Diversity and Infection Frequency of Symbiotic Bacteria in Different Populations of the Rice Brown Planthopper in China[1]

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Abstract Genetic sequences (16S rRNA) demonstrated that the symbiotic bacteria associated with different populations of the rice brown planthopper, *Nilaparvata lugens* Stål, were clustered into 18 operational taxonomic units. Molecular phylogenetic analyses revealed that the majority of these bacteria belong to *Alphaproteobacteria* (*Wolbachia*), *Gammaproteobacteria* (*Arsenophonus*, *Acinetobacter*, *Serratia*, *Arthrobacter*), and the *Bacteroidetes* (*Chryseobacterium*, *Sphingobacterium*). Bacteria of the genera *Acinetobacter*, *Serratia*, and *Arthrobacter* were present in all brown planthopper individuals analyzed. The infection frequency (IF) of *Arsenophonus* in brown planthopper reared on the rice variety 'Taichun Native1′ was higher than that observed with the 'Mudgo' and 'ASD7′ varieties. The IF of *Arsenophonus* increased by 50% when the planthoppers were reared on Taichun Native1 for >15 generations, unrelated to the origin of that population of planthoppers. The IF of *Wolbachia*, however, declined when the brown planthoppers were maintained on a single rice variety under laboratory conditions for several generations. These results suggest that the symbiotic bacteria varied with brown planthopper populations and might have an effect on the adaptation and evolution of brown planthopper on different rice varieties.

Key Words symbionts, *Nilaparvata lugens*, *Arsenophonus*, *Wolbachia*, 16S rRNA gene sequences

The brown planthopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is a major pest of rice. Rice varieties resistant to the pest have been successfully developed and widely cultivated; however, virulent strains of *N. lugens* that are capable of feeding and/or reproducing on resistant rice varieties have appeared. For example, the ASD7 population of *N. lugens* is highly virulent to the gene *bph2*-carrying rice variety 'ASD7', while the Mudgo population is highly virulent to the *Bph1*-carrying rice variety 'Mudgo' (Cruz et al. 2011, Pathak and Heinrichs 1982). The mechanisms underlying the adaptation of brown planthopper populations on different resistant rice varieties are not well known.

Symbiotic bacteria play important roles in the insect–plant interactions. A large number of symbiotic bacteria associated with herbivorous insects are commonly divided into two general categories: (a) primary symbionts, such as *Buchnera*

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aphidicola in aphids, *Carsonella ruddii* in psyllids, and *Tremblaya princeps* in mealybugs (Baumann 2005, Douglas 1998); and (b) secondary symbionts, such as *Regiella insecticola*, *Serratia symbiotica*, and *Hamiltonella defensa* in aphids (Chen et al. 2000, Clark et al. 2010, Darby et al. 2001, Fukatsu et al. 2000) and *Cardinium*, *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Enterobacter*, *Paracoccus*, and *Acinetobacter* in whiteflies (Chiel et al. 2007, Singh et al. 2012).

In general, the role of primary symbionts in their hosts is thought to be nutritional enrichment. On the other hand, secondary endosymbionts have various roles such as acting as a mutualist or a reproductive manipulator (Moran et al. 2008) or by enhancing resistance to pathogens or parasitoids, thermal tolerance, and adaptation to new host plants (Feldhaar 2011). For example, *H. defensa* and *S. symbiotica* can increase resistance to parasitoids of the genus *Aphidius* (Ferrari et al. 2004, Oliver et al. 2003). Also, *S. symbiotica* is found to improve the thermal tolerance of aphids (Allen et al. 2007, Montllor et al. 2002, Russell and Moran 2006), synthesize essential amino acids in *Acyrthosiphon pisum* and *Cinara cedri* (Lamelas et al. 2011), and potentially contribute to cellulose digestion in termites (Thayer 1978). In the stable fly, *Stomoxys calcitrans* (L.), the survival rate of larvae was generally higher when the larvae were reared on egg yolk medium inoculated with *Acinetobacter* sp. and *Flavobacterium odoratum* compared with *Escherichia coli* and *Empedobacte* (Lysyk et al. 1999). *Chryseobacterium* sp. and *Sphingobacterium* sp. were associated with cellulose or xylan digestive systems, respectively, in the termite *Reticulitermes speratus* (Kolbe) (Cho et al. 2010) and the longhorn beetle *Batocera horsfieldi* (Hope) (Zhou et al. 2009). *Regiella insecticola* is strongly correlated with its host plant range (Tsuchida et al. 2002, 2004) and its resistance to entomopathogenic fungi (Scarborough et al. 2005). In the sweetpotato whitefly, *Bemisia tabaci* (Gennadius), the association between whitefly biotypes and secondary symbionts indicated a possible contribution of these bacteria to host characteristics, such as insecticide resistance, host range, and speciation (Chiel et al. 2007, Thierry et al. 2011). It is widely accepted that bacteria greatly facilitate both the evolution and the ecological diversification of major hemipteran lineages (Baumann 2005, Moran et al. 2008).

Brown planthopper individuals harbor eukaryotic yeast-like symbionts found in the fat body cells of the abdomen (Chien et al. 1981). These yeast-like symbionts can supply its host with proteins during embryonic and postembryonic development (Lee and Hou 1987) and play a key role in recycling uric acid (Hongoh et al. 2000). The density of these yeast-like symbionts decrease when brown planthoppers are transferred from a susceptible to a resistant rice variety. The yeast-like symbionts increase in abundance in the subsequent second and third generations feeding on the resistant plant variety (Lu et al. 2004). The occurrence of these symbionts, therefore, may be related to the variation in brown planthopper performance and adaptation to rice resistance. Recently, *Wolbachia* or *Arsenophonus* were detected in all 15 brown planthopper populations collected from China and southeastern Asian countries (Qu et al. 2013). Subsequent analysis of the symbiotic bacteria from the different brown planthopper populations using denatured gradient gel electrophoresis (Xu et al. 2014) and the 16S rRNA gene sequences (Tang et al. 2010) showed that these methods might be effective means to study the structure and diversity of bacterial communities in some brown planthopper populations. However, little is known about the infection frequency (IF) and the dynamics of

Table 1. Brown planthopper populations used in this study.

Population	Rice Variety	Resistant Gene	Collection Site*	Collection Date
TN1-1995	Taichung Native1	No R gene	CNRRI field	August 1995
TN1-2009	Taichung Native1	No R gene	CNRRI field	August 2009
TN1-VN	Taichung Native1	No R gene	Shunhua, Vietnam	April 2008
TN1-PP	Taichung Native1	No R gene	Los Baños, Philippines	July 2008
Mudgo	Mudgo	*Bph1*	CNRRI field	August 1995
ASD7	ASD7	*bph2*	CNRRI field	August 1995
IR42	IR42	*bph2*	CNRRI field	August 2009
IR56	IR56	*Bph3*	CNRRI field	August 2009

* CNRRI: China National Rice Research Institute.

these bacterial communities in different populations of brown planthopper. Moreover, the associations between specific bacterial microbes and brown planthopper populations and their virulence to rice are less understood. We present herein a study of the bacterial diversity associated with different brown planthopper populations and the bacterial IF in brown planthopper populations of different generations. The relation between bacteria and brown planthopper adaptation to resistant rice varieties will also be discussed.

Materials and Methods

Insect source and rearing. Four populations of brown planthoppers were established by collecting adults from field-grown rice at the China National Rice Research Institute (CNRRI, Fuyang, Zhejiang, China). One thousand insects were transferred to each of four different varieties of rice grown in the laboratory and reared exclusively on the respective variety for at least 30 generations prior to use in this study. Each of these varieties possessed either the *bph1* gene (Mudgo), the *bhp2* gene (ASD7, IR42), or the *bph3* gene (IR56) that impart host resistance for brown planthopper. The name designating each population corresponded to the name of the rice plant variety (Table 1). An additional four populations were established from field-collected insects at CNRRI in 1995 (population TN1-1995); at CNRRI in 2009 (population TN1-2009); Shunhua, Vietnam (population TN1-VN); and Los Baños, Philippines (population TN1-PP) (Table 1). These latter four populations were reared exclusively on the TN1 rice variety. The temperature in the insectary where these populations and plants were reared was maintained at 25°C. About 100 individuals were collected and frozen at −70°C from each generation.

DNA extraction. Thirty planthoppers were randomly chosen from each of the populations TN1-1995, Mudgo, and ASD7, as well as individuals collected from field-grown rice at CNRRI. These were frozen at −70°C for 5 min, immersed in 70%

ethanol for 1 min, and then rinsed with sterile distilled water five times to remove surface microorganisms. Total DNA from each planthopper population was extracted using a DNeasy blood and tissue kit (Qiagen, The Netherlands) and used for 16S rRNA amplification and quantitative real-time polymerase chain reaction (qRT-PCR). The protocol for DNA extraction from a single brown planthopper is as follows: An insect was crushed in 150 μl of extraction buffer (1.0% SDS, 0.1 M Tris-HCl, pH 9.0, 0.05 M EDTA, 0.1 M NaCl, and 6.486% sucrose) using a grinding rod in a 1.5-mL centrifuge tube and then lysed at 56°C for 1 h, after which 28 μL of 8.0 M KAC (pH 9.0) was added and the tube was placed on ice for 30 min. Total DNA was then extracted with equal volumes of chloroform–isoamyl alcohol (24:1) and precipitated with 2 volumes of cold ethanol. The DNA extracted from a single brown planthopper was used for IF analysis.

16S rDNA amplification and endonuclease digestion. The 16S rRNA genes of bacteria associated with brown planthopper were amplified by polymerase chain reaction (PCR) with the 16S rDNA universal primers (Table 2). The PCR reaction mixture was 20 μL containing 100 ng of template DNA, 0.5 mmol/L primers, 1× PCR buffer, 0.2 mmol/L of dNTPs, and 0.125 U/μL *Taq* polymerase (Takara, Japan). The PCR program was as follows: 5 min at 95°C, 40 cycles of 45 s at 94°C, 1 min at 52°C, and 1 min at 72°C, followed by 10 min postamplification at 72°C. The template DNA was extracted, having been randomly selected from 30 brown planthoppers from the TN1-1995, Mudgo, ASD7, and CNRRI field-collected populations. Each DNA sample was repeated three times for PCR amplification to obtain sufficient PCR products for subsequent purification and cloning. Primers that are specific to the ß-actin gene (EU179846) of brown planthopper and 18S rDNA of the yeast-like symbiont were used to confirm DNA extraction. PCR products of almost full-length 16S rDNA were purified from agarose gel and ligated into TOPO TA cloning vector (Invitrogen, USA) and used to transform *E. coli*. Sixty *E. coli*–positive colonies for each brown planthopper population were subjected to an additional PCR with the standard vector primers M13, and bands of 1,500 bp were amplified. Enzymatic digestion was performed by incubating 10 μL of the amplified product with 10 U of the enzyme and 2 μL of the 10× buffer R in a total volume of 20 μL. The four endonucleases—*Mse*I, *Eco*RI, *Hind*III, and *Msp*I (MBI Fermentas, Germany)—were tested. The reaction mixture was incubated at 65°C for 3 h (*Mse*I) and at 37°C for 1 h (*Msp*I). Restriction fragments were separated on 3% agarose gels.

Sequencing and analyses of 16S rDNA. The corresponding *E. coli* clones, which were identified to have different restriction patterns with *Mse*I or *Msp*I digestion, were then sequenced. Only sequences >1,200 bp were considered for this study. The CHECK_CHIMERA program (http://rdp.cme.msu.edu) was utilized to remove chimeric sequence. The remaining sequences were blasted at the National Center for Biotechnology Information (NCBI). Operational taxonomic units (OTUs) for the sequences were estimated by the computer program DOTUR (Schloss and Handelsman 2005). The homological sequences were selected and downloaded from NCBI. Multiple sequence alignment was performed using the software Clustal X 1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/clustalx1.83.zip). Phylogenetic trees were constructed using the software mega4.1 (http://www.megasoftware.net) by the neighbor-joining method, and the robustness of the tree topology was tested by bootstrap analysis with 1,000 resamplings (Felsenstein 1985).

Table 2. Polymerase chain reaction primers used in this study.

Bacteria	Primers	References	Product Size (bp)
16S rRNA universal	F: AGAGTTTGATCCTGGCTCAG	Agron et al. 2001	1,500
	R: GGTTACCTTGTTACGACTT		
Total bacterium	F: GCAGGCCTAACACATGCAAGTC	Castillo et al. 2006	315
	R: CTGCTGCCTCCCGTAGGAGT		
Enterobacteriace	F: ATGGCTGTCGTCAGCTCGT	Castillo et al. 2006	384
	R: CCTACTTCTTTTGCAACCCACTC		
Bph-actin	F: CCCCATCGAGCACGGTATCATCA	This study	157
	R: TCTGGGTCATCTTCTCACGGTTGG		
Serratia	F: TGGTGAACTTAATACGTTCATC	This study	201
	R: CTCTAGCTTGCCAGTTTCAAAT		
Acinetobacter	F: ACTTTAAGCGAGGAGGAGGCT	Zouache et al. 2009	426
	R: GCGCCACTAAAGCCTCAAAGGCC		
Chryseobacterium	F: GTACTGAGACACGGACCA	This study	255
	R: GACCCTTTAAACCCAATA		
Arsenophonus	F: CGTTTGATGAATTCATAGTCAAA	Chiel et al. 2007	582
	R: GGTCCTCCAGTTAGTGTTACCCAAC		
Rickettsia	F: GCTCAGAACGAACGCTATC	Chiel et al. 2007	900
	R: GAAGCAAAGCATCTCTGC		

Table 2. Continued.

Bacteria	Primers	References	Product Size (bp)
Hamiltonella	F: TGAGTAAAGTCTGGGAATCTGC	Chiel et al. 2007	736
	R: AGTTCAAGACCGCAACCTC		
Flavobacterium	F: ATTGTTAAAGTTCCGGCG	Hurst et al. 1997	800
	R: CTGTTTCCAGCTTATTCGTAGTAC		
Cardinium	F: GGCTTATTAAGTCAGTTGTGAAATCCTAG	Nakamura et al. 2009	544
	R: TCCTTCCTCCCGCTTACACG		
Wolbachia	F: TTGTAGCCTGCTATGGTATAACT	O'Neill et al. 1992	896
	R: GAATAGGTATGATTTTCATGT		
Yeast-like symbiont	F: ACAAGTTATCGTTTATTTGATAGCACCTTAC	Hughes et al. 2011	469
	R: GGCTGCTGGCACCAGACTTGC		

Analysis of bacterial density in different brown planthopper populations with qRT-PCR. The density of total bacteria, *Enterobacteria*, *Chryseobacterium*, *Arsenophonus*, and yeast-like symbiont was assayed by qRT-PCR analysis of rDNA with specific primers listed in Table 2. qRT-PCR was conducted on ABI7500, and the accompanying software was used for data normalization and quantification. The PCR reaction mixture was 20 μL, which contains 100 ng of template DNA, 1× SYBR Green PCR mix (ABI), and 0.4 μM of primer. The amplification consisted of the following four steps: initial denaturation for 10 min at 95°C, 40 cycles of 20 s at 94°C, 1 min at 60°C (with single data acquisition), and followed by melting curve analysis starting from 60°C and slowly heating to 95°C with a transition rate of 0.1°C/s (with continuous data acquisition). Each population DNA has three biological replicates. Each DNA sample was extracted from 30 emergent adults (15 females, 15 males) of each population. A ß-actin gene (EU179846) in brown planthopper was used as internal control and the sample TN1 population for calibration. The data analysis was according to the 2-ΔΔCT method reported by Livak and Schmittgen (2001), where $\Delta Ct = Ct\ (target) - Ct\ (ß\text{-}actin)$, $\Delta\Delta Ct = \Delta Ct\ (Sample) - \Delta Ct\ (Calibrator)$, Relative quantity $= 2\text{-}\Delta\Delta Ct$. Thus, the calibrator becomes the 1× sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator.

Bacterial IFs in different brown planthopper populations. The bacterial IF was tested by PCR amplification of rDNA using genus-specific PCR primers (Table 2). More than 95 adults (equal number of females and males) for each population were collected and ground individually for DNA extraction. The PCR reaction mixture was 20 μL containing 2.0 μL 10× PCR buffer, 0.2 mmol/L of each dNTP, 0.5 mmol/L of each primer, 0.5 U of *Taq* DNA polymerase (Takara, Japan), and 1.0 μL of template DNA. β-actin was used as the internal control. The PCR program was as follows: 5 min at 95°C, 40 cycles of 45 s at 94°C, 30 s at 58°C, and 45 s at 72°C, and followed by 5 min postamplification at 72°C. The PCR products were separated on 2% ethidium bromide–stained agarose gel by electrophoresis.

Results

Symbiotic bacterial diversity in brown planthopper. PCR analysis of three laboratory populations (TN1-1995, ASD7, and Mudgo) and one field population from CNRRI yielded 200 positive *E. coli* clones verified by using the M13 primer and digested with four endonucleases (*Mse*I, *Eco*RI, *Msp*I, and *Hind*III). *Mse*I and *Msp*I digestion of these positive clones generated 13 and 22 restriction patterns, respectively, some of which are shown in Fig. 1. Digestion with *Hind*III indicated that no *Hind*III restriction site exists. Only two kinds of restriction patterns were obtained upon digestion with *Eco*RI.

Twenty-four sequences, ranging in length from 1,466 to 1,507 bp, were obtained and deposited in GenBank under accession numbers GU124492–GU124507 and JQ975877–JQ975885 (Table 3). The guanine-cytosine contents of those fragments were mostly from 50.2% to 56.6%, except one of 46.9%. These sequences were grouped into 18 OTUs based on 97% similarity cutoff values (Fig. 2). The symbiotic bacterial communities in the brown planthopper populations appeared to be

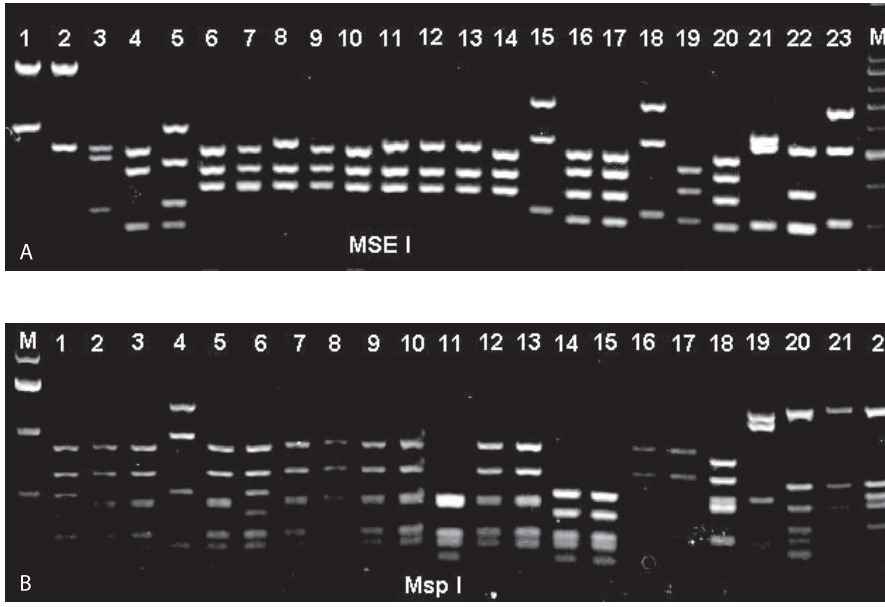

Fig. 1. Restrictive patterns of 16S rDNAs of endosymbiotic bacteria associated with brown planthopper populations. Polymerase chain reaction (PCR) products digested with (A) *Mse*I or (B) *Msp*I, with the numbers 1 to 23 indicating positive clones.

dominated by *Proteobacteria* (164 clones, more specifically, gamma- and alpha-proteobacterial) and *Bacteroidetes* (31 clones) (Table 3, Fig. 2).

In the CNRRI field population, 41 positive *E. coli* clones were digested with 35 of those possessing the same restriction patterns. One of those, W269 shares >99% sequence similarity with AY754820 (*Wolbachia* endosymbiont of the phytoseiid mite *Metaseiulus occidentalis* (Nesbitt)), indicating that *Wolbachia* is the dominant community in the brown planthopper population collected from field-grown rice at CNRRI. The TN1-1995 population had 52 of 57 *E. coli* clones exhibiting the same restriction pattern. Among those, TN68 was found to share >99% similarity with EU043378 (*Arsenophonus* endosymbiont of the psyllid *Glycaspis brimblecombei* Moore), indicating that *Arsenophonus* is the dominant bacterial community in the TN1-1995 population. In the Mudgo population, two dominant communities (clones M157 and M142) were found to share 98% similarities with *Bacteroidetes* symbionts of *Chryseobacterium* and *Sphingobacterium*, respectively. The dominant bacterial communities were found belong to five genera—*Arsenophonus* (58 of 200), *Wolbachia* (35 of 200), *Serratia* (17 of 200), *Acinetobacter* (36 of 200), and *Chryseobacterium* (27 of 200).

Bacterial density in brown planthopper populations. The qRT-PCR analysis showed that the densities of *Enterobacteriace* and *Arsenophonus* in TN1-1995 and ASD7 populations were significantly higher than those in Mudgo population (Table 4). There were no significant differences in density of *Chryseobacterium* and yeast-

Table 3. Bacteria identified in different brown planthopper populations.

OTU*	RC**	Class Affiliation	Closest Match†	PC‡			
				T	M	A	F
1	T68 GU124504	*Gammaproteobacteria*	*Arsenophonus* (EU043378)	52		2	4
2	T257 GU12450	*Gammaproteobacteria*	*Enterobacter aerogenes* (DQ857896)	1	2	3	
3	M280 GU124498	*Gammaproteobacteria*	*Serratia marcescens* (EU233275)	1	8	2	1
	M149 GU124496	*Gammaproteobacteria*	*Serratia marcescens* (DQ417332)		4		
4	M157 GU124501	*Bacteroidetes*	*Chryseobacterium* (AY278484)		20		
	M151 GU124499	*Bacteroidetes*	*Chryseobacterium* sp. (HQ895718)		5		
	M293 JQ975885	*Bacteroidetes*	*Chryseobacterium* sp. (HQ895718)		2		
5	M142 GU124507	*Bacteroidetes*	*Sphingobacterium* (EU483665)		3		
	M386 JQ975884	*Bacteroidetes*	*Sphingobacterium* (JN860316)		1		

Table 3. Continued.

OTU*	RC**	Class Affiliation	Closest Match†	PC‡			
				T	M	A	F
6	M144	*Gammaproteobacteria*	*Pseudomonas* (HQ154547)		2		
	JQ975880						
7	M400	*Betaproteobacteria*	*Acidovorax* sp. (HQ857618)		1		
	JQ975881						
8	A299	*Gammaproteobacteria*	*Acinetobacter baumannii* (FJ550344)		2	21	
	GU124494						
9	A206	*Gammaproteobacteria*	*Acinetobacter* sp. (EF031061)	1		10	
	GU124492						
	A304	*Gammaproteobacteria*	*Acinetobacter* sp. (FR677019)			2	
	JQ975879						
10	A393	*Gammaproteobacteria*	*Erwinia* sp. (GQ131594)	1	1	2	
	GU124495						
11	AH10	*Gammaproteobacteria*	*Pantoea ananatis* (DQ517335)		2		
	JQ975883						
12	A300	*Actinobacteria*	*Arthrobacter* sp. (AY940423)		1	1	
	GU124493						
	A211	*Actinobacteria*	*Arthrobacter nicotianae* (KC153125)			1	
	JQ975878						

Table 3. Continued.

OTU*	RC**	Class Affiliation	Closest Match†	PC‡ T	PC‡ M	PC‡ A	PC‡ F
13	M305 JQ975882	*Betaproteobacteria*	*Massilia timonae* (NR026014)			1	
14	A302 JQ975877	*Firmicutes*	*Staphylococcus* (AY940424)			1	
15	W269 GU124506	*Alphaproteobacteria*	*Wolbachia* (AY754820)				35
16	T274 GU124503	*Proteobacteria*	*Bacterium* (KC119316)	1			
17	M285 GU124500	*Betaproteobacteria*	*Massilia* sp. (FJ005057)		1		
18	M214 GU124497	*Gammaproteobacteria*	*Serratia marcescens* (AP013063)		1		

* Clones with the similarity above 97% were classified as the same operational taxonomic unit (OTU).
** RC: Representative Clone and NCBI number.
† Clones with the similarity above 97% with the cultured species were classified with the corresponding species and GenBank accession.
‡ PC: Different population and clone number. The total clone number TN1 (T) is 57, Mudgo (M) is 56, ASD7 (A) is 46, Field (F) is 40.

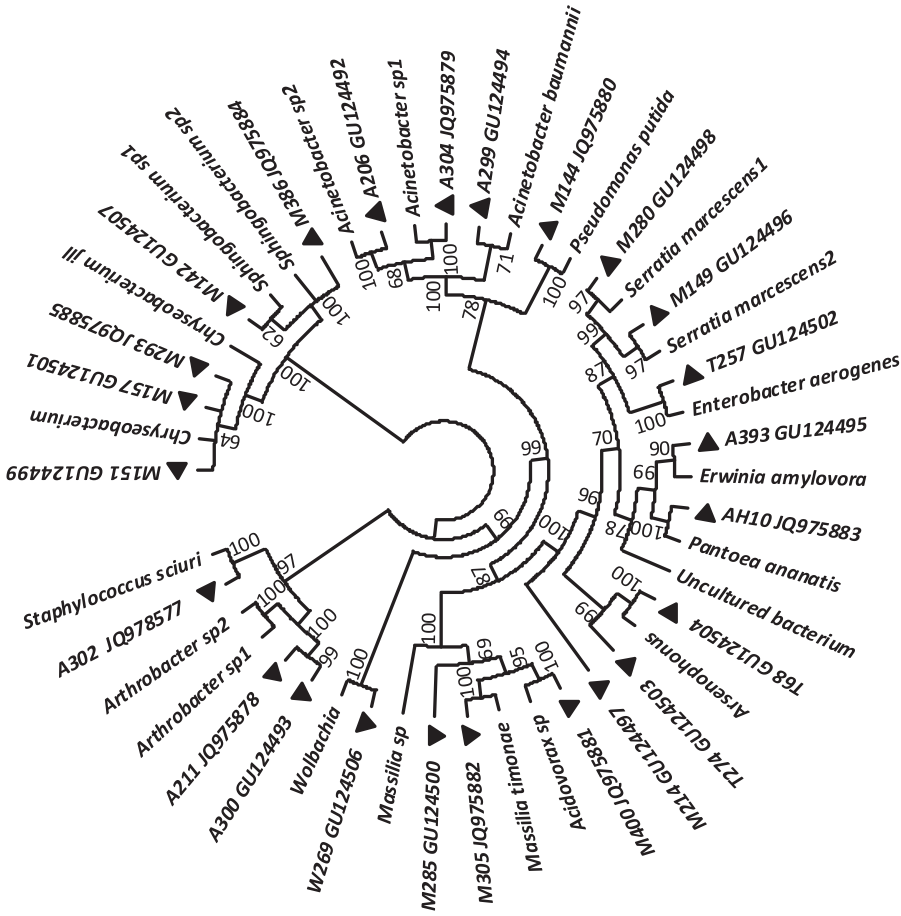

Fig. 2. Phylogenetic tree (without outgroups) of the endosymbiotic bacteria associated with brown planthopper based on 16S rRNA gene nucleotide sequences. Numbers above branches indicate parsimony percent bootstrap values after performing 1,000 bootstraps. Sequences in this study are indicated by a triangle. Other sequences were obtained from GenBank, with accession numbers in parentheses: *Erwinia amylovora* (GQ131594), *Chryseobacterium* sp. (HQ895718), *Chryseobacterium jll* (AY278484), *Sphingobacterium* sp1 (EU483665), *Sphingobacterium* sp2 (JN860316), *Arthrobacter* sp1 (EF379937), *Arthrobacter* sp2 (AY940423), *Serratia marcescens* 1 (EU233275), *Serratia marcescens* 2 (DQ417332), *Enterobacter aerogenes* (DQ857896), *Pantoea ananatis* (DQ517335), *Arsenophonus* (EU043378), *Acidovorax* sp. (HQ857618), *Acinetobacter* sp1 (FR677019), *Pseudomonas putida* (HQ164547), *Wolbachia* (AY754820), *Staphylococcus sciuri* (AY940424), *Massilia timonae* (NR026014), uncultured bacterium (KC119316), *Massilia* sp. (FJ005057), *Acinetobacter baumannii* (FJ550344), *Acinetobacter* sp2 (EF031061).

Table 4. Mean (± SD) relative abundance of selected endosymbiotic bacteria in three brown planthopper laboratory populations as tested by quantitative polymerase chain reaction.*

Lab Population	Total Bacteria	*Entero*	*Ars*	*Chry*	YLS
TN1	1.02 ± 0.73a	1.00 ± 0.50a	1.01 ± 0.21a	1.00 ± 0.47a	1.05 ± 0.21a
ASD7	1.06 ± 0.38a	1.01 ± 0.09a	0.95 ± 0.14a	1.94 ± 1.45a	0.93 ± 0.29a
Mudgo	0.09 ± 0.03b	0.09 ± 0.13b	0.00 ± 0.00b	1.83 ± 1.33a	1.01 ± 0.25a

* Means within columns followed by the same lowercase letter are not significantly different (Duncan's multiple range test; $P = 0.05$). *Entero* = *Enterobacteriace*; *Ars* = *Arsenophonus*; *Chry* = *Chryseobacterium*; YLS = yeast-like symbiont.

like symbiont among the four populations evaluated, although the average density of *Chryseobacterium* was slightly higher in the Mudgo and ASD7 populations than in the TN1-1995 population (Table 4). No *Wolbachia* was detected in the laboratory populations because it can only be cloned from the field population.

Bacterial IFs in brown planthopper populations. Genus-specific primers for nine bacterial endosymbionts (five prevalent in brown planthoppers, four from other insects) were used in PCR analysis to assess infection frequency of the bacterial symbionts in brown planthopper. Based on these analyses, *Arsenophonus* occurred at a higher frequency (57.3%) in TN1 populations (TN1-1995, TN1-2009, TN1-VN, TN1-PP) than in the Mudgo (3.18%) and ASD7 (35.7%) populations. Furthermore, the IF of *Arsenophonus* increased by 50% when the planthoppers were reared on the TN1 rice variety for >15 generations, irrespective of where and when the original population was collected. The IF of *Wolbachia*, however, was reduced by continued experience and rearing on Mudgo (G161), TN1-1995 (G152), and ASD7 (G161) (Table 5). Using Student's *t*-test, no significant difference was observed in either *Arsenophonus* or *Wolbachia* IF in response to insect gender. All, or almost all, of the planthopper individuals (total 850) tested harbored *Acinetobacter* (848), *Serratia* (800), *Chryseobacterium* (847), and yeast-like symbiont (850). *Hamiltonella defense*, *Rickettsia*, *Cardinium*, and *Flavobacterium*, previously reported from whitefly or aphid, were not detected in brown planthopper.

The IF dynamics of *Arsenophonus* and *Wolbachia* were assessed in the TN-2009, IR42, and IR56 populations to explore whether harboring *Arsenophonus* and *Wolbachia* was related to virulence to planthopper-resistant varieties of rice. Approximately 100 insects were randomly selected and assayed from generation 0, 8, 18, 24, and 30 of those laboratory-established populations. The IF of *Arsenophonus* varied with respect to different planthopper populations. In TN1-2009, the IF of *Arsenophonus* increased from 36.7% in generation 9 to 63.9% in generation 30, and then remained at 50–60% thereafter (Table 3). Similar results were seen with the *Arsenophonus* IF dynamics in the IR56 population, which increased from 29.7% in generation 8 to 83.3% in generation 30, and then remained at 80% thereafter. However, the IF of *Arsenophonus* in IR42 did not change dramatically (Fig. 3A). The IF of *Wolbachia* was 35.8% in the CNRRI field population and 5.0% in the laboratory populations (IR56, IR42, TN1-2009) tested. A decline of *Wolbachia* IF was also observed when planthoppers were maintained under laboratory conditions (Fig. 3B). Occurrence of both *Arsenophonus* and *Wolbachia* in the same individual was extremely low, with detection of both in only 5 of 1,500 insects tested.

Discussion

Our results show that the symbiotic bacteria associated with the brown planthopper populations examined in this study were predominately proteobacteria, more specifically gamma- and alpha-proteobacteria and *Bacteroidetes*. Gene sequences were grouped into 18 OTUs (Fig. 2). The OTU1, OTU9, OTU12, and OTU15 identified were similar to OTU1, OTU9, OTU15, and OTU3 reported by Tang et al. (2010), which belong to genus *Arsenophonus*, *Acinetobacter*,

Table 5. Infection frequencies (IFs) of *Arsenophonus* and *Wolbachia* in brown planthopper laboratory populations.*

	Initial IF (%)				*Ars*		*Wol*	
Population	***Ars***	***Wol***	**Generation**	***N***	**Positive**	**Percentage**	**Positive**	**Percentage**
TN1-1995	—	—	G152	200	111	55.5	0	0.0
TN1-2009	13.0	35.8	G27	100	60	60.0	3	3.0
TN1-VN	16.7	31.2	G15	96	58	60.4	10	10.4
TN1-PP	26.3	20.3	G15	96	53	55.2	5	5.2
ASD7	—	—	G161	200	71	35.5	0	0.0
Mudgo	—	—	G161	158	5	3.16	0	0.0

* *Ars* = *Arsenophonus*; *Wol* = *Wolbachia*.

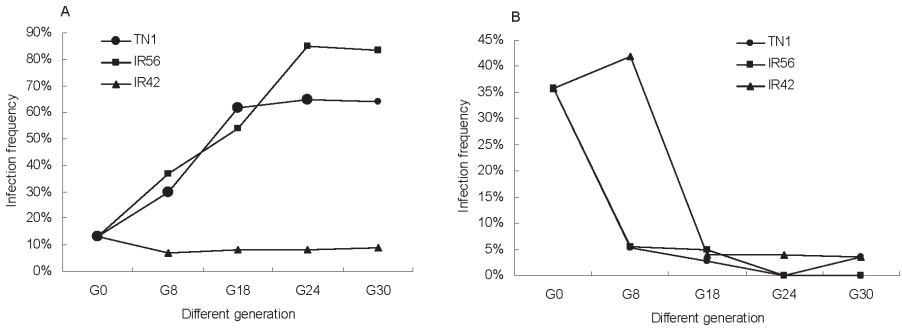

Fig. 3. Infection frequencies of (A) *Arsenophonus* and (B) *Wolbachia* in three brown planthopper laboratory populations (TN1, IR56, and IR42) reared on the respective rice varieties Taichung Native1, IR56, and IR42 for numbers of generations indicated.

Arthrobacter, and *Wolbachia*, respectively. We did not obtain OTU2, which corresponds to *Asaia krungthepensis* cloned from planthoppers on the rice variety Mudgo by Tang et al. (2010). The reason for this inconsistency is possibly due to an inadequate number of samples or contamination. In addition, different environmental conditions and nutritional supplies for brown planthopper may affect the diversity of bacteria they may harbor. The OTU4 and OTU5 were only cloned from the Mudgo population, but a diagnostic PCR-based survey confirmed that these bacteria were also present in TN1-1995 and ASD7 populations at frequencies of 98%, suggesting that the dominant bacterial communities have masked the detection of those in low abundance. Future use of high-throughput sequencing-based metagenomics approaches for individual insects are likely to provide more comprehensive information on the composition of the microbiota in brown planthopper.

The incidence of *Wolbachia*, *Arsenophonus*, and more recently *Cardinium*, has received much attention in studies of arthropod biology (Duron et al. 2008). The proportion of *Wolbachia*-infected species was estimated to be 66% (Hilgenboecker et al. 2008). The genera *Arsenophonus* and *Cardinium* are estimated to be harbored by approximately 5–6% of arthropods (Gherna et al. 1991, Zchori-Fein and Perlman 2004). These symbionts are manipulators of insect reproduction (Engelstädter and Gregory 2009), may be involved in physiological responses of hosts to external stress (e.g., host immune response to parasitoid and virus attacks) (Bian et al. 2010, Fytrou et al. 2006, Teixeira et al. 2008), and host cost related to insecticide resistance (Duron et al. 2006), phytopathogenicity (Zreik et al. 1998), or obligatory mutualism (Burke et al. 2010, Perotti et al. 2007). We did not detect *Cardinium* in this study, while *Acinetobacter*, *Serratia*, *Chryseobacterium*, *Wolbachia*, and *Arsenophonus* were the predominant symbionts detected. *Serratia*, *Acinetobacter*, and *Chryseobacterium* were harbored by almost all planthopper individuals tested in our study. In general, individuals harboring *Arsenophonus* were mostly from the IR56 or TN1 populations, while those harboring neither *Arsenophonus* nor *Wolbachia* were from the Mudgo population.

The *Wolbachia* and *Arsenophonus* IF factors differed among the brown planthopper populations examined in this study. The IF of *Arsenophonus* was higher in the TN1 and IR56 planthopper populations than in the IR42, Mudgo, or ASD7 populations (Fig. 3, Table 3). Furthermore, a decline in the IF of *Wolbachia* was observed when the planthoppers were maintained continuously under laboratory conditions. And, planthoppers from the TN1 population are virulent to nonresistant rice variety 'Taichun Native1′, while the IR56 population insects are virulent to the IR56 variety which possesses the *Bph3* resistant gene for brown planthopper. Therefore, this suggests that the maintenance of the high *Arsenophonus* IF in the TN1 and IR56 populations may not be related to brown planthopper virulence but rather to some unknown traits. Facultative symbionts with the capacity to supply host insects with essential nutrients might enable insect herbivores to feed on nutritionally poor plants. Conversely, symbionts that compete with insects for nutrients provided by the obligate symbiont might force insects to feed on plants of high nutritional quality (Clark et al. 2010). So, we proposed that the nutritional quality in rice variety TN1 and IR56 is similar, but different from rice variety Mudgo and IR42.

Our results reported herein demonstrate that not all brown planthoppers tested in this study harbored *Arsenophonus* or *Wolbachia* and, thus, we propose that these bacteria are not essential for survival of brown planthopper on rice varieties included in this study. We also provided a relatively complete profile of the bacterial communities associated with brown planthopper from these populations. While many questions remain unanswered, these bacteria may affect the host in a number of ways. These results will help to improve our understanding of the complicated symbiosis system in the brown planthopper. Further investigation is now required to document changes in bacterial profiles generated from brown planthopper populations to correlate these variations with virulence to rice varieties.

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