Bradyrhizobium elkanii, Bradyrhizobium yuanmingense and Bradyrhizobium japonicum are the main rhizobia associated with Vigna unguiculata and Vigna radiata in the subtropical region of China

Yong Fa Zhang1,2,5, En Tao Wang1,3, Chang Fu Tian1, Feng Qin Wang1,4, Li Li Han1, Wen Feng Chen1 & Wen Xin Chen1

1The Key Laboratory of Agro-Microbial Resource and Application (Ministry of Agriculture), College of Biological Sciences, China Agricultural University, Beijing, China; 2College of Life Science, Northwest Science and Technology University of Agriculture and Forestry, Yangling, Shaanxi Province, China; 3Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México D. F., México; 4College of Life Science, Henan Agricultural University, Zhengzhou, Henan Province, China; and 5Department of Food and Bioengineering Science, Henan University of Science & Technology, Luoyang, Henan Province, China

Correspondence: Wen Xin Chen, The Key Laboratory of Agro-Microbial Resource and Application (Ministry of Agriculture), College of Biological Sciences, China Agricultural University, Beijing, 100094, China. Tel.: +86 10 6273 1854; fax: +86 10 6273 4008; e-mail: wenxin_chen@263.net

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Abstract

Cowpea (Vigna unguiculata) and mung bean (Vigna radiata) are important legume crops yet their rhizobia have not been well characterized. In the present study, 62 rhizobial strains isolated from the root nodules of these plants grown in the subtropical region of China were analyzed via a polyphasic approach. The results showed that 90% of the analyzed strains belonged to or were related to Bradyrhizobium japonicum, Bradyrhizobium liaoningense, Bradyrhizobium yuanmingense and Bradyrhizobium elkanii, while the remaining represented Rhizobium leguminosarum, Rhizobium etli and Sinorhizobium fredii. Diverse nifH and nodC genes were found in these strains and their symbiotic genes were mainly coevolved with the housekeeping genes, indicating that the symbiotic genes were mainly maintained by vertical transfer in the studied rhizobial populations.

Introduction

Cowpea (Vigna unguiculata) and mung bean (Vigna radiata) are important legumes cultivated in China; each can fix nitrogen through nodule symbiosis with rhizobia. Their drought tolerance, nitrogen-fixation capacity and shade tolerance make these plants important components in intercropping with maize or sorghum. Cowpea is native to Africa, but has been cultivated in China as a vegetable and grain crop and as a herbal medicine for centuries since it was first recorded in the literature some 500 years ago. Mung bean is native to India, but has been cultivated for around 3000 years in China. The seeds of mung bean are commonly used as a cooling food in Chinese cuisine and as a herbal medicine.

Previous studies have classified cowpea and mung bean rhizobia as cowpea miscellany belonging to the genus Bradyrhizobium (Jordan, 1982), but the species status of these taxa was unclear. Several Vigna rhizobial strains have been classified recently as representing Bradyrhizobium spp. and Rhizobium spp. based on 16S rRNA gene sequence phylogeny (Wolde-Meskel et al., 2005; Germano et al., 2006; Yokoyama et al., 2006). However, little information is available regarding the rhizobia associated with cowpea and mung bean in Chinese soils.

Given the importance of cowpea and mung bean in sustainable agriculture and the lack of data on their rhizobia, we decided to collect and characterize the rhizobia naturally associated with these legumes grown in the subtropical region of China. This region has a humid climate and acid soils in which rhizobial populations may be different from those isolated in other regions. For example, Sinorhizobium fredii was predominant in soybean nodules (Camacho et al.,...
Materials and methods

Isolates and strains

Sixty-two new isolates and 28 reference strains of rhizobia were used (Table 1). The Vigna rhizobia were isolated using a routine method and yeast mannotol agar (YMA) (Vincent, 1970) from root nodules collected in the fields of nine subtropical provinces in China. Nodulation of each strain on its original host was confirmed by nodulation tests (Vincent, 1970). The nitrogen-fixing ability of the isolates was verified after 1 month based on the presence of red color (leghemoglobin) inside the nodules. All strains were maintained on YMA medium and incubated at 28 °C.

Amplified 16S rDNA restriction analysis (ARDRA) and restriction fragment length polymorphism (RFLP) of 16S–23S rRNA gene internal transcribed spacer (ITS)

Primers P1 and P6 and DNA extracted from each strain were used to amplify the 16S rRNA gene as described previously (Tan et al., 1997). The PCR products were digested separately with MspI, HinfI, HaeIII and AluI (Laguerre et al., 1996). The ITS fragments were amplified with primers FGPS1490 and FGPL132 according to the procedure of Laguerre et al. (1996) and digested separately with MspI, HaeIII and AluI. The rRNA gene and ITS restriction fragments were separated by electrophoresis and visualized as described (Laguerre et al., 1996). The RFLP profiles were analyzed using the GELCOMPAR II software package. In the cluster analysis of RFLP patterns, the Dice similarity coefficient (S_d) of each strain pair, which was subsequently used in cluster analysis with UPGMA (Sneath & Sokal, 1973).

Sequencing and phylogenetic analyses of 16S rRNA, nifH and nodC genes

Amplification of the 16S rRNA gene was performed as described above. nifH gene fragments were amplified with primers nifH40F and nifH817R according to the procedure of Vinuesa et al. (2005a). The nodC gene was amplified using primers NodCfor540 and NodCrev1160 according to the protocol described by Sarita et al. (2005). The PCR products were purified and directly sequenced as described by Vinuesa et al. (2005a). The resulting sequences, together with the related sequences obtained from the GenBank database, were aligned using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were constructed using the neighbor-joining method with the kimura two-parameter model and were bootstrapped based on 1000 replicates using the programs in the MEGA3.1 package (Kumar et al., 2004).

Results

Isolation and nodulation of bacteria

A total of 62 isolates were obtained, including eight fast-growing, acid-producing bacteria that produced colonies >2 mm in diameter after 2–3 days of incubation, and 54 slow-growing, alkali-producing bacteria with colonies ≤1 mm in diameter after 5–7 days of incubation (Table 1). The nodulation results demonstrated that most of the isolates formed nitrogen-fixing nodules on their hosts of origin (supplementary Table S1).
| Strain                  | Phenon          | rRNA type | ITs Phylogeny  | Host plant | Geographical origin |
|------------------------|-----------------|-----------|----------------|------------|--------------------|
| B. japonicum           |                 |           |                |            |                    |
| B. japonicum USDA 6    |                 |           |                |            |                    |
| B. japonicum USDA 18   |                 |           |                |            |                    |
| CCBAU 33135            |                 |           |                |            |                    |
| CCBAU 33080            |                 |           |                |            |                    |
| CCBAU 43197            |                 |           |                |            |                    |
| B. japonicum-related   |                 |           |                |            |                    |
| CCBAU 51172            |                 |           |                |            |                    |
| CCBAU 43073, CCBAU 43135 |               |           |                |            |                    |
| CCBAU 33041            |                 |           |                |            |                    |
| CCBAU 23236            |                 |           |                |            |                    |
| CCBAU 61321, CCBAU 61323 |              |           |                |            |                    |
| CCBAU 23111            |                 |           |                |            |                    |
| CCBAU 51377            |                 |           |                |            |                    |
| CCBAU 33074            |                 |           |                |            |                    |
| CCBAU 33088            |                 |           |                |            |                    |
| B. liaoningense        |                 |           |                |            |                    |
| USDA 3622              |                 |           |                |            |                    |
| CCBAU 60066            |                 |           |                |            |                    |
| CCBAU 43031, CCBAU 43121 |              |           |                |            |                    |
| CCBAU 61318            |                 |           |                |            |                    |
| B. yuanmingense        |                 |           |                |            |                    |
| B. yuanmingense CCBAU 10071T |             |           |                |            |                    |
| CCBAU 51117            |                 |           |                |            |                    |
| CCBAU 61316            |                 |           |                |            |                    |
| CCBAU 35186-1          |                 |           |                |            |                    |
| CCBAU 35272            |                 |           |                |            |                    |
| CCBAU 51269            |                 |           |                |            |                    |
| CCBAU 51263, CCBAU 51357 |               |           |                |            |                    |
| CCBAU 61313            |                 |           |                |            |                    |
| CCBAU 45066            |                 |           |                |            |                    |
| CCBAU 61067, CCBAU 61068, CCBAU 61069, CCBAU 61070, CCBAU 61072 | | | | | |
| CCBAU 33068            |                 |           |                |            |                    |
| CCBAU 43088            |                 |           |                |            |                    |
| B. elkanii             |                 |           |                |            |                    |
| B. elkanii USDA 76      |                 |           |                |            |                    |
| CCBAU 35121            |                 |           |                |            |                    |
| CCBAU 35113, CCBAU 35256-2 |              |           |                |            |                    |
| CCBAU 51254            |                 |           |                |            |                    |
| CCBAU 51177            |                 |           |                |            |                    |
| CCBAU 33137            |                 |           |                |            |                    |
| CCBAU 33012-1, CCBAU 33012-2 |           |           |                |            |                    |
| CCBAU 51159            |                 |           |                |            |                    |
| CCBAU 33042            |                 |           |                |            |                    |
| CCBAU 33011            |                 |           |                |            |                    |
| CCBAU 23001            |                 |           |                |            |                    |
| CCBAU 33174            |                 |           |                |            |                    |
| CCBAU 33011            |                 |           |                |            |                    |
| CCBAU 51012            |                 |           |                |            |                    |
| CCBAU 35140-2, CCBAU 35192 |              |           |                |            |                    |
| CCBAU 35140-1          |                 |           |                |            |                    |
| R. leguminosarum       |                 |           |                |            |                    |
| R. leguminosarum 127K17 |                 |           |                |            |                    |
| CCBAU 43018-1, CCBAU 43018-2 |           |           |                |            |                    |
| CCBAU 33038-3          |                 |           |                |            |                    |
| R. etli                |                 |           |                |            |                    |
| R. etli CFN 42T        |                 |           |                |            |                    |
| CCBAU 655118           |                 |           |                |            |                    |
| CCBAU 51379            |                 |           |                |            |                    |
| S. fredii             |                 |           |                |            |                    |
| USDA 205T              |                 |           |                |            |                    |
| CCBAU 31015            |                 |           |                |            |                    |
| CCBAU 31030            |                 |           |                |            |                    |

*Twenty-three other reference strains were also used in different analyses. Strains in bold type indicate that these were used in sequencing.

1ND, not done; Bj, B. japonicum; Bl, B. liaoningense; Sl, single lineage; By, B. yuanmingense; Be, B. elkanii; Rlt, R. leguminosarum bv. trifolii; Re, R. etli; Sf, S. fredii.

2Provinces of China.
ARDRA

In ARDRA, eight rRNA gene types were distinguished among the 62 isolates (Table 1, supplementary Fig. S1). The eight fast-growing isolates were found in five rRNA types identical or similar to *Rhizobium etli*, *Rhizobium leguminosarum* and *S. fredii*. The 54 slow-growing isolates were found in three rRNA types, which were respectively identical to the reference strains for *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*–*Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense*.

ITS PCR–RFLP

In this analysis, 29 ITS types were identified among the 62 isolates (Table 1), and these were grouped into 11 clusters at a similarity level of 63% (Table 1, supplementary Fig. S2). At this level of similarity, most of the reference strains were divided into different clusters corresponding to their species, but the reference strains of four *Sinorhizobium* species were found in the same cluster (cluster 9). These grouping results were consistent with the rRNA types in most cases, except for strains in rRNA types 20 and 21. Several ITS clusters were found in rRNA type 20, and ITS clusters 1 and 2 were found in both RNA types.

16S rRNA gene phylogeny

For each rRNA type, 1–4 representative isolates were used in 16S rRNA gene sequence analysis. In the reconstructed phylogenetic tree (supplementary Fig. S3), these isolates were respectively grouped together with reference strains of *R. etli*, *R. leguminosarum*, *S. fredii*, *B. elkanii*, *B. japonicum* and *B. yuanmingense*.

Numerical taxonomy

A subset of 50 isolates and nine reference strains were examined by phenotypic characterization. Seventeen features were the same for the test strains and only the 112 variable features were used in cluster analysis, and all the strains were divided into eight phena corresponding to species in general and four single strains at a similarity level of 80% (Table 1, Fig. 1).

The eight fast-growing isolates and reference strains for *R. etli*, *R. leguminosarum* and *S. fredii* were respectively found in phena 1, 2 and 3 (Table 1). Phena 4–8 were members of the genus *Bradyrhizobium*. Phenon 5 had three isolates of rRNA types 20 and 21. Phenon 6 contained eight isolates in rRNA type 20 and two *B. japonicum* reference strains. Phenon 7 had 14 isolates that were further divided into two subgroups: 7a with 10 isolates of rRNA type 21 and the type strain of *B. yuanmingense*; and 7b with four isolates of rRNA type 20. Fifteen isolates in rRNA type 19 were found in phenon 8 together with *B. elkanii* USDA 76 and in phenon 4.

**Phylogeny of nifH and nodC gene sequences**

In the present study, *nifH* and *nodC* gene fragments were amplified from several representative isolates. In the *nifH* and *nodC* phylogenetic trees (Fig. 2), the isolates were grouped according to their genera and to species in most cases, although two groups were found in the *B. japonicum* strains. The exceptions were *B. elkanii* isolates CCBAU 51159 and CCBAU 23011, which were in the *B. yuanmingense* and *B. japonicum* groups, respectively, in the *nifH* tree, and in the *nodC* tree.
and *B. japonicum* CCBAU 51377, which was in the *B. yuanmingense* group in the nodC tree.

**Phylogeny of housekeeping genes**

According to the grouping results of the previous analyses, representative strains were used in sequencing the atpD, glnII and recA genes. The phylogenetic relationships obtained from these three genes (Fig. 3 for recA, supplementary Figs S4 and S5 for atpD and glnII) were generally similar to each other and were congruent with the groupings in the other analyses. Seven phylogenetic groups corresponding to *B. elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, *R. etli*, *R. leguminosarum* and *S. fredii* were defined among the *Vigna* rhizobia. Five strains (marked in bold type in Fig. 3) showed unstable relationships that were closer to *B. japonicum* in the glnII tree or between *B. liaoningense* to *B. yuanmingense* in the atpD tree, with bootstrap values of 42–66, while two of them were grouped with *B. liaoningense* with a bootstrap value of 51% and three were in the *B. japonicum* group.

**Discussion**

Previously, cowpea and mung bean rhizobia have not been systematically studied and their taxonomic positions were not clear, although several rhizobial strains associated with these two legumes grown in tropics, including the center of origin for cowpea (Wolde-Meskel et al., 2005) and other regions (Germano et al., 2006; Yokoyama et al., 2006), have been studied based on 16S rRNA and nod gene sequencing. The present study revealed that *Bradyrhizobium* spp. occupied 90% of the cowpea and mung bean nodules in the

![Fig. 2. Neighbor-joining phylogenetic tree based on nifH (a) and nodC (b) genes showing the relationships of *Vigna* rhizobia isolated from the subtropical region of China. GenBank accession numbers and host plant of the strain are given in parentheses. Scale bar = 0.2% substitutions per site. Bootstrap values greater than 50% are indicated in the corresponding nodes.](image-url)
subtropical region of China and the remaining proportion was fast-growing rhizobia.

Currently, a polyphasic approach including 16S–23S rRNA gene ITS-RFLP, numerical taxonomy, DNA–DNA hybridization experiments, multilocus sequence analysis (Willems et al., 2003; Martens et al., 2008), ARDRA and 16S rRNA gene sequence analyses has been used to define rhizobial species. According to the consensus of the results from different analyses, *Vigna* isolates could be assigned to seven species (Table 1). All the isolates in rRNA type 21 were identified as representing *B. yuanmingense* based on the results of numerical taxonomy, ITS–RFLP, multilocus sequence analysis and 16S rRNA gene sequence phylogeny. The eight fast-growing isolates were defined as representing *R. leguminosarum*, *R. etli* and *S. fredii*. In the present study, the *S. fredii* and *Sinorhizobium xinjiangense* strains were very similar in all the sequence analyses and they might represent the same species as reported recently (Lloret et al., 2007; Martens et al., 2008). Previously, both *R. etli* (Hernandez-Lucas et al., 1995) and *S. fredii* (Pueppke & Broughton, 1999) have been reported to nodulate and fix nitrogen in *V. unguiculata* under laboratory conditions. The results of the current study verified that these three rhizobial
Fig. 3. Neighbor-joining phylogenetic tree based on recA gene sequences showing the relationships of Vigna rhizobia isolated from the subtropical region of China. GenBank accession numbers are presented in parentheses. Scale bar = 0.2% substitutions per site. Bootstrap values greater than 50% are indicated in the corresponding nodes.
species were also microsymbionts of *V. unguiculata* in fields, but their minor proportion indicated that they were not preferable rhizobia for that host.

As described previously, species definition within the genus *Bradyrhizobium* is difficult and *B. japonicum* and *B. liaoningense* are very closely related species (Willems et al., 2001, 2003). In the present study, four isolates could be defined as representing *B. liaoningense* based on consensus numerical taxonomy (phenon 7b), ITS–RFLP (group 5) and multilocus sequence analysis. Three isolates could be confirmed as representing *B. japonicum* based on numerical taxonomy and multilocus sequence analysis. Although 11 isolates were listed as *B. japonicum*-related strains (Table 1) because most belonged to the *B. japonicum* phenon, their phylogenetic positions in multilocus sequence analysis were unstable, switching between the *Bradyrhizobium* genus and valuable suggestions. We are grateful for critical comments on this manuscript by Drs Karl Cuddy (University of Western Ontario) and Yongqiang Wang (University of Toronto). E.T.W. was supported by projects of SIP 20060213 and SIP 20070538 authorized by IPN, Mexico.

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**Supplementary material**

The following supplementary material is available for this article:

**Table S1.** Nodulation results of *Vigna* rhizobia.

**Fig. S1.** UPGMA dendrogram showing the rRNA types distinguished among the *Vigna* rhizobia isolated from the subtropical region of China.

**Fig. S2.** UPGMA dendrogram based on PCR-RFLP patterns of the 16S–23S rRNA ITS showing the relationships of *Vigna* rhizobia isolated from the subtropical region of China.

**Fig. S3.** Neighbor-joining tree showing the 16S rRNA gene phylogeny of *Vigna* rhizobia isolated from the subtropical region of China.

**Fig. S4.** Neighbor-joining phylogenetic tree based on atpD gene sequences showing the relationships of *Vigna* rhizobia isolated from the subtropical region of China.

**Fig. S5.** Neighbor-joining phylogenetic tree based on glnII gene sequences showing the relationships of *Vigna* rhizobia isolated from the subtropical region of China.

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