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ORIGINAL ARTICLE

Host genotype changes bidirectional to unidirectional cytoplasmic incompatibility in *Nasonia longicornis*

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Wolbachia are the most abundant maternally inherited endosymbionts of insects and cause various reproductive alterations in their hosts. One such manipulation is cytoplasmic incompatibility (CI), which is a sperm–egg incompatibility typically resulting in zygotic death. *Nasonia longicornis* (Hymenoptera: Pteromalidae) has an A supergroup and two closely related B supergroup *Wolbachia* infections. The B supergroup bacteria co-diverged in this host genus. Both triple (*w*NlonA*w*NlonB1*w*NlonB2) and double infections (*w*NlonA*w*NlonB1, *w*NlonA*w*NlonB2) have been obtained from the field. In the present study, CI was determined among the three *Wolbachia* types in different host genetic backgrounds. Results show that host genetic background determines whether bidirectional CI or unidirectional CI occurs between the two closely related B group *Wolbachia*. Results show that the *w*NlonB1-infected males are bidirectionally incompatible with *w*NlonB2 in their 'native' nuclear genetic background, whereas *w*NlonB1 males are compatible with *w*NlonB2 in two other *N. longicornis* genetic backgrounds, resulting in unidirectional CI. In contrast, *w*NlonB2-infected males are incompatible with *w*NlonB1 females in all three host genetic backgrounds. These changes in incompatibility are not due to the loss of the bacteria. We hypothesize that a repressor gene for sperm modification by *w*NlonB1 is segregating in *N. longicornis* populations. The relevance of these findings to the potential role of *Wolbachia* in host-reproductive divergence and speciation is discussed.
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Introduction

Wolbachia are one of the most abundant endosymbionts of arthropods and nematodes, infecting about 66% of all terrestrial arthropods (Hilgenboecker *et al*., 2008). The principal mode of transmission across generations is through the infected female cytoplasm, where the bacteria are transmitted from the maternal ovaries to the eggs (Werren, 1997; Stouthamer *et al*., 1999; Stevens *et al*., 2001; Werren *et al*., 2008). As they are maternally transmitted and are endosymbiotic, *Wolbachia* are selected to increase their fitness through the transmitting sex, that is, infected females. This is achieved either by mutualism or by manipulating the host-reproductive machinery to produce more infected females relative to the non-transmitting sex (males). Some of the mechanisms by which *Wolbachia* achieve this are killing of infected males, induction of parthenogenesis in infected females, feminization of genetic males and cytoplasmic incompatibility (CI) (reviewed in Werren *et al*., 2008). As a result, *Wolbachia*-induced host-reproductive manipulation has profound implications for many fundamental biological processes, such as sex determination (Rigaud *et al*., 1997; Werren and Beukeboom, 1998), sexual selection (Jiggins *et al*., 2000; Koukou *et al*., 2006), speciation (Laven, 1959; Breeuwer and Werren, 1990; Bordenstein *et al*., 2001; Jaenike *et al*., 2006) and organization of host genome structure (Kondo *et al*., 2002b, Dunning-Hotopp *et al*., 2007).

Among the various *Wolbachia*-induced phenotypes, CI seems to be the most common and has received the most attention. According to the 'modification-rescue' model of CI (Werren, 1997), *Wolbachia* 'modify' the sperm of an infected male and the female must also be infected with the same bacteria to 'rescue' this particular modification. If the female is uninfected or infected with a *Wolbachia* of a different CI type, then this particular 'modification' cannot be 'rescued' and CI is expressed. When CI occurs reciprocally between two different *Wolbachia* strains, they are referred to as bidirectionally incompatible. Apparent 'non-functional' and partially functional (for example, modification-deficient but rescue-competent) CI types are also known (Giordano *et al*., 1995; Hoffmann *et al*., 1996; Bourtzis *et al*., 1998; Charlat *et al*., 2003; Zabalou *et al*., 2008). Presence of more than one incompatibility type has been found in a number of different insects including mosquitoes, fruit flies, beetles and wasps (Laven, 1959; Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Montchamp-Moreau *et al*., 1991; Perrot-Minnot *et al*., 1996; Bordenstein *et al*., 2001). Bidirectional CI (bi-CI) has attracted particular attention because of its potential role in preventing gene flow between two

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different incompatibility types and thus contributing to reproductive isolation and speciation (Breeuwer and Werren, 1990; Werren, 1998; Bordenstein *et al*., 2001; Bordenstein, 2003; Telschow *et al*., 2002, 2005a, b).

Host genotypes can influence *Wolbachia* density and phenotypes (reviewed in Jaenike, 2009). In the haplodiploid wasp *Nasonia*, there are two distinct types of CI: conversion of diploid fertilized eggs to haploid males and mortality of fertilized eggs. With introgression of inter-specific genotypes, Bordenstein *et al*. (2003) showed that the expression of these two types of CI is controlled by host genotype. Sinkins *et al*. (2005) showed that introgression into a common genetic background can alter bidirectional to unidirectional CI (uni-CI) in *Culex quinquefasciatus*. Host genotype can also transform *Wolbachia* induced phenotypes, for example, converting CI to male-killing (Jaenike, 2007) and vice-versa (Hornett *et al*., 2010). Effects of host genotype on *Wolbachia* infection density are well established. In the adzuki bean beetle, *Callosobruchus chinensis*, which is infected with multiple *Wolbachia* strains, the density of the bacteria is controlled by both intra-specific host genotype, as well as other co-infecting *Wolbachia* strains (Kondo *et al*., 2005). In the wasp *Leptopilina heterotoma*, *Wolbachia* densities vary based on intra-specific genotypes of two divergent laboratory cultures (Mouton *et al*., 2007). The mosquito *Culex pipiens* has a polymorphism for an allele conferring insecticide resistance (Berticat *et al*., 2002). Mosquitoes carrying the resistant allele have higher *Wolbachia* densities compared with the ones carrying the susceptible allele (Duron *et al*., 2006). The role of host genotype in regulating *Wolbachia* densities has also been empirically established, by transinfection experiments, at an inter-specific level (Ikeda *et al*., 2003), and at the intra-specific level in *Drosophila* (Clark *et al*., 2003).

The parasitoid wasp genus *Nasonia* has been used extensively in *Wolbachia* research. It is a genus of four closely related species (Raychoudhury *et al*., 2010a) that harbor 11 different *Wolbachia* infections (Raychoudhury *et al*., 2009). The four species—*N. vitripennis*, *N. longicornis*, *N. giraulti* and *N. oneida*—are infected with multiple *Wolbachia* infections belonging to supergroups A and B. *N. longicornis* has two infections from the B supergroup (named *w*NlonB1 and *w*NlonB2) and one from the A (*w*NlonA) supergroup (Raychoudhury *et al*., 2009). Moreover, these two B supergroup infections occur as a polymorphism in the field where host strains infected with *w*NlonB1*w*NlonA, *w*NlonB2*w*NlonA and *w*NlonB1*w*NlonB2*w*NlonA have been found (Raychoudhury *et al*., 2009).

The B supergroup *Wolbachia* in *N. longicornis* and *N. giraulti* show one of the few documented cases of host-*Wolbachia* co-divergence. The *w*NlonB1 and *w*NlonB2 infections are estimated to have diverged ~1.5 million years ago in the most recent common ancestor of *N. longicornis* and *N. giraulti* (Raychoudhury *et al*., 2009). Since most *Wolbachia* infections are transient, within a particular host species, over an evolutionary time scale (Vavre *et al*., 1999; Baldo *et al* 2006b; Frost *et al*., 2010), the *N. longicornis* system provides an opportunity to study the long-term effects of *Wolbachia*-host association.

In the present study we investigate the level and type of CI between these strains, specifically focusing on the host strains with the *Wolbachia* genotype of *w*NlonB1*w*NlonA and *w*NlonB2*w*NlonA. We ask the following questions: (1) Do the closely related *w*NlonB1 and *w*NlonB2 cause CI against each other?, (2) Do the closely related *w*NlonB1 and *w*NlonB2 cause CI against *w*NlonA? and (3) Are there effects of host genotype on CI?

Materials and methods

Nomenclature and strains used

The preferred method of denoting a particular host strain and its *Wolbachia* infection in *Nasonia* is to indicate its supergroup, as well as its host genotype. For example, [*w*NlonB1 *w*NlonA]L indicates that the host strain is *N. longicornis*, which has two infections, one each from the two supergroups of A and B. In the present study we only use different *N. longicornis* host strains and their associated *Wolbachia*. Therefore, we have simplified the nomenclature of the bacterial infections by removing the species name, but have included host genotype information. Moreover, as the effects of host genotype have been found to be important for the expression of CI, we have also indicated the source of the bacterial infections. Five host genetic backgrounds (*UT1*, *UT2*, *UT3*, *UT4* and *CA*) have been used and the particular *Wolbachia* infections from these genetic backgrounds have been designated as a subscript. For example, $(wAwB1)_{UT1}$ indicates that the strain has two *Wolbachia* infections and were originally obtained from the UT1 genetic background. The host genetic background is indicated in brackets at the end of the strain designation. For example, $(wAwB1)_{UT1}$[*UT1*] indicates that these *Wolbachia* infections have UT1 host genetic background. Most of these strains were introgressed in different genetic backgrounds to test for the effect of host genotype on CI, and the designations change accordingly.

Many of the strains were treated with antibiotics to test the effects of host genotype on the crosses in the absence of *Wolbachia* infections. The nomenclature of cured strains is indicated by 0 followed by the strain origin in brackets, for example, 0[*UT1*] for the cured strain from the *UT1* host genetic background. The different strains used, along with the new nomenclature, are detailed in Supplementary Table S1.

A confounding factor for the determination of the CI relationship between *w*NlonB1 and *w*NlonB2 is that they are present as double and triple infections along with the *w*NlonA *Wolbachia*. Repeated efforts to make single B-infected strains failed, probably because of their low titer compared with the *w*NlonA. This was consistent with previous attempts by Bordenstein and Werren (2007). But fortunately we had access to a single *w*NlonA-infected strain, $(wA)_{UT4}$[*UT4*] obtained by Bordenstein and Werren (2007) from a double-infected host strain (Supplementary Table S1). This strain was used to distinguish the effects of the A supergroup infection in the crosses. To establish that the A infections in all the host strains are the same Multi Locus Sequence Type (MLST) (Baldo *et al*., 2006b), we amplified and sequenced the A MLST loci from each of the strains. No variation was found in any of the six MLST genes from the four host strains (Raychoudhury *et al*., 2009). Similarly, all the six MLST loci were amplified from the three strains with B supergroup infections and no

sequence difference were found between strains for *w*NlonB1 or *w*NlonB2 (Raychoudhury *et al*., 2009).

Introgression lines

To investigate the effect of host genetic background, introgression lines were produced for most of these strains, where the bacterial genotypes were backcrossed in a different host genetic background. The crosses were done for six generations, whereby, theoretically at least, 98% of the native genome was replaced by the new host genome. Thus, when $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$ females were backcrossed with males from the 0[*UT2*] strain for six generations it produced the strain $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT2]$. To make these introgression lines, five females were initially chosen with a particular bacterial polymorphism and mated to the uninfected males with the desired host genotypes. Mating was observed and these females were individually hosted for 2 days. Then the DNA was extracted to check for the presence of the desired bacterial polymorphisms with specific primers. The progeny of all the females with the desired bacterial polymorphism were mixed together. Five virgin females were randomly chosen from this pooled set of offspring to start the next generation of introgression and this was continued for a further five generations. Thus, there were five different isofemale lines for each set of introgression. These strains were allowed to sib-mate for two generations before being used for the experimental crosses. Out of the five sets of strains for each particular introgression only one strain was used for the crosses.

Crossing design

All crosses were set up as single-pair mating between a virgin male and a virgin female within 2 days of emergence. Individual pairs were observed until they mated, which was mostly within the first 5 min of observation. They were kept overnight at 25° with a drop of honey for feeding. The next day the males were discarded and the females were provided with two *Sarcophaga bullata* hosts. The females were allowed to parasitize the hosts for 48 h, upon which they were discarded and the progeny was allowed to emerge. Upon the death of the progeny they were scored for sex ratio and total family size. To measure levels of cytoplasmic incompatibility, male and female offspring numbers are compared with the females of a strain who are mated to males of different *Wolbachia* infection types versus to uninfected males. This is a valid approach because females do not alter the number of eggs they lay based on their mating status (Breeuwer and Werren, 1995), making comparisons in the number of male and female progeny produced by females of the same strain who are mated to males of an infection type relative to uninfected males a reliable index of offspring mortality and sex conversion due to cytoplasmic incompatibility. Individual crosses where the females died before the 48 h of parasitization or produced diapause larvae were not counted and were discarded from the analysis. All of the crosses were repeated and were consistent with each other. We have presented all the data from the two trials in the online Supplementary Material (Supplementary Tables S2 and S3) and have presented a summary of these crosses (Table 1) and figures for the first trial of each cross in the main text. The analysis presented in the main text is based on the first set of crosses (summarized in Supplementary Table S2).

Determining infection status

Individuals to be used in the crosses were established from single-mated females. The female was allowed to parasitize a host for 48 h and then the DNA was extracted. The presence of *Wolbachia* strains was confirmed by supergroup-specific primer pairs for each strain. Specific primer pairs for *w*B1 were wsp-3_flkBF3/wsp-3_flkBR3, and for *w*B2 were wsp-3_flkBF3A/wsp-3_flkBR3A (Raychoudhury

Table 1 Summary of the crosses done indicating which were incompatible (CI) and compatible (No CI)

Male	*Host genotype*	*Female*	
Wolbachia polymorphism		*CI*	*No CI*
$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$	[*UT1*]	$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$, 0[*CA*], 0[*UT1*], $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$ [*UT1*]	Self, $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$
$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$	[*UT1*][a]	0[*UT1*], $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$ [*UT1*]	Self
$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$	[*CA*]	0[*CA*]	Self, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[CA]$
$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$	[*UT2*]	0[*UT2*]	Self, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$
$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$	[*UT2*]	$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$, $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$, 0[*CA*], 0[*UT2*], $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT2]$	Self, $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$
$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$	[*UT2*][a]	0[*UT2*], $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT2]$	Self
$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$	[*CA*]	0[*CA*], $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[CA]$	Self
$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$	[*UT1*]	$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$, 0[*UT1*]	Self
$(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}$	[*UT3*]	$(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$, 0[*CA*], 0[*UT3*]	Self
$(w\mathrm{A})_{\mathrm{UT4}}$	[*UT4*]	0[*CA*], 0[*UT4*]	Self, $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$,$(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$
0	[*CA*]		Self, $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$, $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$
0	[*UT1*]		Self, $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$, 0[*UT2*], 0[*UT3*], 0[*UT4*]
0	[*UT2*]		Self, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, 0[*UT2*], 0[*UT3*], 0[*UT4*]
0	[*UT3*]		Self, $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$, 0[*UT2*], 0[*UT3*], 0[*UT4*]
0	[*UT4*]		Self, $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$, 0[*UT2*], 0[*UT3*], 0[*UT4*]

Self indicates crosses done within a particular strain.
[a]Indicates that the *Wolbachia* polymorphisms were re-introgressed into their native host genetic backgrounds.

et al., 2009). To further confirm infection status, the amplified product of the supergroup B-specific *ftsZ* primer pair (Baldo *et al*., 2006b; Raychoudhury *et al*., 2009) was sequenced and checked for strain-specific polymorphisms, as *w*NlonB1 and *w*NlonB2 differ by a single base pair (C–A) in position 45 of the allele (Baldo *et al*., 2006b). The A supergroup *Wolbachia* was also similarly confirmed by PCR amplification and sequencing of the products of the A-specific *ftsZ* primer (Raychoudhury *et al*., 2009). The primer sequences, PCR conditions and sequencing protocol are given in Raychoudhury *et al*. (2009). To check for incomplete transmission of *Wolbachia* from the single-mated female to its progeny, some of the daughters were checked with specific primers. No loss of *Wolbachia* polymorphism was found during the crosses. Thus, the resulting crosses were not biased by the loss of the bacteria. To check for antibiotic curing of some of these strains the absence of any *Wolbachia* infections were tested with *wspec* primer pairs (Baldo *et al*., 2006b), which is sensitive for both the A and B infections. The presence of other endosymbionts, such as *Cardinium*, *Rickettsia* and Spiroplasma, has not been found in *Nasonia.* This is based on both bacterial group-specific PCR surveys and bacterial 16*S* ribosomal sequencing (JH Werren, unpublished data).

Statistical analysis

CI in *N. longicornis* is expressed primarily as a reduction in the number of daughters and a slight increase in the number of sons in an incompatible cross (Bordenstein *et al*., 2001). As *Nasonia* has a haplodiploid mode of sex determination the zygotic death manifests itself in the diploid daughters, but not in the haploid sons. CI results in the loss of paternal chromatin, whereby most of the diploid zygotes are killed but some get transformed into males, which increases the number of sons in the progeny family size of an incompatible cross (Bordenstein *et al*., 2001). Thus, incompatible crosses in *N. longicornis* can be distinguished from a compatible cross by a reduction in the number of daughters and a slight increase in the number of sons. To test for statistical significance we used the non-parametric Mann–Whitney *U* test (MWU) as employed in the program StatistiXL (Broadway–Nedlands, Western Australia). The summary data for the crosses are indicated as means ± s.e. of the family size.

Results

Do (*w*A*w*B1)$_{UT1}$[*UT1*] and (*w*A*w*B2)$_{UT2}$[*UT2*] show bidirectional incompatibility in their native nuclear background?

One of the major questions that we wish to answer is whether the two B infections are distinct incompatibility types. To test this we crossed these two strains, first, with the triple-infected males from the strain (*w*A*w*B1*w*B2)$_{UT3}$[*UT3*] and then with each other. As Figure 1a indicates, there is significant CI between the females of these two strains and the males of the triple-infected strain. The cross between the females of (*w*A*w*B1)$_{UT1}$[*UT1*] and (*w*A*w*B2)$_{UT2}$[*UT2*] and (*w*A*w*B1*w*B2)$_{UT3}$[*UT3*] males produces progeny sizes that are significantly smaller (MWU, $U=746.0$, $P<0.001$ and $U=750.0$, $P<0.001$, respectively) when compared with the intra-strain controls indicating CI (Cross nos. 22 and 23, Supplementary Tables S2 and S3). Thus, the two B supergroup infections together produce a modification in the males, which cannot be rescued in the females by the individual B infections. This indicates that the two B infections represent different incompatibility types.

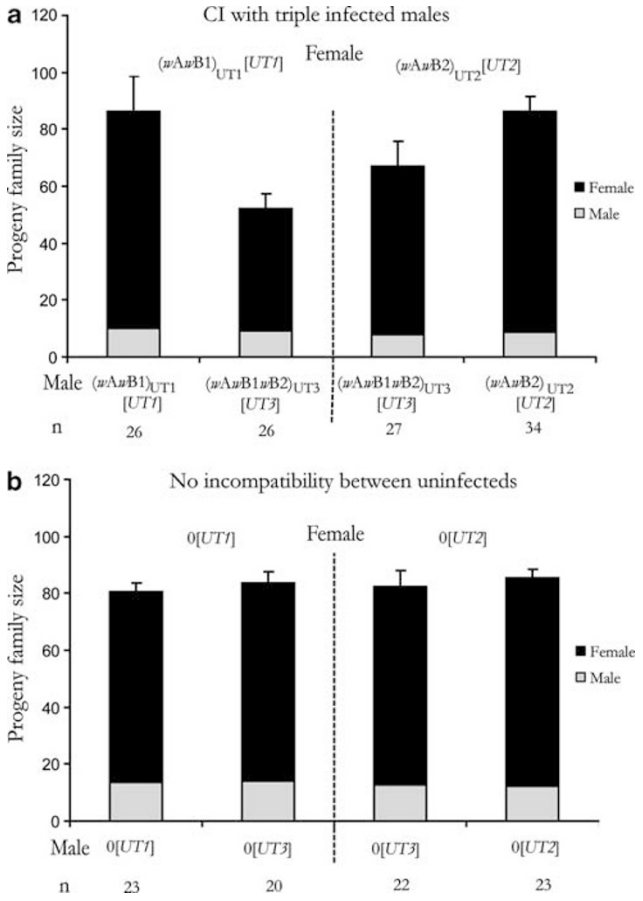

Figure 1 CI between double-infected females and triple-infected males. (**a**) CI seen between the females of the two B-infected strains and triple-infected (*w*A*w*B1*w*B2) males. The columns on the left of the horizontal dotted line show CI between the males of (*w*A*w*B1*w*B2)$_{UT3}$[*UT3*] and females of (*w*A*w*B1)$_{UT1}$[*UT1*]. The columns on the right show CI between the triple-infected males and (*w*A*w*B2)$_{UT2}$[*UT2*]-infected females. In both these sets of crosses the triple-infected males induce a significant reduction in the progeny family size when compared to the intra-strain controls (see text for details). (**b**) The reduction in progeny numbers is not due to nuclear genetic incompatibilities, as the same strains when cured of their *Wolbachia* show no significant reduction in progeny family size (MWU: $U=259.5$, $P=0.656$ and $U=280.5$, $P=0.536$, respectively).

One explanation of the above results could be that the incompatibility observed is not due to the effects of the *Wolbachia* infections, but are strictly due to genetic incompatibilities between host strains (for example, they are cryptic species). To determine whether the incompatibilities are due to the effects of the bacteria and not due to host genetic background alone, we cured these strains of their *Wolbachia* infections by antibiotic treatment and repeated the crosses. The results (Figure 1b and Table 1, Cross nos. 24 and 25, Supplementary Tables S2 and S3) show that none of these crosses produces a significant reduction in the family size, indicating that the incompatibilities are produced due to effects of the bacteria and not by nuclear genetic incompatibilities between the

strains. This supports the view that the two B infections consist of two distinct incompatibility types.

Based on the results of the cross with the males of the triple-infected strain ((*w*A*w*B1*w*B2)$_{UT3}$[*UT3*]), we predicted that (*w*A*w*B1)$_{UT1}$ and (*w*A*w*B2)$_{UT2}$ should be bidirectionally incompatible (that is, each one not able to rescue the modification of the other). To test this we crossed the two strains (*w*A*w*B1)$_{UT1}$[*UT1*] and (*w*A*w*B2)$_{UT2}$[*UT2*] with each other (Figure 2a). The results indicate that there is partial bi-CI between these two strains. When compared with the controls, the females of (*w*A*w*B1)$_{UT1}$[*UT1*] can only partially rescue the modification produced by (*w*A*w*B2)$_{UT2}$[*UT2*] males (MWU, $U=693.0$, $P<0.001$). Similarly, females of (*w*A*w*B2)$_{UT2}$[*UT2*] also partially rescue the modification produced by (*w*A*w*B1)$_{UT1}$[*UT1*] males (MWU, $U=454.5$, $P<0.001$) based on reduction in progeny family size. No significant reduction in family sizes could be detected when these crosses were repeated with cured strains (Figure 2b, Cross nos. 31 and 32, Supplementary Tables S2 and S3), showing that the effect is not due to any intrinsic nuclear genetic incompatibility between the host strains. Thus, the two B infections produce bi-CI against each other and represent two distinct incompatibilities, at least in their native nuclear genetic backgrounds.

Do all the A supergroup infections have a similar incompatibility type?

One of the causes of the bi-CI seen in the two B-infected strains could be due to the differences in their resident A supergroup infections, even though each is identical in sequence at six strain typing genes (see Materials and methods). If the different A infections all represent distinct incompatibility types, then the bi-CI seen between these strains would be caused by these A infections and would be indistinguishable from the effects of the B *Wolbachia*, especially as these infections could not be separated from each other. If, on the other hand, the A infections share similar incompatibility types, then the double- and triple-infected females should be able to rescue the modification produced in males by the single A infection. To test whether the A infections present in the different strains have a similar incompatibility type we crossed males from the single A-infected strain with females of the triple-infected, as well as both the double-infected strains. As Figure 3 shows there is no significant reduction in progeny family size, indicating that multiple-infected females can all rescue the modification produced by the single A infection. In particular, females of the two double-infected strains, (*w*A*w*B1)$_{UT1}$[*UT1*] and (*w*A*w*B2)$_{UT2}$[*UT2*] can rescue modification of the males produced by (*w*A)$_{UT4}$[*UT4*], as there are no significant reductions in progeny family sizes (MWU, $U=421.5$, $P=0.619$ and $U=340.0$, $P=0.162$, respectively). Similarly, the modification produced by the single A-infected males can also be rescued by triple-infected females (MWU, $U=36$, $P=1.0$). This implies that either the (*w*A)$_{UT4}$[*UT4*] strain is not producing any modification or all the A's have similar or the same CI type with respect to the ability to rescue the modification produced by males of the single A-infected strain. To test the possibility that the (*w*A)$_{UT4}$[*UT4*] strain does not produce any incompatibility, we crossed males from this strain with the

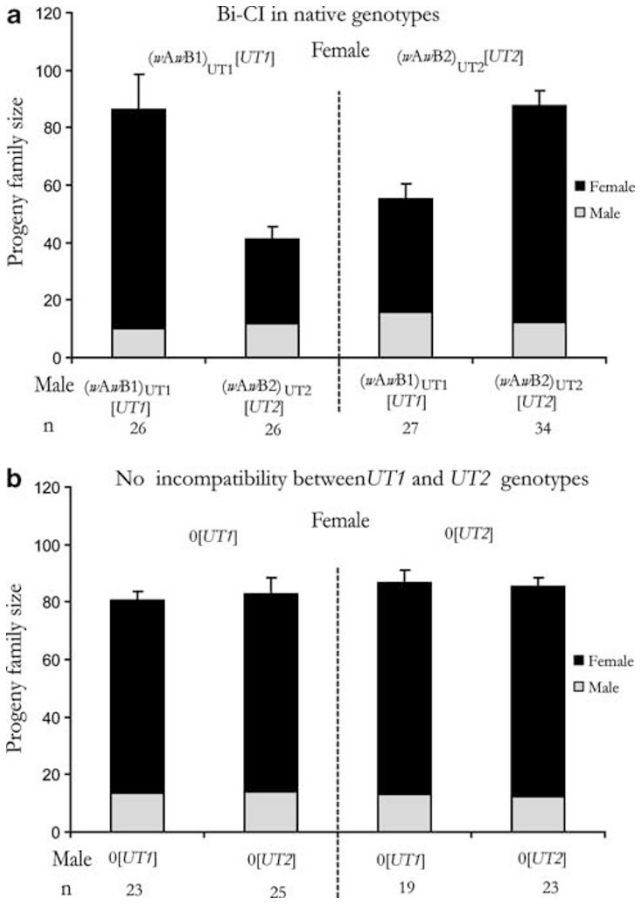

Figure 2 Bi-CI between the two double-infected strains. (**a**) Bi-CI seen between (*w*A*w*B1)$_{UT1}$ and (*w*A*w*B2)$_{UT2}$-infected strains in their own native host genotypes (see text for details). (**b**) The reduction in progeny numbers is not due to nuclear genetic incompatibilities, as the same strains when cured of their *Wolbachia* show no significant reduction in progeny family size. (MWU: $U=312.0$, $P=0.623$ and $U=238.0$, $P=0.635$, respectively).

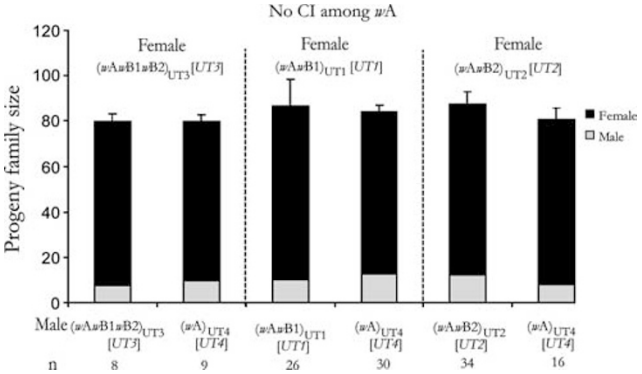

Figure 3 All the A supergroup infections share the same incompatibility type as the modification produced by the (*w*A)$_{UT4}$[*UT4*]-infected males can be rescued by all the other three infection polymorphisms. (MWU: $U=36$, $P=1.0$, $U=421.5$, $P=0.619$ and $U=340.0$, $P=0.162$, respectively).

Wolbachia-cured version of the same strain (0[*UT4*]), as well as with 0[*CA*] females. Results indicate that in both these sets of crosses the (*w*A)$_{UT4}$[*UT4*] males do induce CI (Table 1, Cross nos. 20 and 21, Supplementary Tables S2 and S3). Thus, the most parsimonious explanation is that the bi-CI seen in the previous crosses, involving the

two B infections, is due to differences in the B infection types, and not due to differences in the A infection.

Do the A and the two B infections have different incompatibility types?

One of the key questions about the *Wolbachia* infections in *N. longicornis* is whether they all represent different incompatibility types. We have already established that the two B infections are distinct incompatibility types as they produce bi-CI against each other (Table 1). Having established that all the A infections are similar in their incompatibility, we now asked whether this infection produces CI against the two B infections. As the individual infections could not be separated we used multiple *Wolbachia*-infected males against females from the single A-infected strain ($(w\mathrm{A})_{\mathrm{UT4}}[UT4]$). Males from $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$ and $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$ strains would have their sperm modified by two different *Wolbachia* and males from $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$ would have three infections modifying their sperm but the female would only have the *w*NlonA infections to rescue that modification. Therefore, if these are different incompatibility types there will be a significant reduction in the progeny family size owing to CI. As Figure 4a indicates, the females show significant CI with $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$ males (MWU, $U=380.0$, $P=0.002$), as well as with $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$ males (MWU, $U=441.0$, $P<0.001$). Moreover, there is also CI when the females of $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$ are crossed with males from $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$ (MWU, $U=454.0$, $P<0.001$). The uninfected control cross (Figure 4b, Cross nos. 15, 16 and 17, Supplementary Tables S2 and S3) shows that these results are not influenced by intrinsic host nuclear incompatibilities. Thus, the three infections, *w*NlonB1, *w*NlonB2 and *w*NlonA, represent three different incompatibility types, at least in their native genetic backgrounds.

Does the bi-CI between $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$ and $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$ change in an uniform genetic background?

A confounding factor during the expression of CI is the effect of host genetic background. Host genetic effects are known to moderate CI levels in diverse host taxa (Boyle *et al*., 1993; Bordenstein and Werren, 1998; McGraw *et al*., 2001; Ikeda *et al*., 2003; Kondo *et al*., 2005; Mouton *et al*., 2007). Therefore, to minimize the effects of different host backgrounds, we introgressed all these *Wolbachia* genotypes to a single genetic background by back-crossing them into the 0[*CA*] background for six generations (see Materials and methods: Introgression lines). When the crosses were repeated in this uniform genetic background the results (Figure 5b) indicated that bi-CI was transformed to unidirectional CI. Specifically, $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[CA]$ males show CI when crossed to $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[CA]$ females (MWU, $U=385.0$. $P<0.001$) but not the other way around (MWU, $U=133.5$, $P=0.665$). Thus, there is a pronounced effect of host genetic background on CI produced by $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$ males but not by $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$ males. One of the causes of the lack of CI on the part of $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[CA]$ males could have been loss of one or both their *Wolbachia* infections. But the presences of both *Wolbachia* were confirmed by PCR (see Materials and methods) before and after the crosses were done. The other reason for this conversion

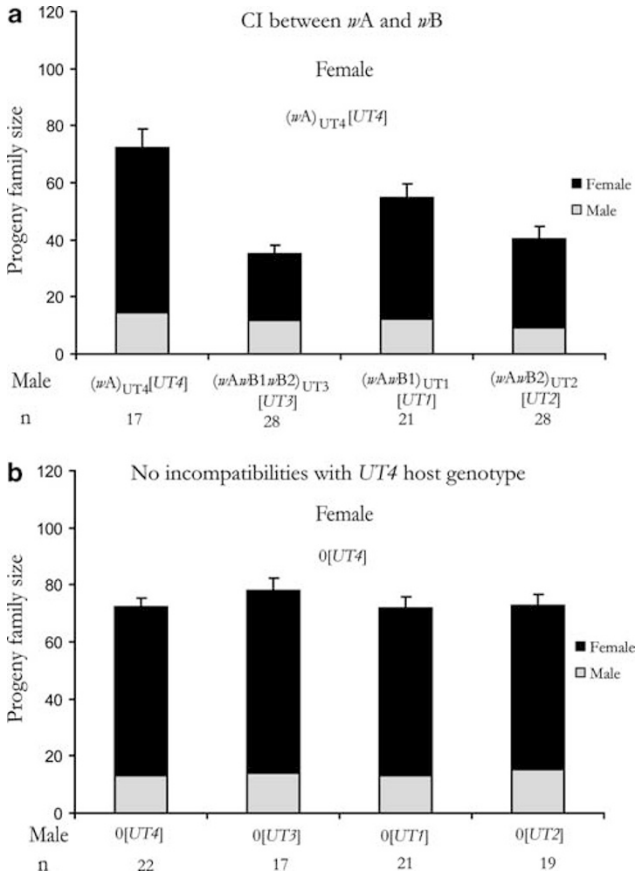

Figure 4 All the three *Wolbachia* in *N. longicornis* represent distinct incompatibility types. (**a**) The A and the two B supergroup infections all represent distinct incompatibility types. All the three multiply infected males produce CI against the single *w*A-infected females. The first column is the intra-strain control of the single A-infected strain $(w\mathrm{A})_{\mathrm{UT4}}[UT4]$. The second, third and fourth column represents the progeny family size when the females of this strain were crossed with males from $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$, $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$ and $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$, respectively. There is significant reduction in the progeny family size in each of these crosses (see text for details). (**b**) The reduction in progeny number is not caused by host nuclear incompatibilities, as the same strains when cured of their *Wolbachia* show no significant reduction in progeny size. (MWU: $U=253$, $P=0.063$, $U=242.0$, $P=0.801$ and $U=227.0$, $P=0.651$, respectively).

of CI could be that the $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$ males were not modifying sperm in this new host genetic background. We crossed the males of this strain with 0[*CA*] females and the results indicate that there is CI, and therefore modification of sperm (Cross no. 34, Supplementary Table S2 and S3).

Does the bi-CI between $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$ and $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}$ change in reciprocal genetic backgrounds?

One of the reasons why the $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[CA]$ males failed to produce CI could be owing to an interaction between the new host genotype and the resident *Wolbachia* infections. This particular genotype was collected from California, although the native genotype of the $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}[UT1]$ strain is from Utah (online Supplementary Table S1). Therefore, to investigate the host effect we reciprocally introgressed both these strains into the

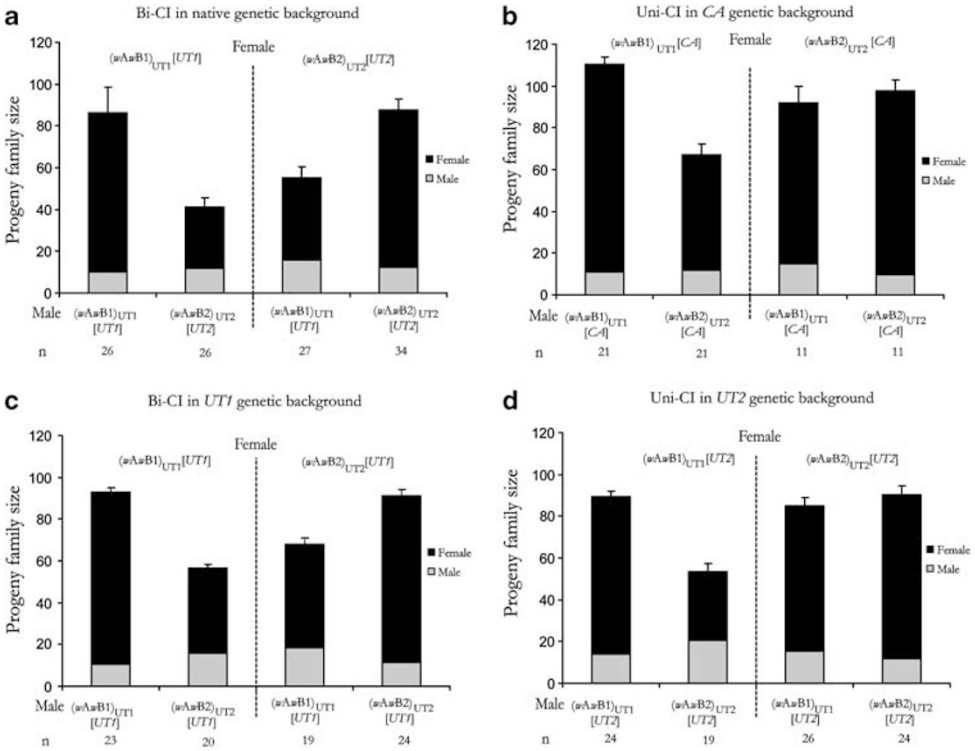

Figure 5 CI between the two strains $(wAwB1)_{UT1}$ and $(wAwB2)_{UT2}$ in different host genetic backgrounds. (**a**) Bi-CI between $(wAwB1)_{UT1}$ and $(wAwB2)_{UT2}$ in their native host genetic background. (**b**) Uni-CI between $(wAwB1)_{UT11}$ and $(wAwB2)_{UT2}$ in the *CA* genetic background. (**c**) Bi-CI between $(wAwB1)_{UT1}$ and $(wAwB2)_{UT2}$ in the *UT1* genetic background. (**d**) Uni-CI between $(wAwB1)_{UT1}$ and $(wAwB2)_{UT2}$ in the *UT2* genetic background. Panel **a** is the same as Figure 2a and is repeated here for comparison.

native genotypes of each other. This was also done to establish whether re-introgressing the $(wAwB1)_{UT1}$ strain back to its native genotype would enable the recovery of bi-CI. As Figure 5c indicates, there is bi-CI between these two strains in the *UT1* genetic background. What has to be noted is that the $(wAwB1)_{UT1}[UT1]$ is in its native genotype whereas the $(wAwB2)_{UT2}$ strain has been introgressed into the former's host genotype (that is *UT1*). There is a significant reduction in family size when $(wAwB2)_{UT2}[UT1]$ males were crossed to $(wAwB1)_{UT1}[UT1]$ females (MWU, $U=459.0$. $P<0.001$), as well as in the reciprocal cross (MWU, $U=430.0$, $P<0.001$). In contrast, when the strain $(wAwB1)_{UT1}[UT1]$ was introgressed into the native host genetic background of $(wAwB2)_{UT2}$ (that is *UT2*), there was no bi-CI (Figure 5d, Table 1). Males from $(wAwB2)_{UT2}[UT2]$ produced a significant reduction in the progeny family size, when compared with the controls (MWU, $U=394.5$, $P<0.001$). But no significant reduction in progeny family size was seen between the crosses involving $(wAwB1)_{UT1}[UT2]$males and $(wAwB2)_{UT2}[UT2]$ females (MWU, $U=374.5$, $P=0.227$). Thus, what is clear from all of these crosses is that males with *w*NlonB1 *Wolbachia* only induce CI when they have the *UT1* genetic background. This has been established further by re-introgression to its native host genetic background. The *w*NlonB2 *Wolbachia*, however, produces CI irrespective of the genetic background it is in.

Discussion

The two supergroup B infections in *N. longicornis* were documented to have diverged from each other approximately 1.5 million years ago (Raychoudhury *et al*., 2009). Our results indicate that these two *Wolbachia* strains are bidirectionally incompatible in one genetic background while unidirectionally incompatible in others. The host genetic background has in the past been implicated to have a very significant role in the expression of CI (reviewed in Jaenike, 2009), and this study further demonstrates that host genotypes can determine whether CI is unidirectional or bidirectional. A similar effect was found between two strains of *Culex* mosquitoes (Sinkins *et al*., 2005), suggesting that host genetic alteration between bidirectional and uni-CI may be widespread.

Two distinct hypotheses can be put forward to explain the switch from bi-CI to uni-CI. The first hypothesis is that *w*NlonB1 and *w*NlonB2 are reciprocally incompatible in their native genetic backgrounds. However, modification of sperm by *w*NlonB1 is 'repressed' in the two other genetic backgrounds (*UT1* and *CA*), resulting in uni-CI. An alternative to the above hypothesis involves a more complex scenario of bacterial density changes causing incompatibility between the A and B infections. It posits that the *w*NlonB1 infection does not cause CI with *w*NlonB2, but rather, differential changes in the density between *w*NlonA and *w*NlonB2 causes the changes in CI. In this model, in the *UT1* genetic background *w*NlonA has its density increased and the *w*NlonB1density decreased. This manifests itself as bi-CI between $(wAwB1)_{UT1}$ and $(wAwB2)_{UT2}$ strains. In the other two host genetic backgrounds that density of the *w*NlonA is not increased and there is uni-CI with *w*NlonB2 bearing males. However, this second hypothesis poses several problems. If the *w*NlonB1 infection

is not producing CI, then, why is it being maintained? One of the explanations can be that it is a mutualist and is providing some benefits to the host. But this is not very apparent in the laboratory, as far as progeny number of the infected and cured host strains are concerned, because there is no significant difference between the two (MWU, $U=381.5$, $P=0.103$, Cross nos. 2 and 11, Supplementary Tables S2 and S3). It, however, could provide other benefits, such as protection against viruses, as has been found recently in *D. melanogaster* (Hedges *et al*., 2008; Teixeira *et al*., 2008). Another problem with the second hypothesis is that it cannot readily explain the occurrence of CI between $(w\mathrm{A}w\mathrm{B1}w\mathrm{B2})_{\mathrm{UT3}}[UT3]$ males and $(w\mathrm{A}w\mathrm{B2})_{\mathrm{UT2}}[UT2]$ females (Figure 1a and Table 1). If the *w*NlonB1 is not involved in producing any incompatibility then the above cross should be compatible. Thus, the most likely explanation for the switch of CI from bidirectional to unidirectional remains the first hypothesis, which posits nuclear genetic 'repression' of sperm modification by *w*NlonB1 in two different genetic backgrounds. Such a repression could be mediated through reduction in *w*NlonB1 bacterial densities (for example, in testes). Although PCR results have established that none of the bacteria are lost in the different genetic backgrounds, quantitative PCR of the densities of these different *Wolbachia* strains can help to elucidate whether host genotype effects are mediated through changes in density of the different bacterial types.

The present study is also relevant to the debate concerning the potential role of *Wolbachia* in producing reproductive barriers between populations. Although controversial, *Wolbachia* has been suggested to promote speciation by preventing gene flow between infected and uninfected or differently infected populations (reviewed in Werren, 1998; Bordenstein, 2003). In *Nasonia*, *Wolbachia* has been shown to be a major contributor to reproductive incompatibility between *N. longicornis* and *N. giraulti* (Bordenstein *et al*., 2001). One of the ways that *Wolbachia* can promote speciation is by inducing bidirectional CI, where gene flow is stopped entirely or restricted substantially between populations infected with different CI causing *Wolbachia*. Various theoretical studies have shown that such an event is plausible under some conditions (Telschow *et al*., 2002, 2005a, b).

The present study shows that bi-CI is also dependant on host genotype. The $(w\mathrm{A}w\mathrm{B1})_{\mathrm{UT1}}$ strain produces bi-CI only in one host genetic background (*UT1*) and not in the other two. Thus, bi-CI is a result of the interaction between the *Wolbachia* and host genotype. Therefore, the present study shows another novel way of producing bidirectional CI, where interactions between *Wolbachia* and host genotype together, and not singly, cause bidirectional CI. A question that follows from this observation is whether these *Wolbachia* are producing any substantial reduction in gene flow among *N. longicornis* populations? Various clues indicate that this is probably not true. First, the two double infections are present as polymorphisms along with the triple-infected strains. Raychoudhury *et al*. (2009) showed that two different infection polymorphism (that is, double and triple) can share the same mitochondrial haplotype, indicating loss of one of the B infections from the triple-infected strains. Therefore, any genetic divergence that builds up from bi-CI between the two double-infected strains would be swamped by the individuals from the triple-infected strains, because the females of latter strain would be compatible with males from both the double-infected strains. Second, no significant pre-mating isolation could be detected between these strains when they were crossed. All the inter-strain crosses happened readily, indicating no significant behavioral isolation has evolved in these strains. Moreover, no F1 post-zygotic incompatibility could be detected in these strains when cured of their respective *Wolbachia* infections (Supplementary Tables S2 and S3).

A further question that emerges is what maintains these infection polymorphisms? Basic theory indicates that triple-infected strains would replace all the double-infected polymorphisms (Vautrin *et al*., 2008), because triple-infected females are compatible with both the double-infected males, whereas, both the double-infected females are incompatible with the triple-infected males. However, incomplete transmission of *Wolbachia* can maintain infection polymorphisms via a balance between loss of triple infections owing to incomplete transmission and selection for them by CI (Baldo *et al*., 2008). Indeed, Raychoudhury *et al*. (2009) found in a survey of *N. longicornis* across the west coast of North America that the frequency of *w*NlonB1*w*NlonA, *w*NlonB2*w*NlonA and *w*NlonB1*w*NlonB2*w*NlonA were 8, 30 and 58%, respectively, and that triple infections were associated with both of the major mitochondrial haplotypes. This indicates that the triple-infected strains likely predate divergence of the mitochondrial clades of *N. longicornis*, supporting the view that the double infections are derived by stochastic loss or competitive exclusion of one of the B infections.

One of the crucial facts that emerge from the present study is the importance of host-genetic influences on the expression of CI. But what is surprising is that this effect manifests itself even within a species, that is, within *N. longicornis*. Moreover, most of these strains come from one of the two major mitochondrial haplogroups in *N. longicornis* (Raychoudhury *et al*., 2009), which is found mainly in Utah (called the Great Basin haplotype whereas the other, found mainly in California, is called the West Coast haplotype). Thus, even within the same mitochondrial haplotype there is a pronounced effect of host nuclear genotype on CI. An example of switch from bi-CI to uni-CI has been previously reported by Sinkins *et al*. (2005) in *Culex quienquefasciatus* mosquitoes. They found that the bi-CI between two strains, *Bei* and *Pel*, was lost when the latter's genome was introgressed into the former's cytoplasm. Theoretical studies predict that host suppressors of incompatibility will be selected for (Rousset *et al*., 1991; Turelli, 1994; Koehncke *et al*., 2009) and there are recent examples of such repressors being found in nature (Hornett *et al*., 2006). Koehncke *et al*. (2009) showed theoretically that selection particularly favors the evolution of nuclear suppressors of sperm modification in males, because such genotypes are reproductively compatible with both infected and uninfected types. The apparent loss of sperm modification by the *w*NlonB1 *Wolbachia* in some host nuclear genotypes could be due to selection for a nuclear suppressor of *w*NlonB1 sperm modification. How such a suppressor polymorphism is maintained and why it would be specific to the *w*NlonB1 bacterium remains unclear.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)