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ORIGINAL PAPER

Bradyrhizobium brasilense sp. nov., a symbiotic nitrogen-fixing bacterium isolated from Brazilian tropical soils

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Abstract Four strains of rhizobia isolated from nodules of *Vigna unguiculata* (UFLA03-321^T, UFLA03-320 and UFLA03-290) and *Macroptilium atropurpureum* (UFLA04-0212) in Brazilian soils were previously reported as a new group within the genus *Bradyrhizobium*. To determine their taxonomic position, these strains were characterized in this study using a polyphasic approach. The analysis of the 16S rRNA gene grouped the four strains with *Bradyrhizobium pachyrhizi* PAC48^T. However, the concatenated sequence analysis of the two (*recA* and *glnII*) or three (*atpD*, *gyrB* and *recA*) housekeeping genes indicated that these strains represent a novel species of *Bradyrhizobium,* which is very closely related to *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T. Genomic relatedness analyses between the UFLA03-321^T strain and *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC48^T revealed an average nucleotide identity below 96% and values of estimated DNA–DNA hybridization below 70%, confirming that they represent genomically distinct species. Analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles and phenotypic characteristics also allowed differentiation of the novel species from its two neighboring species. In phylogenetic analysis of *nodC* and *nifH* genes, UFLA03-321^T exhibited maximum similarity with *B. tropiciagri* CNPSo 1112^T. The data suggest that these four UFLA strains represent a novel species, for which the name *Bradyrhizobium brasilense* sp. nov. is proposed, with UFLA03-321^T (=LMG 29353 =CBAS645) as type strain. G + C content in the DNA of UFLA03-321^T is 63.9 mol %.

Keywords *Bradyrhizobium* · *Vigna unguiculata* L. · Polyphasic taxonomy · Genomics · MALDI-TOF MS

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Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is a legume species cultivated in tropical and subtropical regions of the world, and it is a major source of vegetable protein for low income populations. This species can greatly benefit from biological nitrogen fixation in symbiosis with rhizobia (Soares et al. 2006, 2014; Costa et al. 2014). Additionally, cowpea is a promiscuous legume that can be used as a trap plant to access rhizobia diversity (Guimarães et al. 2012; Costa et al. 2013; Jaramillo et al. 2013; Grönemeyer et al. 2015a, 2016). Siratro (*Macroptilium atropurpureum*) is another legume species that is able to establish symbiosis with nitrogen-fixing bacteria, and it is nodulated by a large number of rhizobia species. That is why it is recommended to access rhizobia diversity (Lima et al. 2009).

Studies carried out in soils of different Brazilian ecosystems, using cowpea and siratro as trap plants, have indicated species of the *Bradyrhizobium* genus as the major symbiont of these legumes. In addition, high phenotypic and/or genotypic (intra- and interspecific) diversity among *Bradyrhizobium* strains have been observed (Florentino et al. 2010; Guimarães et al. 2012, 2015; Silva et al. 2012; Jaramillo et al. 2013; Rufini et al. 2014). The first *Bradyrhizobium* species from cowpea nodules was isolated in Brazilian Amazon soil, which was classified as *Bradyrhizobium manausense* (Silva et al. 2014). After that, two *Bradyrhizobium* species isolated from cowpea nodules in South African soils were described and classified: *Bradyrhizobium kavangense* (Grönemeyer et al. 2015a) and *Bradyrhizobium vignae* (Grönemeyer et al. 2016).

In a previous study, 50 *Bradyrhizobium* strains isolated from different legume species, mainly cowpea, in Brazilian soils, were characterized by phylogenetic analyses of the housekeeping genes (*atpD, dnaK, gyrB* and *recA*), which indicated five clusters with strains that are potential representatives of novel species (Guimarães et al. 2015). In this study, one of the clusters, which included four strains (UFLA03-321^T, UFLA03-320, UFLA03-290 and UFLA04-0212), was selected for further analysis by molecular and phenotypic methods.

Materials and methods

Origin of strains

The UFLA03-321^T and UFLA03-320 strains were isolated from cultivated soil in Lavras, Minas Gerais, Brazil (21°20′S and 45°00′W) using cowpea as a trap plant (Rufini et al. 2014). The UFLA03-290 strain was isolated from soil in an agroforestry system in Amazonas, Brazil (4°21′S and 69°36′W), using cowpea as a trap plant (Jaramillo et al. 2013); and the UFLA04-0212 strain was isolated from cultivated soil in Amazonas, Brazil (10°14′S and 62°21′W) using siratro as a trap plant (Florentino et al. 2009). Medium 79 (Fred and Waksman 1928), also known as Yeast Mannitol Agar (YMA) (Vincent 1970), was used for isolation and characterization of these strains. These strains are deposited in the culture collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras, Brazil. The type strain (UFLA03-321^T) was also deposited in the culture collection (BCCM/LMG) of Ghent University, Belgium, and in the Environment and Health Bacteria Collection (CBAS) of the Oswaldo Cruz Foundation (FIOCRUZ), Brazil. The CBAS and BCCM/LMG are registered in the WFCC (World Federation for Culture Collections). The Digital Protologue database TaxonNumber for strain UFLA03-321^T is TA00130.

16S rRNA and housekeeping gene sequencing

In previous studies, the 16S rRNA gene of the four UFLA strains was sequenced, but the nucleotide sequences were too short to derive meaningful phylogenetic conclusions (500–798 bp) (Jaramillo et al. 2013; Rufini et al. 2014; Guimarães et al. 2015). Thus, the 16S rRNA gene of these strains was sequenced again to obtain longer sequences. DNA was extracted from the strains by the alkaline lysis method (Niemann et al. 1997). The 16S rRNA gene (1252–1302 bp) of the UFLA03-320, UFLA04-212 and UFLA03-290 strains, and the *recA* gene (483–501 bp) of the UFLA03-320, UFLA03-321 and UFLA03-290 strains were amplified in this study using the primers and conditions indicated in Table S1. The reaction mixture volume used and the sequencing conditions of these genes have been described elsewhere (Ribeiro et al. 2015). The *atpD* (432 bp) and *gyrB* (645 bp) genes of the four strains and the *recA* (381 bp) gene of UFLA04-0212 were sequenced in a previous study (Guimarães et al. 2015). The 16S rRNA (1477 bp) and *glnII* (1035 bp) gene sequences of type strain UFLA03-321^T were extracted from its genome.

Concatenated sequence analysis included *glnII* and *recA* genes, since their sequences are available for all type strains of *Bradyrhizobium* species and for the type strain UFLA03-321^T. In addition, concatenated sequence analysis with *atpD*, *gyrB* and *recA* genes, including all UFLA strains, is shown in the supplementary material (Supplementary Fig. S1).

The sequences of each gene were aligned using the ClustalW multiple alignment algorithm in BioEdit. Phylogenetic trees were constructed by the neighbor joining method (NJ) (Saitou and Nei 1987) and by the maximum likelihood method (ML) (Felsenstein 1981) using the Kimura 2 parameter model (Kimura 1980). The MEGA 5 software package (Tamura et al. 2011) was used in the construction of phylogenetic trees, with bootstrap values based on 1000 replications. The sequences of type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI) were also included in all phylogenetic trees. The gene accession number for each strain is shown in figures.

Average nucleotide identity (ANI), estimated DNA–DNA hybridization and G + C content

Genomic comparisons were used in this study as an alternative to traditional DNA–DNA hybridization (DDH) to support designation of the novel species. To sequence the

genome, the UFLA03-321^T strain was grown in liquid YMA medium (Vincent 1970). DNA was purified from 10^9 bacterial cells using the phenol–chloroform extraction protocol. The DNA library for Illumina sequencing was constructed from 1 ng of total DNA using the Nextera XT kit (Illumina). Pair-end reads (2 × 250 bases) were sequenced with the MiSeq Reagent kit 500v2 (Illumina) on the MiSeq platform (Illumina). The DNA library for IonProton sequencing was constructed from 200 ng of total DNA using the Ion Xpress Plus Fragment Library kit (Thermo). Single-end reads of 106 bp average were sequenced with the Ion PI Sequencing 200 Kit V3 on the IonProton platform (Thermo). De novo assembly of the sequence of the UFLA03-321^T strain was performed using SPADes 3.6.2 (Bankevich et al. 2012) and finalized with G-finisher (Guizelini et al. 2016).

Average nucleotide identity (ANI) was estimated with the genome sequences of the UFLA03-321^T strain (accession number MPVQ00000000), obtained in this study, and genomes of type strains of *B. pachyrhizi* (accession number SAMN03782120) and *B. elkanii* (accession number NZ_ARAG00000000) available in the GenBank. ANI values were calculated using the online calculator at http://enveomics.ce.gatech.edu/ani/index (Goris et al. 2007).

Since our ANI results were close to the threshold of 96% (Richter and Rosselló-Móra 2009), we verified this result with estimated DNA–DNA hybridization (DDH) data. *In silico* pairwise DDH values were estimated for the UFLA03-321^T strain and the most closely related species using the Genome-to-Genome Distance Calculator (GGDC 2.1) (http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff et al. 2013). The DNA G + C content of the UFLA03-321^T genome was determined based on the draft genome obtained in this study.

Phenotypic characterization

In previous studies, the ability of UFLA strains to grow in medium 79 (Fred and Waksman 1928) was evaluated under different conditions of NaCl concentrations (w/v) (0.01, 0.25, 0.5, 0.75 and 1%) (Guimarães et al. 2015), as well as their resistance to 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}) (Guimarães et al. 2015). For purposes of comparison, in the present study, the growth of *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T in 79 medium were evaluated under the same NaCl concentrations, and their resistance to the nine antibiotics cited was also evaluated, using the methods applied by Guimarães et al. (2015).

In addition, this study evaluated the range and optimum value of pH (4, 5.5, 6.8, 8, 9 and 10) and temperature (5, 15, 20, 28, 34, 37 and 40 °C) for the four UFLA strains, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T grown in 79 medium (Fred and Waksman 1928) according to the previously described methodology (Florentino et al. 2010). The ability of these strains to assimilate 15 carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, mannitol, L-methionine, sodium lactate and sucrose) and eight nitrogen sources (L-arginine, L-asparagine, casein hydrolysate, L-cysteine, glycine, L-glutamic acid, L-methionine and tryptophan) was also evaluated. The assimilation of carbon sources was evaluated in modified medium 79: 10 g carbon source, 0.5 g K_2HPO_4, 0.5 g KNO_3, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.5 g $CaCO_3$, 4 ml Fe-EDTA (1.64%), 2 ml of micronutrient solution (2.86 mg H_3BO_3 L^{-1}, 2.03 mg $MnSO_4{\cdot}4H_2O$ l^{-1}, 0.22 mg $ZnSO_4{\cdot}7H_2O$ l^{-1}, 0.08 mg $CuSO_4{\cdot}5H_2O$ l^{-1} and 0.09 mg $Na_2MoO_4{\cdot}H_2O$ l^{-1}), 5 ml of bromothymol blue solution (0.5% in KOH at 0.2 N), 15 g agar and pH 6.8. The composition of medium 79 to evaluate the assimilation of nitrogen sources was the same as described above, substituting KNO_3 for one of the cited sources and using mannitol as a carbon source. Positive and negative references were the type strains of known *Bradyrhizobium* species (USDA 76^T and PAC48^T), for which information is available in the literature regarding their ability to grow (Ramírez-Bahena et al. 2009; Yao et al. 2015) or not (Yao et al. 2015) in some of these carbon and nitrogen sources. In addition to these tests, the UFLA03-321^T strain was characterized using the API 20NE (bioMérieux), according to manufacturer's instructions, with 5 days of incubation.

The UFLA strains and the type strains of two closely related species, *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC48^T, were also characterized by the MALDI-TOF MS analysis (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry). For this analysis, the strains were grown in YMA medium (Vincent 1970). Sample preparation and data analyses were carried out as previously described (Wieme et al. 2014). As the UFLA04-0212 strain could not be characterized by MALDI-TOF MS analysis, it was not included in the phylogenetic tree.

Fatty acid analyses

To provide additional description for the novel species, cellular fatty acid profiles of the UFLA03-321^T strain were determined, after its growth in YMA (Vincent 1970) at the log phase of growth (5 days). Samples were prepared and fatty acids were extracted as previously described

(Santana-Filho et al. 2012). Gas chromatography coupled with mass spectrometry (GC–MS) analysis was carried out according to Sassaki et al. (2008). In all analyses, the injection volume was 1 µL, with a split ratio of 1:10. Post-run analysis was performed with Saturn Workstation 5.1.

nodC and *nifH* gene sequencing

The phylogeny of the symbiotic genes (*nodC* and *nifH*) of the UFLA strains was also investigated in this study. DNA extraction was carried out as described above for the housekeeping genes. The primers used and the amplification and sequencing of the *nodC* gene were carried out according to Sarita et al. (2005), modified by De Meyer et al. (2011). For the *nifH* gene, analysis was carried out according to Gaby and Buckley (2012). Amplification of both *nodC* and *nifH* genes of the UFLA04-0212 strain was not possible; thus it was not included in the phylogenetic trees. Sequences were aligned and phylogenetic trees were constructed as described above.

Results and discussion

Phylogeny of 16S rRNA and housekeeping genes

Phylogenetic trees of the 16S rRNA gene using the NJ (Fig. 1) and ML methods (data not shown) were very similar. The UFLA03-321^T strain shared more than 99.5% sequence similarity of the 16S rRNA gene with ten *Bradyrhizobium* species (Table S2). The four UFLA strains (UFLA03-320, UFLA04-212, UFLA03-290 and UFLA03-321^T) shared 100% similarity among themselves and with *Bradyrhizobium pachyrhizi* PAC48^T (Fig. 1). Many *Bradyrhizobium* species have identical or almost identical 16S rRNA gene sequences (Fig. 1). The present results confirm the high degree of conservation of nucleotide sequences of the 16S rRNA gene among members of the *Bradyrhizobium* genus, corroborating previous reports (Willems et al. 2001; Ramírez-Bahena et al. 2009; Durán et al. 2014; Guimarães et al. 2015; Ribeiro et al. 2015).

Phylogenetic analysis of the sequences of housekeeping genes *atpD*, *dnaK*, *glnII, gyrB*, *recA* and *rpoB* have been successfully used in addition to analysis of the 16S rRNA gene for discrimination between species of the *Bradyrhizobium* genus (Vinuesa et al. 2005; Ramírez-Bahena et al. 2009; Wang et al. 2012;. Durán et al. 2014; Zilli et al. 2014). In the present study, the results of the concatenated sequence analysis of the *glnII* and *recA* genes were similar when using the NJ (Fig. 2) and ML methods (data not shown) and indicated that the UFLA03-321^T form a separate cluster, very closely related to *B. pachyrhizi* PAC48^T, supported by a high bootstrap value (99%) (Fig. 2). The similarity among UFLA03-321^T and *B. pachyrhizi* PAC48^T was 98.8% in the concatenated analysis (*glnII* and *recA*) (Table S2). Concatenated sequence analysis, including *atpD*, *gyrB* and *recA* genes, showed *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T as the species most closely related to UFLA03-321^T (Fig. S2), with similarity values of 97.5 and 97.0%, respectively (Table S2).

ANI, estimated DNA–DNA hybridization and G + C content

The ANI values between the UFLA03-321^T strain compared to *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T was 95.5 and 94.0%, respectively, which indicates that they represent genomically distinct species. The recommended cut-off value of 70% genomic relatedness based on DNA–DNA hybridization (DDH) for species delineation (Wayne et al. 1987) has been found to correlate to 95–96% ANI (Goris et al. 2007; Richter and Rosselló-Móra 2009). Recently, the threshold of 96% ANI was applied to delineate a new species of bacteria (Orata et al. 2016). Genomes showing ANI values higher than 96% have also already been reported between different species: *Burkholderia mallei–pseudomallei* and *Bordetella bronchiseptica–parapertussis–pertussis* (Richter and Rosselló-Móra 2009). According to Richter and Rosselló-Móra (2009), taxonomic synonymy (i.e., ANI > 96% between different species) should be maintained only for medical purposes, as the species mentioned above.

Digital DDH between the UFLA03-321^T strain compared to *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T was 62 and 59%, respectively, values which are slightly below the proposed threshold of 70% for species designation (Wayne et al. 1987), supporting our ANI results and strengthening designation of the novel species. Digital DDH estimates, as delivered by the GGDC, provide higher correlations with traditional DDH results (Auch et al. 2010; Meier-Kolthoff et al. 2013) and they have been successfully used to estimate genome relatedness between bacterial species (González-Castillo et al. 2015; Orata et al. 2016). The UFLA03-321^T genome has a G + C content of 63.9 mol %. This value is within the range reported for *Bradyrhizobium* species (59 to 65.1 mol %) (Chahboune et al. 2011; Ramírez-Bahena et al. 2012).

Phenotypic characterization

The main phenotypic characteristics differentiating the four UFLA strains, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T are shown in Table 1. A detailed phenotypic characterization is presented in the description of the novel species. All strains grew under a wide range

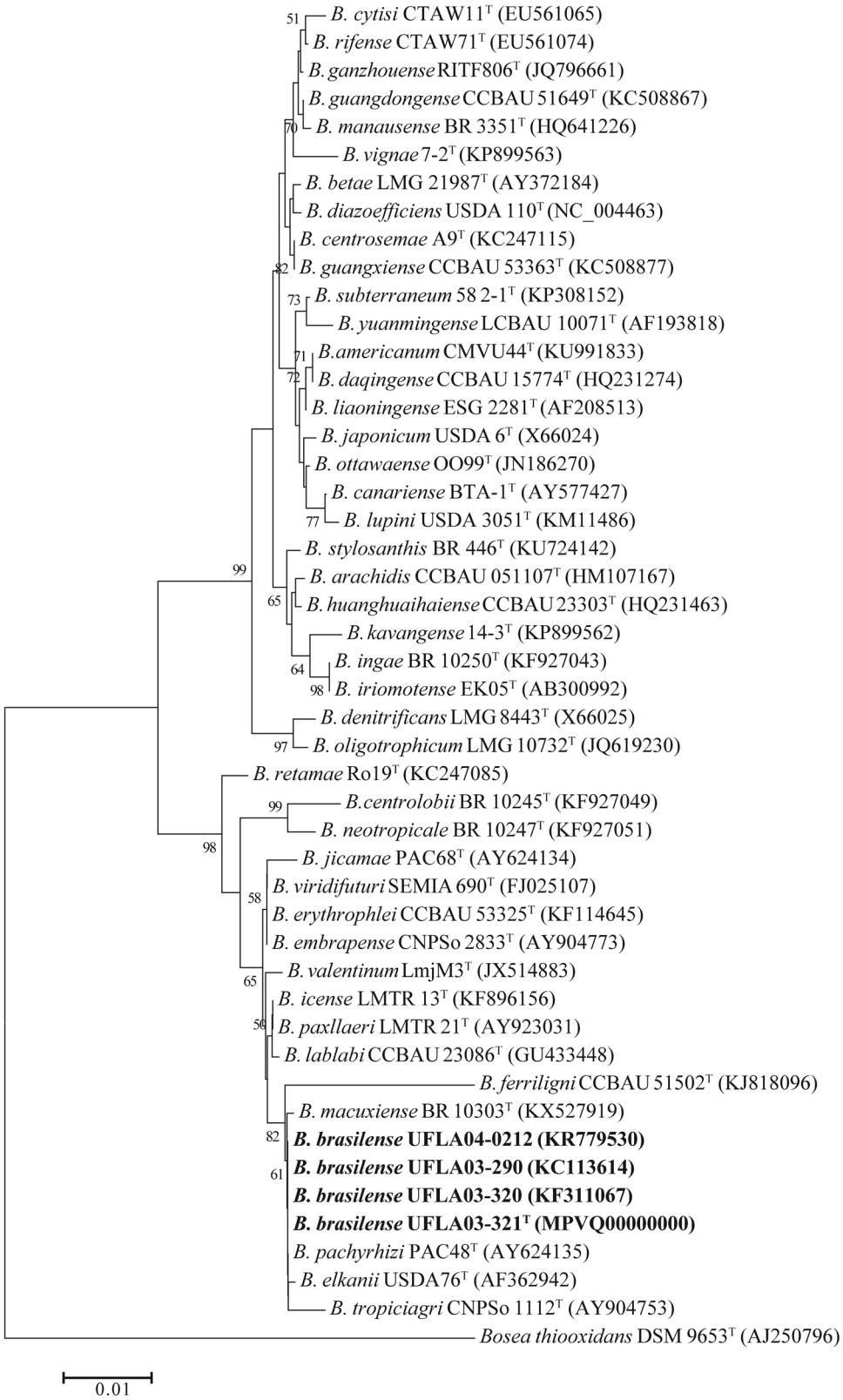

Fig. 1 Neighbor-joining phylogeny based on 16S rRNA gene sequences (1219 bp) showing the relationships between strains of the novel species (shown in *bold*) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. The 16S rRNA sequence of *Bosea thiooxidans* DSM9653^T was used as outgroup. Gene accession numbers for each strain are given in parentheses

of temperature and pH conditions. Salinity tolerance was variable. The strains UFLA03-321^T, UFLA03-320, UFLA04-0212, PAC48^T (*B. pachyrhizi*) and USDA 76^T (*B. elkanii*) were tolerant to salinity levels up to 0.75% NaCl. The use of carbon and nitrogen sources varies among strains. Antibiotic sensitivity did not vary among

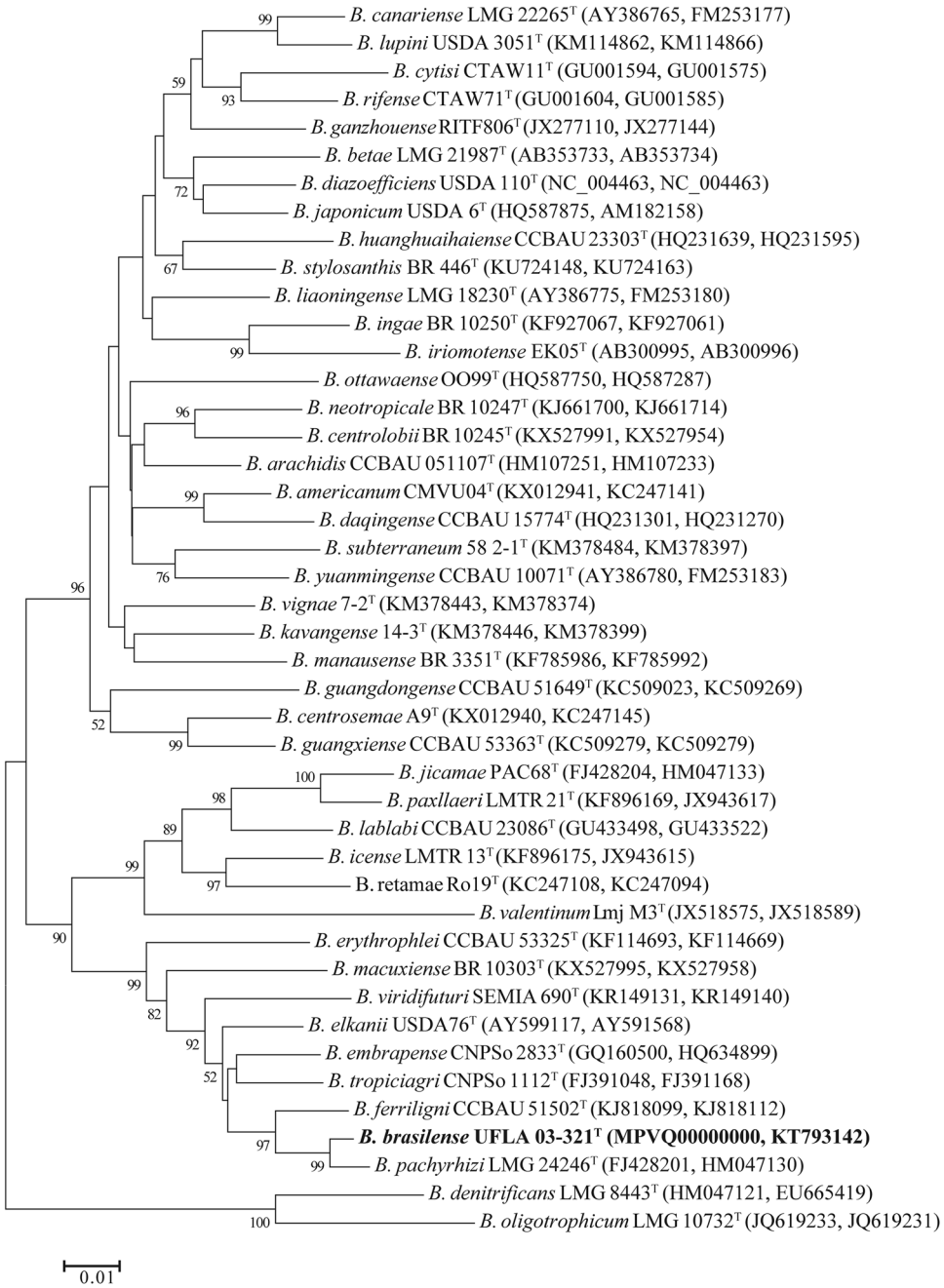

Fig. 2 Neighbor-joining phylogeny based on partial concatenated sequences (798 bp) of the *recA* and *glnII* gene showing the relationships between type strain UFLA03-321^T (shown in *bold*) and other type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene accession numbers for each strain are given in parentheses

UFLA strains. PAC48^T (*B. pachyrhizi*) and USDA 76^T (*B. elkanii*) were sensitive to neomycin (30 μg mL^{-1}), whereas all UFLA strains were resistant to this antibiotic (Table 1).

Results of MALDI-TOF MS analysis were in accordance with those of phylogenetic analysis of housekeeping genes, confirming that UFLA strains form a cluster separate from their closest phylogenetically related strains: *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC48^T (Fig. S2). These results are in agreement with those from recent studies showing good discrimination among different species, and even among strains of the same species, within the genus *Bradyrhizobium* (Sánchez-Juanes et al. 2013; Durán et al. 2014).

Fatty acid analyses

The fatty acids detected in the type strain UFLA03-321^T were C14:0, 0.32%; C15:0, 2.22%; C16:0, 18.31%; C16:1, 1.23%; C17:0, 0.69%; C18:0, 10.45%; C18:1ω9t, 0.79%; C18:1ω9c, 51.17%; C19 cyc-9,10, 12.53%; C20:0, 0.77% and C24:0, 0.56%. The presence of C16:0 and C18:1 is a typical characteristic of the genus *Bradyrhizobium* (Tighe et al. 2000) and the dominance of these two fatty acids is

Table 1 Differential phenotypic characteristics of *Bradyrhizobium brasilense* (UFLA strains) and the most closely related type strains (*Bradyrhizobium elkanii* USDA 76^T and *Bradyrhizobium pachyrhizi* PAC 48^T)

Characteristic	UFLA 03-321^T	UFLA 03-320	UFLA 03-290	UFLA 04-0212	USDA 76Ta	PAC 48Ta
Growth at						
40 °C	−	−	−	−	w	w
0.75% NaCl	+	+	−	+	+	+
Carbon source assimilation						
L-asparagine	w	w	w	w	+	+
D-fructose	+	+	w	+	+	+
D-glucose	w	w	w	w	+	+
L-glutamic acid	+	+	+	w	+	+
L-glutamine	w	w	w	w	+	+
Lactose	−	w	w	+	w	+
Malic acid	−	−	−	−	w	w
L-methionine	−	−	−	−	w	w
Sodium lactate	−	−	−	−	+	+
Sucrose	−	−	−	−	+	+
Nitrogen source assimilation						
L-arginine	w	+	w	w	+	+
Casein hydrolysate	+	+	+	+	w	w
L-cysteine	−	−	−	−	+	+
L-methionine	−	−	−	−	w	w
Resistance to antibiotics (μg ml^{-1})						
Gentamycin (10)	+	+	+	+	w	−
Neomycin (30)	+	+	+	+	−	−

Data represent the means of three biological replicates

+ growth; − no growth; *w* weakly positive

[a] LMG strains (LMG 6134^T = USDA 76^T and LMG 24246^T = PAC48^T) were obtained from the LMG culture collection

consistent with previous reports on the *Bradyrhizobium* species (Durán et al. 2014; Silva et al. 2014; Yao et al. 2015).

Phylogeny of *nodC* and *nifH* genes

In phylogenetic analysis of the *nodC* gene, UFLA03-321^T and UFLA03-320 strains formed a new cluster (Fig. 3), close to *B. tropiciagri* CNPSo 1112^T, isolated from a *Neonotonia wightii* nodule in Brazilian soil, and *B. embrapense* CNPSo 2833^T, isolated from a *Desmodium heterocarpon* nodule in Colombian soil, with similarity of 94.4 and 93.4%, respectively (Table S2). Phylogenetic analysis of the *nifH* gene clustered the UFLA03-321^T and UFLA03-320 strains with *B. tropiciagri* CNPSo 1112^T (Fig. S3), with 99.4% similarity (Table S2).

In contrast, the UFLA03-290 strain grouped separately from UFLA03-321^T and UFLA03-320 in the phylogenetic tree of both genes (Fig. 3 and Fig. S3), and the similarity with these strains was only 78.3 and 87.4% for the *nodC* and *nifH* genes, respectively (Table S2). The *nifH* sequence of the UFLA03-290 strain was most closely related to the *nifH* sequence of *B. subterraneum* 58 2-1^T (isolated from an *Arachis hypogaea* nodule), *B. vignae* 7-2^T, *B. kavangense* 14-3^T (isolated from a *Vigna unguiculata* nodule) from South African soils (Grönemeyer et al. 2015a, b, 2016) and *B. arachidis* CCBAU 051107^T (isolated from an *Arachis hypogaea* nodule) from Chinese soil (Wang et al. 2013) (Fig. S3). The low similarities between UFLA03-290 and the UFLA03-321^T and UFLA03-320 strains indicated an independent evolutionary history for their *nodC* and *nifH* genes, which may be associated with the region and ecosystem of origin.

In previous studies, the UFLA strains were evaluated for nodulation and nitrogen fixation ability in symbiosis with *Vigna unguiculata*, *Phaseolus lunatus*, *Stizolobium aterrimum* and *Acacia mangium* (Jaramillo et al. 2013; Rufini et al. 2014; Costa et al. 2017; Costa et al. unpublished data). The UFLA03-321^T, UFLA03-320 and UFLA04-0212 strains efficiently nodulate and fix nitrogen with *V. unguiculata* and *S. aterrimum* (Rufini et al. 2014; Costa et al. unpublished data). The UFLA03-290 strain nodulates these two species, but has low symbiotic efficiency (Jaramillo et al. 2013; Costa et al., unpublished data). None

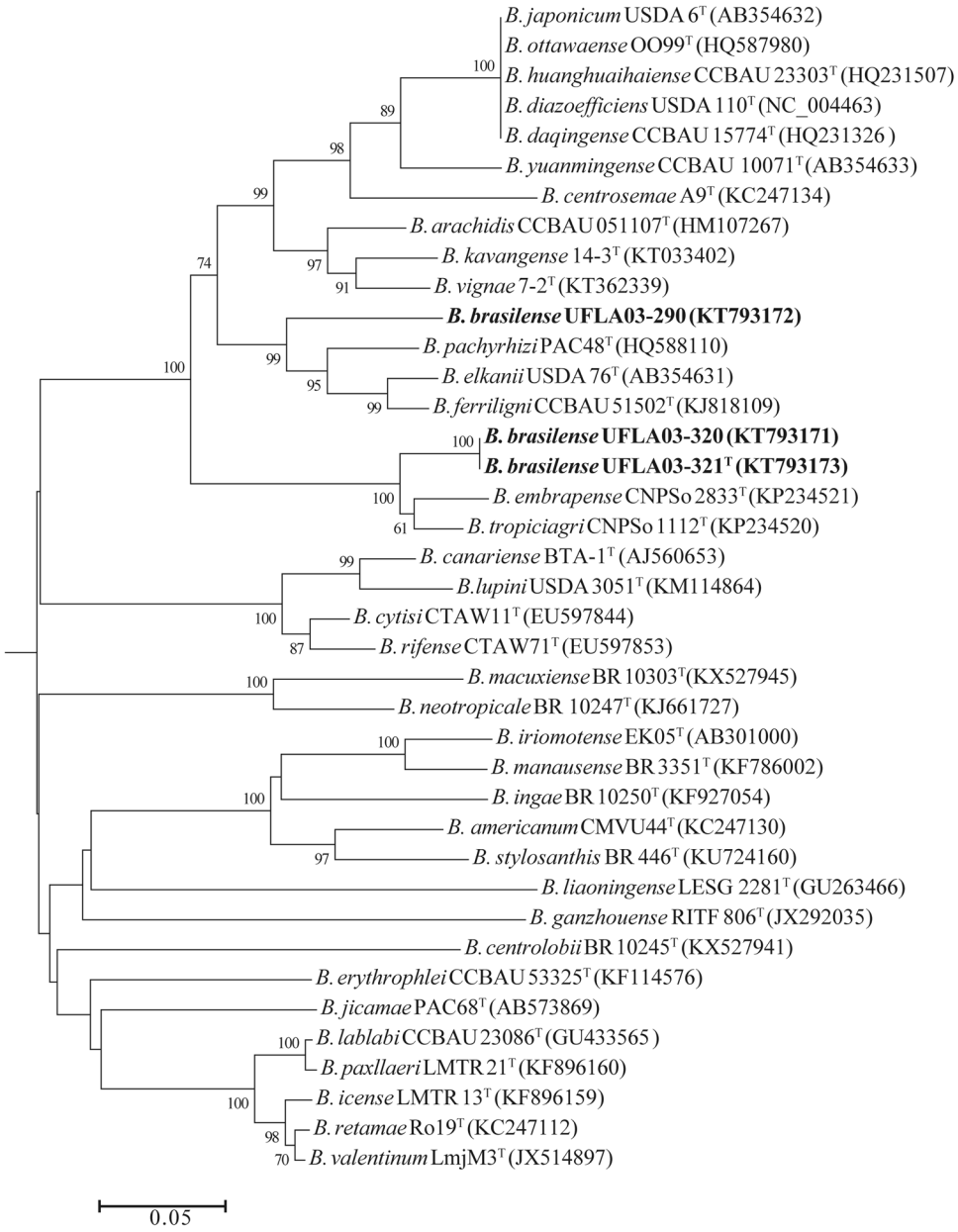

Fig. 3 Neighbor-joining phylogeny based on partial sequences (426 bp) of the *nodC* gene showing the relationships between strains of the novel species (shown in *bold*) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene accession numbers for each strain are given in parentheses

of the strains nodulates *A. mangium* (Costa et al., unpublished data). With regard to *P. lunatus*, the UFLA03-321^T, UFLA03-320 and UFLA04-0212 strains inefficiently nodulate; and the UFLA03-290 strain does not nodulate this legume (Costa et al. 2017). The UFLA03-321^T, UFLA3-320 and UFLA 4-0212 strains also nodulate *Glycine max* (Guimarães et al. 2015). A previous study showed that *B. pachyrhizi* PAC48^T is not able to nodule *Glycine max* (Ramírez-Bahena et al. 2009). These data confirm symbiotic differences between the UFLA strains and *B. pachyrhizi* PAC48^T, as reflected in the phylogenetic analysis of the *nodC* and *nifH* genes (Table S2).

The phenotypic, genotypic and symbiotic characteristics presented in this study suggest classification of the UFLA strains as a novel species, for which the name *Bradyrhizobium brasilense* sp. nov. is proposed, with UFLA03-321^T as the type strain.

Description of *Bradyrhizobium brasilense* sp. nov

Bradyrhizobium brasilense (bra.si.len'se. N.L. neut. adj. *brasilense* of Brazil, referring to the fact that strains were isolated from Brazilian ecosystems).

Cells are gram-negative rods, aerobic and do not form endospores. Colonies have a diameter of 1 mm in medium 79 after 5 days incubation at 28 °C, and are cream-colored. The four strains show alkaline reaction in medium 79 using mannitol as a carbon source and bromothymol blue as an

indicator. They grow in pH from 4 to 10, and temperature from 15 to 37 °C, with optimal growth at 28 °C. Salinity tolerance varies between strains. UFLA03-321^T tolerates up to 0.75% NaCl. UFLA03-321^T shows negative reaction for reduction of nitrate, tryptophan deaminase activity, glucose fermentation, arginine dihydrolase, and esculin hydrolysis; and positive reaction for urease and hydrolysis of gelatin. The four 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}), erythromycin (15 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and neomycin (30 μg ml^{-1}), and are sensitive to kanamycin (30 μg ml^{-1}). They are positive for the use of D-arabinose, glycerol and mannitol, but do not use citric acid, glycine, malic acid, maltose, L-methionine, sodium lactate or sucrose as a carbon source. They show weak use of L-asparagine, D-glucose and L-glutamine. The use of D-fructose, L-glutamic acid and lactose as a carbon source varies among strains. L-asparagine, casein hydrolysate and L-glutamic acid are used as a nitrogen source, whereas L-cysteine, glycine, L-methionine and tryptophan are not used as a nitrogen source. The use of L-arginine as a nitrogen source varies among strains. The type strain UFLA03-321^T (=LMG 29353 =CBAS645) was isolated from effective nodules of cowpea inoculated with soil collected in Lavras, Minas Gerais, Brazil. The G + C content of the DNA of UFLA03-321^T is 63.9 mol %.

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Compliance with ethical standards

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

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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