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'Neill *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'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*, *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 subtillus*, 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_2O, 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 know 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 *ftsZ* DNA was amplified using PCR in a volume of 25 μl (1 μl DNA sample, 2.5 μl 10 × 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_2O 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 °C, 1 min at 55 °C, 3 min at 72 °C) 35 cycles (15 s at °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) (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 ftsZ* sequence, ftsZf1 (5′GTT GTC GCA AAT ACC GAT GC 3′) and ftsZr1 (5′CTT AAG TAA GCT GGT ATA TC 3′), which yield a 1043–1055 b.p. product (depending upon *Wolbachia* strain). Results have shown that these primers are specific for *Wolbachia*. They amplify the *ftsZ* gene from all tested arthropods known to harbour *Wolbachia* infections, and fail to amplify the product from gamma group 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 *Erhlichia canis* (Stouthamer *et al.* 1993).

Primers were also designed for specific amplification of Adm and B group *ftsZ* and 16S rDNA regions. The *ftsZ* primers ftsZAdf (5′CTC AAG CAC TAG AAA AGT CG 3′), ftsZAdr (5′TTA GCT CCT TCG CTT ACC TG 3′), ftsZBf (5′CCG ATG CTC AAG CGT TAG AG 3′) and ftsZbr (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 GTT TCA CAC AG 3′), 16SAr (5′TAA GGG ATT AGC TTA GCC TC 3′), 16SBf (5′TTC GGC CGG ATT TTA CAC AA 3′), and 16SBr (5′TAG GGA TTA GCT TAG GCT TG 3′) amplify a 259 b.p. region of the 3-prime portion of the 16S rDNA gene.

To confirm specificity of the primers an *ftsZ* region was chosen in which B group *Wolbachia* typically have one AciI restriction site, producing fragments of 243 b.p. and 752 b.p., whereas Adm *Wolbachia* typically have two AciI restriction sites, producing fragments of size 178 b.p., 462 b.p. and 297 b.p. Similarly, the 16S region contains a RsaI site absent in the A group but present in the B group, creating fragments of 117 b.p. and 142 b.p. After PCR, 20 μl of amplified reaction product was restriction digested and electrophoresed 3–4% Nusieve (3:1) agarose gel to examine for the appropriate restriction sites that distinguish A and B division bacteria.

(*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 (Bio 101, Inc., La Jolla, California, U.S.A.) and dissolved in 20 μl distilled, deionized H_2O, 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 *ftsZ* primers (1043–1055 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 dITP 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*, *ftsZ* representatives of both types were cloned and sequenced, from products amplified using the A and B specific primers.

(*d*) *Phylogenetic analysis*

The *ftsZ* sequences were manually aligned to previously determined *Wolbachia ftsZ* 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 Genebank under accession numbers U28175–U28211.

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 *ftsZ* gene (see figure 1). As is apparent in the figure, there are two major divisions of *Wolbachia*, designated A and B. The average *ftsZ* 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

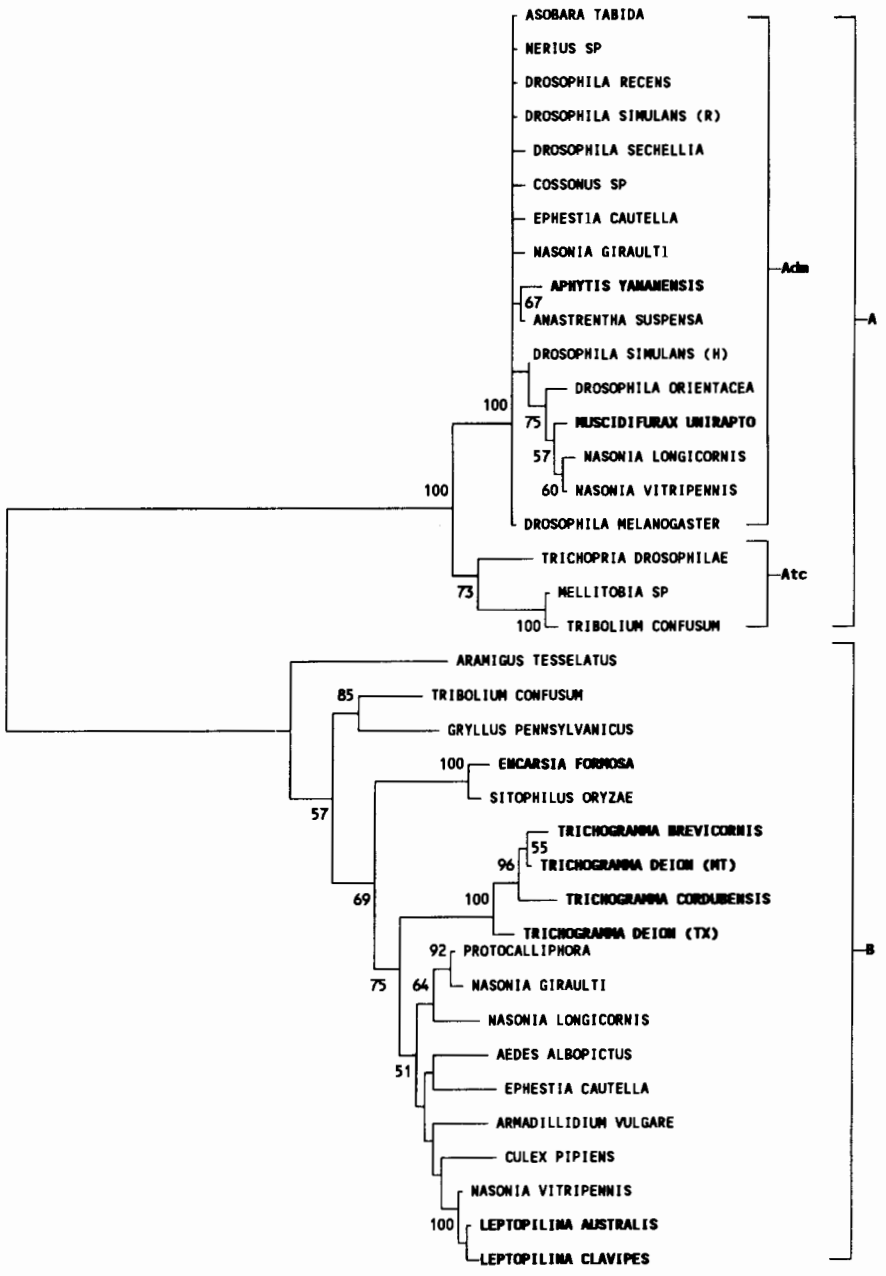

Figure 1. Phylogenetic tree of *Wolbachia* based upon sequences of the *ftsZ* gene. Name of the host arthropod species is followed by the strain designation. Parthenogenesis associated bacteria are shown in bold. The tree was generated by neighbour-joining using the p-distance including insertion–deletions.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

Tribolium 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 §3*c*).

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 9 b.p. and the other 15 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

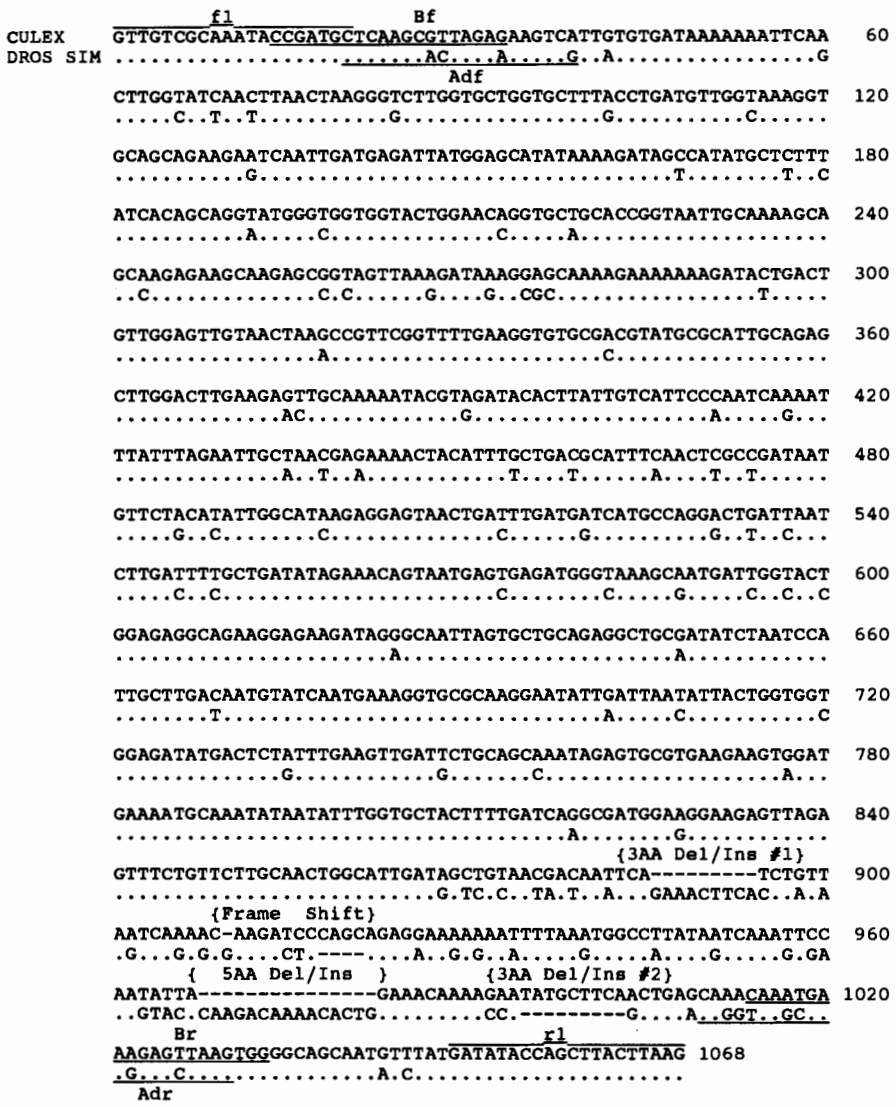

Figure 2. Nucleotide are shown for the *ftsZ* gene from a representative member of the B group (*Culex pipiens* host) and A group (*Drosophila melanogaster* host) bacteria. Primer locations are underlined (or overlined). The actual ftsZBr primer differs from the *Culex Wolbachia* sequence shown by one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

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 gaps places these strains in the same clade (85 out of 100 boot-strap replicates). However, exclusion of insert 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 believe that these two bacterial strains should be placed in the same clade. The phylogeny presented in figure 1 is therefore based upon p-distance including insertions–deletions.

(*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 anthropods (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 *Esphestia cautella* by only one base pair despite the fact that these hosts diverged from each other at least 200 Ma before present (BP) (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 7×10^{-9} 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 (girualti 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. *Nasonia giraulti* and *N. longicornis* are parasitoid wasps 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 $p = (x-1)^{-1}$, 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 & CI* Wolbachia

Parthenogenesis *Wolbachia* are found in both the A and B groups and are typically flanked by CI 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 §3*f*) 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*

(To verify specific amplification, the *ftsZ* PCR product was restriction digested with AciI 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 bacteria between 16S rDNA and *ftsZ*. See text for further discussion.)

	Adm primers for		B primers for		
species (strain)	16S	*fstZ*	16S	*ftsZ*	type
single infections					
Drosophila melangaster (BH6)	A	A	—	—	I
D. simulans (Riverside)	A	A	—	—	I
D. simulans (Hawaii)	A	A	—	—	I
D. recens	A	A	—	—	I
D. orientacea	A	A	—	—	?
Anastretha suspensa	A	A	—	—	?
Trypolyon sp.	A	A	—	—	?
Muscidifurax uniraptor	A	A	—	—	P
Aphytis yananensis	A	A	—	—	P
Asobara tabida	A	A	—	—	I
D. seychellia	A	A	—	—	?
Leptopilina australis	—	—	B	B	P
Leptopilina clavipes	—	—	B	B	I
Trichogramma deion (Mnt)	—	—	B	B[a]	P
Trichogramma deion (Tx)	—	—	B	B[a]	P
Encarsia formosa	—	—	B	B	P
Bangasternus orientalis	—	—	B	B	?
Culex pipiens	—	—	B	B	I
Gryllus pennsylvanicus	—	—	B	[a]	?
Tribolium confusum	—	—	B	[a]	I
double infections					
Spalangia fuscipes	A	A	B	B	P
Ephestia cautella	A	A	B	B	I
Aedes albopictus	A	A	B	B	I
Nasonia vitripennis (LbII)	A	A	B	B	I
N. longicornis (IV7)	A	A	B	B	I
N. girualti (RV2)	A	A	B	B	I
special cases					
Armadillidium vulgare (F)	A	—	—	B[a]	F
Protocalliphora sp.	—	—	A	B	?
Sitophilus oryzae	A	—	A	B	?

[a] Distinctive digestion pattern.

L. clavipes; antibiotic curing indicates that parthenogenesis is bacterially induced in these species (J. van Alphen, personal communication). In addition, we have detected *Wolbachia* in the parthenogenetic weevil *Naupactus tesselatus*, although antibiotic treatments of this species have 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 *Trichropria drosophilae*. These bacteria appear to cause CI, based on curing experiments (J. van Alphen, personal communication). *Wolbachia* were also detected in reproductive tissues of *Nerius*, *Anastrentha* 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) phylogeny. 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 Atc *ftsZ* sequences. Primer specificity was determined by restriction digestion of PCR product with enzymes that reveal restriction site polymorphisms between A and B (AciI for *ftsZ*, RsaI 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 addition-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. *Sitophyllus 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

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, Breeuwer *et al.* 1992) and cloned the product into mp 18^{T2} 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 Atc 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 Atc group; however, by sequencing data and use of Atc 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 pipientis* (the species name given to the B group bacterial found in the mosquito *Culex pipientis*) 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 rapid 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. Rousset 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 CI 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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