Convergent incidences of *Wolbachia* infection in fig wasp communities from two continents

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Wide surveys suggest that *Wolbachia* bacteria infect *ca.* 20% of all insect species, but particular taxonomic or ecological groups may display significantly higher or lower incidences. We studied 61 fig wasp species in Australia and found the highest known incidence (67%) of infection in a targeted study of this nature. A comparable study in Panama reported a similar figure (59%), confirming the exceptionally high incidence of *Wolbachia* in fig wasps. Importantly, these are two independent estimates of *Wolbachia* incidence in fig wasp communities, because no host species, or even genera, are shared between localities. The high level of infection may reflect enhanced opportunities for horizontal transmission inside fig fruits. Although incidence was similar in Panama and Australia, the actual strains involved were different and region-specific. Local strains were shared by several host species, although there was often no obvious (direct) ecological link between two hosts with the same infection.

Keywords: *Wolbachia*; fig wasps; incidence; horizontal transmission; evolution

1. INTRODUCTION

Wolbachia are alpha-proteobacteria related to *Rickettsia* and infect a wide range of arthropods, including insects, arachnids and Crustacea (O'Neill *et al.* 1992; Werren *et al.* 1995a,b; Bouchon *et al.* 1998), as well as occurring in filarial nematodes (Bandi *et al.* 1998). They are transmitted maternally in egg cytoplasm and alter host reproduction in various ways to enhance their own transmission. The most common effect is cytoplasmic incompatibility (Hoffmann & Turelli 1997), but there are also various forms of host sex-ratio distortion, namely parthenogenesis induction (Stouthamer *et al.* 1993), feminization (Bouchon *et al.* 1998) and male killing (Hurst *et al.* 1999). These effects on individual hosts can also have impacts on host population biology (e.g. Jiggins 2003), mating systems (e.g. Jiggins *et al.* 2000) and even speciation (e.g. Bordenstein *et al.* 2001).

Four similar surveys of *Wolbachia* incidence in large numbers of diverse insect species from a particular region have all yielded estimates of between 16% and 23% species infected (Panama, Werren *et al.* 1995a; Berkshire, UK, West *et al.* 1998; Indiana, USA, Werren & Windsor 2000; Thailand, Kittayapong *et al.* 2003). These studies suggest that *ca.* 20% of all insect species harbour *Wolbachia* infections, and Werren & Windsor (2000) raised the possibility that it is a global equilibrium. Alternatively, the proportion of infected species may be increasing or even decreasing. Using a simple birth–death model, the proportion of species infected depends on the balance between the rate of origin of new infections and the rate of loss of existing infections. New infections arise by horizontal transmission

from already-infected species (e.g. Heath *et al.* 1999; Huijgens *et al.* 2003), and are expected to involve some ecological interaction between the two hosts involved (Werren 1997). In addition, horizontal transmission may be more likely between closely related host species, because they share vectors (e.g. predators) through which *Wolbachia* are transferred, as well as having similar physiology, to which certain *Wolbachia* strains may be adapted (Rigaud *et al.* 2001; Jiggins *et al.* 2002). Existing infections may be lost for various reasons, including turnover in infection phenotypes from cytoplasmic incompatibility to sex-ratio distortion, followed by loss of infection (Hurst *et al.* 2002; Jiggins *et al.* 2002).

Although broad surveys suggest some consistency in the incidence of *Wolbachia* infection, there is increasing evidence that certain taxonomic groups may be more prone to infection (Werren & Windsor 2000). For example, infection levels are significantly higher than 20% for Hymenoptera in the broad surveys. They are also generally high in studies focused on particular hymenopteran groups, with estimates of 50% (25/50) of Indo-Australian ant species (Wenseleers *et al.* 1998), 59% (26/44) of Panamanian fig wasp species (Shoemaker *et al.* 2002), 62% (13/21) of rose gall-inducing wasps (Rhoditini), 57% (4/7) of rose gall parasites (multiple tribes), 44% (4/9) of herb gall-inducing wasps ('Aylacini') and 60% (6/10) of gall inquiline wasps (Synergini) (Schilthuizen & Stouthamer 1998; Plantard *et al.* 1999; Rokas *et al.* 2002). A curious exception is provided by oak-gall-inducing wasps (Cynipini), where only 9% (5/53) of species are infected (Rokas *et al.* 2002).

In addition to differences between host taxa, there might be systematic differences in the incidence of infection according to geographical region, habitat or host life history. For example, certain authors (Wenseleers *et al.* 1998; Reuter & Keller 2003) have suggested that the typically small effective population sizes of social insects may facilitate the invasion of new *Wolbachia* strains, because infec-

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tion frequency must exceed a critical level to result in deterministic spread in a host population (Caspari & Watson 1959). By contrast, spread is predicted to be slower in inbreeding hosts (Wade & Stevens 1994), such as fig-pollinating wasps (Herre *et al.* 1997).

If the ecological attributes of given host taxa are important in determining the incidence of *Wolbachia* infections, then comparable sets of species in different continents are predicted to reach similar equilibrium frequencies of infection, although the strains involved may be different. We test this prediction for the first time, to our knowledge, by comparing *Wolbachia* infections from Australian fig wasp communities with those from Panamanian fig wasp communities (studied by Shoemaker *et al.* 2002). Importantly, the incidence of *Wolbachia* in these two localities is essentially independent, because the fig-pollinating wasp taxa involved separated *ca.* 70 Myr ago (Machado *et al.* 2001), so the gain and loss of infections will have happened locally in parallel. Specifically, we ask whether (i) the incidence of *Wolbachia* infections in Australian fig wasps is significantly greater than the global 20% figure, (ii) the incidence is significantly different from that in Panamanian fig wasps, and (iii) there are distinct phylogenetic clusters of Panamanian and Australian *Wolbachia* strains.

(a) Background biology and taxonomy

Figs (*Ficus* spp.) are involved in an obligate mutualism with fig-pollinating wasps (family Agaonidae), in which the wasps disperse fig pollen, while fig flowers provide nutrition for wasp larvae. The mutualism probably arose *ca.* 90 Myr ago (Machado *et al.* 2001) and has since diversified considerably (Cook & Rasplus 2003). There are over 750 extant fig species and most of these are pollinated by a single agaonid wasp species. *Ficus* is divided into some 20 sections whereas Agaonidae contains 20 genera, most of which are associated with one *Ficus* section. Phylogenetic evidence supports a long history of coevolution, with a high degree of cospeciation (Herre *et al.* 1996; Weiblen 2000, 2001; Machado *et al.* 2001; Weiblen & Bush 2002). The mutualism is exploited by many non-pollinating wasp species, which feed upon fig tissue or other wasps. Most fig species harbour several of these parasite species, which belong to several different wasp lineages that have colonized the fig habitat independently (Cook & Rasplus 2003).

The genus *Ficus* is found throughout the tropics and subtropics but each section has a more restricted geographical distribution. Only two fig sections, *Pharmacosycea* (pollinated by the genus *Pegoscapus*) and *Americana* (pollinated by the genus *Tetrapus*), occur in the New World. The other sections all occur in the Old World and diversity is highest in southeast Asia and New Guinea. Australia has 45 *Ficus* species. These represent several *Ficus* sections, but the major component is provided by the endemic Australasian section *Malvanthera* (Dixon 2003). Our study focused on *Wolbachia* in the wasp communities of 16 species of *Malvanthera* figs, which are pollinated by wasps in the genus *Pleistodontes* (Hymenoptera, Agaonidae), whereas Shoemaker *et al.* (2002) focused on 18 Panamanian fig species (from sections *Pharmacosycea* and *Americana*). Importantly, there are no wasp species (pollinators or parasites), or even genera, in common between the two local-

ities, and the pollinator genera involved separated a very long time ago (*ca.* 70 Myr ago; Machado *et al.* 2001). Thus, given ancient host divergence times and the short residence time of *Wolbachia* in fig wasps (Shoemaker *et al.* 2002), the Australian and Panamanian fig wasp species are independent samples with no shared ancestral *Wolbachia* infections.

2. MATERIAL AND METHODS

(a) Species and field sampling

We focused on wasps associated with *Ficus* section *Malvanthera*, but also included some wasps from other Australian fig species (in sections *Conosycea*, *Ficus*, *Sycidium*, *Sycocarpus* and *Sycomorus*), as well as a few species from other Old World localities. Both *Malvanthera* figs and their pollinators (*Pleistodontes* species) are well studied and have been subjected to recent taxonomic revisions (figs, Dixon 2003; wasps, Lopez-Vaamonde *et al.* 2002). The taxonomy of non-pollinating fig wasps is less well advanced (Boucek 1988). The most common genus in our samples was *Sycoscapter* (Hymenoptera: Sycoryctinae). Only two Australian *Sycoscapter* species have been described formally (Boucek 1988), but phylogenetic studies show that each *Malvanthera* species hosts a separate and host-specific *Sycoscapter* species (Lopez-Vaamonde *et al.* 2001).

We collected wasps emerging from ripe fig fruits and stored them at -20°C in 100% ethanol. We identified wasps and then screened 1–5 individuals of most species, but also sampled some common species more extensively, screening 10–70 individuals. We maximized the independence of samples by emphasizing screening of individuals from different localities, trees, sampling periods and fruits, in order of priority. We screened female wasps for *Wolbachia* because males are very small and often difficult to identify.

(b) Molecular methods

We removed the abdomen from each insect with a sterile scalpel blade and extracted DNA from it using a Chelex procedure (West *et al.* 1998). We always prepared a positive control from the abdomen of an insect known to be *Wolbachia*-infected. We screened for *Wolbachia* by polymerase chain reaction (PCR) with primers *wsp*81F and *wsp*691R that amplify part of the *Wolbachia* surface protein gene (*wsp*) (Zhou *et al.* 1998). We scored individuals yielding a product of the expected size as infected, and samples that did not amplify as provisionally uninfected. We tested the quality of the DNA extract in the latter cases by performing PCR for insect cytochrome *b* (Jermin & Crozier 1994). If this was successful, we regarded the sample as uninfected. If it failed, we excluded the sample from our data.

We performed PCR for *wsp* on a Geneamp 2400 machine (Perkin-Elmer Cetus) with 3 min at 95°C , 35 cycles of: 30 s at 95°C , 1 min at 55°C , 1 min 30 s at 72°C , and final elongation of 7 min at 72°C . We used the same conditions for cytochrome *b*, but with the annealing temperature reduced to 45°C . We electrophoresed 10 μl of each PCR product on a 1% agarose gel to determine amplicon presence and size. The PCR products were 500–600 bp for *wsp* and 450 bp for cytochrome *b*.

We excised agarose gel bands and purified them using a GFX DNA Purification Kit (Amersham Pharmacia Biotech Inc.), before sequencing DNA directly using the PCR primers. If direct sequencing failed three times (or repeatedly generated sequences with multiple peaks), we cloned the insert and sequenced 6–10 different clones to test for the presence of multiple *Wolbachia* strains.

For cloning, each PCR product was ligated into a T-tailed vector (pGEM-T Easy Vector system, Promega Ltd) and transformed into *E. coli* JM109. White insert-containing colonies were picked, grown overnight (16 h) in 2X YT broth, and the plasmids purified using the GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech Inc.). The *wsp* inserts were sequenced using M13 vector primers. Sequences were obtained using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Inc.) and an ABI PRISM 3700 DNA Analyzer (Perkin Elmer Inc.). Each isolate was fully sequenced in both directions. *Wsp* sequences representing each *Wolbachia* strain have been deposited in GenBank (AY567542–AY567593).

(c) Sequence alignment and phylogenetic analysis

A 509 bp region of the *wsp* gene was aligned in the SEQUENCHER program with final adjustments by eye, following previous alignments and excluding the third hypervariable region (Zhou *et al.* 1998; Shoemaker *et al.* 2002). The alignment is available from the EMBL-Align database by SRS at <http://srs.ebi.ac.uk>, under the accession ALIGN_000677. We estimated phylogenies using maximum parsimony (MP) in PAUP* version 4.0b10 (Swofford 2002). We treated gaps as missing data and, following Quicke *et al.* (2001), conducted an initial heuristic search with 10 000 random additions and TBR branch swapping, holding one tree per replicate. We then used the trees generated by the initial search as starting trees for a second heuristic search, in which we saved multiple trees. We assessed clade support using 1000 bootstrap replicates. We also estimated phylogenies using Bayesian methods in MRBAYES v. 3.0 (Huelsenbeck & Ronquist 2001), adopting the GTR model of nucleotide substitution. We ran the analysis for 10^6 generations, with one tree retained every 100 generations. Likelihood stationarity was achieved after 2.5×10^5 generations and we excluded this 'burn-in' period.

3. RESULTS

(a) Incidence of *Wolbachia* in fig wasps

We screened 64 species of wasps associated with 24 Australian and two outgroup *Ficus* spp. and detected infections in 67% (43/64) of species (table 1). There was no significant difference between pollinators (75%) and non-pollinators (61%) in the proportion of infected species ($\chi^2 = 1.38$, d.f. = 1, $p > 0.05$). In three cases, one species of fig is associated with two species of pollinator. Both pollinators of *F. crassipes* were infected, whereas both pollinators of *F. obliqua* were uninfected. However, in the case of *F. pleurocarpa*, *P. deuterus* harbours *Wolbachia* whereas *P. regalis* does not (table 1).

(b) Intraspecific variation in *Wolbachia* prevalence

In all but one species where we screened more than one individual, they were either all infected or all uninfected. However, *P. nigriventris* has two geographically separated populations each with a different infection status: the northern is infected, whereas the southern is uninfected (table 1).

(c) Diversity of *Wolbachia* strains

We obtained 63 *wsp* sequences from 47 individuals, representing 37 species. All infected species harbour A-clade *Wolbachia*, except for *Lipporhophalum tentacularis*, which harbours both A- and B-clade *Wolbachia*. This result is consistent with other studies (of Hymenoptera) in which infections by A-clade *Wolbachia* predominate (e.g. West *et al.* 1998). Fifteen out of 37 species harbour double infections (AA or AB) and there is no significant difference

between the incidence of double infections in pollinating and non-pollinating wasps ($\chi^2 = 0.04$, d.f. = 1, $p > 0.05$; table 1). In most cases, *wsp* sequences from individuals from different populations of the same species were identical. However, *P. imperialis* had mixed infections. Some populations harboured a single A-clade *Wolbachia*, whereas others had a double infection (table 1).

(d) Incidence of *Wolbachia*: Australia versus Panama

There were no significant differences between Australian and Panamanian fig wasp communities in *Wolbachia* incidence (table 2). Combining species screened from these two localities, we have an overall incidence of *Wolbachia* in fig wasps of 63% (66/105 species). This combined incidence is significantly higher than the global estimate of 20% infection in insects (121/591 species, $\chi^2 = 81.51$, $p < 0.0001$), and the estimate of 25% infection when considering Hymenoptera only (39/155 species, $\chi^2 = 36.95$, $p < 0.001$).

(e) Phylogenies

The topologies of the MP and Bayesian phylogenies were identical and revealed distinct Australian (5) and Panamanian (3) clades (figure 1). No *Wolbachia* sequences were identical between the Australian and Panamanian fig wasps. However, in two instances Panama/Australia sequences are more closely related to Australia/Panama sequences than to others from the same location (figure 1).

(f) Interspecific horizontal transmission of *Wolbachia*

There are several cases where multiple Australian species share the same *wsp* sequence. For example, the same sequence is shared by *P. blandus*, *P. addicotti*, *P. rigisamos*, *P. imperialis*, *P. nigriventris*, the *Sycoscapter* species found in *F. microcarpa* and the *Platyneura* species found in *F. racemosa*. Out of 273 pairwise comparisons between species sharing very similar (less than 5 bp difference) *Wolbachia* strains, 25% were from the same genus. However, 71% were neither closely related nor shared the same fig community. The remaining 5% (14/273) of species pairs shared the same fig community. In 5/13 fig wasp communities for which we obtained *wsp* sequences from more than one wasp species, two or more wasp species shared very similar *wsp* sequences. For example, in *F. xylosyia*, the pollinator *P. riecki* and the non-pollinating *Sycoscapter* species have identical *wsp* sequences. We tested for an ecological correlate of horizontal transfer by comparing observed and expected numbers of cases where two wasps with the same *wsp* strain (less than 5 bp difference) shared the same host fig species. We used the actual data on host figs and *wsp* sequences and randomized the association between these two traits 100 times to generate a null distribution. The observed number (14) of 'ecological pairs' of wasps that shared the same *wsp* sequence fell within the null distribution of randomized data (range 7–23, mean = 15.0) such that there is no evidence for a host fig correlate of shared infection status ($p = 0.68$).

(g) Do *Wolbachia* sequences show geographical signatures?

To test whether the *Wolbachia* strain harboured by a host is correlated significantly with its geographical origin, we

Table 1. Fig wasp species screened for *Wolbachia* in this study.

<i>Ficus</i> sp. (code)	wasp species	wasp biology	number infected (number screened)	<i>Wolbachia</i> subgroup
section Malvanthera				
<i>F. brachypoda</i> (F1)	<i>Pleistodontes athysanus</i>	pollinator	8 (8)	AA
	<i>Sycoscapter</i> sp.	non-pollinator	0 (2)	— ^a
<i>F. cerasicarpa</i> (F2)	<i>Pleistodontes macrocainus</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	A
<i>F. crassipes</i> (F3)	<i>Pleistodontes addicotti</i>	pollinator	14 (14)	A
	<i>Pleistodontes nitens</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	2 (2)	AA
	<i>Watshamiella</i> sp.	non-pollinator	1 (1)	A
<i>F. destruens</i> (F4)	<i>Pleistodontes rigisamos</i>	pollinator	14 (14)	A
<i>F. glandifera</i> (F5)	<i>Pleistodontes blandus</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	AA
<i>F. hesperidiiformis</i> (F6)	<i>Pleistodontes plebejus</i>	pollinator	1 (1)	AA
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	A
<i>F. lilliputiana</i> (F7)	<i>Pleistodontes proximus</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	A
<i>F. macrophylla</i> (F8)	<i>Pleistodontes froggatti</i>	pollinator	0 (10)	—
	<i>Eukobelea</i> sp.	gall-inducer	0 (1)	—
	<i>Sycoscapter</i> sp.	non-pollinator	0 (3)	—
	<i>Watshamiella</i> sp.	non-pollinator	2 (2)	AA
<i>F. obliqua</i> (F9)	<i>Pleistodontes greenwoodi</i>	pollinator	0 (14)	—
	<i>Pleistodontes xanthocephalus</i>	pollinator	0 (9)	—
	<i>Eukobelea</i> sp.	gall-inducer	0 (4)	—
	<i>Megastigmus</i> sp.	non-pollinator	0 (1)	—
	<i>Philotrypesis</i> sp.	non-pollinator	2 (2)	a
	<i>Pseudidarnes</i> sp.	gall-inducer	0 (2)	—
	<i>Sycoscapter</i> sp.	non-pollinator	5 (5)	A
	<i>Watshamiella</i> sp.	non-pollinator	0 (2)	—
<i>F. platypoda</i> (F10)	<i>Pleistodontes cuneatus</i>	pollinator	1 (1)	AA
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	A
<i>F. pleurocarpa</i> (F11)	<i>Pleistodontes deuterus</i>	pollinator	10 (10)	A
	<i>Pleistodontes regalis</i>	pollinator	0 (7)	—
	<i>Sycoscapter</i> sp.	non-pollinator	2 (2)	A
<i>F. rubiginosa</i> (F13)	<i>Pleistodontes imperialis</i>	pollinator	71 (71)	AA+A ^a
	<i>Herodotia</i> sp.	gall-inducer	1 (1)	—
	<i>Eukobelea</i> sp.	gall-inducer	2 (2)	A
	<i>Philotrypesis</i> sp. (yellow)	non-pollinator	1 (1)	AA
	<i>Philotrypesis</i> sp. (black)	non-pollinator	3 (3)	AA
	<i>Pseudidarnes</i> sp.	gall-inducer	0 (5)	—
	<i>Sycoscapter</i> sp.	non-pollinator	2 (2)	a
	<i>Watshamiella</i> sp.	non-pollinator	0 (3)	—
<i>F. subpuberula</i> (F14)	<i>Pleistodontes astrobocheilus</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	0 (1)	—
<i>F. triradiata</i> (F15)	<i>Pleistodontes schizodontes</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	0 (1)	—
<i>F. watkinsiana</i> (F16)	<i>Pleistodontes nigriventris</i>	pollinator	9 (17)	AA
	<i>Eukobelea</i> sp.	gall-inducer	5 (7)	AA
	<i>Sycoscapter</i> sp.	non-pollinator	0 (3)	—
<i>F. xylosyia</i> (F17)	<i>Pleistodontes riecki</i>	pollinator	1 (1)	A
	<i>Sycoscapter</i> sp.	non-pollinator	2 (2)	A
other <i>Ficus</i> sections				
<i>F. benjamina</i> (F18)	<i>Eupristina</i> sp.	pollinator	0 (2)	—
<i>F. carica</i> (F19)	<i>Blastophaga psenes</i>	pollinator	4 (4)	A
	<i>Philotrypesis</i> sp.	non-pollinator	1 (1)	AA
<i>F. congesta</i> (F20)	<i>Ceratosolen</i> sp.	pollinator	0 (2)	—
<i>F. coronulata</i> (F21)	<i>Ceratosolen</i> sp.	pollinator	4 (4)	a
<i>F. microcarpa</i> (F22)	<i>Eupristina</i> sp.	pollinator	2 (2)	A
	<i>Sycoscapter</i> sp.	non-pollinator	1 (1)	A
<i>F. montana</i> (F23)	<i>Liporrhophalum tentacularis</i>	pollinator	6 (6)	AB
<i>F. opposita</i> (F24)	<i>Kradibia</i> sp.	pollinator	2 (2)	AA
<i>F. pungens</i> (F25)	<i>Sycoscapter</i> sp.	non-pollinator	0 (1)	—

(Continued.)

Table 1. (Continued.)

<i>Ficus</i> sp. (code)	wasp species	wasp biology	number infected (number screened)	<i>Wolbachia</i> subgroup
<i>F. racemosa</i> (F12)	<i>Ceratosolen</i> sp.	pollinator	0 (2)	—
	<i>Platyneura</i> sp.	gall-inducer	2 (2)	A ^a
<i>F. septica</i> (F26)	<i>Ceratosolen bisulcatus</i>	pollinator	1 (1)	AA
	<i>Philotrypesis</i> sp.	non-pollinator	4 (4)	—
	<i>Sycoscapter</i> sp.	non-pollinator	0 (1)	—

^a Sample confirmed positive by PCR but more than six attempts to obtain sequence failed.

Table 2. Comparison of incidence of *Wolbachia* infections in fig wasps between Panama and Australia. (Incidence is expressed as a proportion of species in that category and χ^2 -values are given for d.f. = 1. Non-significance (n.s.) means $p > 0.05$.)

comparison	Panama	Australia	χ^2	significance
total number of fig wasp species screened	44	61		
number of pollinator species	18	26		
number of non-pollinator species	26	35		
incidence of <i>Wolbachia</i> in all species	0.59	0.66	0.46	n.s.
incidence of <i>Wolbachia</i> in pollinators	0.50	0.73	2.45	n.s.
incidence of <i>Wolbachia</i> in non-pollinators	0.65	0.60	0.18	n.s.
incidence of double infections	0.16	0.35	1.89	n.s.

coded each *wsp* sequence in the phylogeny according to the origin (Australia or Panama) of its fig wasp host. We shuffled this character randomly across the tips of the phylogeny 1000 times, using MACCLADE 4 (Maddison & Maddison 2000). For each randomized dataset we calculated the minimum number of changes required, to create a null distribution of the number of steps. The number of changes (or fewer) implied by the actual data (six) occurred only four times in the 1000 randomized samples, so there is a significant ($p = 0.004$) phylogenetic clustering of *Wolbachia* strains according to geography.

4. DISCUSSION

(a) High incidence of *Wolbachia* in fig wasps

We found that 67% of Australian fig wasps harbour *Wolbachia* infections, which is significantly and substantially higher than the putative global equilibrium of 20% for insects in general (Werren & Windsor 2000). In fact, this is the highest level reported in a taxonomically focused study of this kind. Further, our figure is extremely similar to that obtained (59%) in a comparable study of fig wasp communities in Panama. Because these two estimates are independent, with the incidences depending on the local dynamics of region-specific *Wolbachia* strains, it appears that fig wasps have an unusually high incidence of *Wolbachia* infection. This is the first time, to our knowledge, that the incidence of *Wolbachia* has been compared across continents for a well-defined ecological and taxonomic group of insects and allows us to assert that the high infection level is a feature of fig wasps in general, rather than a result of regional factors.

The high incidence recorded in fig wasps could be attributable to features of Hymenoptera in general, or to fig wasps in particular. Most broad surveys of insects (Werren *et al.* 1995a; West *et al.* 1998; Werren & Windsor 2000) and targeted studies of specific taxa (Wenseleers *et al.* 1998; Plantard *et al.* 1999; Rokas *et al.* 2002) have reported

high incidences of infection for Hymenoptera, although the fig wasps (Shoemaker *et al.* 2002 and this study) have the highest incidence of all. Hymenoptera may be unusually prone to *Wolbachia* infections but, as yet, there is no convincing general reason for this. Haplodiploidy might reduce host–parasite conflict, because unfertilized eggs can still develop as males; however, this idea requires testing on haplodiploid taxa other than Hymenoptera (Shoemaker *et al.* 2002). Other ideas, involving social (Wenseleers *et al.* 1998; Reuter & Keller 2003) or parasitoid lifestyles (e.g. West *et al.* 1998; Cook & Butcher 1999; Vavre *et al.* 1999) may be important but do not apply to fig wasps, which are neither social nor have generalist parasitoids (Shoemaker *et al.* 2002).

The peculiar biology of fig wasps offers other potential explanations. High rates of horizontal transmission (HT) both within species (delays loss of infection) and between species (promotes new infections) should contribute to high *Wolbachia* incidence and the fig syconium may favour HT (Shoemaker *et al.* 2002). In most fig species, each syconium (i.e. fig fruit) contains 50–500 wasps, of a few different species, developing together in close proximity. Some species are parasitoids or kleptoparasites of other wasps, while many induce galls (Cook & Rasplus 2003). Direct or indirect interactions during larval development may provide good opportunities for HT (e.g. Huigens *et al.* 2000) and further opportunities may arise when the mature wasps emerge and mate inside the syconium, where some species engage in fights resulting in injuries and deaths (see also Rigaud & Juchault 1995).

Some data from cynipid gall wasps (Plantard *et al.* 1999; Rokas *et al.* 2002) are at least consistent with the idea that large numbers of insects developing together in a small space may facilitate HT. Oak gall wasps (Cynipini) are unusual for Hymenoptera in that only 5/53 (9%) of species screened by Rokas *et al.* (2002) were infected. Many aspects of their biology are comparable to fig wasps, but

Figure 1. MP analysis of 76 *Wolbachia* *wsp* sequences from Australia (open lines), Panama (filled lines), and outgroup (no lines) species of fig wasp. See § 2c for details of heuristic searches conducted. Bootstrap values (1000 replicates)/posterior node probabilities (Bayesian analysis) are shown above branches for each node.

galls of most cynipid species are unilocular and contain only one (if unparasitized) or a few (in some cases of parasitism) individual wasps, which may reduce the opportunity for HT. Interestingly, gall wasps belonging to the tribes Rhoditini (rose gall wasps) and 'Aylacini' (herbaceous plant gall wasps) have higher incidences of infection (Plantard *et al.* 1999), and many of these species form multilocular galls.

Another possibility is that *Wolbachia* infections persist well in fig wasps owing to low levels of host–parasite conflict. Shoemaker *et al.* (2002) suggested two possibilities. First, conflict may be low in fig-pollinating wasps, because selection on wasp nuclear genes favours production of female-biased sex ratios owing to local mate competition. Consequently, there is less discrepancy in the optimal wasp sex ratios from host and parasite viewpoints. However, while all fig-pollinating wasps have female-biased sex ratios (Herre *et al.* 1997), only some non-pollinating species do (West & Herre 1998; Fellowes *et al.* 1999) and there is no significant difference in *Wolbachia* incidence between the two groups (table 1; Shoemaker *et al.* 2002). Second, it is possible that the *Wolbachia* strains infecting fig wasps tend to have direct positive effects on host fitness. This idea is attractive, but untested.

(b) Frequency of infection within a species

In most species, individuals tested were either all positive or all negative (table 1). In several species, sample sizes are too small to be meaningful, but this observation holds for the better-sampled species (table 1) and is largely consistent with the survey in Panama (Shoemaker *et al.* 2002). We found two pollinator species that varied in infection status. *P. nigriventris* and its host fig, *F. watkinsiana*, have two disjunct natural populations in northern and southern Queensland rainforests, separated by a gap of ca. 1000 km of unsuitable arid habitats. All eight southern wasps screened were uninfected, whereas all nine northern wasps were infected, suggesting a population-level difference in infection status. A similar scenario applies in the cynipid gall wasp *Biorhiza pallida*, which has different infection status in northern and southern Europe (Rokas *et al.* 2001). In *P. imperialis*, all 70 individuals screened from a wide geographical range were infected with an A-strain, but some populations also harboured a second A-strain (table 1; figure 1). *P. imperialis* might comprise more than one 'cryptic' species (e.g. Molbo *et al.* 2003); alternatively, the second infection may have been gained or lost in only some geographical areas within the species' range.

(c) Geography and horizontal transmission of infections

We found only A-clade *Wolbachia* in Australian fig wasps, which is consistent with their predominance in Panamanian fig wasps (Shoemaker *et al.* 2002) and in Hymenoptera in general (e.g. Werren & Windsor 2000). However, the strains infecting fig wasps in Panama and Australia are different and several regional clades are observed (figure 1). Consequently, although fig wasp communities on opposite sides of the world have similar incidences of infection, they have reached their current infection situations by independent local *Wolbachia* dynamics. This is consistent with the overwhelming body of evidence that *Wolbachia* infections do not persist in host lineages over many millions of

years, and emphasizes the ongoing role of HT in determining the incidence of *Wolbachia* infections (e.g. Schilthuizen & Stouthamer 1997; Cook & Butcher 1999; Werren & Windsor 2000; Shoemaker *et al.* 2002).

The striking similarity of some *wsp* sequences shared by unrelated fig wasp species provides strong circumstantial evidence for HT, especially when one considers that most of the sequences generated in this study cluster into two of five clades (Australia clades 1 and 2, figure 1). In some cases, we can identify potential ecological contexts for HT; for example, the identical *wsp* sequences shared by the pollinator and a non-pollinator of *Ficus xylosyca* could be an example of HT between different species in the same syconia. In addition, very similar *Wolbachia* strains are harboured by more than one species in wasp communities on five other fig-species. However, like Shoemaker *et al.* (2002), we found a majority of cases where there was no direct ecological link between two host species that shared the same *wsp* strain. Importantly, if we build phylogenies that include *wsp* sequences from insects other than fig wasps (results not shown), some of these strains cluster with fig-wasp-derived *wsp* sequences. This suggests that HT may also involve more distantly related taxa, perhaps some of the many other insect species that live or feed on fig trees, although the mechanisms of transfer remain more difficult to envisage. Future studies to investigate the route of HT could test whether all species, of non-pollinating fig wasps are specific to one host fig or are generalists on different fig species, and investigate the diversity of *Wolbachia* strains in other insects found on figs.

5. CONCLUSIONS

Fig wasps appear to have the highest known incidence of *Wolbachia* infection, regardless of geographical origin, but we do not yet know why. High levels of HT within and between species may be important and future studies should seek the phylogenetic signatures of HT within species, as has been done recently for fire ants (Shoemaker *et al.* 2003). In addition, positive direct fitness effects could be important, but are hard to investigate given the paucity of fig wasp species with mixed infection status and the difficulty of breeding experiments in these coevolved mutualists.

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