Distribution of *Wolbachia* among neotropical arthropods

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SUMMARY

Wolbachia are a group of cytoplasmically inherited bacteria that cause reproduction alterations in arthropods, including parthenogenesis, reproductive incompatibility and feminization of genetic males. Two major subdivisions of *Wolbachia* (A and B) occur. *Wolbachia* are found in a number of well-studied insects, but their overall distribution in arthropods has not been well studied. A survey for *Wolbachia* in 157 Panamanian neotropical arthropod species was done using a polymerase chain reaction assay. *Wolbachia* were detected in 26 of 154 insect species (16.9%) and zero of three arachnids (0%). Extrapolating to the estimated total number of insect species present globally (10–30 million), an estimated 1.69–5.07 million insect species are infected with these bacteria, making *Wolbachia* an extremely abundant bacterial group. *Wolbachia* were found in each of the major insect orders examined, including Coleoptera (6/57 infected), Diptera (5/14), Hemiptera/Homoptera (1/7), Hymenoptera (6/23) Lepidoptera (7/43) and Orthoptera (1/8). Of the 26 positives, eight species were found to be singly infected with A group *Wolbachia*, nine singly with B group *Wolbachia* and nine doubly infected with both A and B group *Wolbachia*. Double infections occur at significantly higher frequencies than expected by chance. The abundance of *Wolbachia* further supports their potential importance as a mechanism for rapid speciation in insects.

1. INTRODUCTION

Wolbachia are 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. *Wolbachia* are associated with reproductive incompatibility (termed cytoplasmic incompatibility or CI), parthenogenesis, and feminization of genetic males (Barr 1980; Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993). These bacteria have been implicated as a possible mechanism for rapid speciation in insects (Laven 1967; Breeuwer & Werren 1990; Coyne 1992).

Sequencing of bacterial 16S rDNA (Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993) and protein coding (Werren *et al.* 1995) genes has shown that cytoplasmic incompatibility, parthenogenesis, and feminizing bacteria form a closely related group in the alpha proteobacteria, the *Wolbachia*. Using a protein coding region (*ftsZ*), Werren *et al.* (1995) found two major groups of *Wolbachia* (designated A and B). Both groups are widespread in insects. Based upon synonymous substitution rates, these two groups are estimated to have diverged 58–67 MaBP, indicating that *Wolbachia* have been around since the late mesozoic to early cenozoic. The phylogenetic data also shows horizontal transmission of *Wolbachia* between insect taxa, including between different orders.

Wolbachia have so far been detected in over 40 arthropod species (O'Neill *et al.* 1992; Stouthamer *et al.* 1993; Werren *et al.* 1995). Most of these are insects, but *Wolbachia* have also been detected in two isopods (Rousset *et al.* 1992) and a mite (Breeuwer, personal commun.). Among insects, they have been found in all the major orders, including Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, and Orthoptera. However, there is little data on the actual distribution of *Wolbachia* among arthropods, and no systematic surveys of *Wolbachia* distribution have been published.

The purpose of this study is to investigate the distribution of *Wolbachia* among a sample of neotropical arthropods from Panama. Arthropods (primarily insects) were collected and tested for presence of *Wolbachia* using an assay based upon polymerase chain reaction (PCR) amplification of the *ftsZ* protein coding gene from *Wolbachia*.

2. METHODS

(*a*) *Arthropod collection and handling*

Arthropods were collected in the vicinity of Barro Colorado Island, Gamboa, Panama City and Cerro Campana from August 1993 to October 1994. Live arthropods or dissected ovaries from live arthropods were fixed in 95% (by volume) ethanol. Specimens were kept under refrigeration (around 4 °C) until shipment to Rochester New York for further analysis. There the fixed specimens were kept under

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refrigeration until DNA extraction. Arthropod remains are stored in the insect voucher collection at the Smithsonian Tropical Research Institute, Panama City, Republic of Panama. Seven drosophilid species were collected by Dr Jan Sevenster and kept in laboratory culture before we tested them.

(*b*) *Template preparation*

DNA was extracted from the fixed material of 1–10 individuals (depending on size) using either: (i) eggs or ovaries; (ii) whole abdomens; or (iii) whole individuals (for small arthropods). The tissues were dissected in sterile double-distilled deionized water on a sterile petri dish and then serially rinsed in droplets of sterile H_2O, before extraction of DNA using our previously published methods (Breeuwer *et al.* 1992; Werren & Jaenike 1995). Extreme care was taken throughout the procedure to avoid contamination. All solutions were filter sterilized (0.22 μm pore size). Control DNA samples were prepared using pupae or ovaries of known infected and uninfected strains of either *Nasonia vitripennis* or *Drosophila simulans*.

(*c*) *Assay for* Wolbachia

To test for presence of *Wolbachia*, PCR was performed using *Wolbachia* specific primers for the *ftsZ* bacterial cell-cycle gene (Werren *et al.* 1995). 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 subdivision of *Wolbachia*. To confirm presence of *Wolbachia ftsZ*, PCR product was screened against A and B *Wolbachia ftsZ* by a dot-blot procedure (see below). In addition, some products were sequenced and compared with known A and B group *Wolbachia*.

Absence of amplification using the general *ftsZ* primers (a presumptive negative) could be caused by either: (i) absence of *Wolbachia* in the insect; (ii) failure in the DNA extraction procedure; or (iii) incorrect concentration of DNA solution. As a control for amplifiablility of the DNA extraction, samples that were negative for *ftsZ* were tested by using primers for highly conserved regions of eukaryotic 28S rDNA (Burke *et al.* 1993).

From experience we have often found positive amplifications following dilution of the DNA. This is caused by either excess template DNA concentration or presence of inhibiting substances. Therefore, samples were tested at various dilutions ranging from one fifth to one hundredth (depending upon the DNA concentrations apparent from ethydium bromide staining intensity). The maximum concentration yielding a 28S rDNA positive was then used to retest for *Wolbachia* with the general *ftsZ* primers. Samples that failed to yield a positive by the 28S rDNA primers for all concentrations (false negatives) were not included in the study. Additional positive controls using known infected insects (*Nasonia vitripennis* or *Drosophila simulans*) were performed with each set of PCR reactions.

(*c*) *PCR methods*

Amplification of the nearly complete *Wolbachia ftsZ* sequence was accomplished with primers 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 to 1055 b.p. product (depending upon *Wolbachia* strain). These primers are specific for *Wolbachia* (Werren *et al.* 1995). Procedures and primers for ftsZ amplification are in Werren *et al.* (1995).

Controls for PCR amplifiability of DNA solutions were done using general eukaryotic 28S rDNA primers, 28Sf (5′ CCC TGT TGA GCT TGA CTC TAG TCT GGC 3′) and 28Sr (5″ AAG AGC CGA CAT CGA AGG ATC 3′). PCR cycling conditions were one cycle (1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C), 35 cycles (15s at 94 °C, 1 min at 55 °C, 2 min at 72 °C) and one cycle (15s at 94 °C, 1 min at 55 °C, 7 min at 72 °C) (Ericomp thermal cycler). These yield a 500–600 b.p. product, depending upon presence of expansion domains.

Primers for specific amplification of A and B group *ftsZ* were also used (Werren *et al.* 1995). The A group *ftsZ* primers ftsZAdf (5′CTC AAG CAC TAG AAA AGT CG 3′), ftsZAdr (5′TTA GCT CCT TCG CTT ACC TG 3′) specifically amplify a 955–957 region of *ftsZ* from the Adm group of *Wolbachia*, but not from the Atc group (Werren *et al.* 1995). The B primers 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 specifically from B group bacteria.

To confirm specificity of the A and B group *ftsZ* primers, restriction digests were performed on some PCR products. B group *Wolbachia* typically have one *AciI* restriction site, producing fragments of 243 b.p. and 752 b.p., whereas Adm *Wolbachia* have two *AciI* restriction sites, producing fragments of size 178 b.p., 462 b.p. and 297 b.p.

(*d*) *Dot blot assay*

To confirm that PCR products that were amplified using the general ftsZ primers actually represent *Wolbachia ftsZ*, PCR products were separately hybridized to ^{32}P labelled A group and B group ftsZ DNA using a dot blot procedure. One μl PCR product was suspended in 75 μl distilled H_2O and 25 μl 20X SSC and the DNA was denatured at 95 °C for 10 min. After addition of 100 μl 20X SSC with bromophenol blue, the solution was put on ice and 50 μl of the mixture was loaded onto a nylon filter using the Minifold I microsample filtration manifold (Schleicher & Schuell). Two filters with the same samples were produced. These were treated with denaturization solution (0.5 M NaOH, 1.5 M NaCl) for five minutes, then with neutralizing solution (0.5 M Tris–HCL (pH 7.5) 1.5 M NaCl) for 5 min, followed by 2X SSC treatment for 5 min. DNA was UV crosslinked to the nylon filters. The filters were then prehybridized for 3 h at 65 °C and then hybridized overnight at 65 °C with ^{32}P labelled ftsZ product amplified from either A group ftsZ or B group ftsZ cloned DNA as template (Gibco BRL kit). The A and B group clones were from *Wolbachia* sequences amplified from the wasp *Nasonia vitripennis* (strain LbII). Both prehybridization and hybridization solutions were 2X SSC, 5X Denhardt's, 1% (by volume) sodium pyrophosphate, 25 mM sodium phosphate, 250 mgml^{-1} denatured ctDNA and 1% (by volume) SDS in distilled water. Following hybridization, filters were rinsed at moderate (55 °C, 0.1 × SSC) and then high stringency (68 °C, 0.1 × SSC). The filters were exposed to autoradiographic film for 15–24 hours at room temperature. Hybridization intensities to group A and group B probes were scored visually.

3. RESULTS

Representatives of 157 different arthropod species (154 insects and three arachnids) were screened for *Wolbachia* by PCR using the general *Wolbachia ftsZ* gene primers. Of the 154 insect species screened, 26 (16.9%) were found to be infected. The three arachnids were uninfected.

Table 1. *Distribution of* Wolbachia *in Panamanian insect orders and Arachnids*

(The distribution of *Wolbachia* infections detected using the general *ftsZ* PCR assay are shown for the different insect orders and arachnids.)

taxon	positive	total no.	% positive
Insecta	26	154	16.9
Coleoptera	6	57	10.5
Diptera	5	14	35.7
Hemiptera/ Homoptera	1	7	14.3
Hymenoptera	6	23	26.1
Lepidoptera	7	43	16.3
Orthoptera	1	8	12.5
Odonata	0	2	0
Arachnida	0	3	0
total	26	157	16.6

Table 1 presents the frequency of detected infections based upon insect order. Infected individuals were found in each of the major orders tested, including Coleoptera (10.5% infected $N = 57$), Diptera (35.7% $N = 14$), Hemiptera/Homoptera (14.3% $N = 7$), Hymenoptera (26.1% $N = 23$), Lepidoptera (16.3% $N = 43$) and Orthoptera (12.5% $N = 8$). The frequency of infected species does not differ significantly between orders ($\chi^2 = 6.5$, d.f. $= 5$, $p > 0.10$). However, sample sizes are too small and sampling of species too sporadic to draw strong conclusions on differences between orders in the frequency of *Wolbachia* infections.

False negatives (testing negative for *Wolbachia* when the bacteria actually occur in the species) have been minimized by using control PCR reactions using general eukaryotic 28S rDNA primers. Samples that were negative for the control reaction were retested using concentrations ranging from one fifth to one hundredth of the original DNA extraction concentration. Samples were then tested for *ftsZ* at the highest concentration that yielded a positive result by the control PCR. Any specimens that failed to amplify the control reaction at any of the tested concentrations were not included in the analysis. Of the 193 samples first tested (from 178 different species), 48 were negative for 28S rDNA amplification at the original DNA concentration. Of these, 22 produced 28S rDNA product at lower concentrations. Sixteen of these remained negative for *Wolbachia ftsZ* at the lower concentration, whereas six yielded *Wolbachia ftsZ*. The remaining 26 did not produce product with the 28S rDNA primers at any of the tested concentrations, and were excluded. Failure to amplify the 28s rDNA control is most likely the result of improper DNA extraction or presence of substances inhibiting the PCR reaction. In some cases amplification may have been prevented by sequence differences in the 28S rDNA domains. However, regions from which these primers were designed are highly conserved among arthropods, suggesting that this is unlikely.

To confirm that the amplified products were indeed *Wolbachia ftsZ*, all reactions that yielded possible *ftsZ* products with the general *ftsZ* primers were dotted onto nitrocellulose filters and separately probed with ^{32}P labelled *Nasonia vitripennis* B group and A group *ftsZ* product using moderate to stringent hybridization criteria (see §2). Product from all 26 positive species cross-hybridized to both A and B group *ftsZ* clone DNA under moderate stringency. Even under highly stringent hybridization conditions, there was cross-hybridization between A and B group ftsz DNA, based upon hybridization to known A and B PCR products. Therefore, the dot blot procedure could not be used to reliably distinguish between A and B group bacteria, although it was successful in detecting *Wolbachia ftsZ* sequences present in PCR product. For example, the ant *Ectatoma tuberculatum* yielded multiple bands from PCR amplification, including a weak band approximately the correct size for *Wolbachia ftsZ*. The dot blot hybridization procedure confirmed presence of *Wolbachia ftsZ* in this product.

Table 2 presents a list of individual species tested for *Wolbachia*. In some cases, arthropods have not yet been identified to species, but only to genus, family or, in a few cases, to order only. For these specimens, our sample identification number is included in table 2. Among the Coleoptera, *Wolbachia* were detected in three chrysomelids, *Chelymorpha alternans*, *Chresinellina heteropunctata* and *Acromis sparsa*. Both *A. sparsa* and *C. alternans* are highly derived tortoise beetles in the subfamily Cassidinae tribe Stolaini (Boroweic 1995), that feed exclusively on convolvulaceous vines. The third species, *C. heteropunctata*, is currently classified within the distant basal tribe Cassidini, but bears sufficient similarity to warrant its placement in the stolaini (D. Windsor, unpublished data). Thus, all three infected species appear to occur in this tribe. Phylogenetic analysis of the host beetles and associated *Wolbachia* could indicate whether the bacteria were acquired before host divergence.

Wolbachia infections were found in two weevils (curculionids), *Cossonus* sp. and an unidentified species (ID# 904J). The *Cossonus* weevil, collected from a dead *Ficus* tree, was found to be doubly infected with A and B group bacteria. The unidentified weevil 904J, appears to be from a genus of South American origin (H. Stockwell, personal comm.) and was found to harbour B group bacteria. A double AB infection of *Wolbachia* was also detected in a clerid beetle of the genus *Priocera* (unidentified species).

Among the Diptera, an unknown species of *Nerius*, the stratiomyid fly *Cynomyia cyanea* and three drosophilid fly species (*D. melanogaster*, *D. tropicalis* and *D. willistoni*) were found to be positive for *Wolbachia*. *D. tropicalis* and *D. willistoni* belong to the *willistoni* species group of *Drosophila*. This species group is particularly interesting because it is highly speciose and cytoplasmically inherited microorganisms (L-form streptococci) have previously been described that cause F1 hybrid sterility in some members (Ehrman & Powell 1982; Somerson *et al.* 1984).

Among Hemiptera, the reduviid 'kissing bug' *Rhodnius pallescens* was found to harbour *Wolbachia*. *R. pallescens* is a vector of *Trypanosoma cruzii*, the protozoan agent that causes Chagas disease. In two different individuals tested, one was found to have B group

Table 2. *Distribution of* Wolbachia *by species*

(Identification of tested arthropods to order, family and species are shown along with results of the PCR assays. Presence of A, B or double (AB) infections were detected based upon PCR using group specific primers. Arthropods that have not been identified to species (or family) are placed in the appropriate family (or order) and indicated by the identification number. If multiple individuals were tested for a species, the number tested is indicated in parentheses.)

taxon	*Wolbachia*
Coleoptera	
Brentidae	
Brentus anchorage	–
Carabidae	
Coptodear	–
Morion sp.	–
Agra sp.	–
Chrysomelidae	
Polychalma multicava	–
Calyptocephela brevicornis	–
Pseudimatidium sp.	–
Disonycha trifasciata	–
Chelymorpha alternans	B (3)
Chersinellina heteropunctata	B
Acromis sparsa	AB
Cephaloleia sp.	–
Cephaloleia sallei	–
Coptocycla leprosa	–
Pseudimatidium sp.	–
Platyphora sp.	–
Diabrotica sp.	–
Colaspis sp.	–
Platyphora encosma	–
Spaethiella sp.	–
Asphaera reichei	–
Imatidium thoracicum	–
Tapinaspis wesmaeli	–
Cephaloleia sp.	–
Aslamidium impurum	–
Prosopodonta dorsata	–
Cleridae	
Priocera sp.	AB
Curculionidae	
Pseudopthalmus sp.	–
Rhinostomus barbirostris	–
Peridinetus subirroratus	–
Cossonus sp.	AB
Stegotes sp.	–
Rhynchophorus palmarum	–
Pachyschelus sp.	–
904J Unid. sp.	B
Xestolabus corvinus	–
Phelypera distigma	–
Elateridae	
Semiotus liqneus	–
Semiotus insignis	–
Endomychidae	
Stenotarsus rotundus	– (2)
Erotylidae	
Pselaphacus vitticollis	–
Pselaphacus conspersus	– (2)
Cypherotylus sp.	–
Lycidae	
JW14 Unid. sp.	–
Other	
010 Unid. sp.	–
Epilachna borealis	–
Passalidae	
Passalus punctiger	–
Scarabeidae	
Canthidium haroldi	– (2)
Canthidium auritex	–
Onthophagus acuminatus	–
Canthon angustatus	–
Cyclocephala corbonarius	–
Diplotaxis sp.	–
Canthon sp.	–
Cyclocephala sp.	–
Cyclocephala sp.	–
Trogositidae	
Temnochila sp.	–
Diptera	
Drosophilidae	
Drosophila sp.	–
D. equinoxialis	–
D. malerkotliana	–
D. melanogaster	A
D. nebulosa	–
D. sturtevanti	–
D. tropicalis	B
Dn. willistoni	A
Neridae	
Nerius sp.	AB
Glyphidops sp.	–
Pantophthalmidae	
Pantophthalmus tabinus	–
Stratiomyidae	
Cynomyia cyanea	AB
Richardiidae	
Automola atomaria	–
Tabanidae	
Tabanus sp.	–
Hemiptera/Homoptera	
Cicadidae	
Zammara smaragdina	–
Fidicina mannifera	–
Pentatomidae	
Euschistus sp	–
Reduviidae	
Montina sp.	–
828O Unid. sp.	–
Rhodnius pallescens	AB(1) B(1)
Leogorrus litura	–
Hymenoptera	
Family?	
007 Unid. sp.	–
827C Unid. sp.	–
Agaonidae	
Tetrapus costaricensis	A
Apidae	
Trigona sp.	A
Chalcidae	
Citrogaster sp.	–
Evaniidae	
902X Unid. sp.	–
P6 Unid. sp.	–
Formicidae	
Cryptoceras	–
Ectatomma tuberculatum	A(1)–(1)
Ichneumonidae	
P2 Unid. sp.(Orthocentrinae)	–
P3 Unid. sp.(Orthocentrinae)	–

Table 2. (*cont.*)

taxon	*Wolbachia*	taxon	*Wolbachia*
P4 Unid. sp.	A	M9 *Cissia* sp. 1	AB
P5 Unid. sp.(Pimplinae)	A	M10,M21 *Cissia* sp. 2	AB(1)–(1)
P7 Unid. sp.(Ichneumoninae)	–	*Heliconius erato*	–(3)
P8 Unid. sp.(Crytinae)	–	*Heliconius melpomene*	–
828M Unid. sp.	–	*Hamadryas amphinome*	–
006 Unid. sp.	–	*Agraulis* sp.	–
Pompilidae		*Eumaeus* sp.	–
P1 Unid. sp.		Papilionidae	
Vespidae	A	*Papilio thoas*	–
Mischocyttarus sp.	–	*Battus* sp.	–
Mischocyttarus sp.	–	*Junonea coenea*	–
Polistes versicolor	–	*Eurema nice*	–(5)
Metapolybia sp.	–	*Eurema daira*	–(2)
Polybia sp.	–	Pyralidae	
Lepidoptera		*Parapoynx* sp.	B
Family?		902P Unid. sp. 1	–
104G Unid. sp.	B	Riodinidae	
Arctiidae		M16 Unid. sp.	–
Virbia birchii	–	Saturnidae	
829D Unid. sp. 1	B	*Adeloneivaia* sp.	–
L14 Unid. sp. 2	–	826E Unid. sp.	–
Castniidae		104K Unid. sp.	–
Unid. sp. 1	–	104J Unid. sp.	–
Hesperiidae		Orthoptera	
Heliopetes (?) sp.	–	Family?	
Lycaenidae		828G Unid. sp.	–
M11 Unid. sp.	–	Acrididae	
Thecla sp.	–	904K Unid. sp.	–
Noctuididae		Blaberidae	
104F Unid. sp.	–	*Pycnoscella sapinamenela*	–
Notodontidae		Blatellidae	
902D unid. sp. 1	–	*Nyctibora* sp.	–
902V unid. sp. 2	–	104C Unid. sp.	–
902AD unid. sp. 3	–	Tettigoniidae	
Josia draconis	–(2)	*Microcentrum* sp. 2	–
Nymphalidae		*Microcentrum* sp. 1	–
Anartia jatrophe	–	*Lophaspis scabricula*	B
Anartia fatima	–(8)	Odonata	
Agraulis vannilae	–(2)	Libellulidae	
Dryas junio	–(2)	JW1, JW8 Unid. sp.	–(2)
M13 *Anathanassa* sp.	–	JW2 Unid. sp. 2	–
M23 Unid. sp.	–	Arachnida	
Dynamine thalassina	–	Salticidae	
Junonia evarete	–(4)	902K Unid. sp.	–
Danaus plexippus	–	Araneidae	
Cissia libye	B	*Eriophora fuliginea*	–(2)
Cissia usitata	AB(1)–(3)	902Q Unid. sp.	–

bacteria and the other to have a double infection of A and B group bacteria. Three hymenopteran species were positive for *Wolbachia*. Stingless bees of the genus *Trigona* are widespread in the tropics. An unidentified species in this genus was found to harbour A group *Wolbachia*. One of two individuals in the ant *Ectatomma tuberculatum* was positive for *Wolbachia*. In addition the fig wasp *Tetrapus costaricensis* harbours A *Wolbachia*, as do two ichneumonid wasps and one vespid wasp.

An extensive set of Lepidoptera (43 species) were examined, and seven were found positive for *Wolbachia*. Of particular interest is the genus *Cissia*, where *Wolbachia* have been detected in all four species examined. This could represent another case where *Wolbachia* were acquired before speciation, and subsequently co-radiated with the host. Alternatively, they may represent independent horizontal transmission events. *Wolbachia* were also detected in the pyralid *Parapoynx sp.*, a rather interesting group of 'aquatic' moths in which larvae feed on submerged aquatic plants. Among the Orthoptera, a single species, *Lophapsis scabricula*, (Tettigoniidae), tested positive.

Results provide information on the distribution of A group and B group *Wolbachia* and the occurrence of single versus double infections. Among the species examined in this study, eight had single A group infections, nine had single B group infections and nine had double AB infections. The double infection

category includes *Rhodnius pallescens*, which was polymorphic, giving one AB individual and one B individual. This gives a frequency of double infections (including *R. pallescens*) among infected species of 34.6% compared with 30.8% for single A infections and 34.6% for single B infections.

The overall frequency of species infected with A (including A and AB infections) is 10.8% and with B (including B and AB infections) is 11.5%. Therefore, the random expectation for frequency of double infections is 1.2%. The observed frequency of 5.7% is significantly greater than expected ($\chi^2 = 24.8$, d.f. = 1, $p > 0.001$).

4. DISCUSSION

This study clearly shows that *Wolbachia* are widespread and common, at least among insects. Indeed, *Wolbachia* appear to be an extremely abundant group of bacteria. There are currently 750000 known insect species globally (Wilson 1993). Extrapolating from 16.9% infected neotropical insect species gives 126750 insect species with *Wolbachia* infections. However, the estimated total number of insect species (known and not yet discovered), ranges from 10–30 million, giving an estimated 1690000– 5070000 species infected with *Wolbachia*. Either estimate clearly makes *Wolbachia* among the most abundant and widespread group of parasitic bacteria.

The approximations above hinge on *Wolbachia* having similar frequencies among insects in different parts of the world. Our unpublished preliminary survey of neotemperate insects supports this view, showing 11.3% infected species. Similarly, an unpublished survey of neotemperate insects also places infection levels around 15% (R. Giordano & H. Robertson, personal comm.). However, systematic surveys in other parts of the world have yet to be performed.

Data on the occurrence of these bacteria in other arthropods is limited. *Wolbachia* have so far been found in isopods (Rousset *et al.* 1992; Juchault *et al.* 1994) and mites (J. A. J. Breeuwer, personal comm.). The findings suggest that these bacteria may be common in other arthropods. We also do not know whether this bacterial group occurs in animals other than arthropods. Discovery of *Wolbachia* in other phyla would be a significant finding.

Species were chosen in this study independent of any prior knowledge of whether *Wolbachia* were present. Thus, it represents a reasonably 'random' sampling of insects, at least with respect to infection status. Nevertheless, a limitation of this study is that, for many of the species examined, only one individual was tested (see table 2). Thus, the estimate of 16.9% of insect species harbouring *Wolbachia* infections is almost certainly an underestimate. Basic theories for the dynamics of CI *Wolbachia* suggest that the infection will rapidly spread to fixation in a population once introduced (Caspari & Watson 1959). Therefore, we may expect that for most species with *Wolbachia*, the infection is near fixation. However, several cases of polymorphisms of *Wolbachia* infection are known, most notably in *Drosophila simulans* (Turelli & Hoffmann 1991) and *Drosophila melanogaster* (Hoffmann *et al.* 1994). In some cases polymorphic equilibria can be expected, particularly where insects are naturally exposed to antibiotics (Stevens & Wicklow 1992) or more generally when transmission of the infection to progeny is incomplete (Turelli 1995). More extensive sampling of infected and 'apparent' uninfected species is needed to determine the distribution of infection levels in different species.

Wolbachia are known to produce a variety of reproductive alterations in arthropods, including cytoplasmic incompatibility, parthenogenesis, and feminization of genetic males (Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993). The neotropical isolate of *Drosophila melanogaster* examined here presumably harbours a CI bacteria, because CI *Wolbachia* have been described in this species (Hoffman *et al.* 1994). However, the etiologies of the other 25 cases described here are unknown. Cytoplasmic incompatibility appears to be the most common phenotype caused by *Wolbachia*, with parthenogenesis induction so far being restricted to *Wolbachia* in some Hymenoptera (Stouthamer *et al.* 1993; Werren *et al.* 1995) and feminization to *Wolbachia* in isopods (Rigaud *et al.* 1991; Rousset *et al.* 1992; Juchault *et al.* 1994). *Wolbachia* have been found in parthenogenetic weevils, although the bacteria have not been established as the cause of parthenogenesis (Werren *et al.* 1995).

Results presented here show that *Wolbachia* are widespread and abundant in arthropods. Given this fact, it is likely that *Wolbachia* have evolved a variety of interactions with their hosts. In addition to cytoplasmic incompatibility, parthenogenesis and feminization, it is reasonable to expect that some strains will have evolved mutualistic symbioses with their hosts. This is expected particularly because these bacteria are routinely cytoplasmically inherited, a transmission pattern that can promote the evolution of mutualism (Fine 1975). In fact, individual *Wolbachia* strains could evolve both reproductive manipulation and mutualistic (beneficial) effects on hosts. These are not mutually exclusive phenotypes for *Wolbachia*.

The phylogenetic distribution of *Wolbachia* clearly shows that horizontal transmission of the bacteria does occur between host taxa, even between different orders (O'Neill *et al.* 1992; Werren *et al.* 1995). Thus, *Wolbachia* have the potential for horizontal transmission and may have evolved increased infectious transmission rates in some strains. Furthermore, given the abundance of this bacteria group, it is not unreasonable to expect that some *Wolbachia* are pathogenic, either to their insect hosts or to other organisms.

The occurrence of double infections is interesting. Breeuwer *et al.* (1992) first proposed the possibility of double infections of *Wolbachia*, based on the detection of multiple 16s rDNA sequences in the parasitoid wasp *Nasonia vitripennis*. Double infections have since been uncovered in a number of species by PCR using either 16s rDNA or *ftsZ* primers (Mercot *et al.* 1995; Perrot-Minnot *et al.* 1995; Rousset *et al.* 1995; Sinkins *et al.*

1995; Werren *et al.* 1995) and have been confirmed in *Nasonia* by southern hybridizations (Perrot-Minnot *et al.* 1995).

Werren *et al.* (1995) found double infections in seven of 29 species specifically examined for AB infections (24.1%). In the current study 34.6% of infected species harbored double AB infections. Furthermore, based upon the overall frequencies of A and B infections, we have found that species with double infections occur significantly more frequently than expected by chance. The finding is interesting, and suggests that either: (i) species that are prone to acquiring infections with one type bacteria are also prone to acquiring the other type; (ii) presence of one infection facilitates the establishment of a second infection (iii) double AB infections are more stably maintained within a species than are single infections; or (iv) double infections occur at higher frequencies within a species, thus leading to a detection bias. Species with double infections may also have higher rates of speciation or lower rates of extinction.

Wolbachia have been implicated as a mechanism for rapid speciation in insects (Laven 1967; Breeuwer & Werren 1990; Coyne 1992). For example, bacteria are involved in bidirectional reproductive incompatibility between sibling species of *Nasonia* (Breeuwer & Werren 1990), geographic races of *Culex* mosquitoes (Laven 1967; Subbarao *et al.* 1974) and strains of *Drosophila simulans* (O'Neill & Karr 1990; Montchamp-Moreau *et al.* 1991). The data presented here, which shows presence of *Wolbachia* in over 15% of insect species, further support the potential importance of these bacteria in speciation. Nevertheless, it has yet to be determined how frequently *Wolbachia* are associated with the formation of reproductive isolation between newly evolving species.

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