Insect science

Insect Science (2010) 17, 101–111, DOI 10.1111/j.1744-7917.2009.01291.x

ORIGINAL ARTICLE

High incidences and similar patterns of *Wolbachia* infection in fig wasp communities from three different continents

Lin-Lin Chen[1,2], James M. Cook[3], Hui Xiao[1], Hao-Yuan Hu[1,2], Li-Ming Niu[1,2] and Da-Wei Huang[1,4]

[1]*Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China,* [2]*Graduate University of Chinese Academy of Sciences, Beijing, China,* [3]*School of Biological Sciences, University of Reading, Reading, UK, and* [4]*College of Plant Protection, Shandong Agricultural University, Tai'an, Shandong Province, China*

Abstract *Wolbachia* are endosymbiotic bacteria that infect numerous arthropod species. Previous studies in Panama and Australia revealed that the majority of fig wasp species harbor *Wolbachia* infections, but that similar patterns of incidence have evolved independently with different wasp species and *Wolbachia* strains on the two continents. We found *Wolbachia* infections in 25/47 species (53%) of fig wasp associated with 25 species of Chinese figs. Phylogenetic analyses of *Wolbachia wsp* sequences indicated that very similar strains are not obviously found in either closely related or ecologically linked fig wasps species. The extremely high prevalence of *Wolbachia* in fig wasps (over 50% of species infected) is not constrained by geographical origin and is a recurrent theme of fig wasp/*Wolbachia* interactions.

Key words fig wasps, horizontal transmission, *Wolbachia*, *wsp*

Introduction

Wolbachia are cytoplasmically inherited endosymbiotic alpha-proteobacteria found in a wide range of insects, filarial nematodes, arachnids and isopods (Bouchon *et al.*, 1998; O'Neill *et al.*, 1992; Werren & Jaenike, 1995; Werren *et al.*, 1995b). *Wolbachia* have attracted considerable recent interest for their widespread distribution and a number of reproductive alterations that they cause in their hosts, such as cytoplasmic incompatibility (CI) (Breeuwer & Werren, 1990; Hoffmann *et al.*, 1986; Yen & Barr, 1971), parthenogenesis induction (Stouthamer *et al.*, 1990; Stouthamer *et al.*, 1993), male-killing (Hurst *et al.*, 1999), and feminization (Rigaud *et al.*, 1991). In theory, these alterations of host reproduction can facilitate or even cause host speciation (Bordenstein *et al.*, 2001; Rokas, 2000).

Correspondence: Da-Wei Huang, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Beijing 100101, China. Tel: +86 10 64807235; fax: +86 10 64807235; email: huangdw@ioz.ac.cn

Previous similar surveys of *Wolbachia* incidence in a range of diverse insect species from four different regions (Panama, Great Britain, North America and Thailand) have all yielded estimates of between 16% and 23% of species infected (Kittayapong *et al.*, 2003; Werren & Windsor, 2000; Werren *et al.*, 1995a; West *et al.*, 1998), which suggest that approximately 20% of all insect species harbor *Wolbachia* infections, consistent with a widespread equilibrium frequency (Werren & Windsor, 2000). Besides these broad surveys, many taxon-specific or ecologically focused surveys for *Wolbachia* have shown that certain taxonomic groups may be more prone to infection (Werren & Windsor, 2000). For example, it appears that some insect groups, especially Hymenoptera, are more prone to harbor *Wolbachia* than others (Vavre *et al.*, 1999; Wenseleers *et al.*, 1998; Werren & Windsor, 2000). They are also generally high in studies focusing on particular hymenopteran groups, with infection 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), 67% (43/64) of Australian fig wasps species (Haine & Cook, 2005), 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) (Plantard *et al.*, 1999; Rokas *et al.*, 2002; Schilthuizen & Gittenberger, 1998). However, there is a curious exception provided by oak gall-inducing wasps (Cynipini), where only 9% (5/53) of species are infected (Rokas *et al.*, 2002).

Studies on *Wolbachia* in fig wasps have concentrated on the prevalence and incidence of *Wolbachia* bacteria across large geographic regions. High incidence of *Wolbachia* was found both in Australia and Panama, which is the highest proportion reported for any group of insects (Haine & Cook, 2005; Shoemaker *et al.*, 2002). Evidence for frequent horizontal transmission of *Wolbachia* bacteria was also found in both continents, while the majority of similar *Wolbachia* strains were found in wasps without known ecological interactions.

Figs (*Ficus*, Moraceae) and their associated fig wasps (Hymenoptera, Chalcidoidea, Agaonidae (Boucek, 1988)) have attracted much attention and have been regarded as a model system for research on topics such as coevolution, sex ratio evolution, virulence evolution and host-parasite interactions (Cook & Rasplus, 2003; Weiblen, 2002). Figs and fig pollinators are a textbook example of obligate pollinating mutualism and an extreme case of coevolution (Ramirez, 1980; Ramirez, 1974; Weiblen & Bush, 2002; Wiebes, 1979). Non-pollinators can be gallers, parasitoids or inquilines which impact on the system by competing for oviposition sites, killing pollinator larvae, or by using fertilized ovaries and thus occupying a portion of seeds (West & Herre, 1994; West *et al.*, 1996).

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 northern Australia. *Ficus* species are diverse in China (98 species; Zhang *et al.*, 1998) and these represent four subgenuses and 11 sections, with most of them distributed in southern China.

Our study focused on *Wolbachia* in the wasp communities of 25 species of figs, and no Chinese fig specimens had been involved in previous studies except three, *F. benjamina*, *F. microcarpa* and *F. racemosa*. Further, all fig species studied here are native in China and incidence of *Wolbachia* in these species would represent the basic status of that in China. There are two main purposes of this paper: (i) to test whether the incidence of *Wolbachia* in Chinese fig wasps is as high as reported for fig wasp communities in Panama and Australia; and (ii) to identify the *Wolbachia* strains in Chinese fig wasps and whether they are shared with any fig wasps from other regions.

Materials and methods

Species and field sampling

From 2003 to 2006, fig wasp species associated with 25 figs were sampled mainly in southern China (Table 1). Ripe syconia were collected from species of wild fig trees. Wasps were allowed to emerge naturally from their syconia and were stored in 95% ethanol at −20°C. Similar species on one host fig species were distinguished on the basis of female body color and ovipositor length. Most species are currently less well described, especially the non-pollinators. Due to the coevolution relationship between fig pollinator wasps and their host fig, pollinators are relatively easily identified and well studied (Jiang *et al.*, 2006a; Jousselin *et al.*, 2003; Machado *et al.*, 2001; Marussich & Machado, 2007), while the taxonomy of non-pollinating fig wasps is less well advanced. The most common genus in our samples were *Sycoscapter* (Hymenoptera: Sycoryctinae) (up to 10 in our samples) and *Philotrypesis* (Hymenoptera: Sycoryctinae) (up to 13) (Table 1). Previous molecular phylogenetic studies suggested *Sycoscapter* (Sycoryctinae) have partially cospeciated with their hosts (Lopez-Vaamonde *et al.*, 2001; Weiblen & Bush, 2002). *Philotrypesis* species in our samples are all involved in the previous molecular phylogenetic study done before in our lab (Jiang *et al.*, 2006b), which helped us in species identification by DNA markers (ITS2/Cyt B/28S rDNA).

Molecular methods

Several female wasps were selected at random from samples preserved in 95% ethanol, with the remains as voucher specimens. Specimens were washed in Tris-EDTA (TE) buffer before DNA extraction in order to get rid of the ethanol and dirt outer layer of the insects. Total genomic DNA was extracted from a single individual using a standard phenol-chloroform extraction method (Sambrook *et al.*, 1989). DNA quality was assessed by amplifying a portion of COI gene (1751–2613) sequences using conserved insect mitochondrial primers C1-J-1751 (5′-GGA TCA CCT GAT ATA GCA TTC CC-3′) (Simon *et al.*, 1994) and COIH2613 (5′-ATT GCA AAT ACT GCA CCT AT-3′) (Castro *et al.*, 2002). We screened for *Wolbachia* by polymerase chain reaction (PCR) with primers *wsp* 81F(5′-TGG TCC AAT AAG TGA TGA

Table 1 Summary of fig wasp samples and species used for phylogenetic study.

Ficus spp. (code)	Fig wasp species	Wasp biology	Number infected (number screened)	Voucher specimen nos. (GenBank accession nos.)	Locality
F. altissima (F1)	*Sycoscapter* sp. 2[a]	Non-pollinator	1 (1)	IOZA0023	XTBG, Yunnan, China
F. auriculata (F2)	*Philotrypesis longicaudata*	Non-pollinator	2 (2)	IOZA0041	XTBG, Yunnan, China
	Ceratosolen emarginatus	Pollinator	1 (1)	IOZA0042 (EU239227)	XTBG, Yunnan, China
F. benjamina (F3)	*Eupristina koningsbergeri*	Pollinator	1 (1)	IOZA0030	XTBG, Yunnan, China
	Sycoscapter sp.[a]	Non-pollinator	0 (1)	IOZA0037	XTBG, Yunnan, China
	Phioltrypesis tridentate	Non-pollinator	1 (1)	IOZA0032	XTBG, Yunnan, China
F. concinna (F4)	*Plastycapca* sp.	Pollinator	1 (1)	IOZA0108	XTBG, Yunnan, China
	Philotrypesis sp. 1[b]	Non-pollinator	0 (1)	IOZA0106	XTBG, Yunnan, China
F. curtipes (F5)	*Philotrypesis* sp.[b]	Non-pollinator	0 (1)	IOZA0005	XTBG, Yunnan, China
	Lipothymus sp.[c]	Non-pollinator	0 (1)	IOZA0028	XTBG, Yunnan, China
F. cyrtophylla (F6)	*Blastophaga* sp.[d]	Pollinator	1 (1)	IOZA0068	XTBG, Yunnan, China
F. drupacea (F7)	*Ceratosolen.* sp.[e]	Pollinator	0 (1)	IOZA0044	XTBG, Yunnan, China
	Philotrypesis sp.[b]	Non-pollinator	0 (1)	IOZA0115	XTBG, Yunnan, China
F. fistulosa (F8)	***Platyneura* sp.**	Non-pollinator	1 (1)	IOZA0089 (EU239231)	XTBG, Yunnan, China
F. hederacea Roxb *(F9)*	*Sycoscapter* sp.[a]	Non-pollinator	0 (1)	IOZA0113	XTBG, Yunnan, China
F. hirta (F10)	*Sycoscapter* sp.[a]	Non-pollinator	1 (1)	IOZA0046	XTBG, Yunnan, China
	***Philotrypesis* sp.**[b]	Non-pollinator	1 (1)	IOZA0047 (EU239228)	XTBG, Yunnan, China
F. hirta var. *roxburghii (F11)*	***Philotrypesis* sp.**[b]	Non-pollinator	1 (1)	IOZA0049 (EU239229)	Guangzhou, Guangdong, China
F. hispida (F12)	***Ceratosolen solmsi***	Pollinator	1 (1)	IOZA0018 (EU239232)	Danzhou, Hainan, China
	Philotrypesis pilosa	Non-pollinator	1 (1)	IOZA0016 (EU239233) (EU239234)	Danzhou, Hainan, China
	Philotrypesis sp.[b]	Non-pollinator	0 (5)	IOZA0017	Danzhou, Hainan, China
	Apocrypta bakeri	Non-pollinator	0 (5)	IOZA0019	Danzhou, Hainan, China
F. ischmopoda (F13)	*Sycoscapter* sp.[a]	Non-pollinator	0 (1)	IOZA0109	XTBG, Yunnan, China
F. langkokensis (F14)	*Sycoscapter* sp.[a]	Non-pollinator	1 (1)	IOZA0057	XTBG, Yunnan, China
	Blastophaga sp.[d]	Pollinator	1 (1)	IOZA0058	XTBG, Yunnan, China
F. microcarpa (F15)	***Eupristina verticillata***	Pollinator	1 (1)	IOZA0050 (EU239230)	XTBG, Yunnan, China
	Philotrypesis sp. 1[b]	Non-pollinator	0 (1)	IOZA0051	XTBG, Yunnan, China
	Philotrypesis sp. 2[b]	Non-pollinator	0 (2)	IOZA0052	XTBG, Yunnan, China
	Sycoscapter sp. 1[a]	Non-pollinator	0 (1)	IOZA0053	XTBG, Yunnan, China
	Sycoscapter sp. 2[a]	Non-pollinator	0 (1)	IOZA0054	XTBG, Yunnan, China
	Sycobia sp. 1	Non-pollinator	0 (1)	IOZA0055	XTBG, Yunnan, China

Continued.

Table 1 Continued.

Ficus spp. (code)	Fig wasp species	Wasp biology	Number infected (number screened)	Voucher specimen nos. (GenBank accession nos.)	Locality
F. nervosa Heyne *(F16)*	*Philotrypesis* sp.[b]	Non-pollinator	1 (1)	IOZA0098	XTBG, Yunnan, China
F. racemosa (F17)	*Platyneura mayri*	Non-pollinator	1 (1)	IOZA0003	XTBG, Yunnan, China
	Platyneura agraensis	Non-pollinator	0 (1)	IOZA0006	XTBG, Yunnan, China
F. semicordata (F18)	*Platyneura dunia*	Non-pollinator	1 (1)	IOZA0008	XTBG, Yunnan, China
	Sycoscapter trifemmensis	Non-pollinator	0 (7)	IOZA0012	XTBG, Yunnan, China
F. squamous (F19)	*Ceratosolen* sp.[e]	Pollinator	1 (1)	IOZA0036	XTBG, Yunnan, China
F. subalata (F20)	*Philotrypesis* sp.[b]	Non-pollinator	0 (2)	IOZA0064	XTBG, Yunnan, China
	Philotrypesis sp.[b]	Non-pollinator	1 (1)	IOZA0066	XTBG, Yunnan, China
F. superba (F21)	*Platyscapa corneri*	Pollinator	1 (1)	IOZA0014	XTBG, Yunnan, China
F. tinctoria (F22)	*Philotrypesis* sp.[b]	Non-pollinator	1 (2)	IOZA0063	XTBG, Yunnan, China
F. variolosa Lindl *(F23)*	*Blastophaga silvestriana*	Pollinator	0 (1)	IOZA0095	XTBG, Yunnan, China
F. virens Ait. *(F24)*	*Sycoscapter* sp.[a]	Non-pollinator	0 (1)	IOZA0076	XTBG, Yunnan, China
F. virens Ait. var. *virens (25)*	*Lipothymus* sp.[c]	Non-pollinator	0 (1)	IOZA0083	Guangzhou, Guangdong, China
	Sycoscapter sp.[a]	Non-pollinator	1 (1)	IOZA0085	Guangzhou, Guangdong, China
	Ormyrus sp.	Non-pollinator	1 (1)	IOZA0086	Guangzhou, Guangdong, China
	Philotrypesis sp.[b]	Non-pollinator	0 (2)	IOZA0091	Guangzhou, Guangdong, China
Total 25	47		25/47 = 53%		

Wolbachia strains from species in bold were used for phylogenetic study.
[a]Undescribed species in *Sycoscapter* genus, add up to 10;
[b]Undescribed species in *Philotrypesis* genus, add up to 13;
[c]Undescribed species in *Lipothymus* genus, add up to 2;
[d]Undescribed species in *Blastophaga* genus, add up to 2;
[e]Undescribed species in *Ceratosolen* genus, add up to 2.

AGA AAC-3′) and *wsp* 691R (5′-AAA AAT TAA ACG CTA CTC CA-3′) (Dobson *et al.*, 1999) which amplify part of the *Wolbachia* surface protein gene (*wsp*). We always prepared a positive control from the genomic DNA of a wasp individual known to be *Wolbachia*-infected in pre-research work and a negative control with equal volume sterile water instead of wasp DNA. To avoid fake negative results, we retested those DNA specimens with negative *wsp* amplifications in a previous step using universal primers of two more conservative gene fragments: *ftsZ* (*ftsZ* F [5′-TAC TGA CTG TTG GAG TTG TAA CTA ACG CGT-3′] and *ftsZ* R [5′-TGC CAG TTG CAA GAA CAG AAA CTC TAA CTC-3′], Jeyaprakash & Hoy, 2000) for *fts Z* gene and 16s rRNA (16SwolF [5′-TTG TAG CCT GCT ATG GTA TAA CT-3′] and 16SwolR [5′-GAA TAG GTA TGA TTT TCA TGT-3′], O'Neill *et al.*, 1992; van Meer *et al.*, 1999) for 16S rRNA gene.

A reaction mix was made for each PCR run; 25 μL of the mixture containing 2.5 μL 10 × Hifi Taq Buffer (with Mg^{++}) (TransGen Biotech, Beijing, China), 0.75 μL 10 mmol/L deoxynucleotide triphosphate (dNTP), 1 μL Hifi Taq polymerase, 1 μL 10 mmol/L each primers and 1–3 μL genomic DNA template. Amplifications were carried out in a Eppendorf thermocycler programmed as follows: 2 min at 94°C for one cycle; 30 s at 94°C, 45 s at 55°C (55°C for *wsp*, *ftsZ* and *16S*, 46°C for *COI*), and

1 min at 72°C for 30 cycles; 10 min at 72°C for one terminal cycle. Sequencing reactions were carried out with ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA).

Sequences of *wsp* PCR products from several individuals clearly revealed the presence of more than one strain of *Wolbachia* (i.e., multiple peaks for 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 T-tailed vector (pEASY-T1 clone vector, TransGen Biotech, China) and transformed into *Escherichia coli* Top 10 cells. Positive colonies were screened for the presence of the desired *wsp* PCR insert using the above *wsp* primers. We chose PCR-amplified products from 3–7 colonies (which presumably had the *wsp* insert) representing each individual for sequencing.

Sequence alignment and phylogenetic analyses

Sequences obtained in our work and those previously published *Wolbachia* sequences from fig wasps found in GenBank (Haine & Cook, 2005; Shoemaker *et al.*, 2002) were initially aligned by using Clustal W (Thompson *et al.*, 1994). Unambiguous alignments were easily acquired for *wsp* sequences. The sequence data were used to construct phylogenetic trees of *Wolbachia* strains using neighbor-joining (NJ) and maximum parsimony (MP) methods, as implemented in PAUP 4.10 (Swofford, 2003). All characters were unordered and equally weighted. Gaps were treated as missing data. For NJ analysis, the most appropriate evolutionary model was determined for a given data set using PAUP 4.10 (Swofford, 2003) and Modeltest 3.7 (Posada & Crandall, 1998). Bootstrap support values were generated using 1 000 replicates and the tree was midpoint rooted. For MP analysis, we used a 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 (EU239227–239234).

Results

Wolbachia *prevalence in Chinese fig-associated wasps*

We screened 47 wasp species associated with 25 Chinese *Ficus* spp. and detected infections in 53% (25/47) of species (Table 1). There was no significant difference between pollinators (82%: 9/11) and non-pollinators (64%: 23/36) in the proportion of infected species ($\chi^2 = 1.246$, df = 1, $P > 0.1$).

Intraspecific variation in Wolbachia *prevalence*

In all species where we screened more than one individual, they were either all infected or all uninfected. According to the eight *wsp* sequences we got, only one fig wasp species, *Philotrypesis pilosa* on *F. hispida*, has multiple *Wolbachia* infections in the same individual, in which two *wsp* sequences of *Wolbachia* with 17% nucleotide diversity were obtained (Accession numbers: EU239233 and EU239234).

Incidence of Wolbachia: *China versus Panama and Australia*

Infections in different continents are compared in Table 2. No significant difference was found in the incidence of *Wolbachia* in all species ($\chi^2 = 0.86$, df = 2, $P > 0.1$), pollinators ($\chi^2 = 3.89$, df = 2, $P > 0.1$) and non-pollinators ($\chi^2 = 0.21$, df = 2, $P > 0.1$) among the three continents.

Phylogeny analyses of Wolbachia *strains*

Unfortunately we only obtained eight Chinese *Wolbachia* sequences, in which only six unique strains were identified. We used these eight sequences from our study plus 74 sequences from strains in other fig wasps available from GenBank (Accession numbers: AF521149–AF521152, AF521154–AF521172,

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

Comparison	Panama	Australia	China	χ^2	Significance
Incidence of *Wolbachia* in all species	0.59 (44)	0.66 (61)	0.53 (47)	0.86	n.s.
Incidence of *Wolbachia* in pollinators	0.50 (18)	0.73 (26)	0.82 (11)	3.89	n.s.
Incidence of *Wolbachia* in non-pollinators	0.65 (26)	0.60 (35)	0.64 (36)	0.21	n.s.

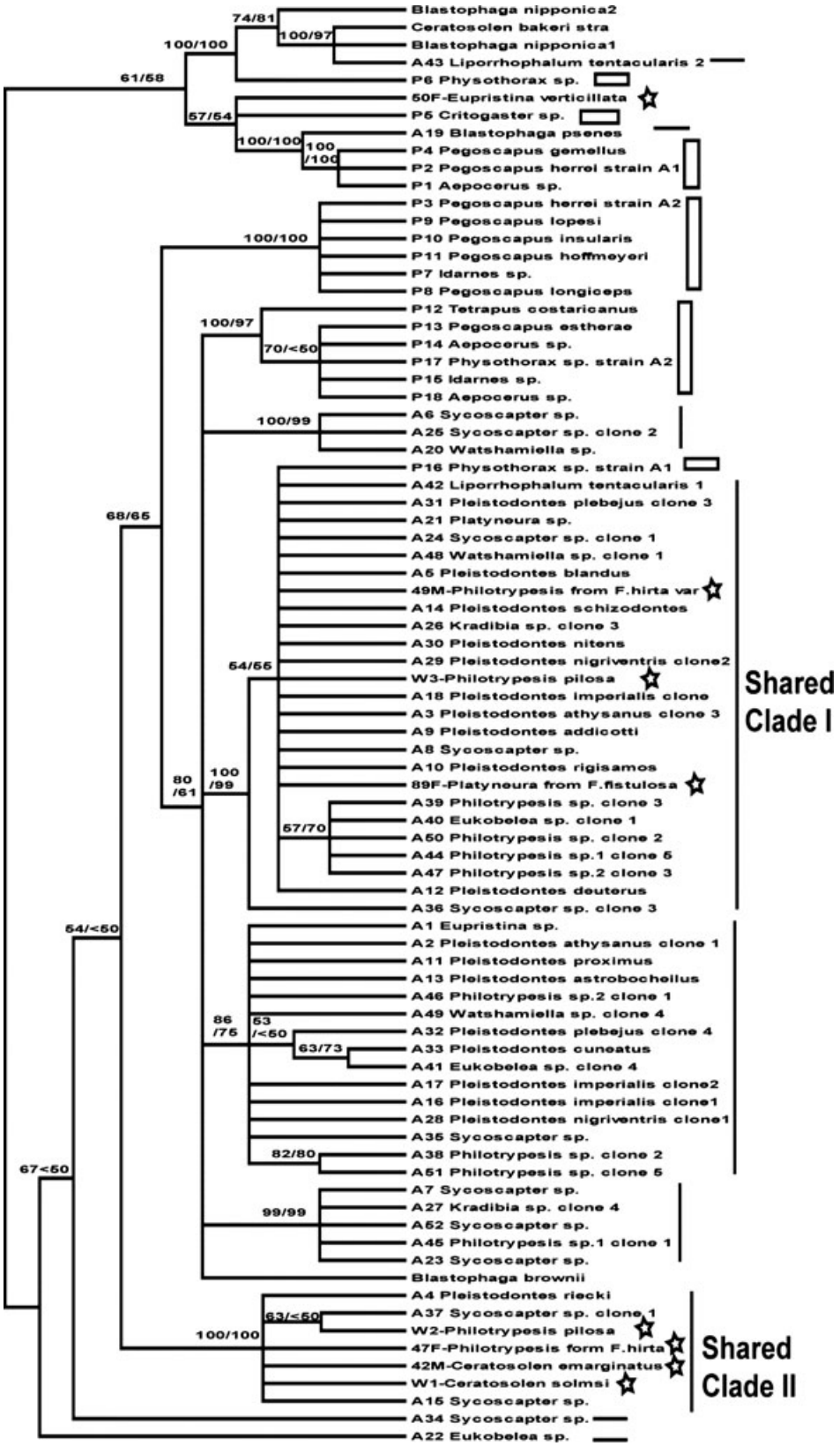

Table 3 Identical *wsp* sequences shared in species of different geographic origin.

Wolbachia strain (in share clade on Tree)	Host wasp species	Geographic origin	Accession number
1 share clade I	*Pleistodontes blandus*	Australia	AY567546
	Sycoscapter sp.	Australia	AY567549
	Pleistodontes addicotti	Australia	AY567550
	Pleistodontes rigisamos	Australia	AY567551
	Pleistodontes imperialis	Australia	AY567559
	Platyneura sp.	Australia	AY567562
	Pleistodontes nigriventris	Australia	AY567570
	Pleistodontes nitens	Australia	AY567571
	Pleistodontes plebejus	Australia	AY567572
	Liporrhophalum tentacularis	Australia	AY567583
	Philotrypesis pilosa	China	EU239234
2 share clade II	*Pleistodontes riecki*	Australia	AY567545
	Sycoscapter sp.	Australia	AY567556
	Ceratosolen solmsi	China	EU239232
	Ceratosolen emarginatus	China	EU239227
	Philotrypesis sp.	China	EU239229

AY567542–AY567593) adding up to 82 *wsp* sequences in the phylogeny study. Closely related *wsp* sequences (defined as < 5 bp differences) were sometimes shared by congeneric species, or those from the same region, but in other cases involved distantly related species from different geographic areas (China and Australia). There were two clades in which Chinese and Australian species have shared *Wolbachia* strains (Fig. 1). Repeated sampling and re-sequencing showed that these results were not due to laboratory contamination.

The NJ tree using the HKY85 as the appropriate distance model is shown in Figure 1. For MP, 217 out of 520 total characters were informative. A total of 101 most parsimonious trees were found. The topologies of the NJ tree and the strict consensus MP tree were very similar and revealed distinct Australian and Panamanian clades but mass Australian and Chinese clades. Three main features are evident from examination of the NJ tree in Figure 1. First, the *Wolbachia* strains from different fig wasps 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 Panama and Australia, and also Panama and China fig-associated wasps. However, identical *wsp* sequences were found between some Chinese and Australian wasps (Table 3). Third, distantly related fig wasp species often had very similar *Wolbachia* strains. These features of similar *Wolbachia* strains across different wasp species indicate frequent interspecific horizontal transmission of *Wolbachia*.

Discussion

High incidence of Wolbachia *in fig wasps*

We found that 53% of Chinese fig wasps harbor *Wolbachia*, which is significantly higher than most broad surveys of insects (John & Donald, 2000; Werren *et al.*, 1995b; West *et al.*, 1998), although it is not the highest incidence record in fig wasps. There is no significant difference between our result and those obtained in Panama (59%, $\chi^2 = 0.795$, df $= 1$, $P > 0.1$) and Australia (67%, $\chi^2 = 0.075$, df $= 1$, $P > 0.5$) (Table 2). Analyses

Fig. 1 Consensus NJ topology of 82 *Wolbachia wsp* sequences (*ca.* 521 bp of DNA) from Australia (A1–52) (marked with solid lines), Panama (P1–18) (marked with open lines), China (marked with pentagram) and outgroup (no lines), bootstrap support values (1 000 replicates) (neighbor-joining/maximum parsimony) are shown above branches for each node in the phylogram. Bootstrap support values < 50 are not indicated.

on *Wolbachia* prevalence from three different independent places allow us to affirm that the high infection level is a feature of fig wasps in general, regardless of geographical origin. Why do fig wasps have so high a level of *Wolbachia* infections? Hymenopteran insects are highly prone to *Wolbachia* infection (John & Donald, 2000; Rokas *et al.*, 2002; Werren *et al.*, 1995b; West *et al.*, 1998) and two common explanations for their high infection frequencies focus on the social and parasitoid lifestyles (Foster & Ratnieks, 2000; Vavre *et al.*, 1999). However, this cannot explain the high infection frequencies observed in fig wasps because none of the wasps are social and currently there are no known parasitoids that could serve as a conduit for transmission of *Wolbachia* between fig wasp species (Shoemaker *et al.*, 2002). No convincing explanation for the prevalent high *Wolbachia* incidence in fig wasps has been proposed, although the high density of fig wasps in the compact fig syconium and complex host–parasite interactions may favor high rates of horizontal transmission both within and between species (Cordaux *et al.*, 2001; Dyson *et al.*, 2002; Sintupachee *et al.*, 2006; Vavre *et al.*, 1999).

There are three shared fig species between Australia and China (*F. benjamina*, *F. microcarpa*, *F. racemosa*); however, we could not confirm whether the fig wasps species on those fig trees screened in different places were the same because of the difficulty in identifying fig wasp species. This, in this study we were not able to compare whether the shared ones had the same infection status in different continents.

Wolbachia *infection within a species*

In all species where we screened more than one individual, they were either all positive or all negative for infection (Table 1), which is consistent with the surveys from both Panama and Australia (Haine & Cook, 2005; Shoemaker *et al.*, 2002). Perhaps because samples of one wasp species were usually collected from the same fig tree, and sometimes the same fig fruit, this may prompt such a result, so samples of fig wasp species of one fig coming from different populations would be welcomed. We found two different *Wolbachia* strains within one fig wasp species, *Philotrypesis pilosa*, with 17% disparity between *wsp* gene sequences. However, another *Philotrypesis* species, *P.* sp., in company with *P. pilosa* on *F. hispida*, was not infected by *Wolbachia* at all. Is this implying that *Wolbachia* might play a role in the speciation events of *Philotrypesis* species (e.g. Molbo *et al.*, 2003)? More population genetics work on this genus should be done in the future.

Geography and horizontal transmission of infections

We found only A-clade *Wolbachia* in Chinese fig wasps, which is consistent with their overwhelming predominance in Panamanian and Australian fig wasps (Haine & Cook, 2005; Shoemaker *et al.*, 2002). Although the strains infecting fig wasps in Panama and Australia are different and appear in several regional clades on the phylogeny tree (Haine & Cook, 2005), strains infecting wasps from China are distributed in several clades, not forming a monophyly and appearing mostly in clades with Australian ones, which was supposed to support a geographically divided status.

Our analyses indicated shared strains not only among wasps of the same genus but also among different species of distant relationships (Table 3). While in some cases *Wolbachia* bacteria showed as genera-specific, for example, related species of the same genera (*Ceratosolen solmsi* and *Ceratosolen emarginatus*) (*Pleistodontes addicotti*, *P. rigasamos* and *P. imperialis*) shared closely related *Wolbachia* strains. Although it seems that horizontal transmission has occurred in the past between these fig wasps, nevertheless there is no apparent ecological link between these fig wasps. We speculated that *Wolbachia* bacteria has infected fig wasps before continental drift and have reached their current infection situations by independent local *Wolbachia* evolutionary trajectories. Unfortunately, in order to compare this with the results in previous work, we chose *wsp* gene sequences of *Wolbachia* for phylogenetic analyses which were not supposed to be suitable markers since they experienced extensive recombination and were subjected to strong diversifying selection (Baldo *et al.*, 2005; Baldo *et al.*, 2002; Jiggins *et al.*, 2002; Werren & Bartos, 2001). Supplements of other genes sequences, *ftsZ*, *groE* and *dnaA etc.* or multi-locus sequence typing (MLST) of *Wolbachia* would be welcomed, which should reflect reality more convincingly (Baldo *et al.*, 2006).

A good model for studying the mechanism of Wolbachia *horizontal transmission in nature*

Fig communities have a reasonably rich biological diversity, both inside and outside of syconia, with complicated ecological intra- and inter-group relationships, of which certainly the most important one is fig-associated wasps, including pollinators and non-pollinators (Weiblen, 2002). There are other kinds of life-forms coexisting in the colony, including species-specific nematode parasites of fig wasps, species of ants as predators or opportunistic foragers and some dipterous insects whose larvae develop in fig fruits and adults prey on fig

wasps, and so on (Compton & Disney, 1991; Giblin-Davis *et al.*, 1995; Herre, 1996; Herre, 1995; Poinar & Herre, 1991; Schatz & Hossaert-Mckey, 2003). Thus, whether and how *Wolbachia* transfer among so many species would be very interesting themes in future studies. Since small, delectable fig fruits have attracted various species of organisms, some for food and some for "housing", what kind of part does *Wolbachia*, as part of this colony, play among them? Since there is a complicated species diversity with complicated ecological relationships and reasonably high prevalence in fig-associated wasps, we suggest the fig community would be a good model for studying mechanisms of *Wolbachia* horizontal transmission.

Conclusion

Fig wasps appear to be prone to *Wolbachia* infection regardless of geographical origin, with no clear reasons as yet. Special biological and ecological features of these insects may offer models for horizontal transmission of *Wolbachia* within or among species. Future studies should seek the phylogenetic characteristic of horizontal transmission within species or among species with close ecological relationships, for example, all fig wasp species hosted in one fig, so as to further understand the mechanism of horizontal transmission. However, because of the difficulty of breeding experiments in these coevolved mutualists, we should consider all the factors related to the complex ecological and biotic environment around fig wasps during these studies.

Acknowledgment

This project was supported by the National Natural Science Foundation of China (NSFC grant no. 30330090, 30770302), partially by National Science Fund for Fostering Talents in Basic Research (Special subjects in animal taxonomy, NSFC-J0630964/J0109), by a grant (No. O529YX5105) from the Key Laboratory of Zoological Systematics and Evolution of Chinese Academy of Sciences, and by Program of Ministry of Science and Technology of the People's Republic of China (2006FY110500).

References

Baldo, L., Lo, N. and Werren, J.H. (2005) Mosaic nature of the *Wolbachia* surface protein. *Journal of Bacteriology*, 187, 5406–5418.

Baldo, L., Bartos, J.D., Werren, J.H., Bazzocchi, C., Casiraghi, M. and Panelli, S. (2002) Different rates of nucleotide substitutions in *Wolbachia* endosymbionts of arthropods and nematodes: arms race or host shifts? *Parassitologia*, 44, 179–187.

Baldo, L., Dunning Hotopp, J.C., Jolley, K.A., Bordenstein, S.R., Biber, S.A., Choudhury, R.R., Hayashi, C., Maiden, M.C., Tettelin, H. and Werren, J.H. (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology*, 72, 7098–7110.

Bordenstein, S.R., O'Hara, F.P. and Werren, J.H. (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, 409, 707.

Boucek, Z. (1988) *Australian Chalcidoidea (Hymenoptera): A Biosystematic Revision of Genera and Fourteen Families, with a Reclassification of Species*, pp. 156–209. CAB, International, Wallingford, UK.

Bouchon, D., Rigaud, T. and Juchault, P. (1998) Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proceedings of the Royal Society of London B*, 265, 1081–1090.

Breeuwer, J.A. and Werren, J.H. (1990) Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, 346, 558–560.

Castro, L.R., Austin, A.D. and Dowton, M. (2002) Contrasting rates of mitochondrial molecular evolution in parasitic Diptera and Hymenoptera. *Molecular Biology and Evolution*, 19, 1100–1113.

Compton, S.G. and Disney, R.H.L. (1991) New species of *Megaselia* (Diptera: Phoridae) whose larvae live in fig syconia (Urticales: Moraceae), and adults prey on fig wasps (Hymenoptera: Agaonidae). *Journal of Natural History*, 25, 203–220.

Cook, J.M. and Rasplus, J.-Y. (2003) Mutualists with attitude: coevolving fig wasps and figs. *Trends in Ecology and Evolution*, 18, 241–248.

Cordaux, R., Michel-Salzat, A. and Bouchon, D. (2001) *Wolbachia* infection in crustaceans: novel hosts and potential routes for horizontal transmission. *Journal of Evolutionary Biology*, 14, 237–243.

Dobson, S.L., Bourtzis, K., Braig, H.R., Jones, B.F., Zhou, W., Rousset, F. and O'Neill, S.L. (1999) *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, 29, 153.

Dyson, E.A., Kamath, M.K. and Hurst, G.D. (2002) *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity*, 88, 166–171.

Foster, K.R. and Ratnieks, F.L.W. (2000) Facultative worker policing in a wasp. *Nature*, 407, 692–693.

Giblin-Davis, R.M., Center, B.J., Nadel, H., Frank, J.H. and Ramirez, B.W. (1995) Nematodes associated with fig wasps, *Pegoscapus* spp. (Agaonidae), and syconida of native Floridian figs (*Ficus* spp.). *Journal of Nematology*, 27, 1–14.

Haine, E.R. and Cook, J.M. (2005) Convergent incidences of *Wolbachia* infection in fig wasp communities from two continents. *Proceedings of the Royal Society of London B*, 272, 421–429.

Herre, E.A. (1996) An overview of studies on a community of Panamanian figs. *Journal of Biogeography*, 23, 593–607.

Herre, E.A. (1995) Factors affecting the evolution of virulence: Nematode parasites of fig wasps as a case study. *Parasitology*, 111, S179–S191.

Hoffmann, A., Turelli, M. and Simmons, G.M. (1986) Unidirectional incompatibility between populations of *Drosophila simulans*. *Evolution*, 40, 692–701.

Hurst, G.D.D., Schulenburg, J.H.G., Majerus, T.M.O., Bertrand, D., Zakharov, I.A., Baungaard, J., Volkl, W., Stouthamer, R. and Majerus, M.E.N. (1999) Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, 8, 133–139.

Jeyaprakash, A. and Hoy, M.A. (2000) Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology*, 9, 393–405.

Jiang, Z.F., Huang, D.W., Zhu, C.D. and Zhen, W.Q. (2006a) New insights into the phylogeny of fig pollinators using Bayesian analyses. *Molecular Phylogenetics and Evolution*, 38, 306–315.

Jiang, Z.F., Huang, D.W., Chen, L.L., Zhen, W.Q., Fu, Y.G. and Peng, Z.Q. (2006b) Rampant host switching and multiple female body colour transitions in *Philotrypesis* (Hymenoptera: Chalcidoidea: Agaonidae). *Journal of Evolutionary Biology*, 19, 1157–1166.

Jiggins, F.M., Bentley, J.K., Majerus, M.E.N. and Hurst, G.D.D. (2002) Recent changes in phenotype and patterns of host specialization in *Wolbachia* bacteria. *Molecular Ecology*, 11, 1275–1283.

Jousselin, E., Rasplus, J.Y. and Kjellberg, F. (2003) Convergence and coevolution in a mutualism: evidence from a molecular phylogeny of *Ficus*. *Evolution*, 57, 1255–1269.

Kittayapong, P., Jamnongluk, W., Thipaksorn, A., Milne, J.R. and Sindhusake, C. (2003) *Wolbachia* infection complexity among insects in the tropical rice-field community. *Molecular Ecolution*, 12, 1049–1060.

Lopez-Vaamonde, C., Rasplus, J.Y., Weiblen, G.D. and Cook, J.M. (2001) Molecular phylogenies of fig wasps: partial cocladogenesis of pollinators and parasites. *Molecular Phylogenetics and Evolution*, 21, 55–71.

Machado, C.A., Jousselin, E., Kjellberg, F., Compton, S.G. and Herre, E.A. (2001) Phylogenetic relationships, historical biogeography and character evolution of fig-pollinating wasps. *Proceedings of the Royal Society of London B*, 268, 685–694.

Marussich, W.A. and Machado, C.A. (2007) Host-specificity and coevolution among pollinating and nonpollinating New World fig wasps. *Molecular Ecolution*, 16, 1925–1946.

Molbo, D., Machado, C.A., Sevenster, J.G., Keller, L. and Herre, E.A. (2003) Cryptic species of fig-pollinating wasps: implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 5867-5872.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2699–2702.

Plantard, O., Rasplus, J.Y., Mondor, G., Le Clainche, I. and Solignac, M. (1999) Distribution and phylogeny of *Wolbachia* inducing thelytoky in Rhoditini and 'Aylacini' (Hymenoptera: Cynipidae). *Insect Molecular Biology*, 8, 185–191.

Poinar, J.G.O. and Herre, E.A. (1991) Speciation and adaptive radiation in the fig wasp nematode, *Parasitodiplagaster* (Diplogasteridae : Rhabditida) in Panama. *Revue Nématol.*, 14, 361–374.

Posada, D. and Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14, 817–818.

Ramirez, B.W. (1980) Evolution of the monoecious and dioecious habit in *Ficus* (Moraceae). *Brenesia*, 18, 207–216.

Ramirez, B.W. (1974) Coevolution of *Ficus* and Agaonidae. *Annals of the Missouri Botanical Garden*, 61, 770–780.

Rigaud, T., Souty-Grosset, C., Raimond, R., Mocquard, J. and Juchault, P. (1991) Feminizing endocytobiosis in the terrestrial crustacean *Armadillidium vulgare* Latr. (Isopoda): recent acquisitions. *Endocytobiosis and Cell Research*, 7, 259–273.

Rokas, A., Atkinson, R.J., Nieves-Aldrey, J.L., West, S.A. and Stone, G.N. (2002) The incidence and diversity of *Wolbachia* in gallwasps (Hymenoptera; Cynipidae) on oak. *Molecular Ecology*, 11, 1815–1829.

Rokas, I.I. (2000) *Wolbachia* as a speciation agent. *Trends in Ecology and Evolution*, 15, 44–45.

Sambrook, J., Fitsch, E.F. and Maniatis, T.C.S.H. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., 1659 pp. Cold Spring Harbor Press, New York, USA.

Schatz, B. and Hossaert-Mckey, M. (2003) Interactions of the ant *Crematogaster scutellaris* with the fig/fig wasp mutualism. *Ecological Entomology*, 28, 359–368.

Schilthuizen, M. and Gittenberger, E. (1998) Screening mollusks for *Wolbachia* infection. *Journal of Invertebrate Pathology*, 71, 268–270.

Shoemaker, D.D., Machado, C.A., Molbo, D., Werren, J.H., Windsor, D.M. and Herre, E.A. (2002) The distribution of *Wolbachia* in fig wasps: correlations with host phylogeny, ecology and population structure. *Proceedings of the Royal Society of London B*, 269, 2257–2267.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. and Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of

conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651–701.

Sintupachee, S., Milne, J., Poonchaisri, S., Baimai, V. and Kittayapong, P. (2006) Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. *Microbial Ecology*, 51, 294–301.

Stouthamer, R., Luck, R.F. and Hamilton, W.D. (1990) Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 2424–2427.

Stouthamer, R., Breeuwert, J.A., Luck, R.F. and Werren, J.H. (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature*, 361, 66–68.

Swofford, D.L. (2003) *PAUP*, Phylogenetic Analysis Using Parsimony (* and Other Methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

van Meer, M.M.M., Witteveldt, J. and Stouthamer, R. (1999) Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Molecular Biology*, 8, 399–408.

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P. and Bouletreau, M. (1999) Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molecular Biology and Evolution*, 16, 1711–1723.

Weiblen, G.D. (2002) How to be a fig wasp. *Annual Review of Entomology*, 47, 299–330.

Weiblen, G.D. and Bush, G.L. (2002) Speciation in fig pollinators and parasites. *Molecular Ecology*, 11, 1573–1578.

Wenseleers, T., Ito, F., Borm, S.V., Huybrechts, R., Volckaert, F. and Billen, J. (1998) Widespread occurrence of the microorganism *Wolbachia* in ants. *Proceedings of the Royal Society of London B*, 265, 1447–1452.

Werren, J.H. and Windsor, D.M. (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proceedings of the Royal Society of London B*, 267, 1277–1285.

Werren, J.H. and Bartos, J.D. (2001) Recombination in *Wolbachia*. *Current Biology*, 11, 431–435.

Werren, J.H. and Jaenike, J. (1995) *Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives. *Heredity*, 75 (Pt 3), 320–326.

Werren, J.H., Windsor, D.M. and Guo, L.R. (1995a) Distribution of *Wolbachia* among Neotropical Arthropods. *Proceedings of the Royal Society of London B*, 262, 197–204.

Werren, J.H., Zhang, W. and Guo, L.R. (1995b) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proceedings of the Royal Society of London B*, 261, 55–63.

West, S.A. and Herre, E.A. (1994) The ecology of the New World fig-parasitizing wasps *Idarnes* and implications for the evolution of the fig-pollinator mutualism. *Proceedings of the Royal Society of London B*, 258, 67–72.

West, S.A., Herre, E.A., Windsor, D.M. and Green, P.R.S. (1996) The ecology and evolution of the New World non-pollinating fig wasp communities. *Journal of Biogeography*, 23, 447–458.

West, S.A., Cook, J.M., Werren, J.H. and Godfray, H.C.J. (1998) *Wolbachia* in two insect host-parasitoid communities. *Molecular Ecology*, 7, 1457–1465.

Wiebes, J.T. (1979) Co-evolution of figs and their insect pollinators. *Annual Review of Ecology and Systematics*, 10, 1–12.

Yen, J.H. and Barr, A.R. (1971) New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. *Nature*, 232, 657–658.

Zhang, S.S., Wu, Z.Y. and Cao, Z.Y. (1998) Angiospermae: Dicotyledoneae: Moraceae. *Flora Reipublicae Popularis Sinicae*, 23(1), pp. 66–69. Science Press, Beijing. (in Chinese)

Accepted June 30, 2009