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Natural Wolbachia Infections in the *Drosophila yakuba* Species Complex Do Not Induce Cytoplasmic Incompatibility but Fully Rescue the *w*Ri Modification

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ABSTRACT

In this study, we report data about the presence of Wolbachia in *Drosophila yakuba*, *D. teissieri*, and *D. santomea*. Wolbachia strains were characterized using their *wsp* gene sequence and cytoplasmic incompatibility assays. All three species were found infected with Wolbachia bacteria closely related to the *w*Au strain, found so far in *D. simulans* natural populations, and were unable to induce cytoplasmic incompatibility. We injected *w*Ri, a CI-inducing strain naturally infecting *D. simulans*, into the three species and the established transinfected lines exhibited high levels of CI, suggesting that absence of CI expression is a property of the Wolbachia strain naturally present or that CI is specifically repressed by the host. We also tested the relationship between the natural infection and *w*Ri and found that it fully rescues the *w*Ri modification. This result was unexpected, considering the significant evolutionary divergence between the two Wolbachia strains.

WOLBACHIA are maternally transmitted intracellular bacteria infecting many arthropods and nematodes (Werren 1997; Bandi *et al.* 1998; Stouthamer *et al.* 1999). Wolbachia infections can induce reproductive alterations such as feminization (Rigaud 1997), thelytokous parthenogenesis (Stouthamer 1997), male killing (Hurst *et al.* 2000), and, most commonly, cytoplasmic incompatibility (CI; Hoffmann and Turelli 1997; Charlat *et al.* 2002a,b). CI is expressed when a male infected by one (or more) Wolbachia strain(s) is crossed with a female that either is uninfected or does not harbor the strain(s) found in the male and manifests itself as embryonic lethality (Bourtzis *et al.* 2003). Embryos resulting from such crosses show elevated mortality rates.

Although the molecular mechanism of CI has not yet been elucidated, it is helpful to describe this phenomenon in terms of the *mod resc* phenomenology (Werren 1997), which emphasizes the sex-specific aspects of CI: in the male germline, Wolbachia somehow modify (*mod* function) nuclear components of the sperm (Presgraves 2000); the anaphase of modified paternal chromosomes is delayed during the first mitotic division, resulting in failure of zygote development unless Wolbachia is present and causes the appropriate *resc* function (for rescue) in the female germline (Lassy and Karr 1996; Callaini *et al.* 1997; Tram and Sullivan 2002). In mechanistic terms, it has been suggested that *mod* and *resc* interact in a lock-and-key manner with a direct inhibition of the *mod* factor (the lock) by the *resc* factor (the key), but other models are as likely (reviewed in Poinsot *et al.* 2003).

On the basis of the *mod resc* model, any Wolbachia/host association can be classified as belonging to one of the four following phenotypic categories: mod^+ $resc^+$, mod^+ $resc^-$, mod^- $resc^+$, and mod^- $resc^-$. The mod^+ $resc^+$ phenotype corresponds to most associations described so far, where Wolbachia induce CI and rescue their own modification. The mod^- $resc^-$ phenotype describes associations where Wolbachia neither induce CI nor rescue that induced by other strains. The mod^- $resc^+$ phenotype is observed when Wolbachia does not induce CI but can rescue that induced by other strains. Finally, the mod^+ $resc^-$ phenotype corresponds to situations in which Wolbachia induce CI without being capable of rescuing their own modification. Such strains have not been found yet, but theory does not preclude their maintenance in natural populations (Charlat and Merçot 2001).

During the past 2 decades, Wolbachia infections and Wolbachia-induced cytoplasmic incompatibility phenom-

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ena have been reported for several Drosophila species. In this article, we focus on species from the *Drosophila melanogaster* subgroup. This clade includes nine species (Lachaise *et al.* 2000, 2003): *D. simulans*, *D. sechellia*, *D. mauritiana*, and *D. melanogaster* (forming the *melanogaster* complex); *D. orena* and *D. erecta* (unassigned at the species complex level); and finally, *D. teissieri*, *D. santomea*, and *D. yakuba* (forming the *yakuba* complex).

To date, most Wolbachia studies have focused on the *melanogaster* subgroup (Hoffmann *et al.* 1986, 1994, 1996; Hoffmann 1988; O'Neill and Karr 1990; Rousset *et al.* 1992, 1999; Boyle *et al.* 1993; Holden *et al.* 1993; Bourtzis *et al.* 1994, 1996, 1998; Solignac *et al.* 1994; Giordano *et al.* 1995; Merçot *et al.* 1995; Rousset and Solignac 1995; Poinsot *et al.* 1998). Within this clade, *D. simulans* appears to be the most diversely infected host, harboring at least five phylogenetically and phenotypically distinct strains. Three of them, *w*Ri (Hoffmann *et al.* 1986), *w*Ha (O'Neill and Karr 1990), and *w*No (Merçot *et al.* 1995), are found to express both the modification and the rescue functions in their natural host (*i.e.*, mod^+ $resc^+$ phenotype) and are all bidirectionally incompatible. The *w*Ma strain (Rousset and Solignac 1995; Charlat *et al.* 2003; also referred to as *w*Ki in earlier publications) does not exhibit modification in the male germline. However, this infection can fully rescue the modification of the *w*No strain (Merçot and Poinsot 1998), thus expressing a mod^- $resc^+$ phenotype. The fifth strain, *w*Au, does not appear to induce (Hoffmann *et al.* 1996; James and Ballard 2000; Reynolds and Hoffmann 2002; Charlat *et al.* 2003) or to rescue CI (Poinsot *et al.* 1998), thus exhibiting a mod^- $resc^-$ phenotype. Wolbachia is also present in *D. sechellia* and *D. mauritiana*. Two strains infect *D. sechellia*, namely *w*Sh and *w*Sn (Rousset and Solignac 1995). On the basis of gene sequences and CI properties, *w*Sh and *w*Sn appear identical to the *w*Ha and *w*No infections, respectively, of *D. simulans* (Zhou *et al.* 1998; Charlat *et al.* 2002a,b). On the other hand, only one Wolbachia strain has been described in *D. mauritiana*, *w*Mau. On the basis of *wsp* sequences and CI properties, *w*Mau appears identical to the *w*Ma strain from *D. simulans* (Giordano *et al.* 1995; Rousset and Solignac 1995; Bourtzis *et al.* 1998; Zhou *et al.* 1998). The last species of the *melanogaster* complex, *D. melanogaster* itself, seems to harbor only one type of Wolbachia strain, *w*Mel, which induces variable levels of CI (0–77%) depending on the host genotype and male age (Hoffmann 1988; Bourtzis *et al.* 1994; Hoffmann *et al.* 1994; Solignac *et al.* 1994; McGraw *et al.* 2001; Reynolds and Hoffmann 2002; Weeks *et al.* 2002). However, the *wsp* gene sequence analysis of *w*Mel strains infecting five different *D. melanogaster* lines indicates that four of them had identical sequences and the fifth one differed from the others by only 2 of 565 bp, all being very closely related to the *w*Au strain with a maximum difference of only 5 bp (Zhou *et al.* 1998).

Information is much scarcer for the remaining five species of the *melanogaster* subgroup. *D. orena* and *D. erecta* are not thought to be infected (Bourtzis *et al.* 1994, 1996) but more systematic surveys could change this view. In particular it should be noted that all knowledge of *D. orena* relies on a single isofemale line. By contrast, Wolbachia was detected by PCR in the three species of the *yakuba* complex (Lachaise *et al.* 2000). Furthermore, the infection in *D. santomea* was reported to be identical to the *w*Au infection from *D. simulans* judged by partial *wsp* gene sequences (Lachaise *et al.* 2000).

In this study, we initially aimed to characterize infections in the *yakuba* complex through CI assays and DNA sequence comparison. Infections in all three species were found to be identical on the basis of *wsp* sequences (and thus closely related to the *w*Au infection from *D. simulans*) and not to express the *mod* function in their natural hosts. Throughout this article, this infection will be referred to as *w*Sty (for *s*antomea, *t*eissieri, and *y*akuba). To test whether this lack of CI is due to bacterial or host factors, we injected *w*Ri, a CI-inducing strain naturally infecting *D. simulans*, into all three species. Additionally, we tested the compatibility relationships between *w*Sty and *w*Ri.

MATERIALS AND METHODS

Insects: All *D. yakuba*, *D. teissieri*, and *D. santomea* used in this study and their origins are presented in Table 1. Flies were routinely grown at 25° on corn flour/sugar/yeast medium as low-density mass cultures. Low-density rearing is preferable since larval crowding can have a negative effect on the expression of CI (Sinkins *et al.* 1995). Tetracycline-treated strains were established by rearing flies for two generations on medium containing tetracycline at 0.025% (w/v) final concentration.

Micro-injections: Micro-injections were carried out as previously reported (Poinsot *et al.* 1998). Using a microcapillary needle (Femtotips, Boehringer), cytoplasm was drawn from infected early embryos and then injected into slightly dehydrated uninfected recipient early embryos.

PCR amplification: Total DNA was extracted from individual Drosophila flies following the STE (100 mm NaCl, 10 mm Tris-HCl pH 8, 1 mm EDTA pH 8) boiling method (O'Neill *et al.* 1992). The presence of Wolbachia was initially determined by PCR using the 16S *rDNA* Wolbachia-specific primers, 99F and 994R, which yield a product of ~900 bp (O'Neill *et al.* 1992). Infection was also confirmed using the *wsp* primers 81F and 691R, which yield a product of ~600 bp. The exact size varies depending on the bacterial strain (Braig *et al.* 1998; Zhou *et al.* 1998). The PCR results of the 16S *rDNA* and *wsp* primers were in complete agreement. PCR control reactions were performed to test the quality of the DNA template using the mitochondrial *cytb* primers, cytb1 and cytb2, which yield a 378-bp product (Clary and Wolstenholme 1985). Of a total of 50 μl of extract, 1 μl was used as template for PCR. All PCR analyses were carried out in 25-μl volumes and involved an initial denaturation step at 94° for 5 min. This was followed by 35 cycles of denaturation at 94° for 1 min, annealing at 55° for 1 min, extension at 72° for 1 min, and a final extension at 72° for 10 min. The PCR reactions included 2.5

mM $MgCl_2$, all four dNTPs (each at 250 μM), 0.5 μM of each primer, 1 unit of DNA Taq polymerase [Promega (Madison, WI) or GIBCO BRL (Gaithersburg, MD)], and buffer supplied by the manufacturers. PCR products were visualized on 1.2% agarose gels stained with ethidium bromide.

Cloning and sequencing: *wsp* PCR fragments were cloned into the pGEM-T vector (Promega) following the manufacturer's instructions. Plasmid DNA was purified using the QIAprep Spin plasmid kit (QIAGEN GmbH, Hilden, Germany). Sequencing reactions were performed using either the d-rhodamine dye-terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) or the Amersham Big Dye sequencing kit and run on an ABI377 sequencer (Perkin-Elmer Applied Biosystems), all according to the manufacturers' protocols and instructions. Three to five independent clones per Wolbachia-infected Drosophila strain listed in Table 1 were sequenced in both directions to identify PCR errors, and the majority-rule consensus was taken as the *wsp* sequence of each Wolbachia strain. No evidence was detected for multiple infections. The *wsp* sequences of this study have been deposited in the EMBL database under accession nos. AJ620679–AJ620681.

CI measurements (individual crosses): All matings were set up with one virgin female (3 days old) and one virgin male (up to 1 day old). Crosses were performed at 25° in bottles upturned on agar/molasses plastic petri dishes. The dishes were replaced daily to monitor the number of eggs laid. Hatching rates were scored 36 hr after egg collection. The parents of each cross were tested by PCR for the presence of Wolbachia. The females from those crosses that did not produce any larval progeny were tested for insemination. Crosses from noninseminated females were excluded from further analysis.

***mod* intensity:** To determine if *w*Sty expresses the *mod* function in its natural hosts and, if so, with what intensity, uninfected females were mated with both infected and uninfected males of the same genetic background. Strains for which embryonic mortality is significantly higher in crosses with infected males are considered mod^+. The same test was performed with *w*Ri-transinfected males. For interspecific comparisons, we used a corrected value of *mod* intensity that eliminates interspecific differences in "background mortality." Following Poinsot *et al.* (1998), CIcorr corresponds to the percentage of embryos that actually do not develop as a consequence of CI and not due to background mortality. If CCM stands for the control cross mortality (observed in crosses between uninfected males and uninfected females), then

$$\text{CIcorr}(\%) = [(\text{unhatched} - \text{CCM})/(\text{total} - \text{CCM})] \times 100,$$

where unhatched is the percentage of unhatched eggs observed in the incompatible cross. Thus, unless CCM is 0%, CIcorr is lower than the raw embryonic mortality rates. CIcorr of a given male was set at 0 whenever the percentage of unhatched was lower than the percentage of CCM.

Compatibility relationships: To test if *w*Sty can rescue the *w*Ri *mod* function, males bearing *w*Ri were crossed with females bearing *w*Sty as well as with uninfected females of the same genetic background. Rescue is detected if embryonic mortality is significantly reduced by the presence of *w*Sty in females.

Statistical analysis: Statistical analysis included ANOVA and *t*-tests on CI levels for the comparison of different crosses. The existence of statistically significant CI levels was tested by comparing the percentage of unhatched eggs observed in the appropriate crosses (noncorrected CI levels were used for such comparisons). CIcorr was used for comparison of *mod* intensity among different species and only when a significant CI level was observed. All percentage values were arcsine root transformed before analysis.

TABLE 1

Drosophila species and strains used in this study and their associated Wolbachia strain

Species	Strain	Source	Wolbachia[a]
D. yakuba	SA3	Bom Successo, Africa[b]	*w*Sty
D. yakuba	0261.0[c]	NDSRC	—
D. yakuba	KB82	This study	*w*Ri
D. yakuba	KB83	This study	*w*Ri
D. teissieri	S-13a	Umea	*w*Sty
D. teissieri	S-33	Cambridge	*w*Sty
D. teissieri	0257.0[d]	NDSRC	*w*Sty
D. teissieri	1015	Bloomington	*w*Sty
D. teissieri	KB114	This study	*w*Ri
D. teissieri	KB115	This study	*w*Ri
D. santomea	STO.2	Bom Successo, Africa	*w*Sty
D. santomea	STO.7	Bom Successo, Africa	*w*Sty
D. santomea	STO.9[e]	Bom Successo, Africa	*w*Sty
D. santomea	STO.4	Bom Successo, Africa	—
D. santomea	STO.8	Bom Successo, Africa	—
D. santomea	STO.10	Bom Successo, Africa	—
D. santomea	KB127	This study	*w*Ri
D. santomea	KB128	This study	*w*Ri

NDSRC, National Drosophila Species Resource Center.

[a] Based on partial *wsp* gene sequences.

[b] Collected by Daniel Lachaise on São Tomé Island (Lachaise *et al.* 2000).

[c] The *D. yakuba* strain 0261.0 was used as recipient to establish the two *w*Ri-transinfected *D. yakuba* lines.

[d] The *D. teissieri* strain 0257.0 was used as recipient, after tetracycline treatment, to establish the two *w*Ri-transinfected *D. teissieri* lines.

[e] The *D. santomea* strain STO.9 was used as recipient to establish the two *w*Ri-transinfected *D. santomea* lines.

RESULTS

Wolbachia in *D. yakuba*, *D. teissieri*, and *D. santomea*: We surveyed the three closely related species *D. yakuba*, *D. teissieri*, and *D. santomea* for Wolbachia infection by using PCR amplification of *wsp* sequences. Wolbachia infection is found in all three species (Table 1), confirming previous results (Lachaise *et al.* 2000). We sequenced part of the *wsp* gene of the Wolbachia strains present in infected stocks and lines from the three allied species (four *teissieri*, one *yakuba*, and one *santomea*). The six sequences obtained (EMBL accession nos. AJ620679–AJ620681) were identical to one another and closely related to that of the *D. simulans* Coffs Harbor Wolbachia strain (*w*Au, EMBL accession no. AF020067) analyzed by Zhou *et al.* (1998), which differs by only a single G-to-A transition in nucleotide position 409. Interestingly, *w*Sty presents the same partial wsp gene sequence as *w*Cer2 (AF418557), a Wolbachia strain naturally infecting the cherry fruit fly *Rhagoletis cerasi* (Riegler and Stauffer 2002). These results are consistent with those reported by Charlat *et al.* (2004).

Naturally Wolbachia-infected *D. yakuba*, *D. teissieri*, and *D. santomea* do not express CI: Naturally Wolbachia-infected strains of the three closely related species, *D.*

TABLE 2

Naturally Wolbachia-infected *D. yakuba*, *D. teissieri*, and *D. santomea* and expression of CI

Cross (female × male)	Eggs	Crosses	% mortality	Comparison[a]
1. *D. yakuba* (T) × *D. yakuba* (T)	1456	26	7.6 ± 1.7	1 *vs.* 2 (NS)
2. *D. yakuba* (T) × *D. yakuba* (*w*Sty)	1660	26	9.3 ± 2.6	
3. *D. teissieri* (T) × *D. teissieri* (T)	1424	26	13.9 ± 2.4	3 *vs.* 4 (NS)
4. *D. teissieri* (T) × *D. teissieri* (*w*Sty)	1835	26	13.0 ± 2.6	
5. *D. santomea* (T) × *D. santomea* (T)	807	19	8.8 ± 2.3	5 *vs.* 6 (NS)
6. *D. santomea* (T) × *D. santomea* (*w*Sty)	1991	31	8.4 ± 2.3	

CI is reported as percentage embryo mortality ±SE. Experiments for each Drosophila species were performed simultaneously. NS, no significant difference.

[a] Pairs of crosses were compared using the *t*-test.

yakuba, *D. teissieri*, and *D. santomea*, were tested for the expression of CI. Of the three different Wolbachia-infected *D. teissieri* strains tested, none expressed CI (data not shown). Only data obtained from strain 0257.0 (see Table 1), which was later used in the rescue experiments presented below, were used in the analysis. In addition, the only *D. yakuba* line available to us was tested for CI expression as well as the *D. santomea* STO.9 strain because the other two infected strains, STO.2 and STO.7, were not very fertile. Means and standard deviations are presented in Table 2. None of the three Wolbachia-infected Drosophila species exhibits any detectable levels of CI in the appropriate genetic crosses (*t*-tests: $P = 0.76$ for *D. yakuba*, $P = 0.67$ for *D. teissieri*, and $P = 0.97$ for *D. santomea*). These laboratory estimates are consistent with field data obtained for *D. yakuba*, which were reported by Charlat *et al.* (2004).

Establishment of transinfected lines: Injections of uninfected strains (tetracycline treated) of the three allied species were performed using *D. simulans* Riverside as donor line (infected by *w*Ri). The *w*Ri Wolbachia was successfully transferred and established in the naturally uninfected *D. yakuba* strain 0261.0 from the National Drosophila Species Resource Center (the only strain available to us at the time of the transinfection experiments performed during May–June 1996), in a tetracycline-cured line of *D. teissieri* strain 0257.0 (transinfection experiments performed during May–June 1999), and in the naturally uninfected line of *D. santomea* strain STO.8 (transinfection experiments performed in summer 2000). Two *w*Ri-transinfected lines were obtained for each of the three Drosophila relatives. At the time of this study, all transinfected lines are still stably infected with no evidence of loss of infection for >200 generations for *D. yakuba*, 100 generations for *D. teissieri*, and 70 generations for *D. santomea*.

***w*Ri-transinfected lines of *D. yakuba*, *D. teissieri*, and *D. santomea* express high levels of CI:** All transinfected lines were repeatedly tested for the expression of CI. There was no significant variation between the CI levels induced by *w*Ri in different transinfected lines within a given species (*t*-tests: $P = 0.72$ for *D. yakuba*, $P = 0.14$ for *D. teissieri*, and $P = 0.89$ for *D. santomea*), which allowed us to pool the data of the respective lines. Analysis of the pooled samples of the *w*Ri-infected lines of all species showed significant levels of CI in appropriate crosses (Table 3; *t*-tests: $P < 0.0001$ for *D. yakuba*, $P < 0.0001$ for *D. teissieri*, and $P < 0.0001$ for *D. santomea*). The CI levels ranged from 85 to 100% in *D. yakuba*, from 56 to 100% in *D. teissieri*, and from 62 to 100% in *D. santomea*. On the other hand, using the CIcorr levels for species comparison, ANOVA analysis shows that significant variation of the CI levels is induced by *w*Ri between the three Drosophila species ($F = 13.6$, d.f. = 2, 98, $P < 0.001$). While the *w*Ri-infected *D. yakuba* and *D. santomea* lines expressed similar CI levels, the *w*Ri-infected *D. teissieri* lines showed a lower CI value (Tukey's honest significant difference test; data not shown). Our analysis implies that *w*Ri can completely rescue its own modification in the *w*Ri-infected, closely related Drosophila species (data not shown). On the basis of the aforementioned results, it is clear that none of the three species prevents *w*Ri from causing CI, although *mod* intensity varies among species.

Do *w*Sty Wolbachia strains rescue the *w*Ri modification in Drosophila-infected hosts? Previous work has shown that *w*Au infections cannot rescue modification by *w*Ri in *D. simulans* (Hoffmann *et al.* 1996; Poinsot *et al.* 1998). However, and contrary to our expectations, the naturally occurring *w*Sty Wolbachia strains present in *D. yakuba*, *D. teissieri*, and *D. santomea* can rescue the *w*Ri imprint (Table 4; *t*-tests: $P < 0.0001$ for *D. yakuba* cross 1 *vs.* 2, $P < 0.0001$ for *D. teissieri* cross 5 *vs.* 6, and $P < 0.0001$ for *D. santomea* cross 9 *vs.* 10). In addition, and given that the *w*Sty infections do not induce CI (in Table 4, the crosses 4, 8, and 12 can be considered as a "no modification" control relative to crosses 1, 5, and 9, respectively), this rescue seems to be complete in all three species (Table 4; *t*-tests: $P = 0.50$ for *D. yakuba* cross 2 *vs.* 4, $P = 0.58$ for *D. teissieri* cross 6 *vs.* 8, and $P = 0.48$ for *D. santomea* cross 10 *vs.* 12). Our data clearly indicate that the *w*Sty infections present in *D. yakuba*,

TABLE 3

***w*Ri-transinfected *D. yakuba*, *D. teissieri*, and *D. santomea* and expression of CI**

Cross (female × male)	Eggs	Crosses	% mortality	Comparison[a]
1. *D. yakuba* (T) × *D. yakuba* (T)	1130	16	12.0 ± 5.9	1 *vs.* 2 (<0.0001)
2. *D. yakuba* (T) × *D. yakuba* (*w*Ri)	2515	34	94.2 ± 0.8	
3. *D. teissieri* (T) × *D. teissieri* (T)	617	16	14.3 ± 2.5	3 *vs.* 4 (<0.0001)
4. *D. teissieri* (T) × *D. teissieri* (*w*Ri)	1421	32	82.4 ± 3.0	
5. *D. santomea* (T) × *D. santomea* (T)	556	16	11.6 ± 2.9	5 *vs.* 6 (<0.0001)
6. *D. santomea* (T) × *D. santomea* (*w*Ri)	1210	35	94.9 ± 1.3	

CI is reported as percentage embryo mortality ±SE. Experiments for each Drosophila species were performed simultaneously. Crosses 2, 4, and 6 represent the pool of both transinfected lines of each species since there was no intraspecies difference between the lines.

[a] Pairs of crosses were compared using the *t*-test. Values in parentheses are *P*-values.

D. teissieri, and *D. santomea* can very efficiently rescue the *w*Ri imprint.

DISCUSSION

Our study shows that strains of the three species forming the *yakuba* complex (*D. yakuba*, *D. teissieri*, and *D. santomea*) are infected with Wolbachia, thus confirming previous reports (Lachaise *et al.* 2000). Sequence analysis indicates that all three species harbor the same *wsp* gene, closely related to that of the *D. simulans* Coffs Harbor strain (*w*Au) analyzed by Zhou *et al.* (1998), with 1 bp in 588 differing. Appropriate CI crosses demonstrate that this infection, which we refer to as *w*Sty, does not cause CI in any of the three species, equivalent to the *w*Au infection in *D. simulans* (Hoffmann *et al.* 1996). Thus, at first sight, both CI properties and sequence data group *w*Au and *w*Sty together.

To exclude the possibility that the three Drosophila relatives may not be permissive for CI, we established *w*Ri-infected *D. yakuba*, *D. teissieri*, and *D. santomea* lines (two isofemale lines for each species). The *w*Ri strain expresses a clear mod^+ $resc^+$ phenotype in its natural host *D. simulans* (Hoffmann *et al.* 1986), but also in *D. melanogaster* and *D. mauritiana* (Boyle *et al.* 1993; Giordano *et al.* 1995). All six *w*Ri-transinfected lines expressed high levels of CI (*mod* function) and restored embryonic viability in crosses with infected females (*resc* function), demonstrating that the three closely related species are permissive to CI. As discussed below in more detail, it remains possible that the *mod* function of *w*Sty is specifically repressed in these hosts.

A great number of reports indicate that there is considerable variation in the levels of *mod* intensity in different Wolbachia/host interactions. Veneti *et al.* (2003) recently examined the relationship between the level

TABLE 4

Compatibility relationships between naturally infected (*w*Sty) and *w*Ri-transinfected *D. yakuba*, *D. teissieri*, and *D. santomea*

Cross (female × male)	Eggs	Crosses	% mortality	Comparison[a]
1. *D. yakuba* (T) × *D. yakuba* (*w*Ri)	1247	17	95.1 ± 0.9	1 *vs.* 2 (<0.0001)
2. *D. yakuba* (*w*Sty) × *D. yakuba* (*w*Ri)	1321	16	36.9 ± 6.6	2 *vs.* 4 (NS)
3. *D. yakuba* (*w*Ri) × *D. yakuba* (*w*Ri)	1033	10	13.4 ± 5.9	
4. *D. yakuba* (*w*Sty) × *D. yakuba* (*w*Sty)	1277	17	32.4 ± 4.9	
5. *D. teissieri* (T) × *D. teissieri* (*w*Ri)	802	17	88.0 ± 2.5	5 *vs.* 6 (<0.0001)
6. *D. teissieri* (*w*Sty) × *D. teissieri* (*w*Ri)	1314	16	26.2 ± 4.2	6 *vs.* 8 (NS)
7. *D. teissieri* (*w*Ri) × *D. teissieri* (*w*Ri)	1720	18	36.9 ± 4.8	
8. *D. teissieri* (*w*Sty) × *D. teissieri* (*w*Sty)	847	12	22.2 ± 5.5	
9. *D. santomea* (T) × *D. santomea* (*w*Ri)	602	17	94.7 ± 1.8	9 *vs.* 10 (<0.0001)
10. *D. santomea* (*w*Sty) × *D. santomea* (*w*Ri)	1693	21	29.2 ± 3.3	10 *vs.* 12 (NS)
11. *D. santomea* (*w*Ri) × *D. santomea* (*w*Ri)	781	10	11.0 ± 3.3	
12. *D. santomea* (*w*Sty) × *D. santomea* (*w*Sty)	873	11	35.7 ± 8.4	

CI is reported as percentage embryo mortality ±SE. Experiments for each Drosophila species were performed simultaneously. NS, no significant difference.

[a] Pairs of crosses were compared using the *t*-test. Values in parentheses are *P*-values.

of *mod* intensity in a number of naturally infected and transinfected Drosophila hosts and the distribution and density of Wolbachia in testes. Their results indicated the presence of two main groups of Drosophila-Wolbachia associations: group I, which exhibits a positive correlation between CI levels and percentage of infected sperm cysts (*mod*$^+$ phenotype), and group II, which does not express CI (*mod*$^-$ phenotype) irrespective of the infection status of the sperm cysts. Group II can be further divided into two subgroups: the first one containing associations with high numbers of heavily Wolbachia-infected sperm cysts and the second one in which Wolbachia is rarely detected in sperm cysts, being mostly present in somatic cells. On the basis of this classification, all *w*Ri-infected *D. yakuba*, *D. teissieri*, and *D. santomea* associations used in this study belong to group I and express CI (*mod*$^+$ phenotype) while all naturally infected (*w*Sty) lines of the three closely related species belong to group II associations and do not express CI (*mod*$^-$ phenotype; Veneti *et al.* 2003). It has to be noted that natural *w*Sty infections show only a few infected sperm cysts and therefore the *mod*$^-$ phenotype may be the result of this inability of the *w*Sty strains to infect sperm cysts and/or a genetic absence of a *mod* locus (Veneti *et al.* 2003).

Following Veneti *et al.* (2003), there are three requirements for the expression of CI in a host-Wolbachia association: (a) Wolbachia has to be able to modify sperm (*mod*$^+$ genotype), (b) Wolbachia has to infect sperm cysts, and (c) Wolbachia has to be harbored by a permissive host (see also McGraw *et al.* 2001). The question raised is whether the *mod*$^-$ phenotype observed in the three species forming the *yakuba* complex is due to a host or a bacterial property. A potential way to address this question is to transfer these *w*Sty infections to another host, preferably *D. simulans*, and study their infection and CI properties (these experiments are presently in progress). It would be also interesting to perform the reciprocal transfer, that is, to transfer *w*Au from *D. simulans* to its sibling species.

Although sequence data and CI intensity assays group together *w*Au and the *w*Sty infection, the "rescue test" that we performed with males from *w*Ri-transinfected lines reveals that *w*Sty in *D. yakuba*, *D. teissieri*, and *D. santomea* is able to fully rescue the *w*Ri mod function and, at least in *D. teissieri*, as efficiently as the *w*Ri infection itself. This result, which represents the first report of a non-CI-inducing Wolbachia strain rescuing the *w*Ri modification, was not expected because *w*Sty and *w*Ri are not closely related as judged by their *wsp* sequences, although they both belong to the A Wolbachia clade. In addition, we cannot exclude the possibility of recombination events between the *wsp* gene, which is probably unrelated to CI, and gene(s) involved in the mechanism of CI. Furthermore, it has to be noted that in *D. simulans*, the *w*Au strain has been found to be unable to rescue the *w*Ri, *w*Ha, *w*No, and *w*Mel imprints (Hoffmann *et al.* 1996; Poinsot *et al.* 1998). This might suggest that *w*Sty and *w*Au differ genotypically regarding the *resc* determinants. The alternative explanation could be that there is a host effect on the expression of the *resc* function similar to that documented for the expression of the *mod* function (Boyle *et al.* 1993; Breeuwer and Werren 1993; Bordenstein and Werren 1998; Poinsot *et al.* 1998; McGraw *et al.* 2001). This possibility could also be tested once the natural infections of the three sibling species are transferred to a new host *D. simulans* and/or the *w*Au infection is transferred to the *yakuba* species complex (see above).

TABLE 5

A purely quantitative interpretation of compatibility relationships among *w*Mel, *w*Ri, *w*Au, and *w*Sty

	mod		*resc*	
Wolbachia	Quality[a]	Quantity[b]	Quality	Quantity
*w*Ri	A	+ + +	A	+ + +
*w*Mel	A	+ +	A	+ +
*w*Au	A	−	A	−
*w*Sty	A	−	A	+ + +

[a] Letters refer to qualitative variations of compatibility types.
[b] + and − refer to quantitative variations (variations of Wolbachia density and/or variations of concentrations of the *mod* and *resc* factors).

Poinsot *et al.* (1998) reported a unique case of asymmetrical partial CI between *w*Mel and *w*Ri infections in *D. simulans*. In this case, *w*Ri could fully rescue *w*Mel while *w*Mel could only partially rescue *w*Ri. Together with our results, these earlier data would suggest that there has been no qualitative divergence of the compatibility types accompanying the divergence of *w*Mel, *w*Ri, *w*Au, and *w*Sty. Indeed, as illustrated in Table 5, a model assuming purely quantitative variations (variations of Wolbachia density or of *mod* and *resc* concentrations in male and female germlines) can satisfactorily explain the observed pattern. Using this model, one can predict that if *w*Sty can rescue the *w*Ri *mod* function after injection into *D. simulans*, then it should also be able to rescue that induced by *w*Mel.

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