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ORIGINAL ARTICLE

Bidirectional incompatibility among divergent *Wolbachia* and incompatibility level differences among closely related *Wolbachia* in *Nasonia*

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Most insect groups harbor obligate bacterial symbionts from the α-proteobacterial genus *Wolbachia*. These bacteria alter insect reproduction in ways that enhance their cytoplasmic transmission. One of the most common alterations is cytoplasmic incompatibility (CI) – a post-fertilization modification of the paternal genome that renders embryos inviable or unable to complete diploid development in crosses between infected males and uninfected females or infected females harboring a different strain. The parasitic wasp species complex *Nasonia* (*N. vitripennis*, *N. longicornis* and *N. girault*i) harbor at least six different *Wolbachia* that cause CI. Each species have double infections with a representative from both the A and B *Wolbachia* subgroups. CI relationships of the A and B *Wolbachia* of *N. longicornis* with those of *N. girault*i and *N. vitripennis* are investigated here. We demonstrate that all pairwise crosses between the divergent A strains are bidirectionally incompatible. We were unable to characterize incompatibility between the B *Wolbachia*, but we establish that the B strain of *N. longicornis* induces no or very weak CI in comparison to the closely related B strain in *N. girault*i that expresses complete CI. Taken together with previous studies, we show that independent acquisition of divergent A *Wolbachia* has resulted in three mutually incompatible strains, whereas codivergence of B *Wolbachia* in *N. longicornis* and *N. girault*i is associated with differences in CI level. Understanding the diversity and evolution of new incompatibility strains will contribute to a fuller understanding of *Wolbachia* invasion dynamics and *Wolbachia*-assisted speciation in certain groups of insects.
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Introduction

Wolbachia are widespread endosymbiotic bacteria that are found predominantly in the germlines of arthropods and nematodes (Werren, 1997; Stouthamer *et al*., 1999; Stevens *et al*., 2001). Their main mode of transmission within species is maternal from ovaries to eggs, but horizontal transmission must also occur between species to account for the wide range of infected hosts. By manipulating arthropod reproduction through male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI), *Wolbachia* increase the relative number of infected females (that is, the transmitting sex) in a host population, and thereby spread rapidly within a host species (Caspari and Watson, 1959; Turelli and Hoffmann, 1991; Turelli, 1994; Werren and O'Neill, 1997). These reproductive alterations can also have important implications to basic processes such as sex determination (Rigaud *et al*., 1997; Werren and Beukeboom, 1998), sexual selection (Jiggins *et al*., 2000) and speciation (Laven, 1957; Breeuwer and Werren, 1990; Bordenstein *et al*., 2001; Bordenstein, 2003; Jaenike *et al*., 2006; Koukou *et al*., 2006). Between arthropod species, horizontal transmission is common on an evolutionary time scale (Werren *et al*., 1995a; Sintupachee *et al*., 2006) and has been observed in the laboratory under certain circumstances (Heath *et al*., 1999; Boyle *et al*., 1993; Rigaud *et al*., 2001; Huigens *et al*., 2004; Frydman *et al*, 2006).

CI is the most commonly detected type of *Wolbachia*-induced reproductive alteration. It is a sperm–egg incompatibility expressed in crosses between an infected male and uninfected female. Although the genetic and biochemical mechanisms of CI are not known, the cytological effects are clear. Sperms that are 'modified' by *Wolbachia* in the testes show abnormal processing after fertilization of the egg, if the appropriate *Wolbachia* are not present in the egg to 'rescue' the modification (Werren, 1997). In particular, breakdown of the nuclear envelope of the male pronucleus is delayed (Tram and Sullivan, 2002) and the paternal chromatin undergoes improper condensation during early mitotic divisions (O'Neill and Karr, 1990; Reed and Werren, 1995; Callaini *et al*., 1997; Tram *et al*., 2006). As a result, most embryos usually die, but in some haplodiploid species haploidization of the embryo results in male development (Reed and Werren, 1995; Tram *et al*., 2006). When both male and female are infected by *Wolbachia* with the same 'modification-rescue' system, the sperm modification is

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rescued in eggs, and compatibility is restored (Werren, 1997). However, if a male and female harbor strains of *Wolbachia* with different 'modification-rescue' systems, then bidirectional CI results (Perrot-Minnot *et al*., 1996; Werren, 1997; Charlat *et al*., 2001). Such strains are referred to as '(in)compatibility types' and have been observed in various insects, including mosquitoes, fruit flies and parasitic wasps (Laven, 1957; Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Montchamp-Moreau *et al*., 1991; Perrot-Minnot *et al*., 1996; James and Ballard, 2000; Bordenstein *et al*., 2001; Dedeine *et al*., 2004). Bidirectional CI has attracted considerable attention for its potential role in driving rapid speciation, because gene flow between diverging populations that harbor different *Wolbachia* incompatibility types, can be reduced or eliminated due to endosymbionts (O'Neill and Karr, 1990; Werren, 1998; Bordenstein *et al*., 2001; Telschow *et al*., 2002; Bordenstein, 2003). The effect can also select for premating isolation (Telschow *et al*., 2005a, b).

Among the eight major subgroups of *Wolbachia* (Lo *et al*., 2002; Rowley *et al*., 2004; Bordenstein and Rosengaus, 2005), the A and B groups are most commonly found in insects and diverged approximately 60 million years ago (Werren *et al*., 1995a). Multiple infections occur at appreciable frequencies throughout a wide range of insect species (Werren *et al*., 1995a, b; Werren and Windsor, 2000; Jeyaprakash and Hoy, 2000). In the parasitic wasp genus *Nasonia*, all three species (*N. vitripennis*, *N. giraulti* and *N. longicornis*) are coinfected by each of the two major insect *Wolbachia* subdivisions, A and B (Breeuwer *et al*., 1992; Werren *et al*., 1995a; Werren and Bartos, 2001; van Opijnen *et al*., 2005). Nearly all field samples within these three species harbor the double AB infections (Bordenstein *et al*., 2001). This genus is therefore particularly useful for studying the CI phenomenon as they are prone to acquiring and maintaining genetically distinct *Wolbachia*. Some isolates of *N. longicornis* are now known to carry two very closely related B *Wolbachia* strains along with the A *Wolbachia* strain (R Choudury, personal communication). The IV7 isolate used in this study, however, is only infected with one A and one B *Wolbachia*.

These three wasp species are reproductively isolated in the laboratory owing to *Wolbachia*-induced bidirectional incompatibility between the different, double AB infections (Breeuwer and Werren, 1990; Bordenstein *et al*., 2001). CI also produces distinct phenotypes among the *Nasonia* species: embryonic mortality in *N. longicornis* and *N. giraulti* due to missegregation of the paternal chromosomes, and conversion to male development in *N. vitripennis* due to exclusion of the paternal genome from embryonic development (Bordenstein *et al*., 2003; Tram *et al*., 2006). Although there are low levels of conversion (10–20%) in *N. longicornis* and *N. giraulti* and low levels of embryonic mortality in *N. vitripennis*, a genetic analysis showed the major difference underlying the mortality/conversion phenotype is a *Nasonia* host genetic effect rather than *Wolbachia* strain differences (Bordenstein *et al*., 2003).

In this paper, we determine CI among the single A and B *Wolbachia* in *Nasonia* and how the incompatibility relationships associate with genetic divergence among the *Wolbachia* strains. The phylogenetic data thus far suggest that five *Wolbachia* infections entered the *Nasonia* system laterally and one codiverged with its host species (Figure 1). These phylogenetic inferences are based on three lines of evidence. First, each of the three A *Wolbachia* show more *wsp* (*Wolbachia* surface protein gene) nucleotide similarity to strains found in other insects than to those strains infecting other *Nasonia* species (van Opijnen *et al*., 2005). For instance, the A infection in *N. longicornis* shows no synonymous divergence to that of *Drosophila melanogaster* (*w*Mel) and *D. simulans* (*w*Au), yet it shows 14.44 and 8.86% synonymous divergence to the A infection in *N. vitripennis* and *N. giraulti*, respectively. Further, extrapolated divergence times for the A *Wolbachia* in these *Nasonia* species (9.0 and 5.5 Mya, respectively) are greater than the estimated time of the most recent common ancestor of the three *Nasonia* species (Campbell *et al*., 1993). The group B *Wolbachia* show a similar trend except for the strains in *N. giraulti* and *N. longicornis*. These B strains show no *wsp* synonymous divergence, although they are 26.81% divergent to the B infection in *N. vitripennis*. These strains presumably codiverged with the ancestor of the B *Wolbachia* in *N. giraulti* and *N. longicornis*, sister species that are estimated to have diverged only a few 100 000 years ago (Campbell *et al*., 1993; van Opijnen *et al*., 2005). They remain one of only a few documented instances of codivergence of *Wolbachia* and their insect hosts. The above nucleotide patterns are also observed with additional genes, including 16S rDNA and seven protein-coding genes (Breeuwer *et al*., 1992; Werren *et al*., 1995a; van Opijnen *et al*., 2005; Casiraghi *et al*., 2005; Baldo *et al*., 2006). Together, the data suggest that the three A

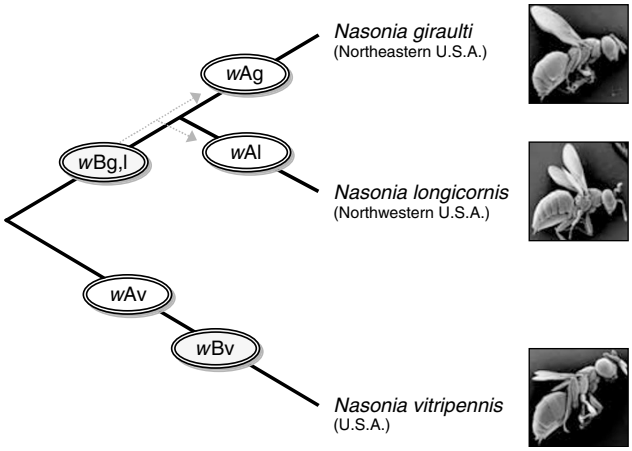

Figure 1 A schematic phylogeny showing the hypothesized origin of A (white circles) and B (gray circles) *Wolbachia* in *Nasonia* (redrawn from van Opijnen *et al*., 2005). All three A and two B *Wolbachia* strains were independently acquired in the three *Nasonia* species by horizontal transfer from another insect. The *w*Bg,l infection then likely codiverged with *N. giraulti* and *N. longicornis*, denoted by the dotted lines with arrowheads. Regional species distributions are noted in parentheses. Scanning electron micrographs of *Nasonia* males (Copyright Dennis Kunkel Microscopy, Inc.) show the major morphological difference (that is, male wing size) between the three closely related species, with *N. giraulti*, *N. longicornis* and *N. vitripennis* having the largest, intermediate and smallest wing sizes, respectively. An italicized lower case *w* followed by a capital A or B denotes the subgroup of *Wolbachia* (for example, *w*A). The lower case v, g or l that follows specifies whether the strain is derived from *N. vitripennis*, *N. giraulti* or *N. longicornis*. For example, *w*Al symbolizes the *N. longicornis* A *Wolbachia*.

Wolbachia and two B *Wolbachia* of *Nasonia* were independently acquired by horizontal transfer from insects outside the genus. The exception of the B group *Wolbachia* in *N. giraulti* and *N. longicornis* indicates codivergence with these sister species. Finally, a low level of nucleotide diversity among each infection suggests that all of the *Wolbachia* were acquired too recently to have had time to accumulate much polymorphism. This scenario is consistent with the proposed origins of the *Nasonia Wolbachia*.

A major question regarding the evolution of CI-inducing *Wolbachia* is how codivergence of closely related strains or lateral acquisition of divergent strains into the same host species influence the expression and evolution of new CI strains. We address three questions related to this topic: (i) Do the distantly related A infections of each species constitute three distinct incompatibility types? (ii) Do the closely related B infections of *N. giraulti* and *N. longicornis* differ in CI? and (iii) Does the host species genotype influence bidirectional incompatibility between double infections of *N. giraulti* and *N. longicornis*? These questions have important implications for the origin of new incompatibility types, the rate at which new ones can evolve, and the significance of host–*Wolbachia* genetic interactions in shaping CI patterns.

Materials and methods

Nasonia are gregarious parasitoid wasps of fly pupae. An introduction to *Nasonia* biology can be found in Whiting (1967). They are raised on *Sarcophaga bullata* (flesh fly) pupae in the laboratory, with constant light and temperature (25°C). Under these conditions, generation time is 14 days for *N. vitripennis* and 15 days for *N. giraulti* and *N. longicornis*. These insects have haplodiploid sex determination, so females are diploid and develop from fertilized eggs, whereas males are haploid and develop from unfertilized eggs.

Nomenclature

Individuals in each *Nasonia* species are double infected with an A and B *Wolbachia* strain, comprising at least six strains in the genus. For the purposes of this paper, we will use a shorthand nomenclature system to refer to these strains in the text and figures. An italicized lower case *w* followed by a capital A or B denotes the subgroup of *Wolbachia* (for example, *w*A). Zero in place of this denotes an uninfected host. The lower case v, g or l that follows specifies whether the strain is derived from *N. vitripennis*, *N. giraulti* or *N. longicornis*. And finally, when describing crosses with wasps harboring the *Wolbachia* of another species (that is, introgression lines), the entire designation is enclosed in brackets, and a capital V, G or L follows to indicate the host species genetic background. Thus, [*w*Al]L symbolizes the *N. longicornis* A *Wolbachia* in the *N. longicornis* host genetic background.

Insect strains

Eight laboratory insect strains were used to test CI when *Wolbachia* occur in their resident species background. Two *N. vitripennis* strains were used: [*w*Av]V is a single A-infected lab strain (named 12.1) and [0v]V is an uninfected strain (named 13.2). Both were derived from a double-infected wild-type strain by spontaneous loss of *Wolbachia* after prolonged diapause (Perrot-Minnot *et al*., 1996). Three *N. giraulti* strains were used: [*w*Ag, *w*Bg]G is double-infected (RV2), [0g]G is uninfected (RV2R) and [*w*Ag]G is single A-infected (16.2 RV2D). The latter two strains were both derived from RV2 through antibiotic treatments in 1996, and diapause treatment in 1998, respectively. Similarly for *N. longicornis*, [*w*Al, *w*Bl]L is double-infected (IV7), [0l]L is uninfected (IV7R3-1B and [*w*Al]L is single A-infected (2.1 IV7D). In this species, the latter two strains were derived from IV7 through antibiotic treatment in 2000, and diapause treatment in 1998, respectively. Attempts to isolate single B infections of *N. giraulti* and *N. longicornis* through antibiotic and diapause treatment were unsuccessful, probably due to low bacterial densities of these infections in diapausing host larvae.

Introgression lines

Introgression lines were produced that harbor the cytoplasm of *N. longicornis* (infected and uninfected) in the genetic background of *N. giraulti*. [*w*Al, *w*Bl]G carries the double-infected *N. longicornis* cytoplasm from IV7 in the *N. giraulti* genetic background of RV2R. [0l]G is comprised of the uninfected cytoplasm of IV7R3-1B and the same *N. giraulti* genetic background of RV2R. These introgression lines were generated by six generations of backcrossing hybrid females to males of *N. giraulti*. This design should theoretically result in at least a 98% genome replacement, and the retaining of the cytoplasm of the parental female (infected or uninfected). Crosses with these introgression lines and pure *N. giraulti* lines that carry a *N. giraulti* cytoplasm were set up according to the methods described below.

Crossing design

All crosses were set up as single pair matings between virgin females and virgin males. Males and females were collected as pupae. Individual female and male adults were paired and observed for 10–15 min. Only those pairings where copulation occurred were used. After 24 h, the males were discarded and each female was provided with four hosts and a drop of honey for feeding. After 48 h, the females were transferred to new vials and given a single host for 6 h. Females were then discarded from each vial and the parasitized hosts were left undisturbed until adult emergence in approximately 2 weeks. Adults were scored upon death for sex and total family size. Crosses producing diapause offspring were not included in the scoring.

Statistics

We present descriptive statistics and significance values from nonparametric Mann–Whitney *U* (MWU) tests using MINITAB 12.23. Summary data are indicated as percentages or as means $\pm$ standard errors (s.e.) of offspring number.

Results

Results can be summarized as follows: (i) all three divergent A *Wolbachia* strains in the three *Nasonia* species constitute different incompatibility types. (ii) The codiverging strains of B *Wolbachia* in *N. giraulti* and *N. longicornis* differ in CI penetrance with the latter

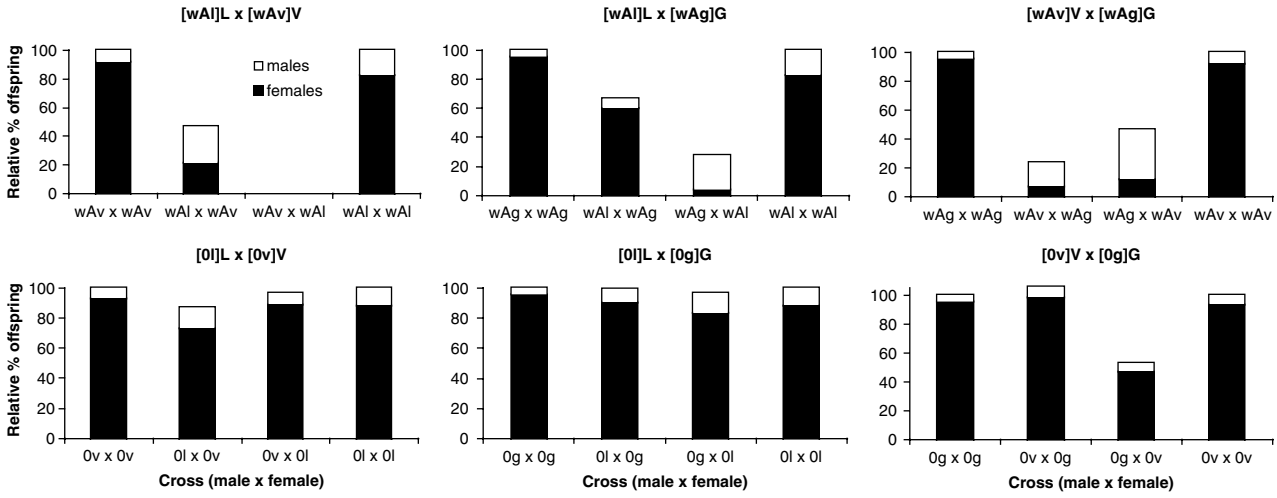

Figure 2 Bidirectional cytoplasmic incompatibility (CI) between each of the distantly related A *Wolbachia* strains of *Nasonia*. Data are represented as percent males and females based on the mean number of male and female offspring produced. Data from compatible self-crosses are standardized so that total offspring produced equates to 100% to control for fertility differences between the three species; incompatible crosses are standardized according to the same scale for compatible crosses with the same maternal parent. Crosses are always listed as male × female.

inducing weak or no CI. (iii) The host genetic background does not influence bidirectional CI of double AB infections between the sister species *N. giraulti* and *N. longicornis*. Previous work had also showed no host genetic effects on bidirectional CI between *N. giraulti* and *N. vitripennis* (Breeuwer and Werren, 1993a). In interpreting the results below, it should be kept in mind that in compatible crosses fertilized eggs normally result in only female offspring, whereas males result from unfertilized eggs. Therefore, CI is documented by a reduction in the number of female progeny, and can be due to mortality of female embryos (which does not increase the number of male progeny) or conversion of diploid embryos into haploid males (which does increase the number of male progeny). The relative level of mortality and conversion CI can therefore be determined by comparing numbers of sons and daughters in incompatible crosses to compatible control crosses.

Bidirectional CI between distantly related A *Wolbachia* strains

The A *Wolbachia* of all three species of *Nasonia* are not closely related, indicating independent acquisition of all three bacteria by horizontal transfer from other sources (Figure 1; Breeuwer *et al*., 1992; Werren *et al*., 1995a; van Opijnen *et al*., 2005). The modification and rescue components of these three A strains were tested for whether they were sufficiently different to render them bidirectionally incompatible. Experiments were done with each strain within its respective host species genetic background.

Figure 2 summarizes the results of these compatibility tests using relative percent offspring produced to standardize differences in fertility between the species. Crosses between all three A *Wolbachia*-infected wasps show significant decreases in diploid female production in comparison to control uninfected crosses and self-crosses that have the same maternal parent. For example, bidirectional CI between the A infections of *N. longicornis* and *N. vitripennis* yields a 76.9% reduction in the number of daughters in the *w*Al male × *w*Av female cross direction (mean ± s.e.: 8.7 ± 3.8 daughters, $N = 9$ vs 37.6 ± 2.3 daughters produced in the *w*Av × *w*Av control self-cross, $N = 27$, MWU, $P = 0.0001$) and a 100% reduction in the reciprocal *w*Av male × *w*Al female cross direction (0.0 ± 0.0 daughters, $N = 4$ vs 10.7 ± 1.92 control daughters, $N = 10$, $P = 0.0053$). In a replicate experiment, we determined that the partial CI in the *w*Al male × *w*Av female cross is repeatable and results from an incomplete *w*Al modification of the sperm rather than partial rescue. The *w*Al male × 0v female cross shows similar levels of partial CI (4.7 ± 1.1 daughters, $N = 17$) when compared to the 0v × 0v control self-cross (28.5 ± 1.6 daughters, $N = 27$, $P < 0.0001$, data not shown). Approximately 21% of the F1 incompatibility in the *w*Al male × *w*Av female cross may be due to interspecific F1 hybrid mortality as 0l males × 0v females gives significantly fewer daughters (24.5 ± 1.7, $N = 30$) than that of the control 0v male × 0v female cross (31.0 ± 0.9, $N = 31$) (MWU, $P = 0.001$).

Bidirectional CI between the A infections of the sister species *N. longicornis* and *N. giraulti* causes a 36.9% reduction in female offspring number in the *w*Al male × *w*Ag female cross (15.9 ± 1.0 daughters, $N = 18$ vs 25.2 ± 1.1 control daughters, $N = 27$, MWU, $P < 0.0001$) and a 95.3% reduction in the reciprocal *w*Ag male × *w*Al female cross (0.5 ± 1.0 daughters, $N = 6$ vs 10.7 ± 1.92 control daughters, $N = 10$, MWU, $P = 0.04$). In a replicate experiment, we found that this asymmetry in incompatibility strength is repeatable and again due to partial sperm modifications of the respective male infections. The incomplete modification of the sperm is apparent when comparing the number of daughters of the *w*Al male × 0g female cross to that of the uninfected 0g male × 0g female control (8.3 ± 1.6, $N = 23$ vs 17.0 ± 1.7, $N = 28$), which shows a 51.2% reduction in female offspring. Because females are uninfected in these cases, there is no rescue to be expected. Similarly, *w*Ag male × 0l female causes a 94.3% reduction in female offspring (0.5 ± 0.2, $N = 31$) relative to that of the uninfected 0l male × 0l female cross (8.7 ± 1.3, $N = 30$).

And finally, bidirectional CI between the A infections of *N. vitripennis* and *N. girault*i causes a 92.5% reduction in *w*Av male × *w*Ag female relative to the control 0v male × wAg female (1.9±0.7, $N=9$ vs 25.2±1.1 control, $N=27$, MWU, $P<0.0001$) and a 87.0% reduction in the reciprocal *w*Ag male × *w*Av female cross vs the control (4.9±3.0, $N=7$ vs 37.6±2.3 control, $N=27$, MWU, $P=0.0001$). Bidirectional incompatibility of these *Nasonia* A infections has been documented previously (Bordenstein and Werren, 1998; Werren, 1998).

In summary, all three divergent A strains from each of the *Nasonia* species are bidirectionally incompatible when in their own respective host genetic background. Partial incompatibility in these crosses results from incomplete A *Wolbachia* sperm modifications, rather than partial rescuing of that modification in the A-infected fertilized eggs.

CI levels of recently diverged B *Wolbachia* strains

In contrast to the horizontally acquired A infections, the closely related B *Wolbachia* in *N. girault*i and *N. longicornis* appear to have codiverged during divergence of their respective host species (Figure 1) (van Opijnen *et al*., 2005). The occurrence of such closely related *Wolbachia* within hosts that can be hybridized presents a rare opportunity to characterize the changes in CI that occur among recently diverged *Wolbachia* variants. Because single B-infected strains of these two species have proven difficult to generate, we were restricted to characterizing unidirectional CI only via crosses between double-infected males (for example, *w*Ag, *w*Bg) and single A-infected females (for example, *w*Ag). The reason is that while the sperm modification of type A *Wolbachia* will be rescued in the A-infected egg, the sperm modification of type B *Wolbachia* will not be rescued because the B infection is absent from the egg (Mercot *et al*., 1995; Sinkins *et al*., 1995; Perrot-Minnot *et al*., 1996; Dobson *et al*., 2001).

Figure 3 summarizes the results of these compatibility tests. *w*Bg induces nearly complete CI, but *w*Bl induces weak or no CI within its resident species genetic background. This finding can be seen by comparing the number of females produced in the following crosses. When *w*Ag, *w*Bg males are crossed to *w*Ag females ($N=34$), the number of females produced are significantly reduced from that of the control *w*Ag male × *w*Ag female self-cross ($N=19$) (MWU, $P<0.0001$). These results indicate that the *w*Bg bacterium in the male has modified the sperm and the *w*Ag-infected egg is incapable of rescuing that modification. The conclusion is further supported by comparing the *w*Ag, *w*Bg male × 0g female cross ($N=15$) to the *w*Ag male × 0g female cross ($N=24$). Here CI is observed in both cases, but when *w*Ag, *w*Bg males are used, the CI is complete (that is, no female production), and when *w*Ag males are used, the CI is incomplete (that is, some females produced). Thus, double-infected males express stronger levels of CI than single A-infected males in *N. girault*i. However, B-infected males do induce complete CI.

The pattern is different in *N. longicornis*. Genetic crosses show that *w*Bl expresses no or very weak CI in its own host genetic background. The *w*Al, *w*Bl male × *w*Al female cross produces many daughters (9.3±1.0, $N=28$) in comparison to those of the control *w*Al male × *w*Al female self-cross (13.9±1.7, $N=7$) (MWU, $P=0.1418$). The lack of significant CI in the former cross can be explained either by the inability of *w*Bl in a double-infected male to induce incompatibility or by *w*Al female rescue of the *w*Bl sperm modification. A comparison of the *w*Al, *w*Bl male × 0l female ($N=21$) cross to the *w*Al male × 0l female cross ($N=15$) shows that *w*Bl does not express significant CI levels (77.1 and 84.0% reduction in the number of females, MWU, $P=0.9014$). Taken together, these results specify that *w*Bl expresses no or weak CI. It is possible that some expression of CI could be detected with larger sample sizes in the crosses. Absence of the B *Wolbachia* in *N. longicornis* cannot account for this difference in CI level as infection status was confirmed by PCR amplification of *Wolbachia* 16S rDNA gene sequences before and after the experiments. *w*Bl may cause a fecundity cost in *N. longicornis* as *w*Al, *w*Bl self-crosses ($N=17$) produce significantly fewer daughters and total offspring than the *w*Al self-crosses ($N=7$) (MWU, $P=0.03$ for both).

Taken together, results indicate that the closely related B *Wolbachia* of *N. girault*i and *N. longicornis* express different levels of CI in their resident species genetic backgrounds. However, we were not able to determine whether they show bidirectional CI to each other, due to failures in producing single *w*Bl and *w*Bg strains. In addition, CI-induced embryonic mortality is the expected, primary CI type in *N. girault*i and *N. longicornis*

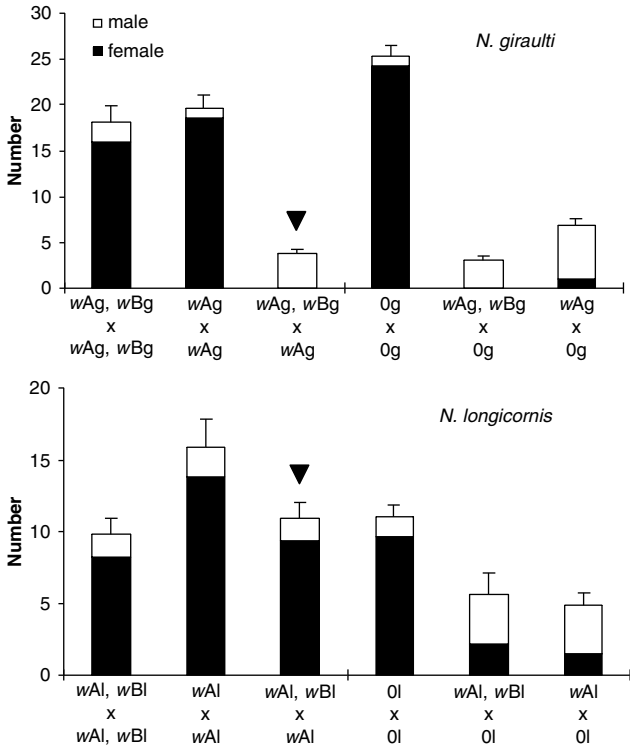

Figure 3 Unidirectional cytoplasmic incompatibility (CI) caused by the single and double infections of *N. girault*i and *N. longicornis*. Data are shown as mean numbers of males and females produced ± s.e. of total offspring for each cross. Cross labels denote male infection status on top line followed by crossing symbol and female infection status on the bottom line. Solid arrowheads denote the two crosses showing a significant CI level difference between the codiverging B *Wolbachia* of these sister wasp species.

(Bordenstein *et al*., 2003), as indicated by the significant reductions in total family sizes of incompatible crosses. We estimate that the average percentage of eggs that die due to embryonic mortality from the crosses showing significant CI is 86.3% in *N. girault*i and 73.7% in *N. longicornis* (see Materials and methods), respectively.

Absence of host genetic influences on *N. girault*i – *N. longicornis* bidirectional CI

Bordenstein *et al*. (2001) previously reported bidirectional CI between the double infections of these two sister species in their normal genetic background. Double-infected individuals were used from each species and incompatibility levels were complete in one cross direction, and incomplete in the other, reflecting the typically incomplete CI of infected *N. longicornis* males. To examine whether the host genome exerts any influence over bidirectional CI and the variation in levels of CI, the *w*Al-, *w*Bl-infected *N. longicornis* cytoplasm was introgressed by backcrossing it for six generations into a *N. girault*i genetic background. This line, denoted [*w*Al, *w*Bl]G as well as its uninfected counterpart [0l]G, was used to retest CI against the pure *N. girault*i lines, [*w*Ag, *w*Bg]G and [0g]G. The experimental design slightly differed from the above experiments in that single females were allowed to oviposit into two hosts for life. Therefore, family sizes are larger than those reported above.

As seen in Figure 4, bidirectional CI between these double infections is still expressed even when the *N. longicornis Wolbachia* are in a *N. girault*i genome. Self-crosses and crosses with uninfected individuals are fully compatible and yield normal female-biased sex ratios. In incompatible crosses, the number of females is reduced by 88.4% in the [*w*Al, *w*Bl]G male × [*w*Ag, *w*Bg]G female cross ($N=23$) (MWU, $P<0.0001$) and 99.4% in the reciprocal [*w*Ag, *w*Bg]G male × [*w*Al, *w*Bl]G female cross ($N=25$) (MWU, $P<0.001$). CI levels are strong and are similar to levels from crosses showing bidirectional CI in non-introgression lines (Bordenstein *et al*., 2001). Therefore, the *N. girault*i host genome does not affect bidirectional CI nor the incomplete levels of CI associated with *N. longicornis Wolbachia* in this introgression line. Previous work had found no host genetic effects on bidirectional CI of double AB infections in the species pair *N. girault*i and *N. vitripennis* (Breeuwer and Werren, 1993a).

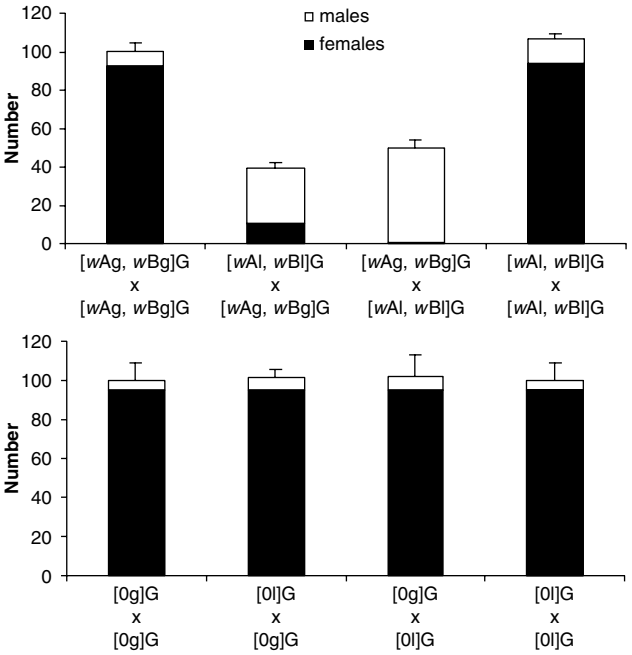

Figure 4 Bidirectional incompatibility between the double infections of *N. longicornis* and *N. girault*i in a common *N. girault*i genetic background. Data are shown as mean numbers of males and females produced ± s.e. of total offspring for each cross. Cross labels denote male infection status on top line followed by crossing symbol and female infection status on the bottom line. Reciprocal and self-cross of infected individuals are shown above those of uninfected individuals.

Discussion

The study set out to answer three questions related to CI in *Nasonia*: (i) Do the distantly related A *Wolbachia* of each species constitute distinct incompatibility types? (ii) Do the closely related B *Wolbachia* of *N. girault*i and *N. longicornis* differ in CI? and (iii) Does the host genome influence interspecific, bidirectional incompatibility between double infections of *N. girault*i and *N. longicornis*?

Experiments demonstrated that all three species-specific A infections in the *Nasonia* genus are bidirectionally incompatible and constitute at least three different incompatibility types. Phylogenetic relationships of these strains, based on several *Wolbachia* gene sequences (Werren *et al*., 1995a; Werren and Bartos, 2001; van Opijnen *et al*., 2005; Casiraghi *et al*., 2005; Baldo *et al*., 2006), indicate that they are genetically divergent and were acquired in separate horizontal transfer events into the *Nasonia* from other unknown insects (Figure 1). Our finding of bidirectional incompatibility is consistent with this hypothesis, because there has been ample time for the modification-rescue systems of these strains to have diverged and become incompatible. Independent acquisition via lateral transfer events tends to be the predominant mechanism for how different incompatibility types arise in a host system (Bordenstein, 2003). Although little is known about the average rate of horizontal transfer for *Wolbachia*, it is apparent that horizontal transfer events into *Nasonia* can sometimes occur frequently – indeed, two separate acquisitions (two A *Wolbachia*) in *N. girault*i and *N. longicornis* have happened in the last 0.2 Mya based on their estimated divergence time (Campbell *et al*., 1993) (Figure 1).

An alternative mechanism for the origin of different incompatibility types is *in situ* evolution. That is, new incompatibility types could evolve within a species or in closely related species that have a *Wolbachia* that has codiverged with the host. Ultimately, new incompatibility types must arise from ancestral incompatibility types to account for the variation in CI observed among *Wolbachia* that have entered species by horizontal transfer. However, the process of new incompatibility type evolution is not well understood, due to a lack of knowledge about the genetic basis of CI. Charlat *et al*. (2001) developed a two-step model for the *in situ* evolution of new incompatibility types. They assume that the modification and rescue components of CI are governed by separate genes. The first step of the model

involves the neutral spread of a mutation creating a new modification type. When this mutation drifts to an appreciable frequency, a second mutation that can rescue this new modification type will be selected for and cause the deterministic spread of this new incompatibility type under a broad set of conditions.

Models of incompatibility type evolution are difficult to test because there are very few natural examples thus far of incipient evolution of new incompatibility types within a species or between sister species. This is in part based on a limited spectrum of fastly evolving genetic markers to infer strain relationships, although transposon and phage genes may prove useful in the future (Sanogo and Dobson, 2004; Duron *et al*., 2005, 2006). Most studies showing bidirectional CI within their natural host species are based on *Wolbachia* strains that are distantly related (O'Neill and Karr, 1990; Clancy and Hoffmann, 1996; Perrot-Minnot *et al*., 1996; Bordenstein and Werren, 1998; James and Ballard, 2000; Dedeine *et al*., 2004; Mouton *et al*., 2005) with only few exceptions (Laven, 1957; Sinkins *et al*., 2005). The reason then for the paucity of data regarding these phenomena is likely a simple one. There are currently few described cases of sister CI-*Wolbachia* strains existing in the same or sister host species. Microinjection experiments may circumvent this problem because closely related *Wolbachia* that are harbored in distantly related species can be moved into a common host background (Charlat *et al*., 2004) and then tested for bidirectional CI. However, results produced from this approach must be interpreted with caution as host–*Wolbachia* interactions in the novel host may lead to confounding effects on incompatibility relationships. Nevertheless, some experiments that moved relatively closely related/identical *Wolbachia* of *D. simulans* and *D. sechellia* into a common *D. simulans* genetic background did not find significant differences in CI phenotype (Charlat *et al*., 2002).

The B *Wolbachia* strains of *N. giraulti* and *N. longicornis* constitute one of the best cases for natural codivergence of host and *Wolbachia* in insects. Sequence relationships of multiple *Wolbachia* genes from these B strains parallel the phylogenetic relationship of the two host species, *N. giraulti* and *N. longicornis*, which shared a direct common ancestor $\sim$0.2 Mya (Campbell *et al*., 1993; Werren *et al*., 1995a; Werren and Bartos, 2001; van Opijnen *et al*., 2005; Choudhury *et al*., unpublished). However, a practical problem remains to determine whether they show bidirectional CI. Each strain must be isolated as a single infection from the typically double-infected individuals of each species. Crosses between them can then be performed to test for bidirectional CI, as done with the single A infections in this study. Attempts to segregate out these single B infections, however, have only been successful in *N. vitripennis* (Perrot-Minnot *et al*., 1996). Both a prolonged diapause treatment (method described in Perrot-Minnot *et al*., 1996) and a low-dose antibiotic treatment have failed to segregate out the single B infections of *N. giraulti* and *N. longicornis* (Bordenstein and Werren, unpublished). Only the A infections and cured individuals have been isolated in these sister species. A likely explanation for this outcome is a higher density of A than B *Wolbachia* in these species.

We therefore cannot yet determine whether bidirectional CI occurs between these two related B infections. CI between the distantly related B strains of *N. vitripennis* and *N. giraulti* has been shown (Bordenstein and Werren, 1998; Werren, 1998), suggesting that at least these B strains represent two distinct incompatibility types. In addition, despite the inability to segregate out the sister B *Wolbachia* strains, we can still test for differences in the expression of unidirectional CI for each of the B infections by mating a double AB-infected male to a single A-infected female. By doing so, we 'expose' the CI associated with the B infection, because the A sperm modification of the double-infected individual will be rescued in the egg, but the B infection will not.

The experiments reported here showed a dramatic difference in CI level between these two closely related B infections, *w*Bl and *w*Bg (Figure 3). The *N. giraulti* B strain expressed complete CI (that is, no daughters are produced), whereas the *N. longicornis* B strain expressed nearly no CI. An important question then is why is there such a drastic difference in CI level between *w*Bl and *w*Bg? There are at least two possible explanations (a) the *w*Bl *Wolbachia* lost the capability of inducing modification or (b) host genetic effects suppress modification in *w*Bl. We have not yet resolved these alternatives.

CI level variation may be due to genetic changes in the *w*Bl and *w*Bg sister strains of *Wolbachia*, and represent early steps of evolutionary divergence in CI. Several models have pointed out that once a *Wolbachia* strain becomes fixed in a species, there is no direct selection to maintain modification function in males because *Wolbachia* in males are not transmitted to future generations (Prout, 1994; Turelli, 1994; Hurst and McVean, 1996). Degradation in modification function is therefore expected, either by mutation accumulation or by selection against modification because of negative pleiotropic effects in infected females. Under this scenario, the ancestral B *Wolbachia* of *N. giraulti* and *N. longicornis* is hypothesized to be a strong CI inducer that lost its ability to induce complete CI in *N. longicornis* after divergence. This conclusion is supported by the fact that the B infections in *N. giraulti* and *N. longicornis* are at near fixation (Bordenstein *et al*., 2001).

The B *Wolbachia* of *N. longicornis* may simply occur at lower densities and therefore cause lower CI levels. Studies in several systems, including *Nasonia*, show bacterial density effects on CI level (Boyle *et al*., 1993; Breeuwer and Werren, 1993b; Clancy and Hoffmann, 1998; Noda *et al*., 2001). Recently, the bacterial density effects in *Nasonia vitripennis* were further shown to be inversely associated with bacteriophage WO-B densities, suggesting that rates of lytic phage development may sometimes underlie the regulation of *Wolbachia* densities in arthropods (Bordenstein *et al*., 2006). If bacterial density is involved, it could be that the natural infection level of *w*Bl has fallen below the threshold for induction of CI, or that this has occurred in the particular laboratory strain used, possibly subsequent to its introduction into the laboratory.

Host genetic influences may also lead to differences in bacterial densities. Host–*Wolbachia* genetic interactions are well known to moderate CI levels in diverse host systems (Boyle *et al*., 1993; Bordenstein and Werren, 1998; Werren, 1998; McGraw *et al*., 2001) and may do so through several routes, including effects on processing of the sperm modification, bacterial densities and tissue tropism (Poinsot *et al*., 1998; Dobson *et al*., 1999; McGraw *et al*., 2001; Clark *et al*., 2002). Indeed, natural selection is

expected to act upon the host genome of males to inhibit the modification action of *Wolbachia*, because their resulting sperm would then be compatible with uninfected eggs (Koehncke *et al*., unpublished). Thus, male host effects on CI are expected to evolve at the host level as well. For example, evidence indicates that *Wolbachia* are largely excluded from the developing sperm cysts in *D. melanogaster* (McGraw *et al*., 2001; Clark *et al*., 2002), probably explaining the low level of modification in this species. If such host-induced tissue tropism is responsible for the apparent absence of CI induction by *w*Bl, then we must assume that it is specific to that strain, because the *w*Al does induce CI. However, detailed cytological studies to determine the tissue specificity of different *Wolbachia* strains in *Nasonia* have yet to be done.

The key results of this study are that all three A infections of *Nasonia* are distinct incompatibility types and that the two closely related B *Wolbachia* differ in CI level when tested in their resident species backgrounds. If we assume that these two B *Wolbachia* are not bidirectionally incompatible, then we can say that at least a total of five different incompatible *Wolbachia* strains (three As and two Bs) infect *Nasonia*, which were all presumably acquired by horizontal transfer from foreign sources. The one case of B *Wolbachia* codivergence has raised several interesting questions that warrant future studies of the incipient evolution of changes in CI, most important is whether codivergence of *Wolbachia* more often leads to loss-of-function mutations rather than evolutionary diversification of new incompatibility types. Finally, it is apparent from these studies that the interspecific postmating isolation caused by double infections of CI-*Wolbachia* in each *Nasonia* species (Breeuwer and Werren, 1990; Bordenstein *et al*., 2001) is reinforced by each single infection comprising its own incompatibility type, with the exception of *w*Bl. Therefore, if stochastic segregation occurs in natural populations leading to the loss of *Wolbachia* infections, both A and B strains would have to be lost to fully restore interspecific postmating compatibility in *Nasonia* hybridizations.

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