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 26138T =CGMCC 1.10948T =HAMBI 3180T) as the type strain. The DNA G+C content of strain CCBAU 23303T is 61.5 mol% (Tm).
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 23303<sup>T</sup>, 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 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′-CTACGGCAAGCGACGCTGACG-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 GUTC method (Terefeework *et al.*, 2001). The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels supplemented with 0.5μg ethidium bromide ml<sup>−1</sup>. 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 23303<sup>T</sup> 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 23303<sup>T</sup> 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 24129<sup>T</sup> (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 23303<sup>T</sup>. Strain CCBAU 23303<sup>T</sup> 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-91-007352<sup>T</sup>, *Rhodospseudomonas palustris* ATCC 17001<sup>T</sup>, *Nitrobacter winogradskyi* ATCC 14123, *Agromonas oligotropha* JCM 1494<sup>T</sup>, *B. denitrificans* LMG 8443<sup>T</sup>, *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 23303<sup>T</sup>, CCBAU 25541 and CCBAU 45502 had identical IGS sequences, which were 99.8% similar to that of CCBAU 051161. Strain CCBAU 23303<sup>T</sup> had IGS sequence similarities of 95.3% with *B. yuanningsense* CCBAU 10071<sup>T</sup> and 95.1, 94.8, 93.7, 93.7,
The novel strain CCBAU 23303T was most similar to B. japonicum USDA 6T and had the same topology as the NJ tree (not shown). The combined sequences of the three housekeeping genes (recA, glnII and atpD) were used for MLSA, and the ML tree reconstructed with these sequences had the same topological structure as the NJ tree (not shown). These results suggest that the novel strain represents a novel species, as supported by the high DNA–DNA relatedness between CCBAU 23303T and the type strains of other Bradyrhizobium species.

The four representative strains showed identical sequences to each of the three genes, the phylogenetic relationships were consistent between the NJ and ML trees (not shown). These data demonstrated that the four strains represented a novel species, according to the conclusion of Willems et al. (2003) that two bradyrhizobial strains that share less than 95.5 % IGS sequence similarity belong to different genomic species.

The four representative strains showed identical sequences for three housekeeping genes (recA, glnII and atpD). For each of the three genes, the phylogenetic relationships were consistent between the NJ and ML trees (not shown). The most similar strains to CCBAU 23303T were ‘B. daqingense’ CCBAU 15774, B. betae LMG 21987T, B. canariense BTA-1T, B. iriomotense LMG 24129T and B. liaoningense USDA 3622T. Strains of the remaining Bradyrhizobium species presented similarities of less than 90 % with CCBAU 23303T (Table S1). These data demonstrated that the four strains represented a novel species, consistent between the NJ and ML trees (not shown). The NJ tree was derived from a distance matrix (Kimura’s two-parameter model), and the bootstrap confidence levels at internodes are indicated at internodes. Bar, 1 % nucleotide substitution.

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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T 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. daqingense’ CCBAU 15774 and B. yuanmingense CCBAU 10071T was 88.2, 90.3 and 91.7 %, respectively, demonstrating that they are members of the same genomic species.
Cellular fatty acids of strain CCBAU 23303T were assayed from 60.8 to 62.4 mol% (which is within the range for 70% DNA–DNA relatedness, detailed data available in Table S1), which are much lower than the threshold value of 70% DNA–DNA relatedness of the genus Bradyrhizobium.

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 ninhydrin, developed at 110°C for 4–6 min).

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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 of the four representative strains and the type strains.
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 23303<sup>T</sup> could form effective nodules with *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. 1 (Zhang *et al.*, 2011) represent a novel species in the genus *Bradyrhizobium*, *Bradyrhizobium huanghuaihaiense* sp. nov.

### Description of *Bradyrhizobium huanghuaihaiense* sp. nov.

*Bradyrhizobium huanghuaihaiense* (huang.huai.hai.en’s. N.L. neut. adj. *huanghuaihaiense* of or belonging to Huang-Huai-Hai Plain, China, referring to the fact that the first strains were isolated from root nodules of soybean grown on the Huang-Huai-Hai Plain).

Cells are Gram-negative, aerobic, non-spore-forming rods, 1.35 μm long and 0.46 μm wide. Colonies are circular, convex and translucent, 1 mm in diameter within 7 days at 28 °C on YMA medium. The generation time is 7–9 h in TY broth. Grows at pH 6–9, with optimum growth at pH 7.0. Growth occurs at 10 °C and between 28 and 37 °C (optimally at 28 °C). Tolerates 60 °C for 10 min. Cannot grow on YMA in the presence of 1 % NaCl. No growth in Tween 80 or in Luria–Bertani broth. Catalase, oxidase and hydrolysis of starch are positive. Nile blue reduction, phenylalanine dehydrogenase production and Voges–Proskauer reaction are negative. Cannot produce hydrogen sulfide. In addition to the carbon sources listed in Table 1, the type strain can metabolize D-arabinose, D-galactose, melibiose, D-ribose, sodium D-glucuronate, hippuric acid, sodium succinate, sorbose, sucrose, calcium gluconate, D-fructose, D-mannose, sodium pyruvate, D-xylene and L-proline as sole carbon sources, but not adipic acid, dulcitol, *meso*-erythritol, lactose, melezitose, D-sorbitol, raffinose, soluble starch, L-arginine, glycine, DL-asparagine or L-methionine. The type strain cannot grow on D-threonine as a sole nitrogen source. Strains of the novel species are resistant to the antibiotics (μg ml<sup>−1</sup>) ampicillin (50), kanamycin (5), neomycin sulfate (50), streptomycin (5), erythromycin (100), chloramphenicol (50) and gentamicin (100). Summed feature 8 (18 : 1<sub>ω6</sub>c and/or 18 : 1<sub>ω7</sub>c) and 16 : 0 are the dominant fatty acids. The type strain contains phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and cardiolipin.

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

### Acknowledgements

Thanks to Yan Li and Xiao Xia Zhang for their help with the determination of polar lipids and fatty acids, respectively. This work

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

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**Utilization of sole nitrogen sources**

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<th>1-L-Cystine</th>
<th>1-D-Glutamic acid</th>
<th>1-L-Isoleucine</th>
<th>1-L-Lysine</th>
<th>1-L-Threonine</th>
<th>Growth on YMA with/</th>
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