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Wolbachia Transfer from *Rhagoletis cerasi* to *Drosophila simulans*: Investigating the Outcomes of Host-Symbiont Coevolution

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***Wolbachia* is an endosymbiont of diverse arthropod lineages that can induce various alterations of host reproduction for its own benefice. Cytoplasmic incompatibility (CI) is the most common phenomenon, which results in embryonic lethality when males that bear *Wolbachia* are mated with females that do not. In the cherry fruit fly, *Rhagoletis cerasi*, *Wolbachia* seems to be responsible for previously reported patterns of incompatibility between populations. Here we report on the artificial transfer of two *Wolbachia* variants (*w*Cer1 and *w*Cer2) from *R. cerasi* into *Drosophila simulans*, which was performed with two major goals in mind: first, to isolate *w*Cer1 from *w*Cer2 in order to individually test their respective abilities to induce CI in the new host; and, second, to test the theoretical prediction that recent *Wolbachia*-host associations should be characterized by high levels of CI, fitness costs to the new host, and inefficient transmission from mothers to offspring. *w*Cer1 was unable to develop in the new host, resulting in its rapid loss after successful injection, while *w*Cer2 was established in the new host. Transmission rates of *w*Cer2 were low, and the infection showed negative fitness effects, consistent with our prediction, but CI levels were unexpectedly lower in the new host. Based on these parameter estimates, neither *w*Cer1 nor *w*Cer2 could be naturally maintained in *D. simulans*. The experiment thus suggests that natural *Wolbachia* transfer between species might be restricted by many factors, should the ecological barriers be bypassed.**

Wolbachia is a maternally inherited α-proteobacterium and symbiont of arthropods (4, 26, 34, 42). This bacterium has an intracellular lifestyle, and infections occur throughout host somatic and germ line tissues of insect species (15). As a reproductive parasite, it manipulates host reproduction and favors in this way its own dispersal in host populations. The most common *Wolbachia* effect described so far is cytoplasmic incompatibility (CI) (8, 21). CI arises when infected males mate with uninfected females and results in embryonic lethality. Reciprocal crosses between infected females and uninfected males do not express CI. This pattern can be interpreted through a two-function model (29, 42): *Wolbachia* would somehow modify the sperm of infected males during spermatogenesis (modification, or *mod* function), leading to embryo death unless *Wolbachia* is present in the egg and restores viability (rescue, or *resc* function). The *mod* and *resc* functions seem to interact in a specific manner, because CI can also be observed in crosses between males and females that are both infected, if the two partners bear different *Wolbachia* variants.

CI allows *Wolbachia* to invade host populations because it increases the fitness of infected females relative to that of uninfected ones. Both theoretical and empirical studies (6, 16, 19, 36) have highlighted the key role of three parameters in the invasion dynamics: (i) CI level (the percentage of embryos killed by CI in incompatible crosses), (ii) the fitness effect of infection on female hosts (apart from CI), and (iii) the bacterial transmission efficiency from mothers to offspring. The studies described above showed that the frequency of infected individuals presents a stable equilibrium depending on these three parameters. The infection frequency reaches this stable equilibrium value only if it first passes a threshold frequency, the level of which also depends upon these three parameters.

CI is known for a variety of insect species, including the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). Early studies demonstrated high levels of incompatibility between populations of *R. cerasi* (1, 2), the basis of which was recently shown to involve *Wolbachia* (32). Populations of *R. cerasi* are either infected by a single *Wolbachia* variant, *w*Cer1, or superinfected by two variants, *w*Cer1 and -2. Incompatibility occurs between males from doubly infected populations and females from singly infected populations, suggesting the *w*Cer2 infection as the cause of CI (32). However, the picture is not perfectly clear. First, although it is likely that *w*Cer1 once invaded the species through CI, the ability of this variant to induce CI cannot be tested, because populations lacking *w*Cer1 have never been found. Second, the direct demonstration that *w*Cer2 is responsible for CI has not yet been provided by a set of replicate crosses with individuals of known infection status. The establishment of standardized infected and uninfected laboratory lines is time-consuming and not straightforward, given the long generation time and specialized biology of *R. cerasi*.

In this paper, we report on the artificial transfer of *Wolbachia* between two different dipteran families, from the true fruit fly, *R. cerasi*, into the geneticist's fruit fly, *Drosophila simulans* (Diptera, Drosophilidae), an extensively studied *Wolbachia* host (24). These experiments were done with two major

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goals in mind. The first objective was to obtain lines singly infected by *w*Cer1 and *w*Cer2 in order to test their ability to induce CI. Cytoplasmic injections have indeed been proven to be an efficient technique for stimulating *Wolbachia* segregation (9). The second objective was to test the prediction regarding the consequences of *Wolbachia*-host coevolution on three key parameters: maternal transmission efficiency, fitness effects, and CI levels. Selection on host factors tends to increase the efficiency of maternal transmission and to decrease CI levels and fitness costs (35). Selection on bacterial factors tends to increase the efficiency of maternal transmission and to decrease fitness costs. Selection on *Wolbachia* factors for CI levels is neutral as long as population structure is not too pronounced (30, 35). Coevolution is thus expected to lead to high transmission rates, low fitness costs, and low levels of CI. Reciprocally, low transmission efficiency, negative fitness effects, and high CI levels are expected after an injection of *Wolbachia* into a new host (11). The results presented are partially in agreement with these predictions. Indeed, a fitness cost to the host and low transmission efficiency are observed, as expected, but the level of CI is clearly reduced.

MATERIALS AND METHODS

***R. cerasi* and *D. simulans* lines.** Larvae of *R. cerasi* were collected from a *w*Cer1- and -2-infected population on honeysuckle (*Lonicera xylosteum*) in Vienna, Austria, in 1999. After pupation, puparia were stored under the optimal conditions (37). Emerging flies were kept in cages with water, adult diet, and artificial egg-laying devices (3). *D. simulans* STC was used as a recipient for the *Wolbachia* from *R. cerasi*. STC is an inbred stock from the Seychelles archipelago, originally infected by two *Wolbachia* strains, *w*Ha and *w*No, that was cured of infection following tetracycline treatment (28).

***Wolbachia* injection and line establishment.** The transfer of *w*Cer1 and *w*Cer2 into the *D. simulans* STC strain was performed by cytoplasmic injection (33). Using a microneedle (Femtotips; Eppendorf), cytoplasm was taken from *R. cerasi* eggs and injected into the posterior part of recipient eggs. Donor eggs were obtained by dissection directly from ovaries, providing fresh and weakly differentiated embryos. Fresh receiver eggs were collected from the egg-laying plates every hour. Recipient eggs were dechorionated manually prior to injection.

D. simulans females developing from injected eggs represent the generation 0 (G_0). Each G_0 female was crossed with one G_0 male and was left for laying before its infection status was determined by PCR. The infection status of the offspring was determined by PCR on a mass extraction of three G_1 females. In lines in which infection was detected in G_1, 10 G_1 sisters were mated to their brothers and left to lay separately before their infection status was determined.

During the experiment, all lines were maintained at 25°C at low larval densities in vials with axenic medium (14). Rates of transmission from mothers to offspring were low in transinfected lines, imposing stringent conditions for maintenance of infection. Thus, at every generation, and for every transinfected line, six females were left to lay independently before their infection status was determined. The next generation was then started by using offspring from infected females only.

CI tests. Individual crosses were done with 3-day-old virgin males and 4- to 5-day-old virgin females. Each cross was initiated by placing one male and one female in a vial with axenic medium. Copulation was monitored, allowing the discarding of pairs in which it lasted less than 15 min, to ensure that sperm was actually transferred. The male was then removed, and the female was supplied with an egg-laying plate for 48 h. Upon removal of the female, the eggs were placed at 25°C for 24 h before the egg hatch was measured by counting all eggs. Laying plates with less than 20 eggs were discarded. All individuals from infected strains were checked by PCR for the presence of *Wolbachia*.

Maternal transmission rates. Maternal transmission was first roughly estimated as the proportion of infected female daughters from infected mothers during the line establishment, up to G_{10}. The proportion of infected males was similarly assessed in G_8, G_9, and G_{10}. If CI occurs, this infection rate is an overestimate of the actual transmission rate: CI will increase the proportion of infected adults, because uninfected eggs tend to die. The actual maternal transmission rate of two lines was thus estimated after crossing infected females with uninfected males in G_{20}.

Measurements of fitness effects. Female fertility and fecundity were taken as parameters for the fitness effects of infections. These were investigated during CI assay experiments and therefore by using the same mating protocol. For fertility assays, uninfected males were crossed to infected and uninfected females, and hatching rates were compared. For fecundity assays, infected and uninfected males were crossed with infected and uninfected females. Fecundity was estimated by counting the eggs laid per female in 48 h.

PCR-RFLP and sequencing. DNA was extracted from flies according to the method described by O'Neill et al. (25). The PCR primers used were general primer 81F-691R of the *Wolbachia* surface protein gene *wsp* (44) as well as *w*Cer1- and *w*Cer2-specific *wsp* primer pairs (32), *fts*Zf1-*fts*Zr1 of the cell cycle gene *ftsZ* (41), and the 16S rRNA-specific primer for *Wolbachia* (25). PCRs were done in reaction volumes of 12.5 μl for the infection screening or in 50 μl for post-PCR procedures: 1 or 4 μl of template DNA, 1× reaction buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 μM forward and reverse primers, and 0.5 or 2 U of *Taq* DNA polymerase (Gibco), and sterile water was added to the final volume. PCR was run under conditions described by Zhou et al. (44). *wsp*, *ftsZ*, and 16S rRNA PCR products from *w*Cer1-infected *R. cerasi*, *w*Cer2-infected *D. simulans*, and *w*Au infected *D. simulans* were cycle sequenced with Big Dye (Perkin-Elmer). *w*Cer2 and *w*Au differ in their 81F-691R *wsp* sequence by one substitution (32). This mutation site proved to be a *w*Cer2-specific restriction site for *Fnu*4H1. *w*Cer2-infected lines were PCR-restriction fragment length polymorphism (RFLP) digested with *Fnu*4HI (New England Biolabs) under the standard conditions recommended by the restriction enzyme provider, in order to exclude any line or strain contamination with *w*Au.

Statistical analysis. CI and fertility data were analyzed with Wilcoxon's nonparametric tests. Fecundity data were analyzed by analysis of variance (ANOVA).

Nucleotide sequence accession number. The *ftsZ* sequences from *w*Cer1, *w*Cer2, and *w*Au have been deposited in the GenBank nucleotide sequence database under accession no. AY227737 to -39, respectively. The 16S rRNA gene sequences from *w*Cer1, *w*Cer2, and *w*Au have been deposited under accession no. AY227740 to -42, respectively.

RESULTS

Line establishment. A total of 1,036 embryos of the uninfected *D. simulans* STC line were injected with cytoplasm of *w*Cer1- and -2-infected *R. cerasi*. From these, 82 embryos developed into adult females, 51 of which were infected. The different infection types were *w*Cer1 and -2 ($n = 31$), *w*Cer2 ($n = 12$), and *w*Cer1 ($n = 8$). Thus, segregation between *w*Cer1 and *w*Cer2 already occurred after injection into generation 0 (G_0). Transmission of *w*Cer1 and/or *w*Cer2 from G_0 to G_1 was found in 18 females. From these G_0 females, about 10 daughters were taken for line establishment. Only 3 out of 187 G_1 females were superinfected with *w*Cer1 and -2, 38 were infected with *w*Cer2, and 8 were infected with *w*Cer1. *w*Cer1 was lost from all lines between G_1 and G_2, despite efforts to detect rare infected G_2 females. In G_6, six isofemale lines remained infected by *w*Cer2: RC20, RC21, RC33, RC45, RC50, and RC78. The six lines were from six different G_0 females injected with *w*Cer1 and -2 cytoplasm. Uninfected lines RC20Ø, RC21Ø, RC33Ø, RC45Ø, RC50Ø, and RC78Ø were founded with uninfected G_1 females, sisters of the infected females used for the establishment of the infected lines.

Transmission rates. The infection rates in offspring from *w*Cer2 mothers were measured during the line establishment from generations 1 to 10, giving the following estimates: 54% in RC20 ($n = 30$; 95% confidence interval, 36.2 to 71.8%), 61% in RC21 ($n = 102$; 95% confidence interval, 51.5 to 70.5%), 65% in RC33 ($n = 50$; 95% confidence interval, 51.8 to 78.2%), 80% in RC45 ($n = 129$; 95% confidence interval, 73.1 to 86.9%), 52% in RC50 ($n = 43$; 95% confidence interval, 37 to 66.9%), 86% in RC78 ($n = 33$; 95% confidence interval, 74.2 to 97.8%). These infection rates are overesti-

TABLE 1. Crossing experiments to test whether *w*Cer2 does induce cytoplasmic incompatibility in *D. simulans*

Generation[a]	Fly[b]		No. of crosses	No. of eggs counted	Mean % embryonic mortality (SE)	Wilcoxon's test result[c]	*P*[d]
	Male	Female					
G_8	RC21 (*w*Cer2)	RC21Ø (Ø)	15	1,613	29.2 (4.5)		
G_8	RC21Ø (Ø)	RC21Ø (Ø)	10	1,093	11.7 (5.9)	2.607	<0.01
G_9	RC21 (*w*Cer2)	RC21Ø (Ø)	4	491	40.5 (9.2)		
G_9	RC21Ø (Ø)	RC21Ø (Ø)	8	791	14.9 (3.7)	2.378	<0.02
G_{10}	RC21 (*w*Cer2)	RC21Ø (Ø)	9	1,011	33.1 (4.6)		
G_{10}	RC21Ø (Ø)	RC21Ø (Ø)	8	849	13.5 (3.6)	2.887	<0.01
G_8	RC33 (*w*Cer2)	RC33Ø (Ø)	5	584	14.7 (4.5)		
G_8	RC33Ø (Ø)	RC33Ø (Ø)	6	593	13.9 (4.3)	0.183	<0.86
G_{10}	RC33 (*w*Cer2)	RC33Ø (Ø)	14	1,465	13.6 (2.6)		
G_{10}	RC33Ø (Ø)	RC33Ø (Ø)	8	722	4.8 (1.3)	2.355	<0.02
G_8	RC45 (*w*Cer2)	RC45Ø (Ø)	19	1,896	36.5 (4.2)		
G_8	RC45Ø (Ø)	RC45Ø (Ø)	8	717	24.5 (9.3)	1.540	<0.13
G_9	RC45 (*w*Cer2)	RC45Ø (Ø)	9	1,077	64.6 (9.5)		
G_9	RC45Ø (Ø)	RC45Ø (Ø)	8	920	11.9 (8.3)	2.983	<0.01
G_{10}	RC45 (*w*Cer2)	RC45Ø (Ø)	9	1,061	44.8 (9.9)		
G_{10}	RC45Ø (Ø)	RC45Ø (Ø)	8	819	5.8 (1.3)	3.464	<0.001
G_8	RC50 (*w*Cer2)	RC50Ø (Ø)	9	784	43.2 (8.2)		
G_8	RC50Ø (Ø)	RC50Ø (Ø)	7	608	14.1 (2.8)	2.699	<0.01
G_{10}	RC50 (*w*Cer2)	RC50Ø (Ø)	4	289	33.8 (8.9)		
G_{10}	RC50Ø (Ø)	RC50Ø (Ø)	5	528	6.8 (1.3)	2.449	<0.02

[a] Generation following injection.
[b] The infecting *Wolbachia* variant is shown in parentheses. Ø, uninfected.
[c] The Wilcoxon's tests were performed by comparing each cross involving infected males with the corresponding control cross, in which the male is not infected.
[d] *P*, associated α probability.

mates of the maternal transmission rate, because the infection status of fathers was not checked. The proportion of infected individuals could be greater in crossings between infected females and infected males than between infected females and uninfected males, because CI selects for higher infection rates in the offspring.

The actual maternal transmission rates in RC21 and RC45 were estimated in G_{20} by crossing infected females with uninfected males. The transmission rates were 77% for RC21 (n = 60; 95% confidence interval, 66.3 to 87.6%) and 55% for RC45 (n = 71; 95% confidence interval, 43.4 to 66.6%).

CI assays. The expression of CI was tested by crossing uninfected females with infected and uninfected males. CI is observed if embryonic mortality is significantly higher when males are infected. This was investigated by using four infected lines (RC21, RC45, RC33, and RC50) and their uninfected counterparts (RC21Ø, RC45Ø, RC33Ø, and RC50Ø). As shown in Table 1, *w*Cer2 was found to induce CI in 8 out of 10 experiments, although at a low level.

The ability of *w*Cer2 to rescue its own CI expression was tested by crossing infected males with infected and uninfected females. Rescue is observed if embryonic mortality is significantly lower when females are infected. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 2, significant rescue was found in both experiments.

To test if this rescue was complete, infected females were crossed with infected and uninfected males. Rescue can be considered as complete if embryonic mortality is not significantly higher when males are infected. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 3, rescue was not found complete in the experiment involving the RC45 and RC45Ø lines, while *P* was found just above the 5%

TABLE 2. Test of whether *w*Cer2 is able to rescue its own modification in *D. simulans*[a]

Fly[b]		No. of crosses	No. of eggs counted	Mean % embryonic mortality (SE)	Wilcoxon's test result[c]	*P*[d]
Male	Female					
RC21 (*w*Cer2)	RC21Ø (Ø)	13	1,502	35.4 (3.9)		
RC21 (*w*Cer2)	RC21 (*w*Cer2)	12	1,249	22.8 (3.0)	2.393	<0.02
RC45 (*w*Cer2)	RC45Ø (Ø)	18	2,138	54.7 (6.9)		
RC45 (*w*Cer2)	RC45 (*w*Cer2)	20	1,744	28.2 (3.3)	2.938	<0.01

[a] Data were pooled from two experiments (performed in G_9 and G_{10}), after testing for homogeneity.
[b] The infecting *Wolbachia* variant is given in parentheses. Ø, uninfected.
[c] The Wilcoxon's tests were performed by comparing each pair of crosses.
[d] *P*, associated α probability.

TABLE 3. Test of whether *w*Cer2 totally rescues its own modification in *D. simulans*[a]

Fly[b]		No. of crosses	No. of eggs counted	Mean % embryonic mortality (SE)	Wilcoxon's test result[c]	*P*[d]
Male	Female					
RC21Ø (Ø)	RC21 (*w*Cer2)	13	1,216	13.7 (3.4)		
RC21 (*w*Cer2)	RC21 (*w*Cer2)	12	1,249	22.8 (3.0)	1.904	<0.06
RC45Ø (Ø)	RC45 (*w*Cer2)	15	1,281	15.5 (6.5)		
RC45 (*w*Cer2)	RC45 (*w*Cer2)	20	1,744	28.2 (3.3)	3.2	<0.02

[a] To increase sample size, data were pooled from two experiments (performed in G_9 and G_{10}), after testing for homogencity. Crosses between infected males and infected females are the same as in Table 2.
[b] The infecting *Wolbachia* variant is given in parentheses. Ø, uninfected.
[c] The Wilcoxon's tests were performed by comparing each pair of crosses.
[d] *P*, associated α probability.

threshold in the experiment involving RC21 and RC21Ø. Thus, the data suggest that *w*Cer2 does not fully rescue its own CI. As discussed below, imperfect transmission is thought to be the likely explanation.

Fitness effects. The effect of *w*Cer2 on female fertility can be tested by crossing uninfected males with infected and uninfected females. A positive or negative effect on fertility is detected if hatching rates differ in the two crosses. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 4, *w*Cer2 was not found to affect female fertility.

The effects of *w*Cer2 on female fecundity were tested by crossing infected and uninfected females with both infected and uninfected males (lines RC21 and RC45 and RC21Ø and RC45Ø, respectively). The results, presented in Table 5, were analyzed by ANOVA (Table 6). In the experiment involving RC21 and RC21Ø, a surprising effect of male infection status was observed. Indeed, females appeared to lay significantly more eggs when mated with infected males. In this experiment, infected females were less fecund than uninfected ones, but this difference was not significant at the 0.05 threshold. In the experiment involving RC45 and RC45Ø, no effect of male infection was found. Again, infected females were less fecund than uninfected ones, and here the difference was significant. Thus, the data suggest that *w*Cer2 reduces fecundity in infected females.

PCR-RFLP and sequencing. Sequenced *wsp* PCR products and PCR-RFLP from single flies of strains RC21 and RC45 confirmed the presence of *w*Cer2 in these lines. Contamination with *w*Au did not occur. *ftsZ* PCR products of *w*Cer2-infected *D. simulans*, *w*Au-infected *D. simulans* Coffs Harbor, and of *w*Cer1-infected *R. cerasi* flies were sequenced. *w*Cer2 and *w*Au shared the same *ftsZ* sequences, confirming their close genetic relationship. *w*Cer1 was more distantly related, and sequence divergences in *ftsZ* (2.23% in 941 bp) and *wsp* (2.38 to 2.55% to *w*Cer2 and *w*Au, respectively, in 588 bp) (32) were similar. Interestingly, substitutions were equally spread through *ftsZ* of *w*Cer1, whereas they were restricted to the 3′ region of *wsp*. Most substitutions in *wsp* of *w*Cer1 were nonsynonymous. All three strains *w*Cer1, *w*Cer2, and *w*Au shared the same 16S rRNA sequences.

DISCUSSION

Injection, segregation, and infection loss. After injection from superinfected *R. cerasi* into *D. simulans*, *w*Cer1 and *w*Cer2 segregated in G_0. In their original host, segregation of *w*Cer1 and *w*Cer2 was observed at a rate of <1% in field populations, whereby in all cases, *w*Cer1 was the leaking variant (32). High segregation rates during injection most probably result from the low number of bacterial cells that are injected within a single recipient egg and actually survive.

Both *w*Cer1 and *w*Cer2 were still detectable by PCR in G_1 following injection, suggesting that both variants reached the germ cells of G_0 females. However, *w*Cer1 was lost from all lines between G_1 and G_2, suggesting that it was unable to develop properly in this new host or to actively maintain itself in the germ line. This loss was unfortunate, because it prevented us from determining the phenotypic effects of *w*Cer1, yet it also proved to be an informative result. The incapacity of *w*Cer1 to develop in a new host might reflect a higher genetic divergence from *w*Cer2 and a very tight and specific adaptation to the original host. This interpretation is consistent with the view that *w*Cer1 is a more ancient infection in *R. cerasi* than is

TABLE 4. Fertility test of *w*Cer2-infected *D. simulans* females[a]

Fly[b]		No. of crosses	No. of eggs counted	% Fertility (SE)	Wilcoxon's test result[c]	*P*[d]
Male	Female					
RC21Ø (Ø)	RC21Ø (Ø)	16	1,640	85.8 (2.4)		
RC21Ø (Ø)	RC21 (*w*Cer2)	13	1,216	86.3 (3.4)	0.395	<0.7
RC45Ø (Ø)	RC45Ø (Ø)	16	1,739	91.2 (4.0)		
RC45Ø (Ø)	RC45 (*w*Cer2)	15	1,281	84.5 (6.5)	1.107	<0.27

[a] To increase sample size, data were pooled from two experiments (performed in G_9 and G_{10}) after testing for homogeneity. Crosses between uninfected males and infected females are the same as in Table 3. Crosses between uninfected males and uninfected females are the same as in Table 1.
[b] The infecting *Wolbachia* variant is shown in parentheses. Ø, uninfected.
[c] The Wilcoxon's tests were performed by comparing each pair of crosses.
[d] *P*, associated α probability.

TABLE 5. Descriptive statistics for fecundity testing of *w*Cer2-infected *D. simulans* females[a]

Fly[b]		No. of crosses	No. of eggs counted	Avg no. of eggs laid/female (% SE)
Male	Female			
RC21Ø (Ø)	RC21Ø (Ø)	16	1,640	102.5 (4.7)
RC21 (*w*Cer2)	RC21Ø (Ø)	13	1,502	115.5 (5.9)
RC21Ø (Ø)	RC21 (*w*Cer2)	13	1,216	93.54 (7.0)
RC21 (*w*Cer2)	RC21 (*w*Cer2)	12	1,249	104.18 (4.8)
RC45Ø (Ø)	RC45Ø (Ø)	16	1,739	108.7 (5.5)
RC45 (*w*Cer2)	RC45Ø (Ø)	18	2,138	118.8 (4.0)
RC45Ø (Ø)	RC45 (*w*Cer2)	15	1,281	85.4 (8.1)
RC45 (*w*Cer2)	RC45 (*w*Cer2)	20	1,744	87.2 (4.3)

[a] To increase sample size, data were pooled from two experiments (performed in G_9 and G_{10}), after testing for homogeneity.
[b] The infecting *Wolbachia* variant is shown in parentheses. Ø, uninfected.

*w*Cer2, as suggested by infection patterns in natural populations (32). On the contrary, *w*Cer2 was still present in G_2. Although the efficiency of maternal transmission is low in *D. simulans*, imposing a stringent protocol for infection maintenance, we still possess, at the time of writing, the six lines derived from six different G_0 females.

CI levels, fitness effects, and transmission efficiency. We found that *w*Cer2 can induce CI in *D. simulans*, although embryonic lethality is far from 100%. This confirms that *w*Cer2 is able to induce CI and strengthens the view that it is responsible for the patterns of incompatibility observed between *R. cerasi* populations (2).

We observed that *w*Cer2 is able to rescue its own CI, but only partially so. This probably results from imperfect maternal transmission (i.e., not all eggs are infected and therefore protected from CI). The transmission rates that would be necessary to explain the imperfect rescue would be 55 to 65% for RC21 and RC45. Similar transmission rate values were observed for both lines at G_{20}. Thus, it seems that *w*Cer2 is not, strictly speaking, self-incompatible. Partial nonrescue is simply due to imperfect maternal transmission.

*w*Cer2 does not affect female fertility, but seems to reduce female fecundity by at least 10%. Negative effects on host fitness have been reported previously in natural as well as artificial *Wolbachia*-host associations (19, 21). Intriguingly, in one data set (involving lines RC21 and RC21Ø), females were found to lay more when mated with infected males—a result that we fail to interpret in adaptive terms.

*w*Cer1 was not transmitted after G_1, while *w*Cer2 had a low transmission rate. This can be seen by the infection frequency observed during line maintenance, giving a mean value of 66% for the six transinfected lines. Transmission efficiency per se was estimated at G_{20} in lines RC21 and RC45, giving a mean value of 65.5%, which is much lower than any maternal transmission rate reported so far for natural *Wolbachia*-host associations. We observed considerable variability within and between the transinfected lines in their infection rates with *w*Cer2 over a long time, here represented by the data from generations 1 to 10 and from generation 20. This variability was not correlated to generation number or lines. We do not yet have an explanation for this finding.

Testing theory. Theory predicts that *Wolbachia*-host coevolution should lead to a decline of CI level and fitness costs and to an increase in maternal transmission (30, 35). Inversely, strong CI, strong costs, and low transmission rates are expected in new associations (11). We tested this prediction by creating a new association and measuring the parameters. As expected, fitness costs to the host and low transmission rates were observed, but CI levels were very low. *Wolbachia* density in male testes has been recognized as a key factor for the expression of CI in *Wolbachia* associations (8, 12, 40). Whether the lower expression of CI of *w*Cer2 in *D. simulans* is correlated with a reduced density still needs to be assessed. However, from an evolutionary perspective, there are two possible explanations why CI levels might be low in the novel *w*Cer2 *D. simulans* association.

First, *D. simulans* might actively repress the expression of *w*Cer2. This is plausible because *w*Cer2 is very closely related to *w*Au, a natural *Wolbachia* variant of *D. simulans*, which does not appear to induce CI in this host (10, 20, 23, 31). Although *w*Au might have lost its ability to induce CI, regardless of the host background, a possibility remains that *D. simulans* actively and specifically represses its expression. This being so, *D. simulans* might recognize *w*Cer2 as *w*Au-like *Wolbachia* and therefore repress it.

Alternatively, the *w*Cer2 infection might be maladapted to the new host and therefore not be able to induce high levels of CI in a new host background. Hence, the prediction that CI should be high in new associations might be incorrect. Levels of CI expressed in different host species have so far only been compared in experiments in which the original and novel host were closely related (5, 11, 27). High levels of CI were observed after the transfer of *w*Ri from *D. simulans* into *Drosophila serrata* (11) and after the transfer of *w*Mel-infected *D. melanogaster* into *D. simulans* (27). However, these results could reflect the evolutionary closeness of *Drosophila* species rather than the ability of *Wolbachia* to express high CI in any background. High CI levels might in fact not always be the sign of a recent *Wolbachia*-host association. Prout (30) and Turelli (35) demonstrated that within panmictic populations, bacterial variants inducing higher CI levels are not selected for, but Frank (17) showed that if the population is structured, bacte-

TABLE 6. ANOVA results for fecundity testing of *w*Cer2-infected *D. simulans* females

Source and line	df[a]	Mean square	F	*P*
RC21 and RC21O				
Male infection	1	1,855.89	4.70	<0.04
Female infection	1	1,390.95	3.52	<0.07
Male-by-female infection	1	20.75	0.05	<0.82
Error	50	394.79		
RC45 and RC45O				
Male infection	1	602.32	1.27	<0.27
Female infection	1	12,824.52	27.00	$<10^{-4}$
Male-by-female infection	1	292.81	0.62	0.44
Error	65	474.91		

[a] df, degree of freedom.

rial variants inducing higher levels of CI are advantaged. Population structure might be sufficiently important for strong CI levels to be maintained in the long term.

The likelihood of horizontal transfers. From phylogenies of *Wolbachia* and their hosts, as well as direct observation, it is now clear that horizontal transfers between species can occur (18, 22, 25, 38, 41, 44). *Wolbachia* in arthropods could be seen as a huge metapopulation with infected host species as habitats for various subpopulations (7). Within host species, extinction and colonization might regularly occur through loss or gain of infection, and the current distribution of *Wolbachia* could represent a global and dynamic equilibrium between these two processes (43).

Following the ideas of Combes (13, 39), it can be generalized that *Wolbachia* must cross three filters (ecological, physiological, and population) before it is established in a new host species. The ecological filter is defined by the interaction between an existing and a potential new host species. It will condition the probability for *Wolbachia* of getting in contact with a new species within an individual's body. The physiological filter is defined by the ability of *Wolbachia* to colonize the germ line of an individual. Finally, the population filter conditions the ability of *Wolbachia* to invade and maintain itself in host populations, which depends on the values of the three main parameters: strength of CI, maternal transmission efficiency, and fitness effects on the host (19, 36).

Here, the ecological filter was bypassed as *Wolbachia* was intentionally injected into the new host. *w*Cer1 and *w*Cer2 were both established in the germ line. However, *w*Cer1 was lost after the first generations, whereas *w*Cer2 was maintained. The three parameters influencing *Wolbachia* invasion dynamics (CI level, transmission efficiency, and fitness effects) were far from optimal. Based on formulas from the model of Hoffmann et al. (19), and using the estimated parameter values, the only possible infection frequency at equilibrium for *w*Cer2 is 0. In other words, should *w*Cer2 cross the ecological barriers by natural means, it would not be able to invade populations of *D. simulans*, nor would it be able to maintain itself starting from a high frequency. Our results thus suggest that the horizontal transfer between evolutionarily distant species was, in this case at least, very unlikely or impossible. Within the *Wolbachia* metapopulation, subpopulations (i.e., *Wolbachia* variants) seem to be adapted to local habitats (species or groups of closely related species). The population filter, the ability to invade host populations, might in fact be the most critical step, preventing *Wolbachia* from invading all arthropod species.

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