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 *ftsZ*, a rapidly evolving bacterial cell-cycle gene. *ftsZ* 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 *ftsZ* 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*; and (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-zygotic reproductive incompatibility (termed cytoplasmic incompatibility or ci) in a wide range of insects (Barr 1980; O'Neil et al. 1992; Breeuwer et al. 1992), parthenogenesis in wasps (Stouthamer et al. 1993), and feminization of genetic males in an isopod (Rousset et al. 1992).

By using polymerase chain reaction (PCR) amplification and sequencing of bacterial 16S rDNA genes, several research groups have established that ci, parthenogenesis, and feminizing bacteria form a closely related group in the alpha proteobacteria, the *Wolbachia* (O'Neil 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'Neil 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*, *ftsZ* and *dnaA* (Holden et al. 1993; Bourtzis et al. 1994). *ftsZ* is a bacterial cell-cycle gene involved in regulation of cell division (Lukenhaus 1990). It contains conserved and highly divergent regions, making it suitable for finer scale phylogenetic analysis within a bacterial genus. Using *ftsZ* sequence information from *Wolbachia* and three other bacterial species (*Escherichia coli, Bacillus 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 *ftsZ* gene from known infected (but not from known uninfected or antibiotically 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 *ftsZ* gene sequences to determine the phylogenetic relationships among ci, parthenogenetic and feminizing *Wolbachia*, (ii) to investigate potential cases of intertaxon (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...
from the live or fixed material using either: (i) ovaries dissected from one to ten individuals (depending upon size); (ii) whole abdomens; or (iii) whole individuals (for small arthropods). The tissues were dissected in sterile double-distilled, deionized water on a sterile slide and then serially rinsed in droplets of sterile H$_2$O$_2$ before DNA extraction using our previously published methods (Breeuwer et al., 1992; Werren & Jaenike, 1995). Extreme care was taken throughout the procedure to avoid bacterial contamination. All solutions were filter sterilized (0.22 µm pore size). Control DNA samples were prepared using pupae or ovaries of known infected and uninfected strains of either Nasonia vitripennis or Drosophila simulans.

Parasitic wasps develop within the tissues of other insects. To avoid the possibility of host tissue contamination during preparation of DNA, parasitic wasps were reared on host strains known to be uninfected with Wolbachia for at least one generation (usually many) before DNA extraction.

(b) PCR amplification

Bacterial flsZ DNA was amplified using PCR in a volume of 25 µl (1 µl DNA sample, 2.5 µl 10x buffer (Promega), 0.75 µl MgCl$_2$ (50 µM), 0.5 µl nucleotide mix (10 mM each), 0.350 µl 20 mM primer 1, 0.350 µl 20 mM primer 2, 0.25 µl tag polymerase (Promega) and distilled, deionized H$_2$O$_2$ was added to a final volume of 25 µl or 50 µl (double constituents). The PCR reaction mix was prepared in one 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 ºC, 1 min at 55 ºC, 3 min at 72 ºC) 35 cycles (15 s at 94 ºC, 1 min at 55 ºC, 3 min at 72 ºC) and 1 cycle (15 s at 94 ºC, 1 min at 55 ºC and 10 min at 72 ºC) (Erincomp thermal cycler). After PCR, 1 µl of amplified reaction product was run on a 1% agarose gel to determine presence and size of amplified DNA.

Primers were used for amplification of the nearly complete Wolbachia flsZ sequence, flsZrf (5’GGT GTC GCA AAT ACC GAT GC 3’) and flsZr1 (5’CCT AAG TTA GCT GGT ATA TC 3’), which yield a 1045–1055 b.p. product (depending upon Wolbachia strain). Results have shown that these primers are specific for Wolbachia. They amplify the flsZ gene from all tested arthropods known to harbour Wolbachia infections, and fail to amplify the product from gamma proteobacteria (E. coli or S. typhimurium), or alpha proteobacteria known to be closely related to Wolbachia based on 16S rDNA phylogeny, such as the rickettsial AB bacterium (Werren et al. 1994) and Ehrlichia canis (Stouthamer et al. 1993).

Primers were also designed for specific amplification of Adm and B group flsZ and 16S rDNA regions. The flsZ primers flsZAdf (5’CTC AAC CAG TAG AAA AGT CG 3’), flsZAdr (5’TGA CCT GCT TCG GCT ACC TG 3’), flsZBf (5’CCG ATG CTC AAG CGT AG 3’) and flsZBr (5’CCA CTT AAC TCT TTC GTT TG 3’) amplify a 955–957 b.p. region of the gene. The 16S rDNA primers 16SAf (5’TTC GGC CGG GCG TTA CAC AG 3’), 16SAr (5’TAA GGG ATT AGC TTA GCC GCC 3’), 16SBf (5’TTG GGC CGG ATT TTA CAC AA 3’) and 16SBr (5’TGA TTT AGC TAT TAG GTG 3’) amplify a 905–907 b.p. region of the 3-prime portion of the 16S rDNA gene.

3. RESULTS

(a) Wolbachia phylogeny

Phylogenetic relationships of 38 different Wolbachia strains were investigated by a neighbour joining algorithm (Saitou & Nei 1987) using a 970 b.p. region of the flsZ gene (see figure 1). As is apparent in the figure, there are two major divisions of Wolbachia, designated A and B. The average flsZ divergence (p-distance) between these two groups ranges from 13.03–15.69%, whereas the divergence within the A group ranges from 0 to 2.67% and in the B group from 0.72 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 Adm and Atc. Each subgroup is designated by a representative host species of the group, Adm for Drosophila melanogaster and Atc for...

(c) Cloning and sequencing

For cloning purposes, a 50 µl PCR reaction was used (doubling of solutions above). PCR products were purified with the Geneclean kit (Biotek, Inc., La Jolla, California, U.S.A.) and dissolved in 20 µl distilled, deionized H$_2$O$_2$ and then directly cloned into a T-tailed M13mp18 derivative (W. Burke, unpublished results). At least two clones were sequenced from each arthropod species (strain) for the complete region between the general flsZ primers (1045–1055 b.p.) or the A and B specific primers (955–957 b.p.). All inconsistencies between clones (which occurred in less than 0.42% of base positions) were resolved by comparison with the subgroup consensus sequence. Two regions of sequence ambiguity (because of secondary structure) were resolved by additional sequencing reactions using diTTP for dGTP in labelling and termination mixtures (Sequenase Version 2.0 kit, U.S. Biochemical).

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

(d) Phylogenetic analysis

The flsZ sequences were manually aligned to previously determined Wolbachia flsZ sequences. For phylogenetic analysis, a 970 b.p. region (including deletion–insertions) between the A and B subdivision specific primers was used, because this region is shared among all sequences available. A phylogenetic analysis was performed for 39 Wolbachia strains using the neighbour-joining program of Tamura (NJBOOT2 v 1.10). Genetic distance was calculated in several different ways, p-distance (proportion different) including deletions–insertions, p-distance excluding deletions–insertions, and Jukes & Cantor (1969) distance (excluding deletions–insertions). Synonymous substitution rates were determined by the mega program (v 1.01, Kumar et al. 1993), which employed the algorithm of Nei & Gojobori (1986). Sequences are available in Genbank under accession numbers U28175–U28211.

Figure 1. Phylogenetic tree of Wolbachia based upon sequences of the ftsZ gene. Name of the host arthropod species is followed by the strain designation. Parthenogenesis associated bacteria are shown in bold. The tree was generated by neighbour-joining using the p-distance including insertions–deletions. Numbers 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.

TriboIium confusum. The Adm 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).

Between 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 3 b.p. and the other 9 b.p. All B group bacteria have both deletions, except Tribolium confusum and Gryllus pennsylvanicus bacteria, 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 B bacteria before the loss of the region in the lineage leading to other B 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

gave similar phylogenies, except for the positions of B group Tribolium confusum and Gryllus pennsylvanicus bacteria. As stated above, these bacteria share an insertion absent in other B group bacteria, with a unique single base substitution. Use of p-distance with bacteria. As stated above, these bacteria share an information (by p-distance without gaps and Jukes-Cantor distance) fails to place these strains within the same clade. Based upon the shared insertion, we estimated divergence times between A and B group bacteria. 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 ftsZ shows the expected level of divergence between these taxa, we used the E. coli ftsZ sequence to design primers and then amplified and cloned the ftsZ region from Salmonella typhimurium. Based on the first 355 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 58.5% (adjusted to 113.4%). This is typical for protein coding regions between these taxa (Ochman

(b) Estimated 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 per cent synonymous substitutions between two representatives of A and B group bacteria (D. simulans and C. pipiens) is 33.4%, and correcting for several hits by the formula of Jukes & Cantor (1969) 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 ftsZ shows the expected level of divergence between these taxa, we used the E. coli ftsZ sequence to design primers and then amplified and cloned the ftsZ region from Salmonella typhimurium. Based on the first 355 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 58.5% (adjusted to 113.4%). This is typical for protein coding regions between these taxa (Ochman

& Wilson 1987), and gives the expected 142–162 Ma divergence. Results indicate that in these bacteria \( ftsZ \) does not show unusual rates of synonymous substitution. The adjusted per cent divergence between A and B \( Wolbachia \) over this same gene region is 44.3\%, similar to the entire gene (46.6\%).

(c) Evidence for horizontal (intertaxon) transmission

The phylogenetic evidence strongly supports horizontal transmission of \( Wolbachia \) among arthropods (see figure 1). Even a cursory examination shows that the \( Wolbachia \) \( ftsZ \) 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 insect 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 \( Cossonus sp \) and lepidopteran \( Esphelista cautella \) by only one base pair despite the fact that these hosts diverged from each other at least 200 Ma before present (Hennig 1981).

Detection of Adm bacteria in diverse host species by PCR is highly repeatable, using different strains and DNA extractions. In addition, southern hybridizations 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 PCR artefact.

Evidence supports a relatively recent range expansion of Adm bacteria into new insect species. Given approximately 265 synonymous substitution sites over the 937 b.p. region, and a \( 7 \times 10^{-8} \) per year divergence rate, the 95\% confidence limits for divergence time among Adm \( Wolbachia \) with identical \( ftsZ \) sequences (e.g. the hymenopteran \( Asobara tabida \) and the dipteran \( Drosophila simulans \) (R)) are 0–1.6 Ma, and 99\% confidence limits are 0–2.5 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 \( Trichogramma \) species cluster as a single phylogenetic unit, as do \( Wolbachia \) strains found in the two \( Leptopilina \) species and two \( Nasonia \) species (\( giuraulti \) and \( longicornis \)).

Nevertheless, intertaxon 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 \( Armadillidium vulgare \), than to the mosquito \( Aedes albopictus \). The closest relative of \( B \) \( Wolbachia \) found in the beetle (order Coleoptera) \( Tribolium confusum \) occurs in the cricket \( Gryllus pennsylvanicus \). The \( Tribolium Wolbachia \) is associated with cytoplasmic incompatibility (Wade & Stevens 1985); etiology of the cricket bacteria is unknown.

(d) Parasitoid–host exchange

We propose that transmission between parasitic insects and their hosts is one vehicle for intertaxon 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 current data set. \( N. giraulti \) and \( N. longicornis \) are parasitoid waps that specialize in attacking protocalliphorid flies living in birdnests (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 fly species in a variety of habitats. Both \( N. longicornis \) and \( N. giraulti \) harbour B group \( Wolbachia \) that are closely related to those of their preferred Protocalliphorid fly hosts.

The probability \( p \) that \( Protocalliphora \) occurs in the same clade with its parasitoids (\( N. longicornis \) and \( N. giraulti \)) is \( p = (x-1)^x \), where \( x \) is the number of bacterial taxonomic units in the phylogeny. A conservative approach is to consider only B group strains and to treat the two \( Leptopilina \) strains and the three \( Trichogramma \) 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 \( (p = 0.071) \), 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 drosophilid larval parasitoid \( Asobara tabida \). These two species share nearly identical \( Wolbachia \) (one difference over 962 b.p.). However, because the Adm group bacteria appear to have undergone considerable recent horizontal transmission, this association has little statistical resolving power.

(e) Distribution of parthenogenesis & \( \alpha \) \( Wolbachia \)

Parthenogenesis \( Wolbachia \) are found in both the A and B groups and are typically flanked by \( \alpha \) inducing \( Wolbachia \) from sexual species. Based upon the current phylogeny, it can be postulated that parthenogenesis has evolved several times independently, in \( Leptopilina \), \( Trichogramma \), \( Encarsia \), \( Muscidifurax \) and \( Aphytis \). It therefore appears that parthenogenesis induction can evolve relatively easily in the \( Wolbachia \), suggesting a simple biochemical mechanism. An alternative interpretation is that parthenogenesis has evolved by exchange of the genetic machinery for parthenogenesis via recombination between \( Wolbachia \) strains. However, concordance between 16S rDNA and \( ftsZ \) (see §3f) suggests that recombination between A and B division bacteria is not common, although recombination within groups could be more frequent. A second, alternative interpretation of the pattern is that parthenogenesis induction is not the result of evolutionary changes in the \( Wolbachia \), but to differences in hosts.

The \( ftsZ \) amplification and sequencing results shown here establish that \( Wolbachia \) are present in the parthenogenetic Hymenoptera \( Leptopilina australis \) and
Table 1. Shown are results of PCR amplifications using Adm and B specific primers for ftsZ and 16S rDNA

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| (To verify specific amplification, the ftsZ PCR 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 16S rDNA patterns. Table 1 shows results of the PCR assay. A general concordance between the A and B subgrouping of the 29 different arthropod species was found. However, in bacterial species with frequent recombination, gene phylogenies do not necessarily parallel bacterial strain (or species) phylogenies. The hypothesis that ftsZ gene phylogeny accurately reflects Wolbachia strain phylogeny can be investigated by determining whether there is concordance between phylogenies of ftsZ 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 subdivision. We investigated general concordance between ftsZ and 16S rDNA by a PCR-based assay, using 29 different arthropod species—strains. Specific primers were designed for the Adm and B groups of Wolbachia, for both the ftsZ and 16S rDNA genes. It should be noted that the A group primers of ftsZ were designed specifically to amplify the Adm group and are not effective in amplification of Acil ftsZ sequences. Primer specificity was determined by restriction digestion of PCR product with enzymes that reveal restriction site polymorphisms between A and B. To verify specific amplification, the ftsZ primer Acil for 16S rDNA, see §2. Table 1 shows results of the PCR assay. A general concordance between ftsZ and 16S rDNA is clear from the analysis. Ten species (two strains of D. simulans) contain Adm group ftsZ and Adm group 16S rDNA, based both upon primer specificity and restriction digestion. Nine species contain B group ftsZ and B group 16S rDNA. Seven of these clearly show the expected B-specific restriction profile of the ftsZ product. Two contain additional—restriction sites that make interpretation of the profile tentative. Six species contain both Adm and B ftsZ, 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. Protocalliphora 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. Stiophillus oryzae, the rice weevil, contains a B group ftsZ, 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 Armadillidium vulgare amplifies an Adm group 16S rDNA, but a B group ftsZ, based both on primer specificity and restriction profiles. The Wolbachia found in Armadillidium vulgare is unusual in several respects. First, this Wolbachia occurs in reproductive tissues of Nerus, Anastretha and Protocalliphora flies, Mellitobia wasps, Cossonus beetles, and Gryllus pennsylvanicus crickets. The etiologies of these Wolbachia are unknown.}

L. clavipes; antibiotic curing indicates that parthenogenesis is bacterially induced in these species (J. van Alphen, personal communication). Wolbachia were also detected in reproductive tissues of Nerus, Anastretha and Protocalliphora flies, Mellitobia wasps, Cossonus beetles, and Gryllus pennsylvanicus crickets. The etiologies of these Wolbachia are unknown.

(f) Congruence of ftsZ and 16S rDNA phylogeny

The ftsZ phylogeny presented in figure 1 could reflect the actual phylogeny of the Wolbachia strains present in the host arthropods. However, in bacterial species with frequent recombination, gene phylogenies do not necessarily parallel bacterial strain (or species) phylogenies. The hypothesis that ftsZ gene phylogeny accurately reflects Wolbachia strain phylogeny can be investigated by determining whether there is concordance between phylogenies of ftsZ 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 subdivision.

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Template from the isopod Armadillidium vulgare amplifies an Adm group 16S rDNA, but a B group ftsZ, based both on primer specificity and restriction profiles. The Wolbachia found in Armadillidium vulgare is unusual in several respects. First, this Wolbachia occurs
within an isopod and is one of the few strains found outside of insects. Secondly, this bacterium is unique among known Wolbachia, because it is implicated as the cause of feminization of genetic males in its host (Rousset et al. 1992; Rigaud et al. 1991), rather than causing cytoplasmic incompatibility or parthenogenesis as do all other known Wolbachia (Stouthamer et al. 1993). To investigate this case further, we PCR amplified 16S rDNA from Armadillidium DNA using general 16S rDNA prokaryotic primers (Weisburg et al. 1991, Breuer et al. 1992) and cloned the product into mp18 vector (W. Burke, unpublished results). Sequence was determined for a region that contains the reverse group specific primer domain. Results show this region to be A-like, and suggest that the Armadillidium Wolbachia may be the product of recombination between A and B group Wolbachia.

(g) Double infections with different Wolbachia

Sequencing of several ftsZ clones from PCR 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 Ate group ftsZ sequences. There were two basic interpretations of this pattern: (i) individual Wolbachia strains have two divergent copies of ftsZ (i.e., ftsZ is a two gene family); or (ii) there is a single ftsZ gene, but some insects harbour infections with several Wolbachia strains.

Evidence strongly supports the hypothesis that some insects harbour 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 is precisely the pattern observed; of 29 species tested (plus two strains of 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 N. vitripennis 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 PCR 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 Ate group; however, by sequencing data and use of Ate specific primers, we have found that Tribolium confusum harbours double infections of these two strains.

4. DISCUSSION

Wolbachia are a widespread group of cytoplasmically inherited 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 upon the PCR assay. Wolbachia have been found in most of the major insect orders (including Hymenoptera, Diptera, Coleoptera, Lepidoptera, Orthoptera, Homoptera and Hemiptera) and in isopods. 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 Aedes albopictus were transferred into Drosophila simulans, where the bacterium succeeded in causing cytoplasmic incompatibility in its new host (Braig et al. 1994). Our work indicates that Aedes 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 (for instance, the A and B groups diverged between 58 to 66 Ma BP), it is likely that many of these will eventually be designated as separate species. Therefore, prematurely assigning the species designation Wolbachia pipiens (the species name given to the B group bacterial 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 lipidopterans, 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 0–2.5 Ma (99% confidence limits), whereas the different orders of insects they are found in diverged at least 200 Ma BP (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 activities. Many of the species in which this strain is found have themselves undergone range expansions because of human disturbance or transport. Disruptions 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 mechanisms of horizontal transmission by Wolbachia are of special interest. Our phylogenetic data show that transmission between parasitic insects and
their hosts may have occurred between *Protocalliphora* flies and *Nasonia* 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. *Nasonia* wasps typically sting and kill their host pupae before laying eggs upon the host, therefore making it difficult for them to transmit *Wolbachia* 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 *Wolbachia* could occur via predators as well, as has been suggested for predatory mites and P-elements (Houck et al. 1991).

*Wolbachia* may promote rapid speciation by causing reproductive incompatibility between populations (Breeuwer & Werren 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. simulans* and *Culex pipiens*, and between sibling species of *Nasonia*. *D. simulans* (Hawaii) and *D. simulans* (Riverside) are two bidirectionally incompatible strains represented in this study (O’Neill & Karr 1990). These two strains show nearly identical ftsZ sequences (one difference over 962 b.p.), indicating that bidirectional incompatibility can evolve relatively rapidly in *Wolbachia*.

The three species of *Nasonia* have similar bacteria, although the picture is complicated by presence of double infections within individuals of these species. Data shown here suggest that *N. vitripennis* may have acquired its B division bacteria from a different source to the two more closely related species *N. giraulti* and *N. longicornis*. 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 *Wolbachia* appear to be relatively common, and could play a role in creating new compatibility types. Of the insects we tested 23% harboured double bacterial infections. Roussol 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 *Wolbachia* strains may favour maintenance of double infections. We have preliminary evidence that double infections in *Nasonia* 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 ftsZ and parthenogenetic *Wolbachia* suggests that parthenogenesis induction has evolved several times independently. However, an alternative interpretation is that parthenogenesis induction is not the result of differences in *Wolbachia*, but of differences in host environment. Although formally possible, we consider it unlikely that a trait such as parthenogenesis, that clearly can be adaptive for the bacteria, is merely the byproduct of host environment.

A second possibility is that the genetic machinery for parthenogenesis has been introduced into *ci* bacteria by genetic exchange. Although our data (showing concordance of 16S rDNA and ftsZ) 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 16S rDNA and ftsZ 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-*Wolbachia*, 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-*Wolbachia* 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 ftsZ gene will be a useful tool, especially when coupled with sequence information from other protein coding regions.

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