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ORIGINAL ARTICLE

Various infection status and molecular evidence for horizontal transmission and recombination of *Wolbachia* and *Cardinium* among rice planthoppers and related species

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Abstract *Wolbachia* and *Cardinium* are widely distributed and are considered important for their ability to disturb reproduction and affect other fitness-related traits of their hosts. By using multilocus sequence typing (MLST), RFLP (restriction fragment length polymorphism) and 16S ribosomal DNA gene sequencing methods, we extensively surveyed *Wolbachia* and *Cardinium* infection status of four predominant rice planthoppers and one kind of leafhopper in different rice fields. The results demonstrated that *Sogatella furcifera* (Horváth) and *Laodelphax striatellus* (Fallén) were infected with the same *Wolbachia* strain (*w*Stri), while *Nilaparvata lugens* (Stål) and its closely related species *Nilaparvata muiri* China were infected with two phylogeneticlly distant strains, *w*Lug and *w*Mui, respectively. Three new *Wolbachia* strains (provisionally named *w*Mfas1, *w*Mfas2 and *w*Mfas3) were detected in the leafhopper *Macrosteles fascifrons* (Stål). Only *S. furcifera* was co-infected with *Cardinium*, which indicated that the distribution of *Cardinium* in these rice planthoppers was narrower than that of *Wolbachia*. Unambiguous intragenic recombination events among these *Wolbachia* strains and incongruent phylogenetic relationships show that the connections between different *Wolbachia* strains and hosts were more complex than we expected. These results suggest that horizontal transmission and host associated specialization are two factors affecting *Wolbachia* and *Cardinium* infections among planthoppers and their related species.

Key words *Cardinium*, infection status, intragenic recombination, rice planthopper, *Wolbachia*

Introduction

Wolbachia and *Cardinium* are two well-known reproductive endosymbionts of arthropods. *Wolbachia* infect at least 20% of all arthropods and some nematode species (Bandi *et al*., 1998; Hilgenboecker *et al*., 2008; Kikuchi & Fukatsu, 2003; Tagami & Miura, 2004; Werren & Windsor, 2000; Werren *et al*., 1995a; West *et al*., 1998), while *Cardinium* are known from only a few orders of Arachnida and Insecta. Double infection with *Wolbachia* and *Cardinium* has also been found in several species of arthropods (Duron *et al*., 2008; Enigl & Schausberger, 2007; Gotoh *et al*., 2007; Liu *et al*., 2006; Perlman *et al*., 2006; Weeks *et al*., 2003; Zchori-Fein & Perlman, 2004).

Rice planthoppers (Hemiptera: Delphacidae) are the most devastating pests in rice fields in many Asian countries. Three of the most important planthopper species in China and its surrounding areas are the brown planthopper (BPH), *Nilaparvata lugens* (Stål), the whitebacked planthopper (WBPH), *Sogatella furcifera* (Horváth) and the small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén). They feed on the stems of rice, causing physiological stress (even causing hopperburn, a noncontagious disease of plants caused by the direct

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feeding damage of certain planthoppers and leafhoppers) (Backus *et al*., 2005; Hayashi & Chino, 1990) and transmit plant-pathogenic viruses that can drastically decrease rice yields (Hibino, 1989; Sōgawa, 1982). Within a wide range of hosts surveyed, BPH was found infected with *Wolbachia* strain *w*Lug (Kittayapong *et al*., 2003), while SBPH and WBPH were infected with a different strain *w*Stri (based on the nucleotide sequences of four genes) (Kittayapong *et al*., 2003; Noda *et al*., 2001b). Two WBPH adult females were also found to be infected with *Cardinium* (Nakamura *et al*., 2009). Horizontal transmission caused by an endoparasite, *Elenchus japonicus* was proposed to explain the identical *Wolbachia* strains infecting these two different planthoppers (Noda *et al*., 2001b). Phylogenetically distant hosts infected with an identical strain of *Wolbachia* cannot be explained by vertical transmission alone.

In the rice agroecosystems in Asia, the present version of the delphacid planthopper food web shows an expanded model of the intricate relationships of the invertebrates (insects, mites and spiders, 218 species), vertebrates (17 species), nematodes (three species), and pathogens (six species). This excludes the 48 taxa of hyperparasites/hyperpredators and the spiders that may behave as hyperpredators as well (Dupo & Barrion, 2009). Planthoppers also share many other kinds of ecto- and endoparasites. Among Hymenoptera that parasitize planthoppers, 56 species infect eggs and 10 species infect the nymphal–adult stage, and seven species share two or three species of BPH, SBPH and WBPH (Dupo & Barrion, 2009). Insect parasitoids were considered as the best candidate vectors for the horizontal transmission of *Wolbachia* (Heath *et al*., 1999; Werren *et al*., 1995a). Since large numbers of the three main species of rice planthoppers (BPH, SBPH and WBPH) and their parasites reside in almost the same environmental habitat, horizontal transmission of endosymbionts among different planthopper species caused by parasitism seems likely. Furthermore, if the different *Wolbachia* strains coexist in the same host, are there exchanges of genetic materials between them?

In this study, by using polymerase chain reaction (PCR) and a simple restriction fragment length polymorphism (RFLP) approach, we conducted a more detailed analysis of infection status and variation of *Wolbachia* and *Cardinium* within natural populations of the predominant rice planthopper species and its related species. We then attempted to reveal the relationships between these different *Wolbachia* strains and the relationships between these endosymbionts and their hosts. Our results will help in the understanding of the diversification, horizontal transmission and host-associated specialization of these two endosymbionts, and may have implications for the biological control of planthoppers (Kang *et al*., 2003; Kawai *et al*., 2009).

Materials and methods

Insect materials

BPH, SBPH, WBPH and a few individuals of the planthopper *Nilaparvata muiri* China and the leafhopper *Macrosteles fascifrons* (Stål) were collected from 17 distant locations in China and two locations in Vietnam between 2007 and 2010 (Table 1). All populations were collected in rice fields. Some individuals were directly preserved in 100% ethyl alcohol, stored at −72°C. A short-term storage of different specimens in ethanol would not compromise the PCR screening programs for *Wolbachia* (Duplouy *et al*., 2009). Some individuals were brought back to the lab alive and reared on rice seedlings at 25 ± 0.5°C and under a controlled photoperiod of 16 : 8 h light: dark.

DNA extractions and template quality assays

Each individual planthopper and leafhopper was washed in alcohol more than six times. After the alcohol was completely evaporated, each individual was placed in 40 μL STE buffer (10 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 0.1 mol/L NaCl, pH 8.0), homogenized with a disposable tissue grinding pestle, mixed with 2.5 μL proteinase K (10 mg/mL), incubated at 37°C for 1 h, and then transferred to 95°C for 5 min to inactivate proteinase K (O'Neill *et al*., 1992). After centrifugation at 3000 × *g* for 30 s, the samples were directly used for PCR or stored at −20°C for later use. Primers specific for insect mitochondrial 12S ribosomal RNA (rRNA), 12Sai and 12Sbi (all primers used in this study are shown in Table 2) were used for PCR checking of the quality of the DNA extraction using previously published protocols (Simon *et al*., 1994).

Procedure for PCR screening for Wolbachia and Cardinium infection

General *wsp* primers *wsp* 81F and *wsp* 691R (Braig *et al*., 1998) which target a DNA fragment of *Wolbachia* outer surface protein (*wsp*) gene, were used for

Table 1 Screening of four species of rice planthoppers and one species of leafhopper for the presence of *Wolbachia* and *Cardinium*.[†]

Species	Collection site/year	Geographic coordinates	*Wolbachia*	*Cardinium*	Double infection
Nilaparvata lugens	Da Nang, Quang Nam Province, Vietnam/2008	15.50°N–108.16°E	1/15	0/15	
	Vinh Linh, Quang Tri Province, Vietnam/2008	16.58°N–107.03°E	2/26	1/26	1/26
	Sanya, Hainan Province, China/2007	18.14°N–109.31°E	5/41	0/41	
	Maoming, Guangdong Province,China/2009	21.40°N–110.53°E	13/8	0/85	
	Nanning, Guangxi Zhuang Autonomous Region, China/2008	22.48°N–108.19°E	5($1^{■} + 1^{•◇}$)/21	1/21	1/21
	Ning'er, Yunnan Province, China/2008	23.04°N–101.01°E	4($1^{■}$)/21	0/21	
	Kaiyuan, Yunnan Province, China/2008	23.43°N–103.13°E	0/2	0/2	
	Baise, Guangxi Zhuang Autonomous Region, China/2008	23.54°N–106.36°E	18($2^{■} + 1^{■◇}$)/80	0/80	
	Shaoguan, Guangdong Province, China/2009	24.48°N–113.37°E	5/40	0/40	
	Wuhan, Hubei Province, China/2008	30.32°N–114.17°E	11($8^{■}$)/34	1/34	
Sogatella furcifera	Vinh Linh,Quang Tri Province, Vietnam/2008	16.58°N–107.03°E	3/5	0/5	
	Sanya, Hainan Province, China/2007	18.14°N–109.31°E	1/2	0/2	
	Nanning, Guangxi Zhuang Autonomous Region, China/2008	22.48°N–108.19°E	22/36	0/36	
	Kaiyuan, Yunnan Province, China/2008	23.43°N–103.13°E	3/5	3/5	3/5
	Baise, Guangxi Zhuang Autonomous Region, China/2008	23.54°N–106.36°E	29/40	19/40	19/40
	Shaoguan, Guangdong Province, China/2009	24.48°N–113.37°E	2/6	5/6	2/6
	Changsha, Hunan Province, China/2008	28.12°N–112.59°E	16/20	19/20	15/40
	Yingtan, Jiangxi Province, China/2009	28.14°N–117.03°E	30/92	35/92	24/92
	Wuhan, Hubei Province, China/2008	30.32°N–114.17°E	0/5	1/5	
	Gaoyou, Jiangsu Province,China/2008	32.47°N–119.27°E	14/23	23/23	14/23
Laodelphax striatellus	Nanning, Guangxi Zhuang Autonomous Region, China/2008	22.48°N–108.19°E	16/18	0/18	
	Baise, Guangxi Zhuang Autonomous Region, China/2008	23.54°N–106.36°E	23/26	0/26	
	Shaoguan, Guangdong Province, China/2009	24.48°N–113.37°E	0/2	0/2	
	Changsha, Hunan Province, China/2008	28.12°N–112.59°E	1/2	0/2	
	Yingtan, Jiangxi Province, China/2009	28.14°N–117.03°E	13/20	2/20	2/20
	Anqing, Anhui Province, China/2008	30.31°N–117.02°E	42/44	0/44	
	Nanhui, Shanghai, China/2009	31.14°N–121.29°E	43/44	0/44	
	Nantong, Jiangsu Province, China/2009	32.01°N–120.51°E	38/40	0/40	
	Nanjing, Jiangsu Province, China/2007	32.03°N–118.46°E	44/44	0/44	
Nilaparvata muiri	Jinhua, Zhejiang Province, China/2010	29.07°N–119.39°E	55/64	0/64	
	Huzhou, Zhejiang Province, China/2009	30.52°N–120.06°E	2/7	0/7	
Macrosteles fascifrons	Jinhua, Zhejiang Province, China/2010	29.07°N–119.39°E	37($11^{♦} + 18^{▲} + 5^{△} + 1^{■} + 1^{■▲} + 1^{♦▲}$) /382	0/382	0/382

[†]The infection status of each population (i.e. presence/absence of *Wolbachia* and *Cardinium*) was shown as the number of infected specimens/total successfully screened for each population. Different *Wolbachia* strains detected from BPH and *M. fascifrons* are distinguished by superscript numbers: •*w*Lug; ■*w*Stri; ◇*w*Waspb; ♦*w*Fas1; ▲*w*Fas2; △*w*Fas3.

Table 2 Primers used in the present study.

Species	Gene	Primer	Sequence (5′–3′)	Annealing temperature
Wolbachia	*gatB*	gatB_F1	GAKTTAAAYCGYGCAGGBGTT	54°C
		gatB_R1	TGGYAAYTCRGGYAAAGATGA	
	coxA	coxA_F1	TTGGRGCRATYAACTTTATAG	55°C
		coxA_R1	CTAAAGACTTTKACRCCAGT	
	hcpA	hcpA_F1	GAAATARCAGTTGCTGCAAA	53°C
		hcpA_R1	GAAAGTYRAGCAAGYTCTG	
	ftsZ	ftsZ_F1	ATYATGGARCATATAAARGATAG	54°C
		ftsZ_R1	TCRAGYAATGGATTRGATAT	
		ftsZf1	GTTGTCGCAAATACCGATGC	55°C
		ftsZr1	CTTAAGTAAGCTGGTATATC	
	ftsZ and its downstream region	inftsZ-F	ARGGAAGAGTTAGAGTTTCT	50°C
		exftsZ-R	ATAGTACRTTAGTTGGCTTA	
	fbpA	fbpA_F1	GCTGCTCCRCTTGGYWTGAT	55°C
		fbpA_R1	CCRCCAGARAAAAYYACTATTC	
	wsp	81F	TGGTCCAATAAGTGATGAAGAAAC	55°C
		691R	AAAAATTAAACGCTACTCCA	
Cardinium	16S rRNA	CLOf	GCGGTGTAAAATGAGCGTG	57°C
		CLOr1	ACCTMTTCTTAACTCAAGCCT	
		27F	AGAGTTTGATCMTGGCTCAG	57°C
		1513R	ACGGYTACCTTGTTACGACTT	
Nilaparvata lugens				
Sogatella furcifera	12S rRNA	12Sai	AAACTAGGATTAGATACCCTATTAT	55°C
Laodelphax striatellus		12Sbi	AAGAGCGACGGGCGATGTGT	
Nilaparvata muiri				
Nilaparvata lugens	COI	BPH-COIF	TACAAGAAAGAGGAAAAAGGG	55°C
		BPH-COIR	AGGACATAGTGAAAATGAGCC	

detecting *Wolbachia* infection. Assays for *Cardinium* infection were performed by PCR amplification of a region of the 16S rDNA gene using the *Cardinium*-specific primers CLOf and CLOr1, which amplified an approximate 450 bp length fragment (Weeks *et al*., 2003). To confirm infection of *Cardinium*, the nearly full-length 16S rDNA gene sequences (~1500 bp) of *Cardinium* were obtained by using the general bacterial 16S rDNA primers 27F and 1513R (Weisburg *et al*., 1991). All PCR reactions followed the published protocols described within the corresponding references. All PCRs were accompanied by positive (recombined plasmids containing target gene fragments) and negative (ddH_2O) controls. Resulting PCR products were electrophoresed on 1.0% agarose gels stained with ethidium bromide, visualized and analyzed using gel image analysis system VersaDoc MP 4000 (BIO-RAD, Hercules, CA, USA).

DNA sequencing

Some PCR products were recovered by using the agarose Gel DNA Fragment Recovery Kit (TaKaRa, Tokyo, Japan) and then inserted into the pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Isolated plasmid DNA or PCR-amplified DNA was used for sequencing templates. A few ambiguous bases and single base pair polymorphisms were assessed again by performing a second amplification using the PrimeSTAR™ HS DNA polymerase (TaKaRa). All sequences were generated using a DNA Sequence System (ABI 3730XI, Applied Biosystems, Foster City, USA). Reliable sequences were generated from these multiple clones and used in further analyses. Sequences generated in this study were deposited in GenBank under the following accession numbers

Table 3 The GenBank accession numbers of the five multilocus sequence typing (MLST) genes and the *wsp* gene of different *Wolbachia* strains involved in this study.

Population information	Host	*Wolbachia* strain	Different genes of *Wolbachia*	GenBank ACCN
Baise, Guangxi Zhuang Autonomous Region, China	*Nilaparvata lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	FJ713749-FJ713754
Nanning, Guangxi Zhuang Autonomous Region, China	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	FJ713755-FJ713760
Da Nang, Quang Nam Province, Viet Nam	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289746-GU289751
Vĩnh Linh, Quang Tri Province, Viet Nam	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289752-GU289757
Sanya, Hainan Province, China	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289758-GU289763
Ning'er, Yunnan Province, China	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289764-GU289769
Wuhan, Hubei Province, China	*N. lugens*	*w*Lug	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289770-GU289775
Baise, Guangxi Zhuang Autonomous Region, China	*Sogatella furcifera*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	FJ713761-FJ713766
Nanning, Guangxi Zhuang Autonomous Region, China	*S. furcifera*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289776-GU289781
Vĩnh Linh, Quang Tri Province, Viet Nam	*S. furcifera*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289782-GU289787
Baise, Guangxi Zhuang Autonomous Region, China	*Laodelphax striatellus*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	FJ713767-FJ713772
Nanning, Guangxi Zhuang Autonomous Region, China	*L. striatellus*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289788-GU289793
Nantong, Jiangsu Province, China	*L. striatellus*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289794-GU289799
Nanjing, Jiangsu Province, China	*L. striatellus*	*w*Stri	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289800-GU289805
Huzhou, Zhejiang Province, China	*Nilaparvata muri*	*w*Mui	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289806-GU289811
Jinhua, Zhejiang Province, China	*N. muri*	*w*Mui	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	HQ404750-HQ404755
Nanning, Guangxi Zhuang Autonomous Region, China	dryinid wasps	*w*Waspb	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289812-GU289817
Baise, Guangxi Zhuang Autonomous Region, China	dryinid wasps	*w*Waspb	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	GU289818-GU289823
Jinhua, Zhejiang Province, China	*Macrosteles fascifrons*?	*w*Mfas1	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	HQ404756-HQ404761
	M. fascifrons?	*w*Mfas2	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	HQ404762-HQ404767
	M. fascifrons?	*w*Mfas3	*gatB/coxA/hcpA/ftsZ/fbpA/wsp*	HQ404768-HQ404773

"?" means the real host of the *Wolbachia* strain was uncertain.

(ACCN): FJ713749-FJ713772, GU289746-GU289823, HQ404750-HQ404773, JN560715-JN560721 (Table 3).

Wolbachia *strain characterization*

Multilocus sequence typing (MLST) was recently developed as a universal and unambiguous genotyping tool for *Wolbachia* (Baldo *et al*., 2006b). The genotype of a strain was determined by cloning and sequencing five MLST genes and the *wsp* gene. We followed the procedure of Baldo *et al*. (2006b) except for the annealing temperature used for the *fbpA* gene. To determine each *Wolbachia* strain, we first directly sequenced the PCR products of the *wsp* gene of each sample. If the sequence result was unambiguous, we directly sequenced the other five MLST genes. Otherwise, we constructed a clone library used for sequencing. We sampled seven populations

of BPH, three populations of WBPH, four populations of SBPH and two populations of *N. muiri*, and the sample number in selected populations ranged from one to five. To confirm the results, we also sequenced more than three individuals of each species of those planthoppers reared in the laboratory. For isolated *Wolbachia* strains *w*Fas1, *w*Fas2 and *w*Fas3, we directly sequenced all the PCR products of the *wsp* gene amplified from each detected positive individual of *M. fascifrons*, and then we chose at least three individuals in which the sequencing result of the *wsp* gene showed only a single strain and were used for sequencing the five MLST genes of each *Wolbachia* strain. All sequences based on PCR products were bi-directional sequenced. Because the *Wolbachia* strain *w*Waspb was accompanied by other *Wolbachia* strains in two BPH individuals collected from two different loci (Table 1), we constructed clone libraries for each of the five MLST genes and the *wsp* gene used for sequencing. For each clone library, we sequenced at least 10 clones to identify each gene sequence of this strain. The GenBank accession numbers of the five MLST genes and the *wsp* gene of different *Wolbachia* strains involved in this study are listed in Table 3. All unique MLST gene sequences were submitted to the MLST database and assigned an allele number (http://pubmlst.org/*wolbachia*/), and each new strain was assigned a sequence type (ST).

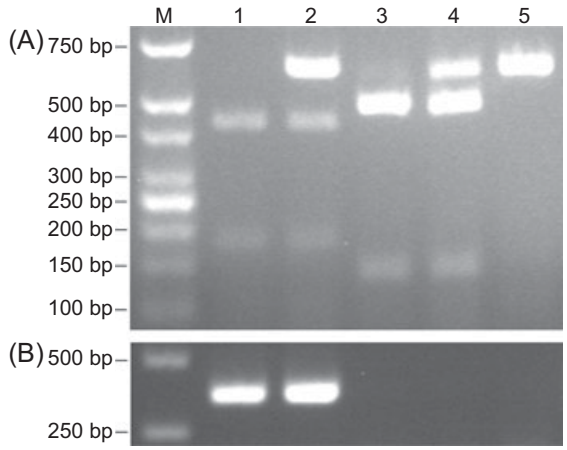

Fig. 1 Restriction analysis of *wsp* polymerase chain reaction (PCR) products amplified from different rice planthopper specimens. Different restriction patterns appeared due to different infection statuses of each individual. (A) Lane M, DNA size marker; Lanes 1 and 2, *wsp* PCR products amplified from brown planthopper (BPH) were digested with *Eco* RV; Lanes 3 and 4, *wsp* PCR products amplified from small brown planthopper (SBPH)/ whitebacked planthopper (WBPH) were digested with *Bgl* II; Lane 5, *wsp* PCR products amplified from rice planthopper cannot be digested by *Eco* RV and *Bgl* II. (B) A specific pair of primers (BPH-COIF and BPH-COIR) to amplify the COI gene of BPH to distinguish the BPH from other morphologically resembled species in the same genus *Nilaparvata*.

Detection of variation of Wolbachia *within natural populations*

The *Wolbachia* strains were characterized with two restriction enzymes, *Eco* RV and *Bgl* II (TaKaRa), which cut the *wsp* gene of the *w*Lug strain and the *w*Stri strain at single sites, respectively. They also cut the target *wsp* amplicon into two fragments of different sizes which could be separated from non-target *wsp* gene amplicons on agarose gels (Fig. 1). The recipe for enzymatic reactions was: 2 μL enzyme, 2 μL 10 $\times$ H buffer, 10 μL PCR product, and adding ddH_2O up to 20 μL. After incubation at 37°C for 1 h, 2 μL 10 $\times$ loading buffer (1% sodium dodecyl sulfate, 50% glycerol, 0.05% bromophenol blue) was added to end the enzymatic reaction. The digestion products were separated on a 2.0% agarose gel. The *wsp* gene PCR products from 64 *Wolbachia*-infected individuals of BPH and most of *Wolbachia*-infected individuals of WBPH and SBPH were digested with *Eco* RV and *Bgl* II, respectively. Different infection statuses would represent different band types (Fig. 1A). If the planthopper was just infected with one strain of *Wolbachia*, the enzyme digestion should have resulted in two fragments (Fig. 1A: lane 1, lane 3). If different strains of *Wolbachia* occurred in the same individual, it should have resulted in three fragments (Fig. 1A: lane 2, lane 4). If the strain of *Wolbachia* could not be digested by these two enzymes, it meant a different *Wolbachia* strain (not *w*Lug or *w*Stri) was present (similar to the digestion result of *w*Mui, Fig. 1A: lane 5). To distinguish the BPH from other morphologically similar species in the same genus *Nilaparvata*, we designed a specific pair of primers, BPH-COIF and BPH-COIR (Table 2), to amplify 450 bp of the cytochrome oxidase subunit I (COI) gene of BPH (Fig. 1B).

Phylogenetic analysis of Wolbachia *and their hosts*

To understand the phylogenetic relationship of *Wolbachia* and its hosts, we separately analyzed the phylogeny of *Wolbachia* and the planthopper species. Known *Wolbachia* MLST sequences (Baldo *et al*., 2006b) were downloaded and aligned with our sequences using ClustalX version 1.83 (Thompson *et al*., 1997). Manual adjustment was not performed for the alignment was very reliable. The model GTR + Γ + I was selected to reconstruct phylogenies. Bayesian inference was performed using

MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001), with 6 000 000 generations and a sample frequency of 200, and the first 7 500 trees were later discarded (a burn-in of 25%). Posterior probabilities were computed from the remaining trees. Maximum parsimony (MP) was also used to reconstruct the phylogeny, performed in PAUP v 4.01 (Swofford, 2002) using the heuristic searches with tree-bisection-reconnection (TBR) branch swapping and with 10 000 bootstrap replicates.

Delphacid relationships from the genus to the subfamily level have been well reconstructed using sequence data from the 3′ end of the 12S gene (Dijkstra *et al*., 2006). In view of this, to analyze the phylogenetic relationship of the planthoppers involved in this article, we sequenced the 3′ end of the 12S rRNA gene of each planthopper species collected from different locations (ACCN: HQ116528-HQ116533). All GenBank accession numbers of 12S rRNA genes of Fulgoroidea used for phylogenetic reconstruction are indicated in Figure 2. Almost all known 12S rRNA gene sequences of species in the family Delphacidae were used to construct phylogenetic trees. Sequences were first aligned within ClustalX v. 1.83 software (Thompson *et al*., 1997) using default settings, and manually aligned in MacClade v. 4.06 (Maddison & Maddison, 2003), and then a neighbor-joining (NJ) tree was constructed. Clade robustness was assessed by bootstrap analysis using 1 000 replicates.

Recombination analysis

To clarify whether horizontal gene exchange had occurred among these *Wolbachia* strains, we conducted recombination detection by using the *wsp* and five MLST genes. A long fragment of the *ftsZ* gene and its downstream region of *Wolbachia* which was amplified by using two pairs of primers ftsZf1/ftsZr1 (Werren *et al*., 1995b) and inftsZ-F/exftsZ-R (Table 2) were also used for recombination analysis. Potential recombination events in alignments of each of these genes were detected using RDP3 Alpha 44 (Martin *et al*., 2005). We chose seven methods implemented in this program for the identification of recombinant sequences and recombination breakpoints: RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN and 3SEQ. Default settings were used throughout and only potential recombination events detected by three or more of the above methods, coupled with phylogenetic evidence of recombination, were considered significant. According to the program manual, the approximate breakpoint positions and recombinant sequences inferred from every potential recombination event were manually checked and adjusted where necessary using the phylogenetic and recombination signal analysis features available in RDP3.

Results

Wolbachia *and* Cardinium *detection*

In total, we screened four species of planthoppers and one species of leafhopper at 19 locations from the Indo-China peninsula to south China. Almost all populations were positive for *Wolbachia* infection (Table 1). The samples included 365 individuals of BPH, 234 individuals of WBPH, 240 individuals of SBPH, 71 individuals of *N. muiri* and 382 individuals of *M. fascifrons*. Different planthopper species exhibited different infection statuses of these two endosymbionts. For *Wolbachia*, nearly 91.7% (220/240) individuals of SBPH were infected. In contrast, the infection rate of WBPH (120/234 = 51.3%) was not as high as SBPH, and BPH showed the lowest infection frequency (64/365 = 17.5%). *Cardinium*, was detected at low levels within BPH, SBPH and WBPH, while exhibiting co-infection with *Wolbachia* within some hosts of the latter species. A high rate of *Wolbachia* infection was also found in *N. muiri* (57/71): all 23 males collected in Jinhua were infected with *Wolbachia*. In addition, 37 individuals of *M. fascifrons* were positive for *Wolbachia* infection.

Wolbachia *and* Cardinium *strain characterization*

After comparison with strains and alleles from the *Wolbachia* MLST database (http://pubmlst.org/*wolbachia*/), complete MLST and WSP (the *Wolbachia* cell surface protein) profiles of *Wolbachia* strains involved in this study are presented in Table 4. Combined with natural and laboratory population sequence results, each of the six *Wolbachia* genes detected from each planthopper species showed identical sequences. It is interesting that different *Wolbachia* strains sharing identical or nearly identical alleles seem to be a common phenomenon among these hosts (Table 4). Aside from an identical ST (sequence type) found in WBPH and SBPH, it is notable that the strain *w*Lug, found in BPH (ST163), was also found in a calyptrate muscoid fly (Diptera: Calyptrate muscoid, the GenBank accession numbers [ACCN] of five MLST genes: FJ969197–FJ9691201) (Stahlhut *et al*., 2010). Although we do not know whether this fly interacts with BPH, this clearly indicated that the *w*Lug strain has undergone recent horizontal transfer between distant insect hosts. In addition, we also found four new *Wolbachia* strains in this study, one from *N. muiri* (*w*Mui) and three from *M. fascifrons* (temporarily named as *w*Mfas1,

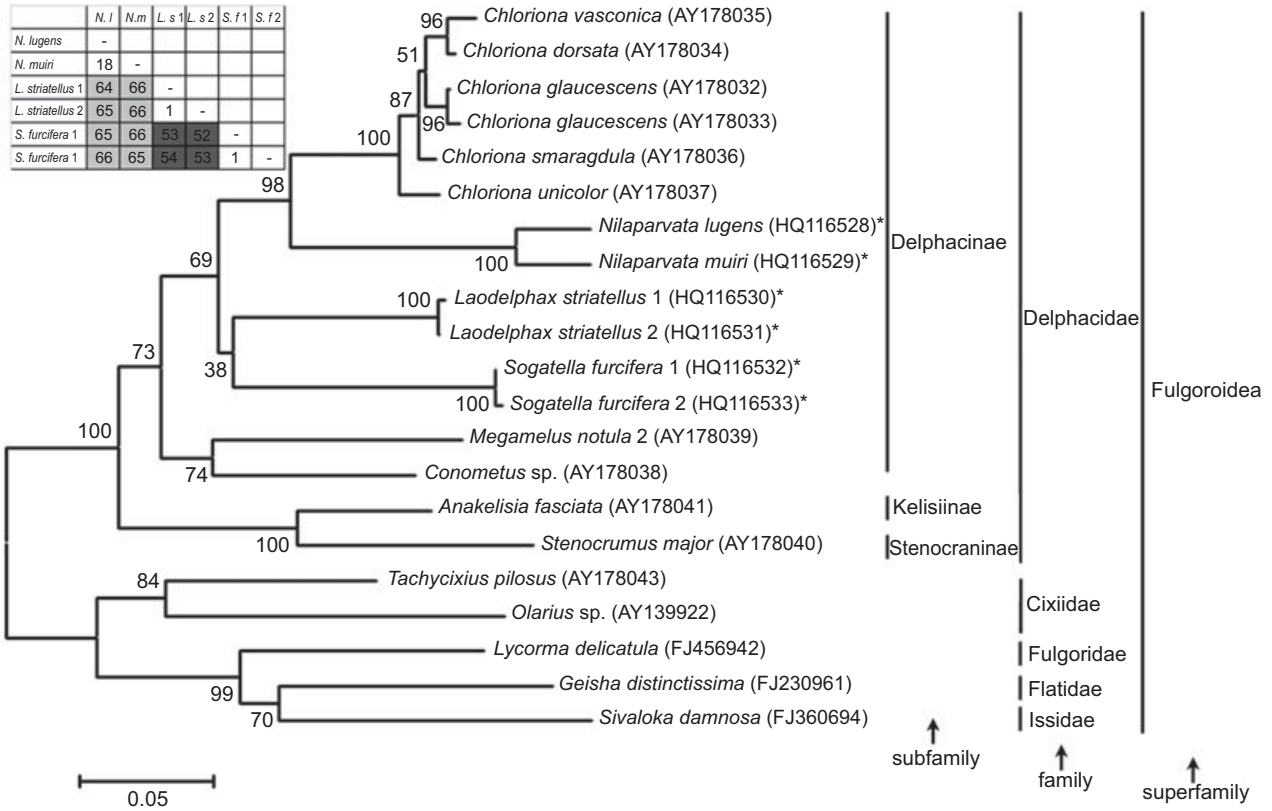

Fig. 2 Phylogenetic tree from neighbor-joining analysis of the sequences of the 3′ end of the 12S ribosomal RNA gene and relationship of the results to the classification of Fulgoroidea based on morphology. Numbers at nodes are for bootstrap percentages from 1 000 replicates. Based on the pairwise analysis of the sequences, the distance matrix on the upper-left shows evolutionary divergence between different planthopper species involved in this study (denoted by asterisks). There is only one nucleotide difference between *Laodelphax striatellus* 1 and *L. striatellus* 2, *Sogatella furcifera* 1 and *S. furcifera* 2.

Table 4 Multilocus sequence typing (MLST) allelic profiles and WSP alleles of *Wolbachia* detected from planthopper and leafhopper species.[†]

Host species	ST	Strain	MLST						WSP			
			gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*	*wsp*	HVR1	HVR2	HVR3	HVR4
N. lugens	163	*w*Lug	107	87	29	35	27	465	112	131	43	32
S. furcifera L. striatellus	**213**	*w*Stri	106	11*	13	**105***	162	463	2	191	192	22
N. muiri	**214**	*w*Mui	**134***	91	**139**	**106**	**194***	**532**	18	**218**	23	16
Unidentified dryinid wasp species	**215**	*w*Waspb	39	14	40	7*	**195***	296	2	17	3	23
M. fascifrons?	**216**	*w*Mfas1	9*	**119**	**140**	**107**	9*	**533**	2	17	3	**44**
M. fascifrons?	**217**	*w*Mfas2	**135**	**120***	**141**	**108**	**197**	**536**	**191**	**220**	23	**16**
M. fascifrons?	**218**	*w*Mfas3	**136**	91	**142**	**109**	9*	**534**	7	**219**	**220**	**215**

[†]Numbers refer to nucleotide alleles of MLST and peptide haplotypes of the four consecutive sections of WSP (each including a hypervariable region, HVR) were assigned by the MLST database. New alleles, STs (ST = sequence type, it identifies a unique allelic profile, assigned only to strains fully characterized by MLST), *wsp* and HVR are highlighted by bold font. Apart from the identical alleles, the nearly identical alleles of MLST (only one nucleotide mismatched) among these *Wolbachia* strains are identified with asterisk. "?" means the real host of the *Wolbachia* strain might originate from the endoparasitoids of *M. fascifrons*.

*w*Mfas2, *w*Mfas3). However, in view of the fact that the infection rates of these three *Wolbachia* strains in *M. fascifrons* were very low (3.1%, 5%, 1.3%, see Table 1), it suggests the possibility that these *Wolbachia* strains originate from endoparasites of *M. fascifrons*. We cannot exclude the possibility that they are low-level infections of the leafhopper itself.

The *Cardinium* 16S rDNA gene sequence from BPH collected in Nanning was found to be identical to that from a spider mite, *Eotetranychus suginamensis* (ACCN: AB506775) and the *Cardinium* 16S rDNA sequences from BPH collected in Vinh Linh and Wuhan are identical to that from WBPH (ACCN: AB506774) (Nakamura *et al*., 2009).

Different infection statuses of Wolbachia *in rice planthoppers*

The *w*Lug or *w*Mui strains were not detected from SBPH or WBPH by RFLP analysis of *wsp* PCR products from the *wsp* gene. However, of 64 *Wolbachia*-infected BPH individuals, PCR products of the *wsp* gene from 50 individuals were completely digested by *Eco* RV but the other 14 (from Baise, Nanning, Ning'er and Wuhan) were not. For SBPH and WBPH, there is a theoretic possibility that some kinds of parasitoids of plant- and leafhopper were also infected with the *Wolbachia* strain *w*Stri (Table 1). Therefore, we cannot completely exclude the possibility that *Wolbachia* in a few infected samples of these two species might have originated from their parasitoids. Furthermore, *Wolbachia* strain *w*Stri was also detected in two individuals of *M. fascifrons*.

Phylogenetic relationships of Wolbachia *and its host*

The phylogeny of different taxa of Fulgoroidea was reconstructed using sequence data from the 3′ end of the 12S gene. At both the family and subfamily level, the relationship of different taxa was very clear (Fig. 2). Among the four planthopper species analyzed in this study, BPH and *N. muiri* showed the closest relationship. Although the phylogenetic relationship of *Nilaparvata*, *Sogatella* and *Laodelphax* was not very clear because of the low bootstrap values and deficient taxon sampling, the distance matrix indicated that the phylogenetic distance between SBPH and WBPH (dark background color) is less than that between SBPH/WBPH and BPH (gray background color).

For phylogenetic analysis of *Wolbachia*, the STs of each *Wolbachia* strain involved in this study were unambiguously assigned to supergroup B based on the concatenated MLST gene phylogeny (Fig. 3). The phylogenetically distant *Wolbachia* strains in related hosts (BPH and *N. muiri*) reflect independent acquisitions of *Wolbachia* from distant lineages (through horizontal transfer). Although the *wsp* gene sequences of *w*Waspb and *w*Mfas1 were very similar to the *wsp* sequence of *w*Stri (>99%), the phylogenic analysis based on the concatenated MLST genes indicated that the two former strains were not related to *w*Stri. Actually, the serious incongruence between the *Wolbachia* phylogenies based on the *wsp* gene and the MLST concatenated sequences (Fig. 4) suggests that the different genes of *Wolbachia* have undergone different evolutionary trajectories.

Recombination analysis

Independent analysis of each *wsp* and the five MLST genes indicated that no intragenic recombination occurred in *gatB*, *coxA* or *hcpA* gene. But intragenic recombination did affect the *fbpA*, *ftsZ* and *wsp* genes, providing clear evidence of horizontal DNA transfer between *Wolbachia* strains in this study (Fig. 5). The *fbpA* gene of *Wolbachia* showed a clear recombination between *Wolbachia* strain *w*Mfas2 detected from *M. fascifrons* and *Wolbachia* strain *w*Lug from BPH. The fragment of the *ftsZ* gene used in the MLST analysis (481 bp excluding the primers) showed no recombination signal in the recombination analysis, in agreement with previous studies (Baldo *et al*., 2006a). However, a longer fragment of this gene and its downstream region revealed an undoubted recombination signal between the *w*Stri and *w*Lug strains (Fig. 5). In order to avoid misidentification of recombination, the *wsp* gene also gave a recombination signal but was not shown for the unknown major parent (parent contributing the larger sequence fraction) and low similarity of the minor parent (parent contributing the smaller sequence fraction). Indeed, analysis of *wsp* genes involved in this study combined with the *wsp* genes of *Wolbachia* detected from other planthoppers, leafhoppers and parasitic wasps (Kittayapong *et al*., 2003; Takiya *et al*., 2006), showed three recombination events of this gene (Fig. 6). All of these recombination events were also supported by the phylogenetic reconstruction implemented in RDP3. Recombination appears to occur among strains from related hosts.

Discussion

There is growing evidence of horizontal transfer of *Wolbachia* among matrilines. Horizontal transfer is a reasonable and reliable explanation for the incongruent

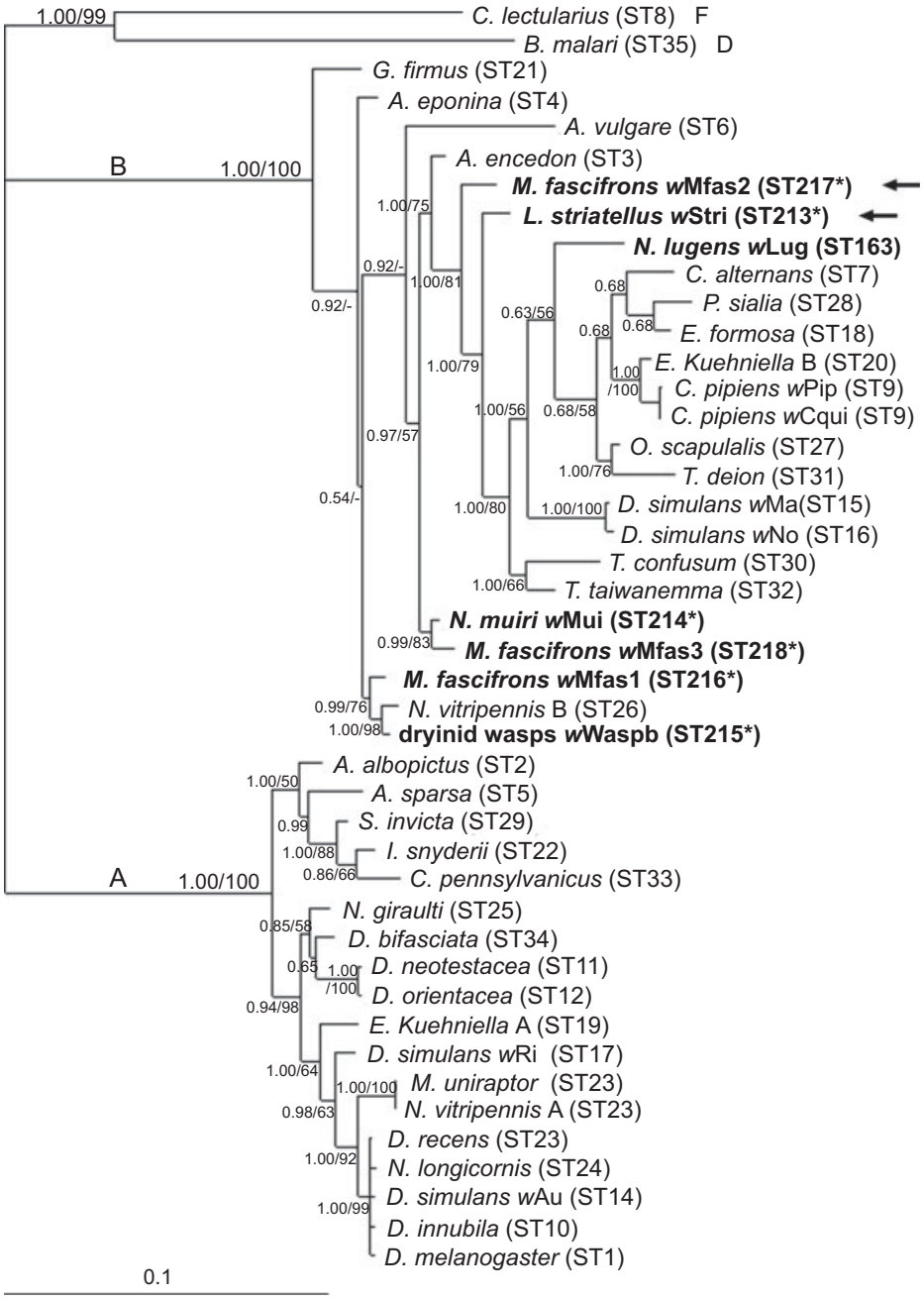

Fig. 3 Bayesian phylogeny based on the concatenated data set for the five multilocus sequence typing (MLST) loci (2 079 bp). Posterior probability (left) and parsimony bootstrap (right) values are shown at each node if they were supported by both clustering algorithms. The nodes having no parsimony bootstrap values mean that parsimony bootstrap (right) values were lower than 50%, "-" means the nodes are inconsistent with the maximum parsimony (MP) tree. The strains involved in this study are highlighted by bold font and new sequence types are denoted by asterisks. Arrows indicate that the corresponding clades might be unreliable because of existing recombination events (Fig. 5).

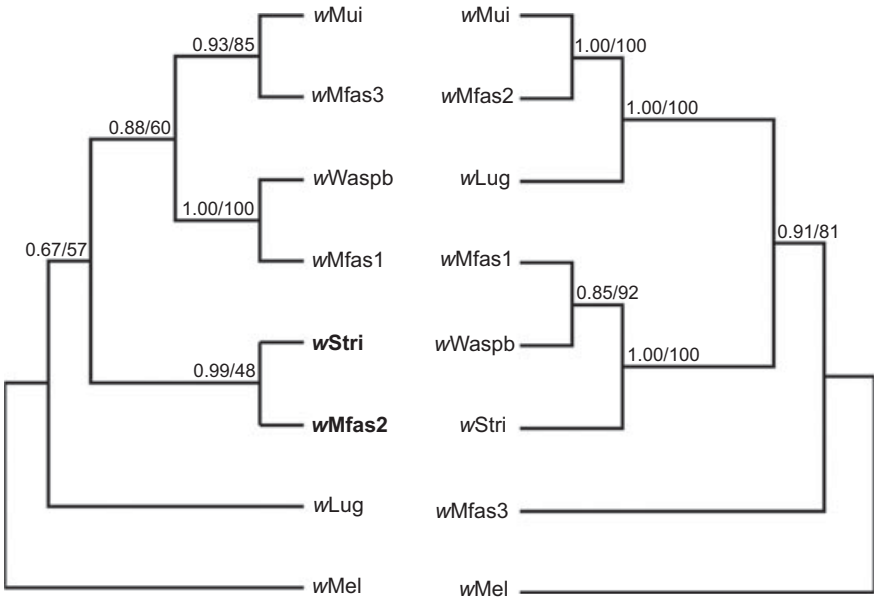

Fig. 4 Comparison of *Wolbachia* phylogenies based on concatenated multilocus sequence typing (MLST) sequences (left) and *wsp* gene sequences (right) show the serious incongruence between these two datasets. Posterior probability (left) and parsimony bootstrap (right) values are shown at each node as they are supported by both clustering algorithms (Bayesian likelihood inference and maximum parsimony). The strains involved in recombination are highlighted by bold font.

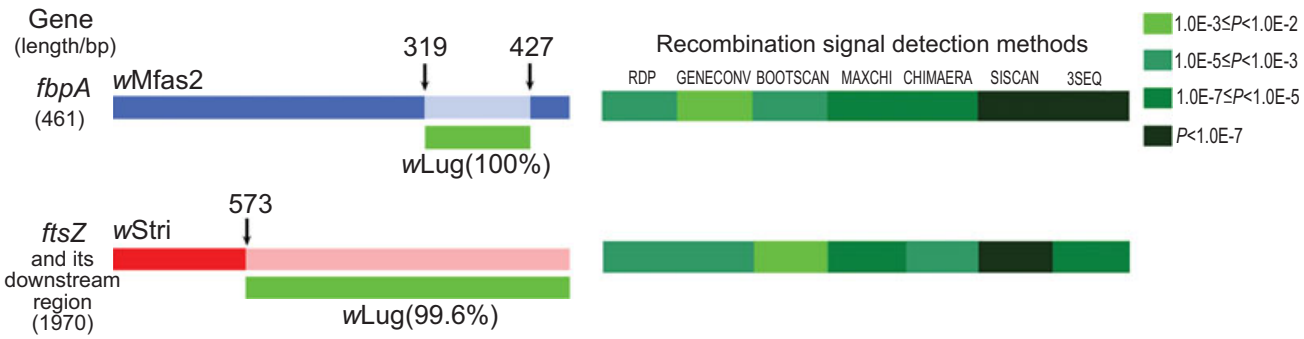

Fig. 5 Summary of positive recombination events of two genes in different *Wolbachia* strains. The left part of figure shows the schematic of each recombination event. The minor parent (parent contributing the smaller fraction of sequence) of each daughter sequence (recombinant) is shown with the name of *Wolbachia* strains and percentage of similarity to daughter sequence. The numbers above the arrow indicate beginning and ending breakpoints of each recombination event. The right part of figure shows the different *P*-values for the seven recombination detection methods implemented in RDP 3.0 using the color coding (as the legend).

molecular phylogenies and the recombination of *Wolbachia* genomes. Transmission via natural enemies or other ecological interactions has long been proposed to mediate the transfer of *Wolbachia* from one host to another, even though only rare cases of horizontal transfer have been observed in experiments (Heath *et al*., 1999; Huigens *et al*., 2004).

Rice fields serve as ecological systems that include a variety of planthoppers, leafhoppers and their related parasitoids. Transfer of endosymbionts between parasitoids and their hosts is a potential mechanism for the horizontal transmission of *Wolbachia* (van Meer *et al*., 1999). Screening and sequence analysis of strains from these samples allowed us to determine whether closely related planthoppers harbored host-specific *Wolbachia* lineages and the possibility of horizontal transmission of *Wolbachia* among different hosts. In general, precautions have to be taken to ensure that endosymbionts detected in a given host or predator do not stem from parasitoids inside the host or prey inside the predator (Enigl *et al*., 2005; Wu

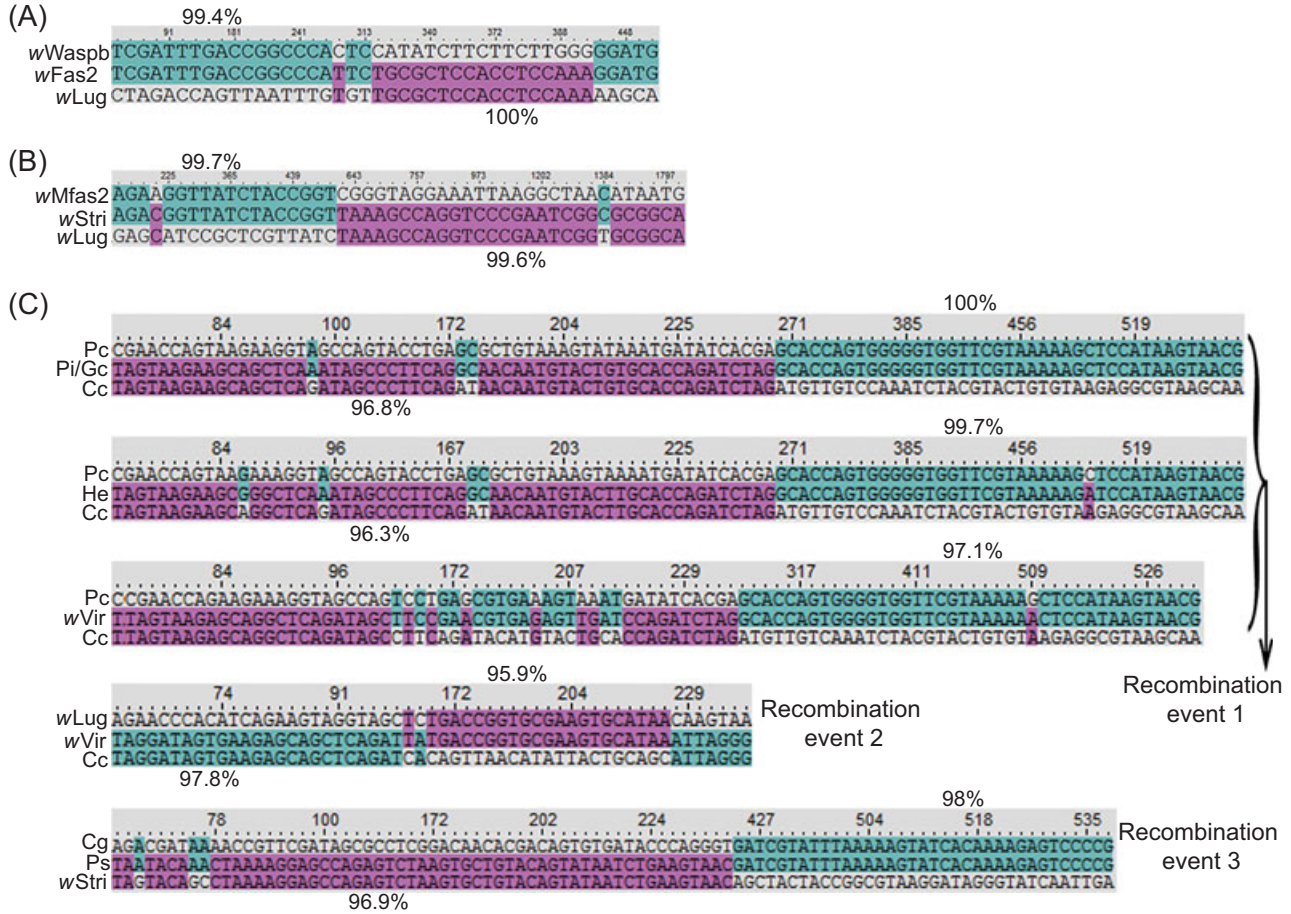

Fig. 6 Description of recombination events of different genes of *Wolbachia*: (A) *fbpA*, (B) *ftsZ* and (C) *wsp* gene. For each alignment, only polymorphic sites around the breakpoints are shown. Numbers above sequence alignments indicate the schematic nucleotide position. Percentages above and under the sequence alignments show the similarities of the daughter sequence to its major and minor parent sequences (marked with the same background color). Among the sequence alignments of *wsp* gene, three recombination events were identified. The recombination event 1 was found in three similar daughter sequences. The recombination event 2 suggests that recombination in the *wsp* gene of *Wolbachia* strain *w*Vir might have occurred twice. The *wsp* gene of each *Wolbachia* strain in the sequence alignments are presented as an existing name or first letter abbreviation of the genus and species name of its host species which was the *Wolbachia* strain detected from (Takiya *et al*., 2006) (Pc: *Proconosama columbica* (Signoret), DQ450160-DQ450162; Pi/Gc: An identical *wsp* sequence was detected from both *Paraulacizes irrorata* (Fabricius) DQ450152, DQ450153 and *Graphocephala coccinea* (Forster), DQ450163; Cc: *Clydacha catapulta* Kramer, DQ450150; He: *Homalodisca elongata* Ball, DQ450154, DQ450155; Cg: *Cuerna gladiola* Oman and Beamer, DQ450149; Ps: *Pamplona spatulata* Young, DQ450159; *w*Vir: *Nephotettix virescens* (Distant), AF481179 (Kittayapong *et al*. 2003); other *Wolbachia* strains were involved in this study).

& Hoy, 2012). Endosymbiont infection originating from parasitoids inside their hosts can be excluded by either testing the legs, wings or other parts of the hosts, which do not contain the parasitoids, or by testing the offspring of the hosts. Here we did not test the legs or wings of planthoppers, because in natural populations of WBPH, *Wolbachia* commonly degenerates in the tissues of adult individuals with increasing age (unpublished data). Sequencing the genes of *Wolbachia* detected by PCR and RFLP methods could also help to clarify whether *Wolbachia* originates from the hosts or parasitoids inside the hosts (Table 1). Furthermore, the results of the endosymbiont infection status of natural populations of the predominant rice planthoppers were confirmed by testing laboratory-reared populations. Indeed, identical or nearly identical *Wolbachia* strains detected from different hosts indicated that horizontal transmission of these bacteria between distant host species might have frequently occurred. Moreover, the finding that each of the MLST genes of *Wolbachia* infecting different hosts (i.e., *w*Stri in SBPH and

WBPH, *w*Lug in BPH and a species of calyptrate muscoid fly) has an identical sequence suggests that horizontal transmission events might have occurred recently in the evolutionary history of *Wolbachia*. Similarly, identical *Cardinium* (based on the sequences of 16S rDNA) detected from different planthopper species suggests that this endosymbiont might also be caused by the horizontal transmission of *Cardinium* between planthoppers.

Parasitism makes the horizontal transmission of *Wolbachia* possible, and in another respect, parasitism may also provide the necessary conditions for the genetic recombination of different *Wolbachia* strains. In this study, we identified unambiguous recombination events in different *Wolbachia* strains (Fig. 5), with "donated" genetic material possibly originating from several common rice field hemipteran pests and their parasites. Previous single gene phylogenies were also found to be unreliable because of intragenic recombination and widespread recombination of *Wolbachia* genomes (Baldo *et al*., 2005; Baldo *et al*., 2006a; Jiggins, 2002; Jiggins *et al*., 2001; Reuter & Keller, 2003; Werren & Bartos, 2001). Among the studied genes, *wsp* has been found to be the most recombinogenic within the *Wolbachia* genome. In this study, we also identified three recombination events within this gene (Fig. 6). All of these results show that gene recombination is an important factor affecting the diversification and evolution of *Wolbachia*. Nonetheless, further studies are needed to understand why some recombinants are more viable than others and whether the functions of recombined genes have changed.

Although horizontal transfer within and between species appears to be frequent for *Wolbachia*, its distributions are far from random (Ros *et al*., 2009). There is increasing evidence that both *Wolbachia* and *Cardinium* show host association and specialization. Recently, a study of 13 inter- and 36 intraspecific transfer cases revealed that host relatedness affected the success of *Wolbachia* transfer (Russell *et al*., 2009). Likelihood-ratio tests demonstrated a positive correlation between transinfection success and relatedness of donor and recipient hosts. For *Cardinium*, the narrower distribution compared with *Wolbachia* indicates more obvious characters of host-associated specialization (Zchori-Fein & Perlman, 2004). In this study, our results also provide evidence of host association of *Wolbachia* and *Cardinium* infecting different planthopper species. For *Wolbachia*, apart from a few cases of *Wolbachia* strain (*w*Stri) emerging in several BPH individuals (potentially originating from endoparasites), we did not find *Wolbachia* strains *w*Lug or *w*Mui in SBPH or WBPH. For *Cardinium*, only WBPH in these four planthopper species was really infected. Moreover, the different planthopper species could be parasitized by the same *Wolbachia*-infected parasite. But under the same conditions for potential horizontal transmission, it seems that only few of them could successfully colonize the new host. For example, the *Wolbachia* strain *w*Stri infecting both SBPH and WBPH also emerged in BPH (Table 1), but this strain could not "settle down" in BPH. The PCR signal of *Wolbachia* strain *w*Stri in BPH could have come from the parasitoids inside the hosts. This was confirmed by detecting the infection status of BPH reared in the lab. This might be explained, at least partly, by the host genetic background playing an important role in horizontal transmission of endosymbionts. Phylogenetic analysis shows that the relationship of SBPH and WBPH is closer than the relationship between SBPH/WBPH and BPH. This relative relation is also supported to some extent by the different chromosome number and sex-determining systems of these three rice planthopper species: 14 autosomal bivalents and two sex chromosomes for BPH (female: 28 + XX/male: 28 + XY), whereas, the chromosome number of WBPH and SBPH is 29 or 30, the male diploid number is 29 (28 + XO) and the female diploid number is 30 (28 + XX) (Noda & Tatewaki, 1990).

On the other hand, host genetic backgrounds also affect the reproductive abnormality induced by *Wolbachia* (Bordenstein *et al*., 2003; Zabalou *et al*., 2008). Rice planthoppers, in spite of SBPH and WBPH, were infected with the same *Wolbachia* strain; previous research showed that the *Wolbachia*-induced cytoplasmic incompatibility (CI) level of SBPH was quite high and even aged males strongly caused CI, while WBPH showed partial CI and males lost their ability to cause CI with increasing age (Noda *et al*., 2001a). There could be many reasons for these different phenotypes. First, only WBPH was found to be co-infected with *Cardinium*. This means that *Cardinium* or other coexisting microorganisms might play a role in the expression of CI in WBPH. Second, in the course of this study, the bacteriophage WO were ubiquitously found in these planthopper species, and different *orf7* gene sequences of bacteriophage WO were obtained from these two kinds of planthoppers (our unpublished data). The bacteriophage WO is thought to affect CI expression (Kent & Bordenstein, 2010) and thus, different bacteriophage WO could explain different CI levels in these planthoppers. Third, the progress of interaction and coadaptation between the endosymbiont and its hosts may have different evolutionary histories: one might come from an archaic host while the other might be acquired by horizontal transmission. Besides CI-inducing strains of *Wolbachia*, we also proved that the *Wolbachia* strain *w*Lug does not cause CI in BPH (Zhang *et al*., 2010). The two different phenotypes in these

planthoppers may also provide a good model for studying the CI mechanism.

The influence of *Wolbachia* and *Cardinium* infection on host populations has attracted considerable interest in their possible roles in speciation and as potential agents of biological control. Some *Wolbachia* strains might have difficulty adapting to the physiological environment of a novel host. On the other hand, some *Wolbachia* lineages (e.g., *w*Ri) are less specialized and could infect other host species more easily (Kang *et al*., 2003). A more effective strategy for biological control might be looking for new *Wolbachia* strains from related species. Clearly, knowing the infection status of *Wolbachia* and *Cardinium* in important hemipteran pests and their potential related parasites in rice fields might help to design methods for biological control.

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Disclosure

We the authors do not have any conflicts of interest, including specific financial interests, and relationships and affiliations relevant to the subject of our manuscript.

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