Bradyrhizobium guangdongense sp. nov. and Bradyrhizobium guangxiense sp. nov., isolated from effective nodules of peanut

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Seven slow-growing rhizobia isolated from effective nodules of Arachis hypogaea were assigned to the genus Bradyrhizobium based on sharing 96.3–99.9 % 16S rRNA gene sequence similarity with the type strains of recognized Bradyrhizobium species. Multilocus sequence analysis of glnII, recA, gyrB and dnaK genes indicated that the seven strains belonged to two novel species represented by CCBAU 51649 T and CCBAU 53363 T. Strain CCBAU 51649 T shared 94, 93.4, 92.3 and 94.9 % and CCBAU 53363 T shared 91.4, 94.5, 94.6 and 97.7 % sequence similarity for the glnII, recA, gyrB and dnaK genes, respectively, with respect to the closest related species Bradyrhizobium manausense BR 3351 T and Bradyrhizobium yuanmingense CCBAU 10071 T. Summed feature 8 and C16 : 0 were the predominant fatty acid components for strains CCBAU 51649 T and CCBAU 53363 T. DNA–DNA hybridization and analysis of phenotypic characteristics also distinguished these strains from the closest related Bradyrhizobium species. The strains formed effective nodules on Arachis hypogaea, Lablab purpureus and Aeschynomene indica, and they had identical nodA genes to Bradyrhizobium sp. PI237 but were phylogenetically divergent from other available nodA genes at less than 66 % similarity. Based in these results, strains CCBAU 51649 T (=CGMCC 1.15034 T =LMG 28620 T) and CCBAU 53363 T (=CGMCC 1.15035 T =LMG 28621 T) are designated the type strains of two novel species, for which the names Bradyrhizobium guangdongense sp. nov. and Bradyrhizobium guangxiense sp. nov. are proposed, respectively.

Peanut or groundnut (Arachis hypogaea L.) belongs to the tribe Dalbergieae in subfamily Papilionoideae of the family Fabaceae, and is believed to have originated in South America (Lavin et al., 2001). Peanut is the third most important leguminous crop by harvest area (2.54 × 107 ha) worldwide, next to bean (3.06 × 107 ha) and soybean (11.2 × 107 ha), and China is the top producer of peanut, followed by India, Nigeria and USA (http://faostat3.fao.org/browse/Q/QC/E). In China, peanut serves as an extremely important source of vegetable oil (Ren, 2008). Screening for and application of a more efficient peanut endosymbiont in an inoculant are critical to improve the nitrogen fixation of peanut and to reduce the use of chemical nitrogen fertilizers. Peanut forms effective root nodules with diverse species within the genus Bradyrhizobium, although the species involved have not been

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CCBAU 51649 T and CCBAU 53363 T are KC508867 and KC508877, respectively; accession numbers are KC509023 and KC509033 for the glnII gene, KC509269 and KC509279 for the recA gene, KC509072 and KC509082 for the gyrB gene, KC508964 and KC508974 for the dnaK gene, KC509176 and KC509186 for the nodA gene, and KC509130 and KC509140 for the nifH gene. Fifty-one nucleotide sequences obtained in this study were deposited in the GenBank database (Table S1, available in the online Supplementary Material).

Five supplementary tables and ten supplementary figures are available with the online Supplementary Material.
clearly defined (Van Rossum et al., 1995; Urtz & Elkan, 1996; Zhang et al., 1999). Several authors have reported a lack of consistency between the results obtained with different taxonomic techniques applied to peanut bradyrhizobia (Van Rossum et al., 1995; Doignon-Bourcier et al., 2000). To date, two novel species of the genus Bradyrhizobium, Bradyrhizobium lablabi and Bradyrhizobium arachidis, have been reported to nodule peanut (Chang et al., 2011; Wang et al., 2013). For a better understanding of the diversity and population patterns of peanut rhizobia, and to apply efficient rhizobial inoculants in peanut production, it is important to improve the classification and identification of peanut rhizobia.

In an analysis of the genetic diversity of peanut rhizobia collected from Guangdong and Guangxi provinces in south-east China, seven isolates were detected as potentially representing two novel species of the genus Bradyrhizobium. Among them, strains CCBAU 51649T, CCBAU 51650, CCBAU 51658 and CCBAU 51773 were collected from two sites from Guangdong, and strains CCBAU 53344, CCBAU 53363T and CCBAU 53429 were collected from two sites from Guangxi; these comprised Groups A and B, respectively (Table S2). In the present study, these seven strains were systematically characterized by using a polyphasic approach and were defined as representing two novel species.

Fresh root nodules of peanut were collected from two fields in Guangdong and Guangxi in China in October 2011 (Table S2). Slow-growing, alkali-producing bacteria were isolated from surface-sterilized effective (red) nodules using a standard method on YMA plates incubated at 28 °C (Vincent, 1970). Colonies were translucent, cream white and convex, with a diameter of 1–2 mm on YMA medium after incubation for 7–10 days. All the isolates formed effective nodules with the host of origin under glasshouse conditions, as tested by using standard methods (Vincent, 1970). The isolates were preserved in YM broth with 20 % (w/v) glycerol at −80 °C and also stored by lyophilization.

Genomic DNA was extracted from each strain using the Wizard Genomic DNA Purification kit A1120 according to the manufacturer’s instructions (Promega). The 16S rRNA gene (Tan et al., 1997), housekeeping genes glnII, recA, gyrB and dnaK (Vinuesa et al., 2005b; Rivas et al., 2009), and symbiosis-related genes nodA and nifH (Chaintreuil et al., 2001; Laguerre et al., 2001) were amplified using the extracted DNA as template and the corresponding published primers and PCR protocols. All the amplicons were sequenced commercially by BGI. The sequences were aligned with those of the corresponding genes for the type strains of recognized Bradyrhizobium species extracted from the NCBI nucleotide sequence database using the BLAST search program. Phylogenetic trees were reconstructed using the maximum-likelihood (ML) and neighbour-joining (NJ) methods with 1000 bootstrap replications for all the single gene sequences, as well as for the concatenated sequence analysis of the housekeeping genes glnII, recA, gyrB and dnaK or glnII and recA. The genetic distances between sequences were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic analyses were performed with programs in the MEGA 5.1 software package (Tamura et al., 2011).

The ML tree (Fig. 1) and NJ tree (Fig. S1) based on 16S rRNA gene sequences (1232 nt) showed a consistent topology with respect to the relationships among the novel strains and recognized Bradyrhizobium species. The four Group A isolates had identical sequences, which shared highest similarity of 99.9 % with Bradyrhizobium ganzhouense RITF806T (Lu et al., 2014) and 96.3–99.8 % with the reference type strains of other Bradyrhizobium species and the Group B strains. The three Group B strains also had identical 16S rRNA gene sequences, which shared highest similarity of 99.8 % with Bradyrhizobium diazoefficiens USDA 110T and 96.6–99.7 % with the other reference type strains of the genus Bradyrhizobium.

As the novel strains showed 16S rRNA gene sequence similarities of more than 97 % with the closest reference species, housekeeping genes were used to further clarify their relationships with defined Bradyrhizobium species. The housekeeping genes glnII (505 nt), recA (380 nt), gyrB (596 nt) and dnaK (229 nt) were partially sequenced and a phylogenetic tree was generated based upon the concatenated sequences with the ML (Fig. 2) and NJ (Fig. S2) methods. The level of similarity of concatenated partial sequences of the glnII, recA, gyrB and dnaK genes (1710 nt) was 100 % for the four Group A isolates, and they showed highest sequence similarity of 94, 93.4, 92.3 and 94.9 %, respectively, with Bradyrhizobium manausense BR 3351T (Silva et al., 2014) (Fig. 2, Table S2), which is also their sister taxon in the 16S rRNA gene tree, although B. ganzhouense RITF806T showed fewer nucleotide differences (Fig. 1). The three strains of Group B also had identical combined sequence for the core genes and shared highest similarity of 91.4, 94.5, 94.6 and 97.7 %, respectively, with Bradyrhizobium yuanmingense CCBAU 10071T (Yao et al., 2002) (Fig. 2, Table S2), although the closest neighbour was B. diazoefficiens USDA 110T in the 16S rRNA gene sequence tree (Fig. 1). Although our results suggest that the 16S rRNA gene phylogeny is not consistent with that of the combined housekeeping genes (Zilli et al., 2014), neither phylogeny is well resolved in this subclade of Bradyrhizobium, and none of the discrepancies is supported by significant bootstrap values (>70 %). The recently described species Bradyrhizobium lupini (Peix et al., 2015) was not closely related to the two groups based on analysis of the combined glnII and recA gene sequences (Figs S3 and S4). All these results, and especially the clearly distinct multilocus sequences (Fig. 2), support the proposal that Groups A and B represent two novel species in the genus Bradyrhizobium.

As a standard method for species delineation recommended by Graham et al. (1991), DNA–DNA
hybridization was performed by the renaturation rate technique (De Ley et al., 1970) with the total DNA of the reference (proposed type) strain of each novel group compared with the other strains in the same group, the type strain of the other novel group, and the type strains of the closest related recognized species. The level of DNA–DNA relatedness ranged from 84.3 to 98.8 % between CCBAU 51649T and the other isolates (CCBAU 51650, CCBAU 51658 and CCBAU 51773) of Group A, and was 91.3 and 94.1 % between CCBAU 53363T and the other two isolates of Group B. The two reference strains CCBAU 51649T and CCBAU 53363T for the novel groups shared 40.2 % relatedness with each other, and 34 and 48 % relatedness with the closest related species B. manausense BR 3351T and B. yuanmingense CCBAU 10071T, respectively (Table S3), values lower than the 70 % species threshold (Graham et al., 1991). These results indicate that the seven strains represent two novel genomic species. The DNA G+C content was 62.33 mol% for CCBAU 51649T and 62.87 mol% for CCBAU 53363T, measured by the thermal denaturation method (De Ley et al., 1970). These values are within the range described for the genus Bradyrhizobium (Jordan, 1982).

Previously, the BOX-PCR procedure has proved a valuable method to distinguish closely related rhizobia, such as strains in the same species (Wang et al., 2013); we used the BOXAIR primer and procedure (Versalovic et al., 1994) for the seven test strains. The fingerprinting profiles (Fig. S5) distinguished the seven isolates from each other, illustrating that they were not clones of the same strain, despite the lack of diversity in the genes that were sequenced.

Cellular fatty acid profiles were determined to provide additional description for the novel species. Representative

Fig. 1. Phylogenetic tree generated with the ML method based on 16S rRNA gene sequences, showing the phylogenetic position of the novel rhizobia associated with Arachis hypogaea. GenBank accession numbers are indicated in parentheses. Bar, 1% nucleotide substitutions per site. Bootstrap values greater than 50% are indicated at branches.
strains CCBAU 51649\(^T\) and CCBAU 53363\(^T\) and the closest related type strains \(B.\) \(yuanmingense\) CCBAU 10071\(^T\) and \(B.\) \(manausense\) BR 3351\(^T\) for each group were used. Each strain was streaked on YMA medium and cultivated at 28 °C. About 40–100 mg of wet cells at the exponential growth phase was harvested, and then cellular fatty acids were extracted following the method described by Sasser (1990) and identified by the MIDI Sherlock Microbial Identification System (Sherlock licence CD v 6.0) in the TSBA6 database. Summed feature 8 (C\(_{18:1}\)\(\omega 6c/C_{18:1}\)\(\omega 7c\)) and \(C_{16:0}\) were detected as the predominant components for strains CCBAU 51649\(^T\) (80.4 and 15.7 %, respectively), CCBAU 53363\(^T\) (84.8, 10.7 %), \(B.\) \(yuanmingense\) CCBAU 10071\(^T\) (82.1, 11.4 %) and \(B.\) \(manausense\) BR 3351\(^T\) (64.3, 21 %) (Table S4), which were similar to the results previously reported for \(Bradyrhizobium\) species, but different from that of \(Mesorhizobium\) and \(Rhizobium\) (Tighe et al., 2000; Wang et al., 2013).

Phenotypic characterization was performed based on the characteristics that have been found to be useful for \(Bradyrhizobium\) species differentiation (Gao et al., 1994; Yao et al., 2002; Vinuesa et al., 2005a; Ramírez-Bahena et al., 2009). Six strains representing the two novel groups, plus \(B.\) \(yuanmingense\) CCBAU 10071\(^T\) and \(B.\) \(manausense\) BR 3351\(^T\) were analysed. Although analysis of the population genomics of \(Rhizobium leguminosarum\) indicated that metabolic characteristics are not good taxonomic (genospecies-level) markers (Kumar et al., 2015), such characters do at least offer more descriptive features for the type strain and reference strains. The assimilation of sole carbon sources was tested by using a Gram-Negative Microplate (Biolog GN2) according to the manufacturer’s instructions. Tolerance to NaCl (1–5 %, w/v), pH (4, 5, 6, 7, 8, 9 and 10) and temperature growth ranges (4, 10, 20, 28 and 35 °C), antibiotic resistance (chloramphenicol, rifampicin, gentamicin, kanamycin, nalidixic acid, streptomycin, gentamicin, kanamycin, nalidixic acid, streptomycin,
B. diazoefficiens was most closely related (93.2% similarity) to that of 51658 (Group A) had a very different relationship for the tested strains compared with those of Bradyrhizobium species, supporting that this nodA gene type was novel, as reported by Muñoz et al. (2011). They formed a deeply distinct clade (Clade VIII) and shared highest nodA gene sequence similarity of 65.3% with Bradyrhizobium sp. NAS144 isolated from Lupinus albus, and 60–64.4% similarity with the reference strains of all recognized Bradyrhizobium species, suggesting that this nodA gene type in peanut rhizobia in China, and suggests that South American peanut-specific nodulation genes may have been imported into China from peanut seeds, as suggested for bean rhizobia (Pérez-Ramirez et al., 1998). Phylogenetic analysis of dnaK gene sequences showed that Bradyrhizobium sp. PI237 and CH81 did not belong the two novel species proposed in this study, as maximal sequence similarity of 98.2% was detected between Bradyrhizobium sp. PI237 and B. arachidis CCBAU 051107\(^T\) and Bradyrhizobium huanhuaihaniense CCBAU 23303\(^T\) and between CH81 and Bradyrhizobium daqingense CCBAU 15774\(^T\) (data not shown).

We cannot determine, on the present evidence, whether the novel species themselves came with the seeds, or are local species that acquired the peanut-nodulation genes from imported rhizobia, a phenomenon that has been described several times (Sullivan & Ronson, 1998; Nandasena et al., 2007; García-Fraile et al., 2010).

To further identify the host range of the novel isolates, we conducted cross-inoculation tests in vermiculite with N-free plant nutrient solution in glasshouse conditions (Vincen, 1970). The seven strains nodulated Arachis hypogaea, Lablab purpureus and Aeschynomene indica, with pink cross-sections of the nodules, indicating that the nodules were effective in fixing nitrogen. These strains induced ineffective nodules on Phaseolus vulgaris, with white cross-section of the nodules. None of the isolates nodulated Medicago sativa (alfalfa), Trifolium repens (clover), Glycine max (soybean) or Vigna radiata (mung bean).

Based upon the phenotypic and genotypic characteristics, two novel species are proposed, represented by the type strains CCBAU 51649\(^T\) for Bradyrhizobium guangdongense sp. nov. and CCBAU 53363\(^T\) for Bradyrhizobium guangxiense sp. nov.

**Description of Bradyrhizobium guangdongense sp. nov.**

Bradyrhizobium guangdongense (guang.dong.en’se. N.L. neut. adj. guangdongense originating from Guangdong Province of China, from where the strains were isolated).

Cells are Gram-negative, non-spore-forming, aerobic rods (Fig. S10). Colonies are translucent, cream white and

![Table 1. Characteristics that distinguish the two novel species and their closest relatives](http://ijs.microbiologyresearch.org)
convex, with a diameter of 1–2 mm on YMA medium after incubation for 7–10 days at 28 °C. Cells of the type strain CCBAU 51649T are 0.34–0.56 × 1.43–2.12 μm in size and have a generation time of 16.94 h when grown in YM medium at 28 °C. Grows at 15–28 °C, with an optimum temperature of 28 °C. The pH for growth ranges from 5 to 7, with optimum of pH 7.0. Grows weakly in the presence of 1.0 % (w/v) NaCl but not in more than 1 % (w/v) NaCl. Resistant to gentamicin (30 μg ml⁻¹) and trimethoprim (100 μg ml⁻¹), but not to chloramphenicol, kanamycin, nalidixic acid, streptomycin, rifampicin or tetracycline at the minimum dose tested (20 μg ml⁻¹). Can use adonitol, D-fucose, D-sorbitol, citric acid, D-ketogluartic acid and propionic acid but not Tween 40, gentiobiose, sucrose or malonic acid as sole carbon sources. Forms effective nodules with peanut plants (their host of origin), and *Lablab purpureus* and *Aeschynomene indica* induces ineffective root nodules on *Phaseolus vulgaris*, but cannot nodulate with *Medicago sativa*, *Trifolium repens*, *Glycine max* or *Vigna radiata*.

The type strain is CCBAU 51649T (= CGMCC 1.15034T = LMG 28260T). The DNA G+C content of the type strain is 62.33 mol% (Tm). CCBAU 51650, CCBAU 51658 and CCBAU 51773 are additional strains of the species.

**Description of Bradyrhizobium guangxiense sp. nov.**

*Bradyrhizobium guangxiense* (guang.xi.en’se. N.L. neut. adj. guangxiense originating from Guangxi Province of China, from where the strains were isolated).

Cells are Gram-negative, non-spore-forming, aerobic rods (Fig. S10). Colonies are translucent, cream white and convex, with a diameter of 1–2 mm on YMA medium after incubation for 7–10 days at 28 °C. Cells of the type strain CCBAU 53363T are 0.31–0.48 × 1.43–2.01 μm in size and have a generation time of 13.49 h when grown in YM medium at 28 °C. Grows at 15–28 °C well, but weakly at 37 °C, with an optimum temperature of 28 °C. The pH for growth ranges from 5 to 7, with optimum of pH 7.0. Grows weakly in the presence of 1.0 % (w/v) NaCl but not in more than 1 % (w/v) NaCl. Resistant to (100 μg ml⁻¹) chloramphenicol, gentamicin, nalidixic acid, streptomycin and tetracycline, but not to kanamycin, trimethoprim or rifampicin at the minimum dose tested (20 μg ml⁻¹). Utilizes Tween 40, D-fucose, citric acid, D-ketogluartic acid and propionic acid but not adonitol, D-sorbitol, gentiobiose, sucrose or malonic acid as sole carbon sources. Induces effective nodules on peanut plants, *Lablab purpureus* and *Aeschynomene indica* and ineffective root nodules on *Phaseolus vulgaris*, but cannot nodulate with *Medicago sativa*, *Trifolium repens*, *Glycine max* or *Vigna radiata*.

The type strain is CCBAU 53363T (= CGMCC 1.15035T = LMG 28261T). The DNA G+C content of the type strain is 62.87 mol% (Tm). CCBAU 53344 and CCBAU 53429 are additional strains of the species.

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**References**


Two novel Bradyrhizobium species


