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Efficient Nitrogen-Fixing Bacteria Isolated from Soybean Nodules in the Semi-arid Region of Northeast Brazil are Classified as *Bradyrhizobium brasilense* (Symbiovar Sojae)

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Abstract

Soybean (*Glycine max* L.) is an important legume that greatly benefits from inoculation with nitrogen-fixing bacteria. In a previous study, five efficient nitrogen-fixing bacterial strains, isolated from nodules of soybean inoculated with soil from semi-arid region, Northeast Brazil, were identified as a new group within the genus *Bradyrhizobium*. The taxonomic status of these strains was evaluated in this study. The phylogenetic analysis of the 16S rRNA gene showed the high similarity of the five strains to *Bradyrhizobium brasilense* UFLA03-321^T (100%), *B. pachyrhizi* PAC48^T (100%), *B. ripae* WR4^T (100%), *B. elkanii* USDA 76^T (99.91%), and *B. macuxiense* BR 10303^T (99.91%). However, multilocus sequence analysis of the housekeeping genes *atpD*, *dnaK*, *gyrB*, *recA,* and *rpoB*, average nucleotide identity, and digital DNA–DNA hybridization analyses supported the classification of the group as *B. brasilense.* Some phenotypic characteristics allowed differentiating the five strains and the type strain of *B. brasilense* from the two neighboring species (*B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T). The *nodC* and *nifH* genes' analyses showed that these strains belong to symbiovar sojae, together with *B. elkanii* (USDA 76^T) and *B. ferriligni* (CCBAU 51502^T). The present results support the classification of these five strains as *Bradyrhizobium brasilense* (symbiovar sojae).

Introduction

Soybean (*Glycine max* L.) is an important protein source used worldwide. In Brazil, the second largest world soybean producer, this crop occupies about 56% of the planted area, playing a significant economic and social role. A factor that decisively contributes to the success of soybean production in Brazil and its market competitiveness is the exploitation of biological nitrogen fixation (BNF) by the inoculation with strains of the *Bradyrhizobium* genus—SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*), and SEMIA 5019 (*B. elkanii*).

Soybean has been a major host from which species of the *Bradyrhizobium* genus are isolated. Nine out of the 54 species of this genus have been isolated from soybean: *B. japonicum* USDA 6^T, from Japan [1]; *B. elkanii* 76^T, and *B. diazoefficiens* USDA 110^T, from the United States [2, 3]; *B. liaoningense* LMG 18230^T, *B. yuanmingense* CCBAU 10071^T, *B. huanghuaihaiense* CCBAU23303^T, and *B. daqingense* CCBAU 15774^T from China [4–7]; *B. ottawaense* OO99^T and *B. amphicarpaeae* 39S1MB^T, from Canada [8, 9]. Besides, only 8 out of 23 *Bradyrhizobium* species isolated from other hosts and tested in soybean had their ability to nodulate this species confirmed, of which 2 formed efficient symbiosis: *B. ferriligni* CCBAU 51502^T and *B. shewense* ERR11^T [10, 11]. Despite the economic importance of

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soybean throughout Brazil, no new *Bradyrhizobium* species have been described based on native isolates nodulating this crop in Brazilian soils.

The use of integrated phenotypic, genotypic, and phylogenetic information is necessary to define the true taxonomic position of a strain or group of strains. In recent years, housekeeping genes (*atpD*, *dnaK, glnII*, *gyrB*, *recA*, and *rpoB*) as phylogenetic markers for bacteria have been widely used for the separation of genetically close strains into different species of the *Bradyrhizobium* genus [4–6, 8, 12, 13]. This analysis, together with new methods for genome-to-genome comparisons, such as average nucleotide identity (ANI) and digital DNA–DNA hybridization (DDH) analyses [14, 15], has contributed significantly to improving the taxonomy of *Bradyrhizobium* and delineate new species [12, 13, 16].

In a previous study [17], 46 strains isolated from nodules of soybean inoculated with soils from different Brazilian regions (Midwest, Northeast, Southeast and South) were classified within the *Bradyrhizobium* genus, based on the partial sequencing of the 16S rRNA gene, and allocated within two new phylogenetic groups, based on the concatenated sequence analysis of the five housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA* and *rpoB*). In the present study, five strains of one of these groups (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22), efficient in nitrogen fixation in symbiosis with soybean [17], were selected for the definition of their taxonomic position.

Materials and Methods

Origin of the Strains

The five strains were isolated from effective nodules of soybean inoculated with soil from the semi-arid region, Northeast Brazil, collected in Bom Jesus (9° 19′ 21″ S and 44° 48′ 55″ W), in a previous study [17]. The soil used as an inoculum was, during previous soybean cultivation, inoculated with the following soybean-inoculant strains: SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*), and/or SEMIA 5019 (*B. elkanii*). Isolation was carried out on plates with 79 culture medium [18], also known as YMA (Yeast Mannitol Agar) [19].

Phylogenetic Analysis of the 16S rRNA Gene and the Housekeeping Genes

Sequences of 16S rRNA (1288 to 1331 bp), *atpD* (510 pb), *dnaK* (280 pb), *gyrB* (669 pb), *recA* (474 to 559 pb), and *rpoB* (903 to 957 pb) genes of the five strains were obtained in a previous study [17]. Sequences of each gene were aligned using the ClustalW Multiple Alignment algorithms in the BioEdit software. For comparison, the alignment included the sequences of type strains of the *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI). The sequences of four strains currently used as soybean inoculants in Brazil (SEMIA 5079 *B. japonicum*, SEMIA 5080 *B. diazoefficiens*, SEMIA 587 *B. elkanii*, and SEMIA 5019 *B. elkanii*) were also included in the alignment of *dnaK* and *recA* genes.

The first multilocus sequence analysis (MLSA) was conducted using the sequences of the *dnaK* and *recA* genes since their sequences are the only available, among the five housekeeping genes we studied, for the four strains currently used as soybean inoculants in Brazil. An MLSA with *atpD, dnaK*, *gyrB, recA,* and *rpoB* genes, without the inoculant strains, was also performed. Phylogenetic trees were constructed by the maximum likelihood method (ML) [20], using the Kimura 2 parameter model [21]. The MEGA 6 software package [22] was used in the construction of trees, with bootstrap values based on 1000 replications. The accession numbers of the gene sequences used in this study are indicated in the phylogenetic trees. *Bosea thiooxidans* DSM 9653^T was used as an outgroup in the phylogenetic analysis of the 16S rRNA gene.

Genome Sequencing, Average Nucleotide Identity, and Digital DNA–DNA Hybridization

Genomic comparisons were used in this study to support the taxonomic position of the new group. The average nucleotide identity (ANI) and digital DNA–DNA hybridization were estimated with the genomic sequences of a representative strain of the group (UFLA06-13) and of the phylogenetically closest type strains (*Bradyrhizobium brasilense* UFLA03-321^T, *Bradyrhizobium pachyrhizi* PAC48^T, and *Bradyrhizobium elkanii* USDA 76^T) in MLSA with *atpD, dnaK*, *gyrB, recA,* and *rpoB* genes.

Strain UFLA06-13 was grown in liquid 79 medium for genome sequencing. Genomic DNA was purified from 10^9 bacterial cells using the phenol–chloroform extraction protocol. The DNA library was constructed from 1 ng of total DNA with the Nextera XT kit (Illumina), following the conditions suggested by the manufacturer. Pair-end reads (2 × 250 bases) were sequenced with the MiSeq Reagent kit 500v2 (Illumina) on the MiSeq platform (Illumina). The estimated sequencing depth was 30×, and the raw reads were subject to de novo assembly in SPAdes 3.12, with default parameters [23]. The resulting assembly consisted of 8.59 Mb distributed in 561 contigs, with N50 of 188,493. Using checkM 1.0.11 [24], the completeness of the assembled genome, based on 824 lineage-specific marker genes, was estimated as 100%, whereas the contamination was estimated as only 0.52%. The authenticity of the sequenced genome was checked by verifying that

the five sequences of housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*) reported by Ribeiro et al. [17] for this strain were identical to those extracted from the genome.

Average nucleotide identity (ANI) values were calculated using an online calculator (available at https://enve-omics.ce.gatech.edu/ani/index) [14] to compare the strain UFLA06-13 (accession number SNUC00000000), obtained in this study, with the strains UFLA03-321^T (accession number MPVQ00000000), PAC48^T (accession number SAMN03782120), and USDA 76^T (accession number NZ_ARAG00000000).

Phenotypic Characterization

The five strains were phenotypically characterized based on parameters previously used to differentiate *Bradyrhizobium* species. Two soybean inoculants strains classified as *Bradyrhizobium elkanii* (SEMIA 587 and SEMIA 5019) were also included in the analyses. The growth of these strains in 79 medium was evaluated under different conditions of pH (4, 5.5, 6.8, 8, 9 and 10), NaCl (w/v) (0.01, 0.25, 0.5, 0.75 and 1%), and temperature (5, 15, 20, 28, 34, 37 and 40 °C), according to the methodology previously described by Florentino et al. [25].

Their ability to assimilate different carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, maltose, mannitol, L-methionine, sodium lactate, and sucrose) and nitrogen sources (L-arginine, L-asparagine, casein hydrolyzed, L-cysteine, glycine, L-glutamic acid, and L-methionine and tryptophan) was evaluated in modified 79 medium, based on the composition described in the study of Costa et al. [12].

Antibiotic resistance was also tested in 79 medium on plates with bio-disks containing the following antibiotics: ampicillin (10 µg mL^{-1}), cefuroxime (30 µg mL^{-1}), ciprofloxacin (5 µg mL^{-1}), chloramphenicol (30 µg mL^{-1}), doxycycline (30 µg mL^{-1}), erythromycin (15 µg mL^{-1}), gentamicin (10 µg mL^{-1}), kanamycin (30 µg mL^{-1}), and neomycin (30 µg mL^{-1}), according to the protocol previously described by Guimarães et al. [26].

In previous studies, the growth of the type strains UFLA03-321^T (*B. brasilense*), PAC48^T (*B. pachyrhizi*), and USDA 76^T (*B. elkanii*) in 79 medium was evaluated under the same conditions of pH, NaCl, temperature, carbon, and nitrogen sources, as well their resistance to those nine antibiotics above, using the same protocols for strain characterization adopted in this study [12, 26]. These strains were included in the phenotypic characterization table for comparison.

Phylogenetic Analysis of the Symbiotic Genes (*nodC* and *nifH*)

For the phylogenetic analysis of the symbiotic genes (*nodC* and *nifH)*, the DNA of five strains was extracted by the alkaline lysis method [27]. The protocol of Sarita et al. [28], modified by De Meyer et al. [29], was used for amplification and sequencing of the *nodC* gene. The amplification and sequencing of the *nifH* gene were performed according to Gaby and Buckley [30]. For strains UFLA06-21 and UFLA06-19, the amplification of the *nodC* and *nifH* genes, respectively, was not possible. The type strains of the *Bradyrhizobium* species available in the GenBank and the four strains used as soybean inoculants were included in the alignment of sequences of the *nifH* and *nodC* genes (SEMIA 5079, SEMIA 5080, SEMIA 587 and SEMIA 5019). Phylogenetic trees were constructed as described above.

Results

Phylogenetic Analysis of the 16S rRNA Gene and the Housekeeping Genes

The five strains showed 16S rRNA gene sequences identical to SEMIA 587 (*B. elkanii*), SEMIA 5019 (*B. elkanii*), UFLA03-321^T (*B. brasilense*), PAC48^T (*B. pachyrhizi*), and WR4^T (*B. ripae*) and shared 99.91% similarity with USDA 76^T (*B. elkanii*) and BR 10303^T (*B. macuxiense*) (Fig. 1). In the first MLSA, performed with the sequences of the housekeeping genes *dnaK* and *recA*, the five strains had identical sequences and formed a separate group, which shared 97.41% of similarity with UFLA03-321^T (*B. brasilense*), 97.28% of similarity with USDA 76^T (*B. elkanii*), 97.27% of similarity with PAC48^T (*B. pachyrhizi*), 97.08% of similarity with WR4^T (*B. ripae*), and 97.84% of similarity with SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*) (Supplementary Fig. 1). However, concatenated sequence analysis of the five housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA,* and *rpoB*) revealed that the five strains share greater similarity with *B. brasilense* UFLA03-321^T (98.95%), followed by *B. pachyrhizi* PAC48^T (97.63) and *B. elkanii* USDA 76^T (97.62) (Fig. 2).

Genome Sequencing, Average Nucleotide Identity, and Digital DNA–DNA Hybridization

The ANI values estimated from the genome sequences of the representative strain of the group (UFLA06-13) and of the phylogenetically closest type strains UFLA03-321^T (*B.*

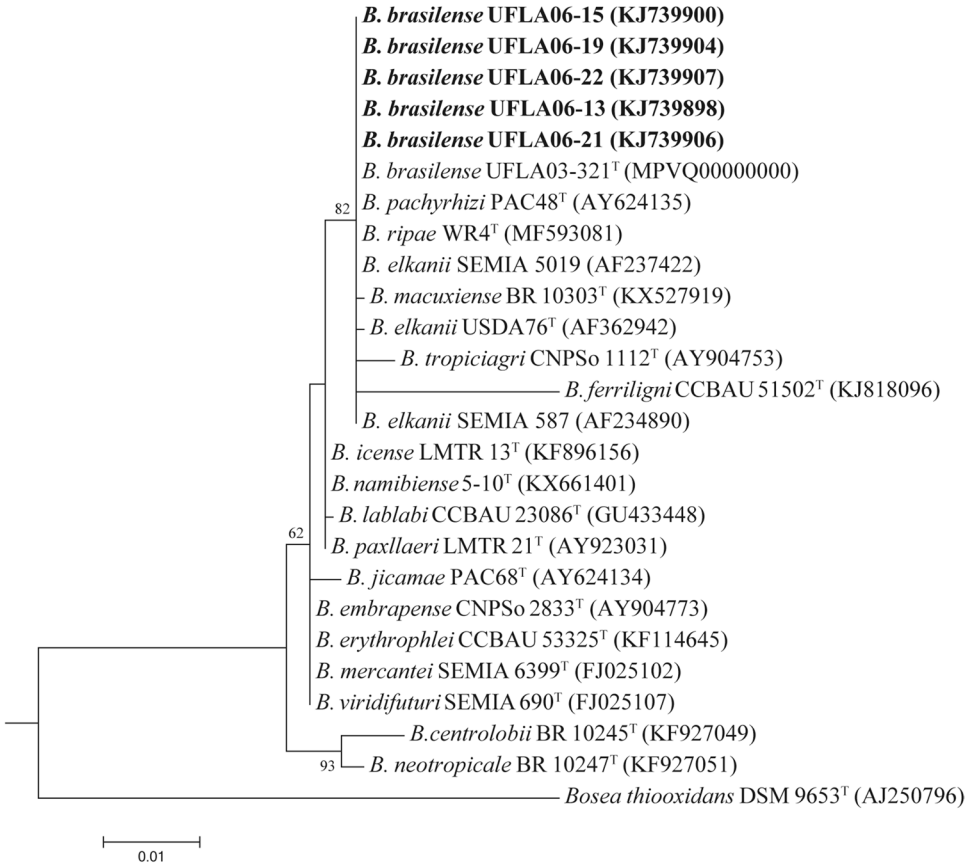

Fig. 1 Maximum Likelihood phylogeny based on 16S rRNA gene sequences (1226 bp) showing the relationships between strains UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22 (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values higher than 50% are indicated at nodes. GenBank accession numbers are provided in parentheses

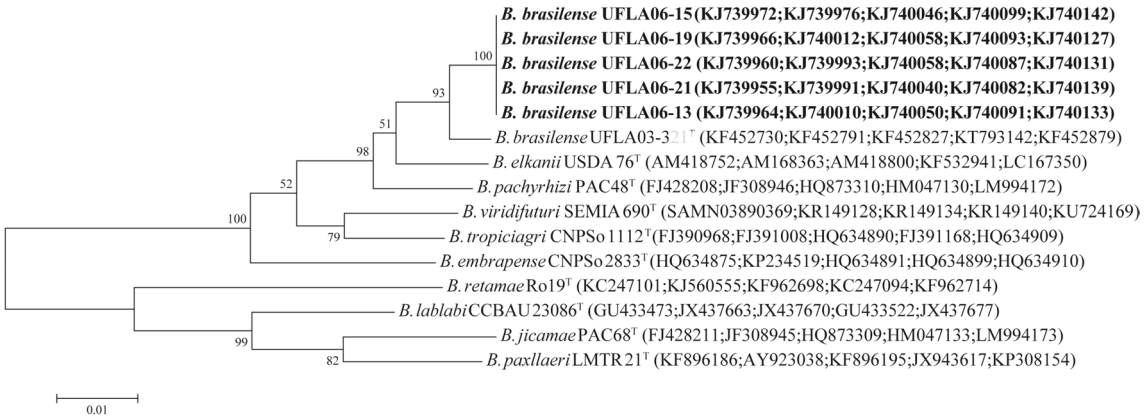

Fig. 2 Maximum Likelihood phylogeny based on partial concatenated sequences (2036 bp) of housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*) showing the relationships between strains UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22 (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values higher than 50% are indicated at nodes. GenBank accession numbers are provided in parentheses

brasilense), PAC48^T (*B. pachyrhizi*), and USDA 76^T (*B. elkanii*) were 98.28, 95.50, and 95.70%, respectively.

Phenotypic Characterization

All strains grow at a wide range of pH levels (4–10) and temperatures (15–37 °C); however, they do not grow at 1% NaCl in 79 culture medium (Table 1). Strains PAC 48^T (*B. pachyrhizi*), USDA 76^T, and SEMIA 587 (*B. elkanii*) show weak growth at

Table 1 Phenotypic characteristics of the *Bradyrhizobium brasilense* strains isolated from *Glycine max* nodules (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22) and *Vigna unguiculata* nodule (UFLA03-321^T) from Brazilian soils and phylogenetically close *Bradyrhizobium* strains

Characteristic	UFLA 06–13	UFLA 06–15	UFLA 06–19	UFLA 06–21	UFLA 06–22	UFLA 03-321Ta	PAC 48Ta	USDA 76Ta	SEMIA 5019	SEMIA 587
Growth at										
15 ºC	+	+	+	+	+	+	+	+	+	+
37 ºC	+	+	+	+	+	+	+	+	+	+
40 ºC	−	−	−	−	−	−	w	w	−	w
0.75% NaCl	w	w	w	w	w	+	+	+	+	+
1% NaCl	−	−	−	−	−	−	−	−	−	−
pH 4.0	+	+	+	+	+	+	+	+	+	+
pH 10.0	+	+	+	+	+	+	+	+	+	+
Carbon source assimilation										
D-Arabinose	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+
L-Asparagine	w	w	w	w	w	w	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	w	w
Citric acid	−	−	−	−	−	−	−	−	−	−
Glycerol	+	+	+	+	+	+	+	+	+	+
Glycine	−	−	−	−	−	−	−	−	−	−
D-Glucose	+	+	+	+	+	w	+	+	+	+
L-Glutamine	+	+	+	+	w	w	+	+	+	+
L-Glutamic acid	w	w	w	w	w	+	+	+	+	+
Lactose	w	−	−	w	w	−	+	w	+	+
Malic acid	−	−	−	−	−	−	w	w	+	w
Mannitol	+	+	+	+	+	+	+	+	+	+
L-Methionine	−	−	−	−	−	−	w	w	w	w
Sodium lactate	−	−	−	−	−	−	+	+	+	+
Sucrose	−	−	−	−	−	−	+	+	+	+
Nitrogen source assimilation										
L-Arginine	−	−	−	−	−	w	+	+	+	+
L-Asparagine	+	+	+	+	+	+	+	+	+	+
Casein hydrolysate	+	+	+	+	+	+	w	+	+	+
L-Cysteine	−	−	−	−	−	−	+	+	+	+
Glycine	−	−	−	−	−	−	−	−	−	−
Glutamic acid	+	+	+	+	+	+	+	+	+	+
L-Methionine	−	−	−	−	−	−	w	w	−	−
Tryptophan	−	−	−	−	−	−	−	−	w	w

Table 1 (continued)

Characteristic	UFLA 06–13	UFLA 06–15	UFLA 06–19	UFLA 06–21	UFLA 06–22	UFLA 03-321Ta	PAC 48Ta	USDA 76Ta	SEMIA 5019	SEMIA 587
Resistance to antibiotics (μg mL^{-1})										
Ampicillin (10)	+	+	+	+	+	+	+	+	+	+
Cefuroxime (30)	+	+	+	+	+	+	+	+	+	+
Ciprofloxacin (5)	+	+	+	+	+	+	+	+	+	+
Chloramphenicol (30)	+	w	+	+	+	+	+	+	+	+
Doxycycline (30)	+	w	+	+	+	+	+	+	+	+
Erythromycin (15)	+	w	+	+	+	+	+	+	+	+
Gentamycin (10)	−	−	−	−	−	+	−	+	+	−
Kanamycin (30)	−	−	−	−	−	−	−	−	−	−
Neomycin (30)	−	−	−	−	−	+	−	w	w	w

+, positive; −, negative; w, weak

[a]Data of the strains UFLA03-321 ^{T}, USDA 76^{T}, and PAC 48 ^{T} were extracted from Guimarães et al. [26] and Costa et al. [12]

40 °C, while *B. brasilense* strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, UFLA06-22, and UFLA03-321^{T}) and the strain SEMIA 5019 (*B. elkanii*) does not grow at this temperature (Table 1). All strains use D-arabinose, L-arabinose, glycerol, and mannitol but do not use citric acid and glycine as a carbon source (Table 1).

B. brasilense strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, UFLA06-22, and UFLA03-321^{T}) weakly use L-asparagine and do not use malic acid, L-methionine, sodium lactate, and sucrose. Conversely, strains PAC48^{T} (*B. pachyrhizi*), USDA 76^{T}, SEMIA 587, and SEMIA 5019 (*B. elkanii*) use L-asparagine, sodium lactate, and sucrose and weakly use L-methionine as a carbon source. The use of D-glucose, L-glutamic acid, L-glutamic acid, and lactose as a carbon source varied among *B. brasilens* strains.

In relation to the nitrogen sources, all strains assimilate L-asparagine and L-glutamic acid, but none of them use glycine as a nitrogen source (Table 1). Likewise, *B. brasilense* strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, UFLA06-22, and UFLA03-321^{T}) do not use L-cysteine, L-methionine, and tryptophan as N source. All strains are tolerant to the antibiotics ampicillin, cefuroxime, ciprofloxacin, chloramphenicol, doxycycline, and erythromycin, except for UFLA06-15, which showed weak tolerance to chloramphenicol, doxycycline, and erythromycin. Strains UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, UFLA06-22, and PAC 48^{T} are sensitive to the antibiotics gentamycin, kanamycin, and neomycin.

Phylogenetic Analysis of the Symbiotic Genes (*nodC* and *nifH*)

Strains UFLA06-13, UFLA06-15, UFLA06-19, and UFLA06-22 had *nodC* gene sequences identical to USDA 76^{T} and SEMIA 5019 (*B. elkanii*) and shared 99.45% similarity with SEMIA 587 (*B. elkanii*), forming a separate group, close to *B. ferriligni* CCBAU 51502^{T} (96.33% similarity) (Supplementary Fig. 2). In the phylogenetic analysis of the *nifH* gene, strains UFLA06-13, UFLA06-15, UFLA06-21, and UFLA06-22 showed identical sequences and shared 99.43% of similarity with USDA 76^{T} (*B. elkanii*), 98.84% of similarity with SEMIA 5019 and SEMIA 587 (*B. elkanii*), and 98.25% of similarity with *B. ferriligni* CCBAU 51502^{T}) (Supplementary Fig. 3). The new *B. brasilense* strains shared only 78.39 and 89.74% of similarity with the type strain of *B. brasilense* (UFLA03-321^{T}) in the phylogenetic analysis of the *nodC* and *nifH* genes, respectively.

Discussion

This study investigated the taxonomic position of a group of *Bradyrhizobium* strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22) isolated from nodules of soybean inoculated with soils from the semi-arid region, Northeast Brazil. The fact that the five strains show 16S rRNA gene sequences identical to five *Bradyrhizobium* species confirms that the 16S rRNA gene does not have sufficient discriminatory power to distinguish members of the *Bradyrhizobium* genus, corroborating previous studies [10, 12, 13, 17, 26, 31].

Multilocus sequence analysis of housekeeping genes (*atpD*, *dnaK*, *glnII, gyrB*, *recA,* and *rpoB*) has been successfully employed for better discrimination among closely related strains within the *Bradyrhizobium* genus [6–8, 16, 32]. In the present study, the MLSA performed with the sequences of the housekeeping genes *dnaK* and *recA* was not sufficient to define the taxonomic position of the five strains (Supplementary Fig. 1). Conversely, MLSA with five genes (*atpD*, *dnaK*, *gyrB*, *recA,* and *rpoB*) indicated that these strains belong to the *Bradyrhizobium brasilense* species (Fig. 2). This result reveals the possibility of getting a better taxonomic resolution by employing a larger number of housekeeping genes in the MLSA.

Taxonomic groups separated by MLSA are usually evaluated by DNA–DNA hybridization (DDH) analysis to confirm their taxonomic position. DDH analysis has been used as the standard method for species delineation, considering a cutoff score of 70% genomic relatedness [33]. Instead of using the traditional DDH, this study used a new method to compare bacterial genomes, the average nucleotide identity (ANI). This method has been extensively used to replace the traditional DDH analysis, avoiding the high costs, labor, and specialized infrastructure. The recommended cutoff score of 70% based on traditional DDH for species delineation [33] has been found to correlate to 95–96% of the ANI values [14, 34].

A recent study applied a threshold of 96% ANI to delineate a new species of *Bradyrhizobium* (*B. brasilense* UFLA03-321^{T}), which showed ANI values of 95.5% with *B. pachyrhizi* (PAC48T) and 94.0% with *B. elkanii* (USDA 76^{T}) [12]. The cutoff score of 96% has also been previously used to delineate species in other genera [35]. According to this cutoff score, the ANI values of 95.50 and 95.70% between the strain UFLA06-13 and strains PAC48^{T} (*B. pachyrhizi*) and USDA 76^{T} (*B. elkanii*), respectively, in this study, indicate that they represent distinct species. Conversely, the ANI value of 98.28% between the strain UFLA06-13 and the strain UFLA03-321^{T} supports the classification of the group as *Bradyrhizobium brasilense*, confirming the taxonomic position indicated by MLSA of the housekeeping genes *atpD*, *dnaK*, *gyrB*, *recA,* and *rpoB*. The present study reports for the first time the classification of strains isolated from nodules of soybean within a new *Bradyrhizobium* species (*B. brasilense*) native from Brazilian soils.

Phenotypic characteristics usually have little taxonomic value since they vary within the same bacterial species [36]. However, the information on phenotypic variability can be useful to verify that strains within a given species are not merely clones. Although the *B. brasilense* strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, UFLA06-22, and UFLA03-321^{T}) showed similar phenotypes in relation to most of the evaluated characteristics, they differed regarding the growth at 0.75% NaCl; the use of D-Glucose, L-Glutamine, L-Glutamic acid, and Lactose as a carbon source; the use of L-Arginine as a nitrogen source; and the tolerance to the antibiotics Chloramphenicol (30 µg mL^{-1}), Doxycycline (30 µg mL^{-1}), Erythromycin (15 µg mL^{-1}), Gentamycin (10 µg mL^{-1}), and Neomycin (30 µg mL^{-1}). The main differential phenotypic characteristics between *B. brasilense* strains and phylogenetically closest strains were the absence of growth at 40 °C; weak use of L-asparagine and no use of malic acid; L-methionine, sodium lactate, and sucrose as a carbon source; and L-cysteine, L-methionine, and tryptophan as a nitrogen source.

The tolerance of the strains to a wide range of pH levels and temperatures and their resistance to several antibiotics corroborate previous studies on *Bradyrhizobium* strains [13, 16, 26, 37]. These characteristics are desirable in the selection of rhizobia since the inoculant strains are recommended for use under different soil and climate conditions. These strains also exhibited good symbiotic efficiency with soybean in a previous study, showing their potential for selection and use as inoculants in this crop [17].

Genes involved in nodulation and nitrogen fixation processes usually do not provide relevant taxonomic information for species classification. However, phylogenetic analyses of symbiotic genes have been used for the delineation of symbiovars, based on similar symbiotic behaviors of rhizobia groups with a determined host range [38]. The symbiovars within the *Bradyrhizobium* genus have been mainly defined according to the analysis of the *nodC* gene [32, 39–41]. However, other genes, such as *nodA* and *nifH,* have also been recommended for this purpose [41, 42].

In this study, phylogenetic analyses of *nodC* and *nifH* genes clustered the strains UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22 with *B. elkanii* (strains USDA 76^{T}, SEMIA 587, and SEMIA 5019) and *B. ferriligni* (strain CCBAU 51502^{T}). These *B. elkanii* strains, isolated from *Glycine max* nodules in soils from the United States (USDA 76^{T}) [2], from South (SEMIA 587) and Southeast (SEMIA 5019) regions of Brazil [43], and the strain of *B. ferriligni* (CCBAU 51502^{T}), isolated from

Erythrophleum fordii nodule in soils from China [10], have been recently classified within a new symbiovar denominated sojae, based on the phylogenetic analysis of the genes *nodC* and *nifH* [41]. Thus, this study proposes that the new *B. brasilense* strains (UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22) must be allocated within the symbiovar sojae.

Predominance of slow-growing non-acid producing rhizobia type (now assigned to *Bradyrhizobium* genus) in tropical areas was hypothesized by Norris [44]. Indeed, for instance *Bradyrhizobium japonicum* and *B. elkanii* are widespread symbionts of diverse Brazilian native legume species from Amazonia and Atlantic forests [45, 46] in non-inoculated areas, many of them similar to *B. elkanii* USDA 76^T. Thus, it is unlikely that these native *Bradyrhizobium* species had acquired their symbiotic genes from soybean nodulating bacteria because this exotic species was introduced in the end of the nineteen century in Brazil. This is also supported by the fact that *Bradyrhizobium* is being found to be the predominant rhizobia genus in evaluations by culture-independent techniques throughout the world [47] indicating its ubiquity and a high saprophytic ability. Interestingly, *B. brasilense* belongs to *B. elkanii* superclade [48]. Thus, the high affinity of *nodC* and *nifH* genes of *B. brasilense* sv. sojae with *B. elkanii* USDA 76^T is coherent with the affinity of their genomes. It is possible that our strains acquired symbiotic genes by horizontal gene transfer (HGT) from soybean strains in the area as hypothesized by Ferreira and Hungria [49] and Barcellos et al. [50] to explain the variability among isolates observed in their study. However, other strains of *B. brasilense* isolated from non-inoculated fields were efficient, for instance, in siratro [12], which unlike soybean is a promiscuous host capable of nodulating with bradyrhizobia containing a wide range of nod genotypes. Moreover, symbiotic genes are in the chromosome (not so easily transferred, although rapid transfer has been demonstrated, for example, in Germany [51]), and the fields in our study were inoculated recently [17]. In spite of this evidence of HGT from inoculant soybean strains to local *B. brasilense* strains, there is a possibility that *B. brasilense* had already harbored the symbiotic genes, although we have yet to observe these genes in *B. brasilense* strains isolated from native legumes.

The ability of the five new *B. brasilense* strains to effectively nodulate and fix nitrogen with their original host (*Glycine max*) was confirmed in a previous study [17]. The strain UFLA06-13 has also been evaluated for nodulation capacity in other hosts (*Vigna unguiculata*, *Phaseolus lunatus*, *Stizolobium aterrimum,* and *Acacia mangium*) [52, Costa et al., unpublished results]. This strain formed nodules in *V. unguiculata* and *S. aterrimum* but did not nodulate *P. lunatus* and *A. mangium*. Interestingly, the four *B. brasiliense* strains described in a previous study (UFLA03-321^T, UFLA03-320, and UFLA03-290 from *V. unguiculata* nodules; and UFLA04-0212, from *Macroptilium atropurpureum* nodules) also did not nodulate *A. mangium* [52, Costa et al., unpublished results]; however, they were able to nodulate *V. unguiculata*, *S. aterrimum,* and *Glycine max* [12, 26, 53, Costa et al., unpublished results], except for strain UFLA03-290, which was not evaluated for its nodulation capacity in soybean. Similarly, strains UFLA06-13 and UFLA03-290 do not nodulate *P. lunatus*, while strains UFLA03-321^T, UFLA03-320, and UFLA04-0212 inefficiently nodulate this legume [52]. Although the *B. brasilense* strains used in this study belong to a symbiovar (sv. sojae) different from those of the *B. brasilense* strains previously described (sv. tropici) [9], the data regarding the ability of nodulation with legumes clearly show that these *B. brasilense* strains share some symbiotic similarity.

Conclusions

Results from the multilocus sequence analysis of five housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*), the average nucleotide identity analysis, and the phenotypic characterization support the classification of strains UFLA06-13, UFLA06-15, UFLA06-19, UFLA06-21, and UFLA06-22 as *Bradyrhizobium brasilense*. Phylogenetic analyses of *nodC* and *nifH* genes show that these strains belong to the symbiovar sojae, together with *B. elkanii* (USDA 76^T) and *B. ferriligni* (CCBAU 51502^T).

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int J Syst Bacteriol 32:136–139. https://doi.org/10.1099/00207713-32-1-136
2. Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can J Microbiol 38:501–505. https://doi.org/10.1139/m92-082
3. Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Melo IS, Martínez- Romero E, Hungria M (2013) Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group

Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. Int J Syst Evol Microbiol 63:3342–3351. https://doi.org/10.1099/ijs.0.049130-0
4. Xu LM, Ge C, Cui Z, Li J, Fan H (1995) *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int J Syst Bacteriol 45:706–711. https://doi.org/10.1099/00207713-45-4-706
5. Yao ZY, Kan FL, Wang ET, Chen WX (2002) Characterization of rhizobiathat nodulate legume species within the genus Lespedeza and description of *Bradyrhizobium yuanmingense* sp. nov. Int J Syst Bacteriol 52:2219–2230. https://doi.org/10.1099/00207713-52-6-2219
6. Zhang YM, Li JY, Chen WF, Wang ET, Sui XH, Li QQ, Zhang YZ, Zhou YG (2012) Chen WX (2012) *Bradyrhizobium huanghuaihaiense* sp nov, an effective symbiotic bacterium isolated from soybean (*Glycine max* L) nodules. Int J Syst Evol Microbiol 62:1951–1957. https://doi.org/10.1099/ijs.0.034546-0
7. Wang JY, Wang R, Zhang YM, Liu HC, Chen WF, Wang ET, Sui XH, Chen WX (2013) *Bradyrhizobium daqingense* sp. nov., isolated from soybean nodules. Int J Syst Evol Microbiol 63:616–624. https://doi.org/10.1099/ijs.0.034280-0
8. Yu X, Cloutier S, Tambong JT, Bromfield ESP (2014) *Bradyrhizobium ottawaense* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of soybeans in Canada. Int J Syst Evol Microbiol 64:3202–3207. https://doi.org/10.1099/ijs.0.065540-0
9. Bromfield ESP, Cloutier S, Nguyen HDT (2019) Description and complete genome sequence of *Bradyrhizobium amphicarpaeae* sp. nov., harbouring photosystem and nitrogen fixation genes. Int J Syst Evol Microbiol. https://doi.org/10.1099/ijsem.0.003569
10. Yao Y, Sui XH, Zhang XX, Wang ET, Chen WX (2015) *Bradyrhizobium erythrophlei* sp. nov. and *Bradyrhizobium ferriligni* sp. nov., isolated from effective nodules of *Erythrophleum fordii*. Int J Syst Evol Microbiol 65:1831–1837. https://doi.org/10.1099/ijs.0.000183
11. Amsalu AA, Woyke T, Kyrpides NC, Whitman WB, Lindström K (2017) Draft genome sequences of *Bradyrhizobium shewense* sp. nov. ERR11^{T} and *Bradyrhizobium yuanmingense* CCBAU 10071^{T}. Stand Genomic Sci 5:12–74. https://doi.org/10.1186/s40793-017-0283-x
12. Costa EM, Guimarães AA, Vicentin RP, Ribeiro PRA, Leão ACR, Balsanelli E, Lebbe L, Aerts M, Willems A, Moreira FMS (2017) *Bradyrhizobium brasilense* sp. nov., a symbiotic nitrogen-fixing bacterium isolated from Brazilian tropical soils. Arch Microbiol 199:1211–1221. https://doi.org/10.1007/s00203-017-1390-1
13. Costa EM, Guimarães AA, Carvalho TS, Rodrigues TL, Ribeiro PRA, Lebbe L, Willems A, Moreira FMS (2018) *Bradyrhizobium forestalis* sp. nov., an efficient nitrogen-fixing bacterium isolated from nodules of forest legume species in the Amazon. Arch Microbiol 200:743–752. https://doi.org/10.1007/s00203-018-1486-2
14. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91. https://doi.org/10.1099/ijs.0.64483-0
15. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinform 14:60. https://doi.org/10.1186/1471-2105-14-60
16. Durán D, Rey L, Navarro A, Busquets A, Imperial J, Ruiz-Argüeso T (2014) *Bradyrhizobium valentinum* sp. nov., isolated from effective nodules of *Lupinus mariae-josephae*, a lupine endemic of basic-lime soils in Eastern Spain. Syst Appl Microbiol 37:336–341. https://doi.org/10.1016/j.syapm.2014.05.002
17. Ribeiro PRA, Santos JV, Costa EM, Lebbe L, Louzada MO, Guimarães AA, Guimarães AES, Willems A, Moreira FMS (2015) Symbiotic efficiency and genetic diversity of soybean bradyrhizobia in Brazilian soils. Agr Ecosyst Environ 212:85–93. https://doi.org/10.1016/j.agee.2015.06.017
18. Fred EB, Waksman SA (1928) Laboratory manual of general microbiology: with special reference to the microorganisms of the soil. McGraw-Hill, New York
19. Vincent JM (1970) A manual for the practical study of root nodule bacteria. International Biological Programme (IBP Handbook, 15), Oxford
20. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376. https://doi.org/10.1007/bf01734359
21. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/bf01734359
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. https://doi.org/10.1093/molbev/msr121
23. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 9:455–477. https://doi.org/10.1089/cmb.2012.0021
24. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Peer J 25:1043–1055. https://doi.org/10.1101/gr.186072.114
25. Florentino LA, Jaramillo PMD, Silva KB, Silva JS, Oliveira SM, Moreira FMS (2012) Physiological and symbiotic diversity of *Cupriavidus necator* strains isolated from nodules of Leguminosae species. Sci Agric 69:247–258. https://doi.org/10.1590/S0103-90162012000400003
26. Guimarães AA, Florentino LA, Almeida KA, Lebbe L, Silva KB, Willems A, Moreira FMS (2015) High diversity of *Bradyrhizobium* strains isolated from several legume species and land uses in Brazilian tropical ecosystems. Syst Appl Microbiol 38:433–441. https://doi.org/10.1016/j.syapm.2015.06.006
27. Niemann S, Puehler A, Tichy HV, Simon R, Selbitshka W (1997) Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. J Appl Microbiol 82:477–484. https://doi.org/10.1046/j.1365-2672.1997.00141.x
28. Sarita S, Sharma PK, Priefer UB, Prell J (2005) Direct amplification of rhizobial *nod*C sequences from soil total DNA and comparison to *nod*C diversity of root nodule isolates. FEMS Microbiol Ecol 54:1–11. https://doi.org/10.1016/j.femsec.2005.02.015
29. De Meyer SE, Van Hoorde K, Vekeman B, Braeckman T, Willems A (2011) Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol Biochem 43:2384–2396. https://doi.org/10.1016/j.soilbio.2011.08.005
30. Gaby JC, Buckley DH (2012) A Comprehensive evaluation of PCR primers to amplify the *nif*H gene of nitrogenase. PLoS ONE 7:e42149. https://doi.org/10.1371/journal.pone.0042149
31. Willems A, Coopman R, Gillis M (2001) Phylogenetic and DNA-DNA hybridization analyses of *Bradyrhizobium* species. Int J Syst Evol Microbiol 51:111–117. https://doi.org/10.1099/00207713-51-1-111
32. Ramírez-Bahena MH, Flores-Félix JD, Chahboune R, Toro M, Velázquez E, Peix A (2016) *Bradyrhizobium centrosemae* (symbiovar centrosemae) sp. nov., *Bradyrhizobium americanum* (symbiovar phaseolarum) sp. nov. and a new symbiovar (tropici) of *Bradyrhizobium viridifuturi* establish symbiosis with Centrosema

species native to America. Syst Appl Microbiol 39:378–383. https://doi.org/10.1016/j.syapm.2016.06.001
33. Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) International committee on systematic bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematic. Int J Syst Bacteriol 37:463–464. https://doi.org/10.1099/00207713-37-4-463
34. Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106:19126–19131. https://doi.org/10.1073/pnas.0906412106
35. Orata FD, Xu Y, Gladney LM, Rishishwar L, Case RJ, Boucher Y, Jordan IK, Tarr CL (2016) Characterization of clinical and environmental isolates of *Vibrio cidicii* sp. nov., a close relative of *Vibrio navarrensis*. Int J Syst Evol Microbiol 66:4148–4415. https://doi.org/10.1099/ijsem.0.001327
36. Ormeño-Orrillo E, Martínez-Romero E (2013) Phenotypic tests in *Rhizobium* species description: an opinion and (sympatric speciation) hypothesis. Syst Appl Microbiol 36:145–147. https://doi.org/10.1016/j.syapm.2012.11.009
37. Florentino LA, Sousa PM, Silva JS, Silva KB, Moreira FMS (2010) Diversity and efficiency of *Bradyrhizobium* strains isolated from soil samples collected from around *Sesbania virgata* roots using cowpea as trap species. Rev Bras Cienc Solo 34:1113–1123. https://doi.org/10.1590/S0100-06832010000400011
38. Rogel MA, Ormeño-Orrillo E, Martínez Romero E (2011) Symbiovars in rhizobia reflect bacterial adaptation to legumes. Syst Appl Microbiol 34:96–104. https://doi.org/10.1016/j.syapm.2010.11.015
39. Guerrouj K, Ruíz-Díez B, Chahboune R, Ramírez-Bahena MH, Abdel-moumen H, Quinones MA, El Idrissi MM, Velázquez E, Fernández-Pascual M, Bedmar EJ, Peix A (2013) Definition of a novel symbiovar (sv retamae) within *Bradyrhizobium retamae* sp. Nov., nodulating *Retama sphaerocarpa* and *Retama monosperma*. Syst Appl Microbiol 36:218–223. https://doi.org/10.1016/j.syapm.2013.03.001
40. Bejarano A, Ramírez-Bahena MH, Velázquez E, Peix A (2014) *Vigna unguiculata* is nodulated in Spain by endosymbionts of Genisteae legumes and by anew symbiovar (vignae) of the genus *Bradyrhizobium*. Syst Appl Microbiol 37:533–540. https://doi.org/10.1016/j.syapm.2014.04.003
41. Delamuta JRM, Menna P, Ribeiro RA, Hungria M (2017) Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. Syst Appl Microbiol 40:254–265. https://doi.org/10.1016/j.syapm.2017.04.005
42. Vinuesa P, León-Barrios M, Silva C, Willems A, Jarabo-Lorenzo A, Pérez-Galdona R, Werner D, Martínez-Romero E (2005) *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. genistearum, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. Int J Syst Evol Microbiol 55:569–575. https://doi.org/10.1099/ijs.0.63292-0
43. Moreira FMS (2006) Nitrogen- fixing Leguminosae nodulating bacteria. In: Moreira FMS, Siqueira JO, Brussaard L (eds) Soil biodiversity in Amazonian and other brazilian ecosystems. CABI, Wallingford, pp 237–270
44. Norris DO (1965) Acid production by *Rhizobium*: a unifying concept. Plant Soil 22:143–166. https://doi.org/10.1007/BF01373988
45. Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA (1993) Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. Syst Appl Microbiol 1:135–146. https://doi.org/10.1016/S0723-2020(11)80258-4
46. Moreira FMS, Haukka K, Young JPW (1998) Biodiverity of rhizobia isolated from a wide range of forest legumes in Brazil. Mol Ecol 7:889–895. https://doi.org/10.1046/j.1365-294x.1998.00411.x
47. Shah V, Subramaniamb S (2018) *Bradyrhizobium japonicum* USDA110: a representative model organism for studying the impact of pollutants on soil microbiota. Sci Total Environ 624:963–967. https://doi.org/10.1016/j.scitotenv.2017.12.185
48. Avontuur JR, Palmer M, Beukes CW, Chan WY, Coetzee MPA, Blom J, Stepkowski T, Kyrpides NC, Woyke T, Shapiro N, Whitman WB, Venter SN, Steenkamp ET (2019) Genome-informed Bradyrhizobium taxonomy: where to from here? Syst Appl Microbiol 42:427–439. https://doi.org/10.1016/j.syapm.2019.03.006
49. Ferreira MC, Hungria M (2002) Recovery of soybean inoculant strains from uncropped soils in Brazil. Field Crop Res 79:139–152. https://doi.org/10.1016/S0378-4290(02)00119-3
50. Barcellos FG, Menna P, Batista JSS, Hungria M (2007) Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a brazilian savannah soil. Appl Environ Microbiol 73:2635–2643. https://doi.org/10.1128/AEM.01823-06
51. Yuan K, Reckling M, Ramirez MDA, Djedidi S, Fukuhara I, Ohyama T, Yokoyama T, Bellingrath-Kimura SD, Halwani M, Egamberdieva D, Ohkama-Ohtsu N (2020) Characterization of rhizobia for the improvement of soybean cultivation at cold conditions in Central Europe. Microbes Environ. https://doi.org/10.1264/jsme2.ME19124
52. Costa EM, Ribeiro PRA, Lima W, Farias TP, Moreira FMS (2017) Lima bean nodulates efficiently with *Bradyrhizobium* strains isolated from diverse legume species. Symbiosis 73:125–133. https://doi.org/10.1007/s13199-017-0473-8
53. Rufini M, Silva MAP, Ferreira PAA, Cassetari AS, Soares BL, Andrade MJB, Moreira FMS (2014) Symbiotic efficiency and identification of rhizobia that nodulate cowpea in a Rhodic Eutrudox. Biol Fert Soils 50:115–122. https://doi.org/10.1007/s00374-013-0832-4