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Multiple Rescue Factors Within a Wolbachia Strain

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ABSTRACT

Wolbachia-induced cytoplasmic incompatibility (CI) is expressed when infected males are crossed with either uninfected females or females infected with Wolbachia of different CI specificity. In diploid insects, CI results in embryonic mortality, apparently due to the the loss of the paternal set of chromosomes, usually during the first mitotic division. The molecular basis of CI has not been determined yet; however, several lines of evidence suggest that Wolbachia exhibits two distinct sex-dependent functions: in males, Wolbachia somehow "imprints" the paternal chromosomes during spermatogenesis (*mod* function), whereas in females, the presence of the same Wolbachia strain(s) is able to restore embryonic viability (*resc* function). On the basis of the ability of Wolbachia to induce the modification and/or rescue functions in a given host, each bacterial strain can be classified as belonging in one of the four following categories: mod^+ $resc^+$, mod^- $resc^+$, mod^- $resc^-$, and mod^+ $resc^-$. A so-called "suicide" mod^+ $resc^-$ strain has not been found in nature yet. Here, a combination of embryonic cytoplasmic injections and introgression experiments was used to transfer nine evolutionary, distantly related Wolbachia strains (*w*Yak, *w*Tei, *w*San, *w*Ri, *w*Mel, *w*Ha, *w*Au, *w*No, and *w*Ma) into the same host background, that of *Drosophila simulans* (STCP strain), a highly permissive host for CI expression. We initially characterized the modification and rescue properties of the Wolbachia strains *w*Yak, *w*Tei, and *w*San, naturally present in the *yakuba* complex, upon their transfer into *D. simulans*. Confocal microscopy and multilocus sequencing typing (MLST) analysis were also employed for the evaluation of the CI properties. We also tested the compatibility relationships of *w*Yak, *w*Tei, and *w*San with all other Wolbachia infections. So far, the cytoplasmic incompatibility properties of different Wolbachia variants are explained assuming a single pair of modification and rescue factors specific to each variant. This study shows that a given Wolbachia variant can possess multiple rescue determinants corresponding to different CI systems. In addition, our results: (a) suggest that *w*Tei appears to behave in *D. simulans* as a suicide mod^+ $resc^-$ strain, (b) unravel unique CI properties, and (c) provide a framework to understand the diversity and the evolution of new CI-compatibility types.

WOLBACHIA is a group of maternally transmitted intracellular bacteria that infect numerous arthropod as well as filarial nematode species (Werren 1997; Bandi *et al.* 1998; Stouthamer *et al.* 1999). In arthropod hosts, Wolbachia mainly reside in ovaries and testes. In many cases, they manipulate host reproduction to ensure their own transmission by inducing feminization (Rigaud 1997), thelytokous parthenogenesis (Huigens and Stouthamer 2003), male killing (Hurst *et al.* 2003) and, most commonly, cytoplasmic incompatibility (CI) (Bourtzis *et al.* 2003). In diploid species, CI is expressed as embryonic lethality of the progeny of a male infected by one (or more) Wolbachia strain(s) and a female that either is uninfected or carries a different Wolbachia strain (Bourtzis *et al.* 2003).

The molecular mechanism of CI has not yet been elucidated; currently available data, however, suggest that Wolbachia modifies nuclear components of the sperm during spermatogenesis (Presgraves 2000). This is called the modification action of Wolbachia (mod function) (Werren 1997). This modification prevents the paternal set of chromosomes from entering the anaphase of the first mitotic division, resulting in failure of zygote development unless the same Wolbachia strain(s) is/are present in the egg and exert(s) the respective rescue function(s) (resc, for rescue) (Lassy and Karr 1996; Callaini *et al.* 1997; Werren 1997; Tram and Sullivan 2002; Ferree and Sullivan 2006). It has been suggested that mod and resc interact in a lock-and-key

This article is dedicated to the memory of Daniel Lachaise.

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manner, with a direct inhibition of the mod factor (the lock) by the resc factor (the key) (Poinsot *et al.* 2003); recent observations have supported this model (Ferree and Sullivan, 2006). On the basis of this 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^+ $resc^-$, depending on their modification and/or rescue properties (Poinsot *et al.* 2003). The phenotypes mod^+ $resc^+$, mod^- $resc^+$, and mod^- $resc^-$ have been observed in many different Wolbachia/host associations (Werren 1997; McGraw and O'Neill 1999; Charlat *et al.* 2001, 2002a; Weeks *et al.* 2002; Bourtzis *et al.* 2003). The mod^+ $resc^-$ phenotype describes Wolbachia strains, which are able to 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 *et al.* 2001, 2002a).

Wolbachia infections and their association with Wolbachia-induced cytoplasmic incompatibility phenomena have extensively been studied in Drosophila species. *D. melanogaster* seems to harbor a group of very closely related Wolbachia strains, known as *w*Mel, that induce variable levels of CI depending on the bacterial and host genotypes and male age (Hoffmann 1988; Boyle *et al.* 1993; Hoffmann *et al.* 1994; Holden *et al.* 1993; Bourtzis *et al.* 1994, 1996; Solignac *et al.* 1994; McGraw *et al.* 2001; Reynolds and Hoffmann 2002; Weeks *et al.* 2002; Merçot and Charlat 2004; Riegler *et al.* 2005). *D. simulans* harbors at least five phylogenetically and phenotypically distinct strains: *w*Ri, *w*Ha, *w*No, *w*Ma, and *w*Au (Merçot and Charlat 2004). The *w*Ri, *w*Ha, and *w*No strains are able to express both the modification and the rescue function in their natural host and are all bidirectionally incompatible (Hoffmann *et al.* 1986; O'neill and Karr 1990; Merçot *et al.* 1995). The *w*Ma strain is considered a mod^- $resc^+$ strain, unable to express the modification function, but being able to fully rescue the modification of the *w*No strain (Rousset and Solignac 1995; Merçot and Poinsot 1998a; Charlat *et al.* 2003). The *w*Au strain is considered a mod^- $resc^-$ strain (Hoffmann *et al.* 1996; Poinsot *et al.* 1998; Merçot and Poinsot 1998b; James and Ballard 2000; Reynolds and Hoffmann 2002; Charlat *et al.* 2003). Two Wolbachia strains have been described in *D. sechellia*, *w*Sh and *w*Sn; both are considered mod^+ $resc^+$ and they are bidirectionally incompatible (Rousset and Solignac 1995; Charlat *et al.* 2002b). In *D. mauritiana*, only Wolbachia strain *w*Mau has been described, which corresponds to *w*Ma following introgression of the genome of *D. mauritiana* in the siII cytoplasm of *D. simulans* (Rousset and Solignac 1995). The CI properties of *w*Mau appear to be identical to those of *w*Ma from *D. simulans*: *w*Mau has been shown to be incapable of expressing a modification function but it can fully rescue the modification of the *w*No strain, thus expressing a mod^- $resc^+$ phenotype (Giordano *et al.* 1995; Rousset and Solignac 1995; Bourtzis *et al.* 1998; James and Ballard 2000; James *et al.* 2002). The Wolbachia strains *w*Yak, *w*Tei, and *w*San have been reported to infect *D. yakuba*, *D. teissieri*, and *D. santomea*, respectively (Lachaise *et al.* 2000; Zabalou *et al.* 2004a). These strains were shown to be unable to express a *mod* function; however, they can fully rescue the *w*Ri modification upon its transfer into their natural hosts (Zabalou *et al.* 2004a).

Two important points that need to be taken into consideration to determine the CI properties of host–Wolbachia associations are: (a) the host nuclear background and (b) the complete absence of Wolbachia in antibiotic-treated lines (Weeks *et al.* 2002). Another important factor is the typing of the given Wolbachia strain used in the CI crosses. Efficient methods for Wolbachia strain typing were, until very recently, quite limited and mostly based on the Wolbachia surface protein (*wsp*) gene (Zhou *et al.* 1998). However, Wolbachia is prone to high rates of recombination, especially within supergroups, and single gene phylogenetics are unreliable for resolving close relationships (Jiggins *et al.* 2001; Werren and Bartos 2001; Bordenstein and Wernegreen 2004; Baldo *et al.* 2005, 2006a).

Taking a new approach to strain typing, Riegler *et al.* (2005) reported a number of polymorphic markers, such as size polymorphisms for IS5 insertion sites or minisatellites and the orientation of a chromosomal inversion, to detect and discriminate five different Wolbachia variants present in *D. melanogaster* natural populations and laboratory stocks. Research on Wolbachia depends critically on the ability to distinguish closely related strains to provide a solid foundation for understanding the evolution of phenotypic changes of this variable endosymbiont. Toward this goal, we recently developed an MLST system to discriminate closely related Wolbachia strains (from supergroups A and B) infecting Drosophila species, including all bacterial strains infecting species of the *D. melanogaster* subgroup (Paraskevopoulos *et al.* 2006). Baldo *et al.* (2006b) recently developed a second MLST system, thus increasing the availability of markers for typing closely related Wolbachia strains.

In this study, we initially aimed at characterizing Wolbachia infections (*w*Yak, *w*Tei, and *w*San), naturally present in the *yakuba* complex, with respect to their modification and rescue activities in *D. simulans*, a highly permissive host for CI expression. Confocal and MLST analysis were also employed for the evaluation of the CI properties. Additionally, we tested the compatibility relationships of *w*Yak, *w*Tei, and *w*San with all other Wolbachia infections naturally present in *D. simulans* (*w*Ri, *w*Ha, *w*Au, *w*No, and *w*Ma) and with *w*Mel. Up to now, the cytoplasmic incompatibility relationships between different variants could always be explained assuming a single pair of modification and rescue factors specific to each variant. This study shows that a single Wolbachia variant can possess multiple rescue factors corresponding to different CI systems. In addition, our

TABLE 1

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

Species	Strain	Source	Wolbachia[a]
D. yakuba	SA3[b]	Bom Successo, Africa[i]	*w*Yak
D. teissieri	0257.0[c]	NDSRC[j]	*w*Tei
D. santomea	STO.9[d]	Bom Successo, Africa[i]	*w*San
D. simulans	STCP[e]	Mahe Island, Seychelles[e]	Ø[k]
D. simulans	STCP 14 (*w*Yak)[f]	This study	*w*Yak
D. simulans	STCP 18 (*w*Yak)[f]	This study	*w*Yak
D. simulans	STCP 2 (*w*Tei)[f]	This study	*w*Tei
D. simulans	STCP 4 (*w*Tei)[f]	This study	*w*Tei
D. simulans	STCP 1 (*w*San)[f]	This study	*w*San
D. simulans	STCP 41 (*w*San)[f]	This study	*w*San
D. simulans	STCP (*w*Mel)[g]	Poinsot *et al.* (1998)	*w*Mel
D. simulnas	Riverside	Hoffmann *et al.* (1986)	*w*Ri
D. simulans	Hawaii	O'Neill and Karr (1990)	*w*Ha
D. simulans	Coffs Harbor	Hoffmann *et al.* (1996)	*w*Au
D. simulans	Noumea	Merçot *et al.* (1995)	*w*No
D. simulans	Madagascar	James and Ballard (2000)	*w*Ma
D. simulans	STCP [*w*Ri][h]	This study	*w*Ri
D. simulans	STCP [*w*Ha][h]	This study	*w*Ha
D. simulans	STCP [*w*Au][h]	This study	*w*Au
D. simulans	STCP [*w*No][h]	This study	*w*No
D. simulans	STCP [*w*Ma][h]	This study	*w*Ma

[a] Based on partial *wsp* gene sequences and MLST analysis.
[b] The *D. yakuba* strain SA3 was used as donor to establish the *D. simulans* STCP 14 (*w*Yak) and *D. simulans* STCP 18 (*w*Yak) lines.
[c] The *D. teissieri* strain 0257.0 was used as donor to establish the *D. simulans* STCP 2 (*w*Tei) and *D. simulans* STCP 4 (*w*Tei) lines.
[d] The *D. santomea* strain STO.9 was used as donor to establish the *D. simulans* STCP 1 (*w*San) and *D. simulans* STCP 41 (*w*San) lines.
[e] The *D. simulans* strain STCP was used as recipient to establish the *D. simulans* STCP (*w*Yak, *w*Tei, and *w*San) lines (Poinsot *et al.* 1998).
[f] The *D. simulans* STCP (*w*Yak, *w*Tei, and *w*San) lines were produced in this study.
[g] The *D. simulans* STCP (*w*Mel) line was produced by Poinsot *et al.* (1998).
[h] Introgressed line produced by series of backcrosses in this study.
[i] Collected by Daniel Lachaise in São Tomé Island (Lachaise *et al.* 2000).
[j] National Drosophila Species Resource Center.
[k] Ø, uninfected line.

results: (a) suggest that *w*Tei behaves in *D. simulans* as a *mod*$^+$ *resc*$^-$ strain, (b) unravel unique CI properties, and (c) provide the framework to understand the diversity and the evolution of new CI-compatibility types.

MATERIALS AND METHODS

Insects: All Drosophila stocks used in this study and their origins are presented in Table 1. Flies were grown at 25° on cornflour/sugar/yeast medium as low-density mass cultures, 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 (Zabalou *et al.* 2004a, 2004b). Using a microcapillary needle (Femtotips; Boehringer, Indianapolis), cytoplasm was drawn from infected early embryos and then injected into slightly dehydrated uninfected recipient early embryos.

Introgression lines: Introgression lines were produced, harboring the cytoplasm of different *D. simulans* infected lines carrying the Wolbachia strains *w*Ri, *w*Ha, *w*No, *w*Au, and *w*Ma in the genetic background of *D. simulans* STCP line. These introgression lines were generated by six generations of backcrossing Wolbachia-infected females of a given line to males of *D. simulans* STCP. This procedure should theoretically result in at least 98% genome replacement and the maintenance of the cytoplasm of the infected parental female.

Nomenclature: For the purposes of this study, we will use the following nomenclature system to refer to uninfected, transinfected (through micro-injections), and introgression lines. The name of each line starts with the species name and strain indicating the host genetic background followed by an italicized lower case w followed by the name of the Wolbachia strain within parentheses (transinfected lines) or within square brackets (introgression lines). Zero within parentheses or square brackets denotes an uninfected host. Thus, *D. simulans* STCP (*w*Yak) symbolizes a transinfected line, *D. simulans* STCP [*w*Ha] an introgression line, while *D. simulans* STCP (Ø) symbolizes an uninfected line.

Detection, typing, and phylogenetic analysis of Wolbachia strains: Bacterial DNA was extracted using the DNeasy Tissue

Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. 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) and the *wsp* primers 81F and 691R, which yield a product of ~600 bp (Braig *et al.* 1998; Zhou *et al.* 1998). 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). PCR conditions have been described in detail previously (Paraskevopoulos *et al.* 2006). The typing of the Wolbachia strains was based on a recently developed MLST approach (Paraskevopoulos *et al.* 2006) and partial sequencing of the *wsp* gene. Furthermore, the same sequence gene data were concatenated and phylogenetic relationships were determined with PhyloBayes, a Bayesian Monte Carlo Markov Chain (MCMC) sampler (Lartillot and Philippe 2004). The CAT mixture model was used to account for site-specific features of protein evolution. Seven independent runs were performed with a total length of 10,000 cycles. The burn-in value was set at 0.95; the posterior consensus was computed on the 9500 remaining trees.

CI measurements: 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. Males were removed after mating to avoid remating and females left to lay eggs for 2–3 days. The dishes were replaced daily to monitor the number of eggs laid. Females that laid <25 eggs were not included in the analysis. 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 and males from those crosses that did not produce any larval progeny were tested for fertility by crossing with a compatible partner. Crosses from sterile females or males were excluded from further analysis.

***mod* intensity:** To determine if a given Wolbachia strain expresses the *mod* function in its natural hosts, and if yes, with which penetrance, 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 the transinfected lines.

Compatibility relationships: To test if a given Wolbachia strain (*e.g.*, *w*A) can rescue the *mod* function of another Wolbachia strain (*e.g.*, *w*B), males bearing *w*B were crossed with females bearing *w*A, 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*A in females.

Statistical analysis: Statistical analysis was performed using various generalized linear models (GLM) with normal error and the identity link function (Nelder and Wedderburn 1972; McCullagh and Nelder 1989). Factors used in these analyses include "bacterial strain" (separately in males and in females) and "experimental location" (Greece and France). More details are given at each analysis in the results section. Significance level was set to 5% for all analyses performed. SPSS (SPSS for Windows 15.0; SPSS, Chicago) was used for all these models.

Immunofluorescence: Embryos, ovaries, and testes from 1-day-old flies were stained with the Wolbachia surface protein (WSP) antibody and propidium iodide (PI) (Molecular Probes, Eugene, OR), as described previously (Veneti *et al.* 2003, 2004). Images were taken using a Leica confocal laser-scanning microscope, and Adobe Photoshop 7.0 was used for editing purposes. For each of the three types of transinfected lines used in this study, 10 blastoderm-stage embryos stained with WSP antibody were used for fluorescence quantification. For each embryo, 1.5 μm-thick sections were taken and fluorescent pixels for the image stacks were measured using the ImageJ software (http://rsb.info.nih.gov/ij/).

TABLE 2

Summary of transinfection experiments

Recipient:	Donor		
D. simulans STCP (ø)	*D. yakuba* SA3 (*w*Yak) (%)	*D. teissieri* 0257.0 (*w*Tei) (%)	*D. santomea* STO.9 (*w*San) (%)
Injected embryos	1080	720	1380
Survived G_0 larvae	193 (17.9)[a]	70 (9.7)[a]	400 (28.9)[a]
Survived G_0 females	26 (13.5)[b]	37 (52.9)[b]	44 (11.0)[b]
Fertile G_0 females	22 (84.6)[c]	35 (94.6)[c]	40 (90.9)[c]
Wolbachia-infected G_0 females	2 (9.1)[d]	6 (17.1)[d]	4 (10.0)[d]

[a] Percentage of hatched G_0 larvae.
[b] Percentage of survived G_0 females.
[c] Percentage of fertile G_0 females.
[d] Percentage of Wolbachia-infected G_0 females.

RESULTS

Zabalou *et al.* (2004a) showed that naturally Wolbachia-infected *D. yakuba* SA3 (*w*Yak), *D. teissieri* 0257.0 (*w*Tei), and *D. santomea* STO.9 (*w*San) lines do not express CI. However, upon transfer of *w*Ri in native hosts of the above strains, it was observed that all three are able to fully rescue the *w*Ri modification. The question raised by this study was whether the mod^- phenotype observed in the three species forming the *yakuba* complex is due to a host or a bacterial property. This study attempts to address this question through the transfer of the *w*Yak, *w*Tei, and *w*San infections to another host, *D. simulans,* which is one of the most permissive Drosophila species for the expression of CI, as well as a known natural host of at least five Wolbachia strains.

Establishment of transinfected lines: Injections of an uninfected (tetracycline-cured) line of *D. simulans* (Poinsot *et al.* 1998), called from now on STCP, were performed using the naturally Wolbachia-infected *D. yakuba* SA3 (*w*Yak), *D. teissieri* 0257.0 (*w*Tei), and *D. santomea* STO.9 (*w*San) as donor lines. The Wolbachia strains *w*Yak, *w*Tei, and *w*San were successfully transferred to and established in the STCP strain. Two *w*Yak, six *w*Tei, and four *w*San-transinfected *D. simulans* STCP lines were obtained (Table 2). Transinfections were confirmed by PCR of the 16S *rDNA* and *wsp* genes of Wolbachia. At the time of writing, all transinfected lines are still stably infected with no evidence of loss of infection for >200 generations. Two stably transinfected *D. simulans* STCP lines for each Wolbachia strain were used in crossing experiments in the Greek laboratories, while one of the lines was also independently characterized in the French laboratory (Table 1). Mann–Whitney tests were carried

out, prior to the GLM analysis presented below, to compare these lines in all crossing experiments performed in Greece. No differences were found between the two stably transinfected *D. simulans* STCP lines for each Wolbachia strain (data not shown), and so we decided to pool these data.

Transinfected and introgression lines were used to perform a total of 1710 crosses (Table 3) in an attempt to study the compatibility relationships between nine different Wolbachia strains (*w*Mel, *w*Yak, *w*Tei, *w*San, *w*Au, *w*Ri, *w*Ha, *w*No, and *w*Ma) in a control host genomic background (*D. simulans* STCP). The phylogenetic relationships of these Wolbachia strains, based on the neighbor-joining phylogenetic analysis of the concatenated gene fragments, are shown in Figure 1.

An initial analysis of the embryonic mortalities resulting from these crosses was carried out by a GLM with bacterial strain (in males and in females) and experimental location as factors. A significant interaction was found by this analysis ($P < 0.001$) and therefore separate generalized linear models were run to determine the modification and the rescue properties of the Wolbachia strains, particularly those of *w*Yak, *w*Tei, and *w*San. It is also important to note that no major differences were observed in the results of the crossing experiments between the two locations (Greece and France).

Do *w*Yak-, *w*Tei-, and *w*San-transinfected *D. simulans* STCP lines express CI? All Wolbachia-infected (transinfected and introgression) *D. simulans* STCP lines were repeatedly and independently tested for the expression of the mod function in appropriate single-pair crosses. All data concerning these crosses are presented in Table 3. A GLM statistical analysis was carried out with "bacterial strain in males" and experimental location as factors. The model proved to be highly significant (likelihood ratio = 481.5, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 4. These data suggest that all Wolbachia-infected *D. simulans* STCP lines tested are able to express CI with the exception of the *w*Au- and *w*Ma-infected ones. The Wolbachia-infected *D. simulans* STCP lines that express CI can be classified into three groups according to the 95% confidence intervals of the *b*-parameters: (a) the first group includes the *w*Mel-, *w*Ri-, and *w*Tei-infected *D. simulans* STCP lines that express "high" levels of CI (mean CI 89.8–99.9% as shown in Table 3); (b) the second group includes the *w*Ha and *w*No-infected *D. simulans* STCP lines that express "medium" levels of CI (45.8–75.4%); and (c) the third group includes the *w*Yak- and *w*San-infected *D. simulans* STCP lines that express "low" levels of CI (21.0–26.5%). These data indicate that the *w*Yak, *w*Tei, and *w*San Wolbachia strains are able to induce CI in *D. simulans* STCP genomic background while they were unable to induce this reproductive alteration in their natural host (Zabalou *et al.* 2004a). It is worth noting that the *w*Tei- transinfected *D. simulans* STCP lines express very high levels of CI (nearly 100%). Is this phenotypic change due to a host or to a bacterial factor(s)?

The rescue properties of the Wolbachia strains, particularly those of *w*Tei, *w*Yak, and *w*San, used in this study were assessed by different GLMs carried out with "bacterial strain in females" and experimental location as factors. The results of these analyses are presented below and in Tables 5–10.

Which Wolbachia strains rescue the *w*Tei modification in the *D. simulans* STCP background? The GLM analysis was shown to be highly significant (likelihood ratio = 226.24, d.f. = 10, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 5. The data suggest that the *w*Tei, *w*Yak, *w*San, *w*Ri, *w*No, and *w*Ma strains can rescue the *w*Tei modification while the *w*Mel, *w*Au, and *w*Ha cannot. The Wolbachia strains that rescue the *w*Tei modification can be classified into two groups according to the 95% confidence intervals of the *b*-parameters: the first group includes the *w*Tei, *w*Yak, *w*San, and *w*Ma strains that exhibit high levels of rescue capacity (mean CI 37.3–66.3% as shown in Table 3), while the second group includes the *w*No and *w*Ri strains that exhibit low levels of rescue potential (mean CI 79.5–80.6%). It should be noted that the crosses performed in France showed slightly lower levels of rescue potential; however, no qualititative differences were observed (Tables 3 and 5).

The efficiency of the *w*Tei, *w*Yak, and *w*San strains to rescue the *w*Tei modification was also assessed by a GLM analysis. A comparison between the "rescue" crosses (*w*Tei-, *w*Yak-, and *w*San-infected *D. simulans* STCP females × *w*Tei-infected males) to the control ones (*w*Tei-, *w*Yak-, and *w*San-infected *D. simulans* STCP females × *D. simulans* STCP males), taking into account the experimental location, was performed. A significant difference was observed in the comparison between the "rescue crosses and the control crosses (Wald's $\chi^2 = 28.56$, d.f. = 1, $P < 0.001$). These data clearly suggest that the *w*Tei, *w*Yak, and *w*San strains cannot completely rescue the *w*Tei modification.

Do the *w*Yak, *w*Tei, and *w*San Wolbachia strains rescue the *w*Ri modification in the *D. simulans* STCP background? Zabalou *et al.* (2004a) showed that naturally Wolbachia-infected *D. yakuba* SA3 (*w*Yak), *D. teissieri* 0257.0 (*w*Tei), and *D. santomea* STO.9 (*w*San) lines could fully rescue the *w*Ri modification upon its transfer in their native hosts. Is this rescue function also observed in the *D. simulans* STCP background? To address this question, it was necessary to study all Wolbachia strains in the same host background. The *w*Ri strain was transferred into *D. simulans* STCP through a series of backcrosses. A comparison between the rescue crosses (Wolbachia-infected *D. simulans* STCP females × *w*Ri-infected males) to the control ones (Wolbachia-infected *D. simulans* STCP females × *D. simulans* STCP males) was performed as shown in Table 3. The GLM statistical

TABLE 3

Wolbachia-infected *D. simulans* STCP lines and expression of CI

Female infection	Male infection									
	STCP	*w*Yak	*w*Tei	*w*San	*w*Ri	*w*Mel	*w*Au	*w*Ha	*w*No	*w*Ma
A. Expression of cytoplasmic incompatibility (expressed as percentage embryonic mortality ± SE) in transinfected and introgression *D. simulans* STCP lines carrying different Wolbachia strains										
Greece										
STCP	13.6 ± 3.0	26.5 ± 4.2	97.2 ± 1.3	24.0 ± 4.1	89.8 ± 4.5		15.6 ± 2.9	75.4 ± 6.5	45.8 ± 7.3	11.7 ± 3.4
*w*Yak	5.3 ± 0.8	15.7 ± 4.3	39.8 ± 7.1		34.4 ± 5.6		21.2 ± 4.6	76.0 ± 5.6	45.8 ± 7.3	8.8 ± 1.8
*w*Tei	11.3 ± 2.0	25.3 ± 6.9	37.3 ± 3.3	10.1 ± 2.8	25.2 ± 2.3		15.0 ± 4.6	79.5 ± 7.7	56.9 ± 5.0	6.6 ± 1.5
*w*San	10.3 ± 2.8		45.2 ± 7.6	12.2 ± 1.6	40.3 ± 4.1		9.4 ± 4.3	69.6 ± 6.4	40.3 ± 5.0	9.0 ± 2.2
*w*Ri	23.7 ± 4.0	27.8 ± 4.6	79.5 ± 6.0	24.5 ± 3.5	34.9 ± 8.7		23.8 ± 4.1	64.8 ± 5.7	64.4 ± 5.9	23.5 ± 6.7
*w*Au	15.3 ± 2.0	12.8 ± 2.8	94.2 ± 2.7	16.3 ± 4.5	96.5 ± 1.7		10.5 ± 2.5		59.8 ± 6.3	
*w*Ha	12.5 ± 2.5	11.3 ± 2.2	93.5 ± 3.0	8.5 ± 1.4	91.4 ± 3.6			6.0 ± 0.8		4.1 ± 0.8
*w*No	19.1 ± 3.0	17.1 ± 6.0	80.6 ± 4.8	16.1 ± 4.0	88.2 ± 3.7		39.0 ± 8.4		9.3 ± 1.8	
*w*Ma	13.7 ± 2.3	25.0 ± 4.6	57.2 ± 6.9	15.2 ± 2.5	88.7 ± 5.1			62.7 ± 5.5		13.2 ± 3.9
France										
STCP	12.5 ± 2.9	21.0 ± 4.5	99.9 ± 0.1	24.2 ± 6.4		99.6 ± 0.2				
*w*Yak	16.6 ± 2.8	17.9 ± 6.4	66.3 ± 6.8			93.1 ± 3.0				
*w*Tei	13.1 ± 1.7		53.5 ± 4.5			37.0 ± 5.3				
*w*San	16.5 ± 4.2		55.5 ± 7.9	6.7 ± 1.3		96.2 ± 1.7				
*w*Mel	49.1 ± 7.1		99.0 ± 0.3			39.0 ± 7.4				
B. No. of eggs (no. of crosses) used to determine the levels of cytoplasmic incompatibility presented in A										
Greece										
STCP	1668 (27)	2635 (33)	1907 (30)	2441 (32)	1322 (19)		1460 (16)	917 (14)	1395 (17)	1447 (24)
*w*Yak	2683 (31)	1794 (21)	545 (10)		1660 (23)		1567 (19)	1207 (16)	1164 (16)	1627 (24)
*w*Tei	1409 (24)	658 (10)	3156 (43)	835 (13)	3124 (35)		1045 (15)	1101 (15)	1650 (18)	1349 (16)
*w*San	1641 (21)		1114 (17)	2389 (31)	2215 (33)		924 (16)	894 (14)	2038 (28)	1278 (19)
*w*Ri	1981 (24)	1894 (24)	1094 (18)	1631 (22)	1000 (13)		1650 (24)	1421 (23)	1090 (14)	931 (17)
*w*Au	1272 (17)	1092 (16)	1566 (21)	1211 (18)	1404 (18)		1315 (20)		1047 (14)	
*w*Ha	882 (16)	1298 (18)	1429 (24)	898 (16)	882 (16)			1063 (19)		1283 (21)
*w*No	1322 (15)	1127 (17)	1592 (26)	1013 (16)	1226 (17)		1092 (14)		872 (13)	
*w*Ma	1386 (19)	1656 (23)	1572 (25)	1522 (21)	1327 (19)			1433 (22)		1141 (18)
France										
STCP	1455 (15)	1898 (18)	1598 (19)	1664 (17)		2312 (23)				
*w*Yak	1466 (15)	917 (9)	1026 (12)			1346 (16)				
*w*Tei	1247 (15)		1251 (15)			961 (11)				
*w*San	1389 (14)		959 (12)	858 (9)		1921 (23)				
*w*Mel	588 (8)		901 (12)			639 (9)				

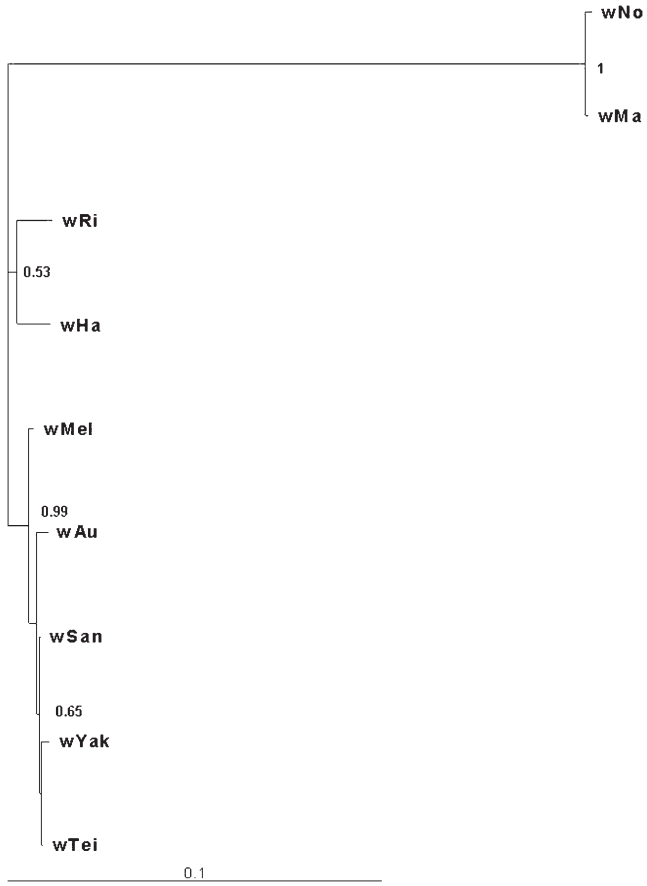

FIGURE 1.—Phylogenetic tree of the Wolbachia strains, constructed using the program MEGA 4.0 on the basis of the neighbor-joining method. Values on the branches represent the percentage of 10,000 bootstrap replicates.

analysis was highly significant (likelihood ratio = 223.73, d.f. = 8, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 6. These data suggest that *w*Tei, *w*Yak, *w*San, and *w*Ri can rescue the *w*Ri modification while *w*Au, *w*Ha, *w*No, and *w*Ma cannot.

The data also suggest that the *w*Tei, *w*Yak, and *w*San strains may equally efficiently rescue the *w*Ri modification in the *D. simulans* STCP background and that the rescue of the *w*Ri modification is as efficient as the one performed by *w*Ri itself (Table 6).

Do the *w*Yak, *w*Tei, and *w*San Wolbachia strains rescue the *w*Mel modification in the *D. simulans* STCP background? The *w*Mel Wolbachia strain has been reported as a *mod*$^+$ *resc*$^+$ strain in previous studies (HOFFMANN 1988; BOURTZIS *et al.* 1994, 1996). About 10 years ago, we transferred the *w*Mel Wolbachia strain into the *D. simulans* STCP background and showed that it induces high levels of CI (POINSOT *et al.* 1998). The fact that all four Wolbachia strains (*w*Yak, *w*Tei, *w*San, and *w*Mel) are present in the same host genomic background, *D. simulans* STCP, provided the opportunity to address the above question through a comparison between the rescue crosses (Wolbachia-infected *D. simulans* STCP females × *w*Mel-infected males) to the control ones (Wolbachia-infected *D. simulans* STCP females × *D. simulans* STCP males) as shown in Table 3. The GLM statistical analysis was highly significant (likelihood ratio = 145.95, d.f. = 4, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 7. These data suggest that only the *w*Tei strain can rescue the *w*Mel modification (equally well as *w*Mel, as is evident from the confidence intervals of their respective *b*-parameters) while the *w*Yak and *w*San strains cannot.

Do the *w*Yak, *w*Tei, and *w*San Wolbachia strains rescue the *w*Ha modification in the *D. simulans* STCP background? Wolbachia strain *w*Ha has been reported as a *mod*$^+$ *resc*$^+$ strain in previous studies (O'NEILL and KARR 1990). The *w*Ha strain was transferred into *D. simulans* STCP through a series of backcrosses, thus providing the potential to address the above question through a comparison between the rescue crosses (Wolbachia-infected *D. simulans* STCP females × *w*Ha-infected males) to the control ones (Wolbachia-infected *D. simulans* STCP females × *D. simulans* STCP males) as shown in Table 3. The GLM statistical analysis was highly significant (likelihood ratio = 89.35 d.f. = 6, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 8. These data suggest that only the *w*Ha strain can rescue its own modification while the *w*Tei, *w*Yak, *w*San, *w*Ri, and *w*Ma strains cannot.

Do the *w*Yak, *w*Tei, and *w*San Wolbachia strains rescue the *w*No modification in the *D. simulans* STCP background? Wolbachia strain *w*No has been reported as a *mod*$^+$ *resc*$^+$ strain in previous studies (MERÇOT *et al.* 1995). The strain was transferred into *D. simulans* STCP through a series of backcrosses, thus providing the potential to address the above question through a comparison between the rescue crosses (Wolbachia-infected *D. simulans* STCP females × *w*No-infected males) to the control ones (Wolbachia-infected *D. simulans* STCP females × *D. simulans* STCP males), as shown in Table 3. The GLM statistical analysis was highly significant (likelihood ratio = 41.41, d.f. = 6, $P < 0.001$). The estimated *b*-parameters (along with their 95% confidence intervals) and *P*-values are presented in Table 9. These data suggest that only the *w*No strain can rescue the *w*No modification while the *w*Tei, *w*Yak, *w*San, and *w*Au strains cannot. The significant difference found for the *b*-coefficient of *w*Ri means that this strain not only fails to rescue the *w*No modification, but it actually increases the observed embryonic mortality.

Typing Wolbachia strains in transinfected lines: We have recently developed and applied an MLST system to type Wolbachia strains infecting different Drosophila species (PARASKEVOPOULOS *et al.* 2006). This MLST approach was used to type the Wolbachia strains present in all donor and transinfected lines used in our study.

TABLE 4

Generalized linear model results on the modification properties of the Wolbachia strains used in this study

Male infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	3.777	3.278	4.275	220.753	1	0.000
*w*Yak	−0.114	−0.188	−0.040	9.188	1	0.002[a]*
*w*Tei	−0.851	−0.925	−0.776	502.798	1	0.000[a]*
*w*San	−0.109	−0.183	−0.034	8.204	1	0.004[a]*
*w*Ri	−0.763	−0.862	−0.663	225.310	1	0.000[a]*
*w*Mel	−0.870	−0.968	−0.772	303.383	1	0.000[a]*
*w*Au	−0.021	−0.126	0.085	0.150	1	0.699[a]
*w*No	−0.323	−0.426	−0.220	37.494	1	0.000[a]*
*w*Ha	−0.619	−0.729	−0.508	119.853	1	0.000[a]*
*w*Ma	0.018	−0.075	0.110	0.143	1	0.706[a]
Location	0.009	−0.044	0.062	0.113	1	0.737[b]

*Significant at 5% level.

[a] *P*-value for the comparison of the "modification" cross (*D. simulans* STCP female × Wolbachia-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *D. simulans* STCP male)

[b] *P*-value for the comparison between the data obtained in Greece and France.

The results were as follows: (a) both donor and transinfected Drosophila lines harbor Wolbachia strains with identical MLST profiles, and (b) no evidence of multiple infections was observed in any of the donor and the transinfected lines. In addition, we sequenced part of the *wsp* gene of the Wolbachia strains present in both the donor and the *w*Yak-, *w*Tei-, and *w*San-transinfected lines: all sequences obtained 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). These results are consistent with those reported by Charlat *et al.* (2004) and Zabalou *et al.* (2004a). Taken together, these data suggest that the donor lines *D. yakuba* (*w*Yak), *D. teissieri* (*w*Tei), and *D. santomea* (*w*San) and the *w*Yak-, *w*Tei-, and *w*San-transinfected *D. simulans* STCP lines carry very closely related Wolbachia strains (Table 1).

Immunofluorescence analysis: Immunofluorescence experiments and confocal analysis were performed in embryos, testes, and ovaries of *w*Yak-, *w*Tei-, and *w*San-transinfected *D. simulans* STCP lines, using an anti-WSP antiserum as described previously (Clark *et al.* 2002, 2003; Veneti *et al.* 2003, 2004). Our analysis shows that

TABLE 5

Generalized linear model results on the rescue potential of different Wolbachia strains against the *w*Tei modification

Female infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	−1.139	−1.693	−0.585	16.223	1	0.000
*w*Yak	0.459	0.353	0.565	71.657	1	0.000[a]*
*w*Tei	0.552	0.471	0.632	180.636	1	0.000[a]*
*w*San	0.491	0.394	0.588	99.376	1	0.000[a]*
*w*Ri	0.141	0.024	0.257	5.573	1	0.018[a]*
*w*Mel	0.066	−0.073	0.206	0.875	1	0.350[a]
*w*Au	−0.006	−0.117	0.105	0.012	1	0.912[a]
*w*No	0.130	0.026	0.233	6.033	1	0.014[a]*
*w*Ha	0.001	−0.105	0.107	0.000	1	0.990[a]
*w*Ma	0.363	0.258	0.468	46.165	1	0.000[a]*
Location	−0.121	−0.191	−0.052	11.771	1	0.001[b]*

*Significant at 5% level.

[a] *P*-value for the comparison of the "rescue" cross (Wolbachia-infected *D. simulans* STCP female × *w*Tei-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *w*Tei-infected *D. simulans* STCP male)

[b] *P*-value for the comparison between the data obtained in Greece and France.

TABLE 6

Generalized linear model results on the rescue potential of different Wolbachia strains against the *w*Ri modification

Male infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	−1.288	−1.956	−0.620	14.276	1	0.000
*w*Yak	0.554	0.434	0.673	81.950	1	0.000[a]*
*w*Tei	0.646	0.536	0.756	132.124	1	0.000[a]*
*w*San	0.495	0.383	0.606	75.836	1	0.000[a]*
*w*Ri	0.549	0.410	0.688	59.724	1	0.000[a]*
*w*Au	−0.067	−0.194	0.060	1.073	1	0.300[a]
*w*No	0.016	−0.113	0.145	0.060	1	0.806[a]
*w*Ha	−0.016	−0.148	0.115	0.060	1	0.806[a]
*w*Ma	0.011	−0.115	0.136	0.027	1	0.868[a]

*Significant at 5% level.

[a] *P*-value for the comparison of the "rescue" cross (Wolbachia-infected *D. simulans* STCP female × *w*Ri-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *w*Ri-infected *D. simulans* STCP male)

the *D. simulans* STCP (*w*Yak), *D. simulans* STCP (*w*Tei), and *D. simulans* STCP (*w*San) lines carried 4000 ± 200, 3900 ± 600, and 1700 ± 200 bacterial counts respectively. ANOVA analysis indicated significant differences between the bacterial densities of embryos of the three transinfected lines ($F = 11.70$, d.f. = 2.27, $P < 0.001$). Tukey's honestly significant differences (HSD) test showed grouping of *w*Yak-infected and *w*Tei-infected *D. simulans* STCP lines together, while the *w*San-infected *D. simulans* STCP line exhibited the lowest numbers. Overall, embryos from all three transinfected lines exhibited relatively low Wolbachia densities and tight posterial localization (Figure 2), similar to those observed in the native hosts (Veneti *et al.* 2004). The vast majority of the sperm cysts of all three transinfected lines used in this study were uninfected. Only a small number (<5%) contained few bacteria, probably scattered in the somatic part of the testes (Figure 2), as it has been described for their native hosts (Veneti *et al.* 2003). Finally, the Wolbachia distribution in ovaries showed bacterial accumulation in the posterior part of the oocyte (Figure 2), as observed for the native hosts (Veneti *et al.* 2004). We therefore conclude that the *D. simulans* genomic background did not significantly affect the distribution of the *w*Yak, *w*Tei, and *w*San bacteria.

DISCUSSION

Zabalou *et al.* (2004a) have shown that the naturally occurring host–Wolbachia associations *D. yakuba* SA3 (*w*Yak), *D. teissieri* 0257.0 (*w*Tei), and *D. santomea* STO.9 (*w*San) do not express CI, but that they are able to fully rescue the *w*Ri modification in the corresponding *w*Ri transinfected native hosts. This poses the question whether the modification function is absent from these Wolbachia strains or merely hidden in the native hosts. Transfers of the Wolbachia strains into the same

TABLE 7

Generalized linear model results on the rescue potential of different Wolbachia strains against the *w*Mel modification

Male infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	−0.335	−0.522	−0.148	12.308	1	0.000
*w*Yak	0.065	−0.007	0.138	3.095	1	0.079[a]
*w*Tei	0.626	0.544	0.708	223.920	1	0.000[a]*
*w*San	0.034	−0.032	0.100	1.004	1	0.316[a]
*w*Mel	0.606	0.518	0.694	182.314	1	0.000[a]*

*Significant at 5% level.

[a] *P*-value for the comparison of the "rescue" cross (Wolbachia-infected *D. simulans* STCP female × *w*Mel-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *w*Mel-infected *D. simulans* STCP male)

TABLE 8

Generalized linear model results on the rescue potential of different Wolbachia strains against the *w*Ha modification

Male infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	−0.184	−0.846	0.479	0.294	1	0.587
*w*Yak	−0.006	−0.173	0.160	0.006	1	0.940[a]
*w*Tei	−0.042	−0.210	0.127	0.232	1	0.630[a]
*w*San	0.058	−0.113	0.230	0.444	1	0.505[a]
*w*Ri	0.106	−0.048	0.260	1.809	1	0.179[a]
*w*Ha	0.694	0.534	0.854	72.275	1	0.000[a]*
*w*Ma	0.127	−0.028	0.282	2.568	1	0.109[a]

*Significant at 5% level.

[a] *P*-value for the comparison of the "rescue" cross (Wolbachia-infected *D. simulans* STCP female × *w*Ha-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *w*Ha-infected *D. simulans* STCP male)

D. simulans (STCP) genomic background, through either embryonic cytoplasmic injections or introgressions, enabled us to address this question as well as to study the compatibility relationships of *w*Yak, *w*Tei, and *w*San with other Wolbachia strains (Weeks *et al.* 2002).

Transinfection experiments are a powerful tool in studies of host–Wolbachia interactions; it should, however, be used with caution. Transinfections may result in the transfer of "hidden" Wolbachia strains to the new host, where they may find a suitable environment for multiplication and persistence (for a documented case see Zabalou *et al.* 2004b). It is therefore important to always type the transferred Wolbachia strain(s). Using a recently developed MLST approach (Paraskevopoulos *et al.* 2006), we typed all Wolbachia strains present in naturally infected, transinfected, and introgressed Drosophila lines used in this study.

The phenotypic shift: The *w*Yak, *w*Tei, and *w*San strains are very closely related judging from their identical *wsp* gene sequences (Lachaise *et al.* 2000; Zabalou *et al.* 2004a) and their position in the same MLST-assigned clonal complex (Paraskevopoulos *et al.* 2006). All three strains were transferred from their native hosts (*D. yakuba*, *D. teissieri*, and *D. santomea*) to *D. simulans* STCP through cytoplasmic injections. The transinfected lines were used in single-pair genetic crosses to study their CI properties (Table 3). A clear phenotypic shift was observed upon the transfer from their native hosts to *D. simulans* STCP. It was observed that the *w*Yak- and *w*San-transinfected *D. simulans* STCP lines expressed low levels of CI, while the transinfected *D. simulans* STCP (*w*Tei) symbiotic association expressed very high levels of CI (nearly 100%). According to Veneti *et al.* (2003), there are three requirements for the expression of CI in a host–Wolbachia association: (i) Wolbachia has to be able to modify sperm (mod^+ genotype), (ii) Wolbachia has to be harbored by a permissive host, and (iii) Wolbachia has to infect sperm cysts. How can this phenotypic shift from mod^- to mod^+ be explained? There are at least four possible explanations:

TABLE 9

Generalized linear model results on the rescue potential of different Wolbachia strains against the *w*No modification

Male infection	*b*	95% Wald confidence interval		χ^2	d.f.	*P*-value
		Lower	Upper			
(Intercept)	0.474	−0.160	1.107	2.146	1	0.143
*w*Yak	0.000	−0.162	0.163	0.000	1	0.995[a]
*w*Tei	−0.111	−0.268	0.047	1.890	1	0.169[a]
*w*San	0.055	−0.088	0.199	0.571	1	0.450[a]
*w*Ri	−0.186	−0.354	−0.018	4.692	1	0.030[a]*
*w*Au	−0.140	−0.308	0.028	2.653	1	0.103[a]
*w*No	0.366	0.194	0.538	17.382	1	0.000[a]*

*Significant at 5% level.

[a] *P*-value for the comparison of the "rescue" cross (Wolbachia-infected *D. simulans* STCP female × *w*No-infected *D. simulans* STCP male) to the control cross (*D. simulans* STCP female × *w*No-infected *D. simulans* STCP male)

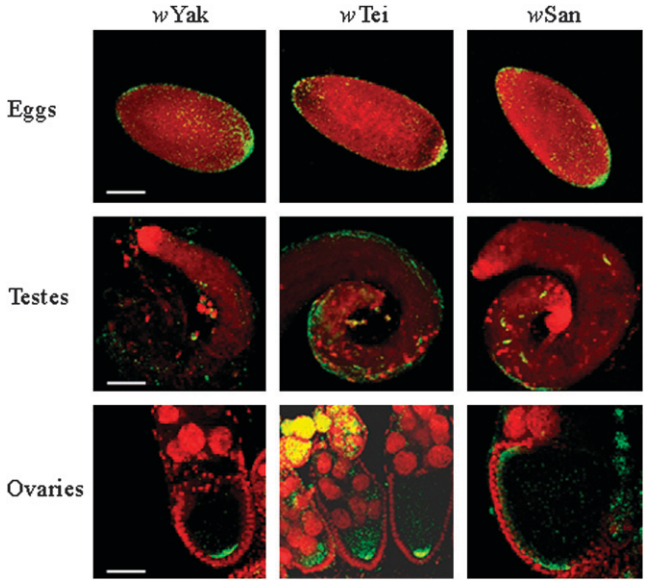

Figure 2.—Representative Wolbachia density and distribution is shown in embryos at syncytial blastoderm stage, testes and ovaries of *w*Yak-infected, *w*Tei-infected, and *w*San-infected *D. simulans* STCP lines. Wolbachia are stained green-yellow and host nuclei red. Most bacteria are concentrated in the posterior part of the eggs and oocytes. Eggs are oriented with the anterior part to the left. A few bacteria are scattered across the testes, and infected sperm cysts are rare, if present at all, in all three lines. Wolbachia cells are abundant in the ovaries, especially in the early stages of oogenesis for all three lines tested (shown only for *w*Tei-infected ovary). For later stages, no real differences between the three lines are observed. Scale bar: embryos, 100 μm; testes, 100 μm; ovaries, 50 μm.

a. It could be that the new host environment of *D. simulans* STCP is permissive for the expression of the modification function of *w*Yak, *w*Tei, and *w*San. Previous reports showed similar phenotypic changes in the behavior of Wolbachia strains upon their transfer to novel hosts (Poinsot *et al.* 1998). Bordenstein *et al.* (2003) also showed that host genotype rather than Wolbachia strain differences determines the type and levels of cytoplasmic incompatibility in the Nasonia species complex (but see also Bordenstein and Werren 2007). In addition, recent transinfection experiments showed that the same Wolbachia variant could induce two distinct reproductive phenotypes, CI and male killing, in different host species (Sasaki *et al.* 2005).
b. Significant differences in the percentage of infected spermatocysts could exist between the native and the transinfected symbiotic associations. Confocal analysis did not provide evidence for this: there was no difference in either the distribution and density or the percentage of the native and the transinfected symbiotic associations (Figure 2; see also Veneti *et al.* 2003). It remains possible, however, that differences in the *w*Tei (*w*Yak or *w*San) replication rate in larval testes could explain the ability of *w*Tei (*w*Yak or *w*San) to modify the paternal chromosomes. Alternatively, sperm could be modified at some point in development, where differences in Wolbachia distribution and density cannot be detected (Clark *et al.* 2002, 2003; Veneti *et al.* 2003).

   Also, the distribution and infection levels of *w*Yak, *w*Tei, and *w*San in embryos and ovaries of the transinfected *D. simulans* STCP lines was not different from that observed in their native hosts (Veneti *et al.* 2004).
c. The transfer of Wolbachia-infected embryonic cytoplasm from the native hosts could result in the establishment of a previously undetected *mod*$^+$ strain(s) in the novel host *D. simulans* STCP. Given the uncultivable nature of Wolbachia, this hypothesis was investigated on the basis of MLST and *wsp* gene sequencing analyses. The results were clear: (a) both naturally infected and transinfected lines carry Wolbachia strains having identical MLST profiles and *wsp* gene sequences and (b) no evidence of multiple infections was observed in any of the donor or the transinfected lines. Thus, a transfer of a previously undetected *mod*$^+$ strain from the native hosts to the novel *D. simulans* STCP is not likely to have occurred.
d. If we assume that *w*Tei (*w*Yak or *w*San) is a genotypically *mod*$^-$ strain in its native host, then the fourth possible explanation could be a genotypic change of *w*Tei (*w*Yak or *w*San) from *mod*$^-$ to *mod*$^+$ upon its transfer to the novel host *D. simulans* STCP. This genetic change could be due to a single point mutation, a chromosomal rearrangement, a recombination event, or a transposable element. All of these explanations are made extremely unlikely by the fact that more than one line with mod$^+$ phenotype for each one of the three Wolbachia strains (*w*Yak, *w*Tei, and *w*San) was generated through their transfer to *D. simulans* STCP.

Bordenstein *et al.* (2006) recently suggested that Wolbachia symbiosis should be considered as a tripartite association between the host, the bacterium, and the phage. If the phage plays indeed a causative role in the modification mechanism, and if the *D. teissieri* host background is more permissive for the lytic action of the endogenous *w*Tei phage(s) compared to the *D. simulans* STCP background, the presence of the same Wolbachia strain could result in low CI levels in *D. teissieri* due to the high lytic action of Wolbachia phase (WO) and high CI levels in *D. simulans* due to the low lytic phage activity. In any case, if phage activity is different in native and transinfected hosts, differences in density should be expected. Our currently available data do not support this hypothesis.

On the basis of the above and considering the available genetic, cellular, and molecular evidence it seems most likely that *D. simulans* STCP is a more permissive host for CI expression by *w*Tei, *w*Yak, and *w*San than their native hosts, *D. teissieri*, *D. yakuba*, and *D. santomea*, respectively.

TABLE 10

Compatibility relationships (expressed in *D. simulans* STCP background) between the Wolbachia strains used in this study

	Male infection								
Female infection	*w*Yak	*w*Tei	*w*San	*w*Ri	*w*Mel	*w*Au	*w*Ha	*w*No	*w*Ma
STCP	Low CI	High CI	Low CI	High CI	High CI	No CI	Medium CI	Medium CI	No CI
*w*Yak	NA[a]	Low CI	ND[b]	No to low CI	High CI	No CI	Medium CI	Medium CI	No CI
*w*Tei	NA	Low CI	NA	No to low CI	Low CI	No CI	Medium CI	Medium CI	No CI
*w*San	NA	Low CI	NA	No to low CI	High CI	No CI	Medium CI	Medium CI	No CI
*w*Ri	NA	Medium to high CI	NA	No to low CI	No to low CI[c]	No CI	Medium CI	Medium CI	No CI
*w*Mel	NA	High CI	NA	Medium CI[c]	No CI	ND	ND	ND	ND
*w*Au	NA	High CI	NA	High CI	ND	No CI	ND	Medium CI	ND
*w*Ha	NA	High CI	NA	High CI	ND	ND	No CI	ND	No CI
*w*No	NA	High CI	NA	High CI	ND	No CI	ND	No CI	ND
*w*Ma	NA	Medium CI	NA	High CI	ND	ND	Medium CI	No CI[d]	No CI

CI, cytoplasmic incompatibility

[a] NA, not assessed (as discussed in the text, the rescue potential of different Wolbachia strains against the *w*Yak and *w*Tei modification cannot be validly determined due to the low levels of CI expressed in *w*Yak- and *w*San-infected *D. simulans* STCP lines).

[b] ND, these crosses have not been performed in the *D. simulans* STCP genomic background.

[c] Based on previous reports (Poinsot *et al.* 1998).

[d] Based on previous reports (Bourtzis *et al.* 1998; Merçot and Poinsot 1998a,b).

Compatibility relationships: The presence of nine different Wolbachia strains belonging to the A and B supergroups (*w*Yak, *w*Tei, *w*San, *w*Ri, *w*Mel, *w*Ha, *w*Au, *w*No, and *w*Ma) in the same host genomic background (*D. simulans* STCP) made genetic crosses possible to study their compatibility relationships. The crosses revealed interesting compatibility patterns between *w*Yak, *w*Tei, *w*San, and the other Wolbachia strains (see Table 10):

a. An unexpected finding was that *w*Tei could not fully rescue its own modification (Table 5). To our knowledge, this is the first fully documented report of a Wolbachia strain that is unable to fully rescue its own modification. Although the incomplete rescue of *w*Cer2 and *w*Cer4 modifications by *w*Cer2 and *w*Cer4 themselves in transinfected medfly lines has previously been reported in *Ceratitis capitata*, the presence of multiple infections in those cases could not be excluded (Zabalou *et al.* 2004b). Riegler *et al.* (2004) also reported that *w*Cer2 cannot fully rescue its own CI, when transferred to *D. simulans* STCP; however, the authors concluded imperfect transmission to be the likely explanation. This is not the case in our study, since *w*Tei exhibits perfect maternal transmission in the transinfected line of *D. simulans* STCP (data not shown). It is worth noting that both *w*Yak and *w*San Wolbachia strains can, with the same efficiency as *w*Tei, partially rescue the *w*Tei modification in the *D. simulans* STCP background, suggesting that all three Wolbachia strains probably share the same genetic rescue properties (see also below). It should be noted at this point that the question of which Wolbachia strains can rescue the *w*Yak and *w*San modification could not be validly addressed due to the low levels of CI induced by these strains.

b. The *w*Yak, *w*Tei, and *w*San Wolbachia strains can fully rescue the *w*Ri modification in the *D. simulans* STCP background, as shown in Table 6. The *w*Yak, *w*Tei, and *w*San strains exhibited the same rescue activity as in their native host background, *D. yakuba*, *D. teissieri*, and *D. santomea*, respectively (Zabalou *et al.* 2004a). On the other hand, *w*Ri exhibits a very low rescue activity of the *w*Tei modification in the *D. simulans* STCP background, as shown in Table 5. Such asymmetrical CI relationships have been reported in the *Culex pipiens*–Wolbachia system (Sinkins *et al.* 2005) as well as for *w*Mel and *w*Ri (Poinsot *et al.* 1998). Poinsot *et al.* (1998) reported a unidirectional CI pattern between the two *mod*$^+$ strains, *w*Mel and *w*Ri: *w*Ri can fully rescue the *w*Mel modification, while *w*Mel can only partially rescue the *w*Ri modification. A similar asymmetrical CI pattern was observed in our study between *w*Tei and *w*Ri in the same host background, *D. simulans* STCP: *w*Tei can fully rescue the *w*Ri modification while *w*Ri can only slightly rescue the *w*Tei modification.

c. The *w*Yak, *w*Tei, and *w*San Wolbachia strains exhibit different compatibility relationships with the *w*Mel strain in the *D. simulans* STCP background, as shown in Tables 5 and 7. The *w*Yak and *w*San strains do not rescue the *w*Mel modification. However, the *w*Tei strain does rescue the *w*Mel modification, although the rescue is not complete. On the other hand, the *w*Mel strain does not rescue the *w*Tei modification. These data suggest the presence of another asymmetrical CI pattern, this time between *w*Tei and *w*Mel: *w*Tei can partially rescue *w*Mel while *w*Mel cannot rescue *w*Tei.

In addition, the data of our study allow us to discuss the compatibility relationships between *w*Mel and *w*Ri.

Poinsot *et al.* (1998) could not address the question of whether the assymetrical CI relationship between *w*Ri and *w*Mel was qualitative or quantitative. In our study, it is demonstrated that *w*San and *w*Yak can rescue the *w*Ri modification but not the *w*Mel modification, suggesting that the genetic determinants of modification are qualitatively different between *w*Mel and *w*Ri.

d. As shown in Table 8, *w*Yak, *w*Tei, and *w*San cannot rescue the *w*Ha modification. In addition, the *w*Ha strain cannot rescue the *w*Tei modification, suggesting that *w*Tei and *w*Ha are bidirectionally incompatible. Also, the *w*Au strain neither induces CI in the *D. simulans* STCP background nor rescues the *w*Tei modification (see Table 5), confirming once again its *mod*$^-$ *resc*$^-$ status (Hoffmann *et al.* 1996).

e. The compatibility relationships of the *w*Yak, *w*Tei, and *w*San (A-supergroup Wolbachia strains) with two B-supergroup Wolbachia strains, *w*No and *w*Ma, were also studied (Tables 5 and 9). Our results showed that the *w*Yak, *w*Tei, and *w*San strains do not rescue the *w*No modification. On the other hand, the *w*No strain can partially rescue the *w*Tei modification. These data suggest that the Wolbachia strains *w*Tei and *w*No are bidirectionally incompatible, exhibiting a unique asymmetrical CI pattern. It should also be noted that this is the first report of a B-supergroup Wolbachia strain, the *w*No, being able to rescue, if only partially, the modification induced by an A-supergroup Wolbachia strain. Similarly, and as shown in Table 5, another B-supergroup Wolbachia strain, *w*Ma, which is considered a *mod*$^-$ *resc*$^+$ strain (Bourtzis *et al.* 1998; Merçot and Poinsot 1998a,b; but see also James and Ballard 2000), was also shown to partially rescue the modification induced by *w*Tei. Thus, both *w*No and *w*Ma are able to partially rescue the *w*Tei modification. Given the fact that *w*Ma fully rescues the *w*No modification (Bourtzis *et al.* 1998; Merçot and Poinsot 1998a,b), these results further support the genetic and phylogenetic evidence that *w*No and *w*Ma are very closely related (Bourtzis *et al.* 1998; Merçot and Poinsot 1998a,b; James and Ballard 2000; Paraskevopoulos *et al.* 2006).

In conclusion, the above discussed observations suggest that *w*Tei exhibits a unique combination of CI properties in the *D. simulans* STCP background: being bidirectionally incompatible with *w*Ha, exhibiting a complex pattern of modification and rescue relationships (partial and/or complete) with both A-supergroup (*w*Yak, *w*San, *w*Mel, and *w*Ri) and B-supergroup (*w*No and *w*Ma) strains and at the same time being unable to fully rescue its own modification. How can such a peculiar CI pattern be explained?

Presence of multiple rescue factors in a Wolbachia strain? A hypothesis that can explain the puzzling combination of CI properties present in the *w*Tei strain is that this Wolbachia strain carries in its genome at least three functional rescue factors for *w*Tei, *w*Ri, and *w*Mel, respectively. This conclusion is based on our genetic crosses, which clearly suggest that *w*Tei can partially rescue *w*Tei and fully rescue *w*Ri, while it can only partially rescue *w*Mel. It is also evident that the *w*Ri strain carries at least two rescue determinants for *w*Ri and *w*Mel: *w*Ri fully rescues wRi and *w*Mel. Similarly, the *w*Mel genome contains at least two rescue determinants for *w*Mel and *w*Ri: *w*Mel fully rescues *w*Mel and partially rescues *w*Ri. In addition, also the *w*No and *w*Ma strains carry at least two rescue factors: (a) the first functional rescue factor is specific for *w*No since both of these strains can fully rescue the *w*No imprint, and (b) *w*No and *w*Ma also carry a second, less functional, rescue factor for *w*Tei, since they can partially rescue the *w*Tei imprint. This study clearly indicates that single Wolbachia strains can carry multiple genetic determinants for rescue functions, belonging to different CI systems. An alternative more qualitative hypothesis could also be proposed. The question is whether "generalist" rescue determinants could exist. Can the degree of specificity of the modification and rescue functions also be questioned? Since the molecular basis of CI is not known, this hypothesis cannot be excluded.

It is worth noting in this context that mosquitoes of the *C. pipiens* complex exhibit very complex CI patterns between populations, with a high frequency of uni- or bidirectional incompatibilities (Subbarao 1982; Magnin *et al.* 1987; O'neill and Paterson 1992; Guillemaud *et al.* 1997; Sinkins *et al.* 2005). Extensive studies on the *w*Pip Wolbachia variants revealed no polymorphism in the nucleotide sequences of 16S rRNA, *ftsZ* and *wsp* genes, the only differences being restricted to the transposons and ankyrin genes (Stouthamer *et al.* 1993; Guillemaud *et al.* 1997; Duron *et al.* 2005; Sinkins *et al.* 2005). On the basis of the above studies, it is evident that the compatibility relationships of the *w*Pip variants infecting species in the *C. pipiens* complex are not in accordance with a single pair of modification and rescue factors, similar to our observations described above for Drosophila; the major difference being that, while all Wolbachia strains are closely related in *C. pipiens* (all closely related members of B supergroup), the strains used in our study are rather divergent (members of both A and B supergroups).

Presence of multiple modification factors: It is difficult to determine if a Wolbachia strain possesses multiple modification factors. However, in the case of *w*Tei, the question arises. The *w*Tei strain appears to bear three independent genetic rescue determinants ($RESC_{TEI}{}^+$, $RESC_{RI}{}^+$, and $RESC_{MEL}{}^+$). Does it also possess the corresponding Mod determinants?

The results obtained using the strain *D. simulans* STCP [*w*No] allow an inference for the RI and MEL systems. *D. simulans* STCP [*w*No] females are completely incompatible with *D. simulans* STCP [*w*Ri] and *D. simulans* STCP [*w*Mel] males. If the *w*Tei variant does possess the functional Mod factors characterizing *w*Ri and *w*Mel, these

D. simulans STCP [*w*No] females should be completely incompatible with *D. simulans* STCP (*w*Tei) males, which is not the case, the crosses being partially compatible. The negative answer is confirmed, in the case of the MEL system, by *D. simulans* STCP (*w*Yak) and *D. simulans* STCP (*w*San) females. Indeed, these females are incompatible with males from the *D. simulans* STCP [*w*Mel] line but are compatible with *D. simulans* STCP (*w*Tei) males. Therefore, the *w*Tei variant seems only to express the TEI mod factors. The alternative hypothesis would be that *w*Tei possesses the genetic Mod factors for RI and MEL but that these determinants are not expressed fully in this Wolbachia variant. However, our results do not support this possibility.

Is *w*Tei a suicide Wolbachia variant? As shown above, *w*Tei can only partially rescue its own imprint. This was an unexpected finding and represents the first documented case of an, even partial, suicide Wolbachia strain reported as yet. How can this partial rescue then be explained?

There are two possible explanations: the first possible explanation is that *w*Tei is a suicide variant in a qualitative manner; that is, it is a true suicide strain. Different mathematical approaches based on the "lock-and-key" model (Poinsot *et al.* 2003) suggest that new CI types can evolve through a two-step process (Charlat *et al.* 2001, 2005; but see also Dobson 2004 for an alternative hypothesis): the first step involves drift on the modification variants, whereas the second step involves selection on the rescue variants. Let us assume a strain that develops a new modification factor (modB) that cannot be rescued by either the wild-type (modA rescA) or the mutant strain (modB rescA). If the modB rescA mutant strain reaches a high frequency in the population, a second mutant, modB rescB, is selectively favored and replaces both the wild-type modA rescA and the first mutant, modB rescA (Charlat *et al.* 2001, 2005). However, it has been determined that even a very small degree of partial compatibility between the new modB and rescA function plays an important role in the likelihood of the evolution of a novel CI type (Charlat *et al.* 2005; Engelstädter *et al.* 2006). The *w*Tei strain may represent the equivalent of such newly evolved modB rescA mutant strain.

The second possible explanation is that *w*Tei is not a suicide variant in a qualitative way but rather in a quantitative way: too much modification factor may be expressed in *D. simulans* STCP (*w*Tei) young males for the rescue function to neutralize it completely. The same kind of phenomenon can be found with other Wolbachia variants, when using very young males (Yamada *et al.* 2007). In the case of *w*Tei, however, it is difficult to test this, since the CI levels expressed by *w*Tei decrease very fast with age in *D. simulans* (our unpublished observations). Alternatively, the female germ line may have less rescue capacity than the one needed for the complete rescue of the *w*Tei modification (*i.e.*, low levels of rescue product being due to low Wolbachia density). Our study shows that the *w*Tei distribution and infection levels in embryos, ovaries, and testes of the transinfected host *D. simulans* STCP (*w*Tei) are similar to those observed in its native host *D. teissieri* (*w*Tei). In addition, the *w*Tei strain infecting *D. simulans* STCP females can fully rescue the modification of heavily *w*Ri-infected *D. simulans* STCP males, thus, a mechanism based on density levels is not likely. However, it should also be noted that another factor, which may be influencing both the modification and the rescue functions, is the lytic state of the Wolbachia phage (WO), as recently reported by Bordenstein *et al.* (2006). The phage may be entering its lytic phase at a particular tissue and/or developmental stage, thus reducing the Wolbachia levels and influencing the modification and/or rescue functions in a tissue- and developmental stage-specific manner.

An alternative hypothesis could also be proposed. Even though *w*Tei might behave as a suicide variant in *D. simulans*, this phenotype is not expressed in the native host where *w*Tei does not induce CI, suggesting that the genetic determinants of CI might evolve neutrally (at least on the CI phenotype).

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