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 23303^T (=LMG 26136^T =CGMCC 1.10948^T =HAMBI 3180^T) as the type strain. The DNA G+C content of strain CCBAU 23303^T is 61.5 mol% (*T_m*).

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*') (Ando & Yokoyama, 1999; Appunu *et al.*, 2008; Han *et al.*, 2009; Kuykendall *et al.*, 1992; Li *et al.*, 2011a; Man *et al.*, 2008; Wang *et al.*, 2012) and two *Ensifer* species, *Ensifer fredii* and *E. sojae* (Chen *et al.*, 1988; Han *et al.*, 2009; Li *et al.*, 2011a, b). Note that the name '*Bradyrhizobium daqingense*' has not yet been effectively or validly published (Wang *et al.*, 2012). In addition to species associated with soybean, other *Bradyrhizobium* species isolated from different sources have been described, including *Bradyrhizobium betae* from roots of sugar beet (Rivas *et al.*, 2004), *Bradyrhizobium canariense* from root nodules of genistoid legumes (Vinuesa *et al.*, 2005a), *Bradyrhizobium denitrificans* from soil (van Berkum *et al.*, 2006), *Bradyrhizobium iriomotense* from tumour-like roots of the legume *Entada koschunensis* (Islam *et al.*, 2008), *Bradyrhizobium pachyrhizi* and *Bradyrhizobium jicamae*

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*, *glnII*, *atpD*, *nodC* and *nifH* sequences of strain CCBAU 23303^T 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 23303^T, 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'-CTACGGCAAGGCGACGCTGACG-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 (Terefework *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 23303^T 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^T 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 *recA*41F/*recA*640R, *glnII*12F/*glnII*689R and *atpD*255F/*atpD*782R, respectively, and protocols described by Vinuesa *et al.* (2005b); (iv) *nodC* (~700 bp) with primer pair *nodC*540/*nodC*1160 and the protocol of Sarita *et al.* (2005); and (v) *nifH* (~800 bp) with the primer pair *nifH*F/*nifH*R 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^T (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, 99.1 and 99.1 %, respectively, with CCBAU 23303^T. Strain CCBAU 23303^T shared 16S rRNA gene sequence similarities of 97.2, 97.8, 98.2, 98.7, 98.8, 99.2 and 99.3 %, respectively, with *Azospirillum felis* B-91-007352^T, *Rhodopseudomonas palustris* ATCC 17001^T, *Nitrobacter winogradskyi* ATCC 14123, *Agromonas oligotrophica* JCM 1494^T, *B. denitrificans* LMG 8443^T, '*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^T, CCBAU 25541 and CCBAU 45502 had identical IGS sequences, which were 99.8 % similar to that of CCBAU 051161. Strain CCBAU 23303^T had IGS sequence similarities of 95.3 % with *B. yuanmingense* CCBAU 10071^T and 95.1, 94.8, 93.7, 93.7,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences (1229 nt) showing the phylogenetic relationships of representative strains of *Bradyrhizobium huanghuaihaiense* sp. nov. with other *Bradyrhizobium* species. *Ensifer fredii* USDA 205^T was used as an outgroup. The NJ tree was derived from a distance matrix (Kimura's two-parameter model). Bootstrap confidence levels $\geq 50\%$ (calculated for 1000 subsets) are indicated at internodes. Bar, 1 % nucleotide substitution.

92.0 and 91.8 %, respectively, with *B. japonicum* USDA 6^T, 'B. daqingense' CCBAU 15774, *B. betae* LMG 21987^T, *B. canariense* BTA-1^T, *B. iriomotense* LMG 24129^T and *B. liaoningense* USDA 3622^T. Strains of the remaining *Bradyrhizobium* species presented similarities of less than 90 % with CCBAU 23303^T (Table S1). 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 23303^T were 'B. daqingense' CCBAU 15774 for the *recA* gene (94.6 % similarity), *B. japonicum* USDA 6^T for the *glnII* gene (95.8 %) and *B. betae* LMG 21987^T for the *atpD* gene (96.2 %). In the multilocus sequence analysis (MLSA), the ML tree reconstructed with the combined sequences of the three housekeeping genes (Fig. 2) had the same topology as the NJ tree (not shown). The novel strain CCBAU 23303^T was most similar to *B. liaoningense* USDA 3622^T and *B. betae* LMG 21987^T in MLSA (94.7 % similarity) (Table S1), which is different from the results of analyses of the 16S rRNA gene and the IGS. The four representative strains in this study formed a distinctive lineage that differed from all defined species.

Symbiotic (*nod* and *nif*) genes are normally located on transferable elements (plasmids or symbiotic islands), so

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 6^T and 'B. daqingense' CCBAU 15774, linked to *B. yuanmingense* CCBAU 10071^T 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 23303^T and other strains was estimated using renaturation rate technology (De Ley *et al.*, 1970). The DNA–DNA relatedness of CCBAU 23303^T 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 23303^T and reference strains *B. liaoningense* USDA 3622^T, *B. betae* LMG 21987^T, *B. japonicum* USDA 6^T, *B. canariense* BTA-1^T, 'B. daqingense' CCBAU 15774 and *B. yuanmingense* CCBAU

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. huanghuaihaiense* sp. nov. under the best-fit model shown. *Ensifer fredii* USDA 205^T 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.

10071^T was 50.0, 44.5, 46.8, 44.5, 37.1 and 36.1 %, respectively (detailed data available in Table S1), which are much lower than the threshold value of 70 % DNA–DNA relatedness recommended for species definition (Wayne *et al.*, 1987).

The DNA G+C content was determined by the thermal denaturation method (Mandel & Marmur, 1968) using DNA from *Escherichia coli* K-12 as a standard. The DNA G+C content of the four representative strains ranged from 60.8 to 62.4 mol% (T_m), which is within the range for recognized members of the genus *Bradyrhizobium*.

Cellular fatty acids of strain CCBAU 23303^T were assayed together with those of *B. liaoningense* USDA 3622^T, *B. betae* LMG 21987^T, *B. japonicum* USDA 6^T, *B. canariense* BTA-1^T, *B. yuanmingense* CCBAU 10071^T and '*B. daqingense*' CCBAU 15774 in order to examine differences between the novel strain and closely related species. The strains were 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 Sasser (1990) and identified with the MIDI Sherlock Microbial Identification System (Sherlock license CD version 6.0), using the TSBA6 database. A total of 27 fatty acids were detected in strain CCBAU 23303^T (Table S2). All tested strains contained the fatty acids 12:0, 16:0, 16:1 ω 5c, 17:0, 17:1 ω 6c, 17:1 ω 8c, summed feature 3 (16:1 ω 6c and/or 16:1 ω 7c) and summed feature 8 (18:1 ω 6c and/or 18:1 ω 7c), 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 23303^T 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 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 23303^T 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 23303^T (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 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).

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

Strains: 1, CCBAU 051161; 2, CCBAU 25541; 3, CCBAU 23303^T; 4, CCBAU 45502; 5, *B. liaoningense* USDA 3622^T; 6, *B. yuanmingense* CCBAU 10071^T; 7, *B. japonicum* USDA 6^T; 8, *B. betae* LMG 21987^T; 9, *B. canariense* BTA-1^T. Data were obtained in this study. +, Growth; –, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9
Utilization of sole carbon sources									
Inulin	+	+	+	+	–	–	+	–	–
D-Galactose	+	+	+	+	–	+	+	+	+
D-Glucose	+	+	+	+	–	–	+	–*	+
Maltose	+	+	+	+	–	–	+	–	–
Melibiose	+	+	+	+	+	–	+	–	–
L-Rhamnose	+	+	+	+	+	–	w	–	–*
D-Ribose	+	+	+	+	+	–	+	w	+
Sodium acetate	+	+	+	+	+	–	+	+	+
Sodium D-gluconate	+	+	+	+	+	–	+	+	+
Hippuric acid	+	+	+	+	–	+	+	+	+
D-Sorbitol	–	–	–	–	–	–	–	–	+
Sorbose	+	+	+	+	–	–	+	+	–
Sucrose	+	+	+	+	–	+	+	w	–
Utilization of sole nitrogen sources									
L-Arginine	–	+	–	–	–	–	+	–	–
L-Aspartic acid	+	+	+	+	+	–	+	w	+
L-Cystine	–	+	+	–	–*	–	–	+	+
D-Glutamic acid	–	+	+	–	–*	–	–	–	–
L-Isoleucine	+	+	+	+	+	–	+	+	+
L-Lysine	–	+	w	–	–	–	+	+	–
L-Threonine	+	+	+	+	–	–	+	+	+
Growth on YMA with/at:									
1 % NaCl	–	–	–	–	–	–	+	+	+
37 °C	+	+	+	–	–	+	–	–	+

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

The representative strain CCBAU 23303^T 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. I (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'se. 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-gluconate, hippuric acid, sodium succinate, sorbose, sucrose, calcium gluconate, D-fructose, D-mannose, sodium pyruvate, D-xylose 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^{–1}) ampicillin (50), kanamycin (5), neomycin sulfate (50), streptomycin (5), erythromycin (100), chloramphenicol (50) and gentamicin (100). Summed feature 8 (18:1ω6c and/or 18:1ω7c) and 16:0 are the dominant fatty acids. The type strain contains phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and cardiolipin.

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

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