sequence was compared with other known sequences by using an advanced National Center for Biotechnology Information (BLAST) search (Altschul et al. 1990). The sequences were aligned using Clustal W program as reported by Thompson et al. (1994) with manual modification, and then the sequences were translated into PAUP* version 4.0b10 by Neighbor-Joining tree by using Kimura's two-parameter distance, with *Rickettsia* and *Wolbachia* used an outgroup (Kimura 1980, Saitou and Nei 1987, Swofford 2002). Symbionts are referred to by the name of their host with the GenBank accession numbers. The robustness of the results was evaluated by 1000 bootstrap replicates.

Antibiotic Treatment. We determined whether male-killing in *H. axyridis* is associated with *Spiroplasma* by treating the beetles with the antibiotic tetracycline. This method is the same standard of evidence commonly used for elimination of bacteria (Hurst et al. 1992). Newly emerged females from both infected and uninfected of Fukuyama lines were mated and allowed to oviposit in petri dishes (6.0 cm in diameter, 1.5 cm in height). After oviposition, the female was placed in a new petri dish. The egg hatch-rate was recorded. Larvae were reared individually and progeny sex ratios were recorded. These same females were fed tetracycline by mixing the antibiotic with honey syrup (100 mg/1 ml) over 2 wk. Then, these females were again allowed to oviposit eggs. We recorded the egg hatch-rate and progeny sex ratios. The progenies from both before and after tetracycline hydrochloride treatment were checked for infection with *Spiroplasma* by PCR by using the specific *Spiroplasma* primers. A Fisher's exact test (Yates 1984) was used to detect male progeny sex ratio increase with posttetracycline treatment in the SR line. As a control test, this treatment was conducted in the N line.

Table 1. Rate of hatching and sex ratio produced by *H. axyridis* females treated with tetracycline hydrochloride for 2 wk and left to lay eggs for 10 d

Line	Statement	No. females tested	No. laid eggs	Rate of hatching	Progeny sex ratio of male
SR	Pretreatment	4	175	0.32[a]	0[c]
	Posttreatment	2	67	0.69[a]	0.22[c]
N	Pretreatment	3	60	0.87[b]	0.48[d]
	Posttreatment	2	40	0.85[b]	0.53[d]

Fisher's exact test to compare rate of hatch and progeny sex ratio of male with pretreatment and posttreatment.
[a] $P < 0.01$.
[b] $P = 0.5344$.
[c] $P < 0.001$.
[d] $P = 0.4099$.

Results

BLAST sequences showed a high homology to 16S rDNA of group VI spiroplasmas (GenBank accession nos. AB127932 and AB127933). We did not find any *Rickettsia* or *Wolbachia*.

Spiroplasma-specific PCR gave a positive result for all individuals tested from SR matrilines; N lines did not have *Spiroplasma*. All positives gave the same sized product, irrespective of phenotypic expression of SR line or geographical origin. The proportion of females infected in Muikamachi and Fukuyama were 0.039 ± 0.017 (mean ± SE) and 0.135 ± 0.032, respectively.

After treatment with tetracycline in honey, the hatch rate of eggs from each of the SR line females increased, and all SR line females produced an increased proportion of male progeny (Table 1). The N line females showed no consistent changes in either the sex ratio or in the hatchability of eggs (Table 1). The changes in egg hatch rate and sex ratio observed after tetracycline treatment of SR line females were not apparent when honey alone was administered.

Discussion

The 16S rDNA of the bacteria detected places them in the *Spiroplasma* group. Egg hatch rates and sex ratio in SR lines treated with antibiotics increased, but in

the N lines treated with antibiotics that were used as controls did not change. We concluded that male-killing is caused by *Spiroplasma,* not by a genetic trait of *H. axyridis.* Coccinellids are known to harbor male-killing bacteria of the genera *Rickettsia* (Werren et al. 1994), *Spiroplasma* (Hurst et al. 1999b), *Flavobacteria* (Hurst et al. 1997), and *Wolbachia* (Majerus et al. 2000). However, from *H. axyridis,* only male-killing *Spiroplasma* is known (Majerus et al. 1999). Based on the 16S rDNA sequence, the bacteria in *H. axyridis* is >99%, similar to that in *Adalia bipunctata* L. (Hurst et al. 1999b).

Male-killers in ladybird species most commonly exist at an intermediate infection frequency of between 2.2 and 23.1% of female individuals (Hurst et al. 1996, Majerus et al. 1999). However, Majerus et al. (1998) showed that the proportion of females infected from a sample collected in Hokkaido, Japan was ≈0.49. The present results show that the prevalence of male-killers in *H. axyridis* is at a lower level in samples from Fukuyama and Niigata. We hypothesize that the Hokkaido sample is unusual. A number of mathematical models have been developed to describe the infection frequency in a host population with male-killing bacteria in terms of vertical transmission efficiency, and positive and negative effects of infection (Hurst 1991, Freeland and McCabe 1997, Hurst et al. 1997, Randerson et al. 2000). Almost all models show that a perfectly transmitted male-killer reaches a frequency in the population of one, and therefore the population goes extinct. In *H. axyridis,* the vertical transmission efficiencies have been observed to be close to perfect (Majerus 2003). The high prevalence found in the Hokkaido sample maybe an indication of a spreading male-killer population, eventually leading the extinction of the population.

Our samples show that the incidence of *Spiroplasma* in the populations studied is lower than that of other areas. Four hypotheses could explain the lower infection levels: 1) inefficient transmission of *Spiroplasma;* 2) presence of a suppressor gene that either kills *Spiroplasma* or balances its sex ratio-distorting effect; 3) another non-Mendelian balancing factor favoring a male-biased sex ratio (Stouthamer et al. 2001, Groenenboom and Hogeweg 2002); and 4) a lower fitness of infected female hosts. *Spiroplasma* in *H. axyridis* has approximately a 100% efficient vertical transmission (K.N. and K.M., unpublished), which supports that the infection frequency should go to 100%. There is also evidence that the male-killers have a negative effect on the infected females. Matsuka et al. (1975) reported that infected *H. axyridis* have some costs in terms of decreased egg laying rate, lower hatchability, and shorter adult life span. Because the value of costs to infected females is higher than the benefits gained, this may result in a lower infection frequency of the male-killer in the field. A detailed analysis of the costs and benefits of the male-killer and a study to find modifiers of the male-killing will be necessary to explain the <100% infection frequency found in the Japanese populations of this beetle.

Acknowledgments

We are grateful to R. Stouthamer (University of California) for critical reading of the original manuscript. We also thank M. Kimura, H. Toyama, and M. Toyama for collecting *H. axyridis* at Muikamachi.

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Received 18 May 2004; accepted 22 September 2004.