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Diversity of Bacterial Symbionts in Populations of *Sitobion miscanthi* (Hemiptera: Aphididae) in China

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ABSTRACT Aphids are a group of insects frequently associated with bacterial symbionts. Although Chinese aphids harbor a high level of species diversity, the associations between Chinese aphids and bacterial symbionts are less known. In this study, we uncovered the diversity of bacterial symbionts in a Chinese widespread aphid, *Sitobion miscanthi* (Takahashi). In this study, we detected the aphid obligate symbiont *Buchnera aphidicola*, and two secondary symbionts, *Hamiltonella defensa* and *Regiella insecticola*, with the diagnostic polymerase chain reaction method in *S. miscanthi* samples. In addition, symbiotic species of *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Pantoea*, and *Pseudomonas*, and the family Enterobacteriaceae were also found. Geographically, sporadic occurrences were detected for *H. defensa* and *R. insecticola*. Moreover, the infection rates of them vary widely among the infected populations: *H. defensa* (5.26–95.2%) and *R. insecticola* (5.26–46.7%). Phylogenetic analyses indicated that the strain of *B. aphidicola* mirrored the history and divergence of *S. miscanthi*; however, the *H. defensa* and *R. insecticola* strains were probably experienced horizontal transmission among *S. miscanthi* and its distantly related species.

KEY WORDS bacterial symbiont, *Hamiltonella defensa*, *Regiella insecticola*, *Sitobion miscanthi*

The symbiotic associations among insects and bacteria are common in nature, and it has been reported that ≈15% of insect species harbor intracellular bacterial symbionts (Douglas 1998, Baumann 2005). Almost all aphids (Hemiptera: Aphididae) harbor an obligate endosymbiont, *Buchnera aphidicola*, which provides essential amino acids to them (Douglas 1998). In addition, aphids harbor a wide variety of bacteria. The three main secondary symbionts, *Serratia symbiotica* (R type), *Hamiltonella defensa* (T type), and *Regiella insecticola* (U type), occur in many aphid species (Sandstrom et al. 2001, Haynes et al. 2003, Russell et al. 2003, Moran et al. 2005). *Rickettsia* is reported in the pea aphid, *Acyrthosiphon pisum* (Harris) (Chen et al. 1996). The bacterium *Wolbachia pipientis* is known from several species of aphids (Jeyaprakash and Hoy 2000, Gomez-Valero et al. 2004, Augustinos et al. 2011).

Aphid symbionts confer diverse biological roles on their hosts, and the compositions of bacterial symbionts have been proposed as another source of genetic variations in aphids (Russell et al. 2013). To date, comprehensive bacterial symbiont detections have been demonstrated in the American, European, and Japanese aphids; however, there is no such research in Chinese aphids yet. China is one of the hot spot regions in aphid species diversity, with ≈1,000 aphid species having been identified in China, which account for 25% of the worldwide records (Liu et al. 2009). Previous studies show that the compositions of bacterial symbionts are divergent among different aphid geographical populations (Tsuchida et al. 2002, Najar-Rodriguez et al. 2009). Therefore, the investigation of bacterial community in Chinese aphids can effectively expand our global views on the species diversity and geographical distribution pattern of aphid bacterial symbionts.

Sitobion miscanthi (Takahashi) is one of the most widespread aphid species in China: it can be found in most Chinese wheat planting areas (Zhang 1999). Moreover, *S. miscanthi* is a member of Macrosiphini, which frequently contains secondary symbionts (Buchner 1965, Sandstrom et al. 2001). In our previous study, a new aphid secondary symbiont named as SMLS (*Sitobion miscanthi* L type symbiont) and *Rickettsia* were detected in *S. miscanthi* populations. However, the bacterial community of *S. miscanthi* has not been fully investigated. In this study, we screened the distribution of three main aphid secondary symbionts, *S. symbiotica*, *H. defensa*, and *R. insecticola*, plus *W. pipientis* in 19 geographical *S. miscanthi* populations. Furthermore, to uncover other bacterial associations in *S. miscanthi*, the restriction fragment length polymorphism (RFLP) method was used to distinguish the 16S rRNA gene (16S) amplicons, which were amplified with universal primers, in the DNA samples of nine randomly chosen aphid geographical populations.

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Table 1. Wheat aphid, *S. miscanthi* (Hemiptera: Aphididae), samples, and their symbiont bacteria

Locality ID	Collection locality	Code	Total no. tested	*H. defensa*	*R. insecticola*	Double infection
1	Qinghai, Xining	XN	17	3	5	1[a]
2	Xinjiang, Shihezi	SHZ	15	1	7	
3	Sichuan, Jiangyou	JY	15		2	2[b]
4	Yunnan, Honghe	HH	21	20		
5	Shaanxi, Yangling	YL	22	2		
6	Shaanxi, Hanzhong	HZ	10			
7	Shaanxi, Baoji	BJ	20			
8	Henan, Zhoukou	ZK	22			
9	Henan, Luoyang	LY	19	1	1	1[c]
10	Henan, Dengzhou	DZ	20			
11	Shandong, Taian	TA	22			
12	Hebei, Baoding	BD	18	1		
13	Hebei, Shijiazhuang	SJZ	18			
14	Hubei, Danjiangkou	DJK	10			
15	Hubei, Zaoyang	ZY	21		1	
16	Jiangsu, Zhenjiang	ZJ	15			
17	Jiangsu, Yancheng	YC	15			
18	Jiangsu, Nanjing	NJ	16			
19	Anhui, Hefei	HF	27			

[a] Double infection with *Rickettsia* and *R. insecticola*.
[b] Double infection with *R. insecticola* and SMLS (*S. miscanthi* L type symbiont).
[c] Double infection with *H. defensa* and SMLS; a heterogeneous bacteria 16S rRNA gene was amplified in nine populations (SHZ, HH, ZK, DZ, BD, SJZ, ZJ, YC, and NJ).

Materials and Methods

Aphids Sampling and DNA Extraction. The aphid samples involved in this study were collected from 19 localities, which cover the Chinese main wheat farming regions (Table 1). One adult aphid was taken within an area of 10 m^2 to avoid repeatedly collecting the offspring of the same mother. The aphids were immediately immersed in 95% ethanol and maintained at −20°C until DNA extraction.

Before DNA extraction, every aphid was initially washed with 70% ethanol, and then washed several times with sterile water to remove the possible surface contaminations. Furthermore, the water for the last time washing of aphid was subjected to bacterial DNA extraction, and then amplified with bacterial 16S universal primers (16SA1 and 16SB1) for bacteria detection. A few aphids were excluded in the subsequent experiments because positive results were obtained in the bacteria detection of their last washing water, and the remaining aphids were defined as effective samples in this study.

In this study, aphid total DNA was extracted using EasyPure Genomic DNA Extraction Kit (TransGen, Beijing, China) following the manufacture's recommendations. The aphid *elongation factor-1α* gene was amplified and used as a reference to evaluate the extracted DNA quality, and the low quality DNA samples were excluded in the following bacterial symbionts detections.

Bacteria Detection and Cloning. Taxon-specific primers were used to detect the three main aphid secondary bacterial symbionts plus *W. pipientis* (Table 2). Forward primers T99F and U99F combined with the reverse primer16SB1 were used to amplify the 16S of *H. defensa* and *R. insecticola*, respectively. Reverse primers R1135R combined with the forward primer 16SA1 was used to amplify 16S of *S. symbiotica.* Primers 81F and 691R were used to amplify the *wsp*

Table 2. The primers used in this study to amplify targeted gene fragments from aphids and their bacterial symbionts

Organism	Gene	Primer name	Primer sequence (5′–3′)	References
Eubacteria	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	Fukatsu and Nikoh 1998
	16S rRNA	16SB1	TACGGYTACCTTGTTACGACTT	Fukatsu and Nikoh 1998
B. aphidicola	16S rRNA	Buch16S1F	GAGCTTGCTYTCTTTGTCGGCRA	Tsuchida et al. 2002
		Buch16S1R	CTTCTGCGGGTAACGTCACRAA	Tsuchida et al. 2002
S. symbiotica	16S rRNA	R1135R	TCCTTTGAGTTCCCGACTTT	Sandstrom et al. 2001
	gltA	RgltaF	GACCAGATCACCCATTTGCT	This study
		RgltaR	TCCAACACCGGGTTCACTAC	This study
H. defensa	16S rRNA	T99F	AGTGAGCGCAGTTTACTGAG	Sandstrom et al. 2001
R. insecticola	16S rRNA	U99F	ATCGGGGAGTAGCTTGCTAC	Sandstrom et al. 2001
	gltA	UgltaF	GACAGTAGCATTTGATGTTCTCC	This study
		UgltaR	CACCAGTGACACCACAGAGC	This study
W. pipientis	*wsp*	wsp 81F	TGGTCCAATAAGTGATGAAGAAAC	Zhou et al. 1998
		wsp 691R	AAA AATTAAACGCTACTCCA	Zhou et al. 1998
Aphid	EF-1α	EF1α-F	GAACGTGAACGTGGTATCAC	Palumbi 1996
		EF1α-R	TGACCAGGGTGGTTCAATAC	von Dohlen et al. 2002

gene of *W. pipientis.* Cycling conditions were 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min, and a final elongation for 10 min. Following polymerase chain reactions (PCRs), positive samples were defined as being those that expressed amplified DNA for a particular bacterium and had sequences of at least 97% similarity to those previously documented for the species (Wille and Hartman 2009). To detect additional bacterial symbionts, the bacterial heterogeneous 16S was amplified using 16SA1 and 16SB1 in nine randomly chosen populations (Table 1). The DNAs were extracted from three aphids for each population. Cycling conditions were the same as those used for the diagnostic PCRs, except that the annealing temperature was 50°C. The DNA of *Aphis glycines* Matsumura infected with *S. symbiotica* and *W. pipientis*, and plasmids containing 16S sequences of *H. defensa* and *R. insecticola*, were chose as templates in the positive controls. Sterile water was chosen as a template in the negative controls.

PCR products were purified using EasyPure PCR Purification Kits (TransGen, Beijing, China). Purified DNA was cloned with the pEASY-T1 vector (TransGen, Beijing, China) and transformed into the *Escherichia coli* TOP10 competent Cell (TransGen, Beijing, China).

RFLP and Sequencing. In this study, the nine clone libraries were individually performed with RFLP analysis. The products amplified from positive clones with the M13F/R (≈1.5 kb) were independently digested with three endonucleases (HaeIII, HhaI, and RsaI; New England BioLabs, Beverly, MA), and then typed the clones using 2.0% agarose gels electrophoresis. The representative clones were sequenced with the Sanger method in BioSune Company (Beijing, China).

Chimeric 16S Gene Sequence Identification and Phylogenetic Analysis. The UCHIME (Edgar et al. 2011) algorithm was used to identify the chimeric sequences, which was implemented in the Mothur software (Schloss et al. 2009). Silva alignments provided by Mothur were used as the reference. Chimeras were excluded from further analyses. The closest relatives of obtained sequences were retrieved in GenBank using BLASTn searches, and then involved into the phylogenetic inferring. Multiple sequences were aligned using the Clustal W method as implemented in MEGA 5.0 with the default parameters (Tamura et al. 2011). A Bayesian inference phylogenetic tree was constructed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The best-fit nucleotide substitution models were selected using jModelTest 0.1.1 (Guindon and Gascuel 2003, Posada 2008) based on Akaike information criterion (Akaike 1974). Two independent runs including four chains were performed with the initial 1,000,000 generations, and stopped when the average deviation of split frequencies fell well below 0.01. Every 100 generations were used to create a tree, and the initial 25% of the total trees were discarded. *Helicobacter anseris* and *Rickettsia bellii* were chosen as outgroup.

Results

Specific Bacteria Detection. In total, 343 effective aphid samples were subjected to the diagnostic PCR analysis (Table 1). The aphid obligate bacterium *B. aphidicola* was detected in all samples (data not shown). Herein, the infections of *H. defensa* and *R. insecticola* were detected; however, no infection of *W. pipientis* and *S. symbiotica* was detected. The infection rates of *H. defensa* and *R. insecticola* in all specimens were 8.16% (28 of 343) and 4.67% (16 of 343), respectively, and no significant difference was observed between them ($\chi^2 = 2.5549$; df = 1; $P = 0.11$). Geographically, both *H. defensa* and *R. insecticola* had sporadic distributional patterns; their infection rates among localities were 31.6% (6 of 19) and 26.3% (5 of 19), respectively. Furthermore, the infection rates of both bacteria among the different symbiont infected aphid populations varied as follows: *H. defensa* (5.26–95.2%) and *R. insecticola* (5.26–46.7%). And in *H. defensa*, the distinctive infection pattern of the Honghe population was revealed; there were significant differences between the infection rates of Honghe and the other populations (Fisher exact test, $P = 0.00$). However, no such differences were observed in *R. insecticola* infected populations with the Bonferroni-corrected *P* values at a significance level of 0.05. Combined with our previous research (Li et al. 2011), three types of double infections were found in several individuals (Table 1): 0.29% (1 of 343) with *Rickettsia* and *R. insecticola*, 0.58% (2 of 343) with *R. insecticola* and SMLS, and 0.29% (1 of 343) with *H. defensa* and SMLS.

RFLP Analysis and Chimera Identification. The 24–48 positive clones of each clone library, with a total of 408 positive clones were subjected to RFLP analyses, of which 39 representative clones were distinguished and sequenced. In these sequences, 17 were identified to be chimeras with UCHIME. After chimeras and sequences of known symbionts excluded, five new groups of 16S sequences were obtained from the RFLP analyses, and lumped into four operational taxonomic units (OTUs) on the basis of a priori criterion of 3% divergence (McCaig et al. 1999; Table 3). BLASTn searches showed that these OTUs represented three bacterial families: Pseudomonadaceae, Aeromonadaceae, and Enterobacteriaceae. OTU1 included two sequences with a 0.4% divergence from each other and a 99% similarity with *Pseudomonas* (Pseudomonadaceae) species. OTU2 shared a 99% similarity with the *Aeromonas* (Aeromonadaceae) species. OTU3 shared a 99% similarity with the *Enterobacter* (Enterobacteriaceae) species, and OTU4 shared a 99% similarity with species in Enterobacteriaceae. In addition, the 16S sequences from other three bacterial strains (RM1, RM2, and RM3;(RM, R type primer misamplification) were occasionally amplified in the diagnostic PCR analysis using the primers for *S. symbiotica;* the three bacterial strains shared high similarity with species of *Pantoea* and *Acinetobacter* and family Enterobacteriaceae, respectively. Moreover, the closest relatives of the OTU4, RM1, and

Table 3. Other symbionts detected in the wheat pest aphid, *S. miscanthi*

Name	Geographical distribution[a]	Most similar sequence in GenBank (accession no.)	Bacterial family	Similarity (%)
OTU1–1	YC\BD\XJ\DZ	*Pseudomonas trivialis* (GU391473)	Pseudomonadaceae	99
OTU1–2	HH\ZK\NJ	*Pseudomonas poae* (FJ937922)	Pseudomonadaceae	99
OTU2	SJZ	*Aeromonas sanarellii* (FJ230076)	Aeromonadaceae	99
OTU3	ZK	*Enterobacter* sp. (EF175731)	Enterobacteriaceae	99
OTU4	NJ	Enterobacteriaceae (EU029106)	Enterobacteriaceae	99
RM1	NJ	*Pantoea* sp. (EU780667)	Enterobacteriaceae	99
RM2	XN	*Acinetobacter* sp. (FJ805432)	Moraxellaceae	99
RM3	TA	Enterobacteriaceae (EU029105)	Enterobacteriaceae	99

[a] The abbreviation of geographical populations are in accordance with that of Table 1; OTU, operational taxonomic unit; RM, R type primer misamplification.

RM3 are isolated from the western flower thrips (*Frankliniella occidentalis* (Pergande)), the cabbage white butterfly (*Pieris rapae* L.), and the Japanese honey bee (*Apis cerana japonica* F.), respectively (Borlee et al. 2008, Chanbusarakum and Ullman 2008, Yoshiyama and Kimura 2009).

Phylogenetic Analysis. Using jModelTest, we selected the GTR substitution model with rate variation among sites (+G) in the Bayesian analysis. In general, the monophyly of Gammaproteobacteria was robustly supported (Fig. 1). In the Gammaproteobacteria clade, the bacterial symbionts were subdivided into four highly supported large branches: Moraxellaceae, Pseudomonadaceae, Aeromonadaceae, and Enterobacteriaceae. In the Enterobacteriaceae branch, the multiple strains of *B. aphidicola*, *H. defensa*, and *R. insecticola* were formed into robustly supported monophyletic groups, respectively. In the other three large branches, the symbionts detected in *S. miscanthi* all clustered with their closest relatives. Consistent with previous reports, *H. defensa* and *R. insecticola* shared sibling relationship (Sandstrom et al. 2001). Strains of *B. aphidicola* from *S. miscanthi* were very similar to those from *Sitobion avenae* (F.), to the extent of being indistinguishable, as previously reported (Choe et al. 2006). In addition, the phylogeny of the *B. aphidicola* reflected the evolutionary relationships among *A. pisum*, *Schizaphis graminum* (Rondani) and *Aphis fabae fabae* Scopoli. The strains of *H. defensa* from *S. miscanthi* showed little divergence, and they shared high similarity with the strains from *A. pisum*, *S. avenae*, and *Periphyllus bulgaricus* (Tashev). Strains of *R. insecticola* varied greatly in *S. miscanthi*. The strain of *R. insecticola* from Xining were clustered with that from *S. avenae*, and the remaining strains were highly similar to those from either *Uroleucon solidaginis* (F.) or *Macrosiphum rosae* (L.).

Discussion

In this study, using the 16S gene diagnostic PCR method, we detected aphid obligate symbiont *B. aphidicola*, and two secondary symbionts, *H. defensa* and *R. insecticola*, in the samples of *S. miscanthi*. In the detection of *W. pipientis*, nothing was yielded with the *wsp* gene primers, and we further retested this result with *W. pipientis*-specific 16S primers. In a few cases, the diagnostic PCR of *S. symbiotica* yield products; however, these products were not specifically amplified with *S. symbiotica* DNA (i.e., RM1, RM2, and RM3). In addition, the partial sequences of OUT2 and OUT4 can also be amplified using the 16S gene diagnostic primers for *R. insecticola*. These nontarget bacterial strains and target bacteria belong to the same bacterial class, and the unspecific amplifications are probably because of the highly conserved of 3′ region in the diagnostic primers. Herein, we further interactively examined the infections of *S. symbiotica* and *R. insecticola* in *S. miscanthi* with *gltA* gene specific primers, and no infection of *S. symbiotica* was found.

Combined with a previous study (Li et al. 2011), we found four secondary bacterial symbionts in Chinese *S. miscanthi*. These symbionts demonstrated divergent infection patterns among geographical aphid populations. SMLS is the most widely distributed symbiont, infecting 103 samples of 14 geographical populations (Li et al. 2011); however, *Rickettsia*, *H. defensa*, and *R. insecticola* are only sporadically detected in the samples. No significant differences were observed between the infection rates of *H. defensa* and *R. insecticola* in whole samples ($P > 0.05$), but each of them was significantly divergent with that of SMLS (SMLS vs. *H. defensa*, $\chi^2 = 35.2148$; df = 1; $P = 2.953e{-}09$; SMLS vs. *R. insecticola*, $\chi^2 = 53.3736$; df = 1; $P = 2.758e{-}13$). Moreover, in this study, significant differences were also observed among the infection rates of some *H. defensa* infected populations. To date, diverse ecological functions of secondary symbionts conferring on aphids have been uncovered, for example, they can improve the fitness of infected aphids under the stresses of heat shock, parasitoid wasps, and fungal pathogens (Montllor et al. 2002, Oliver et al. 2003, Scarborough et al. 2005). It means that local biological or nonbiological environmental selective pressures can probably influence the prevalence of secondary symbionts in aphid populations. Moreover, Russell et al. (2013) suggested that the neutral processes, such as a genetic drift, founder effects, etc., are also responsible for divergence of the secondary symbiont frequency among aphid geographical populations. Further investigations are thus necessary to uncover the underlying influence of environmental pressures and neutral processes on the prevalence of

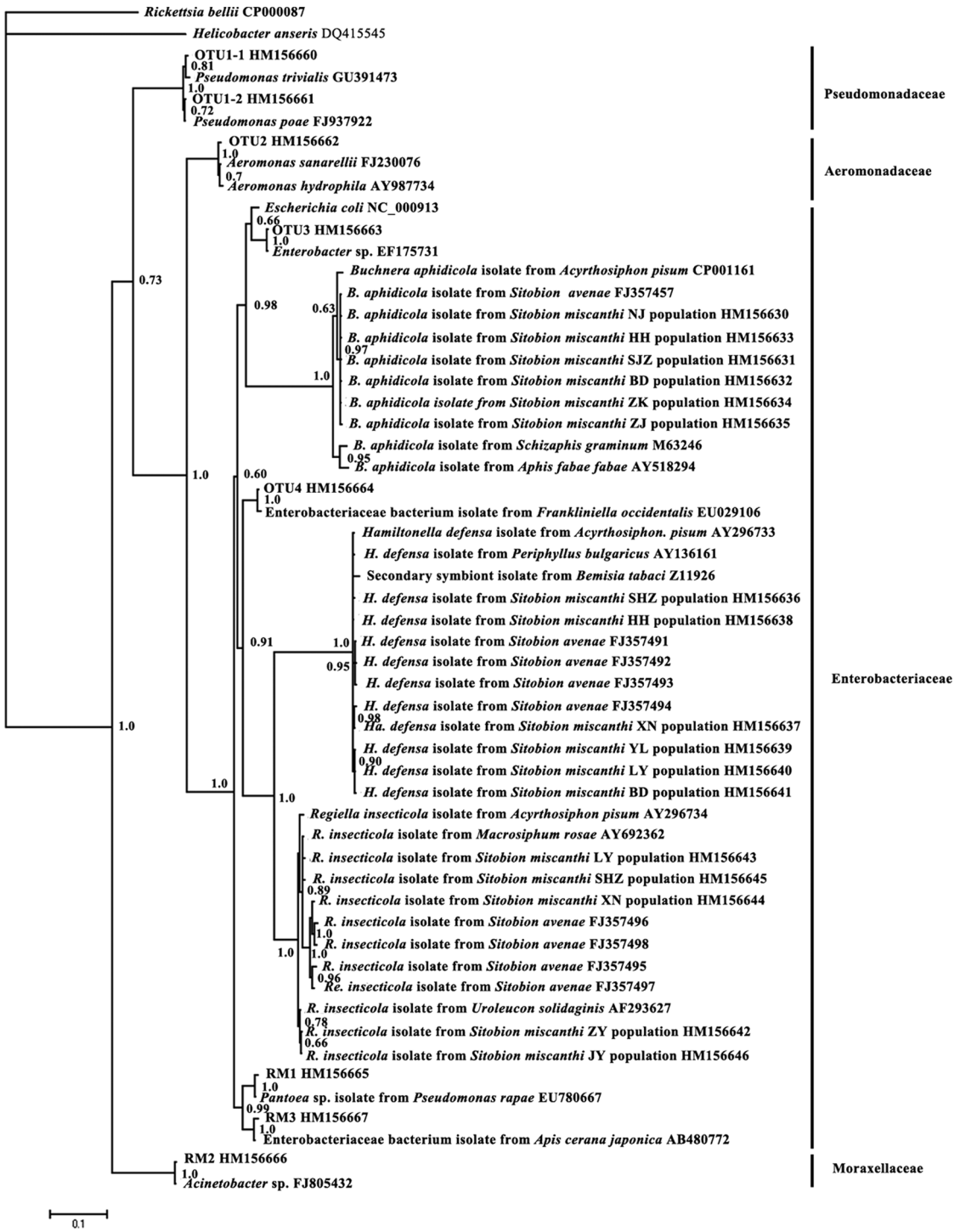

Fig. 1. A Bayesian inference tree based on 16S rRNA gene sequences depicting the phylogenetic relationships of the symbiotic bacteria of wheat pest aphids, *S. miscanthi*, in China. Numbers near interior nodes indicate Bayesian posterior probabilities. The NCBI accession numbers followed the species names. The bar indicates the estimated number of substitutions per site. *B. aphidicola* = *Buchnera aphidicola*; *H. defensa* = *Hamiltonella defensa*; and *R. insecticola* = *Regiella insecticola*.

secondary symbionts in Chinese *S. miscanthi* populations.

In addition, bacterial phylotypes of the genera *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Pantoea*, and *Pseudomonas* and the family Enterobacteriaceae were detected with RFLP analyses. Although their infection patterns are not clear, the *Pantoea* and the Enterobacteriaceae phylotypes share high similarity with those detected in the gut of other insects (Borlee et al. 2008, Yoshiyama and Kimura 2009), which imply their possible infection tropism in *S. miscanthi.* Previous studies have demonstrated that no bacteria-like organism is observed in the gut of aphids, which feed on sterile artificial food, and the bacteria inhabiting in aphid gut will be removed during aphid molt process (Grenier et al. 1994, Douglas 1998). These findings indicate that these bacterial phylotypes detected with RFLP analyses in *S. miscanthi* are probable transient or opportunistic colonizers.

Besides vertical transmission, bacterial symbionts also use horizontal transmission to persist their infections in insect populations. Recently, two routes are verified to mediate the symbiont horizontal transmission among insects. Uninfected whitefly population can acquire *Rickettsia* via plants (Caspi-Fluger et al. 2012); in aphids, *H. defensa* and *R. insecticola* can be transferred through the oviposition process of parasitoid wasps (Gehrer and Vorburger 2012). In this study, the phylogenetic analyses with bacterial 16S gene sequences show that *S. miscanthi* and its distant relatives harbor similar *H. defensa* and *R. insecticola* strains, which indicates that horizontal transfers of these bacterial strains may have occurred among different aphid species. However, further investigations with a large sample size of aphid species and more gene fragments of bacterial symbionts are necessary to retest this hypothesis.

Although a high diversity of aphid species have been found in China, their bacterial associations are still unclear. To our knowledge, this is the first comprehensive study on the bacterial symbionts survey of the Chinese aphid. Furthermore, the origin, transmission patterns, and biological roles of these *S. miscanthi* bacterial symbionts deserve investigation.

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