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Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China

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Abstract

A total of 98 non-symbiotic endophytic bacterial strains isolated from soybean root nodules were classified into eight rDNA types in ARDRA analysis and 21 BOX types in BOX-PCR. The phylogenetic analysis of 16S rDNA identified these strains as *Pantoea, Serratia, Acinetobacter*, *Bacillus*, *Agrobacterium*, and *Burkholderia.* Limited genetic diversity was revealed among these bacteria since most of the strains (85.7%) were found in three very similar rDNA types corresponding to *Pantoea agglomerans*, and many strains shared the same BOX-PCR patterns. The inoculation of nodule endophytes had no significant effects on the growth and nodulation of soybean, but most of the strains produced indoleacetic acid (IAA), could solubilize mineral phosphate, and could fix nitrogen, implying that they are a valuable pool for discovering plant growth promoting bacteria. Our results demonstrated that the nodule endophytes were common in soybean and their diversity was affected by the plant's character and the soil conditions. The 99% similarities found in the *nifH* genes of *Bradyrhizobium japonicum* and of the endophytic *Bacillus* strains strongly indicated that horizontal transfer of symbiotic genes happened between the symbiotic bacteria and the endophytes.

Keywords: Diversity; Endophyte; Root nodule; Soybean; *nifH*; Horizontal transfer; PGPM; Phylogeny; Enterobacteria; *Bacillus*

1. Introduction

The root nodules of leguminous plants are symbiotic organs induced by root nodule bacteria called rhizobia. Inside these root nodules the rhizobia are protected and can obtain carbon from the plant and can supply ammonia to the plant by fixing gaseous nitrogen. As well as the rhizobia, some non-symbiotic bacteria have also been isolated from the root nodules of a wide range of legumes (de Lajudie et al., 1999; Gao et al., 2001; Kan et al., 2007; Zakhia et al., 2006). These non-symbiotic bacteria were endophytes living inside nodules and did not cause visible damage to the plants. These nodule endophytic bacteria have been studied poorly compared with the endophytes living in other plant tissues. The most studied nodule endophytes are *Agrobacterium tumefaciens* strains (de Lajudie et al., 1999; Gao et al., 2001; Mrabet et al., 2006), while diverse bacteria, including *Bacillus* and *Pseudomonas* (Zakhia et al., 2006) and enterobacteria (Kan et al., 2007) were also isolated from nodules. It has been argued that the endophytes only coexist with symbiotic bacteria in nodules and they do not induce nodules (Wang et al., 2006b).

It has been reported that the endophytic bacteria may have two main effects. They may increase the ability of plants to absorb nutrients from the soil by increasing root development and by assisting in solubilizing phosphorus (Kuklinsky-Sobral et al., 2004). They may also control soil-borne pathogens. The inoculation of endophytic bacteria has shown a positive effect on plant growth in contaminated soil (Taghavi et al., 2005). The nodule endophytic *Agrobacterium* strains might specifically reduce the nodulation of *Rhizobium gallicum* in the common bean (Mrabet

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et al., 2006), but they did not affect nodulation of *Sinorhizobium meliloti* with alfalfa (Wang et al., 2006b). Also, the nodule endophytic bacteria might evolve into symbiotic bacteria by acquiring symbiotic genes from the rhizobia by lateral gene transfer inside the nodules, as reported in rhizobia (Trinick et al., 1989) and in endophytic bacteria (Taghavi et al., 2005). In order to understand the plant–bacteria interactions, it is essential to study the diversity of nodule endophytic bacteria and their impacts on and interactions with rhizobia and host plants.

Soybean (*Glycine max* (L.) Merrill) is one of the most important legume crops originated in China. It has been cultivated for more than 5000 years. This plant forms root nodules with different symbiotic bacteria (Chen et al., 2005). Also, diverse endophytic bacteria have been isolated from roots and stems of soybean (Kuklinsky-Sobral et al., 2004). In a survey of soybean rhizobia performed in our laboratory, many non-symbiotic bacteria were isolated from root nodules. We were interested in studying these nodule endophytic bacteria because no information was available about the nodule endophytic bacteria of soybean. The study of these non-symbiotic endophytes could offer information on the bacterial communities associated with root nodules and on the interactions among the symbiotic bacteria, endophytes, host plants, and environmental factors. The aims of our work were to analyze the diversity of endophytic bacteria isolated from root nodules of soybean and to identify the potential of these bacteria for promoting plant growth.

2. Materials and methods

2.1. Isolation of nodule endophytic bacteria

Root nodules were sampled from soybean plants grown in fields at Beian (126°31′E, 48°17′N), Yian (125°18′E, 47°54′N), Yilan (129°35′E, 46°18′N) and Muling (130°33′E, 44°56′N) of Heilongjiang province, in the northeast of China, the original center of cultivated soybean. The nodules were collected in July. Three or four nodules per plant were randomly selected for isolation of rhizobia by a standard procedure and YMA medium (Vincent, 1970) and the non-symbiotic nodule endophytes were obtained as by-products. To test the surface-sterilization process, aliquots of the sterile distilled water used in the final rinse were plated onto YMA medium and the plates were incubated at 28 °C for 4 d (Kuklinsky-Sobral et al., 2004). All strains were incubated at 28 °C. The nodule formation was checked for each isolate by inoculating soybean seedlings as described (Vincent, 1970). The nodule-forming strains were used in another study and the non-symbiotic bacteria were further characterized in this work.

2.2. ARDRA and sequencing of 16S rDNA

Total DNA was extracted from each strain with the method of Terefework et al. (2001) and was used as templates to amplify the 16S rDNA with primers fD1 and rD1 (Weisburg et al., 1991) and PCR procedure of van Berkum et al. (1996). Restriction endonucleases *Msp*I, *Hinf*I, *Alu*I, and *Hae*III recommended by Laguerre et al. (1994) were used separately to digest PCR products. The restriction fragments were separated by electrophoresis in 2.5% (w/v) agarose gels supplied with 0.5 μg ml^{-1} of ethidium bromide (Laguerre et al., 1994). The profiles were photographed under UV light and the restriction patterns of four endonucleases were combined and clustered using the method of unweighted pair grouping with mathematic average (UPGMA) in the Gelcompar II 3.5 software package. Strains sharing identical RFLP patterns were defined as an rDNA type.

The 16S rDNAs amplified with the same primers and procedures from several representative strains were sequenced directly (van Berkum et al., 1996) and the acquired sequences were compared with those of related species found in the GenBank database. All sequences were aligned using MEGA 3.1 software (Kumar et al., 2004). The phylogenetic tree was reconstructed using the Jukes–Cantor distances and the neighbor-joining method, and was bootstrapped using 1000 replicates for each sequence. The similarity criterion for operational taxonomic units (OTUs) defined by 16S rDNA sequence divergence is less than 3% (Vinuesa et al., 2005). The isolation frequency of each OTU was calculated as $F = n/N$, where n is the number of sites where an OTU was isolated; N is the total number of sampling sites. The richness of an OTU in a sampling site was expressed as $R = s/S$, where s is the number of strains in an OTU and S the total strain number obtained in the site.

2.3. BOX-PCR

In order to reveal the genetic diversity of endophytic bacteria, BOX-PCR was performed using the DNA as templates, the BOXA1R primer (5′- CTA CGG CAA GGC GAC GCT GAC G- 3′) and the procedure of Nick et al. (1999). The amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gel. The visualization and pattern analysis were the same as in ARDRA.

2.4. Effects of nodule endophytic bacteria on soybean plants

The isolate alone and the mixture of each isolate with *Bradyrhizobium japonicum* B15 (1:1) were inoculated on the surface of sterilized soybean seeds. Blank controls without inoculation and nodulation control inoculated with *B. japonicum* B15 were included for comparison. The surface sterilization, germination, inoculation, and incubation of the plants were performed as described elsewhere (Vincent, 1970). Number of nodules, fresh weight, and height of seedlings were recorded after 1 month of growth of the plants. All of these data were statistically analyzed t o estimate the effects of endophytic bacteria on the

nodulation and growth of the soybean plants with the model of Post Hoc multiple comparisons, One-Way ANOVA program in the SPSS 11.0 package. The nodule occupancy of endophytic bacteria was estimated by their isolation frequency from the surface sterilized nodules of the inoculated seedlings (Vincent, 1970). Meanwhile, the surface-sterilized roots of seedlings inoculated with the endophytic bacteria alone were ground to estimate the colony forming units (CFUs) of endophytic bacteria (Wang et al., 2006a).

2.5. IAA production assay

Indoleacetic acid (IAA) production was analyzed with the qualitative method of Glickmann and Dessaux (1995). Bacterial culture incubated in King B medium at 28 °C for 36 h was mixed with the Salkowski reagent (1:1 v/v) and incubated in darkness for 30 min. The production of IAA was recognized by the presence of red coloring.

2.6. Phosphate solubilization

We screened for inorganic phosphate solubilization in the endophytic bacteria according to Verma et al. (2001). An aliquot of 10 µl of fresh bacterial culture was spread onto TY medium supplied with 5 g l^{-1} of $Ca_3(PO_4)_2$ and was incubated at 28 °C for 2–3 d. A clear halo around the bacterial colony indicated solubilization of mineral phosphate.

2.7. Screening for nitrogen fixation

To estimate nitrogen fixation ability, a full loop of bacteria was inoculated into 50 ml of nitrogen-free liquid medium (Ashby, 1907): per liter containing mannitol, 10 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.1 g; $CaCO_3$, 5.0 g; pH 7.0–7.5 and was then incubated at 28 °C with shaking for 2–3 d. The bacteria growing in this medium were subcultured by transfer of 1 ml of the culture into another flask with the same medium. Vigorous growth after 3 cycles of subculturing demonstrated that the endophytic bacterium was a diazotroph. In addition, using the primers 34F (5′-AAA GG(C/T) GG(A/T) ATC GG(C/T) AA(A/G) TCC ACC AC-3′) and 491R (5′-TTG TT(G/C) GC(G/C) GC(A/G) TAC AT(G/C) GCC ATC AT-3′) and the procedures of Rosch et al. (2002), the *nifH* fragments were amplified and sequenced directly (van Berkum et al., 1996).

3. Results

3.1. Isolation of nodule endophytes

About 150 soybean nodules were used in the isolation of rhizobia and endophytic bacteria. A total of 98 isolates that could not induce nodules were treated as endophytic bacteria: 28 from Beian, 12 from Yian, 41 from Yilan, and 17 from Muling. In most cases, the endophytic bacteria coexisted with symbiotic *Bradyrhizobium* strains since both of them were isolated from the same nodules. No bacterial colony was observed from the aliquots of the sterile distilled water used in the final rinse of nodule surface sterilization.

3.2. Diversity of nodule endophytes

In the present study, the diversity of nodule endophytes was investigated with ARDRA, BOX-PCR, and 16S rDNA sequencing. Eight rDNA types in ARDRA were distinguished among all the 98 strains (Fig. 1) and they were divided into 21 BOX types (Fig. 2). At the similarity of 82%, the 21 BOX types were grouped into 12 clusters and 2 single isolates. The BOX clusters were basically consistent with the rDNA types, although several BOX clusters could be found within the same rDNA type (Table 1). Eight isolates representing all of the rDNA types were used in 16S rDNA sequencing. Using the criterion of Vinuesa et al. (2005), the isolates within rDNA types III (26 strains), IV (56 strains), and V (2 strains), covering 13 BOX types were identified as an OTU corresponding to *Pantoea agglomerans* by the 16S rDNA sequence analysis (Fig. 3). The remaining 14 strains in 5 rDNA types and 8 BOX types were classified into OTUs corresponding to *A. tumefaciens, Serratia plymuthica*, *Acinetobacter calcoaceticus, Bacillus pumilus*, and *Burkholderia cepacia*.

3.3. Isolation frequency and richness of the OTUs

According to the isolation frequency and richness (Table 2), *P. agglomerans* was the epidemic nodule endophyte that was isolated from all the four sampling sites and it was predominant in all the sites with richness varying between 0.529 and 1.0. *B. cepacia* was isolated from two sites and the remaining OTUs were isolated from one site only. The number of OTUs isolated from each sampling site was 4 in Muling, 3 in Yian, 2 in Beian, and 1 in Yilan.

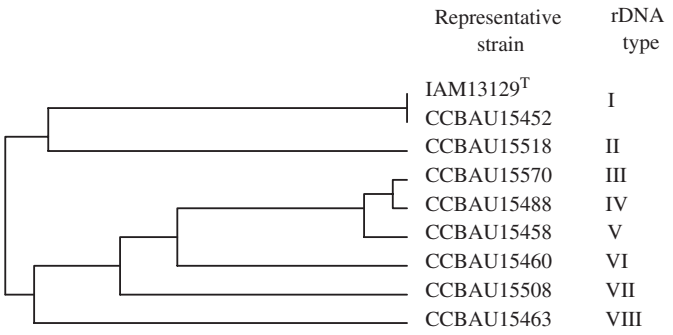

Fig. 1. Simplified UPGMA dendrogram showing the relationships among the rDNA types defined in the soybean nodule endophytic bacteria by ARDRA using 4 restriction endonucleases (*Msp*I, *Hinf*I, *Alu*I, and *Hae*III).

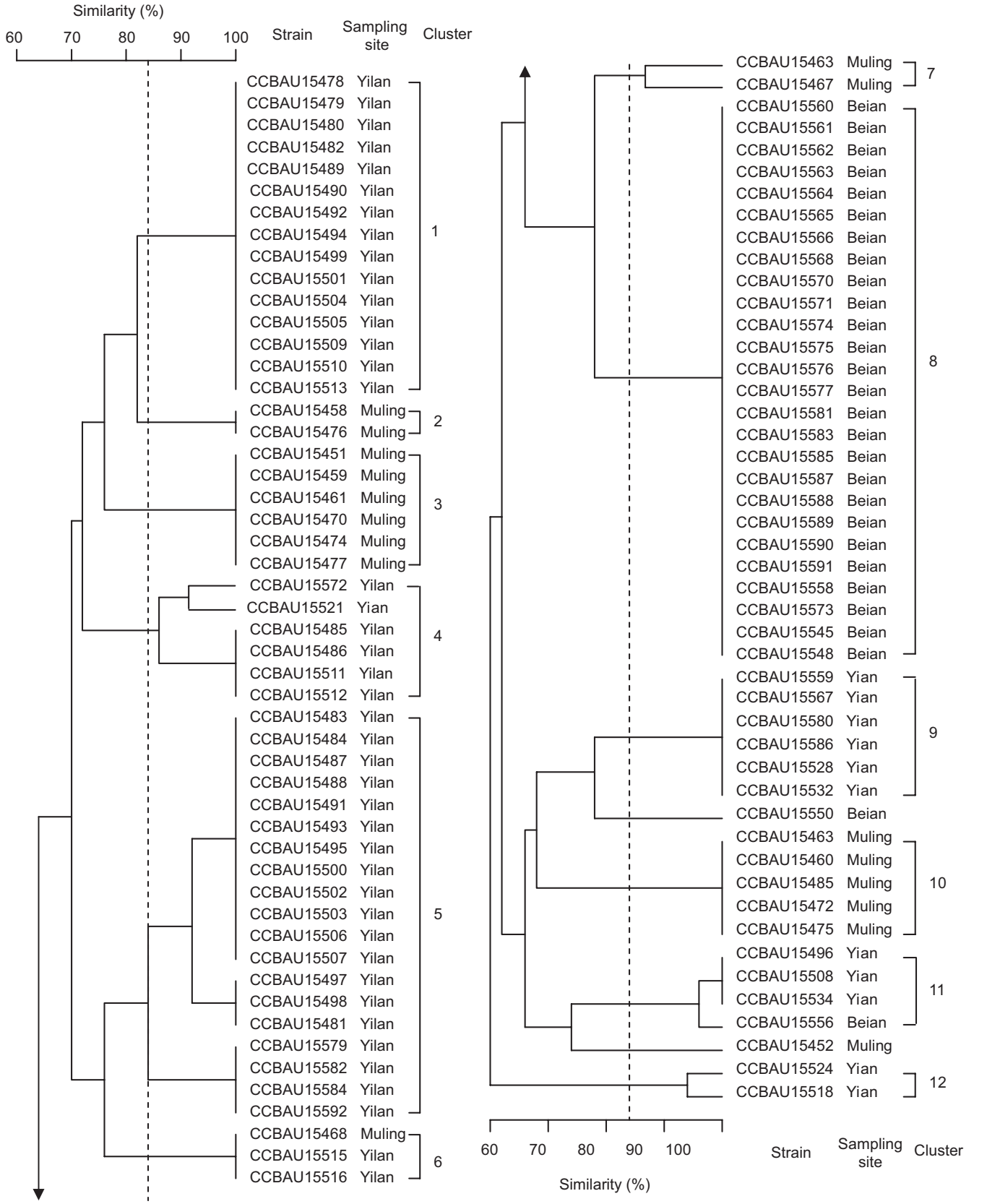

Fig. 2. UPGMA dendrogram resulting from BOX-PCR patterns showing the genomic diversity among the 98 nodule endophytic bacteria. BOX-PCR types of the isolates were indicated by the numbers in each branch.

Table 1
Nodule endophytic bacteria of soybean and reference strains used in the study

CCBAU No.[a]	ARDRA type	BOX group	BOX type	Geographic origin	Related 16S rDNA sequence
15452	I	Single	19	Muling	*Agrobacterium*
15524	II	12	20	Yian	
15518	II	12	21	Yian	*Bacillus*
15560, 15561, 15562, 15563, 15564, 15565, 15566, 15568, **15570**, 15571, 15574, 15575, 15576, 15577, 15581, 15583, 15585, 15587, 15588, 15589, 15590, 15591, 15558, 15573, 15545, 15548	III	8	13	Beian	*Pantoea*
15483, 15484, 15487, **15488**, 15491, 15493, 15495, 15500, 15502, 15503, 15506, 15507	IV	5	7	Yilan	*Pantoea*
15497, 15498, 15481	IV	5	8	Yilan	
15579, 15582, 15584, 15592	IV	5	9	Yilan	
15572	IV	4	4	Yilan	
15521	IV	4	5	Yian	
15485, 15486, 15511, 15512	IV	4	6	Yilan	
15478, 15479, 15480, 15482, 15489, 15490, 15492, 15494, 15499, 15501, 15504, 15505, 15509, 15510, 15513	IV	1	1	Yilan	
15451, 15459, 15461, 15470, 15474, 15477	IV	3	3	Muling	
15559, **15567**, 15580, 15586, 15528, 15532	IV	9	14	Yian	*Pantoea*
15550	IV	Single	15	Beian	
15468	IV	6	10	Muling	
15515, 15516	IV	6	10	Yilan	
15458, 15476	V	2	2	Muling	*Pantoea*
15453, **15460**, 15465, 15472, 15475	VI	10	16	Muling	*Serratia*
15508, 15496, 15534	VII	11	17	Yian	*Burkholderia*
15556	VII	11	18	Beian	
15463	VIII	7	11	Muling	*Acinetobacter*
15467	VIII	7	12	Muling	
Reference strain					
Agrobacterium tumefaciens IAM13129	I				

[a]Strains in boldface were used in 16S rDNA sequencing.

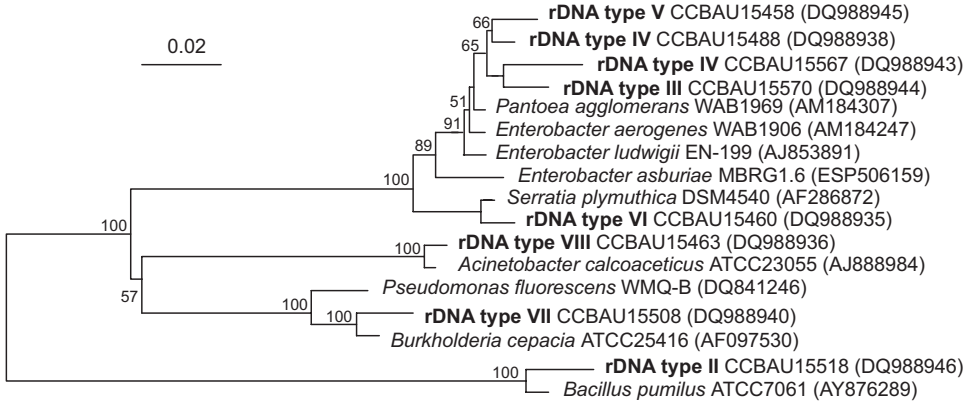

Fig. 3. Phylogenetic tree of the 16S rRNA genes showing the relationships of the endophytic bacteria associated with root nodules. The tree was constructed using Jukes–Cantor distances and neighbor-joining method. Scale bar indicates 2% substitution of nucleotide.

3.4. Inoculation tests

Fifteen representative isolates were used in this analysis: two for each of the rDNA types II–VIII and one for rDNA type I. The results of inoculation tests are presented in Table 3 in which only one isolate for each rDNA type was listed since the results were very similar for the two isolates. No nodule was formed on seedlings inoculated with endophytic bacteria alone and no significant difference was observed between the non-inoculated controls and the inoculated seedlings, demonstrating that the test isolates were not symbiotic or pathogenic bacteria. Furthermore, the nodulation and growth of plants were not different between the seedlings inoculated with *B. japonicum* B15

Table 2
Isolation frequency and richness of the nodule endophytic bacteria

Sampling site	Richness (number of strains) of OTUs						
	Agrobacterium	*Bacillus*	*Pantoea*	*Serratia*	*Burkholderia*	*Acinetobacter*	Total
Beian	0	0	0.964(27)	0	0.036(1)	0	1(28)
Muling	0.059(1)	0	0.529(9)	0.294(5)	0	0.118(2)	1(17)
Yian	0	0.167(2)	0.583(7)	0	0.250(3)	0	1(12)
Yilan	0	0	1.00(41)	0	0	0	1(41)
Frequency	0.25	0.25	1.0	0.25	0.5	0.25	

Table 3
Results of inoculation tests (data were presented as average ± standard variation)*

Strain (rDNA type)	Seedling shoot length (cm)#	Seedling fresh weight (g/plant)#	Number of nodules (per plant)#	Nodule occupation (%) by endophyte	Bacterial UFC/g (fresh root)
CK	25.2 ± 0.7a	7.0 ± 0.211a	0a	ND	0
B15	33.5 ± 1.3b	9.3 ± 0.25b	33 ± 4.5b	100	ND
CCBAU15452 (I)	25.6 ± 1.1a	7.0 ± 0.72a	0a	ND	3.83×10^3
CCBAU15518 (II)	27.2 ± 2.6a	7.3 ± 0.36a	0a	ND	4.76×10^3
CCBAU15570 (III)	26.0 ± 0.4a	6.9 ± 0.36a	0a	ND	1.12×10^4
CCBAU15488 (IV)	27.9 ± 1.7a	7.0 ± 0.75a	0a	ND	1.04×10^4
CCBAU15458 (V)	25.8 ± 0.8a	6.5 ± 0.31a	0a	ND	9.98×10^3
CCBAU15460 (VI)	28.1 ± 0.7a	7.1 ± 0.61a	0a	ND	7.82×10^3
CCBAU15508 (VII)	26.2 ± 1.6a	6.9 ± 0.73a	0a	ND	5.50×10^3
CCBAU15463 (VIII)	27.4 ± 1.9a	7.3 ± 0.55a	0a	ND	5.82×10^3
CCBAU15452 + B15	36.2 ± 0.5b	10.2 ± 0.35b	35 ± 4b	18	ND
CCBAU15518 + B15	37.2 ± 1.2b	11.0 ± 0.91b	38 ± 8b	21	ND
CCBAU15570 + B15	36.4 ± 0.7b	9.6 ± 0.66b	35 ± 4b	29	ND
CCBAU15488 + B15	34.9 ± 1.0b	9.1 ± 0.37b	35 ± 7b	ND	ND
CCBAU15458 + B15	35.8 ± 1.4b	9.7 ± 0.45b	37 ± 5b	ND	ND
CCBAU15460 + B15	36.5 ± 1.2b	9.8 ± 0.26b	34 ± 5b	23	ND
CCBAU15508 + B15	36.5 ± 2.0b	9.6 ± 0.65b	32 ± 6b	20	ND
CCBAU15463 + B15	37.1 ± 1.7b	10.3 ± 0.50b	33 ± 5b	17	ND

ND—not determinate.
*Average was estimated from three seedlings.
#The letters a and b after each number represent the results of statistical analysis. The same letter indicates no significant difference was observed ($\alpha = 0.05$).

alone and with the mixture of endophytic bacteria and B15, demonstrating that the endophytes did not affect the nodulation under the test conditions.

In the re-isolation of the bacteria from roots of seedlings inoculated with the endophyte alone, the endophytic bacteria were isolated from all of the inoculated seedlings varying from 3.83×10^3 to 1.12×10^4 CFU per gram of fresh root (Table 3). No bacteria were detected in the control seedlings (Table 3). In the double-inoculated seedlings, nodule occupancy by the endophytic bacteria was between 17% and 29% (Table 3).

3.5. IAA production

In the present work, the strains *Burkholderia* sp. CCBAU15508, *Acinetobacter* sp. CCBAU15463, *Bacillus* sp. CCBAU15518, *Serratia* sp. CCBAU15465, and *Pantoea* spp. CCBAU15488, CCBAU15570, CCBAU15486, CCBAU15567, and CCBAU15476 could produce IAA with different capacities. According to the intensity of red coloring, the strains *Pantoea* sp. CCBAU15488 and *Burkholderia* sp. CCBAU15508 produced more IAA; *Bacillus* sp. CCBAU15518 and *Serratia* sp. CCBAU15465 produced little; the remaining strains were intermediate.

3.6. Nitrogen fixation

The *Pantoea* spp. CCBAU15488, CCBAU15570, CCBAU15486, CCBAU15567, and CCBAU15476, *Serratia* sp. CCBAU15465 and *Burkholderia* sp. CCBAU15508 could grow in the Ashby N-free medium even after three cycles of subculturing. Strains *Acinetobacter* sp. CCBAU15463 and *Bacillus* sp. CCBAU15518 could not grow in that medium. However, *nifH* gene fragment with the expected size of 460 bp was amplified by PCR from *Bacillus* spp. CCBAU15518 and CCBAU15524, but not from the other strains. The sequences of *nifH* amplified from *Bacillus* spp. CCBAU15524 (EF471734) and

CCBAU15518 (EF471735) were 99% similar to that of *B. japonicum* (AJ563961).

3.7. Phosphate solubilization

As to solubilization of mineral phosphate in vitro, there was a markedly clear halo around the colony of *Serratia* sp. CCBAU15465. The *Burkholderia* sp. CCBAU15508, *Pantoea* spp. CCBAU15488, CCBAU15570, CCBAU15486, CCBAU15567, and CCBAU15476 were also phosphate-solubilizing bacteria, but with less capacity.

4. Discussion

4.1. Isolation and diversity of nodule endophytes

In the present study, the root nodules were sampled in the middle of July, when the soybean plants were at the onset of flowering. All of the sampled nodules were healthy as apparent from their red color and hard texture. So, the endophytic bacteria were not taking advantage of the decay of nodules. Nor were they soil contaminants since the nodule surface sterilization was complete, as was evidenced by the absence of bacteria in the water used in the final rinse of nodule surface sterilization. The high isolation frequency (98 isolates from 150 nodules) demonstrated that the nodule endophytes were common in soybean plants grown in the fields of Heilongjiang. The re-isolation of these bacteria from roots and nodules of the inoculated plants evidenced their endophytic properties. The number of CFU of these bacteria in roots fell in the range of endophytic bacteria (Wang et al., 2006a). Furthermore, the absence of bacteria in the control seedlings evidenced that the seeds did not contain endophytes, implying that the endophytic bacteria were exogenous and obtained from soil.

According to the criterion of Vinuesa et al. (2005), 6 OTUs were defined among the nodule endophytes based upon the 16S rDNA analyses. Among them, *A. tumefaciens* is a well known soil born phytopathogen. Previously, many *Agrobacterium* strains have been isolated from nodules of various legumes and they have been verified as non-symbiotic endophytic bacteria (de Lajudie et al., 1999; Wang et al., 2006b). However, only one Agr*obacterium* strain was isolated from the soybean nodules in the present work, indicating that *Agrobacterium* was not the predominant nodule endophyte for the soybean plants grown in the sampling sites, similar to the report on nodule endophytes in spontaneous legumes (Zakhia et al., 2006).

P. agglomerans strains were common endophytes in root nodules (Kan et al., 2007) and in other plant tissues (Asis and Adachi, 2004; Burch and Sarathchandra, 2006). Furthermore, *P. agglomerans* has been reported to be able to nodulate legumes of the genus *Hedysarum* (Benhizia et al., 2004). Therefore, the *P. agglomerans* was able to live as endophytes and microsymbionts in nodules of some legumes. *B. cepacia* was also recorded as a plant endophyte (Balandreau et al., 2001) and as a nodule occupant (Vandamme et al., 2002). The species *B. pumilus, S. plymuthica*, and *A. calcoaceticus* were never previously recorded as nodule endophytic bacteria, although some other species in the genera *Bacillus* have been found in nodules (Bai et al., 2002; Barrett and Parker, 2006).

The community composition of soybean nodule endophytic bacteria was different from that discovered from root nodules of spontaneous legumes in Tunisia (Zakhia et al., 2006). In that case, the nodule endophytes belonged to the genera *Phyllobacterium*, *Sphingomonas*, *Rhodopseudomonas*, *Pseudomonas*, *Microbacterium*, *Mycobacterium*, and *Bacillus*. Moreover, *Pseudomonas, Ralstonia*, and *Enterobacter* were found in the roots and stems of soybean plants (Kuklinsky-Sobral et al., 2004), but not in nodules as shown in the present study. The differences between nodule endophytes in Chinese soybean and in spontaneous legumes in Tunisia, and between the nodules and the roots/stems of soybean may be related to differences in the plant genotypes, local climates, soil conditions, and human activities as reported in a study of soil bacterial communities (Yannarell and Triplett, 2005) and of different endophytes (Seghers et al., 2004). The media used for isolation and the genetic background of host plants might also cause the differences.

In studies on rhizobia, almost every strain had its unique BOX-PCR pattern even when the strains were isolated from the same site (Gao et al., 2001; Seguin et al., 2001). In contrast, the present study revealed that many endophytic bacteria isolated from the same sites shared identical BOX-PCR patterns, indicating that they might be the same strain or progeny of a single clone. This limited genetic diversity in the nodule endophytic bacteria implied that the nodules might have strong selection for the genomic background of endophytic bacteria in each site, confirming that the plants specifically select endophytic bacterial genotypes (Siciliano et al., 2001).

In the present study, most of the BOX types were restricted in their isolation sites, and only the BOX type 6 was found in two sampling sites (Fig. 3). These results confirmed that the genomic backgrounds of nodule endophytic bacteria were related to the geographical origin. This phenomenon is universal, and had been found in endophytes (Dalmastri et al., 1999) and in rhizobia (Bala et al., 2003; Tlusty et al., 2005). Our data implied that the nodule endophytic bacterial community might be determined by both the geographic origins and the host plants, similar to the rhizobia (Tlusty et al., 2005).

4.2. Potential for promoting plant growth

Previously, Kuklinsky-Sobral et al. (2004) found that some endophytic bacteria of soybean had potential for promoting plant growth by the production of IAA, solubilization of mineral phosphate, and nitrogen fixation. These features were also detected in our nodule endophytes. However, no visible effects on the growth and

nodulation of seedlings were observed in the inoculation tests (Table 3). These results indicated that the nodule endophytes had potential for promoting plant growth, but their real effect on plants was unclear. More study is needed on the interactions among the endophytic bacteria, the symbiotic bacteria, and the host plants, including the impact of nodule endophytic bacteria on the nodulation, fixation of nitrogen, and plant growth. The lateral transfer of symbiotic genes from rhizobia to the nodule-endophytic bacteria is also an interesting researching item.

4.3. Analysis of *nifH* gene

Kuklinsky-Sobral et al. (2004) reported that *nifH* was not able to be amplified from some nitrogen-fixing bacteria, and the failure of amplification of *nifH* may be the result of variability of this gene (Zehr et al., 2003). Therefore, the failure of *nifH* amplification for some nitrogen-fixing bacteria in the present study was explainable. In the present study, the high similarity (99%) detected in the *nifH* genes of *Bradyrhizobium* and endophytic *Bacillus* offered strong evidence to suggest that lateral gene transfer of *nifH* might have happened between the symbiotic and endophytic bacteria because the previously reported *nifH* sequence of rhizospheric *Bacillus* (AJ968717) was closely related to those of *Paenibacillus* but quite different from those of rhizobia (Zakhia et al., 2006). In addition, the horizontal transfer of symbiotic genes from *B. japonicum* to other symbiotic bacteria has been reported (Barcellos et al., 2007), indicating that the symbiotic genes of *B. japonicum* could be transferred into companying bacteria in nodules. The failure to grow in nitrogen-free medium might indicate that the *Bacillus* strains require microaerobic conditions for fixing nitrogen, but also demonstrated the possibility that the *nif* genes of *Bacillus* sp. CCBAU15518 might function only in the symbiotic conditions, similar to those of rhizobia. The horizontal transfer of symbiotic genes from symbiotic bacteria to endophytic bacteria is ecologically important because it offers a mechanism for the emergence of new symbiotic bacteria by one-step evolution. Based upon its importance, both the horizontal transfer and the function of *nifH* in nodule endophytic *Bacillus* need to be studied further.

In conclusion, we found that the existence of non-symbiotic bacteria was common in soybean nodules; both the plants and the soil conditions were factors to analyze the genetic diversity of nodule endophytic bacteria; and the nodule endophytes served as a valuable pool for discovering plant growth promoting bacteria. The predominant nodule endophytic bacteria in soybean plants were *Pantoea*, followed by *Serratia*, *Burkholderia*, *Acinetobacter*, *Bacillus*, and *Agrobacterium* in the fields of Heilongjiang. These results offered basic information for further study on the interactions among the nodule endophytic bacteria, the nodule bacteria, the host plant, and the soil factors.

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