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Bradyrhizobium spp. and *Sinorhizobium fredii* are Predominant in Root Nodules of *Vigna angularis*, a Native Legume Crop in the Subtropical Region of China

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Adzuki bean (*Vigna angularis*) is an important legume crop native to China, but its rhizobia have not been well characterized. In the present study, a total of 60 rhizobial strains isolated from eight provinces of China were analyzed with amplified 16S rRNA gene RFLP, IGS-RFLP, and sequencing analyses of 16S rRNA, *atpD*, *recA*, and *nodC* genes. These strains were identified as genomic species within *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Ochrobactrum*. The most abundant groups were *Bradyrhizobium* species and *Sinorhizobium fredii*. Diverse *nodC* genes were found in these strains, which were mainly co-evolved with the housekeeping genes, but a possible lateral transfer of *nodC* from *Sinorhizobium* to *Rhizobium* was found. Analyses of the genomic and symbiotic gene backgrounds showed that adzuki bean shared the same rhizobial gene pool with soybean (legume native to China) and the exotic *Vigna* species. All of these data demonstrated that nodule formation is the interaction of rhizobia, host plants, and environment characters.

***Keywords*:** phylogeny, adzuki bean, rhizobia, diversity

Adzuki bean or small red bean (*Vigna angularis*) is an important legume crop in China. It was originally domesticated 3,000 years ago in China and has been transported to Japan through Korea. It can fix nitrogen through its nodule symbiosis with rhizobia, and plays an important role in sustainable agriculture. The seeds of adzuki bean are commonly used as a popular health food in Chinese cuisine and as herbal medicine.

Previously, rhizobia of adzuki bean and other *Vigna* species were classified as the cowpea miscellany (Allen and Allen, 1981), a heterogeneous group belonging to *Bradyrhizobium* (Jordan, 1982). However, both *Bradyrhizobium* spp. and *Rhizobium* spp. have been isolated from *Vigna* nodules according to the analysis of 16S rDNA phylogeny (Wolde-Meskel *et al.*, 2005; Germano *et al*., 2006; Yokoyama *et al*., 2006). Recently, 62 rhizobial strains associated with cowpea (*V. unguiculata*) and mung bean (*V. radiata*) were studied and 90% of them belonged to *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, and *B. elkanii* and the remaining were *R. leguminosarum*, *R. etli*, and *S. fredii* (Zhang *et al.*, 2008). Meanwhile, 54 indigenous mung bean rhizobia from different geographic regions of China were clustered into *B. japonicum*, *B. liaoningense*, *B. elkanii*, and a miscellaneous group of *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* (Yang *et al.*, 2008). About 7 bacterial strains isolated from adzuki bean growing in Japan were identified as *Bradyrhizobium* sp. (Fujihara *et al.*, 2002), but we have not found other reports about systematic study on adzuki bean rhizobia with molecular analyses.

Different from most of the other *Vigna* species that originated in Africa or other tropical regions, adzuki bean is originated in the temperate region of China. Considering that the association between rhizobia and their host legumes is determined by the interaction among rhizobia, legumes and environmental factors, study on diversity of adzuki bean rhizobia and comparing them with those of the other *Vigna* species might improve our knowledge of rhizobia diversity and ecology.

Materials and Methods

Nodule collection and isolation of rhizobial strains

The rhizobial strains of *Vigna angularis* were collected in fields of 8 provinces in the subtropical region of China (Table 1). To isolate rhizobia, root nodules were surface sterilized with bleach solution (1% of active chlorine), following by crushing and striking on yeast extract mannitol agar (YMA) (Vincent, 1970) supplied with 0.025% bromothymol blue as pH indicator. A total of 60 isolates were obtained (Table 1), including 20 fast-growing, acid-producing bacteria with colonies appearing on YMA plates after 3 days of incubation and 40 slow-growing, alkali-producing rhizobia whose colonies ($\leq$ 1 mm in diameter) could be seen after at least 4 days of incubation. All the isolates were maintained at 4°C on YMA slants for temporary storage and in 20% (w/v) glycerol at -70°C for long-term storage.

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Table 1. Bacterial strains used in this study, their relevant characters and sampling sites

Test strain (CCBAU number)[a]	rDNA type	IGS		Geographical origin
		Cluster	Type	
***Rhizobium fabae* 23163**	2	I	1	Anhui
***Rhizobium* sp. I 41184**	3	Single	2	Hunan
***Rhizobium* sp. II 03115**	5	Single	8	Shanxi
***Mesorhizobium* sp. I 41182**, 41332	8	V	17	Hunan
***Ochrobactrum* sp. I 61222**	7	Single	20	Sichuan
Sinorhizobium fredii				
21325,	10	II	9	Jiangsu
21330	10	II	10	Jiangsu
21323, 21324	10	II	11	Jiangsu
21327, 21328	10	II	12	Jiangsu
21099, 21108, **21111**	10	III	14	Jiangsu
21329, **21326**,	10	IV	15	Jiangsu
21119, 21088	12	II	11	Jiangsu
***Sinorhizobium* sp. I 51063**	11	Single	19	Guangdong
B. japonicum				
33044,	13	VI	21	Jiangxi
23191	13	VI	21	Anhui
33175, 33355	13	VI	22	Jiangxi
B. liaoningense				
33111, **33113**, **33114**, 33356, 33354	13	VII	23	Jiangxi
33357	14	VII	23	Jiangxi
B. yuanmingense				
43039, **43052**, 43373,	15	VIII	25	Hubei
33027, 33107, 33106	15	VIII	25	Jiangxi
33112	15	VIII	22	Jiangxi
B. elkanii				
51190, 51168, 51262	16	IX	26	Guangdong
61217, 61218, 61417, 61418, 61219, 61419,	16	IX	26	Sichuan
61220, 61223, 61420, 61421, 61422, 61423,	16	IX	26	Sichuan
61424, 61426, 61427, 61425	16	IX	26	Sichuan
33026, 33352, 33353	16	IX	27	Jiangxi
51160	17	IX	27	Guangdong
Reference strain				
R. leguminosarum USDA 2370^T	1		4	USA
R. leguminosarum 162K18			5	USA
R. etli CFN 42^T	3		3	Mexico
R. fabae CCBAU 33202^T		1	1	China
R. sullae IS 123	3		7	Spain
R. tropici B CIAT 899^T	4			Columbia
R. giardini USDA 2914^T	6			France
A. tumefaciense IMA 13129	6			Japan
S. fredii USDA 205	10	II	13	USA
S. fredii USDA 194	11		6	USA
S. fredii 2048	10	II	13	China
S. meliloti USDA 1002^T			6	USA
M. tianshanense CCBAU 3306	9		18	China
M. tianshanense 6	9		18	China
B. yuanmingense CCBAU10071^T	15	VIII	24	China
B. japonicum 6^T	13	VI	22	USA
B. liaoningense USDA 3622^T	13	VII	24	China
B. elkanii USDA 76^T	16	IX	27	USA

[a] The strains marked with boldface were used in 16S rRNA gene sequencing.

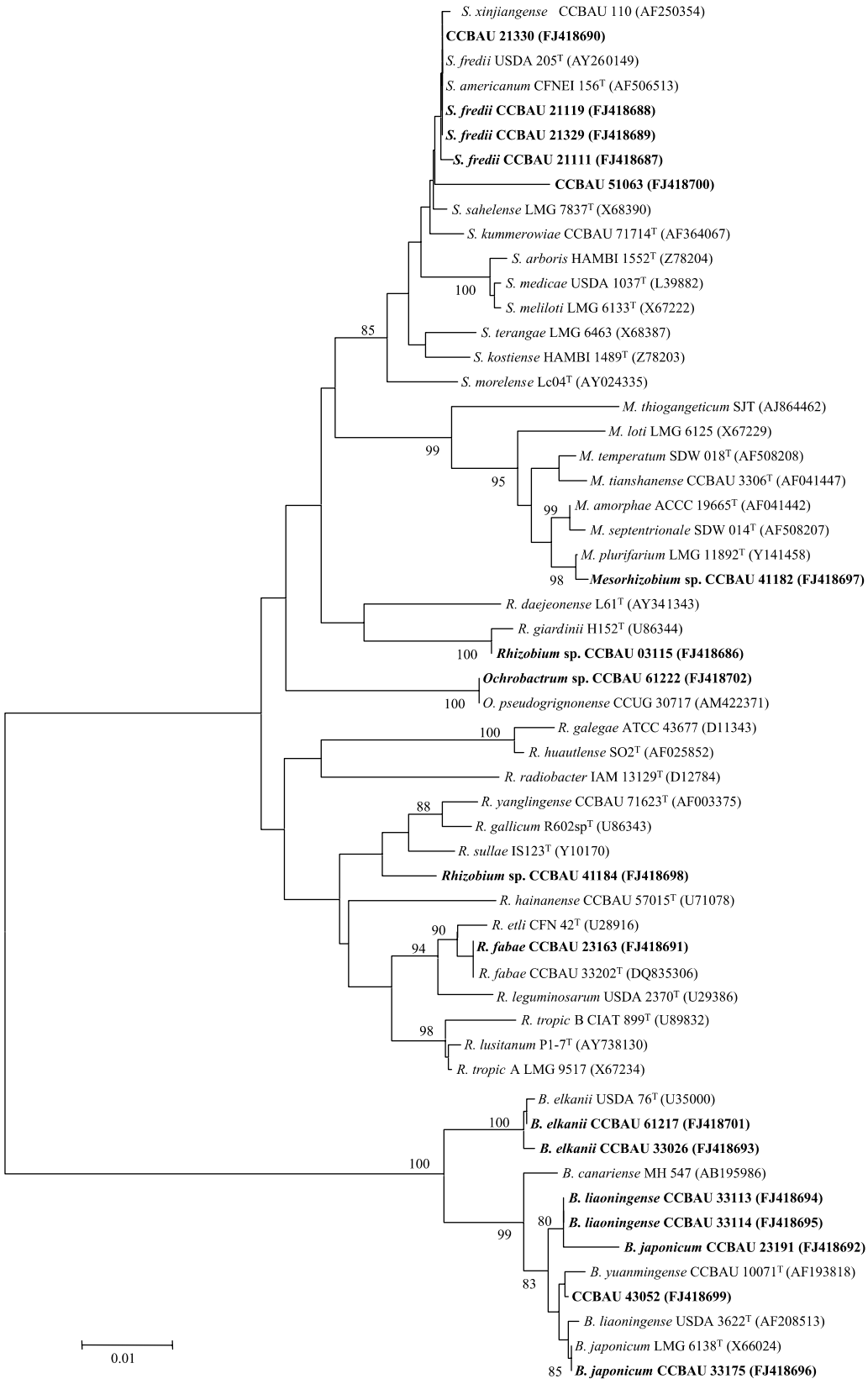

Fig. 1. Neighbour-joining tree of 16S rRNA gene sequences showing the phylogenetic relationships among the test and reference strains. Bootstrap confident levels greater than 70% are indicated at the internodes. The scale bar represents 1% substitutions per site.

Amplified 16S rDNA restriction analysis (ARDRA) and 16S rRNA gene sequence analysis

DNA was extracted from each of the 60 strains according to methods described by Terefework *et al.* (2001). The 16S rRNA genes were amplified with primers: P1; 5'-TGG CTC AGA ACG AAC GCT GGC GGC-3' and P6; 5'-CCC ACT GCT GCC TCC CGT AGG AGT-3' and the PCR procedures reported by Tan *et al.* (1997). The PCR products were digested separately with *Msp*I, *Hinf*I, *Alu*I, and *Hae*III (Laguerre *et al*., 1994). The restriction fragment length polymorphism (RFLP) of 16S rDNA was analysed by electrophoresis of the restriction PCR products in 3% (w/v) agarose gels containing ethidium bromide (0.5 μg/ml) and photographed under UV light. Strains that shared the same RFLP patterns were designated as an rDNA type. The rDNA restriction patterns were used to calculate similarity and a dendrogram was constructed using the UPGMA method in the Gelcompar II software package (Vauterin and Vauterin, 1992). With the same primers and procedures used for ARDRA, the 16S rDNA of the representative strains for different rDNA types was amplified and directly sequenced as described (Hurek *et al.*, 1997). The acquired sequences, together with the related sequences obtained from the GenBank database by Blast program were aligned using MEGA 4.0.1 software (Tamura *et al.*, 2007). Phylogenetic tree was reconstructed using the Jukes-Cantor distances and neighbor-joining method, and was bootstrapped based on 1,000 replicates.

PCR-based RFLP of ribosomal intergenic spacer (IGS)

To further characterize the isolates, a phylogenetic analysis was carried out on the ribosomal IGS region. The IGS fragments were amplified with the primers: FGPS6; 5'-GGA GAG TTA GAT CTT GGC TCA-3' and 23S-38; 5'-CCG GGT TTC CCC ATT CGG-3' and the PCR procedures of Rasolomampianina *et al.* (2005). The IGS PCR products were digested respectively with the restriction endonucleases *Msp*I, *Cfo*I, and *Hae*III. The restriction fragments were separated, visualized and analyzed by the same methods as in ARDRA.

Phylogenetic analyses of symbiotic and housekeeping genes

The *nodC* genes were amplified using the forward primer nodCfor540; 5'-TGA TYG AYA TGG ART AYT GGC T-3' and reverse primer nodCrev1160; 5'-CGY GAC ARC CAR TCG CTR TTG-3' and the PCR protocol of Sarita *et al.* (2005). Partial housekeeping genes *atpD* and *recA* were amplified, respectively, using primer pairs: *atpD*255F; 5'-GCT SGG CCG CAT CMT SAA CGT C-3' /*atpD*782R; 5'-GCC GAC ACT TCM GAA CCN GCC TG-3' and *recA*41F; 5'-TTC GGC AAG GGM TCG RTS ATG-3' /*recA*640R; 5'-ACA TSA CRC CGA TCT TCA TGC-3' and the PCR protocol reported previously (Vinuesa *et al.*, 2005). The PCR products were purified and directly sequenced as in 16S rRNA gene sequencing. Phylogenetic trees were also constructed as described in 16S rRNA gene sequence analysis.

Confirmation of nodulation ability

After all the analyses, the nitrogen-fixing ability of strains representing different rhizobial groups was verified by inoculate surface-sterilized and pre-germinated seeds of *V. angularis* by using a standard method (Vincent, 1970). The inoculated seedlings were grown in vermiculite watered with N-free plant nutrient solution under natural sunlight and room temperature in May. After 1 month of growth, the nodulation was recorded by the existence of nodules and the efficiency was estimated by the presence of red color (leghemoglobin) inside the nodules (Vincent, 1970). The nodulation tests were performed in triplicate and non-inoculated seedlings were included as blank controls.

Results

ARDRA and 16S rRNA sequence analysis

In ARDRA, a total of 17 rDNA types were distinguished among all the test strains and the isolates were found in 13 rDNA types (Table 1). All strains were grouped into four clusters corresponding to *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Supplementary Fig. 1). Isolates CCBAU 23163 (rDNA type 1), CCBAU 41184 (rDNA type 3), and CCBAU 03115 (rDNA type 5) were found in *Rhizobium* cluster. Two isolates CCBAU 41182 and CCBAU 41332 of rDNA type 8 were grouped with *Mesorhizobium* species. CCBAU 61222 (rDNA type 7) was ungrouped. The isolates of rDNA type10, 11, and 12 were distributed in *Sinorhizobium* cluster. The remaining forty isolates belonging to rDNA types 13 through 17 were grouped in *Bradyrhizobium* cluster. The 16S rDNA phylogenetic relationships (Fig. 1) revealed by the sequencing data were well consistent to those estimated from ARDRA. Most of the *Sinorhizobium* isolates showed close relationships with *S. fredii* strains, except CCBAU 51063 which was a divergent lineage within *Sinorhizobium* group. The *Mesorhizobium* isolates were similar to *M. plurifarium*. The *Rhizobium* isolates were respectively found to be similar to *R. giardinii* and *R. fabae* or formed a single lineage in the genus. The ungrouped isolate CCBAU 61222 was identified as *Ochrobactrum* sp. The 40 Bradyrhizobium isolates were divided into two groups respectively similar to *B. elkanii* and *B. japonicum*.

RFLP of ribosomal intergenic spacer (IGS)

The IGS-RFLP analysis revealed greater genetic diversity than ARDRA. In the cluster analysis (Fig. 2), the fast-growing and slow-growing strains were separated clearly. Considering the grouping of the references strains and the groupings in ARDRA, we used different similarity levels to separate the species of fast-growing and slow-growing strains: 85.5% for fast-growing rhizobia and 91% for slow-growing rhizobia. Eighteen IGS types were defined in the 60 test strains and they were grouped in 9 IGS clusters together with some reference strains (Table 1 and Fig. 2). The *Bradyrhizobium* strains were divided into four groups corresponding to *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, and *B. elkanii*. In general, *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* strains were clustered according to their genus. Among the *Rhizobium* isolates, CCBAU 23163 was identical with *R. fabae* reference strain; CCBAU 41184 was a single isolate similar to the *R. fabae*; while CCBAU 03115 was a single isolate similar to *R. sullae*. The *Sinorhizobium* isolates were divided into three clusters and they shared 80% or more similarity

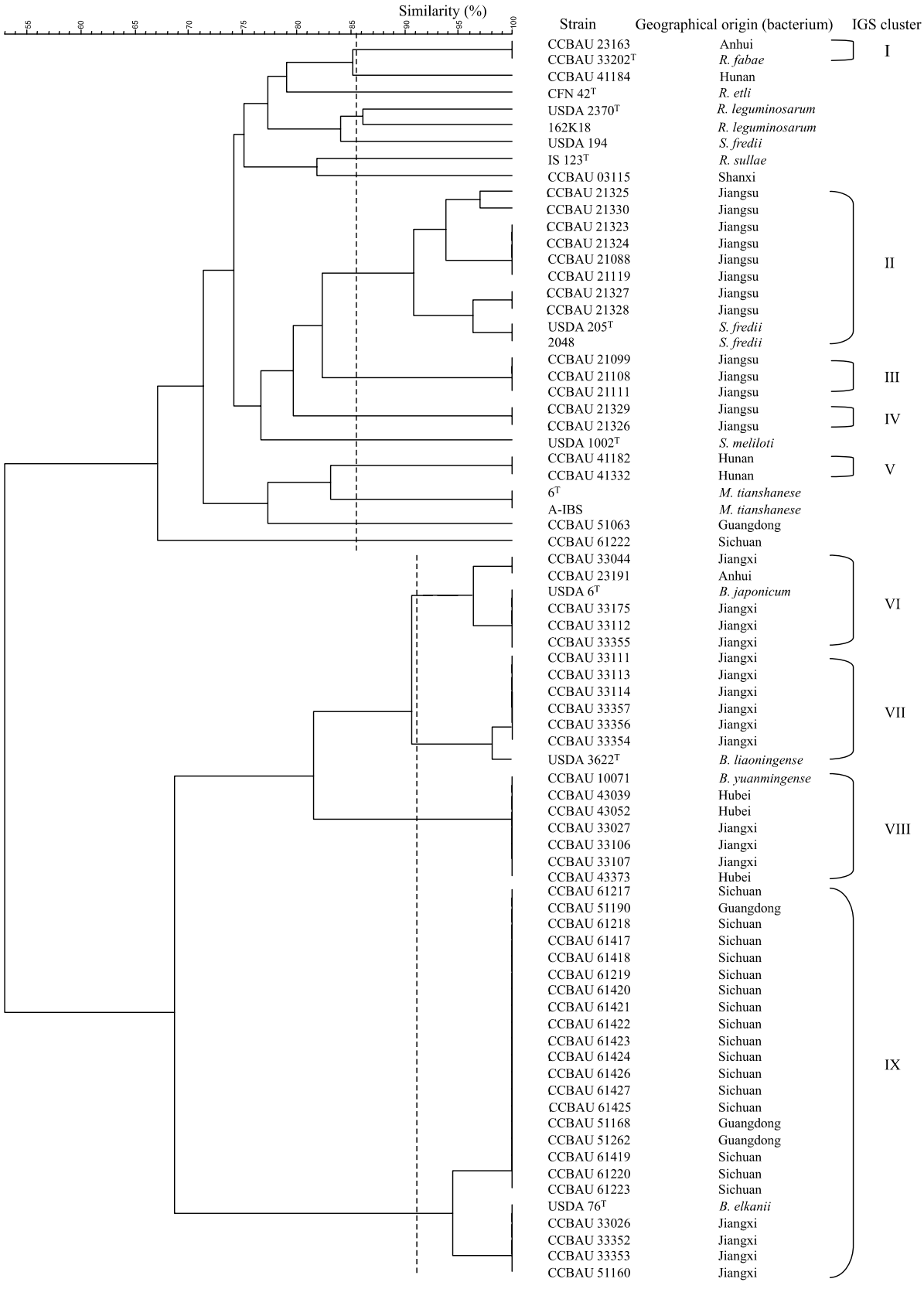

Fig. 2. Simplified dendrogram showing the genetic diversity of *Vigna angularis* rhizobia revealed by ribosomal IGS PCR-RFLP. The Dice coefficient and UPGMA method were used for cluster analysis.

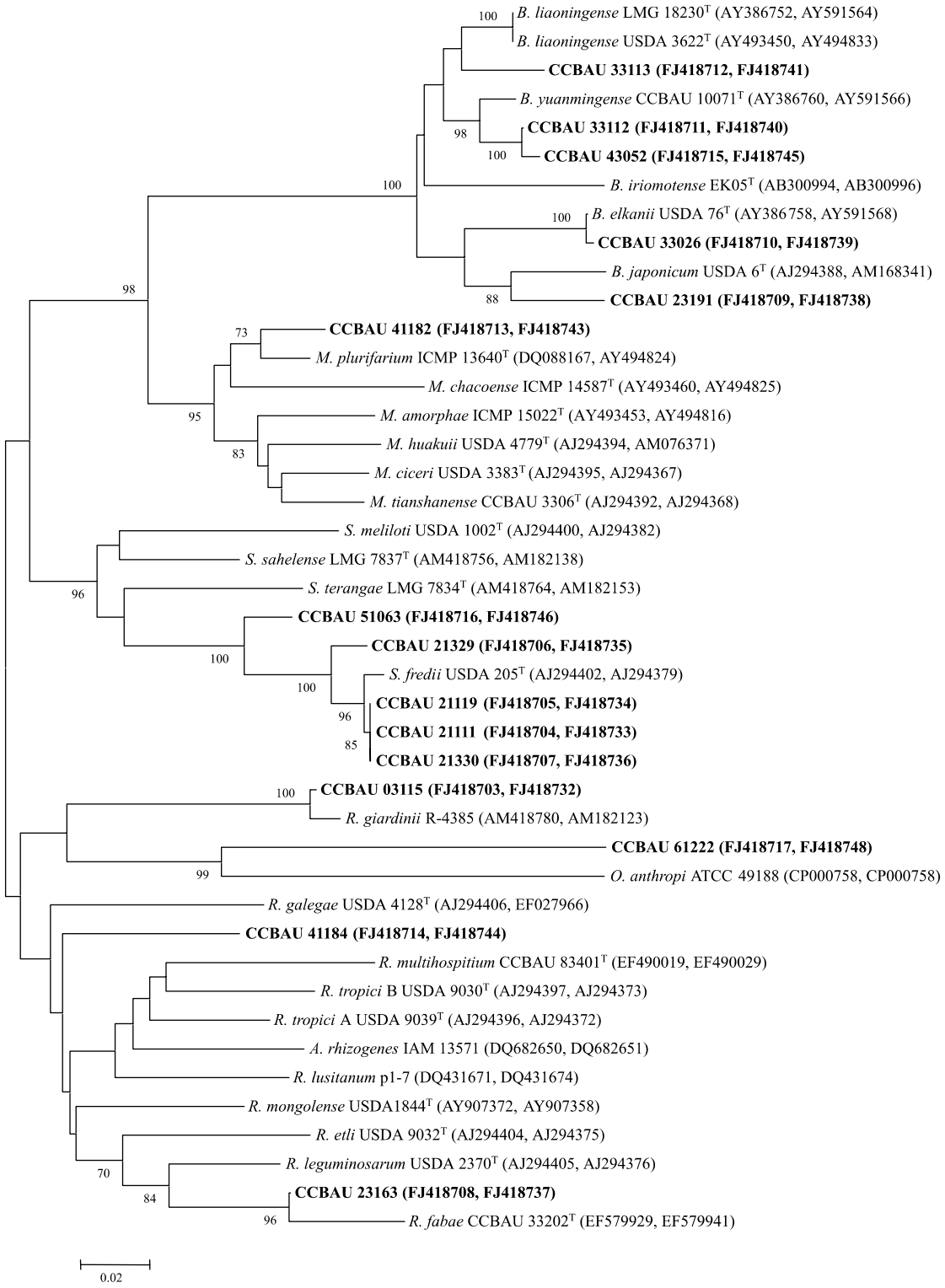

Fig. 3. Neighbour-joining tree of *atpD-recA* gene sequences showing the phylogenetic relationships of the adzuki bean rhizobia (marked with boldface letters). Bootstrap confident levels greater than 70% are indicated at the internodes. The scale bar represents 2% nucleotide substitutions per site. The accession numbers in the parenthesis were *atpD* and *recA*.

with the reference strains for *S. fredii*. Two *Mesorhizobium* isolates were IGS type 17 and were similar to *M. tianshanense*. The *Sinorhizobium* isolate CCBAU 51063 was an exception that was distantly related to the *Mesorhizobium* strains in IGS-RFLP dendrogram.

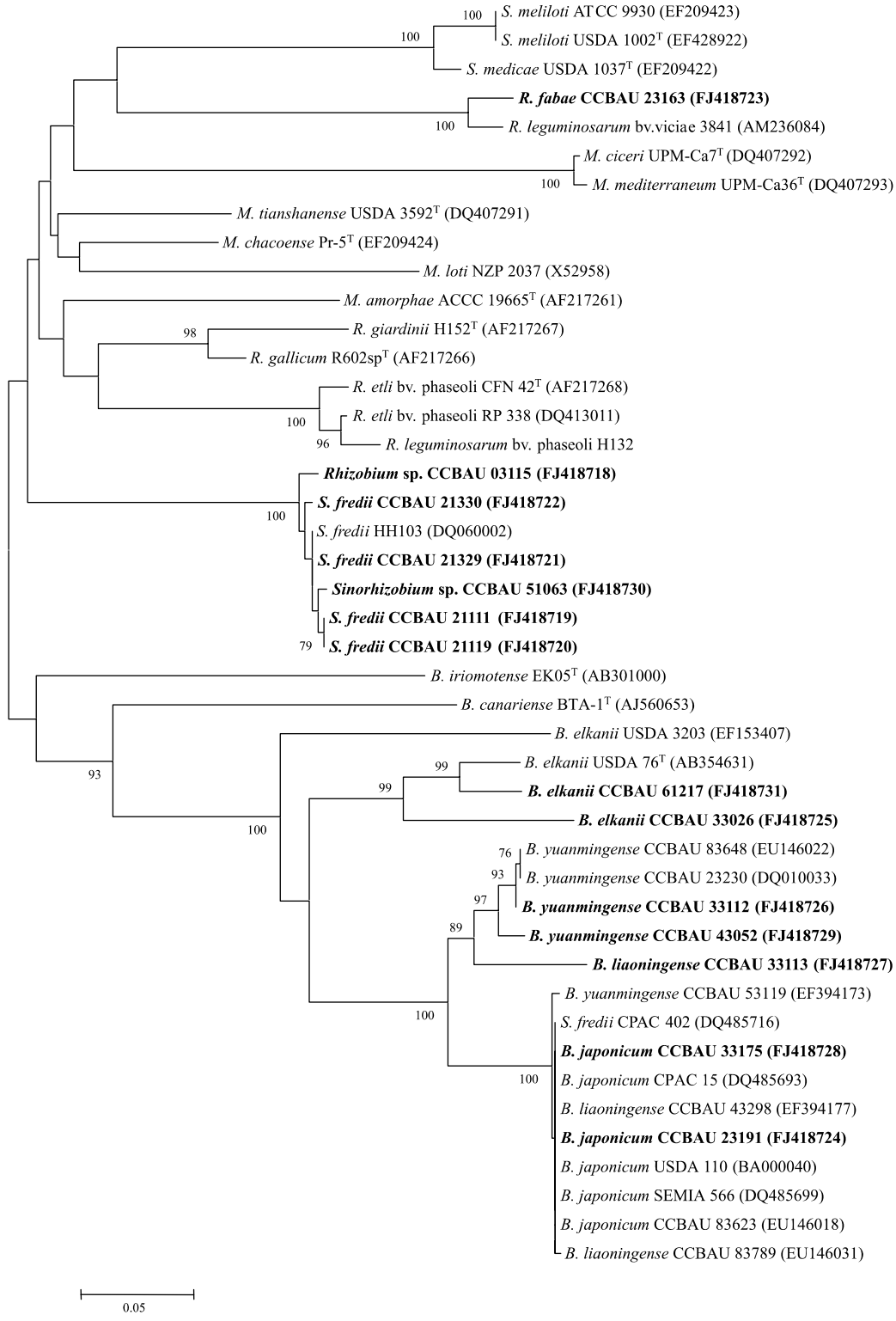

Fig. 4. Neighbour-joining tree of *nodC* gene sequences showing the phylogenetic relationships among the test and reference strains. Bootstrap confident levels greater than 70% are indicated at the internodes. The scale bar represents 5% substitutions per site.

Phylogenetic analyses of *atpD* and *recA* genes

In order to verify the grouping results of ARDRA and IGS-RFLP, representative strains were used in sequencing of *atpD* and *recA* genes. The phylograms obtained from the two housekeeping genes (Fig. 3, Supplementary Fig. 2) showed similar topologies. Only the *atpD-recA* phylogram

was presented (Fig. 3), in which the strains were respectively grouped into genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Ochrobactrum* and consistent with the result of 16S rDNA phylogeny. The *Bradyrhizobium* strains were divided into four groups corresponding to *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, and *B. elkanii*.

Phylogenetic analyses of *nodC* genes and nodulation ability

The *nodC* gene encoding N-acetylglucosaminyltransferase, one of the key enzymes for synthesis of Nod-factor, was chosen to investigate the symbiotic relationships of the rhizobia associated with *Vigna angularis*. The *nodC* genes of 14 representative strains for the rhizobial groups defined with the results of ARDRA, IGS-RFLP, and housekeeping gene sequencing were amplified and sequenced. It was clear that six *nodC* groups were defined among the representative strains of *V. angularis* rhizobia, corresponding to *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, *B. elkanii*, *S. fredii*, and *R. leguminosarum*, in the phylogeny of *nodC* gene (Fig. 4), that was consistent with those of housekeeping genes, except *Rhizobium* sp. CCBAU 03115 which might have acquired *nodC* gene by horizontal gene transfer from *S. fredii*.

In the nodulation tests, no nodule was found on the plants of blank control. All the *Sinorhizobium* and *Bradyrhizobium* representative isolates formed nodules on the host. Among the *Rhizobium* isolates, *R. fabae* CCBAU 23163 and *R. giardinii* CCBAU 03115 formed effective nodules that were evidenced by the pink colour of nodules and green leaves of the host plants, while the *Rhizobium* sp. CCBAU 41184, *Ochrobactrum* sp. CCBAU 61222 and *Mesorhizobium* CCBAU 41182 and CCBAU 41332 occasionally formed ineffective nodules that were evidenced by the white colour of nodules and yellow-green leaves of the host plants or did not form any nodules on adzuki bean.

Discussion

Taxonomically, adzuki bean was named as *Phaseolus angularis* and its classification as *Vigna* species was supported by the phylogenetic data of ITS region of the 18S-26S nuclear ribosomal DNA repeat (Goel *et al*., 2002). Different from the other *Vigna* species that originated in the tropical region of Africa (Allen and Allen, 1981), adzuki bean is native to China. After its domestication 3000 years ago, it was only cultivated in China and in several countries surrounding China. The rhizobia associated with this plant have not been systematically studied using various phylogenetic approaches.

In the present study, the 60 rhizobial strains isolated from root nodules of *Vigna angularis* grown in fields of subtropical region could be defined as six genomic species and six ungrouped strains within *Rhizobium* (3 isolates), *Sinorhizobium* (14 isolates), *Mesorhizobium* (2 isolates), *Bradyrhizobium* (40 isolates), and *Ochrobactrum* (only one) (Table 1), with *S. fredii* (14 isolates), *B. japonicum* (4 isolates), *B. liaoningense* (6 isolates), *B. yuanmingense* (7 isolates), and *B. elkanii* (23 isolates) as the main and effective ones. The species definition was mainly based upon the IGS-RFLP and sequencing analysis of housekeeping genes since they have been proofed valuable for differentiating rhizobia species, especially the *Bradyrhizobium* species (Man *et al*., 2008; Vinuesa *et al*., 2008). According to the results of the present study, the main microsymbionts of adzuki were *Bradyrhizobium*, which supported its classification as a *Vigna* species, because *Phaseolus* species are mainly nodulated with *Rhizobium* species.

The isolation of symbiotic or non-symbiotic *Rhizobium*, *Mesorhizobium* and *Ochrobactrum* strains from the root nodules of *Vigna angularis* demonstrated that the root nodules of adzuki bean could be occupied occasionally by non-specific rhizobia or by endophytes, like the *Agrobacterium* strains in nodules (Wang *et al.*, 2006) and the endophytic rhizobia in roots of maize (Gutiérrez-Zamora and Martínez-Romero, 2001) and rice (Singh *et al.*, 2006). One of the impacts of the non-specific occupation is that it offers potential to develop new symbiotic bacteria by lateral gene transfer from the symbiotic bacteria to the endophytes, as the case of *Rhizobium* sp. CCBAU 03315 that harbored a *nodC* of *S. fredii* and formed effective nodules with adzuki bean.

The composition of rhizobial community associated with adzuki bean in subtropical region of China was similar to that of soybean rhizobia in the same region (Yang *et al*., 2006; Man *et al*., 2008), and to that of *Vigna* species in tropical Asia (Yokoyama *et al.,* 2006), except the *S. fredii* strains that were isolated from adzuki bean in China but not from the *Vigna* species grown in tropical Asia (Yokoyama *et al*., 2006). It was clear that most of the soybean rhizobia and the *Vigna* rhizobia had the same origin or were from the same gene pool in China, based upon their housekeeping gene phylogeny and the *nodC* gene phylogeny. These results and the close phylogenetic relationships between the genera *Glycine* and *Vigna* (Chappill, 1995) demonstrated that the rhizobia from each plant could be useful genetic resource to improve the symbiotic system of plants in both genera.

In general, the *nodC* phylogeny was consistent to that of the housekeeping genes in the adzuki bean rhizobia, but some exceptions were observed. Firstly, the strain *Rhizobium* sp. 03115 had the *nodC* gene almost identical with those of *S. fredii* strains, demonstrating a case of lateral gene transfer from *Sinorhizobium* to *Rhizobium*. Secondly, the *B. liaoningense* strains originated from adzuki bean had *nodC* different from those of *B. japonicum* originated from soybean and adzuki bean, that was different from the case of soybean *B. liaoningense* that had symbiotic genes identical or very similar to those of soybean *B. japonicum* (Man *et al*., 2008). Thirdly, the *nodC* gene of *B. elkanii* strains originated from adzuki bean was different from those of soybean *B. elkanii* strains. These results demonstrated that the *Vigna*-associating *B. elkanii* and *B. liaoningense* strains might have acquired the *nodC* genes from different resources, or their *nodC* genes have divergently evolved to adapt to different hosts. This case was similar to the results reported by Yokoyama *et al*. (2006) because they found that the *Vigna* bradyrhizobia isolated from Thailand formed clades different from those isolated from soybean grown in temperate regions in the *nodC* phylogenetic tree, therefore they estimated that some genetic differentiation has evolved in their host range.

A large body of research supports the idea that nodule formation is the interaction of rhizobia, host plants and geographic environments. With our results in the present and

previous studies on *Caragana* rhizobia in Liaoning Province (Yan *et al.*, 2007), faba bean (*Vicia faba*) rhizobia in temperate and subtropical regions (Tian *et al.*, 2007), and soybean rhizobia in subtropical regions (Man *et al*., 2008), we concluded that the clear geographical distribution of rhizobia could be found only when the rhizobia were collected from distinctive regions which have specific soil types and climates.

In this study, the majority of adzuki bean rhizobia were *S. fredii* in Jiangsu, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense* in Jiangxi, and *B. elkanii* in Sichuan. These results confirmed that the nodule symbiotic bacteria of adzuki bean had biogeography, similar to the rhizobia associated with soybean (Man *et al*., 2008), *Caragana* (Yan *et al*., 2007), and faba bean (Tian *et al*., 2007). The isolation of *S. fredii* from Jiangsu might be related to the soil pH in that zone, since the same bacterium was found to be dominant in soybean root nodules in alkaline-saline soils (Camacho *et al*., 2002). Furthermore, *S. fredii* associated with *Vigna* species was only found in China, but the bradyrhizobia were universal in other countries, demonstrating that both the host legumes and the soil conditions affected the biogeography of symbiotic bacteria, as estimated in several other studies (Moulin *et al*., 2001; Diouf *et al*., 2007; Lu *et al*., 2008).

Conclusively, the results in the present study demonstrated that *S. fredii* and *Bradyrhizobium* species are the main microsymbionts for adzuki bean in China and the symbiotic genes have been maintained in these bacteria mainly by vertical transfer and rarely by lateral transfer. The geographical peculiarity was exhibited by adzuki bean rhizobia in this study and the biogeography of rhizobia was affected by both the host legume and the soil conditions.

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