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INCIPIENT EVOLUTION OF *WOLBACHIA* COMPATIBILITY TYPES

SYLVAIN CHARLAT,[1,2] MARKUS RIEGLER,[3,4] ISABELLE BAURES,[1] DENIS POINSOT,[5] CHRISTIAN STAUFFER,[3] AND HERVÉ MERÇOT[1]

[1]*Institut Jacques Monod, CNRS-Universités Paris 6,7, Laboratoire Dynamique du Génome et Evolution, 2 place Jussieu, 75251 Paris Cedex 05, France*

[3]*Institute of Forest Entomology, Forest Pathology and Forest Protection, Department for Forest and Soil Sciences, BOKU, University of Natural Resources and Applied Life Sciences, Vienna, Hasenauerstrasse 38, 1190, Austria*

[5]*Université de Rennes 1, Equipe d'écobiologie des insectes parasitoïdes, Campus Beaulieu, bâtiment 25, 35042 Rennes Cedex, France*

Abstract.—Cytoplasmic incompatibility (CI) is induced in arthropods by the maternally inherited bacterium *Wolbachia*. When infected males mate with uninfected females or with females bearing a different *Wolbachia* variant, paternal chromosomes behave abnormally and embryos die. This pattern can be interpreted as resulting from two bacterial effects: One (usually termed *mod*, for modification) would affect sperm and induce embryo death, unless *Wolbachia* is also present in the egg, which implies the existence of a second effect, usually termed *resc*, for rescue. The fact that CI can occur in crosses between males and females infected by different *Wolbachia* shows that *mod* and *resc* interact in a specific manner. In other words, different compatibility types, or *mod*/*resc* pairs seem to have diverged from one (or a few) common ancestor(s). We are interested in the process allowing the evolution of *mod*/*resc* pairs. Here this question is addressed experimentally after cytoplasmic injection into a single host species (*Drosophila simulans*) by investigating compatibility relationships between closely related *Wolbachia* variants naturally evolving in different dipteran hosts: *D. simulans*, *Drosophila melanogaster*, and *Rhagoletis cerasi*. Our results suggest that closely related bacteria can be totally or partially incompatible. The compatibility relationships observed can be explained using a formal description of the *mod* and *resc* functions, implying both qualitative and quantitative variations.

Key words.—Compatibility types, cytoplasmic incompatibility, evolution, *mod resc* model, *Wolbachia*.

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Among the various known effects of the endocellular bacterium *Wolbachia* in its arthropod hosts is cytoplasmic incompatibility (CI; reviewed in Hoffmann 1997; Charlat et al. 2001a; Bourtzis et al. 2003). It occurs when males bearing the bacterium mate with uninfected females; such a cross results in embryo death. On the contrary, hatching rates are normal if the female is also infected or if the male is not infected. Thus, in mixed populations, infected females have a reproductive advantage over uninfected ones, which leads to increased infection frequencies.

The phenomenon is well characterized cytogenetically (Breeuwer and Werren 1990; Callaini et al. 1996, 1997; Lassy and Karr 1996; Tram and Sullivan 2002). In incompatible crosses, paternal chromosomes fail to condense normally or at a sufficiently high speed, so that maternal chromosomes segregate on their own at the first mitosis. In diploid organisms, this typically results in developmental arrest. In haplodiploids, where males naturally develop from unfertilized haploid eggs, CI-induced haploidy either results in male development from fertilized eggs or embryo death (Vavre et al. 2000; Bordenstein et al. 2003).

The bacterial molecules involved are still unknown. The current framework is that of the modification-rescue model (Werren 1997), according to which two phenomena must be distinguished: one occurring in the male germline (termed *mod*, for modification) disrupting paternal chromosomes behavior and one occurring in infected eggs (termed *resc*, for rescue) restoring normal development. Attempts have been made to translate *mod* and *resc* into more concrete factors. It has been argued that a lock-and-key model, assuming that *mod* (the lock) and *resc* (the key) are controlled by different genetic determinants and directly interact with each other, is the most likely to be valid (Poinsot et al. 2003).

Besides the incompatibility between infected males and uninfected females (often termed unidirectional, because the reverse cross is compatible), bidirectional incompatibility can also occur if males and females bear different *Wolbachia* variants. This more complex form of CI demonstrates that *mod* and *resc* interact in a specific manner. This means different compatibility types (or *mod*/*resc* pairs) can diverge from a common ancestor (assuming, as is most likely, that not all CI inducing *Wolbachia* derive from a new evolution of the CI phenomenon itself). We are interested in the process behind the divergence of compatibility types. A theoretical analysis focusing on this issue has suggested that compatibility types can evolve if *mod* and *resc* are controlled by different genetic determinants (Charlat et al. 2001b). Empirically, this question has been investigated in *Drosophila simulans* and *Drosophila sechellia*, which are infected by closely related *Wolbachia* variants having evolved separately for not more than half a million years (Rousset and Solignac 1995). Relatedness between the bacteria of the two species is such that no molecular divergence is detectable, based on the 16S rRNA locus or the faster evolving *wsp* gene (Zhou et al. 1998; Charlat et al. 2002). The compatibility relationship between these *Wolbachia* sister-strains was investigated by injecting the bacteria from *D. sechellia* into *D. simulans* (Charlat et al. 2002) and it was found that the two strains remained fully compatible after this period of isolation. The present study goes one step further in the empirical inves-

[2] Present address: University College London, Department of Biology, 4 Stephenson Way, London NW1 2HE, United Kingdom; E-mail: s.charlat@ucl.ac.uk.

[4] Present address: Department of Zoology and Entomology, University of Queensland, St. Lucia QLD 4072, Australia.

tigation of the evolution of the *mod-resc* interaction: closely related, but molecularly distinguishable *Wolbachia* were placed in a single host genetic background (*D. simulans*) and their relationships tested.

The study involves three dipteran species: *Rhagoletis cerasi* (Tephritidae), *Drosophila melanogaster*, *D. simulans* (Drosophilidae) and some of their symbionts. *Rhagoletis cerasi* is infected by two *Wolbachia* variants (namely *w*Cer1 and *w*Cer2). *w*Cer2 is known to induce strong CI in this species because males from doubly infected populations (with individuals bearing two *Wolbachia* variants) are incompatible with females from populations bearing only *w*Cer1 (Riegler and Stauffer 2002). After transfer into *D. simulans*, *w*Cer2 was found to induce low but significant levels of CI (about 40% embryonic mortality; Riegler et al. 2004). *Drosophila melanogaster* is infected by a *Wolbachia* called *w*Mel that can induce CI in its original host, although at a low level, unless very young males are used in experiments (Hoffmann 1988; Hoffmann et al. 1994, 1998; Solignac et al. 1994; Olsen et al. 2001; Reynolds and Hoffmann 2002). After transfer into *D. simulans*, *w*Mel was found to induce very strong CI (near 100% embryonic mortality; Poinsot et al. 1998). *Drosophila simulans* is naturally infected by five different *Wolbachia* (reviewed in Merçot and Charlat 2003). The one studied here is called *w*Au. In the populations where this has been investigated directly, *w*Au was not found to induce CI (Hoffmann et al. 1996; James and Ballard 2000; Reynolds and Hoffmann 2002; Charlat et al. 2003). An intriguing case is the observation in the Lantana population from Florida (Ballard et al. 1996), where two *Wolbachia* infected lines induced significant CI. Later sequencing revealed the presence of *w*Au in these lines (James and Ballard 2000), suggesting *w*Au was responsible for this phenotype. The *w*Cer2, *w*Mel, and *w*Au triangle is of interest regarding the evolution of CI because these three *Wolbachia* are very closely related as indicated by the *wsp* gene and confirmed by the ftsZ and 16S loci (Zhou et al. 1998; Riegler and Stauffer 2002; Riegler et al. 2004). Specifically, based on 588 bp of the *wsp* locus, *w*Mel and *w*Au differ by five substitutions, *w*Mel and *w*Cer2 by four substitutions, and *w*Cer2 and *w*Au by one substitution. Figure 1 shows the most parsimonious phylogeny that can be inferred based on this limited variation.

In addition to these three *Wolbachia*, the *w*Ri variant (a natural infection of *D. simulans* inducing high levels of CI) was included in this study. This was prompted by earlier results, having revealed intriguing compatibility relationships between *w*Ri and *w*Mel (Poinsot et al. 1998). Based on *wsp* sequences, the *w*Ri variant clearly falls out of the *Mel* clade, the group including *w*Mel, *w*Cer2, and *w*Au (Zhou et al. 1998). Actually, *w*Ri is even more distant from this group than is the *w*Cer1 variant, used as an outgroup in Figure 1.

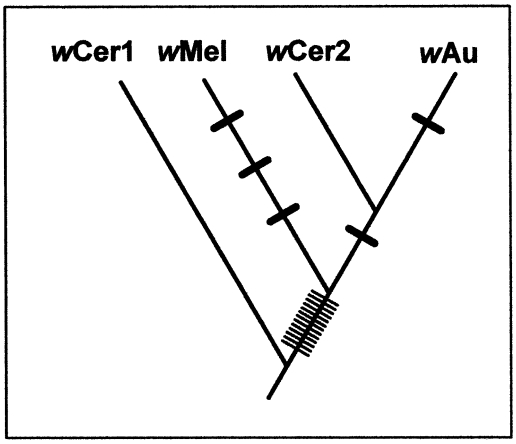

FIG. 1. Phylogenetic relationships between *w*Cer2, *w*Mel, and *w*Au based on *wsp* sequences. The gene region upon which this phylogeny is based is highly variable and thus cannot be aligned confidently with most *Wolbachia* sequences. The *w*Cer1 sequence (Riegler and Stauffer 2002), however, is sufficiently close to the *Mel* clade (the group including *w*Mel, *w*Cer2, and *w*Au) for a good alignment to be obtained and was used as an outgroup here. In this tree, the monophyly of the *Mel* clade is supported by 13 substitutions. Among the five substitutions that occurred within the *Mel* clade, four are noninformative (three autapomorphies of *w*Mel and one autapomorphy of *w*Au) but one supports the monophyly of the *w*Au + *w*Cer2 group. Thin tics symbolize synapomorphies of the *Mel* clade, thick tics symbolize substitutions within the *Mel* clade.

MATERIALS AND METHODS

Drosophila simulans Lines

RC45 and RC50 are two lines infected by *w*Cer2, obtained by cytoplasmic injection into the STC strain (Riegler et al. 2004). STC is an inbred stock from the Seychelles Archipelago, originally infected by two *Wolbachia* (*w*Ha and *w*No) and cured from its infection following a tetracycline treatment (Poinsot et al. 2000). Coffs Harbour S20 is an Australian strain founded using flies from a 1993 collection infected by *w*Au (Hoffmann et al. 1996). Y6 is an isofemale lines from Yaounde (Cameroon) infected by *w*Au (Charlat et al. 2003). ME29 is infected by *w*Mel, following cytoplasmic injection from *D. melanogaster* into a tetracycline-treated *D. simulans* line from New Caledonia (Poinsot et al. 1998). ME29TC is an uninfected line, cured from infection following a tetracycline treatment on the ME29 line (this study). DSR is a Californian strain infected by *w*Ri (Hoffmann et al. 1986). DSRTC is an uninfected line, cured from infection following a tetracycline treatment on the DSR line (this study). Antibiotic treatments were performed at least 10 generations before the experiments. Curing was performed on 10 females isolated from each other, which allowed us to check infection status in each female's offspring. The deriving uninfected lines were pooled a few generations later.

Wolbachia Detection

In all experiments, detection of *Wolbachia* was done by polymerase chain reaction (PCR). DNA was obtained according to O'Neill et al. (1992). The *wsp* gene was amplified according to Zhou et al. (1998) the 16S gene according to O'Neill et al. (1992). Cured lines were checked for five generations following treatment. In addition, individuals from uninfected lines used in CI assays were analyzed and never found infected.

Rearing Conditions

Flies were routinely maintained at 18°C, on axenic medium (David 1962). For two generations before each experiment,

flies were maintained at 25°C at low larval densities. One generation before each experiment, instead of rearing mass strains in bottles, 10 fertilized females of each line were left to lay in separate vials, so that their infection status could be controlled before choosing virgin flies for mating experiments. This procedure was necessary for *w*Cer2 lines, where maternal transmission is low (about 50%; Riegler et al. 2004) and it was generalized to all lines for homogeneity.

Compatibility Relationships Assays

Compatibility relationships were investigated by crossing males and females of different infection status in both directions. For example, consider one is studying compatibility between two CI-inducing *Wolbachia* A and B. Comparing levels of embryonic mortality in the two following crosses: (1) male A × female B and (2) male A × female 0 (where 0 stands for uninfected), allows one to test if *Wolbachia* B can rescue *Wolbachia* A. Under the *mod-resc* model, this is to ask: IS mod_A compatible with $resc_B$? The level of compatibility can be quantified as the percentage of embryos that are saved by the presence of *Wolbachia* B in females. The opposite direction of cross allows to test if mod_B is compatible with $resc_A$.

To avoid possible variations of genetic background effects that could confuse interpretations, experiments involving different *Wolbachia* variants in different genetic backgrounds were performed using F_1 hybrids. For example, if *Wolbachia* A infects line 1 and *Wolbachia* B infects line 2, crosses between lines 1 and 2 were performed before starting CI assays, so that, on average, the genetic background was the same in all the individuals that were to be compared (if one neglects possible variations of mitochondrial genomes and X chromosomes in males, that are not homogenized by this method). F_1 hybrids can be difficult to obtain when males bear a CI-inducing *Wolbachia* that is not present in the female. To circumvent this problem, males were taken from uninfected lines bearing the same genetic background obtained by antibiotic treatment.

Experiments were performed using virgin males aged 3–4 days and virgin females aged 4–7 days. Mating was controlled and crosses where copulation lasted for less than 15 min were discarded to ensure insemination. Inseminated females were individually placed at 25°C, on axenic medium colored with neutral red, making egg counting easier. Females were removed after 48 h of laying and eggs left for an additional 24 h at 25°C to allow hatching of all viable embryos, and finally placed at 4°C until egg counting. Embryonic mortality was then determined as the percentage of unhatched eggs. For statistical rigor and consistency with earlier work, samples with less than 20 eggs were discarded. For crosses showing 0% hatching, a fertility test was performed by crossing each parent with individuals of compatible infection status to distinguish between crosses where CI is 100% and crosses involving intrinsically sterile individuals, which were excluded from analysis. Finally, the infection status of both parents was checked by PCR. It must be noted that experiments involving the *w*Cer2 bacterium require double sampling effort in comparison with classic CI assays, because maternal transmission in its novel host *D. simulans* is only 50% (Riegler et al. 2004).

Statistical Analysis

The data were analyzed using nonparametric Kruskall-Wallis and Wilcoxon two-sample tests. For all crosses presented in Table 1, the Wilcoxon tests were performed by comparing each cross involving infected females with the corresponding control cross, where the female is not infected. Sidak's adjustment was used in case of multiple comparisons (Tables 1c, d, e).

RESULTS

The wAu/wCer2 Relationship

Although *w*Au does not appear to induce CI (Hoffmann et al. 1996; James and Ballard 2000; Reynolds and Hoffmann 2002; Charlat et al. 2003), it has been hypothesized that it could rescue the CI induced by another *Wolbachia* if the two variants were sufficiently closely related (Bourtzis et al. 1998). Indeed, in *D. simulans*, another non CI-inducing *Wolbachia* (Rousset and Solignac 1995; Reynolds and Hoffmann 2002; but see James and Ballard 2000) has been found to express such a $mod^-/resc^+$ phenotype (Merçot and Poinsot 1998). Earlier experiments have revealed that *w*Au cannot rescue the CI induced by *w*Mel (Poinsot et al. 1998). We were interested in testing if *w*Au could rescue the CI induced by *w*Cer2, its closest known relative. To do so, females from two *w*Au lines (Coffs, from Australia, and Y6, from Cameroon) and uninfected females were crossed with *w*Cer2-infected males (lines RC45 and RC50).

As shown in Table 1a, *w*Cer2 was found to induce moderate but marked levels of embryonic mortality in this experiment. This is consistent with earlier studies (Riegler et al. 2004), where *w*Cer2 induced 27–54% embryonic mortality (as compared to 5–24% in the control cross between uninfected males and uninfected females). Most importantly, the female infection status (*w*Au versus uninfected) was not found to affect embryonic mortality significantly in this experiment. Thus, *w*Au does not appear to rescue the *w*Cer2 *mod* function.

The wMel/wCer2 Relationship

To test if *w*Mel can rescue the CI induced by *w*Cer2, females from the ME29 line infected by *w*Mel as well as uninfected females were crossed with *w*Cer2-infected males (lines RC45 and RC50). As shown in Table 1b, *w*Cer2 was found to induce moderate but marked levels of embryonic mortality, consistent with earlier work (Riegler et al. 2004). Most importantly, embryonic mortality was not found significantly reduced by the presence of *w*Mel in females. Thus, *w*Mel does not appear to rescue the *w*Cer2 *mod* function.

To test if *w*Cer2 can rescue the CI induced by *w*Mel, RC45 and RC50 females (bearing *w*Cer2) as well as uninfected females were crossed with ME29 males bearing *w*Mel. As shown in Table 1c, embryonic mortality was significantly reduced by the presence of *w*Cer2 in females. However, the difference was quantitatively very small (7.2% with RC45 females, 2.9% with RC50 females). Thus, *w*Cer2 can rescue

TABLE 1. Results of compatibility assays. To avoid variations of genetic background effects, experiments were performed using F_1 hybrids between lines. For males and females of each cross category, we mention infection status followed by maternal and paternal line in parentheses. Note that RC45 and RC50 have the same genetic background as STC (see Materials and Methods). Abbreviations: 0, uninfected; Neg, total number of eggs counted; Nc, number of crosses; Mea, mean embryonic mortality (%); Med, median embryonic mortality (%); Q25 25th quartile (%); Q75 75th quartile (%); W, result of the Wilcoxon's two-sample test; P, associated α probability. The Wilcoxon tests were performed by comparing each cross involving infected females with the corresponding control cross, where the female is not infected. In sections (c), (d), and (e), Sidak's adjustment was used for multiple comparisons; the control cross is given in the first line.

Male: Infection (mother, father)	Female: Infection (mother, father)	Neg	Nc	Mea	Med	Q25	Q75	W	P
(a) Does *w*Au rescue *w*Cer2?									
*w*Cer2 (RC45, Coffs)	0 (STC, Coffs)	1388	12	37.8	37.1	30.0	46.4		
*w*Cer2 (RC45, Coffs)	*w*Au (Coffs, STC)	1517	13	30.3	25.7	10.8	56.1	1.19	>0.2
*w*Cer2 (RC50, Coffs)	0 (STC, Coffs)	1177	9	27.0	23.2	14.9	39.8		
*w*Cer2 (RC50, Coffs)	*w*Au (Coffs, STC)	1026	7	29.0	25.5	19.6	47.4	0.37	>0.7
*w*Cer2 (RC45, Y6)	0 (STC, Y6)	1202	13	49.0	52.1	35.0	60.5		
*w*Cer2 (RC45, Y6)	*w*Au (Y6, STC)	1290	14	45.3	42.1	33.4	56.2	0.78	>0.4
*w*Cer2 (RC50, Y6)	0 (STC, Y6)	1446	16	46.3	46.1	18.7	73.5		
*w*Cer2 (RC50, Y6)	*w*Au (Y6, STC)	1327	15	44.8	37.8	23.7	66.7	0.20	>0.8
(b) Does *w*Mel rescue *w*Cer2?									
*w*Cer2 (RC45, ME29)	0 (STC, ME29TC)	1296	12	40.2	30.8	27.2	60.2		
*w*Cer2 (RC45, ME29)	*w*Mel (ME29, STC)	910	10	46.3	32.2	22.3	79.1	0.07	>0.9
*w*Cer2 (RC50, ME29)	0 (STC, ME29TC)	1120	11	39.2	27.9	12.4	54.4		
*w*Cer2 (RC50, ME29)	*w*Mel (ME29, STC)	1156	13	41.0	39.0	27.8	48.8	0.61	>0.5
(c) Does *w*Cer2 rescue *w*Mel?									
*w*Mel (ME29, STC)	0 (STC, ME29TC)	1708	17	99.6	100.0	99.1	100.0		
*w*Mel (ME29, STC)	*w*Cer2 (RC45, ME29TC)	1642	16	92.8	98.5	85.1	100.0	2.45	<0.05
*w*Mel (ME29, STC)	*w*Cer2 (RC50, ME29TC)	1713	16	96.7	99.0	93.1	100.0	2.25	<0.05
*w*Mel (ME29, STC)	*w*Mel (ME29, STC)	1071	11	23.3	26.1	8.4	32.5	3.29	<0.01
(d) Does *wR*i rescue *w*Mel (verification)?									
*w*Mel (ME29, DSRTC)	0 (DSRTC, ME29TC)	1245	12	99.5	100.0	99.1	100.0		
*w*Mel (ME29, DSRTC)	*w*Ri (DSR, ME29TC)	1297	14	7.2	5.8	3.9	10.3	4.32	$<10^{-4}$
*w*Mel (ME29, DSRTC)	*w*Mel (ME29, DSRTC)	337	7	34.5	27.9	18.6	50.0	3.54	$<10^{-3}$
(e) Does *w*Mel rescue *w*Ri (verification)?									
*w*Ri (DSR, ME29TC)	0 (ME29TC, DSRTC)	1306	11	97.4	97.7	96.1	100.0		
*w*Ri (DSR, ME29TC)	*w*Mel (ME29, DSRTC)	708	13	69.3	66.2	58.6	82.6	4.14	$<10^{-4}$
*w*Ri (DSR, ME29TC)	*w*Ri (DSR, ME29TC)	974	9	5.3	5.2	3.5	6.0	3.73	$<10^{-3}$
(f) Does *w*Ri rescue *w*Cer2?									
*w*Cer2 (RC45, DSRTC)	0 (STC, DSRTC)	1706	15	50.3	46.2	39.8	63.3		
*w*Cer2 (RC45, DSRTC)	*w*Ri (DSR, STC)	2411	22	31.7	22.3	12.7	46.9	2.45	<0.02
*w*Cer2 (RC50, DSRTC)	0 (STC, DSRTC)	1420	14	19.5	22.3	9.7	30.0		
*w*Cer2 (RC50, DSRTC)	*w*Ri (DSR, STC)	1248	12	26.9	13.9	4.4	50.4	0.31	>0.4
(g) Does *w*Cer2 rescue *w*Ri?									
*w*Ri (DSR, STC)	0 (STC, DSRTC)	3416	24	96.6	98.8	94.0	100.0		
*w*Ri (DSR, STC)	*w*Cer2 (RC45, DSRTC)	2400	19	90.9	92.7	87.0	95.5	2.93	$<10^{-2}$
*w*Ri (DSR, STC)	0 (STC, DSRTC)	2617	20	83.4	85.3	77.7	91.0		
*w*Ri (DSR, STC)	*w*Cer2 (RC50, DSRTC)	798	7	84.1	95.2	91.6	100.0	1.88	>0.05

a very small proportion of the embryos when faced with the *w*Mel *mod* function.

In this experiment, males bearing *w*Mel were also mated with females bearing *w*Mel. As expected, *w*Mel was found able to rescue its own *mod* function much more efficiently so than *w*Cer2.

Verification of the wRi/wMel Relationship

Earlier studies reported an unexpected and complex pattern of compatibility between *w*Ri (a strong CI inducer, naturally infecting *D. simulans*) and the *w*Mel variant injected from *D. melanogaster*: *w*Ri was found fully efficient at rescuing the *w*Mel *mod* function, while *w*Mel was found only partially efficient at rescuing the *w*Ri *mod* function (Poinsot et al. 1998). These results made the *w*Cer2/*w*Ri relationships worth investigating. Before doing so, we tested whether these initial observations could be retrieved.

To test if *w*Ri can rescue the CI induced by *w*Mel, males bearing *w*Mel were crossed with females bearing *w*Ri as well as uninfected females. As expected, a significant reduction of embryonic mortality was observed when females carried *w*Ri (Table 1d). In this experiment, *w*Mel males were also mated with *w*Mel females. As expected, *w*Mel was found to rescue its own *mod* function, but embryonic mortality was still higher than in crosses with *w*Ri females. Comparing these two crosses allows to show that in this experiment females bearing *w*Ri were more fertile when mated with males bearing *w*Mel than were females bearing *w*Mel itself (Wilcoxon $W = 3.65$, $P < 10^{-3}$).

To test if *w*Mel can rescue the CI induced by *w*Ri, males bearing *w*Ri were crossed to females bearing *w*Mel as well as uninfected females. As shown in Table 1e, the presence of *w*Mel in females was found to reduce embryonic mortality significantly, although it was still high (near 70%). A comparison with crosses between *w*Ri males and *w*Ri females shows that females bearing *w*Ri are more efficiently protected from the *w*Ri *mod* function than females bearing *w*Mel (Wilcoxon $W = 3.9$, $P < 10^{-4}$). Thus, as observed previously, *w*Mel can rescue the *w*Ri *mod* function but only partially so.

The wRi/wCer2 Relationship

To test if *w*Ri can rescue the CI induced by *w*Cer2, males bearing *w*Cer2 (lines RC45 and RC50) were crossed with females bearing *w*Ri as well as uninfected females. As shown in Table 1f, *w*Cer2 was found to induce moderate but marked levels of embryonic mortality in the RC45 line, consistent with earlier reports (Riegler et al. 2004). On the contrary, *w*Cer2 induced unexpectedly low levels of embryonic mortality in the RC50 line. Not surprisingly, the two different *w*Cer2 lines thus lead to different conclusions. In crosses involving RC45 males, the presence of *w*Ri in females was found to reduce embryonic mortality significantly, whereas this was not the case in crosses involving RC50 males.

To test if *w*Cer2 can rescue the CI induced by *w*Ri, males bearing *w*Ri were crossed with females bearing *w*Cer2 (RC45 and RC50) as well as uninfected females. The results are presented in Table 1g. Here the rescue capabilities of the RC45 and RC50 lines were analyzed in two different experiments, realized one month apart (explaining why the control cross ''male *w*Ri × female 0'' is presented twice). A similar pattern as in the reciprocal experiment was observed: The presence of *w*Cer2 was found to reduce embryonic mortality weakly but significantly in crosses involving RC45 females but not RC50 females.

DISCUSSION

Did wAu Lose Its resc Function?

Theoretical investigations have revealed that CI levels are not directly subject to selection (Prout 1994; Turelli 1994; Hurst and McVean 1996), as long as population structure is not too strong (Frank 1998). In other words, although high levels of CI facilitate the initial invasion of uninfected populations, there is no selective pressure among compatible *Wolbachia* variants in favor of higher embryonic mortality in crosses between infected males and uninfected females. This nonintuitive conclusion can be simply understood within the framework of the *mod-resc* model, by noting that because *mod* is expressed only in males and *Wolbachia* is transmitted only by females, it derives that variations affecting the *mod* function are neutral. This rationale has led to the prediction that non-CI-inducing *Wolbachia* (the $mod^-/resc^+$ phenotype) could arise and invade infected populations, either by drift or with the help of selection if the ancestral mod^+ phenotype was selected against through pleiotropic effects (Turelli 1994; Hurst and McVean 1996). Validating this view, a non-CI-inducing *Wolbachia* naturally infecting *D. simulans* (namely *w*Ma, also called *w*Ki in some publications) has been found to rescue the CI induced by the closely related strain *w*No (Poinsot and Merçot 1999; Charlat et al. 2003; Merçot and Charlat 2003). Once a $mod^-/resc^+$ *Wolbachia* has reached fixation, thus eliminating CI-inducing variants, the next predicted evolutionary change is the loss of its *resc* function, which has become useless. Indeed, if no CI is expressed in the population, maintaining a functional rescue is not of any help. The $mod^-/resc^+$ *Wolbachia* can then be gradually replaced by a $mod^-/resc^-$ phenotype, either by drift, or with the help of selection if the $resc^+$ phenotype is selected against through pleiotropic effects.

Which of these two steps does *w*Au illustrate? When faced with other CI-inducing *Wolbachia* (including the close relative *w*Mel found in *D. melanogaster*), *w*Au is not found to rescue embryos (Poinsot et al. 1998). Here we challenged this $resc^-$ status by testing if *w*Au could rescue the CI induced by *w*Cer2, its closest known relative. Our results suggest it cannot, consistent with the view that *w*Au has lost its rescue ability, or that *resc* is specifically repressed by the host. However, it must be noted that a minute level of rescue of the kind expressed by *w*Cer2 when faced with the *w*Mel *mod* function cannot be excluded. Indeed, as visible in Table 1a, interquartile ranges are such that small differences could remain undetected.

Compatibility Relationships between Cytoplasmic-Incompatibility-Inducing Variants

We investigated the relationship between *w*Mel and *w*Cer2, two closely related CI-inducing *Wolbachia*, after injection into *D. simulans*. At first sight, this relationship appears asymmetrical. Indeed, *w*Mel was found unable to rescue the *w*Cer2 *mod* function, while *w*Cer2 rescued a tiny proportion of embryos when faced with the *w*Mel *mod* function. It should be noted, however, that the levels of CI induced by *w*Cer2 and *w*Mel are such that rescue of *w*Mel by *w*Cer2 is more easily detected than the reverse. Indeed, *w*Mel typically induces almost 100% CI, with very low variability (interquartile range: 99.1–100.0%). Thus, even a tiny rescue can be detected here. On the contrary, *w*Cer2 induces low and variable CI, so that a small rescue of *w*Cer2 by *w*Mel could remain hidden unless very large samples are used.

Data from a previous experiment provides insights into the ability of *w*Cer2 to rescue its own *mod* function following injection into *D. simulans* (Riegler et al. 2004). In two different injected lines, embryonic mortality was significantly lower in the cross male *w*Cer2 × female *w*Cer2 than in the cross male *w*Cer2 × female 0 (embryonic mortality dropped from 35.4% to 22.8% in the first line and from 54.7% to 28.2% in the second line), providing evidence that *w*Cer2 can rescue its own *mod* function. However, embryonic mortality was significantly higher in the cross male *w*Cer2 × female *w*Cer2 than in the control cross male 0 × female *w*Cer2 (where embryonic mortality was 13.7% and 15.5% in the first and second line, respectively), demonstrating that self-rescue by *w*Cer2 is not perfect. Riegler et al. (2004) arguably suggest that such incomplete self-rescue is caused by imperfect maternal transmission of *w*Cer2 in *D. simulans* rather than actual imperfect rescue at the molecular level. Indeed, because *w*Cer2 is transmitted to about 50% of the

offspring, only 50% of the eggs are supposedly protected from CI.

Inefficient transmission is likely to have the same effect in the present work: if *w*Cer2 was more efficiently transmitted, rescue of *w*Mel-induced CI would probably be higher. But, still, it would be far from complete. If one assumes that transmission efficiency was 50% in our experiment, then *w*Cer2 should have rescued 10% of the embryos affected by the *w*Mel *mod* function instead of the 5% we observed, on average.

Earlier reports on the asymmetrical compatibility relationships between *w*Mel and *w*Ri (Poinsot et al. 1998) prompted us to include *w*Ri in the present study, although this variant is, on the basis of *wsp* sequences, much less closely related to *w*Cer2 than is *w*Mel (Zhou et al. 1998; Riegler and Stauffer 2002). We confirmed that *w*Ri can fully rescue the *w*Mel *mod* function, while *w*Mel can rescue the *w*Ri *mod* function only partially. Surprisingly, we found that females bearing *w*Ri were more fertile than females bearing *w*Mel when mated with males bearing *w*Mel, a finding that had not been observed previously (Poinsot et al. 1998). Two hypotheses can be proposed to account for this result. First, *w*Mel might reduce female fertility regardless of CI. This could be tested by crossing uninfected males with uninfected females as well as *w*Mel-bearing females. Unfortunately, this cross was not necessary for our initial plans and therefore not performed in our experiment. Second, *w*Mel might be partially suicidal, that is, imperfectly rescuing its own CI. As suggested above for *w*Cer2, imperfect maternal transmission might be responsible. However, from the PCR results obtained during CI assays, *w*Mel appears efficiently transmitted. Imperfect self-rescue could also result from insufficient bacterial density in the eggs or an insufficient production of the *resc* factors, as previously suggested (Breeuwer and Werren 1993). Finally, the *w*Mel clone used in this experiment might represent the intermediate $mod_B resc_A$ stage predicted by theory for the evolution of compatibility types (Charlat et al. 2001b).

After confirming the *w*Ri/*w*Mel relationship, we investigated the *w*Cer2/*w*Ri relationships. We found that *w*Ri can partially rescue the *w*Cer2 *mod* function of the RC45 line. However, rescue by *w*Ri was not detected for the *mod* function of the RC50 line. This discrepancy might result from the very low CI expression of RC50 in this experiment (19% embryonic mortality): obviously, if CI expression is low, rescue is difficult to detect because of natural background mortality. Similarly, *w*Cer2 was found to rescue the *w*Ri *mod* function in the RC45 but not the RC50 line. This parallel makes it tempting to assume similar causes: a low density of *w*Cer2 in both male and female germlines in RC50. Overall, our results suggest that *w*Ri and *w*Cer2 are not totally incompatible in both directions of cross.

Attempting a Synthesis

The molecular basis of CI is currently unknown, but several models have been proposed in the literature. When critically confronting them with all the CI patterns known to date, it appears to us that a lock-and-key model (where *mod* and *resc* are determined by different bacterial genes and where the rescue of embryos is resulting from a physical interaction between their products) is the most parsimonious (Poinsot et al. 2003). We will try here to interpret our observations within this framework, using a symbolism modified from an earlier model (Charlat et al. 2001b). We should point out that the main aim here is to propose a formal system for treating data resulting from CI assays. We do not explore the entire parameter space that would fit with our data.

We describe the male side of CI using three parameters: (1) mod_I (*mod* intensity, often referred to as CI level), the percentage of embryonic mortality in crosses between infected males and uninfected females. Physically, mod_I equals the proportion of sperm where the bacterium is still present at the stage where modification takes place, multiplied by the probability that a modified sperm will fail unless rescued: (2) *lock*, the identity of the *mod* function (equivalent to the mod_C parameter in Charlat et al. [2001b], but hopefully more explicit) is a qualitative trait, symbolized here as a linear sequence of 10 characters, with *n* possible states for each character (1, 2. . . , *n*); and (3) N_{Locks} is the number of *locks* deposited in sperm that will have to be inhibited by the *key* for development to proceed; here we arbitrarily define that N_{locks} varies between zero and 100. We also describe the female side of CI using three parameters: (1) *TE* (maternal transmission efficiency) is the average proportion of eggs bearing *Wolbachia* in a clutch laid by an infected female; (2) *key* (equivalent to the $resc_C$ parameter in Charlat et al. 2001b) is the female counterpart of the *lock* parameter. Aligning the *lock* and *key* sequences allows us to calculate a compatibility score (percentage of identity between the two sequences) varying from 0% to 100%. In the present simplified model (10 sites only) each identical site translates into a 10% increase in the compatibility score. (3) N_{keys} is the number of *key*s available in an infected egg. If $TE = 100\%$ and compatibility between *lock* and *key* is complete (identical sequences), all embryos develop normally as long as $N_{keys} \geq N_{locks}$. If $N_{locks} > N_{keys}$, then a proportion N_{keys}/N_{locks} is rescued.

With these parameters in mind, let us try and characterize

TABLE 2. A possible combination of *mod* and *resc* properties inferred from our experiments.

	*w*Au	*w*Mel	*w*Cer2	*w*Ri
mod_I	0	99	40	95
lock	?	1111111111	2222222222/*1112222222*	1111111111
N_{locks}	?	40	20	100
$resc_I$	1	70/100	50	100
key	?	1111111111/*2221111111*	2222222222/*1112222222*	1111111111
N_{keys}	?	40	20	100

the four *Wolbachia* variants under study (*w*Au, *w*Cer2, *w*Mel, and *w*Ri) by filling in Table 2, step by step. mod_I can be directly measured by crossing infected males with uninfected females, yielding the first row of Table 2. In the first column of the table, some traits of *w*Au cannot be inferred from our data: $lock_{(wAu)}$ could be anything, including a total absence of *lock* sequence, and $N_{locks(wAu)}$ could have any value between zero and 100. $TE_{(wAu)}$, however, can be estimated as close to 100% from previous studies (Hoffmann et al. 1996). Thus, we do not know if the apparent *resc-* phenotype of *w*Au is due to a total absence of the *key* sequence, the *key* sequence being incompatible with all the *locks* tested so far, or a very low value of the N_{keys} parameter.

Consider now *w*Mel, where we arbitrarily define $Lock_{(wMel)}$ as 1111111111. As a first hypothesis, (symbolized in bold in Table 2), we will assume that the imperfect self-rescue of *w*Mel (30% mortality in the intrastrain cross) is simply caused by imperfect maternal transmission (i.e., $TE_{(wMel)} = 70\%$), and not by a difference between $lock_{(wMel)}$ and $key_{(wMel)}$. Consequently, $key_{(wMel)} = 1111111111$.

We now turn to *w*Cer2. Because *w*Mel does not appear to rescue *w*Cer2, $lock_{(wCer2)}$ has to be 100% different from $key_{(wMel)}$. Thus, $lock_{(wCer2)}$ can be coded for example 2222222222. $TE_{(wCer2)}$ is known to be approximately 50% (Riegler et al. 2004). Because the level of imperfect self-rescue of *w*Cer2 is in line with this low maternal transmission, $key_{(wCer2)}$ has to be perfectly identical to $lock_{(wCer2)}$; $key_{(wCer2)} = 2222222222$. Yet, because *w*Cer2 is known to rescue *w*Mel partially, $key_{(wCer2)}$ cannot be completely different from $lock_{(wMel)}$. To circumvent this problem, we must reconsider the assumption that the imperfect self-rescue of *w*Mel was caused by imperfect maternal transmission ($TE_{[wMel]} = 70\%$, hypothesis in bold in Table 2). We will now consider another possibility (the italic hypothesis in Table 2), where $TE_{(wMel)} = 100\%$ but where 30% of the sites are different between the *key* and the *lock*; $key_{(wMel)} = 2221111111$. Then, because *w*Mel cannot rescue *w*Cer2 at all, $lock_{(wCer2)}$ must be totally different (e.g. 1112222222), in which case $key_{(wCer2)}$ must also be 1112222222 to insure self-compatibility in *w*Cer2. However, a 30% similarity between $lock_{(wMel)}$ and $key_{(wCer2)}$ together with the 50% maternal transmission of *w*Cer2 imply that *w*Cer2 should rescue $30\% \times 0.5 = 15\%$ of the embryos when faced with the *w*Mel *mod* function, while we observe it rescues no more than 7.5%. This observation can be explained by our model if we allow for N_{locks} and N_{keys} to differ between the two strains, with $N_{locks(wMel)} = N_{keys(wMel)} = 40$, and $N_{locks(wCer2)} = N_{keys(wCer2)} = 20$.

Now consider *w*Ri. Since *w*Ri can totally rescue *w*Mel, then $key_{(wRi)}$ must be identical to $lock_{(wMel)}$, that is $key_{(wRi)} = 1111111111$, and $lock_{(wRi)}$ must be 1111111111 as well because *w*Ri totally rescues its own CI. Now we need to explain why *w*Mel does not rescue 70% of the embryos when faced with the *w*Ri *mod* function, which would be expected since $key_{(wMel)}$ (2221111111111) would share 70% similarity with $Lock_{(wRi)}$. We must again assume a quantitative difference, where $N_{locks(wRi)}$ is higher than $N_{keys(wMel)}$. More precisely, the model fits the data if $N_{locks(wRi)} = 100$, the expected proportion of rescued embryos being: $[1 - mod_{I(wRi)}] + mod_{I(wRi)} \times 0.7 \times (40/100) = 0.32$.

We thus end up with a possible interpretation that can be used to examine how compatibility types have evolved following the divergence of *w*Ri, *w*Mel, and *w*Cer2. In Figure 2, we present the most parsimonious distribution of character changes and ancestral character states for the *lock* and *key* sequences deduced from our hypotheses. The figure suggests that most changes have occurred within the *w*Cer2 lineage. This can be put in relation with the fact that the natural host of *w*Cer2 is a tephritid fruit fly and not a drosophilid. In other words, host traits might also play a role in the evolution of compatibility types.

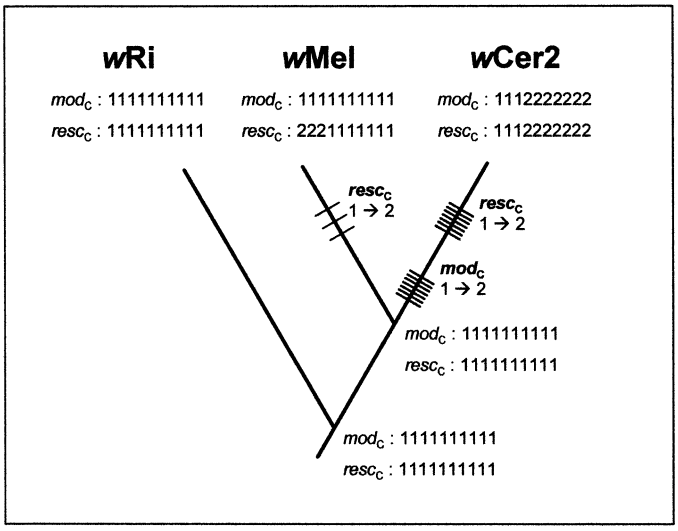

FIG. 2. Most parsimonious distribution of character changes and ancestral character states within the *lock* and *key* sequences. The phylogenetic tree is based on Zhou et al. (1998). Tics symbolize character changes.

The parameter set used is possibly one of several that would account for our experimental results. But the kind of data processing we propose is explicit and can be falsified or improved by additional experiments. CI is now known as a very widespread phenomenon induced in many arthropod groups, not only by *Wolbachia* but also by other distant intracellular bacteria (Hunter et al. 2003). With the accumulation of data, it will become necessary to describe CI relationships as formally as possible, using models such as the one proposed here. Only then will we be able to connect such information with molecular mechanisms that will hopefully be elucidated soon, now that the *Wolbachia* genome has been fully sequenced and analyzed (Wu et al. 2004).

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Corresponding Editor: M. Noor