Bradyrhizobium arachidis sp. nov., isolated from effective nodules of *Arachis hypogaea* grown in China

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**A B S T R A C T**

Twenty-three bacterial strains isolated from root nodules of *Arachis hypogaea* and *Lablab purpureus* grown in five provinces of China were classified as a novel group within the genus Bradyrhizobium by analyses of PCR-based RFLP of the 16S rRNA gene and 16S–23S IGS. To determine their taxonomic position, four representative strains were further characterized. The comparative sequence analyses of 16S rRNA and six housekeeping genes clustered the four strains into a distinctive group closely related to the defined species Bradyrhizobium liaoningense, Bradyrhizobium yuanmingense, Bradyrhizobium huanghuaiaeense, Bradyrhizobium japonicum and Bradyrhizobium daqingense. The DNA–DNA relatedness between the reference strain of the novel group, CCBAU 05110\(^7\), and the corresponding type strains of the five mentioned species varied between 46.05% and 13.64%. The nodC and rpiH genes of CCBAU 05110\(^7\) were phylogenetically divergent from those of the reference strains for the related species. The four representative strains could nodulate with *A. hypogaea* and *L. purpureus*. In addition, some phenotypic features differentiated the novel group from the related species. Based on all the results, we propose a new species Bradyrhizobium arachidis sp. nov. and designate CCBAU 05110\(^7\) (=CGMCC 1.12100\(^T\) = HAMBI 3281\(^T\) = LMG 26795\(^T\)) as the type strain, which was isolated from a root nodule of *A. hypogaea* and had a DNA G+C mol% of 60.1 (Tm).

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*Arachis hypogaea* L. (peanut or groundnut) is an important crop that provides food for direct human consumption and materials for the food industry. It plays a significant part in the economy of many countries of the world, including China which is the world's largest peanut producer ([http://en.wikipedia.org/wiki/Peanut](http://en.wikipedia.org/wiki/Peanut)). Peanut mainly forms effective root nodules with the bradyrhizobia, such as the species *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium lablabi*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium iriomotense* [1,15,23], but rarely with the fast-growing rhizobia [3,23].

In the present study, 23 bacterial strains isolated from effective nodules of *A. hypogaea* and *Lablab purpureus* were analyzed by a polyphasic approach including molecular and phenotypic analyses. Based on the results, a new species *Bradyrhizobium arachidis* sp. nov. is proposed.

Root nodules of *A. hypogaea* and *L. purpureus* were collected from the fields of five provinces (temperate and subtropical regions) of China ([Supplementary Fig. S1](#)).

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**Abbreviations:**
IGS, 16S–23S rRNA intergenic spacer; NJ, neighbor-joining; ML, maximum likelihood; MSA, multilocus sequence analysis; RFLP, restriction fragment length polymorphism.


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order to clarify their taxonomic position further, the strains CCBAU 051107T, CCBAU 45332, CCBAU 23155 and CCBAU 33067, representing the two IGS types, two hosts and different geographic regions were chosen for subsequent studies.

Previously reported primers and procedures were adopted for the amplification and sequencing of the 16S rRNA gene [22], atpD, recA and glnII genes [28], dnaK, gyrB and rpoB genes [17], and partial nodC and nifH genes [11]. These sequences were aligned with those of related Bradyrhizobiun species obtained from the NCBI database using the Clustal W program in the MEGA 5.0 software package [21]. The sequences were also analyzed using the MEGA 5.0 software to generate the Jukes-Cantor (for 16S RNA) or Kimura 2-parameter (other genes) distance [7,8], and to construct unrooted phylogenetic trees by the neighbor-joining (NJ) method [18], which was bootstrapped based on 1000 replications. Maximum likelihood (ML) trees were constructed using the PhyML 3.0 package [6] and the robustness of ML tree topologies was inferred by non-parametric bootstrap tests based on 100 pseudo-replicates. The nucleotide substitution model was selected by the Akaike information criterion (AIC), as implemented in Modeltest 3.7 [16]. For deduced amino acid sequences, the pairwise evolutionary distances used in the ML trees were produced by the Jones–Taylor–Thornton (JTT) model in MEGA 5.0.

The NJ tree of the 16S rRNA genes (Fig. 1) presented relationships similar to those revealed in the ML tree (Supplementary Fig. S3). The species of Bradyrhizobium were clustered into two groups, similar to those in previous reports [13]. The four test strains shared similarities of >99.92% and were found in group I (represented by B. japonicum), and showed similarities between 98.6% and 99.85% with the type strains of the ten defined species in the group. On the other hand, the similarities of the test strains with the four type strains in group II (represented by B. elkanii) were between 96.62% and 96.78% (Supplementary Table S1).

Currently, twelve symbiotic species and two non-symbiotic species have been described in the genus Bradyrhizobium [32]. The high similarities among the 16S rRNA gene sequences of the defined Bradyrhizobium species [31] demonstrated the necessity for additional differentiation analyses. Previously, the phylogenetic analyses of the housekeeping genes atpD, glnII, recA, dnaK, gyrB and rpoB have been used to define the Bradyrhizobium species and the deduced relationships were in agreement with the results of DNA–DNA hybridization [17,28]. In the present study, the phylogenetic relationships derived from the concatenated sequences of these six genes (Fig. 2) were similar to those obtained in the 16S rRNA analysis (Fig. 1). The four representative strains occupied a clearly separated branch within the genus Bradyrhizobium, which was supported by 100% bootstrap values and showed closer relationships to the species Bradyrhizobium liaoningense, Bradyrhizobium huanghuaihaense, Bradyrhizobium yuanmingense, and Bradyrhizobium daqingense [29] than to the other species.

In the ML trees constructed separately for each of the six housekeeping genes, the relationships in the glnII, gyrB or recA trees (Supplementary Fig. S4) were generally congruent with each other, the concatenated trees of all six genes (Fig. 2) and the 16S rRNA genes tree (Fig. 1). Similar to previous results [17,28,33], incongruent phylogenetic relationships for some species were observed in the atpD, dnaK and rpoB single gene phylogenetic trees (Supplementary Fig. S4), which may be related to recombination, migration or lateral gene transfer [17,28,33].

The phylogenetic tree (ML) of the deduced amino acid sequences of the six housekeeping genes showed similar relationships with those of the corresponding genes (Supplementary Fig. S5).

As a standard method for species definition [5,25], DNA–DNA hybridization was performed in triplicate between the reference strain CCBAU 051107T and representative strains of the novel group (CCBAU 45332, CCBAU 23155, CCBAU 33067), and the type strains for the related species by the renaturation-rate technique [2]. The DNA–DNA relatedness (Supplementary Table S1) between CCBAU 051107T and the other three representative strains was from 76.32% to 94.56%. However, these values varied between 13.64% and 46.05% with the type strains of related Bradyrhizobium species, which were much lower than the species threshold (70%) [30]. These data indicated that the new strains represented a novel genomic species within the genus Bradyrhizobium. The DNA G + C mol% of the four novel strains, measured by the thermal denaturation method [2], varied between 60.1 and 60.5, which was within the range for the genus Bradyrhizobium.

BOX-PCR was performed by the reported procedure [26] in order to characterize the four tested strains and type strains for five related species. A unique BOX-PCR fingerprint was obtained for each strain (Supplementary Fig. S6), indicating that the four representative strains were not clones.

Phenotypic features of two representative strains and the type strains of closely related species were determined according to Gao et al. [4]. The tested features included utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance to NaCl and pH, and temperature growth ranges. Biochemical tests, including activities of catalase, urease, oxidase and nitrate reductase, reduction of litmus milk, Nile blue and methylene blue were performed according to Smibert and Krieg [20]. Twenty different features for the novel group and reference strains are shown in Table 1 and the detailed features are presented in the subsequent description of the novel species.

Fatty acid profiling has been suggested for identifying root-nodule bacteria [24]. Cellular fatty acids of strain CCBAU 051107T were assayed in comparison with B. yuanmingense CCBAU 1007T, B. daqingense CCBAU 15774T, B. liaoningense USDA 3622T and B. japonicum USDA 6T. The strains were grown to the late logarithmic phase in TY broth (tryptone, 5 g; yeast extract, 3 g; CaCl2, 0.6 g; distilled water, 1 L; pH 7.2) at 28 °C, and they were then centrifuged to obtain the bacterial cells, followed by washing three times with 0.8% NaCl solution. Fatty acid methyl esters were prepared and separated by the method of Sasser [19], and were identified by the MIDI Sherlock Microbial Identification System (Sherlock license CD v 6.0) in the TSBA6 database. The detailed results are available in Supplementary Table S2. Eight to twelve fatty acids were detected from different strains and eleven were found in strain CCBAU 051107T. Most of the fatty acids were common in the Bradyrhizobium strains, while summed feature 8 (18:1ω7c/18:1ω7c, 76.71–85.04%) and 16:0 (7.28–10.51%) were the dominant compounds. Quantitative differences between the strains were observed in the components of 16:1ω5c, 17:1ω8c, 17:1ω6c and 17:0. The concentrations of 17:1ω6c (4.73%), 17:1ω7c (3.91%) and 17:0 (3.18%) in CCBAU 051107T were much higher than those (0.67–1.94%, 0.48–1.19% and 0.47–1.11%, respectively) in other reference strains.

Respiratory quinones and polar lipids for strain CCBAU 051107T were analyzed by HPLC, as described by Lee et al. [12], and by two-dimensional TLC following the procedures of Minnikin et al. [14]. The respiratory quinone was ubiquinone-10 (Q-10). The major polar lipids were cardiolipin or diphosphatidylglycerol (CL or DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC). In addition, an unknown amino lipid (AL) and four unknown minor polar lipids (PL) were detected (Supplementary Fig. S7). The result was similar to a previous report for Bradyrhizobium species [29].

nodC and nifH are symbiotic genes required for the successful establishment of highly specific nitrogen-fixing symbiosis with the host legumes, therefore, the comparison of these genes may reveal the host ranges of rhizobia [11]. The results of phylogenetic analysis are presented in Supplementary Figs. S8 and S9 for nifH and nodC, respectively. The nifH gene of strain CCBAU 33067 isolated
Fig. 1. Neighbor-joining tree reconstructed from 16S rRNA gene sequences showing the phylogenetic relationships of the strains of *B. arachidis* sp. nov. Bootstrap values greater than 70% are indicated at the nodes. The sequence of *Rhizobium leguminosarum* USDA 2370T was used as an out group. Bar: 1% substitution. Sequence accession numbers of the 16S rRNA genes are presented in parenthesis.

Fig. 2. ML tree based on partial concatenated sequences of *atpD*, glnII, recA, dnaK, gyrB and rpoB genes, showing the phylogenetic position of *B. arachidis* sp. nov. within the genus *Bradyrhizobium*. Bootstrap values greater than 50% based on 100 replicates are shown at the nodes. The sequence of *Rhizobium etli* CFN 427 was used as an out group. Bar: 10% substitution. Sequence accession numbers in the order of *atpD*, glnII, recA, dnaK, gyrB and rpoB genes are presented in parenthesis.
from *L. purpureus* was different from the strains originating from *A. hypogaea*. The topology of the *nodC* phylogenetic tree was very similar to that of the *nifH* tree. The low similarities of *nifH* (81.4–94%) and *nodC* (64.1–88.1%) to those of the reference strains, such as strains originating from soybean and *Lespedeza*, indicated an independent evolutionary history for these genes in the peanut rhizobia.

To confirm the symbiotic ability of the strains further, cross-nodulation tests were performed in vermiculate moistened with N-free solution [27]. The four representative strains could effectively nodulate with *A. hypogaea* and *L. purpureus*, as shown by the red nodules and healthy plants with green leaves, but they did not nodulate with *Medicago sativa*, *Mellilotus officinalis*, *Trifolium repens*, *Phaseolus vulgaris* or *Glycine max*.

Based on the phenotypic and genotypic characteristics, we propose that the twenty-two strains isolated from *A. hypogaea* and one strain isolated from *L. purpureus* represent a novel species, *B. arachidis* sp. nov. with CCBAU 051107T as the type strain.

**Description of *B. arachidis* sp. nov.**

*B. arachidis* (a.ra.chi.dis. N.L. gen. n. arachidis, of Arachis, referring to the fact that the bacterium was mainly isolated from root nodules of *A. hypogaea* L.).

The cells are Gram-negative, aerobic, non-spore-forming rods of 0.46–0.53 µm × 1.30–1.97 µm (Supplementary Fig. S10). Colonies on YMA medium are circular, convex and translucent, and have a diameter of 1 mm within 7–10 days incubation at 28°C. The generation time is 8.8 h in YM broth. The pH range for growth is 6–8, with optimum growth at pH 7.0. Growth occurs between 20°C and 30°C, with optimum growth at 28°C. The bacteria do not grow in the presence of 1% NaCl or higher. The type strain can utilize d-glucose, sodium acetate, sodium citrate, sodium formate and sodium pyruvate, but cannot utilize adipic acid, d-melezitose, l-threonine, l-glycine, salicin, sorbitol, maltose and mycose as sole carbon sources. It can use l-arginine, l-phenylalanine and l-threonine, but cannot use d-glutamic acid, l-lysine and l-methionine as sole nitrogen sources. The type strain is resistant to (µg mL⁻¹) streptomycin sulfate (5), erythromycin (5, 50), gentamycin sulfate (5, 50) and chloramphenicol (5, 50), but sensitive to ampicillin (100) and streptomycin sulfate (50). Negative for sodium nitrate reduction, but positive for catalase. Growth in nutrient broth, YMA medium with 0.1% Congo red or with 0.1% bromothymol blue, respectively. Summed feature 8 (C₁₈:₁ω₇c/C₁₈:₁ω₇c) and C₁₀₀₆ω were the most dominant fatty acids. Detailed additional features are shown in Table 1 and Supplementary Table S2. The strains can be distinguished from related species by their housekeeping genes and by DNA–DNA hybridization (Supplementary Table S1).

The type strain CCBAU 051107T (=CGMCC 1.12100T = LMG 26795T = HAMBI 3281T) was isolated from an effective nodule of *A. hypogaea* grown in Hebei Province, China. The DNA G+C content of the type strain is 60.14% (Tm).

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.10.009.

**References**


