Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods

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SUMMARY

Wolbachia are cytoplasmically inherited bacteria found in reproductive tissues of many arthropod species. These bacteria are associated with reproductive alterations in their hosts, including parthenogenesis, reproductive incompatibility and feminization. A fine-scale phylogenetic analysis was done using DNA sequences from fliZ, a rapidly evolving bacterial cell-cycle gene. fliZ sequences were determined for 38 different *Wolbachia* strains from 31 different species of insects and one isopod. The following results were found: (i) there are two major division of *Wolbachia* (*A* and *B*) which diverged 58-67 millions years before present based upon synonymous substitution rates; (ii) a general concordance is found between the fliZ and 16S rDNA phylogenies, indicating that these represent bacterial strain (rather than simply gene) phylogenies; however, a possible example of recombination between *A* and *B* division bacteria may have occurred in the feminizing *Wolbachia* present in an isopod; (iii) extensive horizontal transmission of *Wolbachia* has occurred between insect taxa, including different insect orders; one strain in particular (designated Adm) shows extensive recent horizontal transmission; (iv) there is an association between the *Wolbachia* found in a parasitic wasp (*Nasonia*) and its fly host (*Protocalliphora*), suggesting exchange of bacteria between these species; (v) parthenogenesis induction has evolved several times among the *Wolbachia*; (vi) some insects harbour infections with more than one *Wolbachia* strain, even within individual insects.

1. INTRODUCTION

The *Wolbachia* are a group of alpha proteobacteria that infect the reproductive tissues of arthropods. These bacteria are transmitted through the egg cytoplasm and alter reproduction in their arthropod hosts in various ways. So far, *Wolbachia* have been found to be associated with post-zygote reproductive incompatibility (termed cytoplasmic incompatibility or *CI*) in a wide range of insects (Barr 1980; O'Neill et al. 1992; Breeuwer et al. 1995), parthenogenesis in wasps (Stouthamer et al. 1993), and feminization of genetic males in an isopod (Rousset et al. 1992).

By utilizing polymerase chain reaction (PCR) amplification and sequencing of bacterial 16S rDNA genes, several research groups have established that *ca*. parthenogenesis, and feminizing bacteria form a closely related group in the alpha proteobacteria, the *Wolbachia* (O'Neill et al. 1992; Breeuwer et al. 1992; Rousset et al. 1992; Stouthamer et al. 1993). Because of low 16S rDNA sequence divergence (1-2%), between *Wolbachia* found in distantly related arthropods, it has been proposed that *Wolbachia* undergo horizontal transmission between insect taxa (O'Neill et al. 1992). However, the very low rate of 16S rDNA sequence evolution makes it difficult to determine the pattern or rate of horizontal transmission between host taxa, or the relationships among *Wolbachia* causing different reproductive alterations. 16S rDNA sequence divergence is estimated to be 1-2%, per 50 million years (Ma) (Ochman & Wilson 1987; Moran et al. 1994).

Two protein coding genes from *Wolbachia* were recently sequenced from infected strains of *Drosophila melanogaster*, fliZ and dnaA (Hollings et al. 1993; Bourer et al. 1994). fliZ is a bacterial cell-cycle gene involved in regulation of cell division (Lukenshahn 1990). It contains conserved and highly divergent regions, making it suitable for finer scale phylogenetic analysis within a bacterial genus. Using fliZ sequence information from *Wolbachia* and three other bacterial species (*Escherichia coli*, *Bazillus subtilis*, and *Rhizobium meliloti*), we designed *Wolbachia* specific primers for PCR amplification of the gene from infected arthropods. Testing of the primers revealed that they amplified the fliZ gene from known infected (but not from known uninfected or bacterially cured) arthropod strains. Preliminary sequence information also indicated that some arthropod strains are infected with more than one *Wolbachia* strain.

The purpose of this study is (i) to use fliZ gene sequences to determine the phylogenetic relationships among *ca*. parthenogenetic and feminizing *Wolbachia*, (ii) to investigate potential cases of interspecific (horizontal) transmission of *Wolbachia*, and (iii) to determine the extent and distribution of infections of arthropods by several *Wolbachia* strains.

2. METHODS

(a) Template preparation

Arthropod material was obtained live, fixed in 70-95% ethanol or as previously extracted DNA. DNA was extracted


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from the live or fixed material using either: (i) swarcs dissociated from one to ten individuals (depending upon size); (ii) whole abdomens; or (iii) whole individuals for small arthropods. The tissues were dissociated in sterile double distilled, deionized water on a sterile slide and then serially rinsed in sterile 80% ethanol before DNA extraction using our previously published methods. (Bereczki et al. 1982; Wetter & Jaekne 1995). Extreme care was taken throughout the procedure as it was not always clear what DNA was actually being isolated and extracted from the mosquito, the nematode, or the arthropod. DNA was isolated from individual mosquito abdomens or femora of the nematode Drosophila simulans. Parasitic wasps develop within the tissues of other insects. To avoid, post-mortem, loss of tissue concentration during preparation of DNA: parasitic wasps were reared on host strains known to be infected with Wolbachia for at least one generation (usually many) before DNA extraction.

4 PCR amplification

Bacterial frzDZNA was amplified using PCR in a volume of 25 μl of 1× PCR buffer (Promega), 0.75 μM MgCl₂, (50 μM), 0.5 μM nucleotide mix (10 mm each), 0.33 μM of 20 μm primer 1, 0.33 μM of 20 μm primer 2, 0.25 μM salt polymerase (Promega) and distilled, deionized H₂O was added to a final volume of 25 μl of 50 μl (double concentration). The PCR reaction mix was prepared in a fresh batch and then added to each sample. The remainder was run as a control for contamination. PCR cycling conditions were one cycle, 1 min at 94°C, 1 min at 55°C, 3 min at 72°C; 35 cycles (15 s at 94°C, 1 min at 55°C, 3 min at 72°C) and 1 cycle (15 s at 94°C, 1 min at 55°C and 10 min at 72°C).

Primers were used for amplification of the nearly complete Wolbachia frzD sequence. frzD2f (5’GCTT GTG AAT ACG CAT GTC 3’), and frzD2r (TTC GCC ATG TCA GGT TCT TGC CTT G 3’), which yield a 1049-1053 bp. product, depending upon Wolbachia strain. Results have shown that these primers can be used to amplify the frzD gene from all tested arthropods known to harbour Wolbachia infections, and fail to amplify the products from gamma proteobacteria (Eubacterium, or alpha bacteria known to be closely related to Wolbachia based on 16S rDNA phylogeny, such as the rickettsiaceae (Wetter et al. 1994) and Ehrlichia et al. (Soussanier et al. 1995).

First, we were also designed for specific amplification of Adm and B group frzD and 16S rDNA regions. The frzD primers frzDAdm (3’TCTT CAG ACG CAG AAA AGG GT 3’), frzDAdm (5’TTC GCC ATG TCA GGT TCT TGC CTT G 3’), frzD2f (TTC GCC GGT GGT TCT TGC CTT G 3’), and frzD2r (TTC GCC ATG TCA GGT TCT TGC CTT G 3’), which yield a 1049-1053 bp. product, depending upon Wolbachia strain. Results have shown that these primers can be used to amplify the frzD gene from all tested arthropods known to harbour Wolbachia infections, and fail to amplify the products from gamma proteobacteria (Eubacterium, or alpha bacteria known to be closely related to Wolbachia based on 16S rDNA phylogeny, such as the rickettsiaceae (Wetter et al. 1994) and Ehrlichia et al. (Soussanier et al. 1995).

To confirm specificity of the primers an frzD region was chosen in which the group Adm Wolbachia typically have one AciI restriction fragment where as B group Wolbachia have two AciI restriction sites, producing fragment sizes of 175 bp, 462 bp and 297 bp. Similarly, the 16S region contains a BamH I site absent in the Adm group but present in the B group, creating fragments of 117 bp, 112 bp. After rea, 20 μl of amplified reaction product was restriction digested and electrophoresed 3-4 % NuSieve (1:3) agarose gel to examine for the appropriate restriction sites that distinguish A and B division bacteria.

5. RESULTS

For cloning purposes, a 50 μl PCR reaction was used (dH2O of solution)+, PCR products were purified with the GeneClean kit (Bio 101, Inc., La Jolla, California, USA) and then directly cloned into a Tailed M13mp18 derivative (W. Burke, unpublished results). At least two clones were sequenced from each arthropod species (E. coli) for the complete region between the general frzD primers (1043-1053 bp) and the A and B specific primers (955-957 bp). All mismatches between clones (which occurred in less than 0.42% of base positions) were resolved by comparison with the same group consensus sequence. Two regions of sequence ambiguity (because of secondary structure) were resolved by additional sequencing reactions using dITP for dGTP in labelling and termination mixtures (Sequence Version 2.0 k, US. Biological).

For some arthropod strains found to be infected with both A and B group Wolbachia, frzD representatives of both types were cloned and sequenced, from products amplified using the A and B specific primers.

frzD phylogeny

The frzD sequences were manually aligned to previously determined Wolbachia frzD sequences. For phylogenetic analysis, a 970 bp. region (including deletions-insertions) between the A and B division specific primers was used, because this region could be aligned among all sequences available. A phylogenetic analysis was performed for 30 Wolbachia strains using the neighbour-joining program of Tamura (1992) with the JNT (v. 1.3). Genetic distance was calculated in several different ways, p-distance (proportion differences) including deletions-insertions, p-distance excluding deletions-insertions, and JNT (v. 1.3) (distance excluding deletions-insertions). Synonymous substitutions were excluded from the calculations. The number of sites were determined by eye (Wheeler et al. 1999), which employed the algorithm of Nei & Gojobori (1986). Sequences are available in Genbank under accession numbers U28172-U28211.

A. Wolbachia phylogeny

Phylogenetic relationships of 38 different Wolbachia strains were investigated by a neighbour-joining algorithm (Saitou & Nei 1987) using a 970 bp. region of the frzD gene (see figure 1). As apparent in the figure, there are two major divisions of Wolbachia, designated A and B. The average frzD divergence (p-distance) between these two groups ranges from 0.03%--5.60% whereas the divergence within the A group ranges from 0.00% to 2.67% whereas in the B group from 0.2% to 5.74%. The A group bacteria show a much greater level of sequence similarity than do B group bacteria. The A division is further divided into two subgroups, designated Adn. and Aec. Each subgroup is designated by a representative host species of the group. Adn, for Drosophila melanogaster and Aec for

**Figure 1. Phylogenetic tree of Wolbachia based upon sequences of the ftsZ gene.** None of the host arthropod species is followed by the strain designation. Paenarthogenetic associated bacteria are shown in bold. The tree was generated by neighbour-joining using the p-distance including insertion-deletions. Number next to nodes indicate the number of replicates confirming the node out of 100. Replicate numbers less than 50 are not included in the figure.

*Wolbachia* species. The A/B group shows a high level of sequence similarity despite being found in diverse insect hosts, suggesting relatively recent horizontal transmission of this group of strains (see §3c).

The A and B groups, the amino acid sequence of the 5-prime end of *ftsZ* is considerably more conserved than that of the 3-prime end. This is apparent by dividing the gene into two approximately equal regions. The first 170 amino acids (AA) show 6 AA differences between the two, whereas the second shared 170 AA show 25 differences. DNA sequences for a representative of the A and B group bacteria are shown in figure 2. The B group contains two deletions relative to A in the variable 3-prime region, one of 9 b.p. and the other 15 b.p. All B group bacteria have both deletions, except *Wolbachia* species and *Glyciphilus* species, which retain the 9 b.p. region present in A. In addition, these two *Wolbachia* show a unique single base-pair change in the second position of the insert relative to A bacteria. They are clearly B, with respect to the other deletions and numerous single base-pair substitutions throughout the *ftsZ* gene. This suggests that these two strains diverged from other *Wolbachia* before the loss of the region in the lineage leading to other *Wolbachia* bacteria. Group A bacteria show two deletions relative to B in the 3-prime region one of 3 b.p. and the other of 9 b.p.

**Phylogenetic relationships among the *Wolbachia* were derived using various genetic distance estimates, p-distance (proportion difference) with insertions–deletions, p-distance without insertions–deletions, and Jukes–Cantor genetic distance. Each of these methods**

Figure 2. Nucleotide sites are shown for the fctZ gene from a representative member of the B group (Cubes piperis host) and A group (Drosophila melanogaster host) bacteria. Primer locations are underlined (or overlined). The actual fctZ primer differs from the Cubes Wolbachia sequence shown by one base (CAAACG in the primer complement versus CAAATG).

The fctZ gene has been used to estimate divergence times between Wolbachia strains. Synonymous substitution rates can be used to estimate divergence times between A and B group Wolbachia. Ochman & Wilson (1987) have estimated the synonymous substitution rate for bacteria to be 0.7-0.8% per Ma. The percent synonymous substitutions between two representatives of A and B group bacteria (D. simulans and C. pipiens) is 33.4%, correcting for several bias in the formula of Jukes & Cantor (1960) yields 46.6%. Based on this rate, the estimated divergence time between A and B group Wolbachia is 58.3-66.6 Ma.

The rate of synonymous substitutions in bacteria was estimated by Ochman & Wilson (1987) based on comparisons between E. coli and Salmonella protein coding regions and using an estimated divergence time between these taxa of 140-160 Ma. To determine whether fctZ shows the expected level of divergence between these taxa, we used the E. coli fctZ sequence to design primers and then amplified and sequenced fctZ from Salmonella typhimurium. Based on the first 350 sequenced base pairs that are shared among these taxa and the two Wolbachia, per cent synonymous substitutions between E. coli (K12) and S. typhimurium (LT2) is 59.5% (adjusted to 115.8%). This is typical for protein coding regions between these taxa (Ochman et al. 1995).
& Wilson (1987), and gives the expected 142-162 Ma divergence. Results indicate that in these bacteria fZ does not show unusual rates of synonymous substi-
tution. The adjusted per cent divergence between A and B Wolbachia over this same gene region is 43.3%, similar to the extant gene (46.6%).

c. Evidence for horizontal (interracial) transmission

The phylogenetic evidence strongly supports hori-
zontal transmission of Wolbachia among arthropods (see figure 1). Even a cursory examination shows that the Wolbachia fZ phylogeny does not parallel that of the hosts.

The Adm group is interesting in this regard, because it shows a high degree of sequence similarity despite being found in a wide range of insect hosts. Adm bacteria are found in host species from the inset orders Coleoptera, Diptera, Hymenoptera and Lepidoptera. Yet, the Adm Wolbachia sequence found in the dipteran Drosophila simulans (R) is identical to that found in the hymenopteran Asobara tabida and differs from the coleopteran Cuscuta sp and lepidopteran Euphyes castalia by only one base pair despite the fact that these hosts diverged from each other at least 200 Ma before present (48) [Hennig 1981].

Detection of Adm bacteria in diverse host species by rec is highly repeatable, using different strains and DNA extractions. In addition, southern hybridization using restriction digested genomic DNA from infected D. simulans and N. vitripennis confirms the presence of Adm Wolbachia in these two species (one dipteran and one hymenopteran). Thus, the presence of Adm bacteria in diverse insect hosts is not the result of a rec artifact.

Evidence supports a relatively recent range ex-
pan-
dion of Adm bacteria into new insect species. Given approximately 265 synonymous substitution sites over the 937 b.p. region, and a 7 x 10^-6 per year divergence rate, the 95% confidence limits for divergence time among Adm Wolbachia with identical fZ sequences (e.g. the hymenopteran Asobara tabida and the dipteran Drosophila simulans [R]) are 0.56 Ma, and 999,99% confidence limits are 1.06 Ma.

In contrast to Adm, B group bacteria show a higher level of sequence divergence between strains, and in some cases phylogenetic concordance with host taxa occurs. For example, the three bacterial strains from Triatoma species cluster as a single phylogenetic unit, as do Wolbachia strains in the two Leptopilina species and two Nasonia species (giruinaldii and lanceicornis).

Nevertheless, interstrain transmission has clearly also occurred among the B group bacteria. For example, Wolbachia in the mosquito Culex pipiens are more closely related to those in the isopod Armillulahus valgus, than to the mosquito Anis albitarsis. The closest relative of B Wolbachia found in the beetle (order Coleoptera) Tribolium onyga occurs in the cricket Gryllus pennsylvanicus. The Tribolium Wolbachia is associated with cytoplasmic incompatibility (Wade & Stevens 1985); etiology of the cricket bacteria is unknown.

We propose that transmission between parasitic insects and their hosts is one vehicle for horizontal transmission of Wolbachia. An obvious test of this hypothesis is to show that parasitoids and their host insects share Wolbachia strains that are more closely related than would be expected by chance. A possible example of this expectation is present in the curculio data set. Nasonia giruinaldii and N. lanceicornis are parasitoids wasps that specialize in attacking protocalliphorid flies living in bird nests (Darling & Werren 1990; J. H. Werren, unpublished results). This contrasts with the congener N. vitripennis, which is a generalist parasitoid known to parasitize a wide range of 5y species in a variety of habitats. Both N. lanceicornis and N. giruinaldii harbour B group Wolbachia strains that are closely related to those of their preferred Protocalliphora fly hosts.

The probability (p) that Protocalliphora occurs in the same clade with its parasitoids (N. lanceicornis and N. giruinaldii) is p = (x - 1)/x, where x is the number of bacterial taxonomic units in the phylogeny. A con-
servative approach is to consider only B group strains and to treat the two Leptopilina strains and the three Triatoma strains as single units, based on the taxonomic affinity of the insect hosts and associated bacteria. By this method, x = 15, and the association is not significant (p = 0.061), although it is the lowest p-
value possible, given the sample size.

A second possible exchange between parasitoid and host involves Drosophila melanogaster and the drosophilalike parasitoid Asobara tabida. These two species share nearly identical Wolbachia (one difference over 862 b.p.). However, because the Adm group bacteria appear to have undergone considerable recent hori-
zontal transmission, this association has little statistical resolving power.

d. Distribution of pathogenesis of Wolbachia

Pathogenesis Wolbachia are found in both the A and B groups and are typically ranked by its induction on host species. Based upon the current phylogeny, it can be postulated that this pathogen has evolved several times independently, in Leptopilina, Triatoma, Encarsia, Maculiferum and Aphidi. It therefore appears that pathogenesis induction can evolve relatively easily in the Wolbachia, suggesting a simple biochemical mechanism. An alternative in-
terpre-
tation is that pathogenesis has evolved by ex-
change of the genetic machinery for pathogenesis via recombination between Wolbachia strains. How-
ever, concordance between 16S rDNA and fZ does (48) suggests that recombination between A and B division bacteria is not common, although recom-
bin-
at-
ation within groups could be more frequent. A second, alternative interpretation of the pattern is that pathogenesis induction is not the result of evol-
u-
 tionary changes in the Wolbachia, but to differences in hosts.

The fZ amplification and sequencing results shown here establish that Wolbachia are present in the pathogenic Hymenoptera Leptopilina and
Table 1. Shaws are results of rca amplifications using Adm and B specific primers for fzoZ and 16S rDNA
(To verify specific amplification, the fzoZ rca product was restriction digested with Acil and 16S rDNA was restriction digested with RsaI. Products were visualized on agarose gels and examined for characteristic Adm and B group restriction profiles. Adm or B within the table indicates that the characteristic restriction site(s) for the respective group were found. As can be seen, there is general concordance between presence of A and B type bacteriophage fzoZ and 16S rDNA. See text for further discussion.)

<table>
<thead>
<tr>
<th>species (strain)</th>
<th>16S fzoZ</th>
<th>16S fzoZ type</th>
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<tr>
<td>Adm primers</td>
<td>B primers</td>
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<tr>
<td>Drosophila melanogaster (i66)</td>
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* Distinctive digestion pattern.

L. clausii: antibiotic curing indicates that parthenogenesis is baccularly induced in these species (J. van Alphen, personal communication). In addition, we have detected Wolbachia in the parthenogenetic weevil Nasonus tessellatus, although antibiotic treatments of this species has not been performed. This case is particularly interesting because all other known examples of Wolbachia-associated parthenogenesis occur in Hymenoptera (Stouthamer et al. 1993). Additional new cases of Wolbachia infections detected here include those in the parasitoids Asobara tabida and Trichoiraphis drosophilae. These bacteria appear to cause cas, based on curing experiments (J. van Alphen, personal communication). Wolbachia were also detected in reproductive tissues of Naria, Anastrepha and Protocalliphora flies, Melichar wasps, Cucuio beetles, and Gypis paenuniceus crickets. The etiologies of these Wolbachia are unknown.

(f.) Congresses of fzoZ and 16S rDNA phylogeny

The fzoZ phylogeny presented in figure 1 could reflect the actual phylogeny of the Wobachia strain present in the host arthropods. However, in bacterial species with frequent recombination, gene phylogeny do not necessarily parallel bacterial strain (or species) phylogeny. The hypothesis that fzoZ gene phylogeny accurately reflects Wolbachia strain phylogeny can be investigated by determining whether there is concordance between phylogenies of fzoZ and 16S rDNA genes. The 16S rDNA does not evolve quickly enough for a fine scale analysis, but can be used to look for general congruence between the A and B subdivisions. We investigated general concordance between fzoZ and 16S rDNA by a pce-based assay, using 29 different arthropod species strains. Specific primers were designed for the Adm and B groups of Wolbachia, for both the fzoZ and 16S rDNA genes. It should be noted that the A group primers of fzoZ were designed specifically to amplify the Adm group and are not effective in amplification of Ant fzoZ sequences. Primer specificity was determined by restriction digestion of rca product with enzymes that reveal restriction site polymorphisms between A and B (Acil for fzoZ, RsaI for 16S rDNA, see [2]). Table 1 shows results of the pce assay. A general concordance between fzoZ and 16S rDNA is clear from the analysis. Ten species (two strains of D. simulans) contain Adm group fzoZ and Adm group 16S rDNA, based upon primer specificity and restriction digestion. Nine species contain B group fzoZ and B group 16S rDNA. Seven of these clearly show the expected B specific restriction profile of the fzoZ product. Two contain addition-restriction sites that make interpretation of the profile tentative. Six species contain both Adm and B fzoZ, and also contain both Adm and B 16S rDNA.

Of the 28 species (plus two strains of D. simulans) examined, only three give discordant results. Proto- calliphora shows amplification with B-specific primers for both genes. However, the 16S rDNA restriction profile lacks the RsaI site found in most B bacteria. This incongruity could be caused by the loss of a restriction site as a result of mutation, and is being investigated. Staphylinus orizae, the rice weevil, contains a B group fzoZ, but amplifies 16S rDNA with both Adm and B specific primers. Restriction digestion of the B primer 16S rDNA product reveals an Adm group restriction pattern, suggesting nonspecific amplification of Adm 16S rDNA by the B primers in this species.

Template from the isopod Armilladillum caligatum amplifies an Adm group 16S DNA, but a B group fzoZ, based on both primer specificity and restriction profiles. The Wolbachia found in Armilladillum caligatum is unusual in several respects. First, this Wolbachia occurs
double infections with different Wolbachia

Sequencing of several ftsZ clones from rcr amplifications, using the general ftsZ primers, revealed the presence of both A and B group ftsZ genes in some insects. For example, all three species of the parasitic wasp Nasonia contain ftsZ sequences from the B group and the Adm group. The beetle Tribolium confusum contains B group and Aec group ftsZ sequences. There were two basic interpretations of this pattern: (i) individual Wolbachia strains have two divergent copies of ftsZ (i.e., ftsZ A and B is a two gene family); or (ii) there is a single ftsZ gene, but some insert harbour infections with several Wolbachia strains.

Evidence strongly supports the hypothesis that some insect harbours infections with several Wolbachia strains. Table 1 shows the results of screening 30 different species/strains for the presence of B group and Adm group ftsZ and 16S rDNA sequences. If the presence of both Adm and B ftsZ sequences is the result of double infection, then it is predicted that insects with single ftsZ types should have single 16S rDNA types, whereas those with both ftsZ types should have both 16S rDNA types. This precisely the pattern observed: of 29 tested species with two strains, in D. simulans 22 have single ftsZ and 16S rDNA and seven have both A and B for ftsZ and 16S rDNA. To account for this pattern by gene duplication in single Wolbachia strains, one would have to assume that whenever ftsZ was duplicated, the 16S rDNA locus was also duplicated. We have confirmed by southern hybridizations of genomic DNA from infected hosts, that some strains of S. striipennis harbour double infections (A and B), whereas others harbour single infections. Crossing experiments confirm that these differences are associated with compatibility differences between the strains (M. Perrot-Minnot & J. H. Werren, unpublished results).

The rcr assay presented in table 1 reveals double infections with Adm and B group bacteria in strains of six different species, or 20% (six out of 30) of those tested. The assay would not detect double infections between B and the Aec group, however, by sequencing data and use of Aec specific primers, we have found that Tribolium confusum harbour double infections of these two strains.

4. DISCUSSION

Wolbachia are a widespread group of cryptic intracellular bacteria that alter reproduction in arthropods. In this study, 11 insect species not previously known to harbour these bacteria were found to carry Wolbachia, based on the rcr assay. Wolbachia have been found in most of the major insect orders (including Hymenoptera, Diptera, Coleoptera, Lepidoptera, Orthoptera, Homoptera and Hemiptera) in invertebrates. The ability of different Wolbachia strains to alter host reproduction in a wide range of arthropods is also shown by recent microinjection experiments. Wolbachia from Ano satiparum were transferred into Drosophila simulans, where the bacterium succeeded in causing cryptic incompatibility in its new host (Bräu et al. 1994). Our work indicates that Ano harbour both B and Adm strains, whereas the resident bacterium of D. simulans is an Adm strain. The study reveals considerable diversity among Wolbachia strains. It is uncertain at what level of divergence a species designation should be assigned to these different Wolbachia types. Therefore we have taken the cautious approach of calling these "strains" of Wolbachia, and not assigning species designations. Given the level of divergence among Wolbachia species, the A and B groups diverged between 50 to 60 Ma, it is likely that many of these will eventually be designated as separate species. Therefore, prematurely assigning the species designation Wolbachia pipientis (the species name given to the B group bacterial strain found in the mosquito Culex pipiens) to all Wolbachia, should be avoided.

The evidence for horizontal transmission is clear-cut. The Adm strain, in particular, has undergone considerable horizontal transmission, being found in hymenopterans, dipterans and lipitodeps, with virtually no sequence divergence between bacteria found in these different hosts. Natural transmission of Adm bacteria into new hosts has occurred within the last 2-3 Ma. Current evidence indicates that the different orders of insects they are found in diverged at least 200 Ma nap (Hennig 1981). The Adm bacteria are likely to be undergoing a rapid host range expansion. Evidence for this includes the apparent recent acquisition of Adm bacteria by Drosophila simulans (Turelli & Hoffmann 1991). We suspect that the widespread distribution of Adm may have been facilitated by human activity. Many of the species in which this strain is found have themselves undergone range expansions because of human disturbance or transport. Disrupts of ecological associations could bring new species into contact and facilitate exchange of their associated Wolbachia. It is unclear what particular characteristics of Adm have permitted it to spread so rapidly between different insect taxa.

Possible mechanism of horizontal transmission by Wolbachia are of special interest. Our phylogenetic data show that transmission between parasitic insects and

their tests may have occurred between *Pseudomonas* spp. and *Naumia* parasitic wasps. The data are insufficient to indicate direction of the transfer; however, the biology of this host-parasite relation suggests transmission occurred from the fly to the parasite. *Naumia* spp. typically sting and kill their host pupae before laying eggs on the host, therefore making it difficult for nematodes to Wasbusha to their host populations; however, the intimate association of developing wasps with host tissues, could facilitate transmission from the host to the wasp. Horizontal transfer of *Wasbusha* could occur via predators as well, as has been suggested for predatory mites and *P.-element* (Hosek et al. 1991).

Wasbusha may promote rapid specialization by causing reproductive incompatibility between populations (Breehon & Warren 1990; Coyne 1992), particularly when bidirectional incompatibility occurs between populations harbouring different bacteria. Partial-to-complete bidirectional incompatibility has been found between strains of *D. similus* and *C. pisipiper*, and between sibling species of *Naumia* (D. similus; Hawaii) and *B. similus* (Riverside) are two bidirectionally incompatible strains represented in this study (O'Neill & Karr 1990). These two strains show nearly identical *fix* sequences (one difference over 962 bp.), indicating that bidirectional incompatibility can evolve relatively rapidly in Wasbusha.

The three species of *Naumia* have similar bacteria, although the picture is complicated by presence of double infections within individuals of these species. Data shown here suggest that *N. atra* may have acquired its *D. similus* bacteria from a different source to the two more closely related species, *N. grimaldi* and *N. longiseta*. It is not known whether bidirectional incompatibility promoted speciation in this complex, or was merely a consequence of divergence.

Infections of individual insects with two different strains of *Wasbusha* appear to be relatively common, and could play a role in creating new compatibility types. Of the insects we tested 21% harboured double *Naumia* bacterial infections. Rosset and Solignac (1995) have also found double infections in the fly *Drosophila simulans*. Occurrence of double infections is surprising, because stochastic processes are expected to cause the eventual loss of one strain during successive generations of cytoplasmic transmission within eggs. However, synergistic interactions between different *Wasbusha* strains may favor maintenance of double infections. We have preliminary evidence that double infections in *Naumia* can create novel compatibility types, which may promote their spread through host populations. Double infections also create an arena in which genetic exchange between bacterial strains could occur.

The phylogeny of *Naumia* and *parthenogenetic Wasbusha* suggests that parthenogenesis induction has evolved several times independently. However, an alternative interpretation is that parthenogenesis induction is not the result of differences in *Wasbusha*, but differences in host environment. Although formally possible, we consider it unlikely that a trait such as pathogenesis, that clearly can be adaptive for the bacteria, is merely the byproduct of host environment.

A second possibility is that the generic machinery for parthenogenesis has been introduced into cp bacteria by genetic exchange. Although our data (showing concordance of *16S* DNA and *fix*; *fix* strongly suggest that recombination between the two divisions of these bacteria is uncommon; even rare recombinational events could introduce novel sequences and mechanisms into particular bacterial lineages. One such possible example is the *Armadillidium* F. bacterium, in which exchange between an *A- and B-*division bacteria may have occurred, based on our *fix* DNA sequences. A second possible case is in the rice weevil, *Sitophilus oryzae*. These preliminary findings should be approached cautiously. The arthropods mentioned may actually harbour both *A- and B-Wasbusha*, but divergence in primer sites could result in disparate amplifications. More definitive proof of genetic exchanges requires southern hybridizations to rule out the possibility of double infections in these organisms. Nevertheless, genetic exchange between *A- and B-Wasbusha* is an intriguing possibility.

The ability to identify bacterial strains and their phylogenetic relationships, will greatly aid studies of these interesting bacteria. It is clear from this study that the *fix* gene will be a useful tool, especially when coupled with sequence information from other protein-coding regions.

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**References**


