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Distribution and diversity of *Wolbachia* in different populations of the wheat aphid *Sitobion miscanthi* (Hemiptera: Aphididae) in China

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Abstract. *Wolbachia* is a widely distributed intracellular symbiont in the reproductive tissues of arthropods. The wheat aphid *Sitobion miscanthi* (Takahashi) is an important agricultural pest worldwide. *Wolbachia* was detected in different populations of *S. miscanthi* in China using 16s rDNA and *wsp*-specific primers. Of eighteen populations eleven were infected with *Wolbachia*. Several strains of *Wolbachia* infected these *S. miscanthi* populations. Of the eleven infected populations, four were infected with only one *Wolbachia* strain and seven with double infections. This is the most systematic survey of the distribution of *Wolbachia* in the wheat aphid.

INTRODUCTION

Wolbachia is a common cytoplasmic symbiont that resides in the reproductive tissues of many arthropods (Juchault et al., 1994; Johanowicz & Hoy, 1995; Sironi et al., 1995; Werren et al., 1995; Rowley et al., 2004). Recent surveys have found *Wolbachia* in over 20% of insect species, including each of the major insect orders (Lo et al., 2007).

The wheat aphid, *Sitobion miscanthi* (Takahashi) (Hemiptera: Aphididae), is a major and widespread pest of cereal crops in China. It inflicts economic damage directly by sap-sucking and indirectly by transmitting the barley yellow dwarf and millet red leaf persistent luteoviruses (Blackman & Eastop, 2000). Because of the considerable reproductive potential, life cycle and behavioural plasticity of this wheat aphid, further characterization of the *Wolbachia* infection of this aphid is needed in order to understand the effect of this symbiont on aphid reproduction and evolution. West et al. (1998) used 16s rDNA and *fts*Z genes to survey four species of aphid: *Aphis jacobaeae*, *Capitophorus carduinus*, *Microlophium carnosum* and *Sitobium fragariae*, but did not find *Wolbachia* in any of these species. This was the first report on *Wolbachia* in aphids. Two years later, Jeyaprakash & Hoy (2000) tested sixty-three arthropod species distributed in sixteen orders using the long PCR method with *wsp* gene, which encodes a surface protein of *Wolbachia*. Among these arthropods *Wolbachia* was detected in two species of aphids: *Toxoptera citricida* (Kirkaldy) and *Aphis craccivora* (Koch), with A-*Wolbachia* identified in *T. citricida* (Kirkaldy). This A-*Wolbachia* sequence is the same as the strain wSus-A1 (GB No. AF217713). Tsuchida et al. (2002) conducted an investigation of the distribution of some endosymbiotic bacteria in Japanese populations of the pea aphid, *Acyrthosiphon pisum*. In spite of the prevalence of secondary endosymbiotic bacteria, *Wolbachia* was not detected. Nirgianaki et al. (2003) analysed twenty-four DNA samples of aphids provided by Paul Baumann (University of California, Davis, USA). These aphids included *Acyrthosiphon pisum*, *Aphis craccivora*, *Diuraphis noxia*, *Myzus persicae*, *Rhopalosiphum padi*, *Uroleucon* spp. etc. None of these species was infected with *Wolbachia*. Then Kittayapong et al. (2003) investigated tropical rice-field community insects in Thailand. Forty-nine of 209 rice-field insect species were infected with *Wolbachia*. Of these insects most were Homoptera (54.2%), but the aphid *Hysteroneura setariae* (Thomas) was not infected with *Wolbachia*. In 2004, Gómez-Valero et al. amplified and sequenced the 16s rDNA and *wsp* genes of *Wolbachia* in the aphid *Cinara cedri*. The phylogenetic analysis based on the *wsp* gene indicated that this kind of *Wolbachia* belonged to group *Con* of supergroup B. In addition, their results indicate that *Wolbachia* coexists with two other endosymbionts: *Buchnera aphidicola* (the primary endosymbiont in aphids) and S symbiont (a secondary symbiont). This is the first record of *Wolbachia* in an aphid observed using electron microscopy.

In this paper, we characterized the pattern of *Wolbachia* infection in natural populations of wheat aphid in China by identifying the strains and determining their relationships with the supergroups of *Wolbachia* already described. Eighteen natural populations of wheat aphid, *S. miscanthi*, were screened for infection using *Wolbachia*-specific 16s rDNA and *wsp* genes.

MATERIAL AND METHODS

Aphid samples

All the aphid samples used in this study were collected from eighteen areas of China where wheat is grown (Fig. 1). In order to avoid collecting offspring of the same mother, only one aphid was collected from each location, which were ten meters apart.

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These aphids were kept in 100% ethanol at –20°C until DNA extraction.

DNA extraction

DNA was extracted according to the protocols described by Vavre et al. (1999) with slight modifications. An aphid was washed in double distilled water and then ground in 200 µl extraction buffer (100 mmolL^{-1}Tris-HCl, PH7, 1.4 molL^{-1}NaCl, 20 mmolL^{-1}EDTA, 2% CTAB) and incubated at 65°C for 1 h. Then 1 µl RNase was added and the solution incubated at 37°C for 1 h. 500 µl chloroform-isoamylic alcohol (24 : 1) was added before centrifugation for 15 min at 13,000 rpm. The supernatant was collected and gently mixed with double volumes of 100% ethanol and tenth of volume of Na-acetate (3 mol L^{-1}, PH 5.2). After precipitation over night at –20°C and centrifuged for 20 min at 13,000 rpm and the precipitate of DNA collected. The precipitate was washed with 70% ethanol and air dried. Finally 20 µl 1 × TE buffer was added to dissolve the DNA sample, which was then stored at –20°C until tested.

***Wolbachia* detection**

Three diagnostic PCRs were performed to amplify a fragment of the 28s rDNA gene of the aphid and of the 16s rDNA and *wsp* genes of *Wolbachia*.

The 28s rDNA gene is universally present in eukaryotes and highly conserved. The primers based on the 28s rDNA gene were used to check for the quality of DNA extraction. The primers were forward (5'TAC CGT GAG GGA AAG TTG AAA) and reverse (5'AGA CTC CTT GGT CCG TGT TT). PCR cycling conditions were a 2 min pre-dwell at 94°C followed by 38 cycles of 30 s at 94°C, 50 s at 58°C, 90 s at 72°C and a post-dwell period of 10 min at 72°C. Samples negative for 28s rDNA gene were discarded. The 16s rDNA primers, which were forward (5'CAT ACC TAT TCG AAG GGA TAG) and reverse (5'AGC TTC GAG TGA AAC CAA TTA), were used to screen for *Wolbachia* infection. PCR cycling conditions were a 2 min pre-dwell at 94°C followed by 38 cycles of 30 s at 94°C, 45 s at 55°C, 90 s at 72°C and a post-dwell period of 10 min at 72°C. The aphid samples that were positive were reamplified using 16s rDNA and *wsp* primers (81F/522R; 136F/691R) using the PCR conditions described above Zhou et al. (1998).

PCRs were performed in 25 µl reaction volumes: 2.5 µl 10 × PCR buffer, 2.5 µl 25 mM $MgCl_2$, 2 µl dNTPs (10 mM each), 15 µl dd H_2O, 1.5 µl 10 µM forward and reverse primers and 1 unit *Taq* DNA polymerase. DNA extracts of *Wolbachia*-infected *Trichogramma evanesceus* were used as positive controls. Negative controls containing only double-distilled water were also included to check for contamination.

Cloning and sequencing

PCR products of the 16S rDNA and *wsp* gene segment were purified using a DNA Fragment Purification kit (Sangon). Purified PCR products were cloned in the plasmid vector pMD19-T (TaKaRa) and transformed into *Escherichia coli* DH5α-competent cells. The nucleotide sequences of selected clones were sequenced on an ABI automated sequencer (ABI Prism 377, USA). Both strands of plasmids were sequenced using universal primers (M13+, M13–) with forward and reverse reads. At least three independent clones were sequenced from each *Wolbachia* strain in order to identify polymerase errors.

Alignments and genetic analyses

Similar sequences to these of the 16S rDNA and *wsp* genes obtained from the wheat aphid were searched for in GenBank, using BLAST. The 16S rDNA sequences were aligned with the representative dataset of sequences from all supergroups described except supergroup G. Sequences of *wsp* were aligned with sequences from 33 A- and B-*Wolbachia* strains downloaded from GenBank (Table 2) followed by manual adjustments based on the amino acid translation of the different genes. Phylogenetic analyses were conducted with neighbour-joining (NJ) and maximum parsimony (MP) methods using MEGA 4.0 (Tamura et al., 2007). For maximum parsimony analysis, the close-neighbour-interchange (CNI) search method was used with the initial tree using random addition trees (10 repetitions). Alignment gaps were excluded and bootstrap analysis carried out with 1,000 replications. For NJ analysis, distances were calculated using the Kimura 2-Parameter model and bootstrap tests performed with 1,000 replications. The phylogenetic tree was constructed using the NJ method.

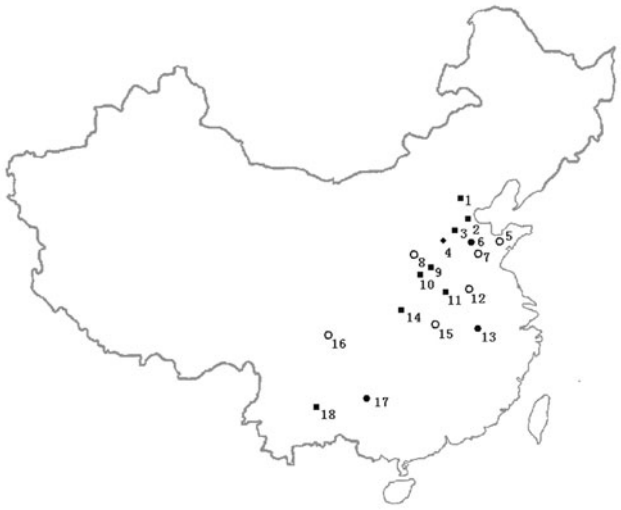

Fig. 1. Map of the sampling locations of *S. miscanthi* (Takahashi) in China. (Note: The islands of South China Sea that belong to China are not included in this map.) 1 – Beijing, 2 – Cangzhou, 3 – Hengshui, 4 – Xingtai, 5 – Weifang, 6 – Jinan, 7 – Taian, 8 – Lingfen, 9 – Xinxiang, 10 – Louyang, 11 – Luohe, 12 – Mencheng, 13 – Anqing, 14 – Shiyan, 15 – Xiaogan, 16 – Chengdu, 17 – Duyun, 18 – Kunming. ♦ – population infected with A-*Wolbachia*; ● – population infected with B-*Wolbachia*; ■ – population infected with A and B-*Wolbachia*; ○ – population not infected with *Wolbachia*.

Recombination analyses of *wsp* gene

Analysis of recombination was done using the part of the *wsp* gene. Four *wsp* sequences, *w*MisBJA1, *w*MisBJA1, *w*MisBJA2, *w*MisBJA3 and *w*MisBJB, from infected wheat aphids were aligned with 33 published sequences (Table 2) for recombination analyse. Automated RDP tool implemented in the program RDP2 was used. Default parameters were used and the highest acceptable P value cutoff was 0.01.

RESULTS

Prevalence of *Wolbachia* in *S. miscanthi*

This extensive targeted survey for *Wolbachia* infection in *S. miscanthi* using PCR amplification of the 16S rDNA gene revealed two different 16S rDNA genes in this wheat aphid. The phylogenetic analysis indicated that the two 16S rDNA sequences belonged to the separate supergroups A and B (Fig. 2).

Of the eighteen geographical populations of *S. miscanthi* sampled in China, eleven (61%) were infected with

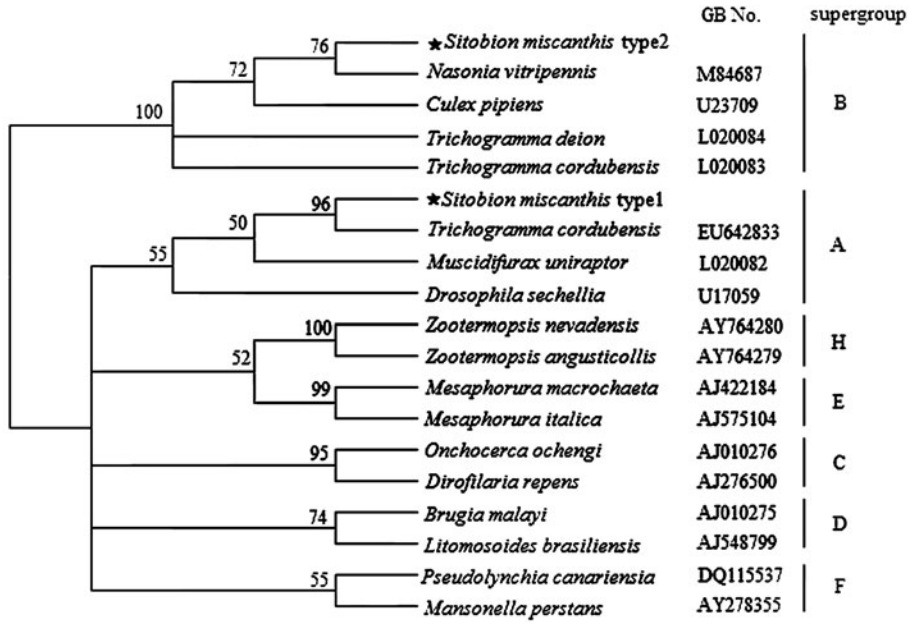

Fig. 2. Phylogenetic tree of *Wolbachia* based on 16S rDNA sequences constructed using NJ method in MEGA. *S. miscanthi* indicated by "★".

Wolbachia, with all the individuals in the population infected. The same phenomenon is also found in whiteflies and leafhoppers (Nirgianak et al., 2003). The reason for this is not clear. Four populations (HBXT, SDJN, AHAQ and GZDY) were infected with only one strain of *Wolbachia*. There were double infections of all individuals in seven populations: BJ, HBCZ, HBHS, HNXX, HNLY, HNLH and YNKM, and seven populations (SDWF, SDTA, SXLF, AHMC, HBSY, HBXG and SCCD) were not infected by any type of *Wolbachia* (Table 1).

Phylogeny and recombination of *wsp* gene

The twenty *wsp* sequences obtained belong to group Kue, Eva, Mis and Pip, respectively, of which Kue, Eva and Mis belong to supergroup A, Pip to supergroup B (Fig. 3). Group Kue, Eva and Pip are described but Mis is a new group. Intragenic recombination within the *wsp* gene was shown using the Siscan and RDP methods in RDP2. One recombination fragment was detected in *w*MisBJA3. The major and minor parents were identified as *w*Mors and *w*Ha. The size of the recombination frag-

TABLE 1. Distribution of *Wolbachia* in different populations of *S. miscanthi* in China based on 16S rDNA gene.

Location		Code	Longitude	Latitude	Total no. tested	Type of *Wolbachia* infection
Province	City					
Beijing		BJ	E116.46	N39.92	30	A, B
Heibei	Cangzhou	HBCZ	E116.83	N38.33	30	A, B
	Hengshui	HBHS	E115.72	N37.72	30	A, B
	Xingtai	HBXT	E114.48	N37.05	24	A
Shandong	Weifang	SDWF	E119.1	N36.62	16	None
	Jinan	SDJN	E117.0	N36.65	12	B
	Taian	SDTA	E117.95	N37.50	16	None
Shanxi	Linfen	SXLF	E111.5	N36.08	16	None
Henan	Xinxiang	HNXX	E113.85	N35.31	30	A, B
	Luoyang	HNLY	E112.44	N34.70	30	A, B
	Luohe	HNLH	E114.02	N33.56	24	A, B
Anhui	Mengcheng	AHMC	E116.55	N33.25	20	None
	Anqing	AHAQ	E117.03	N30.52	12	B
Hubei	Shiyan	HBSY	E110.79	N32.65	16	None
	Xiaogan	HBXG	E113.91	N31.92	22	None
Sichuan	Chengdu	SCCD	E104.06	N30.67	12	None
Guizhou	Duyun	GZDY	E107.53	N26.72	12	B
Yunnan	Kunming	YNKM	E102.73	N25.04	30	A, B

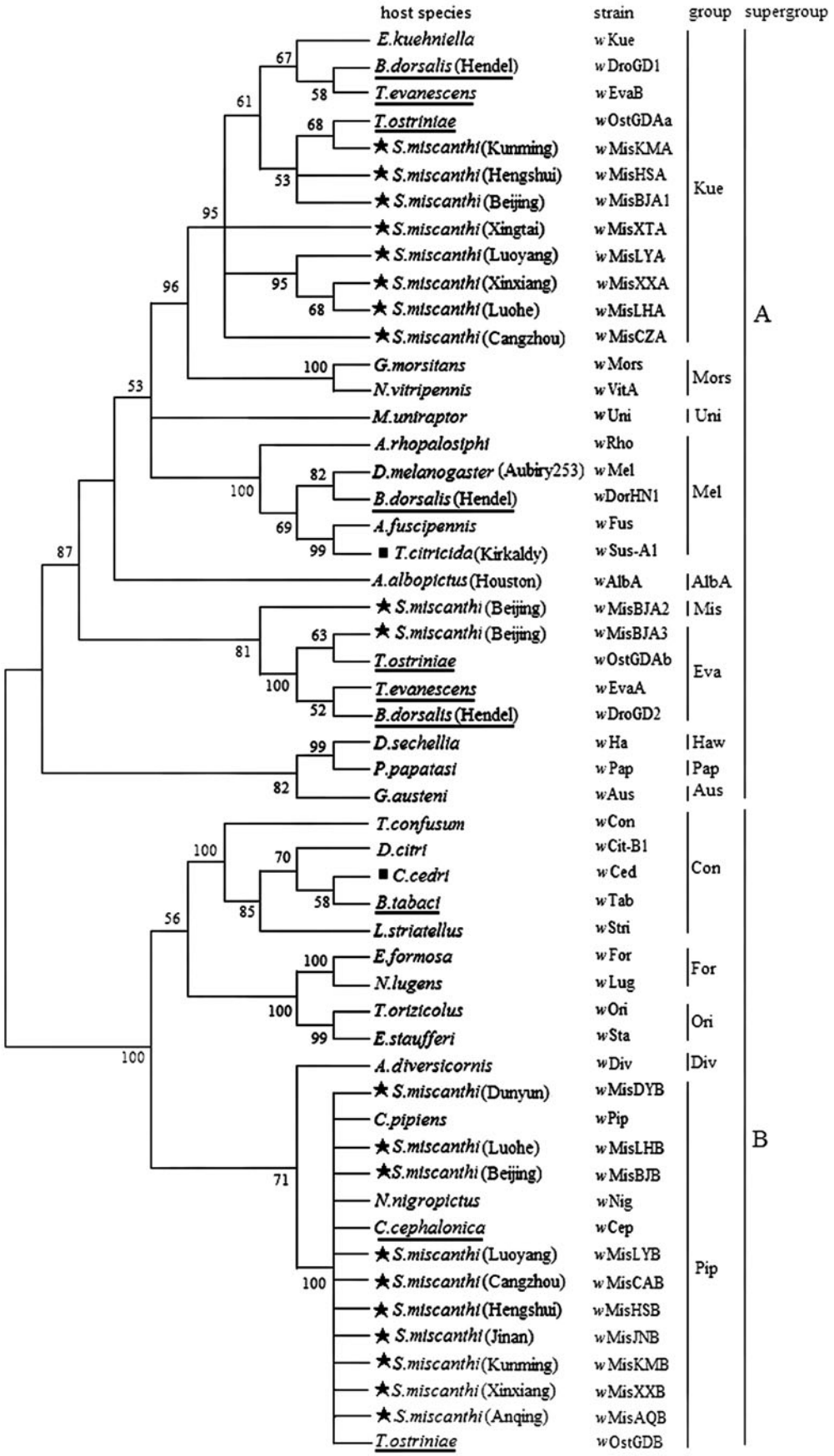

Fig. 3. Phylogenetic tree of *Wolbachia* based on *wsp* gene constructed using NJ method. *S. miscanthi* indicated by "★", other aphids by "■", other insects from China are underlined.

TABLE 2. Host, associated *Wolbachia wsp* gene sequences and GenBank accession numbers.

Supergroup	Group	*Wolbachia* host species	*Wolbachia* strain	GenBank accession
A	Mel	*Drosophila melanogaster* Meigan (Aubiry253)	*w*Mel	AF020063
		Amitus fuscipennis MacGown & Nebeker	*w*Fus	AF071909
		Toxoptera citricida (Kirkaldy)	*w*Sus-A1	AF217713
		Bactrocera dorsalis (Hendel)	*w*DorHN1	DQ834379
		Aphidius rhopalosiphi De Stefani Perez	*w*Rho	AJ631306
	AlbA	*Aedes albopictus* (Skuse) (Houston)	*w*AlbA	AF020058
	Mors	*Glossina morsitans* Westwood	*w*Mors	AF020079
		Nasonia vitripennis Walker	*w*VitA	AF020081
	Kue	*Ephestia kuehniella* (Zeller)	*w*Kue	AF071911
		Bactrocera dorsalis (Hendel)	*w*DroGD1	DQ288282
		Trichogramma ostriniae (Pang & Chen)	*w*OstGDAa	EU157103
		Trichogramma evanescens Westwood	*w*EvaB	AY390280
		Sitobion miscanthi (Takahashi) (Beijing)	*w*MisBJA1	EU302498
		Sitobion miscanthi (Takahashi) (CangZhou)	*w*MisCZA	EU302501
		Sitobion miscanthi (Takahashi) (HengShui)	*w*MisHSA	EU302502
		Sitobion miscanthi (Takahashi) (XingTai)	*w*MisXTA	EU302503
		Sitobion miscanthi (Takahashi) (XinXiang)	*w*MisXXA	EU302504
		Sitobion miscanthi (Takahashi) (LuoYang)	*w*MisLYA	EU302505
		Sitobion miscanthi (Takahashi) (LuoHe)	*w*MisLHA	EU302506
		Sitobion miscanthi (Takahashi) (KunMing)	*w*MisKMA	EU302507
	Uni	*Muscidifurax uniraptor* Kogun & Legner	*w*Uni	AF020071
	Haw	*Drosophila sechellia* (Tsacas & Bächli)	*w*Ha	AF020073
	Pap	*Phlebotomus papatasi* (Scopoli)	*w*Pap	AF020082
	Aus	*Glossina austeni* Newst	*w*Aus	AF020077
	Eva	*Trichogramma evanescens* Westwood	*w*EvaA	AY390279
		Bactrocera dorsalis (Hendel)	*w*DroGD2	DQ288284
		Trichogramma ostriniae (Pang & Chen)	*w*OstGDAb	EU157104
		Sitobion miscanthi (Takahashi) (Beijing)	*w*MisBJA3	EU302500
	Mis	*Sitobion miscanthi* (Takahashi) (Beijing)	*w*MisBJA2	EU302499
B	Con	*Tribolium confusum* Jacquelin du Val	*w*Con	AF020083
		Laodelphax striatellus Fallen	*w*Stri	AF020080
		Cinara cedri Mimeur	*w*Ced	AY620433
		Diaphorina citri Kuwayama	*w*Cit-B1	AF217721
		Bemisia tabaci (Gennadius)	*w*Tab	AY567791
	Div	*Apoanagyrus diversicornis* (Howard)	*w*Div	AF071916
	For	*Encarsia formosa* Gahan	*w*For	AF071918
		Nilaparvata lugens (Stål)	*w*Lug	AF481181
	Ori	*Tagosodes orizicolus* (Muir)	*w*Ori	AF020085
		Eretmocerus staufferi Rose & Zolnerowick	*w*Sta	AF071919
	Pip	*Culex pipiens* Pallens	*w*Pip	AF020061
		Nephotettix nigropictus (Stål)	*w*Nig	AF481177
		Corcyra cephalonica (Stainton)	*w*Cep	AY634679
		Trichogramma ostriniae (Pang & Chen)	*w*OstGDB	EU157105
		Sitobion miscanthi (Takahashi) (BeiJing)	*w*MisBJB	EU302508
		Sitobion miscanthi (Takahashi) (CangZhou)	*w*MisCZB	EU302509
		Sitobion miscanthi (Takahashi) (JiNan)	*w*MisJNB	EU302510
		Sitobion miscanthi (Takahashi) (XinXiang)	*w*MisXXB	EU302511
		Sitobion miscanthi (Takahashi) (HengShui)	*w*MisHSB	EU302512
		Sitobion miscanthi (Takahashi) (LuoYang)	*w*MisLYB	EU302513
		Sitobion miscanthi (Takahashi) (LuoHe)	*w*MisLHB	EU302514
		Sitobion miscanthi (Takahashi) (AnQing)	*w*MisAQB	EU302515
		Sitobion miscanthi (Takahashi) (DuYun)	*w*MisDYB	EU302516
		Sitobion miscanthi (Takahashi) (KunMing)	*w*MisKMB	EU302517

ment is 43 bp (beginning breakpoint 421/ending breakpoint 464).

DISCUSSION

Distribution of *Wolbachia* in *S. miscanthi*

This survey of *Wolbachia* infections in the wheat aphid, *S. miscanthi*, in the main wheat growing areas of China detected *Wolbachia* in eleven of eighteen populations. Several kinds of *Wolbachia* infected the *S. miscanthi* populations. Of the eighteen populations examined eleven were infected, four with only one and seven with two *Wolbachia* strains. Double infections (at least two *Wolbachia* strains found in one host individual) are only recorded in some homopteran species, such as whitefly (Nirgianaki et al., 2003) and the zig-zag leafhopper (Kittayapong et al., 2003). This is the first report of a double infection in aphids.

The nature of the *Wolbachia* infections in the different populations differed. The reason for this is unknown, but several factors might have contributed. Migration in wheat aphids is universal in China, and may have affected the distribution of *Wolbachia*. The direction of migration of *S. miscanthi* in China is uncertain, so a large-scale investigation of the distribution of *Wolbachia* in this wheat aphid is needed.

The A-*Wolbachia* is rarely detected in Hemiptera but the *w*Sus-A1 strain belonging to group *Mel* is recorded from *Toxoptera citricida* (Kirkaldy) (Jeyaprakash & Hoy, 2000). However, in Chinese populations of *Trichogramma* and fruit flies, A-*Wolbachia* strains are common (Fig. 3). Most of A-*Wolbachia* strains detected in Chinese insect populations belong to group *Kue* or *Eva*. The *w*DroHN1 [*Bactrocera dorsalis* (Hendel)] strain belongs to group *Mel* along with the *w*Sus-A1 strain. A greater diversity of B-*Wolbachia* are recorded for Hemiptera (Fig. 3).

The *Wolbachia* recorded in *S. miscanthi* are very similar to those in other insects, such as trichogramma (*Trichogramma ostriniae*, *Trichogramma evanescens*), fruit fly [*Bactrocera dorsalis* (Hendel)], drosophila [*Drosophila simulans* (mauritiana)], mosquito (*Culex pipiens*), leafhopper (*Nephotettix nigropictus*) and rice moth (*Corcyra cephalonica*) (Fig. 3). These insects have no direct relationship to *S. miscanthi*, and *Wolbachia* is not recorded from plants, however, these insects should be linked with the complex food chain, so the most likely route is horizontal transmission. Another hypothesis is that *Wolbachia* was present in a distant ancestor of *S. miscanthi* and that some populations may have lost it. While this may certainly be the case for the B-*Wolbachia*, the A-*Wolbachia* appears in different groups in the phylogenetic tree indicating at least some degree of horizontal transfer.

Origins and evolution of *Wolbachia* in *S. miscanthi*

In recent years, several studies have revealed that high rates of recombination have occurred in the *wsp* gene (Baldo et al., 2005a, b; Roy & Harry, 2007; Verne et al., 2007), so using this gene for phylogenetic reconstruction could be misleading. Moreover, a Multilocus Sequence Typing (MLST) scheme exists for genotyping *Wolbachia* (Baldo et al., 2006; Baldo & Werren, 2007). MLST is an effective means of detecting diversity among strains within a single host, as well as for identifying closely related strains found in different hosts. In this study, the recombination test performed on *wsp* sequences of *Wolbachia* infecting *S. miscanthi* revealed slight intragenic recombination. However, the sequences of the *wsp* gene used in the analysis of recombination consisted of only part of the whole *wsp* gene sequence. Analysis using the whole *wsp* gene would provide more information about recombination. As recently reported, the complete genome of *w*Mel of A-supergroup encodes the necessary machinery for recombination and has experienced both extensive intragenomic homologous recombination and introduction of foreign DNA (Wu et al., 2004). The implications of recombination are clearly of great interest. It may provide a potential motor for evolutionary change and the acquisition of new mechanisms by bacteria. Intracellular symbiosis in aphids is common. The coexistence of *Wolbachia* with other symbionts in aphids (Gómez-Valero et al., 2004) is recorded. So the patterns of recombination in *Wolbachia* genomes could clarify important aspects of the evolution of this host-symbiont system (Baldo et al., 2005a).

The prevalence and distribution of the *Wolbachia* in the wheat aphid *S. miscanthi* suggest that the effect of *Wolbachia* on aphid populations merits further study.

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