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Bradyrhizobium nanningense sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov., isolated from effective nodules of peanut in Southeast China

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ABSTRACT

Nine slow-growing rhizobia isolated from effective nodules on peanut (*Arachis hypogaea*) were characterized to clarify the taxonomic status using a polyphasic approach. They were assigned to the genus *Bradyrhizobium* on the basis of 16S rRNA sequences. MLSA of concatenated *glnII-recA-dnaK* genes classified them into three species represented by CCBAU 53390^T, CCBAU 51670^T and CCBAU 51778^T, which presented the closest similarity to *B. guangxiense* CCBAU 53363^T, *B. guangdongense* CCBAU 51649^T and *B. manausense* BR 3351^T, *B. vignae* 7-2^T and *B. forestalis* INPA 54B^T, respectively. The dDDH (digital DNA-DNA hybridization) and ANI (Average Nucleotide Identity) between the genomes of the three representative strains and type strains for the closest *Bradyrhizobium* species were less than 42.1% and 91.98%, respectively, below the threshold of species circumscription. Effective nodules could be induced on peanut and *Lablab purpureus* by all representative strains, while *Vigna radiata* formed effective nodules only with CCBAU 53390^T and CCBAU 51778^T. Phenotypic characteristics including sole carbon sources and growth features supported the phylogenetic results. Based on the genotypic and phenotypic features, strains CCBAU 53390^T, CCBAU 51670^T and CCBAU 51778^T are designated the type strains of three novel species, for which the names *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov. are proposed, respectively.

Introduction

Peanut (*Arachis hypogaea* L.) is an important legume grain and oil crop with great economic value which is particularly important in China, of which peanut total output ranks first in the world (http://faostat3.fao.org/browse/Q/QC/E). It has been reported that peanut contributed 40.9 kg ha^{-1} of combined nitrogen by biological nitrogen fixation [28], which offer 35.4%–76.1% of the peanut nitrogen requirement depending on the N-fertilizer levels and mono- or intercropping system [4,42]. Hence, screening of more efficient peanut rhizobia is critical for the promotion of rhizobia as inoculant and to reduce the use of chemical nitrogen fertilizers which are harmful to environment. Rhizobia invade legumes mainly through root hair and crack entry. Similar to *Aeschynomene,* peanuts are infected through crack entry and some strains can form nodules in a Nod Factor-independent way [10,12]. The precise mechanism is still unclear and researches on peanut-rhizobia interactions have attracted extensive attention. The discovery of new rhizobial species may provide better insight into symbiotic rhizobium-peanut interactions. To date, peanut forms effective nodules with diverse species mainly within the genus *Bradyrhizobium* [3,11,25], but many genomic species associated with peanut have not been clearly classified except the species *Bradyrhizobium arachidis*, *B. guangdongense*, *B. guangxiense*, *B. vignae*, and *B. lablabi* [2,22,30,47]. However, the inconsistency of strain naming in different studies has brought some uncertainty to the future application and genetic research of strains.

During a study of genetic diversity of rhizobia collected from the peanut root nodules in Guangdong and Guangxi Provinces located

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in Southeast China, nine strains were identified as three potential novel *Bradyrhizobium* species by using a combination of genotypic and phenotypic methods. The names *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov. are proposed for these species.

Materials and methods

Isolation and culture conditions

The nine strains were isolated and purified from fresh nodules collected from different sites in the Provinces of Guangdong (Cities Guangzhou, Maoming, and Zhanjiang) and Guangxi (Nanning and Wuzhou) (Table S1 for details). The collected nodules were disinfected, crushed and streaked on YMA plates. Incubation was for 7–14 days at 28 °C, as described previously [44]. All nine isolates were stored at −80 °C in YM broth with 20% (w/v) glycerol and preserved by lyophilization.

Sequencing of genes and genomes of strains

Total genomic DNA of nine test strains and three reference strains of the most closely related species were extracted using the Wizard Genomic DNA Purification kit A1120 following the manufacturer's instructions (Promega). DNA quality and quantification were measured by Nanodrop (NanoDrop Technologies, Wilmington, USA) and Qubit fluorometer (Invitrogen by Life Technologies Holdings Pte. Ltd., Singapore) instruments. The DNA integrity and purity were checked using a 1% agarose gel for electrophoresis. The genomic DNA was used as templates to amplify the partial genes of 16S rRNA [38], *glnII*, *recA* [46], *dnaK* [35], *nodC* [33] and *nifH* [27] by PCR with the corresponding primers. The DNA sequences were acquired by sequencing at BGI-Shenzhen. Individual genes were sequenced using Sanger technology. The genomes were sequenced using Illumina HiSeq pair-end and Pacbio technology and assembled using SOAPdenovo and Falcon program, respectively, after which they were annotated based on the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [13,39].

Phylogenetic and phylogenomic analysis and overall genome related index

Individual gene sequences were aligned using the clustal W software [20] integrated in MEGA6 [37], with those of the related *Bradyrhizobium* species downloaded from NCBI database. Phylogenetic trees were constructed with the Maximum Likelihood (ML) and Neighbor-joining method (NJ) [32] using Jukes-Cantor model [15] for 16S rRNA, while Kimura 2-parameter model [17] was used for all the single genes as well as concatenated *glnII*, *recA* and *dnaK* sequences, respectively. The reliability of the trees was estimated by bootstrap test using 1000 pseudoreplicates. The pairwise distances were calculated in MEGA6 using the Kimura 2-parameter model. In particularly, two *nodC* phylogenetic trees were constructed respectively based on the complete *nodC* sequences (1303 nt) of 27 available genomes of known *Bradyrhizobium* species as well as 12 genomes sequenced during this study and the partial *nodC* sequences of 430 nt located from about 600 nt to 1100 nt of the total 55 strains. The *nodC* sequences of 39 available genome strains were used in both *nodC* phylogenetic trees.

The phylogenomic tree was constructed by using the software IQ-TREE [26] with the maximum likelihood method based on the amino acid sequences of the 1550 of single-copy core genes of 26 konwn *Bradyrhizobium* strains and 12 tested strains in this study, which were identified using the software GET_HOMOLOGUES [8], aligned by MAFFT program [16], concatenated and trimmed by gblocks v.0.91b [36] before the resulting alignment (443,198 aa in length).

For the overall genome related index (OGRI) [6], Average Nucleotide Identity (ANI) values were calculated by MUMmer in JSpecies [31]. The digital DNA-DNA Hybridization (dDDH) [24] was calculated with the formula 2 model on the Genome-to-Genome Distance Calculator (GGDC 2.1) web service (https://ggdc.dsmz.de/ggdc.php#).

Phenotypic characterization

Phenotypic characteristics were evaluated for the test strains as well as closely related reference strains. Utilization of sole carbon source was tested by using a Gram-Negative Microplate (Biolog GN2) following the manufacturer's instructions. Other phenotypic tests were conducted at 28 °C, evaluating colony morphology, the tolerance to NaCl (1–5%, w/v), pH gradient of 4–10, as well as the antibiotic resistance (trimethoprim, nalidixic acid, rifampicin, spectinomycin, tetracyclines, gentamycin sulfate, chloramphenicol, kanamycin, streptomycin and apramycin) with concentrations of 20, 30, 50 and 100 $\mu g\, ml^{-1}$ respectively. Generation time was determined by culturing the bacteria in TY medium at 28 °C. Cell morphology was observed by scanning electron microscopy with fresh cells from single colony on YMA plate. Temperature growth ranges were performed by culturing the bacteria on YMA medium at 4, 10, 20, 28, 37, 45 and 60 °C.

Analysis of cellular fatty acids

Cellular fatty acids of all tested strains were extracted followed the method as described previously [22], and determined using the MIDI Sherlock Microbial Identification System with the TSBA6 database.

DNA fingerprinting

BOX-PCR was performed using the BOXAIR primer (5′-CTACGGCAAGGCGACGCTGACG-3′) [43] for the nine test strains. Amplicons were separated on a 1.5% agarose gel containing ethidium bromide for 4–5 h at 75 V.

Gene and genome accession numbers

Genebank accession numbers for the genes and genomes of the tested strains were listed in Table S2 and S3, respectively. The assembly accession numbers of genomes downloaded from NCBI for phylogenomic tree were listed in Table S4.

Results and discussion

Consistent topology of the nine test strains and related *Bradyrhizobium* species was observed in the ML (Fig. 1) and NJ (data not show) phylogenetic trees based on the partial 16S rRNA sequences (1198 nt). All the isolates from this study belonged to the *B. japonicum* clade. In the ML phylogenetic tree (Fig. 1), the test strains were distributed in three separate groups which were named Group A, B and C, respectively (Table S1). The test strains belonged to the same group shared the identical 16S rRNA sequence (Fig. 1 and Table S5). Group A strains showed 100% similarities with *B. guangxiense* CCBAU 53363^T [22], and 96.3%–99.8% similarities with other *Bradyrhizobium* species including those in Groups B and C. In the comparison of three Group B strains with closely related species, sequences similarities values varied from 96% to 100% and clustered closest to *B. guangdongense* CCBAU 51649^T [22]. Additionally, four Group C strains showed similarities of 99.9% with *B. kavangense* 14-

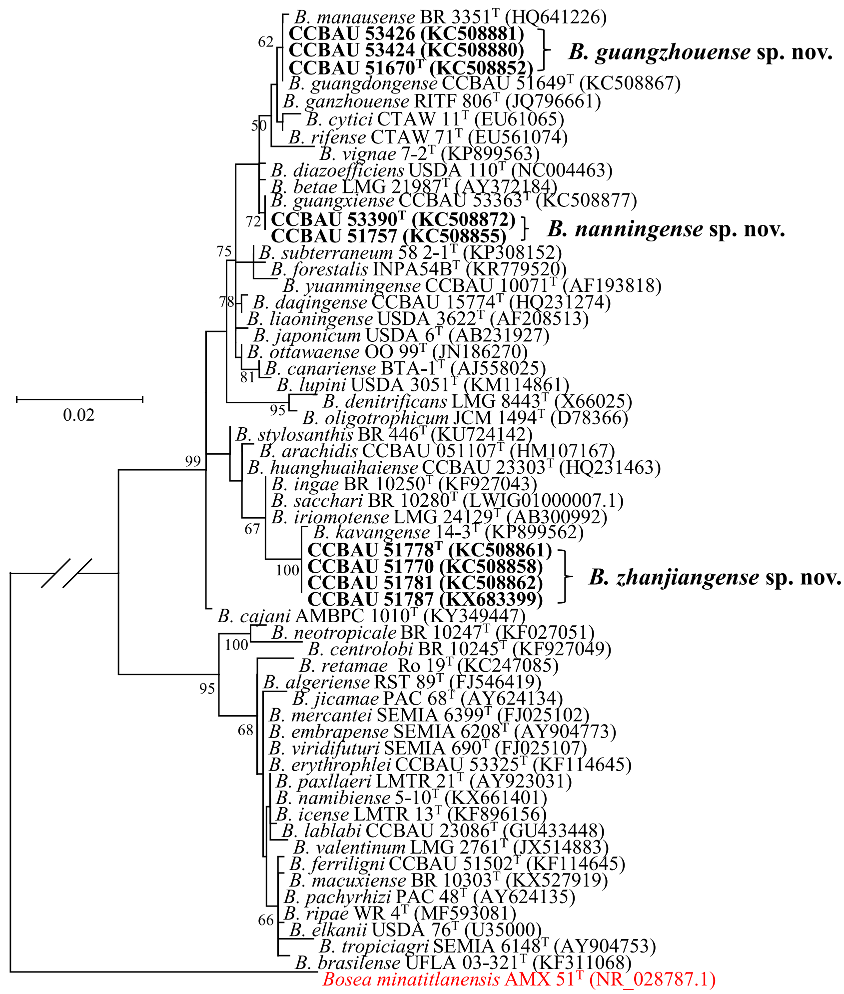

Fig. 1. Phylogenetic tree constructed based on the 16S rRNA gene sequence (1198 nt) using the Maximum-likelihood method. The GeneBank accession numbers were indicated in the brackets. Strains of the novel species are shown in bold. Bootstrap values greater than 50% were shown on the node. Bar: 2% substitutions nucleotides. *Bosea minatitlanensis* AMX 51^T was used as an outgroup.

3^T [21] and 96.0%–99.5% similarities with the rest *Bradyrhizobium* species.

It is well known that 16S rRNA gene sequences could not distinguish the closely related species in the rhizobial genera (like *Bradyrhizobium* and *Rhizobium*), so multilocus sequence analysis (MLSA) of the housekeeping genes *glnII* (479 nt), *recA* (374 nt) and *dnaK* (222 nt) were conducted and similar phylogenetic trees were obtained by using ML (Fig. 2) and NJ (data not show) methods. In the ML phylogenetic tree (Fig. 2), Group A strains CCBAU 53390^T showed 100% similarities to CCBAU 51757, and they showed the highest similarities of 95.9% (*glnII*), 94.2% (*recA*), 95.2% (*dnaK*) and 95.2% (concatenated sequences) to *B. guangxiense* CCBAU 53363^T [22], respectively. The three Group B strains also showed similarities of more than 99.5% to each other, and 94.4% to the closest reference strains of *B. guangdongense* CCBAU 51649^T [22] in the MLSA. The Strains among Group C shared more than 99.2% similarities in the MLSA, less than 95.7% with the nearest strain *B. vignae* 7-2^T [30] and although they were closest to *B. kavangense* 14-3^T in the 16S rRNA phylogenetic tree. The discordance found between 16S rRNA tree and MLSA was also reported previously in the identification of *B. guangdongense* [22] and *B. neotropicale* [50], suggesting that lateral gene transfer (LGT) or recombination event might have happened in their evolution histories. The difference could also be due to the poor resolution of the 16S gene. The discordance in the phylogeny of housekeeping genes is common since 90% of the core genes of genomes, including the housekeeping genes, were inconsistent with their species tree [40].

The genomes of the nine isolates and three type strains of closely related species were sequenced for genomes, among which four strains' were completely sequenced without gaps and the detailed genome information of 12 strains was listed in Table S3. Three of the four complete genomes (CCBAU 53363^T, CCBAU 51649 ^{T} and CCBAU 51670^T) possess a megaplasmid with 0.98 M in size. All the *Bradyrhizobium* genomes have one ribosomal operon and the GC contents determined according to the genome sequences were within the range for species in the genus of *Bradyrhizobium* [48]. The G + C% differences vary from 0.03 to 0.44 between the test genomes within the same species, supporting that recent research conclusion that differences in genomic G + C% cannot be more than one within the same species [18].

The use of average nucleotide identity (ANI) to estimate genome relatedness is also being increasingly used as an alternative to DNA–DNA hybridization (DDH) [1,9,14]. And ANI values of 95–96% would correspond to the 70% DDH [31]. All ANI values of strain pairs belonging to the same species were ranging from 97.7% to 99.72% (Table 1). The highest ANI values between Groups A, B, C and the type strains of the rest available known Bradyrhizobial species were 90.68% (*B. guangxiense* CCBAU 53363^T), 91.20% (*B. manausense* BR 3351^T [34]) and 90.04% (*B. forestalis* INPA 54B ^{T} [23]) respectively, supporting that the group A, B, C strains rep-

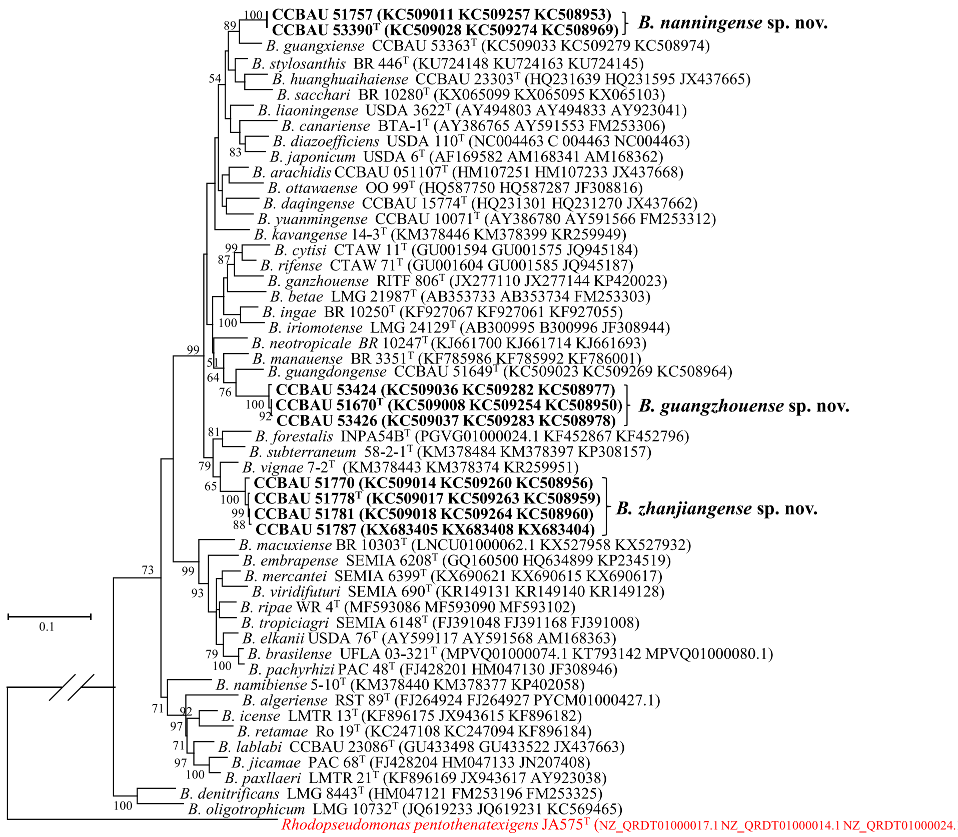

Fig. 2. Maximum-likelihood phylogeny based on the concatenated *glnII, recA* and *dnaK* gene sequences (1075 nt) showing the relationships between the novel species and the recognized species of the genus *Bradyrhizobium*. The GeneBank accession numbers are indicated in the brackets. Strains of the novel species are shown in bold. Bootstrap values greater than 50% were shown on the node. Bar: 10% substitutions nucleotides. *Rhodopseudomonas pentothenatexigens* JA575^T was used as an outgroup.

Table 1
ANI values (percentages) between *B. nanningense* sp. nov., *B. guangzhouense* sp. nov., *B. zhanjiangense* sp. nov. and the type strains of the closely related species of the genus *Bradyrhizobium.*

Genome ANI (CCBAU NO.)	53390^T	51757	53363^T	51670^T	53424	53426	51649^T	BR 3351^T	51778^T	51770	51781	51787	7-2^T	INPA 54B^T
B. nanningense **sp. nov.**														
CCBAU 53390^T	..	99.45	90.68											
CCBAU 51757	99.47	..	90.7											
B. guangxiense CCBAU 53363^T	90.66	90.68	..											
B. guangzhouense **sp. nov.**														
CCBAU 51670^T				..	98.86	99.61	90.53	91.18						
CCBAU 53424				98.94	..	98.93	90.47	91.20						
CCBAU 53426				99.72	98.95	..	90.52	91.20						
B. guangdongense CCBAU 51649^T				90.5	90.42	90.45	..	88.69						
B. manausense BR 3351^T				91.17	91.20	91.19	88.69	..						
B. zhanjiangense **sp. nov.**														
CCBAU 51778^T									..	99.61	98.21	97.9	89.63	90
CCBAU 51770									99.27	..	98.16	97.7	89.62	90.02
CCBAU 51781									98.36	98.14	..	97.9	89.62	90.04
CCBAU 51787									97.96	97.91	97.92	..	89.57	89.96
B. vignae 7-2^T									89.63	89.6	89.61	89.56	..	91.98
B. forestalis INPA 54B^T									90	90.02	90.04	89.96	91.98	..

resent three novel species, although the closest species of Group B and C were different to that in MLSA. DNA–DNA hybridization (DDH) is currently still used as the taxonomic gold standard for distinguishing species in *Archaea* and *Bacteria*. With the development of sequencing technology, digital DNA–DNA hybridization (dDDH) calculated based on the genome sequences is an alternative of wet-lab DDH, and still takes 70% similarities as the standard threshold for species boundaries [5,24]. In this study, the dDDH values within the same species strains ranged in 82.9%–98.6% while the values of three novel groups with the most closely species were of 37.6%–40.1% (Table S5), also supporting that the Group A, B and C strains are novel species. This result was consistent with that in ANI analysis.

In addition, multigene-based phylogenomic tree was expected to define genera and higher taxa, as well as resolving the poorly classified taxa [5]. Here, phylogenomic tree was constructed based on amino acid sequences of the 1550 single copy genes that were derived from comparative genomics of 9 test strains and 29 reference strains. In the phylogenomic tree (Fig. S1), Group A strain CCBAU 53390^T showed 100% similarities to CCBAU 51757, and were closest to *B. guangxiense* CCBAU 53363^T with 96.2% similarities, which was similar to the results in MLSA analysis. The three Group

B strains showed 99.6%–99.9% similarities with each other and the four Group C strains shared 99.4%–99.8% similarities. The Group B strain CCBAU 51670^T was most closely related to *B. manausense* BR 3351^T and *B. guangdongense* CCBAU 51649^T which was the closest one in MLSA, with 96% and 95.6% similarities respectively. Similarly, Group C strain CCBAU 51778^T showed most closely similarities of 95.6% and 95.5% respectively to *B. forestalis* INPA54B^T and *B. vignae* 7-2^T which was the closest species in MLSA. The phylogenomic analysis showed the higher similarities between novel species and the closely related species than that in MLSA. The phylogenetic relationship in Group B and C strains with their closely related species was changed but the difference of the similarities was very small as the above detail descriptions. Even so, the high similarities of strain pairs in Group A, B and C and relatively low similarities shared between each group and their closest related species further confirmed the three novel species classification and the data in this analysis were accordant to that in ANI and dDDH.

Fatty acid composition and abundance varied among the test strains and their closely related species (Table S6). $C_{18:1}$ $\omega 6c$ and $C_{16:0}$ were the most predominant components for the CCBAU 53390^T and CCBAU 51670^T as well as the reference strain CCBAU 53363^T and BR 3351^T, which is consistent with most *B. japonicum* strains [41]. While in CCBAU 51778^T, more kinds of fatty acid were detected, and $C_{19:0}$ cyclo $\omega 8c$ were the second major component accounting for 21.96%, which is similar to *B. elkanii* group I strains [41]. Differences were also found in *B. vignae* LMG 28791^T, in which $C_{18:0}$ $\omega 7c$ (78.2%) but not $C_{18:1}$ $\omega 6c$ was detected as the major fatty acid [30].

Closely related rhizobia, such as strains in the same species can be distinguished by the valuable method BOX-PCR [49]. Here, the BOX-PCR results showed that similar but different BOX-PCR fingerprintings were obtained among the strains in each novel group, indicating that the strains were not clones of the same strain (Fig. S2).

All the test strains as well as their closely related species (except of *B. forestalis* INPA54B^T) can utilize 18 kinds of carbon resources including Tween 40, Tween 80, D-Arabitol, α-D-Glucose, D-Mannitol, D-Mannose, D-Galactonic Acid Lactone, Bromo Succinic Acid, L-Leucine, Glycerol, L-Pyroglutamic Acid, D-Saccharic Acid, Quinic Acid, D,L-Lactic Acid, β-Hydroxy Butyric Acid, D-Gluconic Acid, D-Galacturonic Acid and D-Fructose. Distinctive phenotypic characteristics between the type strains of group A, B, C and their closely related species are listed in Table 2, and a full characterization of sole carbon source is listed in Table S7. 15 distinctive phenotypic characteristics were found between the type strains of group A and the closest related species *B. guangxiense* CCBAU 53363^T. 28 distinctive phenotypic characteristics were found between type strains of group B and closely related species *B. manausense* BR 3351^T. 39 and only 6 distinctive phenotypic characteristics were found between type strains of group C and closely related species *B. vignae* 7-2^T and *B. forestalis* INPA 54B^T as most of the carbon sources were not detected for *B. forestalis* INPA 54B^T (Table 2).

A large amount of biolog results involving *Rhizobium leguminosarum* strains showed that the utilization patterns of carbon resources are not strongly associated with either the genospecies or the symbiovar [19], which is confirmed by that although the four Group B strains belonged to the same species, CCBAU 51770 has distinct different utilization patterns (Table S7). Cells of three new proposed species are Gram-negative, aerobic, non-spore-forming rods (Fig. S3). All the test strains can grow at 14–45 °C with an optimum temperature of 28 °C. The type strain CCBAU 53390^T, CCBAU 51670^T and CCBAU 51778^T have a generation time of 9.11 h, 14.33 h and 15.12 h, respectively, grown in TY medium at 28 °C. The type strain CCBAU 53390^T is resistant to trimethoprim (100 μg ml^{-1}), nalidixic acid (100 μg ml^{-1}), gentamycin sulfate (30 μg ml^{-1}), apramycin (30 μg ml^{-1}), streptomycin (20 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}), but not to rifampicin, spectinomycin, tetracyclines or kanamycin at the minimum dose tested (20 μg ml^{-1}). The type strain CCBAU 51670^T is resistant to trimethoprim (100 μg ml^{-1}), nalidixic acid (100 μg ml^{-1}), but not to rifampicin, spectinomycin, tetracyclines, gentamycin sulfate, chloramphenicol, kanamycin, streptomycin or apramycin at the minimum dose tested (20 μg ml^{-1}). The type strain CCBAU 51778^T is resistant to trimethoprim (100 μg ml^{-1}), nalidixic acid (30 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}), but not to rifampicin, spectinomycin, tetracyclines, gentamycin sulfate, kanamycin, streptomycin or apramycin at the minimum dose tested (20 μg ml^{-1}).

Nodulation and nitrogen fixation genes, including *nodC* and *nifH* are required for effective symbiosis. Generally, phylogenies of these genes are discordant with that of housekeeping genes, as they may group rhizobial strains according to their host range or geographic origin due to lateral gene transfer (LGT) events [45]. Phylogenetic trees were constructed based on the partial *nodC* (430 nt), complete *nodC* (1320 nt) and *nifH* (203 nt) sequences of test strains as well as closely related reference strains of *Bradryzhizobium* species. In the *nodC* gene tree based on partial *nodC* sequences (Fig. S4), the Group A strain CCBAU 53390^T showed 97.6% similarities to the strains of symbiovar glycinearum [45] – several soybean-nodulating strains including *B. japonicum* USDA 6^T, but it shared 91.4% similarity with another Group A strain CCBAU 51757. Although the CCBAU 51757 clustered with the symbiovar centrosemae [29], it showed the highest similarity with CCBAU 53390^T. The three Group B strains had *nodC* genes identical to *B. guangdongense* CCBAU 51649^T and *B. guangxiense* CCBAU 53363^T, which were also isolated from nodules of peanut grown in the same region, showing the highest similarity of 66.8% with symbiovar sierranevadense strain *Bradyrhizobium* sp. GV137 [7]. The four strains within Group C shared 93.5%–99.8% similarities of *nodC* sequences and grouped together with the clade of *B. arachidis* CCBAU 051107^T, *B. shewense* err 11^T, *B. cajani* AMBPC 1010^T and *forestalis* INPA54B^T with the 91.6%–94.8% similarities. In the complete *nodC* tree (Fig. S5), similar phylogenetic topology of Group A, B and C strains were observed, but the clades of the three Groups with other reference species were more divergent, for example, the clade of Group B strains, *B. guangdongense* CCBAU 51649^T and *B. guagnxiense* CCBAU 53363^T showed 53.1%–59% similarities to all other species. The results showed that the *nodC* genes in the three novel genospecies of peanut rhizobia might have different origins even they were isolated in the same ecoregion, and they should be the different symbiovars compared with the known symbiovars of rhizobia.

In the *nifH* gene tree (Fig. S6), relationships similar to that in *nodC* tree were observed, but the representative strain of group A, CCBAU 53390^T, showed greater divergence with the reference strains for soybean-nodulating *Bradyrhizobium*. The detection of identical housekeeping genes but different symbiosis genes in the two strains of Group A, as well as the discrepancy of the phylogenies in their *nodC* and *nifH*, demonstrated the possibility that the nodulation and nitrogen fixation genes might be adopted by LGT together or separately.

To further evaluate the nodulation and nitrogen-fixation abilities of the novel isolates with different host legumes, we conducted cross-inoculation tests in modified Leonard jars containing vermiculite with N-free plant nutrient solution in glasshouse conditions [44]. All test strains of the three groups nodulated *Arachis hypogaea* and *Lablab purpureus* with pink cross-sections of the nodules, indicating that the nodules were effective in fixing nitrogen. These strains induced ineffective malformed nodules on *Phaseolus vulgaris*. None of the isolates nodulated *Medicago sativa*, *Trifolium repens* or *Glycine max*. Effective nodules were formed on *Vigna radi-*

Table 2
Phenotypic characteristics distinguishing among the type strains of the new proposed species and those of their closely related species. 1, *B. nanningense* sp. nov. CCBAU 53390^T; 2, *B. guangxiense* CCBAU 53363^T; 3, *B. guangzhouense* sp. nov. CCBAU 51670^T; 4, *B. manausense* BR 3351^T; 5, *B. zhanjiangense* sp. nov. CCBAU 51778^T; 6, *B. vignae* 7-2^T; 7, *B. forestalis* INPA 54B^T. All data were obtained in the same condition of the same Lab including *B. guangxiense* [22] except of *B. manausense* [34], *B. vignae* [30] and *B. forestalis* [23]. +, positive; -, negative; w, weakly positive; nd, not detected.

Characteristics	1	2	3	4	5	6	7	Characteristics	1	2	3	4	5	6	7
Dextrin	w	–	w	–	+	w	nd	p-Hydroxy Phenylacetic Acid	w	–	–	–	–	nd	nd
Glycogen	–	–	–	–	+	–	nd	Itaconic Acid	+	–	–	–	+	+	nd
N-Acetyl-D-Galactosamine	–	–	–	–	w	–	nd	α-Keto Butyric Acid	+	–	–	–	–	+	nd
Succinamic Acid	+	+	+	–	+	+	nd	α-Keto Valeric Acid	+	+	+	–	–	+	nd
Adonitol	+	–	–	+	–	–	nd	Malonic Acid	w	–	–	+	+	+	nd
L-Arabinose	+	+	+	+	–	+	nd	Propionic Acid	+	+	w	–	+	+	nd
L-Fucose	+	+	+	–	+	+	nd	Sebacic Acid	+	+	+	+	–	+	nd
Gentiobiose	–	–	–	+	+	–	nd	α-Keto Glutaric Acid	+	+	+	–	+	+	nd
α-D-Glucose	+	+	+	+	+	+	w	Glucuronamide	+	+	+	–	+	+	nd
m-Inositol	–	–	+	–	+	–	nd	L-Alaninamide	+	+	–	–	+	–	nd
α-D-Lactose	–	–	–	–	+	–	–	D-Alanine	+	–	+	+	–	+	nd
Lactulose	–	–	–	–	+	–	nd	L-Alanine	–	–	+	w	+	nd	nd
Maltose	–	–	–	–	+	–	nd	L-Alanyl-Glycine	–	–	–	–	+	–	–
D-Melibiose	–	–	–	–	w	–	nd	L-Asparagine	+	+	–	+	+	–	w
β-Methyl-D-Glucoside	–	–	–	–	+	–	nd	L-Aspartic Acid	+	+	–	+	+	+	nd
D-Psicose	w	–	–	–	+	w	nd	L-Glutamic Acid	+	+	–	+	+	+	+
D-Raffinose	–	–	–	–	+	–	nd	Glycyl-L-Aspartic Acid	–	–	–	–	+	+	nd
L-Rhamnose	+	–	+	–	+	nd	nd	Glycyl-L-Glutamic Acid	–	–	–	–	–	+	nd
D-Sorbitol	w	–	–	+	+	–	nd	L-Histidine	–	–	–	–	+	–	nd
Sucrose	–	–	–	w	+	–	w	Hydroxy-L-Proline	–	–	–	–	+	–	nd
D-Trehalose	–	–	–	–	+	–	nd	L-Ornithine	–	–	–	–	+	–	nd
Turanose	w	–	–	+	+	–	nd	L-Proline	+	–	–	w	+	w	nd
Xylitol	+	–	–	–	+	–	nd	D-Serine	w	–	+	+	–	–	nd
Mono-Methyl-Succinate	+	+	+	+	–	+	nd	L-Serine	–	–	–	w	w	–	nd
Cis-Aconitic Acid	+	+	+	–	+	+	nd	L-Threonine	+	+	–	–	+	w	nd
Citric Acid	+	+	–	–	+	+	–	D,L-Carnitine	–	–	–	–	+	–	nd
Formic Acid	+	+	+	–	–	+	nd	Urocanic Acid	+	+	–	–	–	+	nd
D-Glucuronic Acid	+	+	–	–	+	+	nd	Uridine	–	–	–	–	w	–	nd
α-Hydroxy Butyric Acid	+	+	+	–	–	nd	nd	2,3-Butanediol	–	–	–	–	w	–	nd
Succinic Acid	+	+	+	–	+	+	nd	2-Aminoethanol	–	–	–	+	–	–	nd
Putrescine	–	–	–	+	–	–	nd	Phenyethylamine	–	–	–	+	–	–	nd
γ-Hydroxy Butyric Acid	w	–	w	w	w	+	nd								

ata by strains of Group A and C but no nodule was formed when it was inoculated with the strains of Group B, which shared the same host ranges with *B. guangdongense*, as they have the same *nodC* and *nifH* genes.

Based upon the distinct phenotypic and genotypic characteristics, three novel species are proposed, represented by the type strains CCBAU 53390^T for *Bradyrhizobium nanningense* sp. nov., CCBAU 51670^T for *Bradyrhizobium guangzhouense* sp. nov. and CCBAU 51778^T for *Bradyrhizobium zhanjiangense* sp. nov.

The cells of *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov. and *Bradyrhizobium zhanjiangense* sp. nov. are Gram-negative, non-spore-forming rods. In general, all strains of *Bradyrhizobium nanningense* sp. nov. (Table 3) utilize Tween 40, Tween 80, Adonitol, D-Arabitol, L-Fucose, D-Galactose, α-D-Glucose, D-Mannitol, D-Mannose, L-Rhamnose, Xylitol, Methyl Pyruvate, Cis-Aconitic Acid, Citric Acid, Formic Acid, D-Galactonic Acid Lactone, D-Glucosaminic Acid, D-Glucuronic Acid, Itaconic Acid, α-Keto Butyric Acid, Propionic Acid, Sebacic Acid, Bromo Succinic Acid, Glucuronamide, L-Alaninamide, D-Alanine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, L-Leucine, L-Phenylalanine, L-Proline, L-Threonine, Glycerol, L-Pyroglutamic Acid, Succinamic Acid, Succinic Acid, D-Saccharic Acid, Quinic Acid, D,L-Lactic Acid, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid, D-Gluconic Acid, D-Galacturonic Acid and D-Fructose, but not *N*-Acetyl-D-Galactosamine, *N*-Acetyl-D-Glucosamine, D-Cellobiose, i-Erythritol, L-Histidine, D,L-Carnitine, Uridine, Thymidine, 2-Aminoethanol, 2,3-Butanediol, Glucose-1-Phosphate, Glucose-6-Phosphate, Putrescine or Phenyethylamine. All strains of *Bradyrhizobium guangzhouense* sp. nov. (Table 4) utilize Tween 40, Tween 80, L-Arabinose, D-Arabitol, D-Galactose, α-D-Glucose, D-Mannose, Methyl Pyruvate, D-Glucosaminic Acid, Bromo Succinic Acid, Glycerol, L-Pyroglutamic Acid, Succinamic Acid, Succinic Acid, D-Saccharic Acid, Quinic Acid, D,L-Lactic Acid, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid, D-Gluconic Acid, D-Galacturonic Acid and D-Fructose, but not Glycogen, *N*-Acetyl-D-Galactosamine, *N*-Acetyl-D-Glucosamine, Adonitol, D-Cellobiose, i-Erythritol, Gentiobiose, α-D-Lactose, Lactulose, Maltose, β-Methyl-D-Glucoside, D-Psicose, D-Raffinose, Sucrose, D-Trehalose, Turanose, Xylitol, Acetic Acid, Citric Acid, Itaconic Acid, α-Keto Butyric Acid, Malonic Acid, L-Alaninamide, L-Alanyl-Glycine, L-Asparagine, L-Glutamic Acid, Glycyl-L-Aspartic Acid, Glycyl-L-Glutamic Acid, L-Histidine, Hydroxy-L-Proline, L-Ornithine, L-Proline, L-Serine, L-Threonine, D,L-Carnitine, γ-Amino Butyric Acid, Urocanic Acid, Inosine, Uridine, Thymidine, 2-Aminoethanol, 2,3-Butanediol, D,L-α-Glycerol Phosphate, Glucose-1-Phosphate, Glucose-6-Phosphate, Putrescine or Phenyethylamine. All strains of *Bradyrhizobium zhanjiangense* sp. nov. (Table 5) utilize Tween 40, Tween 80, L-Fucose, D-Mannitol, L-Rhamnose, Cis-Aconitic Acid, D-Galactonic Acid Lactone, Itaconic Acid, Bromo Succinic Acid, Glucuronamide, L-Alaninamide, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycyl-L-Aspartic Acid, L-Leucine, L-Phenylalanine, L-Proline, L-Threonine, L-Pyroglutamic Acid, Succinamic Acid, Succinic Acid, D-Saccharic Acid, Quinic Acid, D,L-Lactic Acid, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid, D-Gluconic Acid, D-Galacturonic Acid and D-Fructose but not D-Serine, Putrescine or Phenyethylamine. The detailed descriptions were listed in formal proposal of the new species "*Bradyrhizobium nanningense* sp. nov.", "*Bradyrhizobium guangzhouense* sp. nov." and "*Bradyrhizobium zhanjiangense* sp. nov." as Tables 3, 4 and 5 with the Taxonumber TA00810, TA00812 and TA00811, respectively.

Table 3
Description of *Bradyrhizobium nanningense* sp. nov. according to Digital Protologue TA00810 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00810
Date of the entry	2018/11/22
Draft number/date	1
Version	Draft
Type of description	New Description
Species name	*Bradyrhizobium nanningense*
Genus name	*Bradyrhizobium*
Specific epithet	*nanningense*
Species status	sp. nov.
Species etymology	nan.ning.en'se. N.L. neut. adj. nanningense originating from Nanning City in Guangxi Province of China, from where the type strain was isolated
Corresponding author	Xinhua Sui
E-mail of the corresponding author	suixh@cau.edu.cn
Submitter	Yonghua Li
E-mail of the submitter	lyhsierra@foxmail.com
Designation of the type strain	CCBAU 53390
Strain collection numbers	LMG 29278 = HAMBI 3651
16S rRNA gene accession number	KC508872
Genome accession number [RefSeq]	LBJC00000000
Genome status	draft
Genome size	8098 kb
GC mol %	63.44
Data on the origin of the sample from which the strain had been isolated	
Country of origin	CHN
Region of origin	Nanning City, Guangxi Province
Source of isolation	Nodules on *Arachis hypogaea*
Sampling date	2011/12/1
Geographic location	Nanning
Latitude	108º14′44″N
Longitude	22º51′08″E
Number of strains in study	2
Source of isolation of non-type strains	Nodules on *Arachis hypogaea*
Growth medium, incubation conditions [temperature, pH, and further information]	YMA (per liter: 10 g mannitol, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract, 0.1 g NaCl, 15 g Agar) 28 °C, pH 6.8–7.0
Alternative medium 1	TY (per liter: Tryptone 5.0 g, yeast extract 3.0 g, $CaCl_2 \cdot H_2O$ 0.7 g)
Gram stain	Negative
Cell shape	rod
Cell size (length or diameter)	1.08-1.73 × 0.316-0.431 μm
Sporulation (resting cells)	none
Colony morphology	circular, convex, beige-whitish
Temperature range	14-45 °C
Lowest temperature for growth	14 °C (YMA, pH 6.8–7.0)
Highest temperature for growth	45 °C (YMA, pH 6.8–7.0)
Temperature optimum	28 °C
Lowest pH for growth	5 (YMA, 28 °C)
Highest pH for growth	8 (YMA, 28 °C)
pH optimum	7
pH category	neutrophile
Lowest NaCl concentration for growth	0 (YMA, 28 °C, pH 7.0)
Highest NaCl concentration for growth	1% (YMA, 28 °C, pH 7.0)
Salinity optimum	0.80%
Salinity category	nonhalophile (NaCl inhibitory at <1 % NaCl)
Relationship to O_2	aerobe
O_2 conditions for strain testing	Aerobiosis
Carbon source used [class of compounds]	Sugars, amino acids
Positive tests with BIOLOG	Adonitol, Bromo Succinic Acid, Cis-Aconitic Acid, Citric Acid, D,L-Lactic Acid, D-Alanine, D-Arabitol, D-Fructose, D-Galactonic Acid Lactone, D-Galactose, D-Galacturonic Acid, D-Gluconic Acid, D-Glucosaminic Acid, D-Glucuronic Acid, D-Mannitol, D-Mannose, D-Saccharic Acid, Formic Acid, Glucuronamide, Glycerol, Itaconic Acid, L-Alaninamide, L-Asparagine, L-Aspartic Acid, L-Fucose, L-Glutamic Acid, L-Leucine, L-Phenylalanine, L-Proline, L-Pyroglutamic Acid, L-Rhamnose, L-Threonine, Methyl Pyruvate, Propionic Acid, Quinic Acid, Succinamic Acid, Sebacic Acid, Succinic Acid, Tween 40, Tween 80, Xylitol, α-D-Glucose, α-Keto Butyric Acid, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid
Negative tests with BIOLOG	2-Aminoethanol, 2,3-Butanediol, *N*-Acetyl-D-Galactosamine, *N*-Acetyl-D-Glucosamine, D-Cellobiose, i-Erythritol, L-Histidine, D,L-Carnitine, Uridine, Thymidine, Glucose-1-Phosphate, Glucose-6-Phosphate, Putrescine, Phenyethylamine
Major fatty acids	$C_{18:1}$ *ω6c*, $C_{16:0}$, $C_{16:1}$ *ω5c*

Table 4
Description of *Bradyrhizobium guangzhouense* sp. nov. according to Digital Protologue TA00812 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00812
Date of the entry	2018/11/22
Draft number/date	1
Version	Draft
Type of description	New Description
Species name	*Bradyrhizobium guangzhouense*
Genus name	*Bradyrhizobium*
Specific epithet	*guangzhouense*
Species status	sp. nov.
Species etymology	guang.zhou.en'se. N.L. neut. adj. guangzhouense originating from Guangzhou City in Guangdong Province of China, from where the type strain was isolated
Corresponding author	Xinhua Sui
E-mail of the corresponding author	suixh@cau.edu.cn
Submitter	Yonghua Li
E-mail of the submitter	lyhsierra@foxmail.com
Designation of the type strain	CCBAU 51670
Strain collection numbers	LMG 29,280 = HAMBI 3650
16S rRNA gene accession number	KC508852
Genome accession number [RefSeq]	CP030053/CP030054
Genome status	complete
Genome size	8138 kb
GC mol %	63.95
Data on the origin of the sample from which the strain had been isolated	
Country of origin	CHN
Region of origin	Guangzhou City, Guangdong Province
Source of isolation	Nodules on *Arachis hypogaea*
Sampling date	2011/12/1
Geographic location	Guangzhou
Latitude	113°22′17″N
Longitude	23°20′59″E
Number of strains in study	3
Source of isolation of non-type strains	Nodules on *Arachis hypogaea*
Growth medium, incubation conditions [temperature, pH, and further information]	YMA (per liter: 10 g mannitol, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, 0.2 g $MgSO_4·7H_2O$, 3 g yeast extract, 0.1 g NaCl, 15 g Agar) 28 °C, pH 6.8–7.0
Alternative medium 1	TY (per liter: Tryptone 5.0 g, yeast extract 3.0 g, $CaCl_2·H_2O$ 0.7 g)
Gram stain	Negative
Cell shape	rod
Cell size (length or diameter)	2.32-3.16 × 0.365-0.462 μm
Sporulation (resting cells)	none
Colony morphology	circular, convex, beige-whitish
Temperature range	14-45 °C
Lowest temperature for growth	14 °C (YMA, pH 6.8–7.0)
Highest temperature for growth	45 °C (YMA, pH 6.8–7.0)
Temperature optimum	28 °C
Lowest pH for growth	5 (YMA, 28 °C)
Highest pH for growth	8 (YMA, 28 °C)
pH optimum	7
pH category	neutrophile
Lowest NaCl concentration for growth	0 (YMA, 28 °C, pH 7.0)
Highest NaCl concentration for growth	1% (YMA, 28 °C, pH 7.0)
Salinity optimum	0.80%
Salinity category	nonhalophile (NaCl inhibitory at <1 % NaCl)
Relationship to O_2	aerobe
O_2 conditions for strain testing	Aerobiosis
Carbon source used [class of compounds]	Sugars, amino acids
Positive tests with BIOLOG	Bromo Succinic Acid, D,L-Lactic Acid, D-Arabitol, D-Fructose, D-Galactose, D-Galacturonic Acid, D-Gluconic Acid, D-Glucosaminic Acid, D-Mannose, D-Saccharic Acid, Glycerol, L-Arabinose, L-Pyroglutamic Acid, Methyl Pyruvate, Quinic Acid, Succinamic Acid, Succinic Acid, Tween 40, Tween 80, α-D-Glucose, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid
Negative tests with BIOLOG	2,3-Butanediol, 2-Aminoethanol, Acetic Acid, Adonitol, Citric Acid, D,L-Carnitine, D,L-α-Glycerol Phosphate, D-Cellobiose, D-Psicose, D-Raffinose, D-Trehalose, Gentiobiose, Glucose-1-Phosphate, Glucose-6-Phosphate, Glycogen, Glycyl-L-Aspartic Acid, Glycyl-L-Glutamic Acid, Hydroxy-L-Proline, i-Erythritol, Inosine, Itaconic Acid, Lactulose, L-Alaninamide, L-Alanyl-Glycine, L-Asparagine, L-Glutamic Acid, L-Histidine, L-Ornithine, L-Proline, L-Serine, L-Threonine, Malonic Acid, Maltose, *N*-Acetyl-D-Galactosamine, *N*-Acetyl-D-Glucosamine, Phenyethylamine, Putrescine, Sucrose, Thymidine, Turanose, Uridine, Urocanic Acid, Xylitol, α-D-Lactose, α-Keto Butyric Acid, β-Methyl-D-Glucoside, γ-Amino Butyric Acid
Major fatty acids	$C_{18:1}$ *ω6c*, $C_{16:0}$, $C_{16:1}$ *ω5c*

Table 5
Description of *Bradyrhizobium zhanjiangense* sp. nov. according to Digital Protologue TA00811 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00811
Date of the entry	2018/11/22
Draft number/date	1
Version	Draft
Type of description	New Description
Species name	*Bradyrhizobium zhanjiangense*
Genus name	*Bradyrhizobium*
Specific epithet	*zhanjiangense*
Species status	sp. nov.
Species etymology	zhan.jiang.en'se. N.L. neut. adj. zhanjiangense originating from Zhanjiang City in Guangdong Province of China, from where the type strain was isolated
Corresponding author	Xinhua Sui
E-mail of the corresponding author	suixh@cau.edu.cn
Submitter	Yonghua Li
E-mail of the submitter	lyhsierra@foxmail.com
Designation of the type strain	CCBAU 51778
Strain collection numbers	LMG 29,279 = HAMBI 3648
16S rRNA gene accession number	KC508861
Genome accession number [RefSeq]	CP022221
Genome status	complete
Genome size	9342 kb
GC mol %	62.94
Data on the origin of the sample from which the strain had been isolated	
Country of origin	CHN
Region of origin	Zhanjiang City, Guangdong Province
Source of isolation	Nodules on *Arachis hypogaea*
Sampling date	2011/12/1
Geographic location	Zhanjiang
Latitude	110º21′01″N
Longitude	21º13′15″E
Number of strains in study	4
Source of isolation of non-type strains	Nodules on *Arachis hypogaea*
Growth medium, incubation conditions [temperature, pH, and further information]	YMA (per liter: 10 g mannitol, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract, 0.1 g NaCl, 15 g Agar) 28 °C, pH 6.8–7.0
Alternative medium 1	TY (per liter: Tryptone 5.0 g, yeast extract 3.0 g, $CaCl_2 \cdot H_2O$ 0.7 g)
Gram stain	Negative
Cell shape	rod
Cell size (length or diameter)	1.28-2.45 × 0.441-0.486 μm
Sporulation (resting cells)	none
Colony morphology	circular, convex, beige-whitish
Temperature range	14-45 °C
Lowest temperature for growth	14 °C (YMA, pH 6.8–7.0)
Highest temperature for growth	45 °C (YMA, pH 6.8–7.0)
Temperature optimum	28 °C
Lowest pH for growth	5 (YMA, 28 °C)
Highest pH for growth	8 (YMA, 28 °C)
pH optimum	7
pH category	neutrophile
Lowest NaCl concentration for growth	0 (YMA, 28 °C, pH 7.0)
Highest NaCl concentration for growth	1% (YMA, 28 °C, pH 7.0)
Salinity optimum	0.80%
Salinity category	nonhalophile (NaCl inhibitory at <1 % NaCl)
Relationship to O_2	aerobe
O_2 conditions for strain testing	Aerobiosis
Carbon source used [class of compounds]	Sugars, amino acids
Positive tests with BIOLOG	Bromo Succinic Acid, *cis*-Aconitic Acid, D,L-Lactic Acid, D-Fructose, D-Galactonic Acid Lactone, D-Galacturonic Acid, D-Gluconic Acid, D-Mannitol, D-Saccharic Acid, Glucuronamide, Glycyl-L-Aspartic Acid, Itaconic Acid, L-Alaninamide, L-Asparagine, L-Aspartic Acid, L-Fucose, L-Glutamic Acid, L-Leucine, L-Phenylalanine, L-Proline, L-Pyroglutamic Acid, L-Rhamnose, L-Threonine, Quinic Acid, Succinamic Acid, Succinic Acid, Tween 40, Tween 80, α-Keto Glutaric Acid, β-Hydroxy Butyric Acid
Negative tests with BIOLOG	D-Serine, Phenyethylamine, Putrescine
Major fatty acids	$C_{18:1}$ *ω6c*, $C_{19:0}$ cyclo *ω8c*, $C_{16:0}$

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.126002.

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