



Screening of reproductive endosymbionts indicates *Wolbachia* flourish alone across fig wasps (Hymenoptera: Chalcidoidea)

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Abstract

Reproductive endosymbionts including *Wolbachia* are important heritable bacteria. They can manipulate the reproduction of their hosts, as well as affect the fitness and evolution of hosts. Many insects are co-infected by more than one reproductive endosymbionts. Detection of such multiple infections plays a significant role to comprehensively understand the association between endosymbionts and their hosts. Fig wasps live in the enclosed syconia and exhibit high incidence of *Wolbachia*. However, we have no knowledge about whether fig wasps are infected by other reproductive endosymbionts. We screened seven common reproductive endosymbionts, *Cardinium*, *Arsenophonus*, *Rickettsia*, *Spiroplasma ixodetis*, *Spiroplasma poulsonii*, *Flavobacterium* and *Wolbachia* in fig wasps using standard PCR. Except *Wolbachia*, we found no other reproductive bacteria in 1215 individuals of 66 fig wasp species. The prevalence of *Wolbachia* is up to 79%. The results indicate that the enclosed syconia may cut off the horizontal transmission of some reproductive bacteria from outside as a physical barrier. Meanwhile, it implies that *Wolbachia* may be the only heritable endosymbiont in fig wasps, which makes them an ideal model to dip into the tight relationship between *Wolbachia* and fig wasps.

Key words: Co-infection, fig wasp, PCR, reproductive endosymbiont, syconia, *Wolbachia*.

Introduction

Heritable bacteria that are transmitted vertically from mother to offspring play important roles in many arthropods biology. They have formed adaptively tight associations with hosts during coevolution. Hosts offer habitats for the inherited bacteria, while the inherited bacteria work well in the development and propagation of hosts, such as supplying nutrients ¹⁻⁵, enhancing immunity ^{6,7}, increasing fitness ⁵, resisting tolerance ⁸, manipulating reproduction ⁹⁻¹³ and so on. In general, reproductive endosymbiont manipulate the host reproduction in various ways that enhance their own transmission ^{12, 14}.

Wolbachia, as one of the earliest discovered endosymbiont of arthropods, have received great attention subsequently. It is reported that more than 20% insect species are infected by *Wolbachia* ¹⁴, while up to 76% using "long PCR" method ¹⁵. Besides its pandemic distribution, *Wolbachia* could manipulate hosts reproduction manners by cytoplasmic incompatibility ¹⁶, parthenogenesis induction ¹⁷, killing male progeny from infected females ¹², feminization of genetic males ¹⁸ and even cause host speciation ¹⁹. *Wolbachia* and its insect host has become a good model to study diverse reproduction manipulation mechanisms.

Recently, several other reproductive bacteria, including *Cardinium*, *Arsenophonus*, *Rickettsia*, *Flavobacterium* and *Spiroplasma*, have been gradually found besides *Wolbachia*, which play similar roles in manipulating hosts reproduction as *Wolbachia* does, although they are ignored before. Some of these

reproductive bacteria have been reported from insects of Homoptera, Diptera, Lepidoptera, Hemiptera, Coleoptera and Hymenoptera, causing male killing, cytoplasmic incompatibility, parthenogenesis and other distorters like *Wolbachia* ^{2, 20-24}. It must be cautious for researchers when studying the influence of endosymbiont on hosts ²¹. Moreover, *Cardinium*, *Arsenophonus* and *Rickettsia* have been found in hymenopteran insects, like *Encarsia*, *Nasonia*, *Neochrysocharis*, *Polistes* and *Diplolepis* ^{19, 24-32}, while the other two have not been reported in Hymenoptera.

More and more studies reveal that a number of insect hosts turn out to be double or multiple infections, which mean that insect hosts harbore more than one reproductive symbiont or one symbiont infected by different strains. For example, *Bemisia tabaci* was found to be infected by *Wolbachia*, *Cardinium*, *Rickettsia* and *Arsenophonus* simultaneously ^{9, 33}. Some fig wasp species are infected by different *Wolbachia* strains ^{34, 35}. As far as we know, these co-infected parasites would not work together on manipulating host reproduction; only one clade of them would play dominant roles through some unknown competing mechanisms ³³. Then it is very necessary to know the whole reproductive flora associated with host first ^{32, 36, 37}. Although the transfer mechanisms are not well understood, double or multiple infections probably connect with horizontal transmission through physical contact or food resources. Before dipping into the transmission route of the reproductive bacteria or how they act

on their hosts, it is very necessary to know the bacteria infection status first.

Fig-fig wasp system is a good model to study the phylogeny and coevolution between plant and parasitic insects. Fig wasps almost spend their whole life in figs, inside of which is almost completely closed and black, resulting in almost no communication with the outside. Figs not only supply fig wasps well-fed food and wonderful habitat, but also impose them high survival and evolution stress that result in kinds of interesting phenomena with fig wasp, including the ultra sexual dimorphism, female biased sex ratio and so on. It's estimated that symbiont associated with fig wasps potentially display important functions in increasing the fitness of host's survival and manipulating reproduction^{38,39}.

To date, the research of endosymbiont in fig wasps has been mainly focused on *Wolbachia*. Broad screening for fig wasps infected with *Wolbachia* have been finished in Panama, Australia and China, with infection rates of 59, 67 and 53% respectively^{32,36,37}, moreover, it appears multiple infections with different *Wolbachia* strains. So far, there is no relevant report about the endosymbiont of fig wasps besides *Wolbachia*. In order to uncover the infection status of 7 kinds of common reproductive endosymbiont of fig wasps, we made a thorough survey of seven common reproductive endosymbiont from 66 fig wasp species associated with 20 different fig species.

Materials and Methods

Samples: Sixty six fig wasps species associated with 20 species of fig, including 9 dioecism and 11 monoecism, were haphazardly collected from Hainan, Yunnan, Guangxi and Guangdong provinces of China, from 2003-2012 (Table 1). Fig wasps were identified morphologically and stored in 95% ethanol separately after emerging from mature figs, and stored at -80°C immediately.

Some insects, *Trialeurodes vaporariorum*, *Drosophila hydei* and *Blattella germanica*, collected from China and America separately, were chosen as positive controls.

DNA extraction, PCR amplification and sequencing: All individuals of each fig wasp species were pooled together for DNA extraction. Total DNA of each fig wasp species was extracted using Genomic Quick Extraction Kit (Transgen, Beijing, China) following the manufacturer's instructions. DNA extraction was performed on the entire body of fig wasps, to reduce the risk of missing infection with reproductive bacteria.

The DNA quality was confirmed by amplification of a partial fragment of *CO I* gene using primers LCO1490: 5'-GGTCA ACAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAAC TTCAGGGTGACCAAAAAATCA-3'. Infection rates were investigated for 7 reproductive parasites: *Cardinium*, *Arsenophonus*, *Rickettsia*, *Spiroplasma ixodetis*, *Spiroplasma poulsonii*, *Flavobacterium* and *Wolbachia*. First of all, a part of 16S rRNA gene per each endosymbiont was amplified with specific primers in each specimen to assay the infection. Secondly, the second genes for some endosymbionts were amplified for the further investigation with negative specimen, to avoid the false negatives of PCR amplification (Table 2). The infected specimen of *Trialeurodes vaporariorum*, *Drosophila hydei* and *Blattella germanica* were used as positive control. In addition, negative controls were carried out for each PCR process.

PCR was performed with 12.5 µl reaction. PCR procedures were

as follows: initial denaturation at 95°C for 4 min, 35 cycles of denaturation (94°C, 30 s), annealing (45 to 59°C, depending on primers, 45 s), extension (72°C, 1 min to 1min 30 s) and a final extension at 72°C for 10 min. PCR products were run on 1% agarose gel which was stained by EB, and lighted by UV. The PCR products corresponding to the same size electrophoretic bars as positive controls were purified and automated DNA sequencing was performed by Biosune Biotech Center, Shanghai, China. The sequences were aligned using BLAST to ensure they are the targeted sequences.

Data statistics: The comparisons of different infection rates of *Wolbachia* from fig wasps were performed using SPSS 16.0.

Results and Discussion

Fig wasps (Hymenoptera: Chalcidoidea) are worldwide spread, with more than 640 described species. The universal spread of *Wolbachia* within fig wasps has exceeded the rate of horizontal transmission among species and indicates a complicated interaction between *Wolbachia* and fig wasps^{32,37,40,41}. In recent years, several other reproductive endosymbionts also have been found gradually and even coexisted with *Wolbachia* in some insects, which disturbs the independent biological interaction and phylogenetic relationship between *Wolbachia* and their hosts^{9,33,42}.

In this study, we screened 1215 fig wasp individuals from 66 species (20 genera) associated with 20 fig species (4 subgenera) in China for the presence of 7 reproductive endosymbionts (*Cardinium*, *Arsenophonus*, *Rickettsia*, *Spiroplasma ixodetis*, *Spiroplasma poulsonii*, *Flavobacterium* and *Wolbachia*), using standard PCR. We detected about 18 individuals for each fig wasp species (1215/66) which was much more than the past three detection (Panama 8 (350/44), Australia 4.6 (283/61) and China 1.4 (66/47)). Our much larger sampling size for those broad fig wasp species in this study would reveal the much accurate incidence rate of the 7 reproductive endosymbionts in fig wasps in China.

To avoid probable false negative results, we have finished the two following procedures in our work: firstly, the DNA fragment of *CO I* gene with each DNA sample was amplified, which confirmed the DNA quality of each sample was well enough for our extensive research; secondly, each specific primer pairs for these reproductive endosymbionts, except *Spiroplasma ixodetis*, were confirmed by amplifying the corresponding positive controls (Fig. 1). All the other six reproductive endosymbionts worked well in positive controls, which indicated that there was no problem for these selected primers in this study.

Overall, our survey result improved on the high infection rate of *Wolbachia* in fig wasps (79%), allied with the past three surveys. In surprise, we got zero infection rates of the other six reproductive endosymbionts. However, several false positive sequences have occurred in our results, which were identified as genus *Pantoea* through Blastn program on NCBI.

Given both these false positive results in our PCR assay and higher sampling sizes for both intraspecies (*ca.* 18 individuals for each species) and interspecies (66 fig wasp species), we would get the true estimate for the several reproductive endosymbionts.

In this study, the infection rate of *Wolbachia* was 79% (52/66) with fig wasp species in this study. There was no significant difference between pollinators (93%, 13/14) and non-pollinators

Table 1. Summarised results of fig wasps screened for reproductive endosymbionts.

Subgenus	Section	Ficus spp.	Fig wasp species	Wasp biology	No. collected	Infected by <i>Wolbachia</i>	Locality	Collecting data
<i>Ficus</i>	<i>Eriosycea</i>	<i>F. hirta</i>	<i>Blastophaga javana</i>	P +	4	Y§	HN, China	2004
<i>Ficus</i>	<i>Eriosycea</i>	<i>F. hirta</i>	<i>Sycoscapter hirticola</i>	NP ‡	1	Y	HN, China	2004
<i>Ficus</i>	<i>Eriosycea</i>	<i>F. hirta</i>	<i>Sycoscapter</i> sp.1	NP	1	Y	HN, China	2004
<i>Ficus</i>	<i>Ficus</i>	<i>F. ischnopoda</i>	<i>Sycoryctes callosa</i>	NP	10	Y	YN, China	2004
<i>Pharmacosycea</i>	<i>Oreosycea</i>	<i>F. nervosa</i>	<i>Dolichoris nervosae</i>	P	3	Y	HN, China	2010
<i>Pharmacosycea</i>	<i>Oreosycea</i>	<i>F. nervosa</i>	<i>Philotrypes</i> sp.1	NP	10	Y	HN, China	2010
<i>Pharmacosycea</i>	<i>Oreosycea</i>	<i>F. vasculosa</i>	<i>Dolichoris vasculosae</i>	P	5	Y	HN, China	2008
<i>Sycidium</i>	<i>Palaeomorphe</i>	<i>F. subulata</i>	<i>Philotrypes</i> sp.1	NP	6	Y	YN, China	2004
<i>Sycidium</i>	<i>Palaeomorphe</i>	<i>F. tinctoria</i> subsp. <i>gibbosa</i>	<i>Kradibia gibbosae</i>	P	2	Y	YN, China	2007
<i>Sycidium</i>	<i>Palaeomorphe</i>	<i>F. tinctoria</i> subsp. <i>gibbosa</i>	<i>Neoscyophila omeomorpha</i>	NP	3	N¶	YN, China	2007
<i>Sycidium</i>	<i>Palaeomorphe</i>	<i>F. tinctoria</i> subsp. <i>gibbosa</i>	<i>Philotrypes ravii</i>	NP	20	Y	YN, China	2007
<i>Sycidium</i>	<i>Palaeomorphe</i>	<i>F. tinctoria</i> subsp. <i>gibbosa</i>	<i>Sycoscapter .nayoshorum</i>	NP	12	Y	YN, China	2007
<i>Sycidium</i>	<i>Sycidium</i>	<i>F. cyrtophylla</i>	<i>Blastophaga</i> sp.1	P	13	N	YN, China	2004
<i>Sycomorur</i>	<i>Hemicardia</i>	<i>F. semicordata</i>	<i>Apocryptophagus</i> sp.1	NP	15	N	YN, China	2004
<i>Sycomorur</i>	<i>Hemicardia</i>	<i>F. semicordata</i>	<i>Sycoscapter trifemmiensis</i>	NP	20	Y	YN, China	2004
<i>Sycomorur</i>	<i>Sycocarpus</i>	<i>F. hispida</i>	<i>Apocryta bakeri</i>	NP	26	N	HN/YN, China	2010-2012
<i>Sycomorur</i>	<i>Sycocarpus</i>	<i>F. hispida</i>	<i>Ceratosen solmsi</i>	P	324	Y	HN/YN, China	2010-2012
<i>Sycomorur</i>	<i>Sycocarpus</i>	<i>F. hispida</i>	<i>Philotrypes pilosa</i>	NP	41	Y	HN/YN, China	2010-2012
<i>Sycomorur</i>	<i>Sycocarpus</i>	<i>F. hispida</i>	<i>Philotrypes</i> sp.1	NP	87	N	HN/YN, China	2010-2012
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. auriculata</i>	<i>Ceratosen emarginatus</i>	P	20	Y	HN/YN, China	2008-2011
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. auriculata</i>	<i>Philotrypes longicaudata</i>	NP	20	Y	HN/YN, China	2008-2011
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. auriculata</i>	<i>Sycoscapter</i> sp.1	NP	20	Y	HN/YN, China	2008-2011
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. auriculata</i>	<i>Sycoscapter</i> sp.2	NP	20	Y	HN/YN, China	2008-2011
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. racemosa</i>	<i>Apocryptophagus agraeensis</i>	NP	13	Y	YN, China	2003
<i>Sycomorur</i>	<i>Sycomorur</i>	<i>F. racemosa</i>	<i>Apocryptophagus mayri</i>	NP	11	Y	GX, China	2003
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Acophila</i> sp.1	NP	3	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Eufroggatisca</i> sp.1	NP	10	Y	HN, China	2003-2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Eupristina altissima</i>	P	20	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Eupristina</i> sp.1	P	20	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Meselatus</i> sp.1	NP	4	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Ormyrus</i> sp.1	NP	12	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Philotrypes</i> sp.1	NP	2	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Sycophila</i> sp.1	NP	7	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Sycophila</i> sp.3	NP	5	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Sycoscapter</i> sp.1	NP	4	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Sycoscapter</i> sp.2	NP	2	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. altissima</i>	<i>Walkerella</i> sp.1	NP	10	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Acophila</i> sp.1	NP	20	Y	HN, China	2008-2011
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Eupristina koningsbergi</i>	P	20	Y	HN/YN, China	2008-2011
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Ormyrus</i> sp.1	NP	20	Y	HN, China	2008-2011
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Philotrypes tridentate</i>	NP	20	Y	HN/YN, China	2008-2011
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Sycophila</i> sp.4	NP	20	N	HN, China	2008-2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Sycoscapter</i> sp.1	NP	20	Y	HN/YN, China	2008-2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Walkerella benjamini</i>	NP	20	Y	HN/YN, China	2008-2011
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Sycophila</i> sp.1	NP	3	N	HN, China	2008-2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. benjamina</i>	<i>Sycophila</i> sp.2	NP	3	Y	HN, China	2008-2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. concinna</i>	<i>Philotrypes</i> sp.1	NP	20	Y	YN, China	2003
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. concinna</i>	<i>Philotrypes</i> sp.1	NP	9	Y	YN, China	2003
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. concinna</i>	<i>Platyscapa</i> sp.1	P	4	Y	YN, China	2003
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. curtipes</i>	<i>Sycoscapter</i> sp.1	NP	4	Y	YN, China	2004
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. curtipes</i>	<i>Lipothymus</i> sp.1	NP	20	Y	YN, China	2004

Subgenus	Section	Ficus spp.	Fig wasp species	Wasp biology	No. collected	Infected by <i>Wolbachia</i>	Locality	Collecting data
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Eupristina verticillata</i>	P	25	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Philotrypes emeryi</i>	NP	1	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Philotrypes taiwanensis</i>	NP	6	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Sycophila</i> sp.1	NP	25	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Sycoscapter gajimaru</i>	NP	20	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Sycoryctes moneres</i>	NP	25	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. microcarpa</i>	<i>Walkerella</i> sp.1	NP	6	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. superba</i>	<i>Platyscapa ishihana</i>	P	7	Y	YN, China	2004
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Acophila</i> sp.1	NP	9	N	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Ormyrus</i> sp.1	NP	15	Y	GD, China	2010
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Oritesella</i> sp.1	NP	6	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Philotrypes</i> sp.1	NP	4	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Platyscapa coronata</i>	P	12	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Sycoscapter</i> sp.1	NP	25	Y	GD, China	2010
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. virens</i>	<i>Camarothorax</i> sp.1	NP	20	Y	HN, China	2012
<i>Urostigma</i>	<i>Urostigma</i>	<i>F. religiosa</i>	<i>Sycoscapter</i> sp.1	NP	4	Y	YN, China	2003

† P: Pollinator, ‡ NP: Non-pollinator, § Y: infected by *Wolbachia*, ¶ N: not infected by *Wolbachia*.

Table 2. Genes and primers used in PCR assays to detect reproductive parasites and control DNA quality.

Organism	Gene	Primer (5'-3')	Annealing temperature	Size	Positive control	References
Insect	<i>CO I</i>	LCO1490 -GGTCAACAAATCATAAAGATATTGG HCO2198 -TAAACTTCAGGGTGACCAAAAAATCA	45°C	708 bp	This study	24
	<i>I6S</i>	27F -AGAGTTTGATCCTGGTCAAG Rssur -GAAAAGCATCTCGCATCCG	55°C	970 bp		44
<i>Rickettsia</i> sp.	<i>rDNA</i>	27F -AGAGTTTGATCMTGGCTCAG Rick16SR -CATTCCATCAGGATAAATCTTTT	55°C	200 bp	<i>Trialeurodes vaporariorum</i>	45
	<i>17-</i>	R1 -GCTCTTGCAACTTCTATGTT	55°C	434 bp		21
	<i>kDa</i>	R2 -CATTGTCGCAGGTTGGCC	55°C			
		ArsF1 -GGGTTGTAAAGTACTTTCAGTCGT ArsR2 -GTAGCCCTRCTGTAAAGGCC	55°C	518 bp		21
<i>Arsenophonus nasoniae</i>	<i>rDNA</i>	ArsF3 -GTCGTGAGGAARGTGTARGGTT ArsR3 -CCTYTATCTTAAAGGTTTCGCTGGATG	55°C	~804 bp	<i>Trialeurodes vaporariorum</i>	
	<i>fbA</i>	fbAaf -GCYGGYAAAAGTTCRTTCICC fbAar -CCWGAACDCCTGGAAAAACAAA	52°C	445 bp		46
<i>Cardinium hertigi</i>	<i>I6S</i>	CLOf1 -GGAACTTACCTGGGCTAGAATGTATT	55°C	466 bp	<i>Trialeurodes vaporariorum</i>	21
<i>Flavobacterium</i> sp.	<i>I6S</i>	CLOr1 -GCCACTGTCTTCAAGCTTACCAAC	55°C	530 bp	<i>Blattella germanica</i>	21
<i>Spiroplasma ixodetis</i>	<i>I6S</i>	FlavF -CGAATAAGTRTCGGCAACTCCG FlavR -CTAAAACRTTTCYAGCTTATTCG	55°C	810 bp		47
<i>Spiroplasma poulsonii</i>	<i>I6S</i>	SPixoF1 -TTAGGGGCTCAACCCCTAACCC SPouIF1 -TCTGGCATTGCCAACCCTC	55°C	421 bp	<i>Drosophila hydei</i>	47
<i>Wolbachia pipientis</i>	<i>I6S</i>	SPouIR1 -CCTGTCTCAATGTTAACCTC 16SwoIF -TTGTAGCCTGCTATGGTATAACT 16SwoIR -GAATAGGTATGATTTTCATGTT 81F -TGGTCCAAATAAGTGATGAAGAAC 691R -AAAAAATTAAACGCTACTCCA	55°C	896 bp 611 bp	This study	48 49

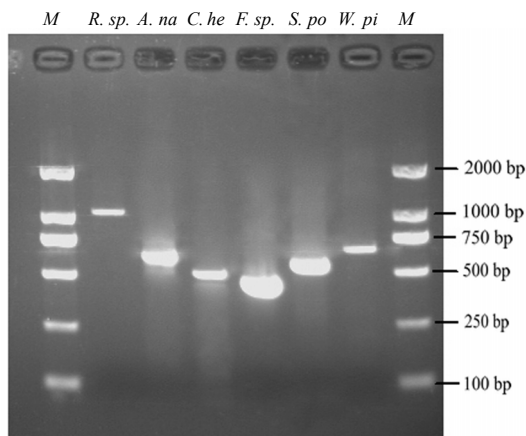


Figure 1. Electrophoretic bars of partial DNA fragment corresponding to positive reproductive endosymbiont. M is the marker; R. sp., A. na, C. he, S. po and F. sp. represent their partial 16S rDNA fragment of *Rickettsia* sp., *Arsenophonus nasoniae*, *Cardinium hertigii*, *Spiroplasma poulsonii* and *Flavobacterium* sp. separately; *W. pi* represents the *wsp* gene fragment of *Wolbachia pipientis*.

(75%, 39/52) in the infection rate ($\chi^2=1.929$, $df=1$, $P>0.05$) (Table 3). Compared to another survey (53%, 25/47) for *Wolbachia* in China, the *Wolbachia* infection rate presented significant difference between the two surveys ($\chi^2=5.121$, $df=1$, $P<0.05$). The infection rate of *Wolbachia* with pollinator and non-pollinator species displayed non significant ($\chi^2=1.691$, $df=1$, $P>0.05$) and significant ($\chi^2=8.076$, $df=1$, $P<0.05$) difference between the two surveys separately in China (Table 4). The infection difference relative to the other two continents (Panama 59% (26/44), Australia 66% (40/61)) was also compared. In contrast to the difference between the two surveys in China, there are no significant difference in all species ($\chi^2=3.029$, $df=2$, $P>0.05$) and non-pollinators ($\chi^2=1.750$, $df=2$, $P>0.05$), while pollinators got ($\chi^2=12.861$, $df=2$, $P<0.05$) significant difference among the three continents (Table 5).

For *Wolbachia*, we got much higher infection rate (79%) in fig wasp species than the past three surveys (ca. 59%), which displayed significant difference either in pollinators ($\chi^2=12.861$, $df=2$, $P<0.05$) or non-pollinators ($\chi^2=8.076$, $df=1$, $P<0.05$). The increasing result should mainly be associated with our large sampling size (18 (1215/66)). There were many single individual

Table 3. Comparison of incidence of *Wolbachia* infections in fig wasps between pollinators and non-pollinators of China 2^a.

Comparison	Pollinators	Non-pollinators	χ^2	Significance
Incidence of <i>Wolbachia</i> in China 2	0.93 (14)	0.75 (52)	1.929	n.s.

Table 4. Comparison of incidence of *Wolbachia* infections in fig wasps between China 1^a and China 2.

Comparison	China 1	China 2	χ^2	Significance
Incidence of <i>Wolbachia</i> in all species	0.53 (47)	0.79 (66)	5.121	s.
Incidence of <i>Wolbachia</i> in pollinators	0.82 (11)	0.93 (14)	1.691	n.s.
Incidence of <i>Wolbachia</i> in non-pollinators	0.44 (36)	0.75 (52)	8.076	s.

a. China 1 indicates previous detection in China.

Table 5. Comparison of incidence of *Wolbachia* infections in fig wasps between Panama, Australia, and China 2.

Comparison	Panama	Australia	China 2	χ^2	Significance
Incidence of <i>Wolbachia</i> in all species	0.59 (44)	0.66 (61)	0.79 (66)	3.029	n.s.
Incidence of <i>Wolbachia</i> in pollinators	0.50 (18)	0.73 (26)	0.93 (14)	12.861	s.
Incidence of <i>Wolbachia</i> in non-pollinators	0.65 (26)	0.60 (35)	0.75 (52)	1.75	n.s.

Incidence is expressed as a proportion of species in that category and χ^2 -values are given for $df=1$ or 2. Non-significance (n.s.) = $P>0.05$.

per species sampling in the past three surveys, which would greatly underestimate the real incidence of *Wolbachia* in fig wasp species. For another words, the four surveys collected samples in different time and locations separately, which would also pay some impact on their infection difference.

For the absence of the other six reproductive endosymbionts in fig wasps in this study, we thought there are two main reasons: Firstly, intimate contact with other species, such as parasitism, predation and physical damage, is an important pathway to obtain new infection of endosymbiont. For example, *Encarsia* parasitoid wasps (Hymenoptera: Aphelinidae) which have been infected three endosymbiont, *Cardinium*, *Rickettsia* and *Wolbachia*, parasites whitefly which harbors all the three endosymbiont^{24,27-30}. *Nasonia* parasitoid wasps (Hymenoptera: Pteromalidae) also harbor three endosymbiont, *Cardinium*, *Arsenophonus* and *Wolbachia*^{25,26,31}. Fig wasps almost spend their whole life in closed fig syconia, with little time to seek other syconia to lay eggs outside syconia³⁹. The closed fig syconia form a naturally physical barrier to limit fig wasps to contact with other insects living outside of fig syconia, which implies that the syconia prevents the horizontal transfer of endosymbiont from outside environment to fig wasps living inside syconia. Secondly, according to previous reports, the average infection rate for the other 6 reproductive endosymbionts in insect is below 7%²¹, much lower than that of *Wolbachia* (20%)¹⁴. Their prevalence in Hymenopteran insects is especially rare besides several parasitoid wasps^{27,28,43}. Compared with the huge transfer resource of *Wolbachia*, the resources of the other 6 endosymbionts are much less, which could also limit the obtained of these endosymbiont for fig wasps.

However, there are still several inherent factors leading to the underestimation of incidence for the 7 reproductive endosymbionts. Firstly, for the limitation of PCR assay with titre of endosymbionts, it is really difficult to detect some low titre endosymbiont in samples, which would produce false negatives in the PCR assay. Secondly, with some low infection rate within host species, it is possible for us to miss some infected individuals and obtained false negative results for limited sample size. Thirdly, since some host species would display dynamic difference on the infection rate of reproductive endosymbionts, according to different sampling data or locations, our insufficient sampling data and locations would miss some positively infected wasp species and resulted in some false negatives for our sampling method.

Totally, our study systematically screened the prevalence of 7 reproductive endosymbionts (*Cardinium*, *Arsenophonus*, *Rickettsia*, *Spiroplasma ixodetis*, *Spiroplasma poulsonii*, *Flavobacterium* and *Wolbachia*) in 1215 fig wasps individuals

associated with 66 fig wasp species in China for the first time. Our study reveals a higher infection rate (78%) of *Wolbachia* with fig wasp species in China, which extensively emphasized the potentially intimate relationship between *Wolbachia* and fig wasp host. Our result presented that the 6 non-*Wolbachia* endosymbionts were zero infection in fig wasps which implied *Wolbachia* walk alone in fig wasps. Further studies on the intimate relationship between *Wolbachia* and fig wasps and the diversity of symbionts in fig wasps living in closed syconia have been planned.

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References

- ¹Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology* **59**:155-189.
- ²Clark, M. A., Baumann, L. and Baumann, P. 1998. *Buchnera aphidicola* (aphid endosymbiont) contains genes encoding enzymes of histidine biosynthesis. *Current Microbiology* **37**:356-358.
- ³Goldberg, C. and Pierre, L. L. 1969. Tyrosinase activity of the symbionts and fat bodies of the cockroach, *Leucophaea maderae*. *Can. J. Microbiol.* **15**:253-255.
- ⁴Moran, N. A., Dale, C., Dunbar, H., Smith, W. A. and Ochman, H. 2003. Intracellular symbionts of sharpshooters (insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. *Environmental Microbiology* **5**:116-126.
- ⁵Moran, N. A. and Degan, P. H. 2006. Functional genomics of *Buchnera* and the ecology of aphid hosts. *Molecular Ecology* **15**:1251-1261.
- ⁶Brennan, L. J., Haukedal, J. A., Earle, J. C., Keddie, B. and Harris, H. L. 2012. Disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of *Wolbachia*-infected *Drosophila simulans*. *Insect Molecular Biology* **21**:510-520.
- ⁷Hedges, L. M., Brownlie, J. C., O'Neill, S. L. and Johnson, K. N. 2008. *Wolbachia* and virus protection in insects. *Science* **322**:702.
- ⁸Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**:1803-1807.
- ⁹Chiel, E., Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Katzir, N., Inbar, M. and Ghanim, M. 2007. Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research* **97**:407-413.
- ¹⁰Clay, K., Klyachko, O., Grindler, N., Civitello, D., Oleske, D. and Fuqua, C. 2008. Microbial communities and interactions in the lone star tick, *Amblyomma americanum*. *Molecular Ecology* **17**:4371-4381.
- ¹¹Gotoh, T., Noda, H. and Ito, S. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity* **98**:13-20.
- ¹²Riparbelli, M. G., Giordano, R., Ueyama, M. and Callaini, G. 2012. *Wolbachia*-mediated male killing is associated with defective chromatin remodeling. *PLoS ONE* **7**:1-14.
- ¹³Stevens, L., Giordano, R. and Fialho, R. F. 2001. Male-killing, nematode infections, bacteriophage infection, and virulence of cytoplasmic bacteria in the genus *Wolbachia*. *Annu. Rev. Ecol. Syst.* **32**:519-545.
- ¹⁴Werren, J. H. and Windsor, D. M. 2000. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? *Proceedings of the Royal Society - Biological Sciences* **267**:1277-1285.
- ¹⁵Jeyaprakash, A. and Hoy, M. A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9**:393-405.
- ¹⁶Veneti, Z., Clark, M. E., Zabalou, S., Karr, T. L., Savakis, C. and Bourtzis, K. 2003. Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations. *Genetics* **164**:545-552.
- ¹⁷Negri, I., Pellicchia, M., Mazzoglio, P. J., Patetta, A. and Alma, A. 2006. Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an xx/x0 sex-determination system. *Proceedings of the Royal Society - Biological Sciences* **273**:2409-2416.
- ¹⁸Adachi-Hagimori, T., Miura, K. and Stouthamer, R. 2008. A new cyto-genetic mechanism for bacterial endosymbiont-induced parthenogenesis in Hymenoptera. *Proceedings of the Royal Society - Biological Sciences* **275**:2667-2673.
- ¹⁹Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E. and Bouletreau, M. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences of the United States of America* **98**:6247-6252.
- ²⁰Bandi, C., Damiani, G., Magrassi, L., Grigolo, A., Fani, R. and Sacchi, L. 1994. Flavobacteria as intracellular symbionts in cockroaches. *Proceedings of the Royal Society - Biological Sciences* **257**:43-48.
- ²¹Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstadter, J. and Hurst, G. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology* **6**:27.
- ²²Haselkorn, T. S. 2010. The *Spiroplasma* heritable bacterial endosymbiont of *Drosophila*. *Genetics* **4**:80-87.
- ²³Leonardo, T. E. and Muir, G. T. 2003. Facultative symbionts are associated with host plant specialization in pea aphid populations. *Proceedings of the Royal Society - Biological Sciences* **270**:209-212.
- ²⁴Zchori-Fein, E. 2004. Characterization of a 'bacteroidetes' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): Proposal of 'Candidatus *Cardinium hertigii*'. *International Journal of Systematic and Evolutionary Microbiology* **54**:961-968.
- ²⁵Darby, A. C., Choi, J. H., Wilkes, T., Hughes, M. A., Werren, J. H., Hurst, G. D. and Colbourne, J. K. 2010. Characteristics of the genome of *Arsenophonus nasoniae*, son-killer bacterium of the wasp *Nasonia*. *Insect Mol. Biol.* **19**(1):75-89.
- ²⁶Ghera, L. R. and Werren, H. J. 1991. *Arsenophonus nasoniae* gen. Nov., sp. Nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. *Society for General Microbiology* **41**:563-565.
- ²⁷Giorgini, M., Monti, M. M., Caprio, E., Stouthamer, R. and Hunter, M. S. 2009. Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harboring the bacterial symbiont *Cardinium*. *Heredity* **102**:365-371.
- ²⁸Harris, L. R., Kelly, S. E., Hunter, M. S. and Perlman, S. J. 2010. Population dynamics and rapid spread of *Cardinium*, a bacterial endosymbiont causing cytoplasmic incompatibility in *Encarsia pergandiella* (Hymenoptera: Aphelinidae). *Heredity* **104**:239-246.
- ²⁹Mahadav, A., Gerling, D., Gottlieb, Y., Czosnek, H. and Ghanim, M. 2008. Parasitization by the wasp *Eretmocerus mundus* induces transcription of genes related to immune response and symbiotic bacteria proliferation in the whitefly *Bemisia tabaci*. *BMC Genomics* **9**:342.
- ³⁰Perlman, S. J., Kelly, S. E. and Hunter, M. S. 2008. Population biology of cytoplasmic incompatibility: Maintenance and spread of *Cardinium* symbionts in a parasitic wasp. *Genetics* **178**:1003-1011.
- ³¹Raychoudhury, R., Grillenberger, B. K., Gadau, J., Bijlsma, R., Van de Zande, L., Werren, J. H. and Beukeboom, L. W. 2010. Phylogeography of *Nasonia vitripennis* (Hymenoptera) indicates a mitochondrial-*Wolbachia* sweep in North America. *Heredity* **104**:318-326.
- ³²Shoemaker, 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 B: Biological Sciences* **269**:2257-2267.
- ³³Singh, S. T., Priya, N. G., Kumar, J., Rana, V. S., Ellango, R., Joshi, A., Priyadarshini, G., Asokan, R. and Rajagopal, R. 2012. Diversity and phylogenetic analysis of endosymbiotic bacteria from field caught *Bemisia tabaci* from different locations of North India based on 16S rDNA library screening. *Infect. Genet. Evol.* **12**:411-419.
- ³⁴Malloch, G., Fenton, B. and Butcher, R. D. 2000. Molecular evidence for multiple infections of a new subgroup of *Wolbachia* in the european raspberry beetle *Byturus tomentosus*. *Molecular Ecology* **9**:77-90.
- ³⁵Vautrin, E., Charles, S., Genieys, S. and Vavre, F. 2007. Evolution and invasion dynamics of multiple infections with *Wolbachia* investigated using matrix based models. *J. Theor. Biol.* **245**:197-209.
- ³⁶Chen, L. L., Cook, J. M., Xiao, H., Hu, H. Y., Niu, L. M. and Huang, D. W. 2010. High incidences and similar patterns of *Wolbachia* infection in fig wasp communities from three different continents. *Insect Science* **17**:101-111.
- ³⁷Haine, E. R. and Cook, J. M. 2005. Convergent incidences of *Wolbachia* infection in fig wasp communities from two continents. *Proceedings of the Royal Society B: Biological Sciences* **272**:421-429.
- ³⁸Janzen, D. H. 1979. How to be a fig. *Annu. Rev. Ecol. Syst.* **10**:13-51.
- ³⁹Weiblen, G. D. 2002. How to be a fig wasp. *Annu. Rev. Entomol.* **47**:299-330.
- ⁴⁰Sun, X. J., Xiao, J. H., Cook, J. M., Feng, G. and Huang, D. W. 2011. Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of *Wolbachia* on host *mtDNA* evolution and diversity. *BMC Evol. Biol.* **11**:86.
- ⁴¹Yang, C. Y., Xiao, J. H., Niu, L. M., Ma, G. C., Cook, J. M., Bian, S. N., Fu, Y. G. and Huang, D. W. 2012. Chaos of *Wolbachia* sequences inside the compact fig syconia of *Ficus benjamina* (Ficus: Moraceae). *PLoS ONE* **7**:1-11.
- ⁴²Goodacre, S. L., Martin, O. Y., Thomas, C. F. G. and Hewitt, G. M. 2006. *Wolbachia* and other endosymbiont infections in spiders. *Molecular Ecology* **15**:517-527.
- ⁴³White, J. A., Kelly, S. E., Perlman, S. J. and Hunter, M. S. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron*: Disentangling the roles of *Cardinium* and *Wolbachia* symbionts. *Heredity* **102**:483-489.
- ⁴⁴Weinert, L. A., Werren, J. H., Aebi, A., Stone, G. N. and Jiggins, F. M., 2009. Evolution and diversity of *Rickettsia* bacteria. *BMC Biology* **7**:6.
- ⁴⁵Kikuchi, Y. and Fukatsu, T. 2005. *Rickettsia* infection in natural leech populations. *Microbial Ecology* **49**:265-271.
- ⁴⁶Duron, O., Wilkes, T. E. and Hurst, G. D. 2010. Interspecific transmission of a male-killing bacterium on an ecological timescale. *Ecology Letters* **13**:1139-1148.
- ⁴⁷Xie, J., Vilchez, I. and Mateos, M. 2010. *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS ONE* **5**:1-7.
- ⁴⁸O'Neill, S. L., Giordano, R., Colbert, A. M., 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.
- ⁴⁹Zhou, W., Rousset, F. and O'Neil, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society - Biological Sciences* **265**:509-515.