

# *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?

John H. Werren<sup>1\*</sup> and Donald M. Windsor<sup>2</sup>

<sup>1</sup>Department of Biology, University of Rochester, Rochester, NY 14627, USA

<sup>2</sup>Smithsonian Tropical Research Institute, Apartado 2072, Balboa-Ancon, Republic of Panama

*Wolbachia* are a group of cytoplasmically inherited bacteria that cause reproduction alterations in arthropods, including parthenogenesis, reproductive incompatibility, feminization of genetic males and male killing. Previous general surveys of insects in Panama and Britain found *Wolbachia* to be common, occurring in 16–22% of species. Here, using similar polymerase chain reaction methods, we report that 19.3% of a sample of temperate North American insects are infected with *Wolbachia*, a frequency strikingly similar to frequencies found in two other studies in widely separated locales. The results may indicate a widespread equilibrium of *Wolbachia* infection frequencies in insects whose maintenance remains to be explained. Alternatively, *Wolbachia* may be increasing in global insect communities. Within each of the three geographic regions surveyed, Hymenoptera are more frequently infected with A group *Wolbachia* and Lepidoptera more frequently infected with B group *Wolbachia*.

**Keywords:** *Wolbachia*; infection frequency; arthropods

## 1. INTRODUCTION

*Wolbachia* are a group of intracellular bacteria related to *Rickettsia*. These bacteria infect the reproductive tissues of arthropods, are transmitted through the egg cytoplasm and alter reproduction in their arthropod hosts in various ways (Werren 1997). *Wolbachia* are associated with cytoplasmic incompatibility (Breeuwer *et al.* 1992; O'Neill *et al.* 1992), parthenogenesis (Stouthamer *et al.* 1993), feminization of genetic males (Rousset *et al.* 1992) and male killing (Hurst *et al.* 1999). They have been implicated as a possible mechanism for rapid speciation in arthropods (Laven 1967; Breeuwer & Werren 1990; Werren 1998; Shoemaker *et al.* 1999).

Sequencing of bacterial 16S rDNA and protein-coding genes has indicated at least four major subdivisions of *Wolbachia*. Two (designated A and B) are found in arthropods (Werren *et al.* 1995a; Zhou *et al.* 1998), including insects, crustacean isopods (Bouchon *et al.* 1998) and mites (Johanowicz & Hoy 1995; Breeuwer 1997), and the other two (designated C and D) are found in nematodes (Bandi *et al.* 1998). A fifth subgroup (designated E) has recently been proposed based on a 16S rDNA sequence for a *Wolbachia* found in a collembolan, a primitive insect (Vandekerckhove *et al.* 1999). The phylogenetic data also show extensive horizontal transmission of *Wolbachia* between insect taxa although the mechanisms are still unclear (Werren *et al.* 1995b).

*Wolbachia* are widespread and common in insects. In the first published systematic survey of *Wolbachia* distributions, Werren *et al.* (1995b) found over 16% of a sample of insect species from Panama were infected with *Wolbachia*. *Wolbachia* were found in all the major orders of insects tested. Extrapolating to the global insect fauna, it was estimated that one to five million insect species are infected with these bacteria (Werren *et al.* 1995b). In a study using similar methods, West *et al.* (1998) found 22% of British insects were infected. The latter study concen-

trated primarily on Lepidoptera (butterflies and moths) and Hymenoptera (wasps, bees and ants). However, systematic surveys of additional localities around the world have yet to be performed.

The objectives of this study were to (i) determine the distribution and type of *Wolbachia* in a sample of Neotemperate North American arthropods, and (ii) compare the results of this survey to two previous studies of lower Central America (Panama) and northern Europe (Britain). The results show a surprising consistency in the overall frequency of *Wolbachia* in these different geographic regions, as well as similar differences in the frequency of *Wolbachia* types between insect orders collected in the different regions.

## 2. METHODS

Arthropods (primarily insects) were collected and tested for the presence of *Wolbachia* using an assay based upon polymerase chain reaction (PCR) amplification of the *ftsZ* protein-coding gene and 16S rDNA from *Wolbachia* using methods similar to our previous surveys (Werren *et al.* 1995b; West *et al.* 1998). Details of the methods are described below.

### (a) Arthropod collection and handling

Arthropods were collected from a single, wooded habitat 10 km south-east of Bloomington, IN (39°10' N, 86°29' W) during August 1994, July 1997, August 1998 and November 1998. Entire animals or dissected ovaries were fixed in 95% ethanol. The fixed specimens were kept under refrigeration (around 4 °C) until shipment to Rochester, NY, for further analysis, where they were kept under refrigeration until DNA extraction. Arthropod remains are stored in the insect voucher collection at the Smithsonian Tropical Research Institute, Panama City, Republic of Panama.

### (b) Template preparation

DNA was extracted from the fixed individuals using either (i) eggs or ovaries, (ii) whole abdomens, or (iii) whole individuals (for small arthropods). The tissues were dissected in

\* Author for correspondence (werr@mail.rochester.edu).

sterile, double-distilled, deionized water on a sterile Petri dish and then serially rinsed in droplets of sterile H<sub>2</sub>O, prior to extraction of DNA using previously published methods (Werren *et al.* 1995b) or Qiaamp tissue extraction protocols (Smith & Kelley 1994). Control DNA samples were prepared using pupae or adults of known infected and uninfected strains of *Nasonia vitripennis*.

### (c) Assay for Wolbachia

A PCR was performed using *Wolbachia*-specific primers for the *ftsZ* bacterial cell-cycle gene to test for the presence of *Wolbachia* (Werren *et al.* 1995a,b). Arthropods yielding a product of the expected size were tentatively scored as positive for *Wolbachia*. Samples positive for *Wolbachia* based on this assay were then retested using primers specific for the A and B subdivisions of *Wolbachia* (*ftsZ*, 16S or both; see § 2(d)). In addition, some products were sequenced and compared to known A and B group *Wolbachia*.

The absence of amplification using the general *ftsZ* primers (a presumptive negative) could be due either to (i) the absence of *Wolbachia* in the insect, (ii) failure of the DNA extraction procedure, or (iii) an incorrect concentration of DNA solution. As a control for the amplifiability of the DNA extraction, samples that were negative for *ftsZ* were tested by using primers for highly conserved regions of eukaryotic 28S rDNA (as described previously in Werren *et al.* (1995b)). From experience, we have often found positive amplifications following dilution of DNA. This is due to either an excess template DNA concentration or the presence of inhibiting substances. Therefore, the samples were tested at various dilutions ranging from 1:10 to 1:100 (depending upon the DNA concentrations apparent from ethidium bromide staining intensity). The maximum concentration yielding a 28S rDNA positive result was then used to retest for *Wolbachia* with the general *ftsZ* primers. Samples that failed to yield a positive result by the 28S rDNA primers for all concentrations (false negatives) were not included in the study. Additional positive controls using known infected insects (*N. vitripennis*) were performed with each set of PCR reactions. To confirm that the products amplified using the general *ftsZ* primers were from *Wolbachia*, the products were hybridized to radioactively labelled *Wolbachia ftsZ* as described previously (Werren *et al.* 1995b).

### (d) Screening using 16S rDNA primers

We conducted a study to compare the efficiency of *Wolbachia* detection with the *ftsZ* primers relative to the 16S rDNA primers. Two sets of 16S rDNA primers were used, which were designated W-Spec (*Wolbachia* specific) and W-E (*Wolbachia Ehrlichia*). The W-Spec primers were designed from the 3' half of the 16S rDNA gene in order to amplify a 438 bp fragment. This region was chosen because it contains restriction sites which differ between A and B group *Wolbachia*, providing second confirmation of bacterial group (Werren *et al.* 1995a).

The W-E primers were designed to detect more divergent bacteria related to *Wolbachia* which may not be detected by the more specific sets. We do know that the general *ftsZ* primers and 16S primers specific for *Wolbachia* amplify a diverse range of A and B *Wolbachia* and also successfully amplify the somewhat more divergent C and D group *Wolbachia* found in nematodes (Bandi *et al.* 1998). However, more divergent bacteria could be present in insects. To screen for such bacteria, primers from the 16S gene were designed, which amplify the product from both *Wolbachia* and related *Rickettsia*, including some *Ehrlichia*,

*Cowdria* and *Anaplasma* (Weisburg *et al.* 1991). A subset of samples were rescreened using these primers (designated W-E for *Wolbachia Ehrlichia*) in order to determine whether more divergent related bacteria were being missed in the samples.

### (e) PCR methods

Amplification of the nearly complete *Wolbachia ftsZ* sequence (1043–1055 bp depending upon the *Wolbachia* strain) was accomplished with primers *ftsZ*f1 and *ftsZ*r1 following previously published methods (Werren *et al.* 1995b; West *et al.* 1998). After the PCR, 8 µl of amplified reaction product was run on a 1% agarose gel in order to determine the presence and size of the amplified DNA. Controls for the PCR amplifiability of the DNA solutions were conducted using the general eukaryotic 28S rDNA primers 28Sf and 28S. In order to determine the *Wolbachia* group, primers for specific amplification of A and B group *ftsZ* were used. To confirm A and B status or to resolve ambiguities, A-specific 16S and B-specific 16S primers were also used in some cases. The methods and primer sequences were as described previously (Werren *et al.* 1995a; West *et al.* 1998).

The 16S W-Spec primers used were W-Specf (CATACC TATTCGAAGGGATAG) and W-Specr (AGCTTTCGAGTGAA ACCAATTC). These amplify a 438 bp fragment and A and B *Wolbachia* can be distinguished by an *RsaI* restriction site present in B *Wolbachia*, which results in 146 and 292 bp digestion. The conditions for the W-Spec amplification were a 2 min period of 95 °C pre-dwell, two cycles of 2 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 35 cycles of 30 s at 95 °C, 1 min at 60 °C and 45 s at 72 °C and a post-dwell period of 5 min at 72 °C.

The primers used for the amplification of *Wolbachia*, *Ehrlichia* and intermediate bacteria (W-E primers) were W-Ef (CAGACGGGTGAGTAATG(C/T)ATAG) and W-Er (TATCA CTGGCAGTTTCCTTAAAG). These amplify a fragment size of 1025 bp. As mentioned, these are designed from the moderately variable regions of 16S rDNA and are relatively conserved among *Wolbachia* and related bacteria (some *Ehrlichia*, *Anaplasma* and *Cowdria*). The primers amplify the products from *Wolbachia* and *Ehrlichia canis*, but not from *Escherichia coli*. Note that better primer design may now be possible with new sequence information on *Ehrlichia* and relatives. The W-E PCR cycling conditions were two cycles of 2 min at 95 °C, 1 min at 64 °C and 2 min at 72 °C, followed by 35 cycles of 30 s at 95 °C, 1 min at 64 °C and 1 min at 72 °C and a post-dwell period of 5 min at 72 °C.

## 3. RESULTS

### (a) Patterns in the Indiana sample

A total of 145 insect species were screened for *Wolbachia* from 13 different orders, of which 19.3% were positive for the bacteria (tables 1 and 2). As in the two previous surveys, *Wolbachia* were found in species of each of the major orders, including Diptera, Coleoptera and Lepidoptera, Hymenoptera and Orthoptera.

Of the 28 insects species positive for *Wolbachia*, 67.9% were singly infected with A *Wolbachia*, 25.0% were singly infected with B *Wolbachia* and 7.1% were doubly infected with A and B *Wolbachia* (tables 3 and 4). The overall infection level with A bacteria (A and A plus B over the total species tested) was 14.5% and for B bacteria it was 6.2%. Based on these frequencies, the overall infection frequency with both bacterial types (1.4%) did not differ significantly from the random expectation if infection with one type were independent of infection with the

Table 1. *Distribution of Wolbachia*

(The identifications of the arthropods tested are shown along with the results of the PCR assays. The presence of A, B or double (A plus B) infections was determined based upon the PCR assays (see §2). Arthropods that were not identified to species (or family) are placed in the appropriate family (or order). If multiple individuals were tested for a species, the number tested is indicated in parentheses. unid. sp., unidentified species; unid. family, unidentified family.)

taxon	<i>Wolbachia</i>	taxon	<i>Wolbachia</i>
Insecta		Bombyliidae	
Coleoptera		unid. sp.	—
Cantharidae		Caliphoridae	
<i>Chauliognathus pennsylvanicus</i> sp.	—	unid. sp.	—
Carabidae		Chironomidae	
<i>Harpalus pennsylvanicus</i>	—	unid. sp.	—
<i>Agra</i> sp.	—	Lonchaeidae	
<i>Galerita</i> sp.	—	unid. sp.	—
Cerambycidae		Micropezidae	
<i>Tetraopes tetraophthalmus</i>	—	unid. sp.	A
<i>Typocerus velutinus</i>	—	Syrphidae	
unid. sp.	—	<i>Milesia virginiensis</i>	—
Cicindellidae		Tabanidae	
<i>Cicindela sexguttata</i>	—(3)	unid. sp.	—
Chrysomelidae		Tachinidae	
<i>Chelymorpha cassidea</i>	—	unid. sp.	—
<i>Diabrotica undecimpunctata</i>	—	Tephritidae	
<i>Lema sexpunctata</i>	—	unid. sp.	A
<i>Charidotella purpurata</i>	A	Tipulidae	
<i>Charidotella sexpunctata</i>	—	<i>Tipula</i> sp. nr. <i>borealis</i>	—
<i>Deloyala guttata</i>	—	<i>Tipula ultima</i>	—
Ciidae		Ephemeroptera	
<i>Cis</i> sp. 1	—	Ephemeridae	
<i>Cis</i> sp. 2	—	<i>Hexagenia</i> sp.	—
Coccinellidae		Hemiptera	
unid. sp.	A	Coreidae	
<i>Cycloneda munda</i>	—	<i>Leptoglossus clypealis</i>	—
<i>Coleomegilla maculata</i>	—	Gerridae	
Curculionidae		<i>Gerris</i> sp. 1	—
<i>Cyrtopistomus castaneus</i>	A	<i>Gerris</i> sp. 2	B
unid. sp. 1	—	Lygaeidae	
unid. sp. 2	—	<i>Oncopeltus fasciata</i>	—
unid. sp. 3	—	Miridae	
Dermestidae		<i>Lygus</i> sp. 1	—
<i>Dermestes lardarius</i>	—	<i>Lygus</i> sp. 2	—
Elateridae		Pentatomidae	
unid. sp.	—	<i>Euschistus variolarius</i>	—
Erotylidae		<i>Murgantia histrionica</i>	—
<i>Megalodacne</i> sp.	—	Rhopalidae	
Lycidae		<i>Leptocoris trivittatus</i>	—
<i>Calypteron terminale</i>	—	Reduviidae	
<i>Calypteron reticulatum</i>	—	<i>Arius cristatus</i>	—
Meloidae		Homoptera	
<i>Epicauta cinerea</i>	B	Aphidae	
Nitidulidae		unid. sp.	—
<i>Lobiopa</i> sp.	—	Cicadellidae	
<i>Carpophilus</i> sp.	—	unid. sp.	—
Scarabeidae		Membracidae	
<i>Phyllophaga</i> sp.	—	unid. sp.	—
Staphylinidae		Hymenoptera	
unid. sp. 1	—	Andrenidae	
unid. sp. 2	A	<i>Panurginae</i> (unid. sp.)	A
Tenebrionidae		Apidae	
<i>Bolitotherus cornutus</i>	—	<i>Ceratina</i> sp.	A
Diptera		<i>Bombus</i> sp.	—
Asilidae		<i>Apis mellifera</i>	—
unid. sp. 1	—	<i>Halictinae</i> sp.	A
unid. sp. 2	—	<i>Melissodes rustica</i>	—

(Cont.)

Table 1. (*Cont.*)

taxon	<i>Wolbachia</i>	taxon	<i>Wolbachia</i>
unid. sp.	—	Ctenuchidae	
unid. sp.	A	<i>Ciseps fulvicollis</i>	—
Bethylidae		unid. sp.	—
unid. sp.	—	Danaidae	
Chrysididae		<i>Danaus plexippus</i>	—
unid. sp.	A	Geometridae	
Cynipidae		<i>Anacamptoides</i> sp.	B
unid. sp.	B	<i>Eubaphe mendica</i>	—(2)
Eumenidae		unid. sp. 1	—
<i>Eumenes fraternus</i>	B	unid. sp. 2	AB
<i>Monobia quadridens</i>	—	Hesperiidae	
<i>Zethus</i> sp.	—	<i>Epargyreus clarus</i>	—
unid. sp.	—	Lycaenidae	
unid. sp.	AB	<i>Plebejus</i> sp.	B
Formicidae		Noctuidae	
<i>Formica</i> sp. 1	—	<i>Pyrrhia</i> sp.	—(3)
<i>Formica</i> sp. 2	A	unid. sp. 1	—
<i>Leptothorax</i> sp.	—	unid. sp. 2	—
unid. sp.	A	Nymphalidae	
Halictidae		<i>Speyeria cybele</i>	—
<i>Augochlora pura</i>	A	<i>Papilio glaucus</i>	—
<i>Lasioglossum</i> sp.	A	<i>Phycoides tharos</i>	—
Ichneumonidae		unid. sp.	—
unid. sp. 1	—	Pieridae	
unid. sp. 2	—	<i>Colias</i> sp.	—
unid. sp. 3	—	Pterophoridae	
unid. sp. 4	—	unid. sp.	—
unid. sp. 5	A	Satyridae	
unid. sp. 6	—	<i>Enodia portlandia</i>	—
unid. sp. 7	—	Mecoptera	
Leucospidae		Panorpidae	
<i>Leucospis</i> sp.	—	<i>Panorpa bichae</i>	—
Mutillidae		Odonata	
<i>Mutilla occidentalis</i>	—	Calopterygidae	
Perilampidae		<i>Calopteryx maculata</i>	—(2)
<i>Perilampus</i> sp.	—	Orthoptera	
Pompilidae		Acrididae	
unid. sp. 1	A	<i>Melanoplus</i> sp.	—
unid. sp. 2	—	Blattidae	
<i>Auplopus</i> sp.	—	<i>Percoblatta</i> sp.	—
Sphecidae		Gryllidae	
<i>Ammophila</i> sp.	—	<i>Phylopalpus</i> sp.	A
<i>Pemphredon</i> sp.	—	<i>Orcharis</i> sp.	—
<i>Chalybion</i> sp.	—	unid. sp.	A
unid. sp. 1	—	unid. sp.	B
unid. sp. 2	—	Mantidae	
Tenthredinidae		<i>Tenodera sinensis</i>	—
unid. sp.	—	Phasmidae	
Torymidae		<i>Diapheromera femorata</i>	—
unid. sp.	—	Tettigonidae	
Vespidae		<i>Scudderia furcata</i>	—
<i>Dolichovespula maculata</i>	—	<i>Conocephalus nemoralis</i>	—
<i>Dolichovespula norvegicensis</i>	—	<i>Amblycorprypha</i> sp.	—
<i>Polistes fuscatus</i>	—	Psocoptera	
Isoptera		Psocidae	
Rhinotermitidae		unid. sp.	—
<i>Reticulitermes flavipes</i>	—	Siphonoptera	
Lepidoptera		Pulicidae	
Arctiidae		<i>Ctenocephalides canis</i>	—
<i>Apantesis virgo</i>	—	Thysanura	
<i>Spilosoma virginica</i>	—	Machilidae	
		<i>Machilis</i> sp.	—

*(Cont.)*

Table 1. (Cont.)

taxon	<i>Wolbachia</i>
Arachnida	
Araneae	
Araneidae	
<i>Micrathena gracillis</i>	—
<i>Araneus</i> sp.	—
<i>Achaeranea tipidariorum</i>	—
Lycosidae	
<i>Lycosa</i> sp.	—
Salticidae	
unid. sp.	—
Theridiidae	
<i>Achaeranea</i> sp.	—
<i>Tidarren</i> sp.	—
Thomisidae	
<i>Misumena</i> sp.	A
Pholcidae	
<i>Pholcus phalangioides</i>	—
Ixodidae	
<i>Dermacentor variabilis</i>	—
Opiliones	
Phalangidae	
<i>Leiobunum</i> (?) sp.	—
Opiliones sp.	—
Chilipoda	
Lithobiidae	
<i>Lithobius</i> sp.	—
unid. family	—
Diplopoda	
unid. family	—
Crustacea	
Isopoda	
unid. family	—
unid. family	—
Amphipoda	
unid. family	—

other (0.9%) (Pearson  $\chi^2 = 1.50$ ,  $p = 0.22$  and d.f. = 1). The pattern of A and B infections in the Indiana sample contrasted with studies in other locations (see below).

Ten arachnid species were tested in the temperate North American sample and one crab spider (*Misumena* sp.) was found to be weakly positive with A *Wolbachia*. This is the first spider that has so far tested positive for *Wolbachia* (zero out of ten from tropical North America). However, follow-up sampling of this species has not yet been performed. Although *Wolbachia* are found in mites (Breeuwer 1997), further work is needed to clarify whether spiders harbour *Wolbachia*.

### (b) Comparing geographic regions

Comparisons between the three surveys are shown in tables 2–4. The surveys were for lower Central America (Panama), temperate Europe (Britain) and temperate North America (Indiana). Some surveys of other insects have recently been published (Bouchon *et al.* 1998; Hariri *et al.* 1998; Wenseleers *et al.* 1998). However, these were focused upon groups where there was some prior knowledge of *Wolbachia* infections and, therefore, the infection frequencies are likely to be biased upwards. In the three surveys compared here, the genera and species were selected arbitrarily with respect to any prior knowledge of

infection status. In addition, similar methods were used in all three locales, making the results more comparable.

All three sites showed remarkable consistency in their overall frequencies of *Wolbachia* infections: 17% for Panama, 22% for Britain and 19% for Indiana. This consistency is surprising because of the stochasticity of the samples. The relative representations of the different orders differed between sites (e.g. 24, 36 and 0% for Coleoptera from Indiana, Panama and Britain, respectively). The European samples were particularly focused. The samples were taken from a community of leaf-mining Lepidoptera and associated parasitoids and from an arbitrary sampling of Lepidoptera arriving at bait traps. Nevertheless, the consistent infection frequencies imply that some general factors may result in a large-scale equilibrium in infection frequency.

Although all three locales showed similar overall frequencies, there were interesting contrasts (table 3). First, among infections, the Indiana samples were significantly more likely to harbour group A than group B bacteria (70% A,  $n = 30$ ) (Pearson  $\chi^2 = 5.35$ ,  $p = 0.021$  and d.f. = 1), whereas Panama (49% A,  $n = 35$ ) and Britain (42% A,  $n = 19$ ) showed similar infection levels with group A and B bacteria.

The frequency of double infections (A and B) detected among infected species also differed significantly between sites (table 3), with 34.6% in Neotropical North America, 5.6% in northern Europe and 7.1% in Neotemperate North America (Pearson  $\chi^2 = 6.2$ ,  $p = 0.045$  and d.f. = 2). In particular, the two temperate regions showed similar levels of double infections. The frequency of double infections in Panama was greater than expected by chance if the probabilities of infection with A and B are independent (Werren *et al.* 1995b), whereas the double infection frequencies were not significantly higher in Britain (West *et al.* 1998) or Indiana (this study). Possible explanations for these differences are discussed later.

### (c) Comparing insect orders

The sample sizes were sufficient for beginning to compare different orders of insects between regions. Although the frequency of infected Hymenoptera was lower in the British sample (13%) than in the Panama and Indiana samples (26 and 31%, respectively), the differences between all three sites were not quite significant (Pearson  $\chi^2 = 5.48$ ,  $p = 0.064$  and d.f. = 2). Similarly, the infection frequencies differed between locales among the Lepidoptera, but not significantly so based on the current sample sizes (Pearson  $\chi^2 = 4.97$ ,  $p = 0.083$  and d.f. = 2). The sample sizes in other orders were not large enough to permit additional comparisons.

Interesting differences occurred between the insect orders in their relative frequencies of A and B infections (table 4). The Hymenoptera had a higher incidence of infection with A *Wolbachia* than with B *Wolbachia* in each location. In contrast, the Lepidoptera were more likely to be infected with B *Wolbachia*. Summing across locations, the Hymenoptera were significantly more likely to be infected with A *Wolbachia* (21.2%) than with B *Wolbachia* (3.5%) (Pearson  $\chi^2 = 12.43$ ,  $p < 0.001$  and d.f. = 1), whereas the Lepidoptera were significantly less likely to be infected with A *Wolbachia* (6.1%) than with B *Wolbachia* (20.4%) (Pearson  $\chi^2 = 6.477$ ,  $p = 0.011$  and

Table 2. Comparison of the numbers of species of Neotemperate, Neotropical and Palaeotemperate insects and arachnids testing positive for *Wolbachia*

group	Indiana			Panama			Britain		
	infected		number tested	infected		number tested	infected		number tested
	n	%		n	%		n	%	
Insecta	28	19.3	145	26	16.9	154	18	21.7	83
Coleoptera	5	14.3	35	6	10.5	57	—	—	—
Hymenoptera	14	31.1	45	6	26.1	23	6	13.3	45
Lepidoptera	3	14.3	21	7	16.3	43	12	35.3	34
other orders	6	13.6	44	7	22.6	31	0	0.0	4
Arachnida	1	8.3	12	0	0.0	3	—	—	—
all arthropods	29	18.5	157	26	16.6	157	18	21.7	83

Table 3. Comparison of the numbers of singly and doubly infected species by region

region	A	B	AB	percentage AB	total	total percentage infected
Indiana	19	7	2	7.1	145	19.3
Panama	8	9	9	34.6	154	16.9
Britain	7	10	1	5.6	83	21.7

Table 4. Comparison of the numbers of singly and doubly infected species in two insect orders by region

region	A	B	AB	percentage AB	total	total percentage infected
Hymenoptera						
Indiana	11	2	1	7.1	45	31.1
Panama	6	0	0	0.0	23	26.1
Britain	5	0	1	16.7	45	13.3
total	22	2	2	7.7	113	23.0
Lepidoptera						
Indiana	0	2	1	33.3	21	14.3
Panama	0	4	3	42.9	43	16.3
Britain	2	10	0	0.0	34	35.3
total	2	16	4	18.2	98	22.4
other insect orders						
Indiana	8	3	0	0.0	79	13.9
Panama	2	5	6	46.2	88	14.8
Britain	0	0	0	0.0	4	0.0
total	10	8	6	0.0	171	14.0

d.f. = 1). The Hymenoptera and Lepidoptera differed significantly in their relative infection frequencies with A and B *Wolbachia* (Fisher's exact test  $p < 0.001$  and  $n = 54$ ).

#### (d) Comparisons of assay methods

Of the initial 89 samples that were screened for *Wolbachia* using the general *ftsZ* primers (and which were positive for 28S rDNA amplification), ten (11.2%) were positive for *Wolbachia*. We rescreened these samples using W-E primers. A total of six additional insects tested positive for the W-E primers. Initially, it was believed that these may represent divergent types of *Wolbachia* or related species, because the W-E primers are more generally designed to amplify bacteria with sequences in the

range of *Wolbachia* to *Ehrlichia*, the most closely related other genera of *Rickettsia* (Breeuwer *et al.* 1992; O'Neill *et al.* 1992). However, partial sequencing of the 16S product revealed these to be typical A group *Wolbachia* (data not shown). Subsequent amplification with A-specific *ftsZ* and 16S primers confirmed this result. Therefore, no *Ehrlichia* or divergent *Wolbachia* were detected in the sample, although the initial screen with the *ftsZ* general primers did miss bacteria detected with the W-E primers. Subsequently, the complete sample set (151) was screened with either the W-E, W-Spec or both sets of primers in addition to an initial screen with the *ftsZ* general primers. Seventeen infections were detected with *ftsZ* and an additional 11 were detected by the W-E or W-Spec

primers. All samples detected by the W-E primers also tested positive for the W-Spec primers (and vice versa). Most of the 11 samples that tested negative in the initial *ftsZ* screen were positive for the A- or B-specific *ftsZ* primers.

The results indicate that, in this particular sample, the 16S rDNA primers were more sensitive in detecting *Wolbachia* infections (18.5%) than the general *ftsZ* primers (11.3%). We attributed this to the relative storage ages of the samples when they were tested. We conducted a study showing that the detection of *Wolbachia* by *ftsZ* declines with the time of the samples in storage (95% ethanol and refrigeration at 4 °C or -20 °C), whereas the detection levels with the W-E and W-Spec primers remained high (C. Kennedy and J. H. Werren, unpublished data). This is most probably due to some degradation of the DNA within the samples, which affects the amplification of the *ftsZ* product more adversely. It should be noted that the British survey found nearly identical infection rates using the *ftsZ* and W-E primers on freshly collected material and the Panama sample was also tested shortly after collection. Therefore, we concluded that using the overall detection level in the Indiana samples was most appropriate for comparisons to the British and Panama surveys.

#### (e) *Large-scale Wolbachia dynamics*

The overall frequencies of *Wolbachia* were similar (*ca.* 20%) in the samples of insects from the three different geographic regions, i.e. tropical North America, temperate Europe and temperate North America. This result provides a first possible glimpse of the global dynamics of *Wolbachia*.

What might be determining the frequency of *Wolbachia* infections on a large scale? In simple terms, the dynamics of infected species will be a balance between the rates of acquisition of infections by species and the rates of loss of infected species. Assuming for the moment that there are no differences in the speciation or extinction rates of infected and uninfected species, then the frequency of infected species ( $f$ ) at equilibrium may be approximated by  $f(1-f)T = fL$ , where  $T$  is the transmission rate to new species and  $L$  is the loss rate of infections in infected species. Normalizing  $L = kT$ , the equilibrium frequency is  $f^* = 1 - k$ . Under these conditions, the loss rate is then expected to be *ca.* 80% of the new infection rate at equilibrium to account for frequencies of *ca.* 20% in the three regions.

## 4. DISCUSSION

The results indicated similar levels of *Wolbachia* infection among insect species from three different locales spanning two continents. However, the statuses of most of the species in these samples were based on a single or few individuals. As a result, species with infections not at fixation were less likely to test positive and the actual frequencies of the infected species were almost certainly higher than those shown in these studies. Nevertheless, testing of one or a few individuals per species is probably the best initial approach to obtaining an estimation of the overall patterns of infected species. A subset of species should be sampled more extensively in order to determine

the frequency distribution of infection polymorphisms. Once this is accomplished, broader scale surveys can be adjusted to estimate the numbers of infected species more accurately.

A second problem with these data concerns the unsystematic sampling scheme. In Panama, insects for which there was a reasonable chance of obtaining identification at least to genus level were collected. Due to the expertise available (and the proclivities of the collectors), the sampling focused more on Coleoptera, Hymenoptera and Lepidoptera. In the British samples, similar principles applied with sampling primarily of Lepidoptera and Hymenoptera. Some of these species were also from a related community of leaf-mining Lepidoptera and associated parasitoids. In the current sample from Indiana, attempts were made to sample a wider spectrum of insect orders. However, most samples came from the common orders mentioned previously. All the sampling efforts were likely to have been biased towards locally abundant species. However, it should be pointed out that the species were selected without prior knowledge of infections in them or closely related species and, therefore, the sample was not biased by such an effect.

Several taxon-focused surveys of insects have recently been performed in mushroom-feeding *Drosophila* (Werren & Jaenike 1995), other *Drosophila* (Bourtzis *et al.* 1996), stalk-eyed flies (Hariri *et al.* 1998), parthenogenetic gall wasps (Schilthuizen & Stouthamer 1998) and ants (Wensleers *et al.* 1998). Several of these have shown frequencies of infection dramatically higher than found in the broader surveys reported here. It should be kept in mind that there was detection bias in several of these surveys that was not present in the ones we conducted. For example, the survey on gall wasps arose from the high frequency of parthenogenesis in these insects, a reproductive mode known to be induced by *Wolbachia* in some Hymenoptera (Stouthamer *et al.* 1993), and the interest in ants resulted partly from earlier detection of *Wolbachia* in these insects (Werren *et al.* 1995b). Thus, groups with a higher frequency of *Wolbachia* infections may be preferentially surveyed due to preliminary results indicating infection incidences.

Such findings suggest that certain taxonomic groups may be more prone to acquiring or maintaining *Wolbachia* infections than others. For example, over 50% of a set of south-east Asian ant species tested positive for *Wolbachia* (Wensleers *et al.* 1998). This could indicate that ants are particularly prone to acquiring the bacteria, although it has not yet been established whether this is a feature of ants in general or of ants (or insects) from the region surveyed. In addition, there are clear differences between insect orders in their relative frequencies of infection with A versus B *Wolbachia*. In particular, Hymenoptera show higher infection levels with A *Wolbachia* and Lepidoptera show higher infection levels with B *Wolbachia*. These results may indicate differences in the ability of A and B *Wolbachia* to infect different taxa, differences in the retention of such infections or historical differences in the emergence and spread of *Wolbachia* within these taxa. The frequency of double (A plus B) infections was found to be higher in the Neotropical sample (Panama) than in either temperate sample (Britain or Indiana). Additional study is needed in order to determine whether this pattern

holds true. If correct, then it implies different horizontal infection dynamics in these different regions.

A major question is whether *Wolbachia* are in some form of global equilibrium where the numbers of newly infected species approximately equal the numbers lost. Alternatively, these bacteria may be undergoing an expansion, in which case the infection frequencies are not at equilibrium. In particular, it has been argued that *Wolbachia* may be undergoing expansions into new host species as a result of human ecological disturbances (Werren 1997). Whether *Wolbachia* are at global equilibrium or undergoing expansion depends upon the rates of gain and loss of infected species.

It is clear that *Wolbachia* are horizontally transmitted between host species, although the mechanisms of the transmission are still uncertain. Werren *et al.* (1995b) found phylogenetic evidence of intertaxon transmission between parasitic wasps (*Nasonia*) and their blowfly hosts (*Protophthora*) and Vavre *et al.* (1999) found similar evidence for some drosophilid parasitoids. In further support of parasitoid–host insect transmission, Heath *et al.* (1999) found that *Wolbachia* can be naturally transmitted from infected *Drosophila simulans* to its larval parasitoid *Leptopilina boulardi* in the laboratory, although the bacteria had seriously reduced transmission in the novel host. However, West *et al.* (1998) and Schilthuisen & Stouthamer (1998) did not find phylogenetic evidence of horizontal transmission between parasitoids and hosts in two different insect guilds. The general view is that ecologically intimate species (e.g. parasites and hosts) are conduits for the transmission of these intracellular bacteria, although the data sets are not yet extensive enough to determine at what rates parasitoid–host versus other mechanisms of transfer occur.

Once established in a species, cytoplasmic-incompatibility-inducing *Wolbachia* can rapidly increase in host populations (Turelli & Hoffmann 1991; Turelli 1994). Similarly, parthenogenesis-inducing and feminizing *Wolbachia* have selective advantages in host populations due to increased production of the (vertically) transmitting sex, i.e. females. The mechanisms of loss of *Wolbachia* from infected species are much less clear. It has been posited that, once cytoplasmic-incompatibility-inducing *Wolbachia* become common in a population, mutation accumulation will result in a slow decline in the bacteria able to cause cytoplasmic incompatibility (Hurst & McVean 1996). However, this is just one possible evolutionary trajectory for the infection (Frank 1997). Others include host genetic changes that suppress modification in the male (Turelli 1994), which could also lead to eventual loss, invasion by a second infection (which can lead to double-infected species) and evolution of new incompatibility types within a species, which can maintain the infection (Werren 1998).

It is currently unknown how frequent loss occurs relative to the infection of new species. However, it is clear that, to achieve global equilibrium in the frequency of infected species, some sort of equilibrium between these processes is necessary. As indicated by the model above, if there is global equilibrium then the rates of loss must be ca. 80% the new infection rate to account for the observed infection frequency of ca. 20%. Therefore, we might expect to find many species where *Wolbachia* are at

intermediate or low frequency and in which hosts have evolved resistance to *Wolbachia*, as has been suggested to occur in *Drosophila melanogaster* (Clancy & Hoffmann 1996). In addition, if a common mechanism of *Wolbachia* loss is evolution of modification rescue-deficient *Wolbachia* (mod– resc+ and mod– resc–) within a species, then we should expect to find many host species in which closely related strains of *Wolbachia* occur, which are mod– resc+, mod– resc– and mod+ resc+. So far, this has not been the case; where ‘defective’ and functional *Wolbachia* are found in the same species, they appear to be from different bacterial strains (Zhou *et al.* 1998). Finally, differential extinction and speciation rates of infected and uninfected species could be a factor influencing the frequency of *Wolbachia* in insect communities.

In conclusion, surveys in three locales on two continents showed similar levels of infection of *Wolbachia* (ca. 20%) and higher levels of A *Wolbachia* infection in Hymenoptera and B *Wolbachia* infection in Lepidoptera. Double infections occurred more frequently in the Neotropical sample (Panama) than in either of the temperate samples (Indiana or Britain). Additional regional-, taxon- and community-based sampling of *Wolbachia* will provide the kinds of data sets needed to draw robust inferences concerning the distribution, evolution and dynamics of *Wolbachia*. In particular, further studies are needed to resolve whether *Wolbachia* are undergoing an expansion or are at global equilibrium.

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