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# The distribution of *Wolbachia* in fig wasps: correlations with host phylogeny, ecology and population structure

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We surveyed for the presence and identity of *Wolbachia* in 44 species of chalcid wasps associated with 18 species of Panamanian figs. We used existing detailed knowledge of the population structures of the host wasps, as well as the ecological and evolutionary relationships among them, to explore the relevance of each of these factors to *Wolbachia* prevalence and mode of transmission. Fifty-nine per cent of these wasp species have *Wolbachia* infections, the highest proportion reported for any group of insects. Further, neither the presence nor the frequency of *Wolbachia* within hosts was correlated with the population structure of pollinator hosts. Phylogenetic analyses of *wsp* sequence data from 70 individuals representing 22 wasp species show that neither the close phylogenetic relationship nor close ecological association among host species is consistently linked to close phylogenetic affinities of the *Wolbachia* associated with them. Moreover, no genetic variation was detected within any *Wolbachia* strain from a given host species. Thus, the spread of *Wolbachia* within host species exceeds the rate of horizontal transmission among species and both exceed the rate of mutation of the *wsp* gene in *Wolbachia*. The presence and, in some cases, high frequency of *Wolbachia* infections within highly inbred species indicate that the *Wolbachia* either directly increase host fitness or are frequently horizontally transferred within these wasp species. However, the paucity of cospeciation of *Wolbachia* and their wasp hosts indicates that *Wolbachia* do not persist within a given host lineage for long time-periods relative to speciation times.

**Keywords:** Agaonidae; fig; fig wasp; horizontal transfer; *Wolbachia*; *wsp*

## 1. INTRODUCTION

*Wolbachia* are alpha-proteobacteria that are found in a wide range of insects, filarial nematodes, arachnids and isopods (Bandi *et al.* 1998; O'Neill *et al.* 1992; Werren *et al.* 1995*b*). Surveys of the distribution of *Wolbachia* across a broad range of arthropods have revealed a striking concordance in overall frequencies of infected species (17–22%) from three different geographical regions (Panama, Britain and North America), consistent with a widespread equilibrium frequency in *Wolbachia* infections (Werren *et al.* 1995*a*; West *et al.* 1998; Werren & Windsor 2000). Extrapolation of these infection frequencies suggests that *Wolbachia* may be among the most abundant group of parasitic bacteria known, infecting up to five million species of insects alone (Werren *et al.* 1995*a*; West *et al.* 1998; Werren & Windsor 2000). In addition to these broad surveys, there have been several taxon-specific or ecologically focused surveys for *Wolbachia* (Werren & Jaenike 1995; Bourtzis *et al.* 1996; Hariri *et al.* 1998; Schilthuizen & Gittenberger 1998; Wenseleers *et al.* 1998; Vavre *et al.* 1999). The proportion of insects infected by *Wolbachia* varies substantially among these more focused surveys. Thus, it appears that some insect groups, especially Hymenoptera, are more prone to harbour *Wolbachia* than others (Wenseleers *et al.* 1998; Vavre *et al.* 1999; Werren & Windsor 2000).

In most insect species that have been examined in detail, *Wolbachia* transmission appears to occur predominantly via the egg cytoplasm. Therefore, many strains are able to enhance their own spread within host populations by a variety of mechanisms that either increase their host's relative investment in daughters, or decrease the reproductive success of uninfected female hosts (Werren 1997). These mechanisms include cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males, male-killing, increased mating success of infected males via sperm competition and complete dependence on bacteria for egg production by the hosts (Breeuwer & Werren 1990; O'Neill *et al.* 1992; Beard *et al.* 1993; Stouthamer & Werren 1993; Sinkins *et al.* 1995*a*,*b*; Wade & Chang 1995; Hurst *et al.* 1996; Dedeine *et al.* 2001; for recent reviews see Werren (1997), Werren & O'Neill (1997) and Stouthamer *et al.* (1999)). Nonetheless, even

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as documented transmission of *Wolbachia* within species appears to be mostly vertical, the nearly complete lack of concordance between phylogenies of *Wolbachia* and their insect hosts demonstrates that pervasive horizontal transmission of *Wolbachia* among species also occurs (O'Neill *et al.* 1992; Werren *et al.* 1995*b*; Werren 1997; Zhou *et al.* 1998; Huigens *et al.* 2000; but see Schilthuizen & Stouthamer (1998); West *et al.* (1998)).

More generally, the distribution of *Wolbachia* strains within and among host species fundamentally depends upon three processes: (i) the rate of spread and the duration of maintenance of given *Wolbachia* infections within host species; (ii) the rate of interspecific horizontal transfer of *Wolbachia* infections across species; and (iii) the rate of mutation of *Wolbachia* genes. The rate of spread within a host species is influenced by the interaction between the various effects that *Wolbachia* induce in their host reproduction (i.e. fitness effects of *Wolbachia* on their hosts, maternal transmission fidelity, etc.) and host population structure. All else being equal, the rate of spread and proportion of infected individuals is expected to be lower within hosts characterized by more inbred population structures (Wade & Stevens 1994). With respect to horizontal transfer of *Wolbachia* among different species, it is generally expected to occur more readily among hosts that live in close proximity with each other or that share intimately connected life cycles (e.g. parasitoids and their hosts).

Importantly, the relative magnitude of genetic differences observed among *Wolbachia* strains infecting host species that are characterized by different evolutionary and ecological relationships will give an indication of the most frequent scenarios of transmission among species. For example, if close ecological association of potential hosts promotes *Wolbachia* transmission among them, we might expect that ecologically related hosts should share genetically similar *Wolbachia* strains. Further, the relative rates of spread both within and among host species can be calibrated roughly against the rate of mutation of *Wolbachia* genes such as *wsp*. For example, if the within-species transmission rate is high relative to among-species transmission and both are high relative to the mutation rate, then we expect to see the most closely related *Wolbachia* genotypes among individuals within a host species. However, to our knowledge, few, if any, studies have taken advantage of these potentially informative relationships and examined the patterns of genetic differentiation of *Wolbachia* strains both among and within a suite of host species for which detailed information on both ecological and evolutionary relationships is available.

Here, we exploit existing detailed ecological and evolutionary information for 18 neotropical (Panama) fig wasp communities to assess their relative influence on the processes of within- and among-species transfer. Importantly, we also assess the relative magnitude of the rates associated with each process with respect to the mutation rate of the widely used *wsp* gene. Within each fig species, there are host-specific species of pollinator wasps for which the population structure (levels of sib-mating) is well understood (Herre 1985, 1987, 1993, 1996). In most cases, each fig hosts a diverse community comprising several species of non-pollinator wasps that also exhibit a high degree of host specificity. The non-pollinators include one or more wasp species that compete with the pollinators for oviposition sites, larger gall-forming wasps, and wasp parasitoids that attack the gall-formers (Boucek 1993; West & Herre 1994; Herre 1996; West *et al.* 1996). All of these wasp species complete their life cycle in the enclosed environment of the fig syconium. Within this community of wasps associated with any particular fig species, some species have very direct interactions with each other (e.g. gall-formers and their parasitoids), while others have less direct interactions (e.g. the gall-formers and the pollinators). The ecological relationships among the representatives of these taxa are maintained across different host fig species and effectively represent replicate communities (Wiebes 1979, 1995; Herre 1989, 1996; West & Herre 1994; Herre *et al.* 1996, 1997; West *et al.* 1996). Further, the phylogenetic relationships among the pollinators and the non-pollinators associated with the Panamanian figs are known (Herre 1996; Herre *et al.* 1996; Machado *et al.* 1996, 2001; Machado 1998). Also, calibrations of fig wasp molecular clocks based on fossil evidence indicate that the fig-pollinating wasps began to radiate roughly 90 Myr ago and that the extant Panamanian pollinator species are very old, usually more than 3–5 Myr (Machado *et al.* 2001). This detailed knowledge allows us to study the distribution of *Wolbachia* strains both within and among host species in the context of varying degrees of spatial proximity, variably direct linkage of life cycles and a continuum of population structures, all across a known set of phylogenetic relationships.

## 2. MATERIAL AND METHODS

### (a) *Collection of insect species*

All Panamanian wasps were collected on or near Barro Colorado Island, Panama, whereas Old World (OW) (Australia–Asia) wasps were used to provide a phylogenetic context for the New World (NW) species (detailed descriptions of localities are available upon request). Fig fruits were collected late in the fruiting cycle but prior to the emergence of any wasps, returned to the laboratory, cut open and placed in Petri dishes to allow all of the wasps to develop and emerge. Upon emergence, all wasps were identified, sexed, counted and stored in 95% ethanol pending DNA analyses.

### (b) *Screening of insects for* Wolbachia

Total genomic DNA was isolated from each individual using the Puregene DNA isolation kit, a simple method for isolating high molecular weight DNA (Ross & Shoemaker 1997). Initially, genomic DNA from one to five individuals for each species was screened for the presence of *Wolbachia* via the polymerase chain reaction (PCR) using the primers *Wsp*81F and *Wsp*691R (Zhou *et al.* 1998). The *wsp* primers amplify a portion of a highly variable gene encoding the bacterial surface protein (Braig *et al.* 1998; Zhou *et al.* 1998). *Wsp* PCRs were performed in 15 μl volumes containing 13 μl of Platinum PCR SuperMIX (Gibco BRL), 0.18 μl of a 25 μM solution of each primer (*Wsp*81f and *Wsp*691R) and 1–3 μl of genomic DNA. Amplifications were carried out in a Perkin Elmer 9700 thermocycler programmed as follows: 1 min at 94 °C for one cycle; 30 s at 94 °C, 30 s at 60 °C (−1/2 °C per cycle) and 1 min at 72 °C for 10 cycles (touchdown PCR); 30 s at 94 °C, 1 min at 53 °C and 1 min at 72 °C for 25 cycles; and 5 min at 72 °C for one terminal cycle. 5 μl of each PCR reaction mixture were electrophoresed

in a 2% agarose gel. Gels were stained with ethidium bromide and bands visualized under UV illumination. Images of stained gels were photographed and stored electronically using a gel photodocumentation system.

Positive DNA controls were run on every sample using separate PCR reactions containing two conserved primers (Pat and Jerry; see Simon *et al.* (1994) for sequences) that amplify a portion of the mitochondrial DNA gene, cytochrome oxidase I. PCR reaction conditions and profiles are described elsewhere (Machado 1998).

After this initial screening, multiple individuals of many species were screened for *Wolbachia* using primers *Wsp81F* and *Wsp691R* (Zhou *et al.* 1998) (see table 1 for sample sizes). In most of these cases, individuals from a number of different fruits as well as several individuals from a given fruit were screened for *Wolbachia* (table 1). We also screened genomic DNA from a subset of individuals representing each infected species to determine whether the *Wolbachia* strains present represented the A or B (or both) subgroups of *Wolbachia* using primers that specifically amplify these two subgroups (16SAf and 16SArDNA, 16SBf and 16SBr; Werren *et al.* (1995*b*)). Primer sequences, PCR conditions and PCR profiles are to be found in Werren *et al.* (1995*b*).

To see if there was any relationship between the population structure of pollinators and the presence of *Wolbachia*, two analyses were performed (table 2). First, we tested for a correlation between the presence of *Wolbachia* and the proportion of single foundress fruits, which is an indicator of the levels of inbreeding of the different pollinator species (Herre 1987, 1995). This analysis was performed using all 18 Panamanian pollinator species. Second, we tested for a correlation between the frequency of *Wolbachia* infections and the proportion of single foundress fruits for a given species. For this analysis, we chose only those species where nine or more individuals were screened for the presence of *Wolbachia*.

### (c) *Sequencing of* Wolbachia *strains*

A 614 bp portion of the *wsp* gene was sequenced from a total of 70 infected individuals (representing a total of 73 sequences from 22 species; figure 1). For sequencing, *Wolbachia* DNA was PCR-amplified as described in § 2b, except that 50 µl volumes were used. Initially, we attempted to sequence all PCR products directly rather than clone PCR products. PCR amplicons were gel-purified using Qiagen gel extraction spin columns and used directly in standard fluorescent cycle-sequencing PCR reactions using the primers *Wsp* 81F and *Wsp* 691R (ABI Prism Big Dye terminator chemistry). Sequencing reactions were cleaned to remove excess dye terminators using sephadex columns (Princeton Separations) and run on an automated ABI Prism 310 sequencer.

Sequences of *wsp* PCR products from several individuals clearly revealed the presence of more than one strain of *Wolbachia* (i.e. multiple peaks or frameshifts in electropherogram profiles). In these cases, *Wolbachia* DNA was PCR-amplified as described above, except that the final extension at 72 °C was increased to 45 min. PCR amplicons then were cloned directly into a vector following the manufacturer's suggestions (Topo TA cloning kit, Invitrogen). Positive (white) colonies were screened for the presence of the desired *wsp* PCR insert using the above *wsp* primers. We chose PCR-amplified products from three to seven colonies (which presumably had the *wsp* insert) representing each individual for sequencing. PCR products were purified using Qiagen PCR spin columns and then used directly in standard fluorescent cycle-sequencing PCR reactions (ABI Prism Big Dye terminator chemistry). Sequencing reactions were cleaned to remove excess dye terminators using sephadex columns (Princeton Separations) and run on an automated ABI Prism 310 sequencer.

Sequences were aligned to a subset of previously published *Wolbachia* sequences from other insects and arthropods (77 sequences found in GenBank (see http://www.ncib.nlm.nih.gov/), submitted by Braig *et al.* 1998; Zhou *et al.* 1998; Hurst *et al.* 1999; van Meer *et al.* 1999; Vavre *et al.* 1999; Schulenburg *et al.* 2000; Shoemaker *et al.* 2000). The sequence data were used to construct phylogenetic trees of *Wolbachia* strains using neighbour-joining (NJ) and maximum parsimony (MP) methods, as implemented in PAUP* v. 4.08b (Swofford 1999). The third hypervariable region (positions 521–573) was excluded for these analyses (Zhou *et al.* 1998; Vavre *et al.* 1999), but were included when we surveyed among individuals within a particular host species for genetic variation in their associated *Wolbachia* strains. The NJ tree was constructed using HKY85 distances and was mid-point rooted. Bootstrap support values were generated using 1000 replicates. Parsimony trees were constructed using the heuristic search option (500 random additions) and TBR branch swapping. Bootstrap support values were generated using the heuristic search algorithm (500 bootstrap replicates with a single random addition search per replicate). *Wsp* sequences representing each *Wolbachia* strain have been deposited in GenBank (nos. AF521148–AF521174).

## 3. RESULTS

### (a) Wolbachia *prevalence in fig wasps*

We screened a total of 44 species of wasps associated with 18 species of NW tropical figs (table 1). Fifty-nine per cent of the wasps were infected with *Wolbachia* (26 out of 44 species). There was no significant difference between the pollinators and non-pollinators in the proportion of species infected by *Wolbachia* (50% versus 67%, respectively; $\chi^2 = 1.02$, d.f. = 1, $p > 0.30$). Infections by A subgroup *Wolbachia* were more common than B subgroup *Wolbachia* (26A versus 5B) in agreement with two previous studies that also showed a higher prevalence of A subgroup *Wolbachia* in Hymenoptera (Werren *et al.* 1995*a*; West *et al.* 1998). In addition, all individuals from five out of the 26 species from Panama carried double infections (three with A and B subgroup (AB) *Wolbachia* infections and two with AA *Wolbachia* infections; table 1).

Our estimate of *Wolbachia* infection frequency for Panamanian fig-associated wasps is significantly higher than the previous estimate based on a broad collection of insects from the same locality (Werren *et al.* 1995*a*). This is true whether one considers all of the species previously surveyed (16.9%; $\chi^2 = 32.55$, d.f. = 1, $p < 0.001$), or excludes all hymenoptera from this earlier survey (15.3%; $\chi^2 = 32.64$, d.f. = 1, $p < 0.001$), or considers only other hymenoptera (22.7%; $\chi^2 = 7.79$, $p < 0.01$; data from Werren *et al.* (1995*a*)). Furthermore, all three comparisons remain significant when we exclude the gall-formers and parasitoids and only consider the 36 species of pollinating wasps (*Pegoscapus* spp. and *Tetrapus* spp.) and non-pollinating wasps (*Critogaster* spp. and *Idarnes* spp.) associated with Panamanian figs ($\chi^2 = 7.00$–$28.27$, d.f. = 1, $p < 0.01$ in every case). Even if we correct for our sampling of multiple individuals within a given host-wasp species, this estimate

Table 1. Frequencies of *Wolbachia* infections in pollinator and non-pollinator insects associated with 18 NW Panamanian *Ficus* species.
(*n*, number of fruits from which wasps were screened for *Wolbachia*; the values in parentheses are the total number of wasp individuals screened. The frequencies of *Wolbachia* infections were determined using a single individual per fruit (*n*) and not the total number of individuals screened.)

| *Ficus* spp. | insect species (code) | interaction | frequency of *Wolbachia* | *n* | A or B?[a] |
|---|---|---|---|---|---|
| *Ficus paraensis* | *Pegoscapus herrei* (W1) | pollinator | 1.0 | 9(11) | AA |
| *Ficus obtusifolia* | *Pegoscapus hoffmeyeri* (W2) | pollinator | 0.33 | 15(25) | A |
| | *Idarnes* sp. (P2A) | non-pollinator | 1.0 | 6 | AB |
| | *Idarnes* sp. (B2) | non-pollinator | 1.0 | 1 | A |
| | *Aepocerus* sp. (A2L) | galler | 0.0 | 6 | — |
| | *Aepocerus* sp. (A2S) | galler | 0.0 | 7 | — |
| | *Physothorax* sp. (PA2) | parasitoid | 0.25 | 4 | B |
| | *Heterandrium* sp. (H2) | galler | 0.0 | 2 | — |
| *Ficus citrifolia* | *Pegoscapus tonduzi* (W3) | pollinator | 0.0 | 10(12) | — |
| | *Idarnes* sp. (P3) | non-pollinator | 1.0 | 1 | A |
| *Ficus popenoei* | *Pegoscapus gemellus* (W4) | pollinator | 1.0 | 41 | A |
| | *Idarnes* sp. (P4A) | non-pollinator | 1.0 | 6 | A |
| | *Idarnes* sp. (P4B) | non-pollinator | 1.0 | 2 | A |
| *Ficus nymphaefolia* | *Pegoscapus piceipes* (W5) | pollinator | 0.0 | 20(21) | — |
| *Ficus trigonata* | *Pegoscapus grandii* (W6) | pollinator | 0.0 | 15(28) | — |
| | *Idarnes* sp. (PF6) | non-pollinator | 0.83 | 6(7) | B |
| | *Aepocerus* sp. (A6L) | galler | 1.0 | 3 | A |
| *F. triangle* | *Pegoscapus lopesi* (W7) | pollinator | 0.90 | 10(20) | A |
| | *Idarnes* sp. (P7) | non-pollinator | 1.0 | 1 | AB |
| *Ficus yoponensis* | *Tetrapus ecuadoranus* (W8) | pollinator | 0.0 | 1 | — |
| | *Critogaster* sp. (P8) | non-pollinator | 0.0 | 1 | — |
| *Ficus maxima* | *Tetrapus americanus* (W9) | pollinator | 0.0 | 1 | — |
| | *Critogaster* sp. (P9) | non-pollinator | 1.0 | 1 | A |
| *Ficus insipida* | *Tetrapus costaricanus* (W10) | pollinator | 1.0 | 28 | A |
| | *Critogaster* sp. (P10) | non-pollinator | 1.0 | 3 | AB |
| *Ficus glabrata* | *Tetrapus* sp. (W11) | pollinator | 0.0 | 1 | — |
| | *Critogaster* sp. (P11) | non-pollinator | 1.0 | 1 | A |
| *Ficus dugandii* | *Pegoscapus longiceps* (W12) | pollinator | 1.0 | 13 | A |
| | *Idarnes* sp. (P12S) | non-pollinator | 1.0 | 6 | A |
| | *Idarnes* sp. (P12I) | non-pollinator | 0.0 | 1 | — |
| | *Aepocerus* sp. (A12) | gall former | 1.0 | 1 | A |
| | *Physothorax* sp. (PA12) | parasitoid | 1.0 | 2 | AA |
| *Ficus turbinata* | *Pegoscapus* sp. (W13) | pollinator | 0.0 | 2 | — |
| | *Idarnes* sp. (P13) | non-pollinator | 1.0 | 1 | A |
| *Ficus pertusa* | *Pegoscapus silvestrii* (W14) | pollinator | 0.0 | 2 | — |
| *Ficus bullenei* | *Pegoscapus gemellus* (W15) | pollinator | 0.29 | 17(27) | A |
| | *Idarnes* sp. (P15) | non-pollinator | 0.0 | 1 | — |
| *Ficus colubrinae* | *Pegoscapus orozcoi* (W16) | pollinator | 0.0 | 1 | — |
| | *Idarnes* sp. (P16) | non-pollinator | 0.0 | 1 | — |
| *Ficus perforata* | *Pegoscapus insularis* (W17) | pollinator | 0.45 | 20(28) | A |
| | *Idarnes* sp. (P17) | non-pollinator | 0.0 | 1 | — |
| *Ficus costaricana* | *Pegoscapus estherae* (W18) | pollinator | 1.0 | 17 | A |
| | *Idarnes* sp. (PS18) | non-pollinator | 0.0 | 4 | — |
| | *Aepocerus* sp. (A18L) | gall former | 1.0 | 1 | A |

[a] Refers to the A and B subgroups of *Wolbachia*.

of *Wolbachia* infection among fig-associated insects remained significantly higher ($\chi^2 = 18.56$, d.f. = 1, $p < 0.001$) than the broad survey of Panamanian insects (Werren *et al.* 1995*a*). The Panama fig wasp estimate is also higher than the estimate from arthropods found in a leaf-miner community in Britain (59% versus 33.3%; $\chi^2 = 5.51$, d.f. = 1, $p = 0.019$; West *et al.* (1998)).

### (b) Wolbachia *prevalence and population structure of hosts*

No relationship was observed between the population structure of each pollinator species (inferred from the proportion of single foundress fruits) and either the presence of *Wolbachia* or frequency of *Wolbachia* infections (Pearson correlation coefficients equal 0.037 and −0.086, respectively and $p > 0.5$ in both cases). Indeed, *Wolbachia* infections were found in species with population structures that fall at the extremes of the distribution of inbreeding levels observed for Panamanian species of fig wasps (table 2). In four out of the nine infected pollinator species, not all individuals harboured *Wolbachia* infections. Interestingly, these four species represent the entire spectrum of population structures among Panamanian fig wasps: *Pegoscapus insularis*, a highly inbred species (more

Table 2. Proportion of single foundress fruits and frequency of *Wolbachia* infections in 18 Panamanian pollinator (Agaonidae) species. Rows with bold print indicate species harbouring *Wolbachia* infections. No correlations were found between the proportion of single foundress fruits, which is an indicator of the level of inbreeding, and either the presence of *Wolbachia* or frequency of *Wolbachia* infection (see § 3b).

| *Ficus* species | fig wasp species | proportion of single foundress fruits[a] | $n$[b] | frequency of *Wolbachia* |
|---|---|---|---|---|
| ***Ficus perforata*** | ***Pegoscapus insularis*** | **0.99** | **20** | **0.45** |
| *Ficus colubrinae* | *Pegoscapus orozcoi* | 0.99 | 1 | 0 |
| ***Ficus paraensis*** | ***Pegoscapus herrei*** | **0.92** | **9** | **1** |
| *Ficus pertusa* | *Pegoscapus silvestrii* | 0.9 | 2 | 0 |
| *Ficus turbinata* | *Pegoscapus* sp. | 0.85 | 2 | 0 |
| ***Ficus obtusifolia*** | ***Pegoscapus hoffmeyeri*** | **0.83** | **15** | **0.33** |
| ***Ficus bullenei*** | ***Pegoscapus gemellus*** | **0.82** | **17** | **0.29** |
| ***Ficus costaricana*** | ***Pegoscapus estherae*** | **0.8** | **17** | **1** |
| *Ficus citrifolia* | *Pegoscapus tonduzi* | 0.78 | 10 | 0 |
| *Ficus maxima* | *Tetrapus americanus* | 0.7 | 1 | 0 |
| *Ficus yoponensis* | *Tetrapus ecuadoranus* | 0.58 | 1 | 0 |
| *Ficus nymphaefolia* | *Pegoscapus piceipes* | 0.55 | 20 | 0 |
| ***Ficus dugandii*** | ***Pegoscapus longiceps*** | **0.54** | **13** | **1** |
| ***Ficus triangle*** | ***Pegoscapus lopesi*** | **0.31** | **10** | **0.9** |
| ***Ficus insipida*** | ***Tetrapus costaricanus*** | **0.27** | **28** | **1** |
| ***Ficus popenoei*** | ***Pegoscapus gemellus*** | **0.24** | **41** | **1** |
| *Ficus glabrata* | *Tetrapus* sp. | 0.1 | 1 | 0 |
| *Ficus trigonata* | *Pegoscapus grandii* | 0.07 | 15 | 0 |

[a] From Herre (1989, 1995).
[b] Number of individuals screened.

than 99% of fruits contain only a single foundress), *P. hoffmeyerii* and *P. gemellus* (pollinating *Ficus bullenei*), two species with lower levels of inbreeding (*ca.* 83% and 82% of fruits, respectively, contain single foundresses), and *P. lopesi*, one of the species of wasps associated with Panamanian figs with the lowest levels of inbreeding (*ca.* 30% of fruits contain single foundresses; table 2; Herre (1987)). Similarly, the five species with complete *Wolbachia* infections also represent the entire spectrum of breeding structures found among Panamanian fig wasps (table 2).

### (c) *Phylogenetic analyses of* Wolbachia *and patterns of interspecific horizontal transmission*

The NJ tree using the HKY85 model of sequence evolution is shown in figure 1. For MP, 280 out of 561 total characters were informative. A total of 128 most parsimonious trees were found. However, the topology of the strict consensus parsimony tree (not shown) was very similar to the NJ tree (figure 1), with differences among the most parsimonious trees mostly confined to placement of taxa near the tips of the tree. From 73 total sequences, only 22 unique strains were identified. There were three cases in which two or more of the Panamanian species share identical *Wolbachia* strains. Two Panamanian species carried more than one strain of *Wolbachia*. We sequenced the *wsp* gene from multiple individuals within 10 different wasp species and detected no sequence variation among *wsp* sequences for any single strain within any of these 10 species. Repeated sampling and re-sequencing showed that these results were not due to laboratory contamination.

Three main features are evident from examination of the NJ tree in figure 1. First, the *Wolbachia* strains from different fig wasp species do not form a single monophyletic group. Many *Wolbachia* strains from given wasp species are more closely related to strains from insects in different orders than to strains from other wasp species associated with figs. Second, no *Wolbachia* strains were identical between the NW and OW fig-associated wasps. However, *wsp* sequences from some NW wasps were closer to OW sequences than to other NW sequences (W6 *Idarnes* sp.). Third, distantly related fig wasp species often had identical or very similar *Wolbachia* strains. This striking similarity of strains across species is quite evident in figure 1, where most of the strains fell within one of three clades of *wsp* sequences. Such a pattern of similar *Wolbachia* strains across different wasp species clearly indicates frequent interspecific horizontal transmission of *Wolbachia*.

We used the program TREEMAP (Page 1996) to compare the phylogeny of 23 *Wolbachia* strains based on *wsp* sequences with the phylogeny of the host wasps carrying these different *Wolbachia* strains (based on partial cytochrome oxidase I and II sequences; Machado (1998); figure 2). The total number of matches between the two cladograms (7 'cospeciation' events) was not significantly different from random expectation ($p = 0.47$). Thus, the trees are not congruent and are consistent with widespread horizontal transmission across species.

To further examine transmission patterns, we constructed a matrix consisting only of species carrying identical or nearly identical (i.e. less than 5 bp changes) *Wolbachia* strains. We then compared the number of instances in which similar *Wolbachia* strains were found in pairs of wasps characterized by different degrees of evolutionary relationship or ecological association. Specifically, we assigned pairs of potential hosts into the following categories: closely related fig wasp species (figure 2), species from the same genus, species from the

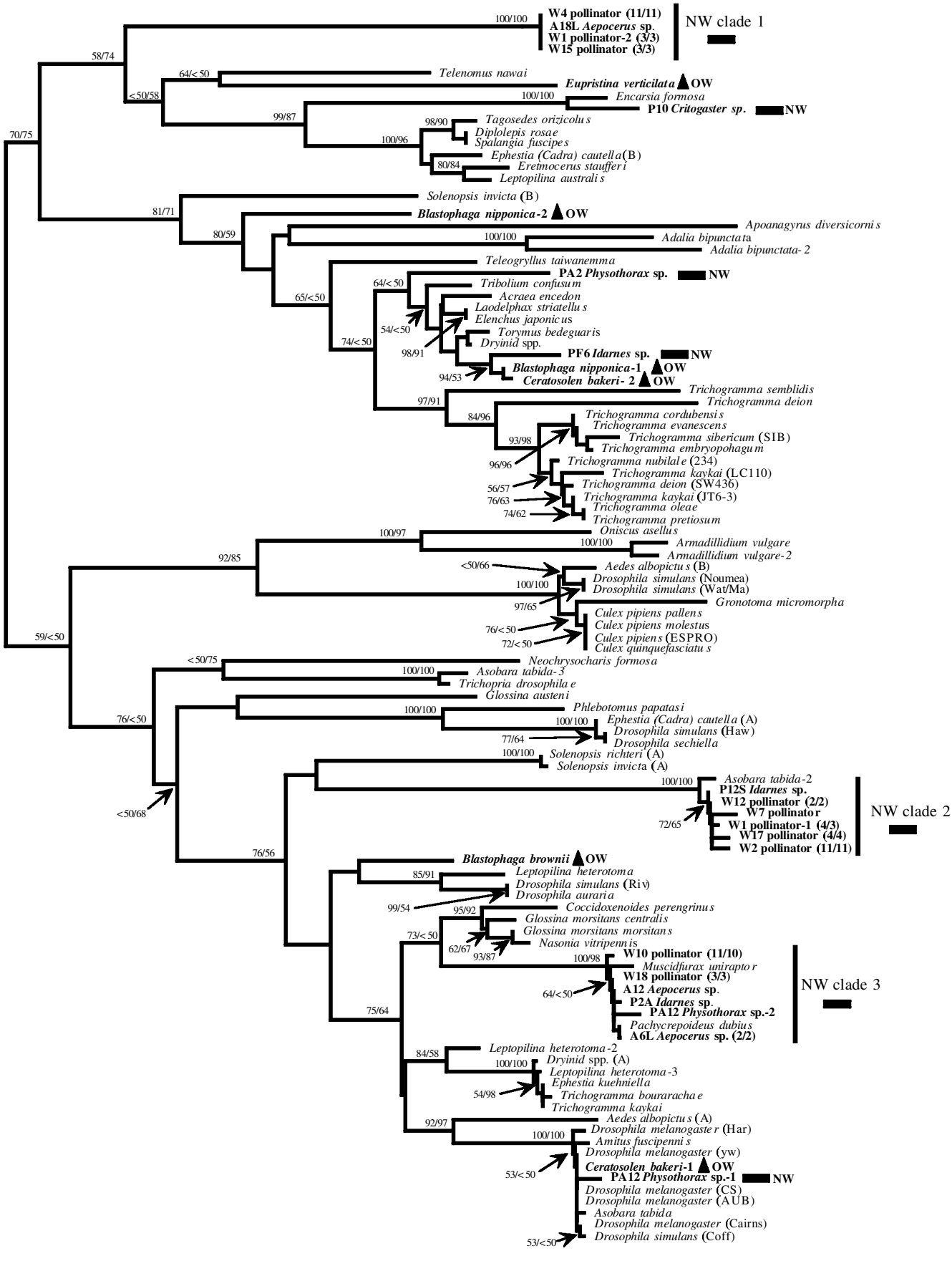


Figure 1. Neighbour joining phylogram of *Wolbachia* strains based on *wsp* sequences (*ca*. 561 bp of DNA). *Wolbachia* strains are identified by the host species from which they were isolated. *Wolbachia* strains from this study are indicated in bold and identified by alphanumeric labels representing specific hosts as shown in table 1. Rectangles, *Wolbachia* strains from NW wasps; triangles, *Wolbachia* strains from OW wasps. Numbers in parentheses indicate number of individual sequences/number of fruits. Bootstrap support values (neighbour-joining/maximum parsimony) are shown above branches for each node in the phylogram. Bootstrap support values less than 50 are not indicated. The three common clades of *Wolbachia* found among NW wasps are indicated by vertical bars (see text).

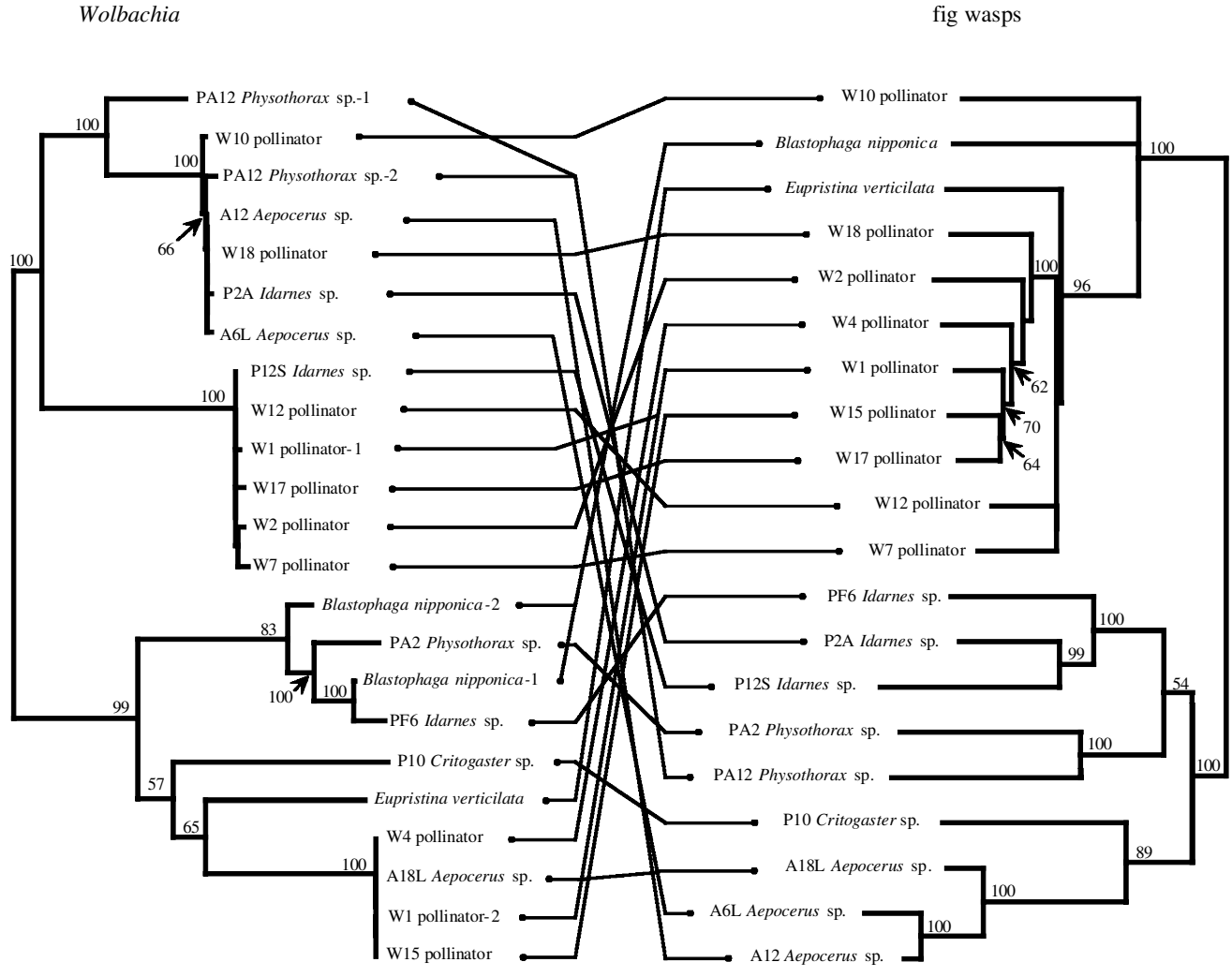


Figure 2. Comparison of phylogenetic trees of *Wolbachia* strains and their hosts (NW and OW fig wasps). *Wolbachia* strains are identified by the host species from which they were isolated. Alphanumeric labels for wasps and *Wolbachia* strains correspond to specific hosts, as shown in table 1.

same fig community (same *Ficus* host), species in a closely related fig community (e.g. pollinators are sister species) or in none of the above (i.e. in species that were not related either phylogenetically or ecologically; table 3). Out of 38 host species pairs that shared very similar *Wolbachia* strains, only six were from closely related host wasp species, again reflecting the high frequency of interspecific horizontal transmission of *Wolbachia* strains among phylogenetically unrelated hosts. Further, only two of these came from the same fig community (same *Ficus* host), showing that horizontal transmission does not necessarily or even frequently correlate well with ecological association. Fundamentally, neither close phylogenic relationship nor close ecological association among host species is related to higher *Wolbachia* transmission (table 3).

## 4. DISCUSSION

Our estimate of the proportion of *Wolbachia*-infected wasps associated with figs from Panama (59%) is higher than previous estimates for insects collected from three different geographical areas, including Panama (Werren *et al.* 1995*a*; West *et al.* 1998; Werren & Windsor 2000). Also, our data are consistent with the previous findings of higher *Wolbachia* infection frequencies in Hymenoptera compared with other insects (Wenseleers *et al.* 1998; Vavre *et al.* 1999; Werren & Windsor 2000). Similar to other studies, we found no evidence to support frequent or even occasional cospeciation between the wasps and their *Wolbachia.* These bacteria clearly can cross species boundaries very rapidly compared with speciation events in the hosts (D. Molbo, C. Machado and E. Herre, unpublished data). Further, the relatively close relationship of some *Wolbachia* strains in NW and OW wasps, as well as to strains from distant insect orders (figure 1), indicates that horizontal transfer might be only slightly constrained by great geographical distance or taxonomic differences. Thus, the high prevalence of *Wolbachia* apparently results from historical processes involving high rates of interspecific horizontal transfer and probable turnover rather than cospeciation and shared ancestry (Schilthuizen & Stouthamer 1997; Werren & Windsor 2000).

Contrary to expectations that hosts with close ecological associations should exchange *Wolbachia* strains frequently and thus tend to share closely related strains, the vast majority of similar *Wolbachia* strains were found in wasps with no known ecological interactions. The most intimate

Table 3. Host species harbouring similar *Wolbachia* strains (identical *wsp* haplotypes (0), or fewer than 5 bp differences in the *wsp* sequences (X)). Lines separate the groups with similar *Wolbachia* strains from the same clade (see figure 1).

| pairs of host species | | closely related species | same genus | in same fig community | in figs pollinated by closely related species | none of these |
|---|---|---|---|---|---|---|
| A18L *Aepocerus* sp. | W4 pollinator | — | — | — | — | 0 |
| A18L *Aepocerus* sp. | W1 pollinator-2 | — | — | — | — | 0 |
| A18L *Aepocerus* sp. | W15 pollinator | — | — | — | — | 0 |
| W4 pollinator | W1 pollinator-2 | 0 | — | — | — | — |
| W4 pollinator | W15 pollinator | 0 | — | — | — | — |
| W1 pollinator-2 | W15 pollinator | 0 | — | — | — | — |
| P12S *Idarnes* (S) | W12 pollinator | — | — | 0 | — | — |
| P12S *Idarnes* (S) | W7 pollinator | — | — | — | X | — |
| P12S *Idarnes* (S) | W1 pollinator-1 | — | — | — | — | X |
| P12S *Idarnes* (S) | W17 pollinator | — | — | — | — | X |
| P12S *Idarnes* (S) | W2 pollinator | — | — | — | — | X |
| W12 pollinator | W7 pollinator | X | — | — | — | — |
| W12 pollinator | W1 pollinator-1 | — | X | — | — | — |
| W12 pollinator | W17 pollinator | — | X | — | — | — |
| W12 pollinator | W2 pollinator | — | X | — | — | — |
| W7 pollinator | W1 pollinator-1 | — | X | — | — | — |
| W7 pollinator | W17 pollinator | — | X | — | — | — |
| W7 pollinator | W2 pollinator | — | X | — | — | — |
| W1 pollinator-1 | W17 pollinator | X | — | — | — | — |
| W1 pollinator-1 | W2 pollinator | — | X | — | — | — |
| W17 pollinator | W2 pollinator | — | X | — | — | — |
| W10 pollinator | W18 pollinator | — | — | — | — | X |
| W10 pollinator | A12 *Aepocerus* sp. | — | — | — | — | X |
| W10 pollinator | P2A *Idarnes* (S) | — | — | — | — | X |
| W10 pollinator | PA12 *Physothorax* sp.-2 | — | — | — | — | X |
| W10 pollinator | A6L *Aepocerus* sp. | — | — | — | — | X |
| W18 pollinator | A12 *Aepocerus* sp. | — | — | — | — | 0 |
| W18 pollinator | P2A *Idarnes* (S) | — | — | — | — | X |
| W18 pollinator | PA12 *Physothorax* sp.-2 | — | — | — | — | X |
| W18 pollinator | A6L *Aepocerus* | — | — | — | — | X |
| A12 *Aepocerus* sp. | P2A *Idarnes* (S) | — | — | — | — | X |
| A12 *Aepocerus* sp. | PA12 *Physothorax* sp.-2 | — | — | X | — | — |
| A12 *Aepocerus* sp. | A6L *Aepocerus* sp. | X | — | — | — | — |
| P2A *Idarnes* (S) | PA12 *Physothorax* sp.-2 | — | — | — | — | X |
| P2A *Idarnes* (S) | A6L *Aepocerus* sp. | — | — | — | — | X |
| PA12 P*hysothorax* sp.-2 | A6L *Aepocerus* sp. | — | — | — | X | — |
| *Ceratosolen bakeri*-2 | *Blastophaga nipponica*-1 | — | — | — | — | X |
| *Ceratosolen bakeri*-1 | W12 *Physothorax* sp.-1 | — | — | — | — | X |
| total number of cases in each category | | 6 | 8 | 2 | 2 | 20 |

interactions are between the parasitoid wasps from the genus *Physothorax* and their host gall wasps (*Aepocerus* spp.; West *et al.* (1996)). Although individuals of two *Physothorax* species were infected, in only one case did the host *Aepocerus* carry a strain similar to its parasitoid (a species associated with *F. dugandii*). Indeed, most of the nearly identical strains are found in species not parasitized by *Physothorax* (figure 1; table 3). Further, transmission of *Wolbachia* among non-pollinators and pollinators sharing the same host fig might occur during their brief physical contact inside the syconium at the time of emergence because all wasp species emerge into the fruit lumen just before the final ripening of the fruits. However, we only observed one case of an identical *Wolbachia* strain found in a pollinator and a non-pollinator associated with the same host (*F. dugandii*). Thus, horizontal transfer of *Wolbachia* in fig wasps (and the majority of insect groups) appears unrelated to any factor that we have measured. The fact that *Wolbachia* strains from several fig wasps are close to strains from other orders of insects indicates that the mechanism of horizontal transmission is quite unspecific. Clearly, other possible mechanisms of horizontal

transfer must exist that neither we nor other research groups have explored. In the future we may be able to begin narrowing down the possible transmission routes by more detailed studies characterizing *Wolbachia* genetic differentiation both among and within host species.

Several explanations have been proposed to account for the observed differences in the prevalence of *Wolbachia* across different taxonomic groups or ecological guilds (West *et al.* 1998; Werren & Windsor 2000). The two most common explanations for higher infection frequencies of the Hymenoptera, which focus on the social and parasitoid lifestyles (Wenseleers *et al.* 1998; Vavre *et al.* 1999), cannot explain the high infection frequencies observed in fig wasps because none of the wasps live in social groups and there are no known generalist parasitoids that could serve as a conduit for transmission of *Wolbachia* between fig wasp species. The only known host–parasitoid interactions in the fig communities that we surveyed (i.e. *Aepocerus–Physothorax*) explain only a minute fraction of all observed cases of interspecific horizontal transfer of *Wolbachia*.

Why are hymenoptera, and especially fig wasps, more prone to *Wolbachia* infections compared with other groups of organisms? It is possible that the haplodiploid sex-determining mechanism of Hymenoptera allows more opportunities for *Wolbachia* to invade than in regular diploid organisms. For example, *Wolbachia* are selected to alter the sex ratio towards female production, and this may occur more readily in haplodiploid organisms because of their maternal control over sex determination. One could test this hypothesis by surveying for *Wolbachia* in other haplodiploid arthropods (e.g. thrips). Similarly, the highly female-biased sex ratio of fig wasps reduces the conflict between host and bacteria, thus leaving little room for *Wolbachia* to alter its transmission rate by affecting the host phenotype. As a consequence, hosts are under less selection to get rid of them. Additionally, it is possible that the *Wolbachia* strains we identified represent strains that are beneficial to their hosts. Once such a variant arises, its spread within newly infected insects with similar life histories may be favoured.

The finding of no relationship between host population structure and either the presence of *Wolbachia* infection or the frequency of infected individuals within species was surprising. The presence and frequency of *Wolbachia* infections within species may be expected to be correlated with the population structure of the hosts because the type of *Wolbachia* that can invade is more limited in highly inbred hosts and because the spread of *Wolbachia* will be slower in more inbred hosts compared with outbred hosts, assuming that transmission of *Wolbachia* is mostly vertical (Wade & Stevens 1994). The breeding biology of fig wasps also constrains the invasion and evolution of sex ratio-distorting *Wolbachia*, especially in species where only a single foundress per fruit is common. Males are required to chew exit holes in the fruit, and therefore fruits that do not contain at least some males will not yield emergent offspring. For *Wolbachia* to successfully invade and persist in these highly inbred wasp populations, the bacteria would have to either directly increase or have neutral effects on host fitness, hitchhike with particularly fit wasp lineages, reduce the fitness of uninfected females to an extent that outweighs the costs of simply harbouring infections (e.g. cytoplasmic incompatibility), or be frequently horizontally transferred within species.

If *Wolbachia* are mutualists (i.e. increase host fitness), then one might predict that the presence of incomplete infections within species is temporary and that *Wolbachia* are in the process of sweeping to fixation or near fixation within species. This prediction can be tested by sampling host populations through time, and is the subject of ongoing research. One also would predict that the *Wolbachia* infections would persist stably through time, perhaps even through speciation events. Counter to this, we observed a relative lack of cospeciation of *Wolbachia* with their hosts. The pattern observed in fig wasps is similar to that found in *Trichogramma* wasps (Schilthuizen & Stouthamer 1997), where a group of related *Wolbachia* are present within the host taxon but the bacteria show little evidence of cospeciation with their hosts, indicating extensive horizontal transmission between host species. However, *Wolbachia* phylogenies based only on the *wsp* gene must be considered tentative because previous studies suggest that this gene may not track organismal phylogeny well due to intragenic (Werren & Bartos 2001) or intergenic (Jiggins *et al.* 2001) recombination.

The lack of *wsp* sequence variation within all wasp species for which the data are available also indicates that *Wolbachia* either do not persist within species for long enough to accumulate sequence diversity, or that there are frequent sweeps of particular *Wolbachia* variants within a species, which obliterate sequence diversity. Given that molecular evidence suggests that the extant wasp species are quite old (more than 2.5 Myr; Machado *et al.* (2001)), the paucity of cospeciation with wasp hosts indicates that the residence time of given *Wolbachia* strains within individual fig wasp species is short relative to speciation.

The relatively high turnover rate of *Wolbachia* has interesting implications for *Wolbachia* as a speciation agent. On the one hand, the potential of *Wolbachia* to act directly as speciation agents may be limited simply because it does not persist for long enough in a given host (Hurst & Schilthuizen 1998). On the other hand, the very high rates of *Wolbachia* infections in fig wasps may increase the potential role of *Wolbachia* in speciation because of the increased probability that two populations coming into secondary contact harbour different *Wolbachia* (Werren 1998) and therefore experience bidirectional incompatibility. Such incompatibility also could select for premating isolation, particularly in the closed mating systems experienced by fig wasps. Thus, although a high turnover in *Wolbachia* infections could reduce the likelihood of *Wolbachia*-induced speciation, under some circumstances, high infection levels could favour it. The relative likelihood of these alternative scenarios can be assessed by extensive temporal and spatial sampling of *Wolbachia* in these wasps.

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