Classification of the inoculant strain of cowpea UFLA03-84 and of other strains from soils of the Amazon region as *Bradyrhizobium viridifuturi* (symbiovar tropici)

Elaine Martins da Costa^{1,2} · Teotonio Soares de Carvalho¹ · Amanda Azarias Guimarães¹ · Aniele Carolina Ribas Leão³ · Leonardo Magalhães Cruz³ · Valter Antonio de Baura³ · Liesbeth Lebbe⁴ · Anne Willems⁴ · Fatima Maria de Souza Moreira¹

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Abstract

Cowpea (*Vigna unguiculata* L.) is a legume species that considerably benefits from inoculation with nitrogen fixing bacteria of the genus *Bradyrhizobium*. One of the strains recommended for inoculation in cowpea in Brazil is UFLA03-84 (*Bradyrhizobium* sp.). The aim of our study was to define the taxonomic position of the UFLA03-84 strain and of two other strains of *Bradyrhizobium* (UFLA03-144 and INPA237B), all belonging to the same phylogenetic group and isolated from soils of the Brazilian Amazon. Multilocus sequence analysis (MLSA) of the housekeeping genes *atpD*, *gyrB*, *recA*, and *rpoB* grouped (with similarity higher than 99%) the three strains with *Bradyrhizobium viridifuturi* SEMIA 690^T. The analyses of average nucleotide identity and digital DNA–DNA hybridization supported classification of the group as *Bradyrhizobium viridifuturi*. The three strains exhibited similar behavior in relation to the most of the phenotypic characteristics evaluated. However, some characteristics exhibited variation, indicating phenotypic diversity within the species. Phylogenetic analysis of the *nodC* and *nifH* genes showed that the three strains are members of the same symbiovar (tropici) that contains type strains of *Bradyrhizobium* species coming from tropical soils (SEMIA 690^T *B. viridifuturi*, CNPSO 1112^T *B. tropiciagri*, CNPSO 2833^T *B. embrapense*, and *B. brasilense* UFLA03-321^T).

Keywords *Bradyrhizobium* · *Vigna unguiculata* L. · Housekeeping genes · Average nucleotide identity · Genome

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✉ Fatima Maria de Souza Moreira
fmoreira@dcs.ufpa.br

¹ Departamento de Ciência do Solo, Universidade Federal de Lavras, Lavras, MG 37200-000, Brazil

² Present address: Universidade Federal do Piauí, Campus Professora Cinobelina Elvas, Bom Jesus, Piauí 64900-000, Brazil

³ Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, Paraná 81531990, Brazil

⁴ Department of Biochemistry and Microbiology, Ghent University, B-9000 Ghent, Belgium

Introduction

Cowpea (*Vigna unguiculata* L.) is a legume species grown in various tropical and subtropical regions of the world. In Brazil, this crop is a staple food and alternative source of income, especially for low-income populations. It is a species that greatly benefits from biological nitrogen fixation in symbiosis with nodulating nitrogen fixing bacteria, and members of the *Bradyrhizobium* genus are the main symbiont [1–5].

The three strains (INPA03-11B, UFLA03-84, BR 3267, and BR 3262) approved by the Brazilian Ministry of Agriculture as inoculants for cowpea in Brazil [6] belong to the *Bradyrhizobium* genus. These strains have been successfully used in inoculation of the crop in different regions of Brazil [7–10]. Recently, the INPA03-11B strain was classified as *Bradyrhizobium elkanii* [11], and the BR 3267 and BR 3262 strains were classified as *Bradyrhizobium yuanmingense*

and *Bradyrhizobium pachyrhizi*, respectively [12]. The UFLA03-84 strain was characterized only partially [11], and more studies are necessary to classify it at species level.

For adequate classification of bacterial strains at species level, the use of integrated genotype and phenotype information is necessary in order to achieve better taxonomic consensus. Due to the high degree of conservation of the nucleotide sequences of the 16S rRNA gene in members of the *Bradyrhizobium* genus [13], various studies have indicated that in addition to the analysis of this gene, analyses with greater power of discrimination, like multilocus sequence analysis (MLSA) of housekeeping genes, such as *atpD*, *dnaK*, *glnII*, *gyrB*, *recA*, and *rpoB*, are necessary to distinguish members of this genus [11, 14–18]. MLSA assists in separation of taxonomic groups that must be evaluated by DNA–DNA hybridization (DDH) analysis for confirmation of taxonomic classification. DDH analysis has traditionally been used, over many years, as the standard method for designation of new species, considering 70% as a cutoff score [19]. However, DDH is a very laborious and costly analysis, and it is performed in few laboratories in the world.

Recently, new methods of comparisons between bacterial genomes, such as average nucleotide identity (ANI) analysis and digital DDH analysis, provided by the GGDC (Genome-to-Genome Distance Calculator) [20], have stood out as important alternatives because they provide high correlations with the results obtained by traditional DDH [20–22]. The cutoff value recommended for DDH (70%) [19] correlates with 95–96% of ANI [21, 23]. ANI and GGDC values have already been successfully used in replacing traditional DDH in definition of the bacterial taxonomic position [15, 18, 24].

In a previous study, analysis of housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*) indicated different phylogenetic groups of *Bradyrhizobium* strains isolated from nodules of legume species, especially cowpea, in Brazilian soils [11]. In this study, one of these groups, including the inoculant strain of cowpea UFLA03-84 and two other strains (UFLA03-144 and INPA237B) coming from soils of the Brazilian Amazon, was selected for definition of its taxonomic position.

Materials and methods

Origin of the strains

The UFLA03-84 and UFLA03-144 strains were isolated from Brazilian Amazon soils under pasture and agricultural systems, respectively, using cowpea as a trap plant [3, 7]. The INPA237B strain was isolated from nodules of *Pterocarpus* sp., also in Brazilian Amazon soil under native forest. For isolation and characterization of the strains, 79 medium [25] (YMA—Yeast Manitol Agar [26]) was used.

Phylogenetic analysis of the 16S rRNA gene and of the housekeeping genes

The three strains had previously been identified as *Bradyrhizobium* sp. based on partial sequencing of the 16S rRNA gene, with the number of base pairs ranging from 616 to 813 [3, 7, 11]. In this study, we once more performed the sequencing of this gene of all the strains to obtain a greater length of nucleotide sequences (1182 to 1300 bp). DNA extraction from the strains was performed by the alkaline lysis method [27]. The primers and the conditions of 16S rRNA gene amplification and sequencing were the same as those used by Guimarães et al. [11].

The sequences of the *recA* gene (519 bp) of the UFLA03-84 and UFLA03-144 strains were obtained in this study by the methodology used by Guimarães et al. [11]. Amplification and sequencing of the *rpoB* gene (915 bp) of the three strains was also performed in this study using the same primers and conditions as in the study of Ribeiro et al. [16]. The sequences of the *atpD* (432 bp) and *gyrB* (645 bp) genes of the three strains and the sequences of the *recA* gene (381 bp) of the INPA237B strains were obtained in a previous study [11]. An MLSA was conducted using the sequences of four housekeeping genes (*atpD*, *gyrB*, *recA*, and *rpoB*). In addition, we also performed an MLSA with the *glnII* (418 pb) and *recA* (381 bp) genes because the sequences of these two genes are available for all the species of *Bradyrhizobium* described up to now and for a strain of the group (UFLA03-84). The sequence of the *glnII* gene of UFLA03-84 was extracted from its genome (accession number NSJY00000000).

The sequences of each gene and the concatenated sequences were aligned using the ClustalW Multiple Alignment algorithm in BioEdit. The phylogenetic trees were constructed with maximum likelihood (ML) methods, using the model with lowest AIC [28] (Akaike information criterion) score as indicated by the automated model selection procedure available in MEGA 6 software package [29]. The sequences of the type strains of the *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI) were included in the alignments. 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 phylogenetic analysis of the 16S rRNA gene.

Phylogenetic analysis of the symbiotic genes (*nodC* and *nifH*)

For analysis of the *nodC* and *nifH* symbiotic genes, the DNA extraction of the three strains was performed as described

above. The primers and the amplification and sequencing conditions of the *nodC* gene (417 to 607 bp) were according to Sarita et al. [30], modified by De Meyer et al. [31]. For amplification and sequencing of the *nifH* gene (288 bp), the IKG3 and DVV primers were used under the conditions described by Gaby and Buckley [32]. Alignment of the sequences and construction of phylogenetic trees were performed as described above. In phylogenetic analyses of both genes (*nodC* and *nifH*), the sequences of the CMVU02 and CMVU30 strains were included, which were recently classified as *Bradyrhizobium viridifuturi* within a new symbiovar called tropici [17].

Genome sequencing, average nucleotide identity, and digital DNA–DNA hybridization

Average nucleotide identity (ANI) and digital DNA–DNA hybridization were estimated with the genomic sequences of a representative strain of the group (UFLA03-84) and of the phylogenetically nearest type strain in MLSA (SEMIA 690^T *Bradyrhizobium viridifuturi*) [33].

To carry out genome sequencing, strain UFLA03-84 was grown in liquid 79 medium [25]. Genomic DNA was purified from 10⁹ 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). De novo assembly was performed in SPAdes 3.6.2 [34] and finalized with GFinisher [35] with *B. elkanii* USDA 76^T as reference, resulting in 21 contigs (N₅₀, 832,094; mean G+C content, 64.1%). To confirm the identity of the strain UFLA03-84 used for genome sequencing, all the sequences from housekeeping genes previously obtained from this strain, as described above, were compared with their counterparts in the genome, resulting in a perfect match among them.

The ANI values were calculated using an online calculator (available at <http://enve-omics.ce.gatech.edu/ani/index>) [21]. The digital DNA–DNA hybridization values were estimated for the strains UFLA03-84 and SEMIA 690^T using the genome-to-genome distance calculator (GGDC 2.1) [20]. The genome accession numbers of the UFLA03-84 (NSJY000000000) and SEMIA 690^T (SAMN03890369) strains are available in the GenBank.

Phenotypic characterization

The growth of the three strains (UFLA03-84, UFLA03-144, and INPA237B) was evaluated in 79 medium [25] under different pH values (4, 5.5, 6.8, 8, 9, and 10) and

temperatures (5, 15, 20, 28, 34, 37, and 40 °C) according to the previously described methodology [36]. We evaluated the ability of these strains to assimilate 17 carbon sources (D-arabinose, L-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 8 nitrogen sources (L-arginine, L-asparagine, casein hydrolysate, L-cysteine, glycine, L-glutamic acid, L-methionine, and tryptophan) in modified 79 medium [25], whose composition is described in the study of Costa et al. [18].

Results

Phylogenetic analysis of the 16S rRNA gene and of the housekeeping genes

Phylogenetic analysis of the 16S rRNA gene (1180 bp) is shown in Fig. 1. In this analysis, the three strains were grouped (100% similarity) with four species of *Bradyrhizobium*: *B. viridifuturi* (SEMIA 690^T), *B. embrapense* (CNPSO 2833^T), *B. erythrophlei* (CCBAU 53325^T), and *B. mercantei* (SEMIA 6399^T) (Fig. 1).

In MLSA, for its part, performed with the sequences of the housekeeping genes *atpD*, *gyrB*, *recA*, and *rpoB* (1814 bp), the three strains form one phylogenetic group with *Bradyrhizobium viridifuturi* (SEMIA 690^T) separated from the other *Bradyrhizobium* species (Fig. 2). In this analysis, the similarities between the strains UFLA03-144, INPA237B, UFLA03-84, and SEMIA 690^T (*B. viridifuturi*) were high (ranging from 99.3 to 99.7%). In MLSA, performed with the sequences of the *glnII* and *recA* genes (799 bp), a similar result was observed (Fig. S1).

Phylogenetic analysis of the symbiotic genes (*nodC* and *nifH*)

Phylogenetic analysis of both genes (*nodC* and *nifH*) grouped the three strains together with the type strains of *Bradyrhizobium embrapense* (CNPSO 2833^T), *Bradyrhizobium tropiciagri* (CNPSO 1112^T), *Bradyrhizobium brasilense* (UFLA03-321^T), and *Bradyrhizobium viridifuturi* (SEMIA 690^T, CMVU02, and CMVU30) (Figs. 3 and 4). The maximum similarity of the sequences of the *nodC* gene of the three strains was with the sequence of *B. viridifuturi* CMVU30 (ranging from 96.8 to 99.7%). In phylogenetic analysis of the *nifH* gene, the strains UFLA03-144 and UFLA03-84 were grouped with *B. embrapense* (CNPSO 2833^T) and *B. viridifuturi* (SEMIA 690^T, CMVU02, and CMVU30), with 100% similarity, whereas the

Fig. 1 Maximum likelihood phylogeny based on 16S rRNA gene sequences (1180 bp) showing the relationships between strains UFLA03-84, UFLA03-144, and INPA237B (in bold) and type strains of *Bradyrhizobium* species. GenBank accession numbers are provided in parentheses

INPA237B strains shared maximum similarity (98.6%) with *B. tropiciagri* CNPSO 1112^T (Fig. 4).

The strains UFLA03-144 and UFLA03-84 share similarity of 99.7% in phylogenetic analysis of the *nodC* gene

and have identical sequences of the *nifH* gene (Figs. 3 and 4). The similarity between the INPA237B strain and the two UFLA strains was 98.5 % in phylogenetic analysis of

Fig. 2 Maximum likelihood phylogeny based on partial concatenated sequences (1816 bp) of housekeeping genes (*atpD*, *gyrB*, *recA*, and *rpoB*) showing the relationships between strains UFLA03-84, UFLA03-144, and INPA237B (in bold) and type strains of the *Bradyrhizobium* species. GenBank accession numbers are provided in parentheses

Fig. 3 Maximum likelihood phylogeny based on partial sequences of *nodC* (400 bp) gene showing the relationships between strains UFLA03-84, UFLA03-144, and INPA237B (in bold) and type strains of *Bradyrhizobium* species. GenBank accession numbers are provided in parentheses

the *nifH* gene and ranged from 97.2 to 97.5% in phylogenetic analysis of the *nodC* gene.

Genome sequencing, average nucleotide identity, and digital DNA–DNA hybridization

Genome quality and completeness was assessed using checkm 1.0.7 [37] which indicated 99.96% completeness (based on 824 lineage-specific marker genes) and only 0.45%

contamination. In addition, complete sequences encoding the 23S, 5S, and 16S rRNA genes, as well as 52 tRNAs for all 20 proteinogenic amino acids were found in the genome. In total, 8092 genes were predicted in RAST 2.0 [38] with a coding density of 85.6% for this draft genome.

In place of traditional DNA–DNA hybridization, in this study we used genome comparison analyses (ANI and digital DDH). The ANI value estimated from the genome sequences of the representative strain of the group (UFLA03-84) and of

Fig. 4 Maximum likelihood phylogeny based on partial sequences of the *nifH* (177 bp) gene showing the relationships between strains UFLA03-84, and UFLA03-144 and INPA237B (in bold) and type strains of *Bradyrhizobium* species. GenBank accession numbers are provided in parenthesis

the type strain of *B. viridifuturi* (SEMIA 690^T) was 98.7%. Digital DDH showed an estimated similarity of 89.3% between the UFLA03-84 and SEMIA 690^T strains.

Phenotypic characterization

The UFLA03-84, UFLA03-144, and INPA237B strains are able to grow in a wide range of pH levels (4 to 10) and temperatures (15 to 37 °C) (Table 1). All strains

commonly use D-arabinose, L-arabinose, and mannitol, but do not use citric acid, glycine, malic acid, maltose, L-methionine, sodium lactate, and sucrose as a carbon source (Table 1). The use of D-fructose, D-glucose, L-glutamic acid, and lactose as a carbon source is variable among the strains. In relation to nitrogen sources, all strains assimilate casein hydrolysate and L-glutamic acid, but no strain uses L-cysteine, glycine, L-methionine, and tryptophan as a nitrogen source (Table 1).

Table 1 Phenotypic characteristics of the *Bradyrhizobium viridifuturi* strains isolated from *Vigna unguiculata* nodules (UFLA03-84 and UFLA03-144) and *Pterocarpus* sp. (INPA237B) in soils from the Brazilian Amazon

Phenotypic characteristics	UFLA03-84	UFLA03-144	INPA237B
Growth at			
15 °C	w	w	w
37 °C	+	+	+
40 °C	–	–	–
0.75% NaCl*	+	+	+
1% NaCl*	–	–	–
pH 4.0	+	+	+
pH 10.0	+	+	+
Assimilation of carbon source			
D-Arabinose	+	+	+
L-Arabinose	+	+	+
L-Asparagine	w	w	w
D-Fructose	w	w	w
Citric acid	–	–	–
Glycerol	w	w	w
Glycine	–	–	–
D-glucose	w	w	–
L-Glutamine	w	w	w
L-Glutamic acid	+	+	w
Lactose	–	–	w
Malic acid	–	–	–
D-Maltose	–	–	–
Mannitol	+	+	+
L-Methionine	–	–	–
Sodium lactate	–	–	–
Sucrose	–	–	–
Assimilation of nitrogen source			
L-Arginine	w	w	w
L-Asparagine	w	w	w
Casein hydrolysate	+	+	+
L-Cysteine	–	–	–
Glycine	–	–	–
Glutamic acid	+	+	+
L-Methionine	–	–	–
Tryptophan	–	–	–
Yeast extract	+	+	–
Resistance to antibiotics (μg mL ⁻¹)*			
Ampicillin (10)	+	+	+
Cefuroxime (30)	+	+	+
Ciprofloxacin (5)	+	+	+
Chloramphenicol (30)	+	+	+
Doxycycline (30)	+	+	+
Erythromycin (15)	+	+	+
Gentamycin (10)	+	+	+
Kanamycin (30)	+	+	+

Table 1 (continued)

Phenotypic characteristics	UFLA03-84	UFLA03-144	INPA237B
Neomycin (30)	+	–	+
Penicillin (10)	+	+	+

+, positive; –, negative; w, weak. * Data for growth in different NaCl concentrations and for antibiotic resistance of the strains UFLA03-84, UFLA03-144, and INPA237B were extracted from Guimarães et al. [11]

Discussion

In this study, we investigated the taxonomic position of a group of *Bradyrhizobium* strains, composed of a cowpea inoculant strain (UFLA03-84) and two other strains (UFLA03-144 and INPA237B) isolated from nodules of legume species, all from soils of the Brazilian Amazon. The grouping, with 100% similarity, of the three strains with *B. viridifuturi* (SEMIA 690^T), *B. embrapense* (CNPSO 2833^T), *B. erythrophlei* (CCBAU 53325^T), and *B. mercantei* (SEMIA 6399^T) shows that the phylogenetic analysis of the 16S rRNA gene does not have sufficient phylogenetic resolution to classify these strains, a fact that corroborates previous studies with the *Bradyrhizobium* genus [11, 13, 14, 17, 18].

Furthermore, in MLSA of the housekeeping genes *atpD*, *gyrB*, *recA*, and *rpoB*, the grouping (with similarity greater than 99%) of the three strains (UFLA03-144, INPA237B, and UFLA03-84) with the strain SEMIA 690^T (*B. viridifuturi*) clearly shows that these strains belong to the same species (*B. viridifuturi*). MLSA of three, four, or more housekeeping genes has been successfully used to assist in designation of the taxonomic position of members of the *Bradyrhizobium* genus [14, 15, 17, 18, 39].

In the place of the traditional DNA–DNA hybridization, new methods of genome comparison, such as ANI and digital DDH (estimated by GGDC) analyses, have been successfully introduced in bacterial taxonomy studies [15, 18, 24, 40]. The cutoff value recommended in traditional DNA–DNA hybridization is 70% for delimitation of species [19]. This value shows high correlation with the 95–96% values of ANI [21, 23] and with the 70% value of digital DDH (provided by the GGDC) [20]. Therefore, the values of 98.7 and 89.3% of ANI and GGDC, respectively, between the UFLA03-84 strain and the SEMIA 690^T strain (*B. viridifuturi*) obtained in our study support classification of the group as *Bradyrhizobium viridifuturi*, confirming the phylogenetic affiliation indicated by MLSA of the housekeeping genes.

Phylogenetic analyses of symbiotic genes normally do not provide relevant information for purposes of taxonomic classification but have frequently been used to investigate rhizobia groups with similar symbiotic activity. According to

information from sequences of symbiotic genes and the ability of establishing symbiosis with a determined range of hosts, rhizobia groups can be classified within a determined symbiovar [41].

In our study, evaluation of the *nodC* and *nifH* genes showed the proximity of the UFLA03-84 strain to other strains of *Bradyrhizobium* species from tropical soils: *B. tropiciagri* CNPSO 1112^T (isolated from the nodule of *Neonotonia wightii*, in Brazil), *B. embrapense* CNPSO 2833^T (isolated from the nodule of *Desmodium heterocarpon*, in Colombia), *B. viridifuturi* SEMIA 690^T (isolated from nodules of *Centrosema pubescens*, in Brazil), CMVU02 and CMVU30 (isolated from nodules of *Centrosema macrocarpum*, in Venezuela), and *B. brasilense* UFLA03-321^T (isolated from *Vigna unguiculata*, in Brazil). These strains of *B. viridifuturi*, *B. tropiciagri*, and *B. embrapense* mentioned were recently included in a new symbiovar, called tropici, based on phylogenetic analysis of the *nodC* gene [17]. Based on analysis of both the *nodC* gene (Fig. 3) and the *nifH* gene (Fig. 4), our results indicate that the strains UFLA03-84, UFLA03-144, and INPA237B and the type strain of *Bradyrhizobium brasilense* (UFLA03-321^T) also belong to the symbiovar tropici. Our study also showed differentiated behavior of the strain isolated from the nodule *Pterocarpus* sp. (INPA237B) in relation to strains isolated from nodules of *Vigna unguiculata* (UFLA03-144 and UFLA03-84), indicating that the INPA237B strain possibly has a different evolutionary history of the *nifH* gene compared to the other strains.

In previous studies, the three strains were evaluated in regard to their ability to nodulate and fix nitrogen in symbiosis with *Vigna unguiculata*, *Glycine max*, *Phaseolus lunatus*, *Stizolobium aterrimum*, and *Acacia mangium* [1, 3, 7, 11, 42, 43]. All strains nodulate and fix nitrogen efficiently with *V. unguiculata*, but none nodulate *G. max*. The UFLA03-84 and UFLA03-144 strains establish efficient symbiosis with *P. lunatus* and *S. aterrimum*, and inefficient symbiosis with *A. mangium*. The INPA237B strain establishes inefficient symbiosis with *P. lunatus* and *S. aterrimum* and does not nodulate *A. mangium*. These data show the symbiotic diversity within the species *Bradyrhizobium viridifuturi*.

The tolerance of the three strains (UFLA03-84, UFLA03-144, and INPA237B) to a wide range of pH levels and temperatures corroborates previous studies with *Bradyrhizobium* strains isolated from nodules of legume species in Brazilian ecosystems [12, 18, 36, 44]. In a previous study, these three strains were also evaluated in regard to ability to grow under different concentrations of NaCl and tolerance to ten antibiotics [11]. This study showed that the UFLA03-84, UFLA03-144, and INPA237B strains tolerate a concentration of up to 0.75% NaCl and that all the strains are resistant to 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), gentamicin (10 µg mL⁻¹), and penicillin (10 µg mL⁻¹) [11] (Table 1).

Tolerance to different conditions of pH, temperature, and NaCl are desirable characteristics in selection of nodulating nitrogen fixing bacteria since the inoculant strains are authorized for use under different soil and climate conditions. It should be highlighted that the good performance of the UFLA03-84 cowpea inoculant strain in different regions of Brazil [7, 8, 10] is related, among other factors, to adaptation of this strain to different soil and climate conditions.

Although the three strains showed the same ability for assimilating most of the carbon and nitrogen sources, for some sources (carbon or nitrogen), there was variation in the assimilating ability of the strains, which indicates phenotypic diversity within the species. According to Ormeño-Orrillo and Martínez-Romero [45] many phenotypic characteristics are variable within a rhizobia species and hence have little taxonomic value. However, the use of D-arabinose and L-arabinose and non-use of D-maltose and sucrose as a carbon source seems to be a characteristic common to the members of *Bradyrhizobium viridifuturi*, as found in our study (Table 1) and in the study of description of this species [46].

Conclusions

The use of integrated genotype and phenotype information supports classification of the UFLA03-84, UFLA03-144, and INPA237B strains as *Bradyrhizobium viridifuturi*. The phylogeny of the *nodC* and *nifH* genes showed that these strains form a group with type strains of *Bradyrhizobium* species from tropical soils (*B. viridifuturi* SEMIA 690^T, *B. tropiciagri* CNPSO 1112^T, *B. brasilense* UFLA03-321^T, and *B. embrapense* CNPSO 2833^T), belonging to the same symbiovar (tropici).

Our results confirm the feasibility of using MLSA of housekeeping genes and of the bacterial genome comparison methods (average nucleotide identity and digital DNA–DNA hybridization) for a reliable taxonomy designation of members of the *Bradyrhizobium* genus.

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Compliance with ethical standards

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

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