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# *Wolbachia* in two insect host–parasitoid communities

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**Abstract**

***Wolbachia* form a group of intracellular bacteria that alter reproduction in their arthropod hosts. Two major phylogenetic subdivisions (A and B) of *Wolbachia* occur. Using a polymerase chain reaction assay we surveyed for the A and B group *Wolbachia* in 82 insect species from two temperate host–parasitoid communities (food webs) and a general collection of Lepidoptera caught at a light trap. One host–parasitoid community was based around leaf-mining Lepidoptera, and the other around Aphids. We found that: (i) 22.0% of insects sampled were infected with *Wolbachia*; and (ii) the prevalence and type (A or B) of *Wolbachia* infection differed significantly between communities and taxonomic groups. We obtained DNA sequences from the *ftsZ* gene for the group B *Wolbachia* found in six leaf-mining species and one of their parasitoids, as well as four of the Lepidoptera caught by a light trap. Taken together, the results of our survey and phylogenetic analyses of the sequence data suggest that host–parasitoid transfer of *Wolbachia* is not the major route through which the species we have examined become infected. In addition, the *Wolbachia* strains observed in five leaf-mining species from the same genus were not closely related, indicating that transfer between species has not occurred due to a shared feeding niche or cospeciation.**

*Keywords*: bacteria, cytoplasmic incompatability, food web, *ftsZ*, parasitoid, *Wolbachia*

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## Introduction

*Wolbachia* form a group of intracellular bacteria that infect the reproductive tissues of arthropods. They are inherited cytoplasmically and have been shown to alter reproduction in their arthropod hosts in a number of ways (Werren 1997). These include causing post-zygotic reproductive incompatibility in a wide range of insects (termed cytoplasmic incompatibility or CI), parthenogenesis in parasitic wasps, and feminization of genetic males in isopods (Barr 1980; Breeuwer & Werren 1990; O'Neil *et al*. 1992; Rousset *et al*. 1992; Stouthamer *et al*. 1993). Consequently, it has been argued that the presence of *Wolbachia* may have several important consequences, such as providing a mechanism for rapid speciation, influencing the evolution of sex-determining systems, and promoting the evolution of eusociality in the haplodiploid Hymenoptera (Breeuwer & Werren 1990; Coyne 1992; Rigaud & Juchault 1993; Turelli 1994; Hurst 1997). Clearly, the importance of *Wolbachia* in all these processes depends ultimately on its prevalence, and how it is transmitted between species.

Molecular phylogenies constructed with *Wolbachia* isolated from different arthropods show very little congruence with the phylogenies of the hosts, suggesting frequent horizontal transfer between species (O'Neil *et al*. 1992; Rousset *et al*. 1992; Stouthamer *et al*. 1993; Werren *et al*. 1995a). The mechanism by which this horizontal transfer has occurred is unknown, but two methods have been suggested. One possibility is that predation or contact after injury may play a role. Rigaud & Juchault (1995) have shown that isopods can become infected with *Wolbachia* by blood contact after injury. Transmission has been demonstrated only between conspecifics or members of closely related species, but transfer between more distantly related individuals might occur at low frequency. In an analogous manner, *P* element (transposon) DNA has been detected in predatory mites fed on *Drosophila* larvae and this has led to the suggestion that predators may be a vector for transposons (Houck *et al*.

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1991). However, the role of predators in the horizontal transfer of *Wolbachia* (or transposons) has yet to be demonstrated in nature.

The second suggestion is that parasitoids may be the agents of horizontal transfer (Werren *et al*. 1995a). Parasitoids are insects whose larvae develop by feeding on the body of a host, normally another insect (Godfray 1994). Most parasitoids attack a set of taxonomically or ecologically related hosts. In many species the parasitoid larvae develop within their host (endoparasitoids) and their eggs are injected into the host by the female using a specialized ovipositor. This intimate association of developing parasitoids with host tissues could facilitate transmission from the host to the parasitoid. Although parasitoid attack is normally fatal, hosts can sometimes mount an immune defence against the parasitoid egg or larva and survive. It is therefore also possible that parasitoids might transmit *Wolbachia* to their host species. Evidence suggestive of a role for parasitoids was provided by the finding that *Wolbachia* isolated from a fly (*Proticalliphora*) and its parasitoid wasp (*Nasonia giraulti*) were phylogenetically closely related with near identical sequences (Werren *et al*. 1995a).

In this study we surveyed for *Wolbachia* in two temperate host–parasitoid communities. Hosts in each community are linked by a known web of common parasitoid wasps and were sampled from a single locality. One community consisted of leaf-mining Lepidoptera and their parasitoids, the other of aphids, their parasitoids and obligate hyperparasitoids (species that develop as parasitoids of other parasitoids). Our first aim was to determine the prevalence of *Wolbachia* in these communities. To date, the most extensive systematic survey of *Wolbachia* prevalence has been that carried out by Werren *et al*. (1995b), who found that *Wolbachia* occurred in 17% of Panamanian neotropical arthropod species. Our second aim was to use data collected by both Werren *et al*. (1995b) and ourselves to test for differences in the distribution of *Wolbachia* between taxonomic groups.

Our third aim was to examine whether parasitoids are vectors in the horizontal transfer of *Wolbachia*. If this is the case then we would expect to find closely related bacteria in hosts and their parasitoids, and in hosts or parasitoids that share a common parasitoid or host. Although such an observation would not prove that parasitoids are vectors (both host and parasitoid occupy similar microhabitats and therefore may be cosusceptible to infection from other sources), it would provide a strong impetus for experimental study of parasitoid transmission. *Wolbachia* isolates can be divided into two groups, A and B, using sequence data (Breeuwer *et al*. 1992; Werren *et al*. 1995a). At present, only the phylogeny of the B group is resolved, and so we specifically looked for group B *Wolbachia* from hosts and parasitoids that were more closely related than expected by chance.

The leaf-mining species that we examined all have extremely similar natural histories. Consequently, our fourth aim was to test for the possibility that species with similar feeding niches will encounter similar *Wolbachia* strains regardless of how they are horizontally transmitted. Sequences of *Wolbachia* from the Lepidoptera are lacking; therefore we also surveyed a small collection of Lepidoptera caught as adults in a UV moth trap.

## Materials and methods

### *Host–parasitoid communities*

*Leaf-miners*. We surveyed 21 species of leaf-mining moths (Lepidoptera: Gracillariidae) and 18 species of their parasitoids that were reared from leaf mines collected in autumn 1995 at Silwood Park, southern UK. All but one host were in the genus *Phyllonorycter* (the exception being a species of *Parornix*). The *Phyllonorycter* we sampled all form tent-shaped mines in the leaves of deciduous trees except for two species that mine *Lonicera*, a vine. *Parornix* mine in their early instars and then inhabit a folded leaf. The parasitoids we reared were all wasps (Hymenoptera), the majority belonging to the family Eulophidae but with two species of Braconidae. About 60% of the parasitoids are *Phyllonorycter* specialists, the remainder also attack other species of leaf-miner. We reared six species of the eulophid genus *Achrysocharoides*, each of which is restricted to a single genus of host plant. A quantitative food web describing this community may be provided by A. Rott & H. C. J. Godfray (unpublished). In addition, Askew & Shaw (1974) and Shaw & Askew (1976) have described *Phyllonorycter* parasitoid communities from northern UK. Hosts were identified using Emmet *et al*. (1985) and parasitoids using Bryan (1980; *Achrysocharoides*), Hanson (1985; *Chrysocharis*), Graham (1959; *Pediobius*), Askew (1968; other Eulophidae) and Shaw & Askew (1976; Braconidae).

*Aphids*. We surveyed six species of aphid, 20 species of primary parasitoid and 7 species of hyperparasitoid, from insects collected in summer 1995 at Silwood Park. The aphids all fed on different host plants. With a single exception (an aphelinid), the primary parasitoids were all Aphidiinae (Braconidae). The hyperparasitoids consisted of Charipidae (five), Megaspilidae (one) and Pteromalidae (two). We were unable to sample all the hosts of the parasitoids we reared. A quantitative food web describing this community may be provided by Muller *et al*. (in press). Aphids were identified using Heie (1980–1985); primary parasitoids by Dr R. Belshaw (Aphidiinae), using Graham (1976; Aphelinidae), and hyperparasitoids using Ferguson (1980; Megaspilidae), and Graham (1969; Pteromalidae). Many Charipidae and

some *Aphidius* require further systematic research and these species are given codes (see Muller *et al*. (in review) for further details).

*General Lepidoptera.* We collected Lepidoptera attracted to a UV moth trap at Silwood Park in autumn 1995. Thirteen species from five families were surveyed for *Wolbachia.*

### *Storage of insects*

Female insects were placed in 100% ethanol and stored with refrigeration. Aphids may be parasitized as adults, and to avoid this possible source of contamination only second-generation individuals, the progeny of wild-caught individuals that had been kept in the laboratory, were tested.

### *DNA extraction*

We dissected the abdomen from each individual insect in sterile distilled deionized water on a sterile Petri dish and then serially rinsed it in droplets of a 5% chlorox solution and sterile water. We then extracted DNA by grinding the abdomens in a 50 μL volume of extraction solution (5% Chelex (Bio-Rad), 0.01% proteinase K, vortexing for 10 s, incubating at 56 °C for 35min, vortexing for 10 s, incubating at 96 °C for 15 min, vortexing for 10 s, and centrifuging for 3 min at 13 000 r.p.m. On all occasions we prepared contemporaneously positive and negative control DNA samples from the abdomens of known infected and uninfected strains of *Nasonia vitripennis.*

### *Assay for* Wolbachia

We tested for the presence of *Wolbachia* by carrying out PCR with *Wolbachia*-specific primers for the *ftsZ* bacterial cell-cycle gene (Werren *et al*. 1995a). Species yielding a product of the expected size were provisionally scored as positive for *Wolbachia*, while species that failed to amplify were provisionally scored as negative. We then retested the species scored as positive using primers specific for the A and B subdivisions of *Wolbachia.* With all sets of PCR reactions we performed positive controls using template from a *Wolbachia*-infected strain of *N. vitripennis.*

Failure of amplification with the general *ftsZ* primers could be due to either: (i) absence of *Wolbachia* in the insect; (ii) failure in the DNA extraction procedure; or (iii) incorrect concentration of DNA solution (Werren *et al*. 1995b). In order to control for the last two possibilities, we tested the samples scored as negative with primers for highly conserved regions of 28S rDNA (Burke *et al*. 1993; Werren *et al*. 1995b). Samples yielding a product of the expected size were then considered to be true negatives for the *Wolbachia* assay. We retested samples which did not yield a 28S rDNA product at a range of dilutions from 1/100 to five times the initial template concentration. We then used the maximum concentration yielding a 28S rDNA positive to retest for *Wolbachia* with the general *ftsZ* primers. Samples that failed to yield a positive by the 28S rDNA primers for all concentrations were not included in the data set.

### *PCR methods*

We amplified the nearly complete *Wolbachia ftsZ* sequence using the *Wolbachia*-specific primers *ftsZ* f1 and *ftsZ* r1, which yield a 1043–1055 bp product (Werren *et al*. 1995a). PCR was carried out in 25 μL volumes containing 1 μL of DNA extract, 0.25 μL of *Taq* (Boehringer), 2.5 μL of 10× PCR buffer (Boehringer), 0.35 μL of 20 μM of each primer, and 2 μL of 2.5 μM dNTPs. The PCR reaction mix was prepared in one batch and then added to each sample, with the remainder run as a control for contamination. PCR cycling conditions were two cycles (2 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C), 35 cycles (30 s at 94 °C, 1 min at 55 °C, 3 min at 72 °C) and one cycle (30 s at 94 °C, 1 min at 55 °C, 10 min at 72 °C) (Perkin-Elmer DNA Thermal Cycler 480). After PCR, we ran 8 μL of the reaction product on a 1.3% agarose gel to determine presence and size of amplified DNA.

We carried out controls for PCR amplifiability of DNA solutions using general eukaryotic 28S rDNA primers, 28sf and 28sr, which yield a product of 500–600 bp (Werren *et al*. 1995b). Reaction volumes were as above and cycling conditions were as in Werren *et al*. (1995b).

We used both *ftsZ* and 16S rDNA primers which specifically amplify from A and B type *Wolbachia* (Werren *et al*. 1995a). The A (*ftsZ* Adf and *ftsZ* Adr) and B (*ftsZ* Bf and *ftsZ* Br) group *ftsZ* primers specifically amplify a 955–957 bp region of *ftsZ* from A and B group *Wolbachia*, respectively. The A (16SAf and 16SAr) and B (16SBf and 16SBr) group 16S rDNA primers specifically amplify a 259 bp region of the 16S rDNA gene from A and B group *Wolbachia*, respectively. Reaction volumes were as above. Cycle conditions for the 16S rDNA primers were two cycles (2 min at 95 °C, 1 min at 64 °C, 2 min at 72 °C), 35 cycles (30 s at 95 °C, 1 min at 64 °C, 1 min at 72 °C) and one cycle (5 min at 72 °C). In all species examined the results of the *ftsZ* and 16S rDNA primers were in complete agreement.

### *Cloning and sequencing*

For cloning purposes, we used a 50 μL PCR reaction with the *ftsZ* B primers (doubling of the solutions above). These were then cloned using the Invitrogen TA cloning kit following the manufacturer's instructions and plasmid DNA was purified using the Wizard Minipreps kit (Promega Ltd). Sequencing was carried out on an Applied Biosystems 373 stretch automated sequencer, using the manufacturer's

*Taq* FS dye terminator sequencing kits. Each isolate was fully sequenced in both directions and the sequences assembled using MacVector and AssemblyLign (Kodak Ltd).

### *Sequence alignment and phylogenetic analysis*

Our sequences were aligned by eye with those used in a previous study (Werren *et al*. 1995a). Alignment was straightforward as *ftsZ* is generally highly conserved in length and sequence. The aligned region contains four unambiguous indels and one frame-shift event. We coded each of these as a separate character to be used in addition to the individual nucleotide characters.

We investigated the phylogenetic relationships of the new type B *Wolbachia* by building trees that included the type B *Wolbachia* from the previous study (Werren *et al*. 1995a). The trees were rooted by including two type A *Wolbachia* from the same previous study. All analyses were performed using test versions 4.0d61–63 of PAUP*, written by David L. Swofford.

Phylogenies were generated using both maximum parsimony (MP) and distance (neighbour-joining (NJ); Saitou & Nei 1987) approaches. In the MP analyses we treated gaps as missing data (but included indels as extra characters as described above) and conducted heuristic searches with 100 random additions and TBR branch swapping. Support for nodes in strict consensus trees was assessed by bootstrapping (100 replicates of 10 random additions). NJ trees were generated using several different distance measures: uncorrected *P*-distances including and excluding gaps, and Jukes-Cantor distances, which are corrected for multiple hits. NJ trees were assessed using 500 bootstrap replicates.

## Results

### *Prevalence of* Wolbachia

We screened 83 different insect species for *Wolbachia*. Of these, 18 (21.7%) were infected. In Table 1 we present the frequency of infections of the different groups in the two food webs, and in Tables 2, 3, and 4 we list the species tested for *Wolbachia*. Three species were excluded from our results because they failed to yield a positive control with the 28S rDNA primers at any of the tested concentrations. These species were two aphids

**Table 1** Distribution of *Wolbachia* in insects surveyed. Shown are the distributions of *Wolbachia* infections detected using PCR assay, the number of species sampled, and the percentage in which *Wolbachia* were detected

| Group | A | B | AB | Total | % infected |
|---|---|---|---|---|---|
| Leaf-miners | 2 | 6 | 0 | 21 | 38.1 |
| Leaf-miner parasitoids | 4 | 0 | 1 | 18 | 27.8 |
| Aphids | 0 | 0 | 0 | 4 | 0 |
| Aphid parasitoids | 0 | 0 | 0 | 19 | 0 |
| Aphid hyperparasitoids | 1 | 0 | 0 | 8 | 12.5 |
| General Lepidoptera | 0 | 4 | 0 | 13 | 30.8 |
| Total | 7 | 10 | 1 | 83 | 21.7 |

**Table 2** The survey of leaf-miners and their parasitoids. The presence of A, B or double (AB) infections was based upon PCR using group-specific primers. If multiple individuals were tested for a species, the number tested is indicated in parentheses

| Leaf-miner / Parasitoid | Host plant | *Wolbachia* |
|---|---|---|
| Leaf-miners | | |
| *Parornix devoniella* | Corylus | B (2) |
| *Phyllonorycter blancardella* | Malus | A (2), – (1) |
| *Phyllonorycter coryli* | Corylus | – (2) |
| *Phyllonorycter corylifoliella* | Crataegus | – |
| *Phyllonorycter emberizaepenella* | Lonicera | – |
| *Phyllonorycter froelichiella* | Alnus | B (1), – (1) |
| *Phyllonorycter geniculella* | Acer | – |
| *Phyllonorycter harrisella* | Quercus | B (2) |
| *Phyllonorycter kleemannella* | Alnus | – |
| *Phyllonorycter lautella* | Viburnum | – |
| *Phyllonorycter maestingella* | Fagus | – (2) |
| *Phyllonorycter messaniella* | Carpinus | – |
| *Phyllonorycter messaniella* | Fagus | – |
| *Phyllonorycter messaniella* | Quercus | – |
| *Phyllonorycter nicellii* | Corylus | A |
| *Phyllonorycter oxyacanthae* | Crataegus | – (2) |
| *Phyllonorycter quinnata* | Carpinus | B (2) |
| *Phyllonorycter rajella* | Alnus | – |
| *Phyllonorycter schreberella* | Ulmus | B |
| *Phyllonorycter sorbi* | Sorbus | – |
| *Phyllonorycter stettinensis* | Ulmus | – |
| *Phyllonorycter tristrigella* | Lonicera | – (2) |
| *Phyllonorycter ulmifoliella* | Betulus | B (1), – (1) |
| Leaf-miner parasitoids | | |
| Eulophidae | | |
| *Achrysocharoides acerianus* | Acer | – |
| *Achrysocharoides atys* | Crataegus | – (5) |
| *Achrysocharoides latereilli* | Quercus | A (1), – (2) |
| *Achrysocharoides niveipes* | Betula | AB |
| *Achrysocharoides splendens* | Alnus | – (3) |
| *Achrysocharoides zwoelferi* | Salix | – |
| *Chrysocharis laomedon* | | – (2) |
| *Chrysocharis nephereus* | | – (2) |
| *Cirrospilus diallus* | | – |
| *Cirrospilus lyncus* | | – (2) |
| *Elachertus inunctus* | | – (2) |
| *Pediobius saulius* | | A (1), – (1) |
| *Pediobius alcaeus* | | A (1), – (1) |
| *Pnigalio longulus* | | – |
| *Sympiesis gordius* | | – |
| *Sympiesis sericeicornis* | | – (3) |
| Braconidae | | |
| *Colastes braconius* | | A |
| *Pholetesor circumscriptus* | | – |

**Table 3** The survey of aphids and their parasitoids. See Table 2 legend for details

| Aphid / Parasitoid | Foodplant | *Wolbachia* |
|---|---|---|
| Aphids | | |
| *Aphis jacobaeae* | *Senecio jacobaea* | – (2) |
| *Capitophorus carduinis* | *Cirsium palustre* | – (2) |
| *Microlophium carnosum* | *Urtica dioica* | – (2) |
| *Sitobium fragariae* | Poaceae | – (2) |
| Primary parasitoids | | |
| (Braconidae: Aphidiinae) | | |
| *Aphidius* sp. A | | – |
| *Aphidius* sp. B | | – |
| *Aphidius eadyi* | | – |
| *Aphidius ervi* | | – |
| *Aphidius microlophi* | | – |
| *Aphidius picipes* | | – |
| *Aphidius rhopalosiphi* | | – |
| *Aphidus urticae* | | – |
| *Binodoxys acalephae* | | – |
| *Dyscritulus planiceps* | | – |
| *Ephedrus plagiator* | | – |
| *Lysiphlebus cardui* | | – |
| *Lysiphlebus fabarum* | British, thelytokous | – |
| *Lysiphlebus fabarum* | German, thelytokous | – (2) |
| *Lysiphlebus fabarum* | German, arrhenotokous | – |
| *Monoctonus pseudoplatani* | | – |
| *Praon dorsale* | | – |
| *Praon abjectum* | | – |
| *Praon volucre* | | – |
| *Trioxys cirsii* | | – |
| *Aphelinus abdominalis* | (Chalcidoidea, Aphelinidae) | – |
| Secondary (Hyper-) parasitoids | | |
| *Alloxysta Gr1** | (Cynipoidea, Charipidae) | – |
| *Alloxysta v2*† | (Cynipoidea, Charipidae) | – (3) |
| *Alloxysta fulviceps* | (Cynipoidea, Charipidae) | – |
| *Alloxysta macrophadna* | (Cynipoidea, Charipidae) | – (3) |
| *Alloxysta victrix* | (Cynipoidea, Charipidae) | – |
| *Asaphes vulgaris* | (Chalcidoidea, Pteromalidae) | – (2) |
| *Coruna clavata* | (Chalcidoidea, Pteromalidae) | A (2), – (1) |
| *Dendrocerus carpenteri* | (Proctotrupoidea, Megaspilidae) | – |

* Unidentified species related to *A. grevis*.
† Unidentified species related to *A. victrix*.

(*Aphis rumicis* and *A. fabae*), and an aphid parasitoid (*Aphidius rhopalosiphi*).

*Leaf-miners.* In the leaf-miner community, eight out of 21 (38.1%) hosts and five out of 18 (27.8%) parasitoids were infected with *Wolbachia.* Amongst the hosts, five *Phyllonorycter* species had B group *Wolbachia*, and two had A group. The only leaf-mining species examined that was not in the genus *Phyllonorycter*, i.e. *Parornix devoniella*, was found to harbour B group *Wolbachia*. Four of the five infected leaf-miner parasitoids had A group *Wolbachia*. The fifth species, *Achrysocharoides niveipes*, had a double AB infection.

*Aphids.* In contrast to the leaf-miner community, *Wolbachia* was extremely scarce in the aphid–parasitoid–hyperparasitoid community. No positives were recorded from aphids (four species) or primary parasitoids (19 species) and only one out of eight (12.5%) of the hyperparasitoids was infected. This species, *Coruna clavata*, contained a group A *Wolbachia*.

*General Lepidoptera.* In our survey of Lepidoptera caught at a light trap, four out of 13 (30.8%) moth species harboured *Wolbachia*, all from group B.

### *Differences between the communities*

The *Wolbachia* infection rates were not significantly different between the leaf-miner food web (33.3%) and the general Lepidoptera sample (30.8%) ($\chi^2_{(1)} = 0.02$, d.f. = 1, $P > 0.05$). However, these two groups had a significantly higher infection rate than the members of the aphid food web (3.2%) ($\chi^2_{(1)} = 11.39$, d.f. = 1, $P < 0.001$).

### *Differences between taxa*

Combining the data from our survey with Werren *et al*. (1995b) gives a total of 236 screened insects. By far the most represented orders are the Lepidoptera (77 species) and the Hymenoptera (67 species). We therefore combined the other insects into a third group (termed other insects), and compared infection rates (Table 5).

The overall proportion of species infected with *Wolbachia* did not differ significantly between the three groups ($\chi^2_{(2)} = 3.06$, d.f. = 2, $P > 0.05$). However, the type of infections did differ. While the proportion of infections

**Table 4** The survey of Lepidoptera caught at a UV moth trap. See Table 2 legend for details

| Species | Family | *Wolbachia* |
|---|---|---|
| *Agriphila tristella* | Pyralidae | B |
| *Amphipyra pyramidea* | Noctuidae | – |
| *Atethmia centrago* | Noctuidae | – |
| *Cyclophora punctaria* | Geometridae | B |
| *Drepana binaria* | Drepanidae | B |
| *Mesapamea secalis* | Noctuidae | – |
| *Mythimna pallens* | Noctuidae | – |
| *Noctua janthina* | Noctuidae | – |
| *Noctua pronuba* | Noctuidae | – |
| *Pandemis corylana* | Tortricidae | – |
| *Tholera cespitis* | Noctuidae | – |
| *Xestia c-nigrum* | Noctuidae | B |
| *Xestia xanthographa* | Noctuidae | – |

**Table 5** Distribution of *Wolbachia* in different taxonomic groups. The data include species surveyed in this study and by Werren *et al*. (1995b). The category 'other insects' includes all species outside the Hymenoptera and Lepidoptera

| Group | A | B | AB | Total | % infected |
|---|---|---|---|---|---|
| Hymenoptera | 11 | 0 | 1 | 67 | 17.9 |
| Lepidoptera | 2 | 14 | 3 | 77 | 24.7 |
| Other Insects | 2 | 5 | 6 | 92 | 14.1 |
| Total | 15 | 19 | 10 | 236 | 18.6 |

that were A group did not differ significantly between the Lepidoptera and other insects ($\chi^2_{(1)}$ = 1.78, d.f. = 1, $P > 0.05$), it was significantly lower in these two groups than in the Hymenoptera ($\chi^2_{(1)} = 16.29$, d.f. = 1, $P < 0.001$). This difference arises because the Hymenoptera have a higher proportion of species infected with A group *Wolbachia* ($\chi^2_{(1)}$ = 13.73, d.f. = 1, $P < 0.001$), and a lower proportion of species infected with B group *Wolbachia* ($\chi^2_{(1)} = 4.85$, d.f. = 1, $P < 0.05$) compared with the other two groups. The Lepidoptera and the other insects do not differ significantly from each other in either the proportion of species infected with group A ($\chi^2_{(1)}$ = 0.29, d.f. = 1, $P > 0.05$) or group B *Wolbachia* ($\chi^2_{(1)}$ = 3.10, d.f. = 1, $P > 0.05$)

### *Double infections with different* Wolbachia

Our survey allowed us to calculate whether double infections of A- and group B *Wolbachia* occurred more frequently than expected by chance. Of the 18 species found to harbour *Wolbachia* in this study, seven had single group A infections, 10 had single group B infections, and one had a double AB infection. The frequency of double infections amongst infected species was therefore 5.6%, compared with 38.9% for single A infections, and 55.6% for single B infections.

Including AB double infections, group A *Wolbachia* occurred in 9.8% of species, and B group in 13.4% of species. Consequently, the random expectation for the frequency of double infections is 1.3%. We observed double infections in 1.2% of species, which was not significantly different from the frequency expected by chance ($\chi^2_{(1)} < 0.01$, d.f. = 1, $P > 0.05$).

### Wolbachia *sequence diversity*

The sequences from this study have been placed in the GenBank/EMBL databases with Accession nos AJ005879 to AJ005889. Sequence diversity in the B group is relatively low, with uncorrected *P*-distances (excluding gaps) ranging from 0.43% to 5.74%. All but one of our *ftsZ* sequences were the same length as most previously described type-B *Wolbachia* sequences. The exception was the sequence from the parasitoid *Achrysocharoides niveipes*. This showed sequence features that clearly link it with an unusual B subgroup that also includes *Wolbachia* from the beetle *Tribolium confusum* and the cricket *Gryllus pennsylvannicus* (Werren *et al*. 1995a). Members of this subgroup lack a 9 bp deletion (relative to group A) that is found in all other group B *Wolbachia*. They also show a unique single-base change (relative to group A) in the fourth position of this 9 bp region. In addition to these common features, the *Wolbachia* sequence from *A. niveipes* has its own unique 18 bp deletion (bases 716–733) and three other surprising features: (i) at base 245 (Werren *et al*. 1995a alignment) there are six adenines (As) rather than seven (all other strains); (ii) at base 888 there are eight As rather than seven (all other strains); (iii) even when indels are used to 'correct' these features the 3' end does not contain a full open reading frame (ORF). To test whether these strange features were artifacts we characterized four different cloned sequences and then obtained two direct sequences from different PCR reactions. All showed the three strange features which appear to be real although they are difficult to explain.

### Wolbachia *phylogenies*

Trees built by MP and NJ algorithms were very similar in their general properties and, most importantly, the minor differences do not influence the conclusions we draw from this study. There were 16 minimum MP trees with 288 steps. The strict consensus of these trees is shown in Fig. 1, which also displays both MP and NJ bootstrap support for different clades. The use of different distance measures made very little difference to the NJ trees generated, which were also similar to the MP trees in most respects. Again, the minor differences do not influence the two main conclusions that we draw from this study.

*Host–parasitoid transfer.* In cases where *Wolbachia* had been transferred between a host and a parasitoid we would expect to find closely related strains. Six leaf-mining moth species and one of their parasitoids, *A. niveipes*, contained group B *Wolbachia*. However, the *A. niveipes* strain was not placed in the same clade as any of the leaf-miner *Wolbachia*, clustering instead with other unusual type-B *Wolbachia* from a cricket and a beetle (Fig. 1). These results, together with the lack of group B *Wolbachia* in the other parasitoid species, provide no evidence that host–parasitoid transfer of group B *Wolbachia* has taken place in this food web.

Wolbachia–*host cospeciation.* If *Wolbachia* are transmitted vertically and cospeciate with their hosts then the phylo-

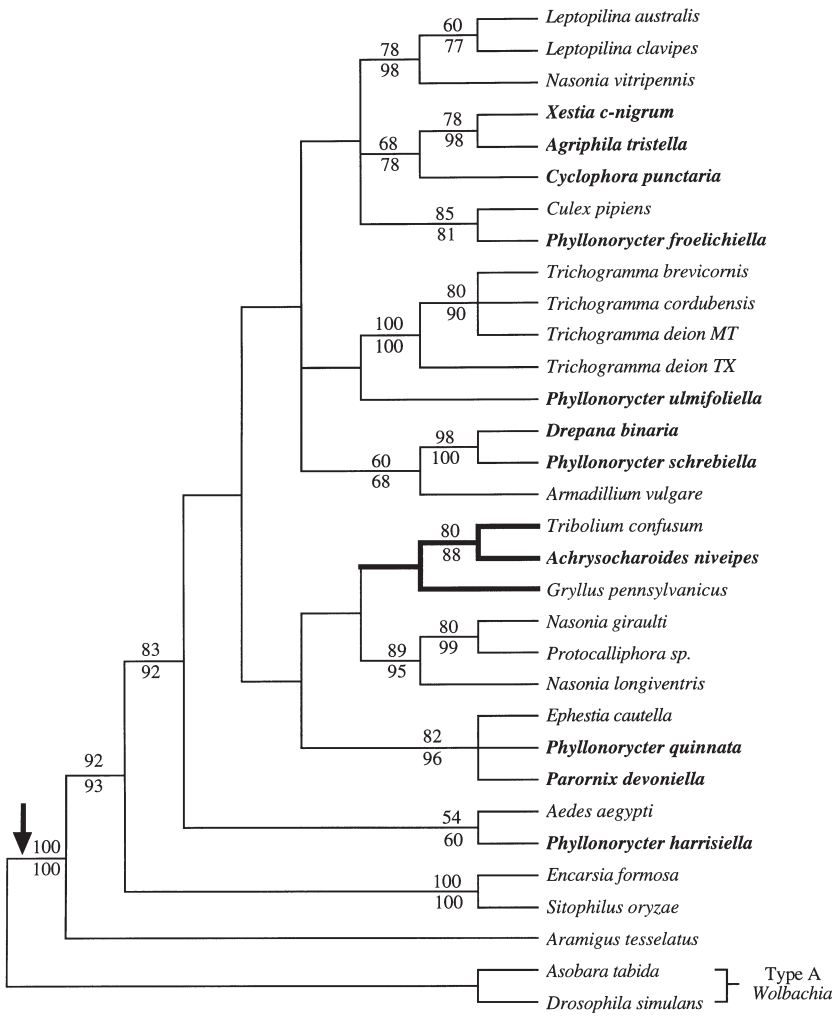


**Fig. 1** Phylogenetic relationships of *Wolbachia* isolates. The figure shows the strict consensus of the 16 minimum unconstrained MP trees. Bootstrap support for nodes is shown for both MP (above the line) and NJ (below the line) analyses. Sequences generated for this study are shown in bold. The unusual B subgroup is shown with bold branches and the arrow shows the alternative basal branch point of this subgroup which is referred to in the text.

geny of *Wolbachia* should be congruent with that of the hosts. In this case we would expect the *Wolbachia* isolated from five different *Phyllonorycter* leaf-mining moth species to form a monophyletic clade. In fact, the extreme opposite is the case; each of these five *Wolbachia* is in a different clade (Fig. 1). This was further investigated using MP analyses which constrained trees to have the five *Phyllonorycter* sequences in a monophyletic group. This resulted in 630 minimum trees of 309 steps. Tree-length comparisons using the Kishino–Hasegawa test showed that all 630 constrained trees were significantly longer than all 16 minimum trees (in all cases $P < 0.0001$). These data clearly contradict the pattern expected if *Wolbachia* were cospeciating with their *Phyllonorycter* hosts.

## Wolbachia *subgroups*

Werren *et al*. (1995a) proposed that the unusual *Tribolium–Gryllus* subgroup was probably basal among group B *Wolbachia*, based upon a 9 bp insertion that it shares with group A *Wolbachia*, but which is absent in all other group B members. Our phylogenetic analyses place these together in a subgroup with the *A. niveipes* bacterium, but not basal in the B group. An MP analysis which constrained this B subgroup to be basal in the B clade gave 783 minimum trees of 301 steps. Tree-length comparisons using the Kishino–Hasegawa test showed that all 783 constrained trees were significantly longer than all 16 unconstrained minimum trees ($0.0092 < P < 0.0236$). Despite this, we believe that it is highly unlikely that a 9 bp deletion occurred in the B group, and was subsequently reinserted in the B subgroup. This is the only indel event that conflicts with the minimum unconstrained trees. To further investigate this issue we generated phylogenies with indels given greater weight than other characters. The B subgroup was only placed basal in the B group when indels were weighted at least 15 times greater than base substitutions. Nevertheless, we think that this may be a true reflection that indels in this coding gene are infrequent events that

should be highly weighted. Again, it is important that trees generated with the constraint and with differential weighting do not influence our main conclusions. These trees had very similar topologies to the unconstrained ones except for the position of the B subgroup.

## Discussion

We surveyed for *Wolbachia* infection in two host–parasitoid communities (food webs), and a small sample of general Lepidoptera caught at a light trap. Overall, 22.0% of the insect species that we examined were infected with *Wolbachia*. This percentage is similar to the 16.9% infection rate found by Werren *et al*. (1995b) in a survey of neotropical insects. However, we also found that the distribution of *Wolbachia* infections differed significantly in several respects between communities and taxonomic groups. Specifically, we found that: (i) the percentage of species infected in the aphid food web (3.5%) was significantly lower than in the leaf-miner food web (33.3%) and the general Lepidoptera sample (30.8%); (ii) the Hymenoptera contain a higher rate of group A infection, and a lower rate of group B infection, compared with all other insects; and (iii) the frequency of AB double infections differed significantly between our study and Werren *et al*. (1995b). We found that AB double infections occurred no more frequently than expected by chance, while Werren *et al*. (1995b) found that double infections occurred more frequently than expected.

Differences in the distribution of *Wolbachia* infections between communities/taxa could occur for at least four nonexclusive reasons. First, some species could be more (or less) resistant to infection (or loss of infection) by any or certain types of *Wolbachia*. A possible point of interest here is that *Wolbachia* have yet to be found in any aphid, and so it is possible that some factor common to this group, such as bacterial endosymbionts, may be preventing *Wolbachia* infection. Alternatively, it is possible that this reflects a detection bias. Adult aphids carry mature embryos and are 'live-bearing'. If *Wolbachia* are restricted to germ cells in aphids, then the concentration of *Wolbachia* DNA within aphids could be quite low, making detection more difficult.

Second, ecological factors may lead to some species being exposed to *Wolbachia* infections more frequently than others. This leads to the general question of whether there are any ecological correlates of *Wolbachia* infection. Third, *Wolbachia* may have infected an ancestral species and then cospeciated. If several ancestors of such a species were surveyed then they would all be infected by very similar strains of *Wolbachia*, and the phylogenetic tree of the *Wolbachia* strains would match that of their hosts (cocladogenesis). Finally, a *Wolbachia* strain could have invaded and subsequently spread amongst the members of a community or taxon. This might occur if a strain became specialized to a taxonomic group or a particular environment. Although this could also lead to closely related species harbouring closely related *Wolbachia* it would not predict cocladogenesis (Schilthuizen & Stouthamer 1997). Controlling for these final two possibilities with appropriate formal comparative analyses (Harvey & Pagel 1991) will be of the utmost importance when trying to identify, across species, any correlates of *Wolbachia* infection.

Our study provides no evidence for horizontal transfer of *Wolbachia* between hosts and parasitoids. Within the leaf-miner community the parasitoids carried predominantly group A *Wolbachia*, while the hosts carried mainly group B *Wolbachia* (Table 1). B group *Wolbachia* were only present in one leaf-miner parasitoid, *A. niveipes* (Table 1). Furthermore, the strain found in *A. niveipes* was not closely related to that in any of the leaf-miners. Two leaf-miner species and five of their parasitoids were infected with A group *Wolbachia*. However, the poorly resolved phylogeny of this group means that we were unable to test for possible horizontal transfer. While one cannot prove a negative, our results suggest that host–parasitoid transfer is not the major route through which the species we have examined become infected. Examination of more host–parasitoid webs, and a better resolved phylogeny of A group *Wolbachia*, is required to determine whether this is a general pattern. Another possibility is that transfer occurs between parasitoid species when more than one species oviposits in the same host (Schilthuizen & Stouthamer 1997). We could not test this possibility because all but one of our infected parasitoid species harboured A group *Wolbachia*.

The B group *Wolbachia* strains in the six leaf-miner species were not closely related. Five of these species were in the genus *Phyllonorycter*. We can therefore reject the possibilities that infections in these species are due to a common route of infection associated with the leaf-mining habit, a taxonomically specialized strain, or infection by *Wolbachia* prior to specialization and subsequent cospeciation. Consequently, the *Phyllonorycter* species provide a clear example of a genus that contains several species that appear to have been infected independently by a diverse range of *Wolbachia* strains. Determining how *Wolbachia* is transmitted horizontally between host species in cases such as this remains a major question.

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