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

Rui Wang[a,1], Yue Li Chang[a,b,1], Wen Tao Zheng[a], Dan Zhang[a], Xiao Xia Zhang[c], Xin Hua Sui[a,*], En Tao Wang[d], Jia Qi Hu[a], Li Ya Zhang[a], Wen Xin Chen[a]

[a] *State Key Laboratory for Agro-Biotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China*
[b] *College of Lab Medicine, Hebei North University, Zhangjiakou 075000, China*
[c] *Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China*
[d] *Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, 11340 Mexico, DF, Mexico*

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ABSTRACT

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 huanghuaihaiense*, *Bradyrhizobium japonicum* and *Bradyrhizobium daqingense*. The DNA–DNA relatedness between the reference strain of the novel group, CCBAU 051107^T, and the corresponding type strains of the five mentioned species varied between 46.05% and 13.64%. The *nodC* and *nifH* genes of CCBAU 051107^T 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 051107^T (=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).

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). Peanut mainly forms effective root nodules with the bradyrhizobia, such as the species *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium lablabi*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium iriomotense* [1,3,15,23], 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) and were used for isolation of rhizobia by the standard YMA medium method [27]. All the isolates grew slowly and produced alkali, forming mucoid colonies with a diameter of approximately 1 mm after 7 days incubation at 28 °C. In the restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene using the method of Laguerre et al. [9], 22 isolates from *A. hypogaea* and one from *L. purpureus* shared the same patterns that were almost identical to those of the strain *B. yuanmingense* CCBAU 10071^T (Supplementary Fig. S1). The RFLP fingerprints of 16S–23S rRNA IGS (intergenic spacer) obtained by the reported method [10] divided these 23 isolates into two IGS types (RFLP patterns), which were clustered together at 77% similarity, and showed close relationships with *B. yuanmingense* CCBAU 10071^T and *B. japonicum* USDA 6^T (Supplementary Fig. S2). In

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

☆ *Accession numbers for type strain*: 16S rRNA gene: HM107167; *atpD* gene: HM107217; *glnII* gene: HM107251; *recA* gene: HM107233; *dnaK* gene: JX437668; *gyrB* gene: JX437675; *rpoB* gene: JX437682; *nifH* gene: HM107283 and *nodC* gene: HM107267.

* Corresponding author. Tel.: +86 10 62734009; fax: +86 10 62734008.
E-mail address: suixh@cau.edu.cn (X.H. Sui).

[1] These two authors contributed equally to this work.

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order to clarify their taxonomic position further, the strains CCBAU 051107^T, 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 *Bradyrhizobium* 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 huanghuaihaiense*, *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 051107^T 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 051107^T 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 051107^T were assayed in comparison with *B. yuanmingense* CCBAU 10071^T, *B. daqingense* CCBAU 15774^T, *B. liaoningense* USDA 3622^T and *B. japonicum* USDA 6^T. The strains were grown to the late logarithmic phase in TY broth (tryptone, 5 g; yeast extract, 3 g; $CaCl_2$, 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 051107^T. Most of the fatty acids were common in the *Bradyrhizobium* strains, while summed feature 8 (18:1 ω6*c*/18:1 ω7*c*, 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 16:1ω5*c*, 17:1ω8*c*, 17:1ω6*c* and 17:0. The concentrations of 17:1 ω8c (4.73%), 17:1 ω6*c* (3.91%) and 17:0 (3.18%) in CCBAU 051107^T 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 051107^T 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 aminolipid (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