Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*

Masato Hiroki^{1*}, Yohsuke Tagami², Kazuki Miura^{2,3} and Yoshiomi Kato¹

¹Department of Biology, International Christian University, Miataka, Tokyo 181-8585, Japan

²Hiroshima University, Higashi-Hiroshima 739-8528, Japan

³National Agricultural Research Center for Western Region, Fukuyama, Hiroshima 721-8514, Japan

Wolbachia are rickettsial intracellular symbionts of arthropods and nematodes. In arthropods, they act as selfish genetic elements and manipulate host reproduction, including sex-ratio distortion and cytoplasmic incompatibility (CI). Previous studies showed that infection of feminizing *Wolbachia* and CI *Wolbachia* sympatrically occurred in the butterfly *Eurema hecabe*. We demonstrate that feminization-infecting individuals can rescue sperm modified by CI-infecting males. Phylogenetic analysis revealed that feminized individuals are infected with two distinct *Wolbachia* strains: one is shared with CI-inducing matriline, and the other is only found in feminized matriline. Therefore, the simultaneous double manipulation, CI rescue and feminization, is caused by different *Wolbachia* strains in feminized individuals, not by a single *Wolbachia* with two functions. This is the first finding of double infection of *Wolbachia* with different reproductive manipulations.

Keywords: *Wolbachia*; *Eurema hecabe*; feminization; cytoplasmic incompatibility; multiple infection

1. INTRODUCTION

Wolbachia are rickettsial intracellular symbionts of many arthropods. In most cases, they are parasitic and manipulate host reproduction to gain reproductive advantage with increasing vertical transmission efficiency (Weeks *et al.* 2002). Cytoplasmic incompatibility (CI) is most common where sperm from infected males induce developmental arrest of uninfected ova. Other reproductive manipulations, where *Wolbachia* bias hosts' sex ratio to females, male killing, parthenogenesis induction (PI) and feminization of genetic males, are known. Male killing is found in female heterogamous and males heterogamous insects, whereas PI is found in haplo-diploid insects and feminization occurs only in female heterogamous arthropods (Stouthamer *et al.* 1999).

Multiple infection of CI *Wolbachia* has been known in several insect taxa including parasitic wasps (Breeuwer *et al.* 1992), fruit flies (Rousset & Solignac 1995), leafcutter ants (Van Borm *et al.* 2001) and bruchid beetles (Kondo *et al.* 2002). Double infections are abundant, but naturally occurring triple or more infection is rare (Jamnongluk *et al.* 2002) despite the experimental establishment of triple infection (Rousset *et al.* 1999). Multiply infected hosts have superior fitness than singly infected ones because sperm modification occurs independently among *Wolbachia* strains. That is, multiply infected females can rescue sperm modification from singly infected males, whereas singly infected females cannot rescue sperm modification from multiply infected males. Therefore, individuals with multiple CI infections will invade singly infected populations. Although empirical and theoretical knowledge about multiple CI infections have been

accumulated, there is no report of simultaneous infection with *Wolbachia* of different types, such as CI and feminization.

The pierid butterfly *Eurema hecabe* is common in most parts of Japan. It was recently demonstrated that this species consists of two types which differ in seasonal wing morph and host-plant use (Kato 1999, 2000). These two types are distinguished by fringe colour (brown and yellow types). On Okinawa-jima Island in the subtropical region of Japan, both types inhabit sympatrically. Infection of feminization *Wolbachia* was recently found in the population of the yellow type on this island (Hiroki *et al.* 2002). M. Hiroki, Y. Ishii and Y. Kato (unpublished data), also clarified that prevalence of CI *Wolbachia* occurs in populations of each type inhabiting Okinawa-jima. This means that feminized individuals may encounter CI-infected males as potential mates. It has therefore been suggested that feminized individuals have the capability to rescue CI. We therefore investigated the suggestion that feminized *E. hecabe* individuals have the capability to rescue CI. Furthermore, we analysed the bacterial phylogenetic relationship of CI and feminized individuals to clarify whether the distinct host manipulations in a single individual are due to one *Wolbachia* strain with two functions or to double infection of different *Wolbachia*.

2. MATERIAL AND METHODS

(a) Animals

Ten female butterflies of the yellow type were collected on Okinawa-jima in May 2001. Their progenies were reared with the fresh cuttings of *Lespedeza cuneata*. Newly eclosed adults were kept individually in plastic cups with 10% sucrose solution. The sex ratio of each brood was determined at adult eclosion. Progeny from two females were all female, whereas those of the other eight females showed normal sex ratios.

* Author for correspondence (hiroki@nt.icu.ac.jp).

(b) Establishment of isofemale lines

An isofemale line was established from one brood of normal sex ratio (OKI-1), and males of this line were crossed with one of the two thelygenic broods (OKI-2). Larvae of the following generations were reared under 16 L : 8 D at 25 °C with an artificial diet including leaf powder of the host plant *Albizia julibrissin* (Kato & Sakakura 1994). For comparison, females of *Wolbachia*-uninfected populations (M. Hiroki, Y. Ishii and Y. Kato, unpublished data) were also caught in May 2001, and isofemale lines were established. *Wolbachia* infection status was tested for all matriline by diagnostic polymerase chain reaction (PCR) using *Wolbachia* *ftsZ*-specific primers (Werren *et al.* 1995).

(c) Examination of infection with feminization Wolbachia

To check whether the thelygenic sex ratio is due to feminization or not, newly eclosed females of the thelygenic brood (OKI-2) were fed on 10% sucrose solution including tetracycline (1 mg ml⁻¹). This treatment leads feminized females to produce all-male broods, but does not affect the progenic sex ratios of normal females (Hiroki *et al.* 2002). These females were crossed with males of the OKI-1 line, and the sex ratio of their progeny was determined.

(d) Examination of compatibility among female lines

Some progeny from the OKI-1 and OKI-2 lines were fed on an artificial diet containing tetracycline throughout the larval stage to cure them of *Wolbachia* infection. After adult eclosion, two kinds of cross experiment were performed: (i) reciprocal crosses were done between infected and cured individuals of the normal sex-ratio line (OKI-1) to test whether the OKI-1 line has CI ability or not. Males of this line were also crossed with *Wolbachia* uninfected females of the Hitachi population. (ii) Cured females of the thelygenic line (OKI-2) were crossed with infected or cured males of the OKI-1 line to examine whether the females of the thelygenic matriline have the ability to rescue CI.

Mated females were allowed to oviposit on fresh cuttings of *L. cuneata* for several days. The number of eggs laid was counted daily. Eggs were kept at 25 °C, and hatchling numbers were counted on day 3.

(e) Phylogenetic analysis of Wolbachia

Several eggs were dissected from adult females, and homogenized in 20 µl of sterile deionized distilled water with 5% Chelex rein (Bio-Rad). The homogenate was incubated for 3 h at 56 °C with 1.4 µl of 20 mg ml⁻¹ proteinase K, and then boiled for 3 min. After a short centrifugation in a microcentrifuge, the supernatant was used as a PCR template. For the *wsp* gene, *wsp* 81F and *wsp* 691R were used as forward and reverse primers (Zhou *et al.* 1998), and for the *ftsZ* gene, *ftsZf1* and *ftsZr1* were used (Werren *et al.* 1995). PCR cycles were as follows: for the *ftsZ* gene, 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 s at 75 °C, followed by 2 min incubation at 75 °C. For the *wsp* gene, cycle numbers were decreased to 30 with 52 °C annealing. Amplified products were ligated with T-easy vector (Promega). Isolated colonies were incubated with ampicillin containing LB medium overnight, and the purified vectors were directly sequenced using SP6 and T7 sequencing primers with BigDye Terminator v.3.1 Cycle Sequencing Kit (PE Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (PE

Figure 1. Phylogenetic tree of the B-group of *Wolbachia* based on *wsp* sequence data with outgroup of A-group *Wolbachia* from *Cadra cautella*. GenBank accession numbers as follows: AF020075, AB094393, AB094394, AB094395, AJ271198, AF020061, AJ307076, AB037895, AF020074, AF020059, AF071918, AF020076, AB094396, AB038331, AF295350, AJ130716 and AF020083.

Applied Biosystems). Sequences of each subclone with previously known sequences obtained from GenBank (accession numbers are shown in figure 1) were aligned by CLUSTAL W (Thompson *et al.* 1994) with manual modification, and then the sequences were translated into PAUP v.4.0b10 (Swofford 2001). Neighbour-joining trees were constructed with 1000 bootstrapping repeats.

3. RESULTS**(a) Sex ratio of thelygenic line**

As shown in table 1, all offspring of tetracycline-treated thelygenic females (OKI-2) were male, whereas untreated control females showed female-biased sex ratios.

(b) Compatibility among matriline

In the normal sex-ratio line (OKI-1 line), *Wolbachia*-infected females produced viable eggs with infected and cured males, whereas all eggs of tetracycline-treated females did not hatch when they mated with infected males (table 2). In the crosses between OKI-1 males and *Wolbachia*-uninfected Hitachi females, no eggs hatched, whereas tetracycline-treated OKI-1 males showed compatibility with Hitachi females (table 3).

Table 4 shows the results of crosses between CI (OKI-1) and the feminized (OKI-2) matriline. Females obtained from thelygenic matriline produced viable eggs when they mated with *Wolbachia*-infected males, whereas tetracycline-treated thelygenic females produced no viable eggs with infected males. When both females and males were treated with tetracycline, thelygenic females produced viable eggs.

Table 1. Sex ratio of OKI-2 matriline from Okinawa-jima population with and without tetracycline treatment.

generations	number of eggs	hatchability (%)	number of adults	proportion male
P			12	0
F ₁				
2-1	139	0.42	19	0.05
2-2	223	0.50	27	0.07
2-3	183	0.67	46	0.04
F ₂				
2-1-1	235	0.76	44	0
2-1-2	120	0.19	—	—
2-3-1	96	0.41	7	0
tetracycline treatment				
2-4	122	0.23	7	1
2-5	115	0.24	12	1

Table 2. Reciprocal crosses between tetracycline-treated and untreated individuals of OKI-1 (normal sex ratio) matriline.

female × male	number of crosses	mean hatchability (range)
infected × infected	4	0.53 (0.20–0.77)
infected × cured	2	0.53 (0.46–0.61)
cured × infected	3	0
cured × cured	1	0.28

Table 3. Crosses between Hitachi females and tetracycline-treated (T) and untreated (U) OKI-1 males.

female × male	number of crosses	mean hatchability
Hitachi × OKI-1 (U)	3	0
Hitachi × OKI-1 (T)	1	0.69

Table 4. Reciprocal crosses between tetracycline-treated (T) and untreated (U) individuals of OKI-1 males and thelygenic OKI-2 females.

female × male	number of crosses	mean hatchability (range)
OKI-2U × OKI-1U	6	0.53 (0.19–0.72)
OKI-2U × OKI-1T	—	—
OKI-2T × OKI-1U	5	0
OKI-2T × OKI-1T	3	0.33 (0.27–0.48)

(c) Phylogeny of *Wolbachia*

We determined 602 bp of the *wsp* gene and 1043 bp of the *ftsZ* gene. These have been deposited in GenBank (*wsp* sequences: WHecCI1, AB094393; WHecCI2, AB094394; WHecFem1, AB094395; WHecFem2, AB094396). For the CI matriline, sequences of 12 clones of the *wsp* gene and 18 clones of the *ftsZ* gene were determined. Single substitutions, which may have been caused by polymerase amplification error, were found in several sequences, but all subclones were monophyletic and grouped into supergroup B (Werren *et al.* 1995).

By contrast, two distinct phyletic sequences were found in the feminizing matriline. Among nine sequences of the *ftsZ* gene, six sequences were identical to CI sequences, whereas the remaining three were phylogenetically distinct. Between these two clades, the base-pair difference was *ca.* 2.5%. For *wsp* sequences, eight out of 10 sequences (WHecFem1) were identical to CI sequences (WHecCI2), and the remaining two (WHecFem2) were grouped into a different clade (figure 1). WHecFem2 sequence is 3 bp shorter than that of WHecFem1.

4. DISCUSSION

In the present study, we found the simultaneous infection by *Wolbachia* with different sexual manipulation. Thelytoky in *E. hecabe* is due to infection by a feminizing *Wolbachia* (table 1), and feminized females rescue sperm modification by CI males (table 4). Phylogenetic analysis of bacterial *wsp* and *FtsZ* genes reveals that CI individuals with a normal sex ratio show single infection, whereas feminized individuals carry two distinct strains of *Wolbachia*; one shared with CI matrilines; the other being found only in feminized matrilines. These facts suggest that the two reproductive alterations, feminization and CI rescue, in feminized individuals are independently caused by different *Wolbachia* strains. This is the first evidence, to our knowledge, of multiple infection by sex-ratio distorting *Wolbachia* and CI *Wolbachia*, whereas a similar case is known in the parasitic wasp *Nasonia vitripennis*: this species is infected with CI *Wolbachia* (Breeuwer & Werren 1990), and small fractions of natural populations also have male-killing bacteria (Skinner 1985).

Many authors have reported *Wolbachia* infection, but often the mechanisms of host manipulation remain unknown. It is thought that the type of host manipulation involved is an association with a host genome. Bacterial divergence is also proposed to cause phenotypic difference in the *Acraea* group, where CI and male killing are found in closely related *Wolbachia* strains (Jiggins *et al.* 2002). In the case of *E. hecabe*, alteration of host reproduction occurs in a single individual. Therefore, the cause of phenotypic difference is due to the symbiont's genetic divergence. Interestingly, the *wsp* sequence of the CI strain in *E. hecabe* is identical to that of male-killing strains in *Bolina* and *Acraea* butterflies (Dyson *et al.* 2002; Jiggins

et al. 2002). This means horizontal transfer of this strain among these three hosts might have occurred, and the manipulation type might be altered because of the host-symbiont interactions.

Frequency of feminizing individuals for the yellow-type population of the Okinawa-jima population is relatively low despite of model prediction: the prevalence of cytoplasmic feminizer easily reaches fixation (Taylor 1990). In 1997, 3 out of 12 matriline were thelygenic (Hiroki *et al.* 2002), and 2 out of 10 in 2001 (this study), whereas the infection frequency of CI *Wolbachia* seems almost fixed in the yellow type of the Okinawa-jima population (M. Hiroki, Y. Ishii and Y. Kato, unpublished data). Hurst *et al.* (2002) also argued in their theoretical studies that CI individuals are easily eliminated by CI-resisting sex-ratio mutants. The polymorphic state of infection in *E. hecabe*, however, is far from the model prediction. This means that: (i) the prevalence of double infection has not reached equilibrium; or (ii) the model assumptions are not satisfied in this case. In *E. hecabe*, prevalence of the feminization mutant seems to reach an equilibrium because of the absence of ZW individuals, which are easily excluded at equilibrium by the doubled advantage of a ZZ female (ZZ females produce ZZ zygotes only, whereas ZW females produce ZW and ZZ zygotes), among feminization *Wolbachia*-infected females.

Competition among *Wolbachia* strains can affect the prevalence of double infection. In the model, Hurst *et al.* (2002) assume that a single mutant plays two functions: CI rescue and sex ratio distortion. In *E. hecabe*, this model assumption is not appropriate because each manipulation is caused by two different strains. If the double infection leads to extra cost for a host (not yet examined in *E. hecabe*), the prevalence of double infections would be depressed. Alternatively, if bacterial intracellular density is depressed by double infection, more frequent segregation of one mutant will be expected. Under such conditions, competition for successful inheritance between strains may occur. However, once either mutant is lost, the cytoplasmic factors of such individuals cannot be passed on to following generations. Feminizer-losing ZZ individuals produce male progeny only, and CI-losing individuals cannot reproduce because of the abundance of CI males. This means that short-term advantage may favour interstrain competition despite of the long-term disadvantage, and such a trade-off may affect the prevalence of feminizer.

Conflicts between *Wolbachia* and their hosts can also affect the prevalence of the feminizer. Sex-ratio suppressor genes in the host's genome will be favoured by both sexes. By contrast, maintenance of the CI strain is advantageous for female hosts because of its high ability to rescue CI (Turelli 1994). As a result, selective loss of the feminizer from female hosts may be an evolutionary advantage to host reproduction. Such conflict may maintain the frequency of the feminizer at its low level.

The authors thank Dr Michael E. N. Majerus and two anonymous referees for critical reading of the manuscript. This work is completed under the Academic Frontier Project (2003-2007), International Christian University, supported by the Ministry of Education, Culture, Sports, Science and Technology.

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