Recombination in *Wolbachia*

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Wolbachia **are widely distributed intracellular bacteria that cause a number of reproductive alterations in their eukaryotic hosts. Such alterations include the induction of parthenogenesis, feminization, cytoplasmic incompatibility, and male killing [1–11]. These important bacteria may play a role in rapid speciation in insects [12–14], and there is growing interest in their potential uses as tools for biological control and genetic manipulation of pests and disease vectors [15–16]. Here, we show recombination in the** ***Wolbachia*** **outer surface protein gene (*wsp*) between strains of** ***Wolbachia*****. In addition, we find a possible ecological context for this recombination. Evidence indicates either genetic exchange between** ***Wolbachia*** **in a parasitoid wasp and in the fly that it parasitizes or horizontal transfer of** ***Wolbachia*** **between the parasitoid and the fly, followed by a recombination event. Results have important implications for the evolution of these bacteria and the potential use of** ***Wolbachia*** **in biological control.**

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Results and discussion

Although *Wolbachia* are routinely inherited cytoplasmically through the eggs of their invertebrate hosts, it is clear from phylogenetic and experimental data and from the widespread distribution of these bacteria that they are also transmitted horizontally between arthropod species [5, 11]. For example, very closely related strains of *Wolbachia* can be found in such diverse hosts as flies, beetles, and wasps [11]. Similarly, microinjection studies reveal that *Wolbachia* can be experimentally transmitted between such diverse hosts as mosquitoes and fruit flies [17]. However, the mechanisms of horizontal transmission of *Wolbachia* in nature are poorly understood. Previous phylogenetic studies using the bacterial protein gene *ftsZ* showed an association between *Wolbachia* that is present in parasitic wasps of the genus *Nasonia* and the blowflies they parasitize. This finding suggests parasitoid-host transfer as a possible mechanism for horizontal transmission [11]. Related research suggests that *Wolbachia*, can be transmitted between parasitoids and hosts under laboratory conditions, although the bacteria are not stably maintained [18]. In addition, closely related *Wolbachia* have recently been found in *Drosophila* and their parasitoids, although a statistically significant association between the two was not established [19]. Other studies of parasitoid-host insect communities have not found an association [20, 21].

The study reported here was originally initiated to determine whether parasitoid-host transmission of *Wolbachia* has occurred between *Nasonia* and the blow flies they parasitize. *Nasonia* is a genus of three parasitic wasp species [22]. One species (*N. vitripennis*, Nv) is found throughout the world and parasitizes the pupae of a wide range of fly species, including flesh flies, blow flies, and house flies. The other two species (*N. longicornis*, Nl and *N. giraulti*, Ng) occur, respectively, in western and eastern North America and are specialists on the pupae of *Protocalliphora* flies, which are themselves ectoparasites of bird nestlings [22]. Nv occurs microsympatrically with Ng and Nl in their respective ranges and often occurs in the same bird nests and occasionally emerges from the same host pupae [22]. Individuals of all three wasp species harbor double infections with divergent *Wolbachia* from the two major subdivisions found in insects (A and B). Previous work using the *ftsZ* gene indicated that *N. vitripennis* had acquired its B group *Wolbachia* via horizontal transmission from another (unknown) source but that *N. giraulti* and *N. longicornis* had similar bacteria that were also closely related to those found in their preferred host, protocalliphorid blowflies [11].

To clarify whether horizontal exchange had occurred, we conducted a more detailed investigation by using the *Wolbachia* outer surface protein (*wsp*) [23, 24]. This protein evolves more rapidly than does *ftsZ* and therefore provides a better phylogenetic assay for recent horizontal transfers. The *wsp* gene was amplified from B group *Wolbachia* in 11 different strains of *N. vitripennis*, 12 strains of *N. longicornis*, 5 strains of *N. giraulti*, and 10 different strains of *Protocalliphora* by polymerase chain reaction with B group–specific primers [24]. *N. vitirpennis* strains included samples from throughout North America and Europe, *N. longicornis* strains were from western North America, and *N. giraulti* and *Protocalliphora* strains came from eastern North America. Direct sequencing of forward and reverse

strands was performed with an ABI PRISM 377 DNA sequencer, and sequences were aligned visually.

The *wsp* sequences of all ten *Protocalliphora Wolbachia* were identical to each other. *N. longicornis* sequences were nearly identical to each other, with only a single base pair difference between two subsets. *N. giraulti* sequences were also nearly identical, with two sequences sharing a single base pair difference and one sequence containing a single unique difference. *N. giraulti* and *N. longicornis* differed from each other by only one additional base pair, indicating a high degree of similarity. It should be noted that this high level of similarity includes one of the rapidly evolving hypervariable regions found within *wsp* [24] and further supports a high phylogenetic affinity between the *Wolbachia* of *N. giraulti* and *N. longicornis*. The 12 sequences from *N. vitripennis Wolbachia* were also identical to each other, although they differed considerably from those found in *N. giraulti* and *N. longicornis* (53 base pair differences). This finding is consistent with the pattern from the *ftsZ* sequences, indicating a different origin for *N. vitripennis* B group bacteria [11].

Analysis of the DNA sequences showed a clear recombination event within the *wsp* gene of *Protocalliphora* B *Wolbachia* (Figures 1 and 2). The first 204 bases (5′ end) of the *Protocalliphora* sequence are nearly identical to those found in the *N. vitripennis Wolbachia* and differ by five bases that are present in the blow fly sequence but absent in Nv and Ng. The remaining 188 bases (3′ end) of the sequence are nearly identical to those found in B *Wolbachia* from the other two *Nasonia* species, Nl and Ng, and differ by only two bases. The 3′ end includes a hypervariable region that evolves so rapidly that it is routinely excluded from phylogenetic analyses of *Wolbachia wsp* because of difficulties in alignment. Considering only shared polymorphisms (where Nv indicates those shared with *N. vitripennis*, and Ng indicates those shared with *N. giraulti*) and excluding indels, the *Protocalliphora* sequence has the following spatial pattern (5′–3′): 17Nv - 34Ng - 1Nv - 1Ng (Figure 1). Statistically significant strings of associated bases are indicative of recombination [25]. The chance probability of getting a string of 34 or more Ng out of 53 shared polymorphisms (35 Ng and 18 Nv) is remarkably low ($p < 10^{-10}$ permutation probability), and this fact strongly indicates a recombination event within the *wsp* gene. Three indels are present, and these are also completely consistent with a recombination event between *wsp* sequences similar to those found in Nv (5′ end) and Ng (3′ end).

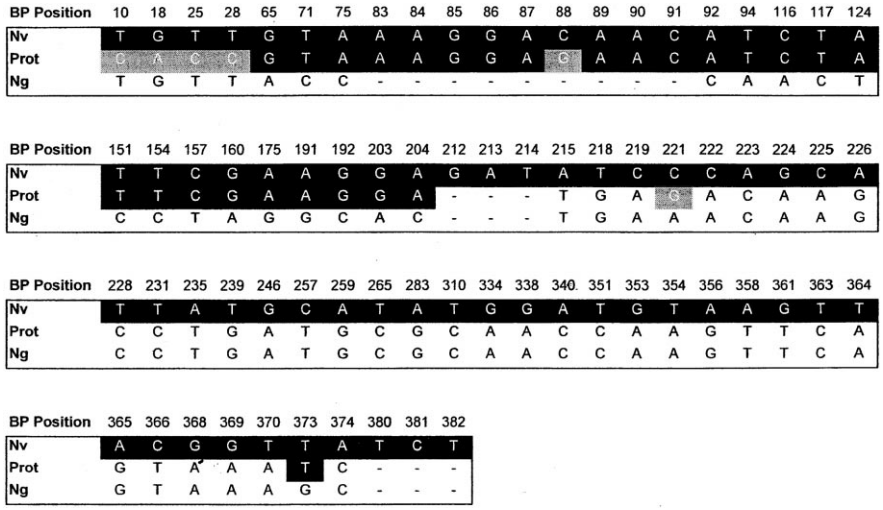
Figure 2 shows a phylogenetic analysis using neighbor joining [26] of the 3′ and 5′ portions of the gene divided at the apparent recombination breakpoint. *wsp* sequences from the other strains of *Wolbachia*, including those most similar to the sequences found in the wasp and blow flies [27], were included in the analysis. As expected, the 5′ region of *wsp* from *Protocalliphora Wolbachia* is more closely related to *Wolbachia* in Nv (5 base pair differences) than to Ng/Nl (16 base pair differences), whereas the 3′ region is more closely related to Ng/Nl (2 base pair differences) than to Nv (36 base pair differences). A number of other methods for inferring phylogeny were also used to evaluate the sequence relationships. These methods include different neighbor-joining algorithms (Kimura 2 parameter, Kimura 3 parameter, and HKY85), maximum parsimony, and maximum likelihood [26]. All methods place Ng/Nl as *Protocalliphora*'s closest relative in the 3′ region; however, there is variation, depending upon the weighting algorithm, as to whether *Wolbachia* from *Protocalliphora* or *Teleogryllus taiwanemma* (an asian cricket) is the closest relative of Ng/Nl *Wolbachia*.

The data clearly show a recombination event within the *wsp* gene of B *Wolbachia* from the protocalliphorid fly. Can we infer the sources of this recombinant gene from the phylogenetic analysis? The 5′ end from the protocalliphorid *Wolbachia* is most closely related to *Wolbachia* in *Tagosedes orizocolus*, *Spalangia fuscipes*, and *Diplolepis rosae*, (two base pair difference, 97% bootstrap value) but is also closely related to *Nasonia vitripennis* (five base pair differences). *Tagosedes orizocolus* is a plant hopper found in Central America [23], *Spalangia fuscipes* is a parthenogenic parasitoid wasp of fruit flies [24], and *Diplolepis rosae* is a parthenogenic gall wasp [24]. No clear ecological or taxonomic association is apparent.

The pattern observed in the 3′ portion of the gene is more indicative of an ecological association. The most similar sequence to that found in *Protocalliphora Wolbachia* is the sequence found in *N. giraulti* and *N. longicornis*, the wasps that parasitize pupae of *Protocalliphora*. The 3′ *Protocalliphora wsp* sequence differs from Ng/Nl by two base pairs. Some Nl sequences have an additional single base pair change. There are currently more than 37 known B group *wsp* sequences, excluding *Protocalliphora* and duplications. The chance probability that the nearest neighbor of the *Protocalliphora Wolbachia* is one from its parasitoid wasps (Nv or Ng/Nl) is therefore $2/37 = 0.054$. These results suggest that the source of the 3′ end of the blow fly *Wolbachia* is that found in one of its parasitoids. Consistent with this view, the *ftsZ* gene also shows a high sequence similarity between *Protocalliphora* and Ng/Nl [11]. When the former study was performed, there were 19 B group *Wolbachia ftsZ* sequences. There are now 55 B group *ftsZ* sequences (excluding duplicates and Ng/Nl) present in the database (NCBI), and the sequences present in *Protocalliphora* and Ng/Nl remain most similar to each other; the chance probability of this occurring is $p = 0.018$. The chance probability that Ng/Nl is the nearest neighbor of *Protocalliphora* in at least two of the three sequenced regions (*ftsZ*, *wsp* 5′, and *wsp* 3′) is $p = .00169$, indicating

Figure 1

Base pair polymorphisms of B *Wolbachia* from *Protocalliphora* (Prot) and its parasitoids (*Nasonia vitripennis*, Nv and *Nasonia girault*i, Ng). Recombination in the B *Wolbachia wsp* gene is shown to have occurred between base pair 204 and base pair 212 in the 392 base pair *Protocalliphora* sequence. The bases unique to *Protocalliphora* are highlighted in gray. Considering these shared polymorphisms and excluding the three indels (in indels, a hyphen [-] indicates one base pair), the *Protocalliphora* sequence has the following spatial pattern (5′→3′): 17Nv - 34Ng - 1Nv - 1Ng. The chance probability of a string of 34 Ng similarities in 53 shared polymorphisms is remarkably low ($p < 10^{-10}$), and this indicates recombination within the gene.

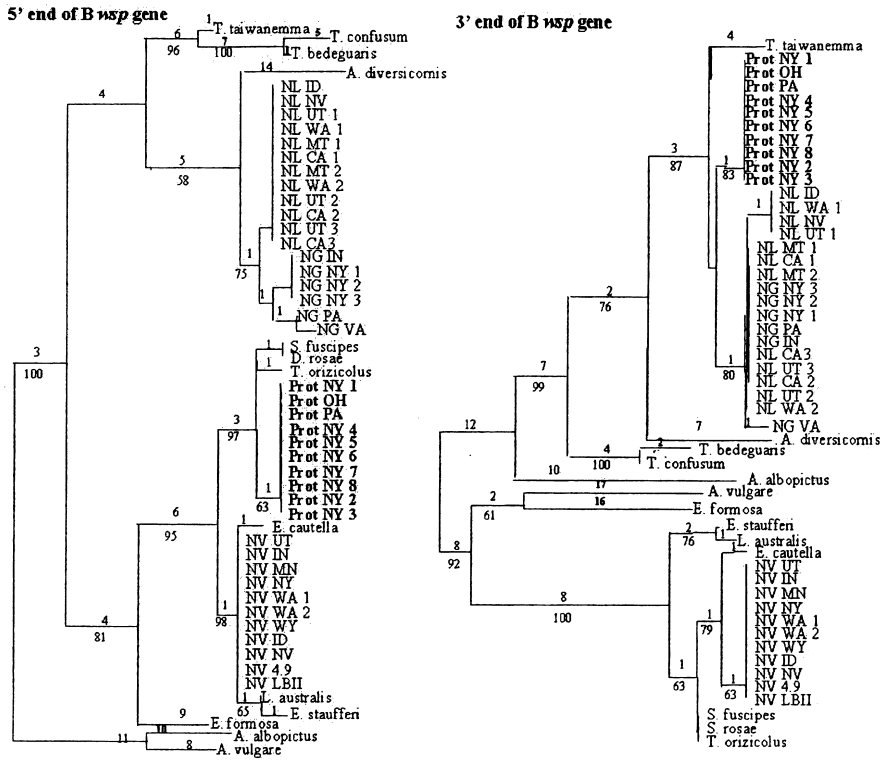


a highly significant association. It should be noted that the parasitic wasps used in these studies were reared for multiple generations on a fly species (*Sarcophaga bullata*) that is not infected with *Wolbachia*. Therefore, there is no chance that the sequences amplified from the wasps came from the host upon which they had developed. In nature, however, *Protocalliphora* flies are the preferred host of *N. girault*i and *N. longicornis*, and these wasp species are almost exclusively found developing from pupae of these flies [22, unpublished data].

Taken together, these results indicate exchange between *Wolbachia* that is present in both the parasitoid and its blow fly host. However, results are consistent with two scenarios. First, a horizontal transfer of *Wolbachia* between *Protocalliphora* and *N. girault*i or *N. longicornis* could have

Figure 2

Midpoint-rooted proportion difference (p-distance) neighbor joining trees showing the recombination event. Host names are used to identify *Wolbachia* sequences. The 5′ tree (left) places *Protocalliphora Wolbachia* in a clade with *Spalangia fuscipes*, *Diplolepis rosae*, and *Torymus orizicolus Wolbachia* (bootstrap value = 97%, 10,000 replicates). The 3′ tree (right) places *Protocalliphora Wolbachia* in a clade with *Nasonia girault*i, *Nasonia longicornis*, and *Teleogryllus taiwanemma Wolbachia* (bootstrap value = 87%, 10,000 replicates).



occurred, and a recombination event with a different *Wolbachia* that is related to that found in *N. vitripennis* (to account for the 5′ end of *wsp*) could have followed. Alternatively, a recombination event could have occurred between the *Wolbachia* resident in *Protocalliphora* and the bacteria present in *N. girault*i or *N. longicornis*. The *fts*Z and 3′ portion of the *wsp* sequences suggest that the "resident" *Wolbachia* that is present in the blow fly is most closely related to that found in *N. girault*i and *N. longicornis*, the parasitoid specialists on these blow flies. However, if correct, this does not indicate whether the original bacterium was transferred from wasp to fly or vice versa. The basic biology would suggest a transmission from the fly to the parasitic wasp because developing offspring of the wasp feed upon the fly (and, thus, possibly pick up the bacteria) and because wasps typically kill their hosts upon stinging them. However, we have observed instances of blow fly survival following stinging by *Nasonia*, particularly when eggs are not subsequently laid.

In an attempt to clarify the "resident" bacterial type found in the blowfly, we sequenced the *dna*A gene, one of the few additional gene sequences known from *Wolbachia* [28], for Nv, Nl, Ng, *Protocalliphora*, and four other insects. Contrary to expectations, the *dna*A gene from *Protocalliphora Wolbachia* was not closely related to the gene found in either Nv (7.4% divergent) or Ng/Nl (7.2–7.4% divergent), whereas Nv, Nl, and Ng were nearly identical (one base pair difference, or 0.2%). These results do not resolve the source of the *Protocalliphora Wolbachia* but are clearly further evidence of genetic recombination among *Wolbachia*. Specifically, whereas *fts*Z and *wsp* of Nv differ considerably from those found in Ng/Nl, their *dna*A sequences are nearly identical. Similarly, although the *fts*Z and the 3′ portion of *wsp* found in Ng/Nl and *Protocalliphora Wolbachia* are nearly identical, the *dna*A gene of *Protocalliphora* is quite divergent.

The genetic structure of bacterial species can range from strictly clonal to highly sexual (i.e., high rates of recombination) [29]. In addition, there is growing evidence of genetic recombination between even distantly related groups of bacteria [30]. However, it has previously been assumed that recombination rates in *Wolbachia* are low or absent. This is based upon the general concordance of gene phylogenies between the major groups of *Wolbachia* (subdivisions A, B, C, and D) and on the biology of these bacteria [3, 5, 11]. Within their host species, *Wolbachia* are typically inherited cytoplasmically, presumably affording few opportunities for horizontal gene transfer. However, these bacteria also clearly undergo horizontal movement between host species, and, in addition, double infections with different *Wolbachia* are not uncommon and possibly afford another avenue for horizontal gene movement [11].

Recombination in *Wolbachia* has several important implications. First, the presence of recombination mechanisms in *Wolbachia* could increase their utility as tools for the genetic manipulation of insect populations by increasing the potential for the introduction of desirable traits into *Wolbachia* [15, 16]. Recombination also complicates phylogenetic analysis of *Wolbachia*. The major phylogenetic subdivisions (e.g., A, B, C, and D) appear to be robust since parallel phylogenies are found for several different genes [2, 11, 24]. However, finer-scale phylogenetic reconstructions should be viewed with caution. In particular, we suspect that recombinant genotypes in the *wsp* gene may be selectively favored, given that this gene codes for the *Wolbachia* outer surface protein, which could be subject to diversifying selection. Given recombination within this gene, the use of *wsp* for *Wolbachia* subgroup designation [24] should therefore be approached cautiously.

Recombination has important implications for the evolutionary interactions of *Wolbachia* with their hosts. These bacteria are very widespread in invertebrates and alter host reproduction in a number of interesting ways. Some *Wolbachia* induce parthenogenetic reproduction in their hosts [8], while others cause feminization of genetic males [7], male killing [9], or reproductive incompatibility between eggs and sperm, known as cytoplasmic incompatibility [10]. It has been proposed that the phylogenetic distributions indicate multiple independent evolutions of these phenotypes (e.g., parthenogenesis induction) in different *Wolbachia* [11]; however, the alternative possibility of horizontal transfer of the relevant genetic machinery by recombination is increasingly possible. Further analysis of the rates of recombination and the role of recombination in *Wolbachia* evolution will emerge from *Wolbachia* sequencing projects [31].

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