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 51649T and CCBAU 53363T. Strain CCBAU 51649T shared 94, 93.4, 92.3 and 94.9 % and CCBAU 53363T 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 3351T and Bradyrhizobium yuanmingense CCBAU 10071T. Summed feature 8 and C16 : 0 were the predominant fatty acid components for strains CCBAU 51649T and CCBAU 53363T. 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 51649T (=CGMCC 1.15034T=LMG 28620T) and CCBAU 53363T (=CGMCC 1.15035T=LMG 28621T) 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 Dalbergiae 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
clearly defined (Van Rossum et al., 1995; Utz & 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 nodulate peanut (Chang et al., 1996; Zhang et al., 1995; Doignon-Bourcier et al., 2002) (Fig. 1, Table S2), although the closest isolate was not closely related to the two groups based on analysis of the concatenated partial sequences (S3 and S4). All these results suggest that the novel species 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 %).

As a standard method for species delineation recommended by Graham et al. (1991), DNA–DNA 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* RITF806\(^{T}\) (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 110\(^{T}\) 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 3351\(^{T}\) (Silva et al., 2014) (Fig. 2, Table S2), which is also their sister taxon in the 16S rRNA gene tree, although *B. ganzhouense* RITF806\(^{T}\) 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 10071\(^{T}\) (Yao et al., 2002) (Fig. 2, Table S2), although the closest neighbour was *B. diazoefficiens* USDA 110\(^{T}\) in the 16S rRNA gene sequence tree (Fig. 1). Although our results suggest that the novel species 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.

http://ijs.microbiologyresearch.org 4657
strains CCBAU 51649T and CCBAU 53363T and the closest related type strains B. yuanmingense CCBAU 10071T and B. manausense BR 3351T 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 (C18:1 v 6.0) was detected as the predominant components of strains CCBAU 51649T (80.4 and 15.7 %, respectively), CCBAU 53363T (84.8, 10.7 %), B. yuanmingense CCBAU 10071T (82.1, 11.4 %) and B. manausense BR 3351T (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; Ramirez-Bahena et al., 2009). Six strains representing the two novel groups, plus B. yuanmingense CCBAU 10071T and B. manausense BR 3351T 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 is most closely related (93.2% similarity) to that of 51658 (Group A) had a very different nodA gene sequence, list in Table 1 and a full characterization based on aBLAST search of the NCBI website. In the nodA gene trees, all strains of both Group A and Group B unexpectedly had identical sequences to those of Bradyrhizobium sp. PI237 and CH81, both of which were isolated from peanut in Argentina (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, supporting that this nodA gene type was novel, as reported by Muñoz et al. (2011). This is the first report to find this novel 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 051107T and Bradyrhizobium huangguanhaiense CCBAU 23303T and between CH81 and Bradyrhizobium daqingense CCBAU 15774T (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 (Vincent, 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 (alfafa), 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 51649T for Bradyrhizobium guangdongense sp. nov. and CCBAU 53363T 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

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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, l-fucose, d-sorbitol, citric acid, α-ketoglutaric 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, l-fucose, citric acid, α-ketoglutaric 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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