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# Genetic diversity and distribution of bradyrhizobia nodulating peanut in acid-neutral soils in Guangdong Province

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ABSTRACT

To reveal the genetic diversity and geographic distribution of peanut (*Arachis hypogaea* L.) rhizobia in Guangdong Province, one of the main peanut producing regions in China, 216 bradyrhizobial isolates were trapped by peanut plants inoculated with soil samples (pH 4.7–7.4) collected from ten sites in Guangdong. Based on BOX-PCR fingerprinting analysis, 71 representative isolates were selected for sequence analyses of ribosomal IGS, *recA*, *atpD* and symbiotic gene *nodA*. As a result, 22 genospecies were detected in the peanut rhizobia, including eight minor groups or single strains corresponding to *Bradyrhizobium diazoefficiens*, *B. japonicum*, *B. yuanmingense*, *B. arachidis*, *B. guangdongense*, *B. guangxiense*, *B. iriomotense* and *B. liaoningense*, as well as 14 novel *Bradyrhizobium* genospecies covering the majority of isolates. Five symbiotic clusters were obtained based on the phylogenetic relationships of *nodA* genes, related to the soybean-nodulating or peanut-nodulating reference strains. Biogeographic patterns, which were mainly correlated with potassium content and pH, were detected in the peanut bradyrhizobial community in Guangdong Province. These findings enriched the diversity of peanut rhizobia, and added the K content as a special determinant for peanut rhizobial distribution in acid soils.



## Introduction

Peanut (*Arachis hypogaea* L.), also known as groundnut, is an important oil and grain crop with great economic significance. This plant forms nitrogen-fixing root nodules with some soil bacteria, mainly the slow-growing rhizobia corresponding to *Bradyrhizobium arachidis*, *B. japonicum*, *B. elkanii*, *B. guangdongense*, *B. guangxiense*, *B. lablabi*, *B. iriomotense*, *B. vignae* and *B. yuanmingense* [4,9,11,21,23,29,32,40,42,44]. In addition, a few fast-growing rhizobia belonging to the genus *Rhizobium* have been isolated from peanut nodules in Moroccan and Argentinean soils, which were closely related to *R. huautlense*, *R. galegae* [9], *R. giardinii* and *R. tropici* [14,32].

Several studies have shown the effects of soil conditions on the legume-rhizobia symbiotic process and nitrogen fixation; and biogeographic patterns have been revealed in the soybean and bean rhizobia that were correlated to soil pH and salinity in China [3,10,19,43,45], or to latitude in the United States [30]. For peanut rhizobia, it has been reported that their genotype richness and diversity depend on the land use system [29], similar to the soybean bradyrhizobia in black soil impacted by land use and crop management [43]. Peanut plants grow preferably in acidic and neutral soils with pH 5.9–7.0. However, the soil physicochemical characteristics have been ignored in many of the previous studies on the diversity of peanut bradyrhizobia, and the peanut bradyrhizobia biogeographic patterns and distribution in the acidic soils in South China are still unclear.

Peanut was introduced to China from South Africa five hundred years ago [46] and the production of peanut in China was more than 16 million tons in 2011 and 2012 (http://faostat3.fao.org/), accounting for approximately 45% of the overall world production. Huanghuaihai (or Northern) Plain, the drainage area of the Yangtze River, and the southeast coastal region are the three main regions for peanut production in China. Although Guangdong is the most important peanut planting region in South China and is one of the five largest planting provinces in China, the unit yield of peanut in

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Guangdong is lower than the average level in the rest of China. In addition, the diversity of peanut rhizobia in this area has not been fully described yet.

Considering all the information mentioned above, the present study was performed to identify the diversity of peanut bradyrhizobia in southern China and to assess their geographic distribution.

## Materials and methods

### *Isolation of symbiotic rhizobial strains*

Soil samples were collected from peanut fields in Guangdong Province after harvesting or before planting in ten different sites with no record of rhizobial inoculation (Table 1, also Supplementary Tables S1 and S2). Physicochemical properties of the soils, including total organic carbon, pH and contents of nitrogen, phosphorus and potassium were measured with routine methods [25]. The soil samples were used as inoculants to trap the rhizobia using peanut plants in a pot plant glasshouse experiment, as described previously [41]. After six weeks, root nodules of the trapping plants were collected, cleaned, and immersed in 95% ethanol for 30 s, 15% (v/v) $H_2O_2$ for 15 min, followed by rinsing six times with sterilized distilled water for surface sterilization, as described previously [23]. The surface-sterilized nodules were crushed and streaked on yeast extract mannitol agar (YMA) [38] and incubated at 28 °C for three days to two weeks, until single colonies occurred. Single colonies were picked and repeatedly streaked on the same medium until the colony morphology was homogeneous. The purified isolates were suspended in YM broth supplemented with 15% (w/v) glycerol and stored at −70 °C.

### *DNA extraction and BOX-PCR fingerprinting*

Total DNA was extracted from each isolate using the method of Terefework et al. [34]. In order to reveal the genetic diversity of rhizobia nodulating with peanut plants, BOX-PCR was performed using the DNA as templates, the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), and the procedure of Nick and Lindström [24]. The amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gel and were visualized under UV light after the gel was stained with 0.5 μg $mL^{-1}$ ethidium bromide. A dendrogram was constructed based on the matrix generated by the UPGMA method and the Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

### *Sequence analyses of ITS,* recA, atpD *and* nodA *genes*

Based on the results of BOX-PCR profiles, isolates representing different genotypes were selected in order to amplify the 16S-23S intergenic spacer (ITS) with primers FGPS132 and FGPS1490 [34]. The PCR products were purified from the gel using the AxyPrep™ DNA Extraction Kit (Axygen Scientific, Inc.) according to the manufacturer's instructions. The gel-purified PCR products were sequenced with the same PCR primers by the Beijing Genomics Institute – Shenzhen. The sequences were compared with those of related species extracted by blasting in the GenBank database. The sequences acquired in this study and the related sequences obtained from the GenBank database by BLASTn and taxonomy searching were aligned with Clustal X [35] and manually edited for obvious errors. Phylogenetic trees were constructed using the maximum likelihood (ML) and neighbor-joining (NJ) methods in the MEGA 6.0 software [31]. The phylogenetic distances were estimated with Kimura's two-parameter ($K_2P$) model [16]. Bootstrap support for each node was evaluated with 500 replicates.

Strains representing different ITS clusters were employed for further characterization. Approximately 550 bp *atpD* and 485 bp *recA* gene fragments were amplified using the primer pairs atpD352F/871R and recA41F/recA640R, respectively, as previously described [39]. For *nodA*, 660 bp gene fragments were amplified with nodA-1/nodA-2, as previously described [13]. For comparison purposes, three strains isolated from wild peanut (*Arachis duranensis*) in a previous study [5] were included in this analysis: *Bradyrhizobium* sp. ADU11, *Bradyrhizobium* sp. ADU22 and *Bradyrhizobium* sp. ADU25. The methods for visualization, purification, sequencing and phylogenetic analyses of the amplified *atpD*, *recA* and *nodA* genes were the same as for ITS. In addition, multilocus sequence analysis (MLSA) was performed by concatenating the sequences of *atpD* and *recA*. Genospecies were identified at the MLSA similarity level of 97%, as suggested in previous studies [6,15,39].

### *Statistical analysis and genetic diversity estimation*

The community structure and species richness of peanut rhizobia were estimated based on genospecies defined mainly by MLSA. Non-parametric multivariate methods are used to evaluate species abundance and biogeography [19,20]. Canonical correspondence analysis (CCA) [33] as a constrained ordination method was utilized in order to understand the relationship between the environmental variables of the sampling sites. Before performing the CCA, a linear or unimodal ordination model was determined by detrended canonical analysis (DCA) [18]. The maximum value of the gradient lengths in the four ordination axes was greater than 3, suggesting that the linear gradient analysis model was not suitable and the unimodal model could be used. The analysis of species abundance and environmental data was performed by CANOCO 5 (Microcomputer Power, Ithaca, NY). The community diversity of bradyrhizobia in different sampling sites was estimated by the Shannon–Wiener ($H'$) and Simpson ($D$) indices in order to reflect the diversity considering both the species richness and evenness in a community. The Pielou ($J$) index was also used to evaluate the species evenness in the same community.

### *Nucleotide sequence accession numbers*

The GenBank accession numbers of the sequences determined in this study are listed in the relevant figures.

## Results

### *Isolation of root nodule bacteria*

A total of 216 pure slow-growing, alkali-producing isolates and nine fast-growing, acid-producing isolates were obtained from 300 peanut nodules. Since the fast-growing isolates could not form nodules on the peanut cultivar Shanyou 523 in nodulation tests, they were excluded from further research. The numbers of isolates from different sampling sites are listed in Table 1, and detailed information is available in Supplementary Table S2.

### *Soil characteristics*

The pH values of the soil samples varied between 4.7 and 7.4 (Table 1). The contents of total nitrogen, phosphorus, potassium and organic carbon in dry soil were (mg $kg^{-1}$) 488–2300, 977–2670, 9660–23,400, and 13,800–42,300, respectively, whereas the available N, P and K were (mg $kg^{-1}$) 29.9–145, 10–119, and 48–250, respectively (detail available in Supplementary Table S1).