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# *Wolbachia* Infections and Cytoplasmic Incompatibility in the Almond Moth and the Mediterranean Flour Moth

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**ABSTRACT**—*Wolbachia* are a group of inherited bacteria found in a number of arthropods and cause various reproductive alterations in their hosts, including feminization, parthenogenesis and cytoplasmic incompatibility. We examined *Wolbachia* infection in three species of moths belonging to the sub-family Phycitinae, the Indianmeal moth *Plodia interpunctella*, the almond moth *Ephestia cautella* and the Mediterranean flour moth *Ephestia kuehniella*. We detected infections in *E. cautella* and two strains of *E. kuehniella*, one from Tsuchiura city and the other from Yokohama city. *Wolbachia* was not detected in *P. interpunctella*. The phylogenetic positions of *Wolbachia* harbored by *E. cautella* and *E. kuehniella* were estimated based on the sequences of the *wsp* gene which encodes a *Wolbachia* surface protein. We also performed crossing experiments to examine cytoplasmic incompatibility. It was shown that *Wolbachia* in *E. cautella* cause complete cytoplasmic incompatibility: no egg-hatch was observed in the cross between infected males and uninfected females. Both Tsuchiura and Yokohama strains of *E. kuehniella* showed partial cytoplasmic incompatibility, but the levels were significantly different between the two strains. The rates of egg hatch in the incompatible crosses within Tsuchiura and Yokohama strains were 60.8% and 16.9%, respectively.

## INTRODUCTION

*Wolbachia* are rickettsia-like bacteria that infect a number of insects and other arthropods. A field survey indicated more than 16% of all insect species are infected with *Wolbachia* (Werren *et al*., 1995a). *Wolbachia* are usually transmitted maternally through the cytoplasm of eggs and cause reproductive alterations in different hosts, including female-producing parthenogenesis (thelytoky) in parasitic wasps (Stouthamer *et al*., 1993), feminization of genetic males into functional females in terrestrial isopods (Rigaud *et al*., 1991) and cytoplasmic incompatibility in diverse insect taxa (Yen and Barr, 1971; Barr, 1980; Noda, 1984; O'Neill and Karr, 1990; Werren, 1997).

Cytoplasmic incompatibility is commonly expressed when *Wolbachia*-infected males mate with uninfected females. Such a cross results in the death of the embryo. Aberrant paternal chromosome condensation/de-condensation processes have been observed in incompatible crosses in *Drosophila* (Lassy and Karr, 1996), *Nasonia* (Reed and Werren, 1995) and *Culex* (Jost 1971). Since *Wolbachia* are excluded during sperm maturation, the mature sperm do not contain *Wolbachia*. It is believed that *Wolbachia* modify the sperm during spermatid development and this modification remains in the sperm in the absence of *Wolbachia*. The infected females can mate successfully not only with uninfected males but also with infected males, indicating that the presence of *Wolbachia* in the egg rescues the modified sperm to normal function. Cytoplasmic incompatibility is a mechanism by which *Wolbachia* invades a host population by decreasing the fitness of uninfected females. Rapid spread of *Wolbachia* infection has been observed in natural populations of *Drosophila simulans* in California (Turelli and Hoffmann, 1991) and the small brown planthopper *Laodelphax striatellus* in Japan (Hoshizaki and Shimada, 1995).

The strength of cytoplasmic incompatibility varies depending on both the host species and *Wolbachia* strains (Bourtzis and O'Neill, 1998). Almost complete incompatibility is observed in flour beetles of the genus *Tribolium*, and the almond moth *Ephestia cautella*. Weak expression of cytoplasmic incompatibility has been reported in *Drosophila melanogaster*, *Drosophila sechellia* and *Drosophila ananassae*. It has also been shown that the strength of cytoplasmic incompatibility varies within a species depending on *Wolbachia* strains (Bourtzis *et al*., 1998).

Phylogenetic trees of *Wolbachia* strains in various hosts have been constructed based on the sequences of 16S rDNA (O'Neill *et al*., 1992), bacterial cell-cycle gene *ftsZ* (Werren *et al*., 1995b) and *groE*-homologous operon (Masui *et al*., 1998). The phylogenetic trees showed that *Wolbachia* clade is divided into two major groups designated as A and B. Sasaki *et al*. (1998) recently detected a *Wolbachia* protein showing size variation between *Wolbachia* strains. The protein is a

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major surface protein and its gene (*wsp*) has been sequenced by Braig *et al*. (1998). Since *wsp* gene is evolving at a faster rate than any other previously reported *Wolbachia* gene, the phylogenetic analysis of *Wolbachia* based on this gene results in an improved phylogenetic resolution (Zhou *et al*., 1998). The phylogenetic analyses have revealed a lack of concordance between host and *Wolbachia* phylogenies, suggesting that *Wolbachia* are horizontally transmitted between host species, though on rare occasions (Moran and Baumann, 1994; Schilthuizen and Stouthamer, 1997).

*Wolbachia* has potential to be used as a means of genetic pest control (Sinkins *et al*., 1997). Laven (1967) noted that cytoplasmic incompatibility can be used to introduce sterility into a wild population. It may also be possible to apply *Wolbachia* to spread useful genes into insect populations by using cytoplasmic incompatibility as a self-spreading mechanism (Beard *et al*., 1993).

Despite the widespread distribution and application potential, little is known about *Wolbachia* infections in Lepidopteran insects, many of which are pest species. In this insect group, *E. cautella* is a relatively well-examined species. Brower (1976) reported the phenomenon of cytoplasmic incompatibility in *E. cautella* and the phenomenon was linked to *Wolbachia* infection by Kellen *et al*. (1981). Werren *et al*. (1995b) found that *E. cautella* harbors both A and B group *Wolbachia*, and Zhou *et al*. (1998) sequenced the *wsp* gene of *Wolbachia* from *E. cautella*. However, it is not clear whether these previous investigations have been conducted on the same strain of *E. cautella*. It should be kept in mind that the host insects collected from different geographical location often contain different *Wolbachia*. (O'Neill and Karr, 1990; Montchamp-Moreau *et al*., 1991)

In this study, we examined *Wolbachia* infections in moths of the sub-family Phycitinae (Lepidoptera: Pyralidae): *Plodia interpunctella*, *E. cautella* and two strains of *Ephestia kuehniella* collected in Japan. *Wolbachia* was not detected in *P. interpunctella*. *E. cautella* was double-infected with both A and B group *Wolbachia*, and the two strains of *E. kuehniella* were single-infected with A group *Wolbachia*. To infer the relative phylogenetic relationships among *Wolbachia* found in the present study, we sequenced the *wsp* genes of these *Wolbachia*. We also report the varying levels of cytoplasmic incompatibility observed in *E. cautella* and *E. kuehniella*.

## MATERIALS AND METHODS

### Insects

Cultures of *Ephestia cautella*, *Ephestia kuehniella* and *Plodia interpunctella* were provided by Dr. H. Nakakita. These moths were originally collected in Tsuchiura city. A strain of *E. kuehniella* from Yokohama city was provided by Dr. Y. Soma.

The moths were reared on a diet consisting of wheat bran, dried yeast and glycerol (20 : 1 : 2 w/w) at 25°C under a 16 hr light : 8 hr dark photoperiod. *Wolbachia*-free strains of *E. cautella* and *E. kuehniella* were established by rearing the moths on the diet containing tetracycline at a final concentration of 0.04% (w/w). The strains were treated for two generations and tested for infection status by PCR (see below).

### Detection of *Wolbachia*

*Wolbachia* infection was examined by PCR using *Wolbachia* specific primers for the *ftsZ* bacterial cell-cycle gene. The template DNA for PCR was extracted by the crude STE boiling method (O'Neill *et al*., 1992). Ovaries were collected by dissection from adult females. The samples were homogenized in 10 volumes of STE (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) containing proteinase K at 0.4 mg/ml and incubated for 90 min at 55°C followed by 15 min at 95°C. After brief centrifugation, 1 µl of the supernatant was used as template DNA for PCR.

PCR was performed in a 20 µl reaction mixture using Takara EX Taq. Three primer sets were used for amplification of *Wolbachia ftsZ* gene according to Werren *et al*. (1995b). The general *Wolbachia ftsZ* primers used were ftsZfl (5'-GTT GTC GCA AAT ACC GAT GC-3') and ftsZrl (5'-CTT AAG TAA GCT GGT ATA TC-3'), the A group specific *ftsZ* primers were ftsZAdf (5'-CTC AAG CAC TAG AAA AGT CG-3') and ftsZAdr (5'-TTA GCT CCT TCG CTT ACC TG-3'), and the B group specific *ftsZ* primers were ftsZBf (5'-CCG ATG CTC AAG CGT TAG AG-3') and ftsZBr (5'-CCA CTT AAC TCT TTC GTT TG-3'). PCR cycling conditions were 5 min at 94°C, 35 cycles (30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C) and 5 min at 72°C.

Samples were also subjected to PCR using primers specific for insect mitochondrial 12S rDNA in order to confirm successful DNA extraction. The primers for mitochondrial 12S rDNA were (5'-AAA CTA GGA TTA GAT ACC CTA TTA T-3') and (5'-AAG AGC GAC GGG CGA TGT GT-3'), known as 12SAI and 12SBI, respectively (Simon *et al*., 1991). PCR cycling conditions were 5 min at 94°C, 35 cycles (30 sec at 94°C, 30 sec at 52°C, 1 min at 72°C) and 5 min at 72°C.

### Phylogenetic analysis

Amplifications of *wsp* gene were performed using the general *wsp* primers (Braig *et al*., 1997): wsp81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') and wsp691R (5'-AAA AAT TAA ACG CTA CTC CA-3'). These primers amplify a DNA fragment of about 600 bp. The cycling conditions were the same as those for the amplification of *ftsZ*. PCR products were directly ligated into pCR II vector (Invitrogen) and sequenced with T7 and M13 reverse primers using an automated sequencer (SQ-5500, Hitachi). The sequences have been submitted to the GenBank database under accession numbers AB024569 for *E. kuehniella* Yokohama, AB024570 for *E. kuehniella* Tsuchiura, AB024571 for *E. cautella* A and AB024572 for *E. cautella* B.

The sequences obtained in this study were aligned, together with other *wsp* sequences from various insects, according to the alignment previously deposited in EMBL alignment database under accession number DS32273 (Zhou *et al*., 1998). A 41 bp region (positions 519–559) corresponding to the third hypervariable region of the gene (Braig *et al*., 1998) was omitted from the analysis because it could not be aligned with confidence (Zhou *et al*., 1998). Sites with gaps were also excluded from the aligned data set. The resulting alignment of approx. 500 bases was used to construct a neighbour-joining tree (Saitou and Nei, 1987) with Kimura's two parameter distance (Kimura, 1980) using the program package Clustal W (Thompson *et al*., 1994). The resulting tree was midpoint-rooted in the absence of a suitable outgroup. Bootstrap test (Felsenstein, 1981) was performed with 1000 replications.

### Crossing experiments

Females and males were separated at late larval stages. The male larvae were easily distinguished by dark patches (the testes) on the back. Crossing experiments were performed using single pairs of virgin females and males. One female and one male were placed in a 30 ml plastic cup and left for three days. During this period, most females laid more than one hundred eggs. Cups in which fewer than 50 eggs had been laid were discarded. From each cup, 50 to 100 eggs were collected and placed onto 1% agarose in a plastic dish (35 mm in diameter). The eggs were incubated at 25°C for 6–7 days and

the numbers of hatched and unhatched eggs were counted. The percentage of eggs which hatched was analyzed by *t*-test following arcsine-squareroot transformation.

## RESULTS AND DISCUSSION

*Wolbachia* infections in *P. interpunctella*, *E. cautella* and two strains of *E. kuehniella*, one from Tsuchiura city and the other from Yokohama city, were examined by PCR assay using primers specific for *Wolbachia ftsZ* gene (Fig. 1). In the PCR assay using the general *ftsZ* primers, fragments of expected size (ca. 1000 bp) were amplified from *E. cautella* and *E. kuehniella*, but not from *P. interpunctella*. PCR assay using primers for specific amplification of A and B group *ftsZ* genes showed that *E. cautella* is double-infected with both A and B group *Wolbachia* and two strains of *E. kuehniella* are infected with only A group *Wolbachia*.

To infer the relative phylogenetic relationships among *Wolbachia* harbored by *E. cautella* and *E. kuehniella*, we sequenced the *wsp* genes of these *Wolbachia* and constructed a phylogenetic tree with known *wsp* sequences of *Wolbachia* from several insects. Two different *wsp* sequences were obtained from *E. cautella*, one of 620 bp and the other 596 bp. These sequences were identical to those of A and B group *Wolbachia* of *E. cautella* Gainesville, respectively (Fig. 2). Since the *wsp* gene is highly variable between *Wolbachia* strains, it is likely that the Gainesville and Tsuchiura strains contain the same *Wolbachia*. We obtained 605 bp fragments from both *E. kuehniella* Tsuchiura and *E. kuehniella* Yokohama. There was only one synonymous nucleotide substitution between the two sequences, indicating that *Wolbachia* of the two strains of *E. kuehniella* are closely related. In the *wsp* phylogeny, *Wolbachia* of *E. kuehniella* are clustered with those of *Nasonia vitripennis*, *Glossina centralis* and *Glossina morsitans*, and this monophyletic group was supported by a bootstrap probability of 87.7%. Although *E. kuehniella* and *E. cautella* are closely related host species, their *Wolbachia* are phylogenetically distant from each other, suggesting that *Wolbachia* infections in these moths differ in their origin. It is likely that these moths have been infected with *Wolbachia* through different pathways of horizontal transmission.

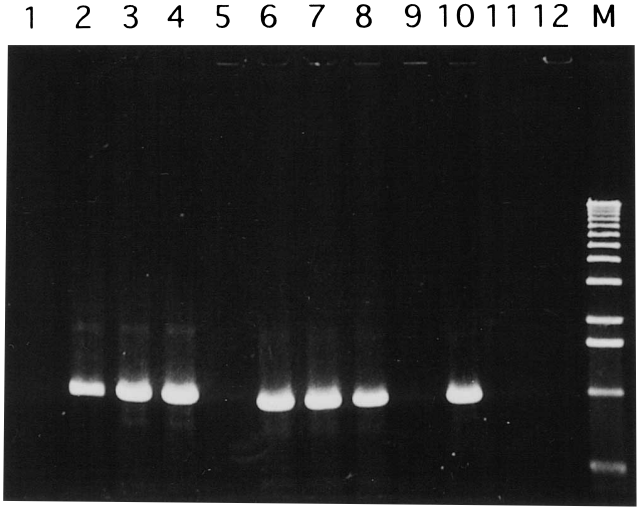


**Fig. 1.** PCR assay for the detection of *Wolbachia* in *Plodia interpunctella*, *Ephestia cautella* and two strains of *Ephestia kuehniella*. The general *ftsZ* primers were used in lanes 1–4. Primers for specific amplification of A and B group *ftsZ* were used in lane 5–8 and lane 9–12, respectively. The PCR products are about 1000 bp in size. Lanes: 1, 5 and 9, *P. interpunctella*; 2, 6 and 10, *E. cautella*; 3, 7 and 11, *E. kuehniella* Tsuchiura; 4, 8 and 12, *E. kuehniella* Yokohama; M, 1 kb DNA ladder (GIBCO BRL).

To examine cytoplasmic incompatibility in *E. cautella* and *E. kuehniella*, we generated *Wolbachia*-free strains by tetracycline treatment and performed crossing experiments. When infected males of *E. cautella* mated with uninfected females, no egg-hatch was observed (Table 1). The crosses of the other three combinations were compatible and more than 80% of the eggs hatched. Thus, *E. cautella* in the present study showed complete cytoplasmic incompatibility, which is consistent with the previous report on the strain collected in the USA (Kellen *et al.*, 1981)

Although the two strains of *E. kuehniella* harbor phylogenetically close *Wolbachia*, they showed significantly different levels of cytoplasmic incompatibility. The rates of egg hatch in the incompatible crosses between infected males and uninfected females were 60.8% in the Tsuchiura strain (Table 2) and 16.9% in the Yokohama strain (Table 3). We also performed crossing experiments between the two strains (Table 4). In the cross between infected Tsuchiura males and uninfected Yokohama females, the hatching rate was 58.4%, which was similar to the hatching rate observed in the cross between infected males and uninfected females of the Tsuchiura strain. In the same manner, the level of cytoplasmic incompatibility observed in the cross between infected Yokohama males and uninfected Tsuchiura females was similar to that in the incompatible cross within the Yokohama strain, suggesting that the intensity of cytoplasmic incompatibility depends on the strain of males, but not females used in the cross. The crosses between infected individuals were always fully compatible. In sum, the infected strains from Tsuchiura and Yokohama differ in the ability to cause cytoplasmic incompatibility, but not in the ability to rescue the modified sperm of infected males.

The different levels of cytoplasmic incompatibility observed in the two strains of *E. kuehniella* may arise through the evolutionary scenario proposed by Hurst and McVean (1996). The spread and maintenance of *Wolbachia* in a host population are determined by the initial frequency of infection, the rate of vertical transmission of *Wolbachia*, fitness cost of the infected host and the intensity of cytoplasmic incompatibility. Cytoplasmic incompatibility is a driving force by which *Wolbachia* spread into host population by decreasing the fitness of uninfected females. Theory suggests that when the frequency of infected hosts is adequately high, a $mod^{-}resc^{+}$ variant, which is incapable of modifying sperm to induce cytoplasmic incompatibility but is capable of rescuing the sperm modified by the resident strain, may arise and spread by para-

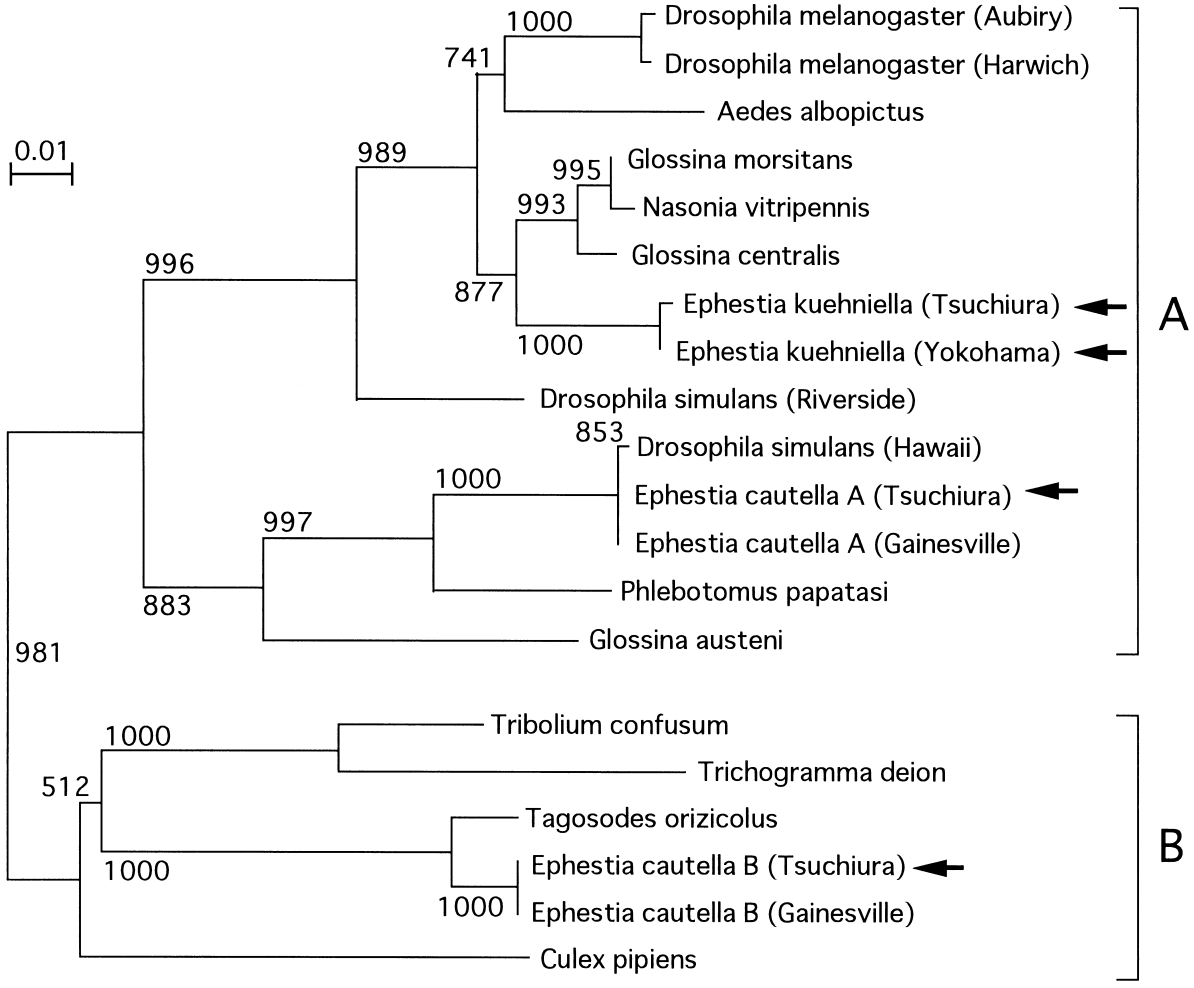


**Fig. 2.** Phylogenetic tree based on *wsp* sequences. The *wsp* sequences from *E. cautella* Tsuchiura, *E. kuehniella* Tsuchiura and *E. kuehniella* Yokohama were aligned with *wsp* sequences from other insects and analyzed by the neighbour-joining algorithm. Bootstrap values out of 1000 replicates are indicated next to nodes. The scale bar indicates genetic distance in units of nucleotide substitution per site. The GenBank accession numbers for *wsp* sequences are AF020058 for *Aedes albopictus*, AF020061 for *Culex pipiens*, AF020063 for *Drosophila melanogaster* Aubiry, AF020066 for *Drosophila melanogaster* Harwich, AF020068 for *Drosophila simulans* Hawaii, AF020070 for *Drosophila simulans* Riverside,AF020075 for *Ephestia cautella* Gainesville (A), AF020076 for *Ephestia cautella* Gainesville (B), AF020077 for *Glossina austeni*, AF020078 for *Glossina centralis*, AF020079 for *Glossina morsitans*, AF020081 for *Nasonia vitripennis*, AF020082 for *Phlebotomus papatasi*, AF020083 for *Tribolium confusum*, AF020084 for *Tricogramma deion* and AF020085 for *Tagosodes orizicolus*.

**Table 1.** Crossing experiments in *Ephestia cautella*

| cross (male × female) | number of crosses | mean % egg hatch | s.d. |
|---|---|---|---|
| uninfected × uninfected | 10 | 86.8 | 9.0 |
| uninfected × infected | 10 | 85.4 | 12.0 |
| infected × uninfected | 10 | 0 | 0 |
| infected × infected | 10 | 85.7 | 9.8 |

**Table 2.** Crossing experiments in *Ephestia kuehniella* Tsuchiura

| cross (male × female) | number of crosses | mean % egg hatch | s.d. |
|---|---|---|---|
| uninfected × uninfected | 10 | 96.9 | 2.6 |
| uninfected × infected | 10 | 95.5 | 1.3 |
| infected × uninfected | 50 | 60.8 | 25.3 |
| infected × infected | 10 | 96.1 | 1.8 |

**Table 3.** Crossing experiments in *Ephestia kuehniella* Yokohama

| cross (male × female) | number of crosses | mean % egg hatch | s.d. |
|---|---|---|---|
| uninfected × uninfected | 10 | 97.9 | 1.9 |
| uninfected × infected | 10 | 97.5 | 2.0 |
| infected × uninfected | 50 | 16.9 | 18.6 |
| infected × infected | 10 | 95.8 | 4.4 |

sitizing the modification effect. In fact, Bourtzis *et al.* (1998) discovered mod$^-$resc$^+$ variants by screening *Drosophila* strains. The two laboratory strains of *E. kuehniella* in the present study harbor phylogenetically close *Wolbachia* which are fully compatible with each other. It is possible that the Tsuchiura strain used to induce strong cytoplasmic incompatibility in the past, but is now changing towards the mod$^-$resc$^+$ phenotype.

**Table 4.** Crossing experiments between Tsuchiura and Yokohama stains of *Ephestia kuehniella*

| cross (male × female) | number of crosses | mean % egg hatch | s.d. |
|---|---|---|---|
| infected Tsuchiura × uninfected Yokohama | 50 | 58.4 | 22.8 |
| infected Yokohama × uninfected Tsuchiura | 50 | 12.8 | 14.4 |
| infected Tsuchiura × infected Yokohama | 10 | 97.7 | 2.2 |
| infected Yokohama × infected Tsuchiura | 10 | 97.0 | 2.8 |

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