Bradyrhizobium huanghuaihaiense sp. nov., an effective symbiotic bacterium isolated from soybean (Glycine max L.) nodules

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In a survey of the biodiversity and biogeography of rhizobia associated with soybean (Glycine max L.) in different sites of the Northern (Huang-Huai-Hai) Plain of China, ten strains were defined as representing a novel genomic species in the genus Bradyrhizobium. They were distinguished from defined species in restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene and the 16S–23S rRNA gene intergenic spacer (IGS). In BOX-PCR, these strains presented two patterns that shared 94 % similarity, demonstrating that they were a homogenous group with limited diversity. In phylogenetic analyses of the 16S rRNA gene, IGS and housekeeping gene sequences, four representative strains formed a distant lineage within the genus Bradyrhizobium, which was consistent with the results of DNA–DNA hybridization. The strains of this novel group formed effective nodules with G. max, Glycine soja and Vigna unguiculata in cross-nodulation tests and harboured symbiotic genes (nodC and nifH) identical to those of reference strains of Bradyrhizobium japonicum, Bradyrhizobium liaoningense and ‘Bradyrhizobium daqingense’ originating from soybean, implying that the novel group may have obtained these symbiotic genes by lateral gene transfer. In analyses of cellular fatty acids and phenotypic features, some differences were found between the novel group and related Bradyrhizobium species, demonstrating that the novel group is distinct phenotypically from other Bradyrhizobium species. Based upon the data obtained, these strains are proposed to represent a novel species, Bradyrhizobium huanghuaihaiense sp. nov., with CCBAU 23303T (=LMG 26136T =CGMCC 1.10948T =HAMBI 3180T) as the type strain. The DNA G+C content of strain CCBAU 23303T is 61.5 mol% (Tm).

Soybean (Glycine max L.) is an important crop worldwide for oil and protein production. It forms nitrogen-fixing root nodules with diverse symbiotic bacteria, including five Bradyrhizobium species (Bradyrhizobium japonicum, B. elkanii, B. liaoningense, B. yuanmingense and B. daqingense).

Abbreviations: IGS, intergenic spacer; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NJ, neighbour-joining; RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, IGS, recA, gdhII, atpD, nodC and nifH sequences of strain CCBAU 23303T are HQ231463, HQ428043, HQ231595, HQ231639, HQ231682, HQ231507 and HQ231551, respectively.

Four supplementary figures and two supplementary tables are available with the online version of this paper.
from nodules of *Pachyrhizus erosus* (Ramírez-Bahena et al., 2009), *Bradyrhizobium lablabi* from root nodules of *Lablab purpureus* (Chang et al., 2011) and *Bradyrhizobium cytisi* from nodules of *Cytisus villosus* (Chahboune et al., 2011).

During an investigation of the biodiversity and biogeography of rhizobia associated with soybean grown in the Northern (Huang-Huai-Hai) Plain of China, ten bacterial strains were defined as ‘*Bradyrhizobium* sp. I’ by PCR-based restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene and 16S–23S rRNA gene intergenic spacer (IGS). Sequencing of the 16S rRNA, *recA*, *glnII* and *atpD* genes of a representative strain CCBAU 051161, confirmed the distinct phylogeny of the group in the genus *Bradyrhizobium* (Zhang et al., 2011). To clarify the taxonomic position of these ten strains, further analyses were performed in the present study.

The ten *Bradyrhizobium* sp. I strains, CCBAU 051145, CCBAU 051158, CCBAU 051161, CCBAU 23303T, CCBAU 23304, CCBAU 23328, CCBAU 23332, CCBAU 25541, CCBAU 45502 and CCBAU 45504, were obtained from seven sites in four Chinese provinces (Hebei, Anhui, Shandong and Henan) (Zhang et al., 2011). In the present study, the bacteria were maintained on YMA slants at 4°C for long-term storage. They were normally incubated at 28°C for short-term storage and in 20% (w/v) glycerol at −80°C for long-term storage. They were normally incubated at 28°C.

To evaluate the genetic diversity among the ten strains, BOX-PCR fingerprinting was performed as described previously (Nick et al., 1999). The primer BOXAIR (5'-CTACCGCAAGGGCAGCTGAG-3') and the procedure of Versalovic et al. (1994) were used to amplify the fragments with genomic DNA as template, which was extracted from each strain with the GUTEC method (Terefeework et al., 2001). The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels supplemented with 0.5 μg ethidium bromide ml⁻¹. RFLP patterns were photographed, normalized, combined and clustered using the GelCompar II software package (Applied Maths) (Vauterin & Vauterin, 1992). In this analysis, two BOX-PCR patterns were distinguished from nine of the ten strains; no PCR product was obtained from strain CCBAU 23332. Strains CCBAU 23303T and CCBAU 23304 had the same pattern, which shared 94% similarity with the pattern displayed by the other seven strains (Fig. S1, available in IJSEM Online), demonstrating that these nine strains comprised at least two clones and that these strains represented a genomic species with little diversity, although they were isolated from different geographical regions.

Based upon their high genetic relatedness, four strains (CCBAU 051161, CCBAU 25541, CCBAU 23303T and CCBAU 45502) representing the two BOX-PCR patterns and originating from the four provinces were subjected to further analyses.

For phylogenetic analyses, genomic DNA extracted as mentioned above from each strain was used as a template to amplify the following targets: (i) the 16S rRNA gene (~1500 nt) with primers P1 and P6 (Tan et al., 1997) and the PCR protocol of Weisburg et al. (1991); (ii) the 16S–23S rRNA gene IGS (~1200 nt) using the primer pair FGPS6/23S-38 (Normand et al., 1992) and the PCR protocol of Rasolomampianina et al. (2005); (iii) partial sequences of the housekeeping genes *recA* (~600 nt), *glnII* (~680 nt) and *atpD* (~530 nt) using primer pairs recA41F/recA640R, glnII12F/glnII689R and atpD255F/atpD782R, respectively, and protocols described by Vinuesa et al. (2005b); (iv) *nodC* (~700 bp) with primer pair nodCF540/nodCR1160 and the protocol of Sarita et al. (2005); and (v) *nifH* (~800 bp) with the primer pair nifHF/nifHR and the protocol of Laguerre et al. (2001). All PCR products were sequenced directly as described previously (Hurek et al., 1997). The sequences acquired in this study and those of related *Bradyrhizobium* strains obtained from GenBank by BLAST searching were aligned using CLUSTAL W in the MEGA 4 software (Tamura et al., 2007). Aligned sequences were analysed by using the MEGA 4.0 software to produce a Kimura two-parameter distance matrix (Kimura, 1980) and to reconstruct an optimal rooted tree using the neighbour-joining (NJ) method (Saitou & Nei, 1987) with bootstrap analysis using 1000 replications. Maximum-likelihood (ML) trees were also reconstructed with the PhyML 3.0 program (Guindon & Gascuel, 2003). The nucleotide substitution model produced by the Akaike information criterion was implemented in Modeltest 3.7 (Posada & Crandall, 1998) and PAUP 4.0b (Swofford, 2000).

The four representative strains had identical 16S rRNA gene sequences and they were most related to *B. iriomotense* LMG 24129T (99.7% similarity) in the NJ tree (Fig. 1). The sequences of the type strains of *B. liaoningense*, *B. betae*, *B. japonicum* and *B. canariense* and the proposed type strain of ‘*B. daqingense*’ presented similarities of 99.2, 99.2, 99.1 and 99.1%, respectively, with CCBAU 23303T. Strain CCBAU 23303T shared 16S rRNA gene sequence similarities of 97.2, 97.8, 98.2, 98.7, 98.8, 99.2 and 99.3%, respectively, with *Afipia felis* B-91-007352T, *Rhodopseudomonas palustris* ATCC 17001T, *Nitrobacter winogradskyi* ATCC 14123, *Agromonas oligotrophica* JCM 1494T, *B. denitrificans* LMG 8443T, ‘*Bradyrhizobium lupini*’ KM50-90 and ‘*B. lupini*’ FN 13. The high degree of sequence similarity supports the conclusion that these species could be classified as members of a single genus (Willems & Collins, 1992). Similar results were obtained when the sequences were analysed using the ML method (not shown).

In sequence analysis of the 16S–23S rRNA gene IGS, the topologies of the NJ and ML phylogenetic trees (not shown) were the same, and the four representative strains showed little variation. Strains CCBAU 23303T, CCBAU 25541 and CCBAU 45502 had identical IGS sequences, which were 99.8% similar to that of CCBAU 051161. Strain CCBAU 23303T had IGS sequence similarities of 95.3% with *B. yunnanense* CCBAU 10071T and 95.1, 94.8, 93.7, 93.7,
The novel strain CCBAU 23303T was most similar to 'B. daqingense' CCBAU 15774, linked to B. yuanmingense CCBAU 10071T at 92 % similarity (Table S1). The nifH phylogenetic tree had the same topological structure as the nodC tree (not shown). These results suggest that the novel Bradyrhizobium strains examined in the present study might have obtained their symbiotic (nod and nif) genes from other soybean-nodulating Bradyrhizobium strains by horizontal gene transfer, as reported for other rhizobia (Moulin et al., 2004).

DNA–DNA hybridization is a standard method for bacterial species definition (Wayne et al., 1987). In the present study, total DNA was extracted using the method of Marmur (1961) from the four representative strains and reference strains. DNA–DNA relatedness between CCBAU 23303T and other strains was estimated using renaturation rate technology (De Ley et al., 1970). The DNA–DNA relatedness of CCBAU 23303T with CCBAU 051161, CCBAU 25541 and CCBAU 45502 was 88.2, 90.3 and 91.7 %, respectively, demonstrating that they are members of the same genomic species. The DNA–DNA relatedness between CCBAU 23303T and reference strains B. liaoningense USDA 3622T, B. betae LMG 21987T, B. japonicum USDA 6T, B. canariense BTA-1T, B. betae USDA 6T (AB231927), 'B. daqingense' CCBAU 15774, and 'B. daqingense' USDA 83T (AF193818) were 92.0 and 91.8 %, respectively, with 'B. japonicum' USDA 110 and 91.8 %, respectively, with 'B. japonicum' USDA 6T (AB231927), 'B. daqingense' CCBAU 15774, and 'B. daqingense' USDA 83T (AF193818), respectively. These data demonstrated that the four strains represented a novel species, which they cannot be used as gene markers for taxonomy. However, they can offer information on symbiotic specificity between rhizobia and legumes, and can reveal the host ranges of rhizobia (Laguerre et al., 2001). In the nodC phylogenetic tree (Fig. S2), the four representative strains showed identical sequences to B. japonicum USDA 6T and 'B. daqingense' CCBAU 15774, linked to B. yuanmingense CCBAU 10071T at 92 % similarity (Table S1). The nifH phylogenetic tree had the same topological structure as the nodC tree (not shown). These results suggest that the novel Bradyrhizobium strains examined in the present study might have obtained their symbiotic (nod and nif) genes from other soybean-nodulating Bradyrhizobium strains by horizontal gene transfer, as reported for other rhizobia (Moulin et al., 2004).

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Cellular fatty acids of strain CCBAU 23303ᵀ were assayed from 60.8 to 62.4 mol% (recommended for species definition (Wayne (detailed data available in Table S1), which are much lower International Journal of Systematic and Evolutionary Microbiology Y. M. Zhang and others recognized members of the genus genus in all tested strains, consistent with previous reports for the feature 8 and 16 : 0 were the two most dominant fatty acids percentages of these fatty acids varied (Table S2). Summed feature 8, 16 : 0, 16 : 1 fatty acids 12 : 0, 16 : 0, 16 : 1 recognized in all tested strains, together with those of Bradyrhizobium (Tighe cultured aerobically on YMA medium at 28 °C and cells were collected during the late-exponential phase of growth. Fatty acid methyl esters were prepared and separated using the method described by Sassar (1990) and identified with the MIDI Sherlock Microbial Identification System (Sherlock CD license version 6.0), using the TSBA6 database. A total of 27 fatty acids were detected in strain CCBAU 23303ᵀ (Table S2). All tested strains contained the fatty acids 12 : 0, 16 : 0, 16 : 1, 17 : 0, 17 : 1, 18 : 0, 18 : 1, 18 : 2, summed feature 3 (16 : 0, 16 : 1, 17 : 0, 17 : 1, 18 : 0, 18 : 1), but the percentages of these fatty acids varied (Table S2). Summed feature 8 and 16 : 0 were the two most dominant fatty acids in all tested strains, consistent with previous reports for the genus Bradyrhizobium (Tighe et al., 2000).

For polar lipid analysis, cells of strain CCBAU 23303ᵀ were grown to late-exponential phase in TY broth (5 g tryptone, 3 g yeast extract and 0.7 g CaCl₂, 2H₂O, pH 6.8–7.2, in 1 l deionized water) at 28 °C with shaking. Polar lipids were extracted from 200–250 mg freeze-dried cells according to Minnikin et al. (1984) and separated by two-dimensional TLC using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/acetic acid/methanol/water (80 : 18 : 12 : 5, by vol.) in the second dimension. The following spray reagents were used for detection of phospholipids, aminolipids and glycolipids, respectively: molybdenum blue spray reagent (results recorded immediately), ninhydrin (0.4 %, w/v, in 1-butanol saturated with molybdenum blue spray reagent (results recorded immediately), ninhydrin (0.4 %, w/v, in 1-butanol saturated with distilled water; developed at 100 °C for 5 min) and anisaldehyde reagent (developed at 110 °C for 4–6 min). Each spot was identified by comparing with standard mixtures of polar lipids (Sigma-Aldrich). The phospholipid profile is shown in Fig. S3. Strain CCBAU 23303ᵀ contained phosphatidylcholine and phosphatidylethanolamine as major components (each representing about 40 % of the total phospholipids) and phosphatidylglycerol and cardiolipin as minor components (each representing about 10 % of the total). Phosphatidylethanolamine was the only aminolipid detected. No glycolipid could be detected. The phospholipid components were completely congruent with those of B. japonicum USDA 110 and Bradyrhizobium sp. strain 32H1 (Miller et al., 1990).

Cell morphology of strain CCBAU 23303ᵀ (Fig. S4) was investigated with scanning electron microscopy after cultivation on a YMA plate at 28 °C for 7 days. Phenotypic features of the four representative strains and the type strains

![Fig. 2. ML tree reconstructed based on concatenated sequences of recA (381 nt), glnII (513 nt) and atpD (423 nt) showing the phylogenetic relationships of representative strains of B. huanghaiense sp. nov. under the best-fit model shown. Ensifer fredii USDA 205ᵀ was used as an outgroup. ML bootstrap support values for 100 pseudoreplicates of the dataset are provided at the corresponding nodes. Bar, 10 % nucleotide substitution.](image-url)
of related *Bradyrhizobium* species were determined according to the methods described by Gao et al. (1994). Biochemical tests including catalase and oxidase production, the Voges-Proskauer reaction and hydrolysis of starch and Tween 80 were performed according to Smibert & Krieg (1994). Production of hydrogen sulfide from cysteine was determined as described by Barrow & Feltham (1993). The four representative strains had the same characteristics in most cases except for utilization of L-arginine, L-cystine, D-glutamic acid and L-lysine as sole nitrogen sources (Table 1). The combination of phenotypic features listed in Table 1 could be used to differentiate the novel strains from the type strains of related species.

Cross-nodulation tests were performed in Leonard jars filled with sterilized vermiculite moistened with nitrogen-free solution using the standard procedure of Vincent (1970).

The representative strain CCBAU 23303T could form effective nodules of *Glycine max*, *Glycine soja* and *Vigna unguiculata*, but not with *Lotus corniculatus*, *Trifolium repens*, *Medicago sativa*, *Pisum sativum*, *Phaseolus vulgaris*, *Leucaena leucocephala* or *Melilotus albus*.

According to the phenotypic and genetic characteristics determined in our tests and the current criteria for definition of rhizobial species (Young, 1996), we propose that the ten strains in *Bradyrhizobium* sp. I (Zhang et al., 2011) represent a novel species in the genus *Bradyrhizobium, Bradyrhizobium huanghuaihainen* sp. nov.

**Table 1. Distinctive features of representative novel strains (B. huanghuaihainen* sp. nov.) and their closest relatives**

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*Data obtained in this study that are not consistent with those reported previously.

The representative strain CCBAU 23303T (=LMG 26136T =CGMCC 1.10948T =HAMBI 3180T), was isolated from effective nodules of soybean (*Glycine max* L.). Its DNA G+C content is 61.5 mol% (*T*<sub>m</sub>).

**Acknowledgements**

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