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ORIGINAL PAPER

Bradyrhizobium forestalis sp. nov., an efficient nitrogen-fixing bacterium isolated from nodules of forest legume species in the Amazon

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Abstract

Three strains of nitrogen-fixing bacteria isolated from nodules of *Inga* sp. (INPA54B^T) and *Swartzia* sp. (INPA86A and INPA01-91A) in soils under native forest in the Brazilian Amazon were previously identified as belonging to the *Bradyrhizobium* genus. In this study, these strains were characterized using a polyphasic approach to establish their taxonomic position. The three strains shared more than 99.5% sequence similarity of the 16S rRNA gene with the type strains of five *Bradyrhizobium* species (*B. japonicum* USDA 6^T, *B. liaoningense* LMG 18230^T, *B. ottawaense* OO99^T, *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T). However, multilocus sequence analysis of two (*recA* and *glnII*) or three (*atpD, gyrB*, and *recA*) housekeeping genes indicated that these three strains represent a new *Bradyrhizobium* species, which is closely related to *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T. DNA–DNA hybridization values between INPA54B^T and *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T were only 41.5 and 30.9%, respectively. Phenotypic characterization also allowed the differentiation of the novel species from *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T. In the phylogenetic analysis of the *nodC* and *nifH* genes, the three strains showed similar sequences that were divergent from those of type strains of all *Bradyrhizobium* species. We concluded that these strains represent a novel species, for which the name *Bradyrhizobium forestalis* is proposed, with INPA54B^T (=LMG 10044^T) as type strain. The G+C content in the DNA of INPA54B^T is 63.7 mol%.

Keywords *Bradyrhizobium* · Biological nitrogen fixation · Polyphasic taxonomy · Symbiotic genes

Introduction

Legume-nodulating bacteria (LNB) are of great socioeconomic and environmental importance. Among the LNB genera currently described, *Bradyrhizobium* stands out due to its wide geographic distribution and host range, besides forming efficient symbiosis with important legume species. In the Amazon biome, which occupies approximately 49% of the Brazilian territory, studies carried out in soils under different land use systems have indicated predominance of *Bradyrhizobium* among the LNB genera isolated from different legume species and high phenotypic and genotypic diversity of *Bradyrhizobium* strains (Moreira et al. 1993, 1998; Guimarães et al. 2012, 2015; Silva et al. 2012; Jaramillo et al. 2013; Baraúna et al. 2014).

Recently, several strains of *Bradyrhizobium* from the Amazon biome have been assigned to new *Bradyrhizobium*

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species: *B. manausense*, isolated from nodules of *Vigna unguiculata* (Silva et al. 2014a); *B. ingae*, isolated from nodules of *Inga laurina* (Silva et al. 2014b), *B. neotropicale, B. centrolobii* and *B. macuxiense*, isolated from nodules of *Centrolobium paraense* (Zilli et al. 2014; Michel et al. 2017); *B. stylosanthis*, isolated from nodules of *Stylosanthes capitata* (Delamuta et al. 2016) and *B. brasilense*, isolated from nodules of *Vigna unguiculata* and *Macroptilium atropurpureum* (Costa et al. 2017a).

In a previous study, 800 strains isolated from nodules of several forest legume species of three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) from the Amazon and Atlantic Forest biomes (Brazil) were phenotypically characterized. Most of these strains showed slow or very slow growth and the ability to alkalize in culture medium 79 (Fred and Waksman 1928). The diversity of 171 of these strains, which are cultural representatives from different divergence groups of Leguminosae was studied by comparison of total protein profiles obtained by SDS–PAGE. Of these, 120 strains were grouped within the genus *Bradyrhizobium* (Moreira et al. 1993). Subsequently, for 44 of these strains the 16S rRNA gene was partially sequenced (Moreira et al. 1998).

Guimarães et al. (2015) carried out sequencing of housekeeping genes of 50 *Bradyrhizobium* strains isolated in different Brazilian ecosystems, including six strains from the Amazon biome, which had been previously characterized by Moreira et al. (1993). Among these six strains, two (INPA54B^T and INPA86A) formed a separate group different from the other strains studied and from *Bradyrhizobium* species currently described. In this study, INPA54B^T and INPA86A were selected for further analysis, by molecular and phenotypic methods. Strain INPA01-91A, which was grouped with INPA54B^T and INPA86 by SDS–PAGE (Moreira et al. 1993), was also included in the analyses.

Materials and methods

Origin of strains

The three strains are derived from soil under native forest of the Brazilian Amazon region. Strain INPA54B^T was isolated from *Inga* sp. (Subfamily: Mimosoideae) nodules, and strains INPA86A and INPA01-91A (=INPA 91A) were isolated from *Swartzia* sp. (Subfamily: Papilionoideae) nodules (Moreira et al. 1993). Isolation and characterization of strains were carried out in culture medium 79. The three strains are deposited in the culture collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras, Brazil, and in the culture collection (BCCM/LMG) of the Ghent University, Belgium (INPA54B^T=LMG 10044^T; INPA86A=LMG 10053; INPA01-91A=LMG 10054). The Digital Protologue database TaxonNumber for strain INPA54B^T is TA00342.

Phylogenetic analysis

The alkaline lysis method was used for DNA extraction from the strains (Niemann et al. 1997). Genetic characterization involved sequence analysis of the 16S rRNA gene, housekeeping genes (*atpD, gyrB, recA* and *glnII*) and symbiotic genes (*nodC* and *nifH*). Sequences of 16S rRNA gene (1284–1290 bp) of the three strains, and partial sequences of the genes *atpD* (453 bp), *gyrB* (594) and *recA* (510 bp) of INPA01-91A were obtained using the same primers and amplification and sequencing cycles used by Ribeiro et al. (2015). The sequences of the genes *atpD* (432 bp), *gyrB* (561 bp) and *recA* (381 bp) of INPA54B^T and INPA86A were obtained by Guimarães et al. (2015). *glnII* gene sequences (1035 bp) of strain INPA54B^T was extracted from its genome (PGVG00000000). Amplification and sequencing of *nodC* (480 to 549 bp) gene were carried out according to Sarita et al. (2005), modified by De Meyer et al. (2011). The analysis of *nifH* (306 to 309 bp) gene was carried out according to Gaby and Buckley (2012).

A multilocus sequence analysis (MLSA) was conducted using the sequences of the *glnII* and *recA* genes, since their sequences are available for all type strains of *Bradyrhizobium* species and for the type strain INPA54B^T. In addition, we also performed a separate MLSA with the *atpD, gyrB* and *recA* genes, including all INPA strains. The sequences of all type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI) were included in the alignment for each gene. The alignment of the sequences was carried out using the ClustalW Multiple Alignment algorithm in BioEdit. Sequences were concatenated and distances were calculated according to the Kimura 2 Parameter method (Kimura 1980). Phylogenetic trees were constructed by the neighbor joining (NJ) (Saitou and Nei 1987) and maximum likelihood (ML) (Felsenstein 1981) methods using the MEGA 5 software package (Tamura et al. 2011), with bootstrap values based on 1000 replications.

DNA–DNA hybridization and G+C content

For confirmation of the novel species, DNA–DNA hybridization experiments were carried out, according to methodology previously described (Ezaki et al. 1989; Willems et al. 2001). First, DNA–DNA hybridization was carried out with strain INPA54B^T, which was isolated from *Inga* sp., and the strain INPA86A, isolated from *Swartzia* sp. Subsequently, strain INPA54B^T was hybridized with *B. yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T. The G+C

content in the DNA of strain INPA54B^T was determined by HPLC (Mesbah et al. 1989).

Average nucleotide identity (ANI)

To perform the genome sequencing, the INPA54B^T strain was grown in liquid culture medium 79. 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 INPA54B^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 INPA54B^T strain (accession number PGVG00000000), obtained in this study, and genome of *B. yuanmingense* LMG 21827^T (accession number SAMN04487810) available in the GenBank. ANI values were calculated using the online calculator at http://enve-omics.ce.gatech.edu/ani/index (Goris et al. 2007). As the genome of *B. subterraneum* 58 2-1^T is not available in the GenBank and type strain (58 2-1^T) is not available to perform their genome sequencing, it was not possible to estimate the ANI between INPA54B^T strain and B. *subterraneum* 58 2-1^T.

Phenotypic characterization

Several phenotypic characteristics were evaluated to compare INPA54B^T, INPA86A and INPA01-91A with *B. yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T. Strains INPA54B^T and INPA86A had been evaluated, in previous studies, for its ability to grow in culture medium 79 under different temperature condition and NaCl concentrations (w/v) (0.01, 0.25, 0.5, 0.75 and 1%) (Guimarães et al. 2015), and regarding their resistance to the following antibiotics: ampicillin (10 µg mL^{-1}), cefuroxime (30 µg mL^{-1}), ciprofloxacin (5 µg mL^{-1}), chloramphenicol (30 ug 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). This dataset was supplemented with tests to establish the pH (pH 4, 5.5, 6.8, 8, 9 and 10) and temperature (5, 15, 20, 28, 34, 37 and 40 °C) ranges for growth. To allow comparison, in the present study we evaluated the growth of INPA01-91A and *B. yuanmingense* LMG 21827^T in culture medium 79 under the same conditions of temperature, pH and NaCl, and their resistance to the nine antibiotics cited, following the same protocols used to INPA54B^T and INPA86A.

The three INPA strains and *B. yuanmingense* LMG 21827^T were also evaluated in this study, for their ability to assimilate 16 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 8 nitrogen sources (L-arginine, L-asparagine, hydrolyzed casein, L-cysteine, glycine, L glutamic-acid, L-methionine and tryptophan) in modified 79 medium, the composition of which is described in the study of Costa et al. (2017a). Strain INPA54B^T was also characterized using the API 20NE kit (bioMérieux), according to the manufacturer's instructions, with five days incubation. Phenotypic data from *B. subterraneum* 58 2-1^T were extracted from previous studies (Grönemeyer et al. 2014, 2015a).

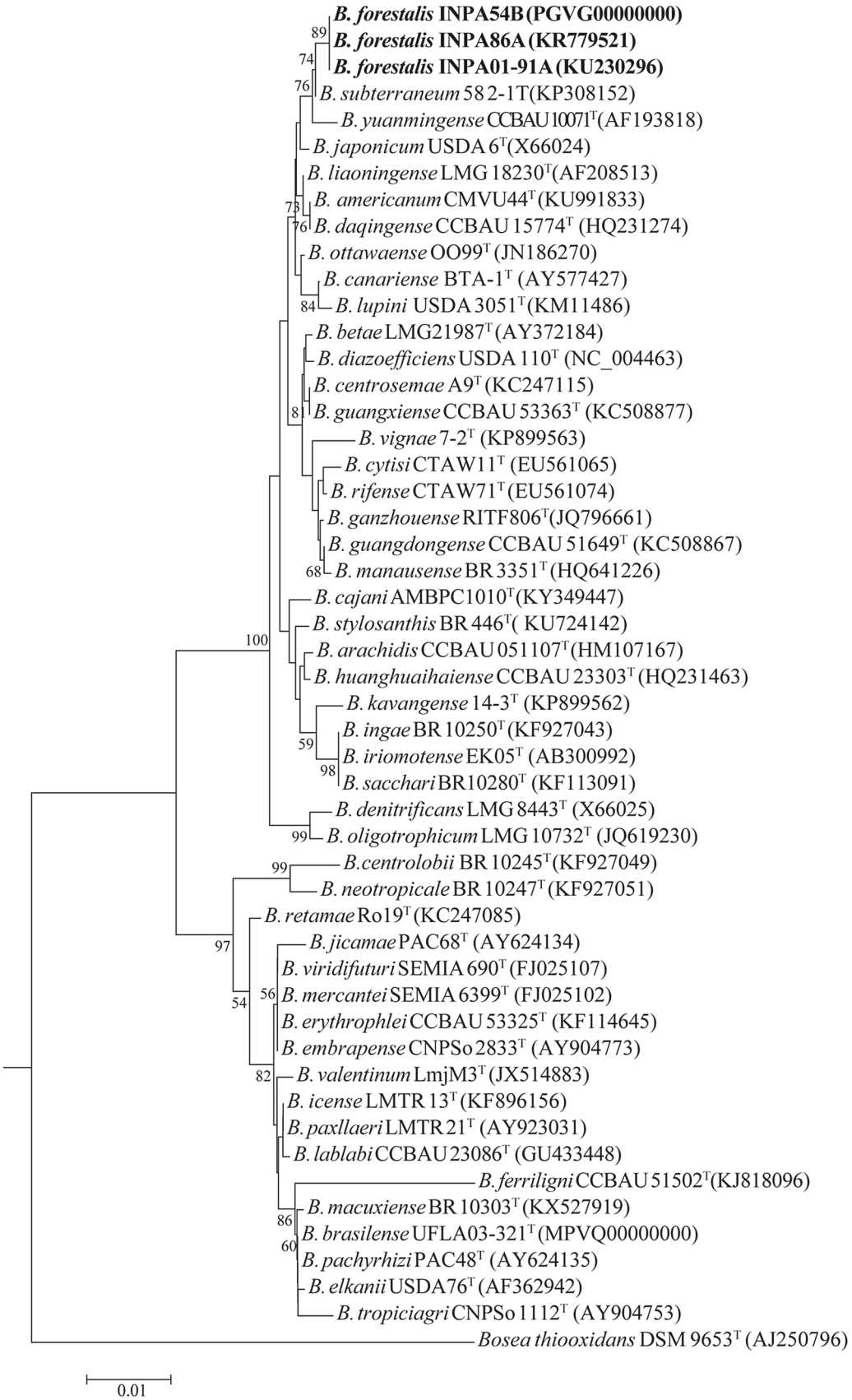
The INPA strains were also compared by analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles. For this analysis, the strains were grown in culture medium 79. Sample preparation and data analyses were carried out as previously described (Wieme et al. 2014).

Results and discussion

Phylogeny of 16S rRNA and housekeeping genes

Results of the phylogenetic analysis of the 16S rRNA gene were similar when using the ML (data not shown) and NJ (Fig. 1) methods. The three INPA strains showed identical 16S rRNA gene sequences (Fig. 1). Strain INPA54B^T shared more than 99.50% similarity with five *Bradyrhizobium* species (*B. japonicum* USDA 6^T, *B. liaoningense* LMG 18230^T, *B. ottawaense* OO99^T, *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T) (Table S1). High similarity between 16S rRNA gene sequences of different *Bradyrhizobium* species have been reported previously (Willems et al. 2001; Wang et al. 2013; Silva et al. 2014b; Costa et al. 2017a), which reflects the high conservation degree of this gene.

For better discrimination between members of *Bradyrhizobium*, MLSA of housekeeping genes has been pointed out as a reliable method (Vinuesa et al. 2005; Ramírez-Bahena et al. 2009; Guimarães et al. 2015; Ribeiro et al. 2015; Costa et al. 2017a). In the present study, two datasets were used for MLSA because not all genes were available for all strains. However, results of the MLSA of two (*glnII* and *recA*) and three (*atpD, gyrB*, and *recA*) housekeeping genes were similar when using the NJ (Fig. 2 and Fig. S1, respectively) and ML methods (data not shown).


B. forestalis INPA54B (PGVG00000000)
B. forestalis INPA86A (KR779521)
B. forestalis INPA01-91A (KU230296)
B. subterraneum 58 2-1T(KP308152)
B. yuanmingense CCBAU10071T(AF193818)
B. japonicum USDA 6T(X66024)
B. liaoningense LMG 18230T(AF208513)
B. americanum CMVU44T (KU991833)
B. daqingense CCBAU 15774T (HQ231274)
B. ottawaense OO99T (JN186270)
B. canariense BTA-1T (AY577427)
B. lupini USDA 3051T(KM11486)
B. betae LMG21987T(AY372184)
B. diazoefficiens USDA 110T (NC_004463)
B. centrosemae A9T (KC247115)
B. guangxiense CCBAU 53363T (KC508877)
B. vignae 7-2T (KP899563)
B. cytisi CTAW11T (EU561065)
B. rifense CTAW71T (EU561074)
B. ganzhouense RITF806T(JQ796661)
B. guangdongense CCBAU 51649T (KC508867)
B. manausense BR 3351T (HQ641226)
B. cajani AMBPC1010T(KY349447)
B. stylosanthis BR 446T(KU724142)
B. arachidis CCBAU 051107T(HM107167)
B. huanghuaihaiense CCBAU 23303T (HQ231463)
B. kavangense 14-3T (KP899562)
B. ingae BR 10250T (KF927043)
B. iriomotense EK05T (AB300992)
B. sacchari BR10280T (KF113091)
B. denitrificans LMG 8443T (X66025)
B. oligotrophicum LMG 10732T (JQ619230)
B.centrolobii BR 10245T(KF927049)
B. neotropicale BR 10247T (KF927051)
B. retamae Ro19T (KC247085)
B. jicamae PAC68T (AY624134)
B. viridifuturi SEMIA 690T (FJ025107)
B. mercantei SEMIA 6399T (FJ025102)
B. erythrophlei CCBAU 53325T (KF114645)
B. embrapense CNPSo 2833T (AY904773)
B. valentinum LmjM3T (JX514883)
B. icense LMTR 13T (KF896156)
B. paxllaeri LMTR 21T (AY923031)
B. lablabi CCBAU 23086T (GU433448)
B. ferriligni CCBAU 51502T(KJ818096)
B. macuxiense BR 10303T (KX527919)
B. brasilense UFLA03-321T (MPVQ00000000)
B. pachyrhizi PAC48T (AY624135)
B. elkanii USDA76T (AF362942)
B. tropiciagri CNPSo 1112T (AY904753)
Bosea thiooxidans DSM 9653T (AJ250796)
89
74
76
73
76
84
8
68
100
59
98
99
99
97
54
56
82
86
60
0.01

◂**Fig. 1** Neighbour-joining phylogeny based on 16S rRNA gene sequences (1228 bp) showing the relationships between strains of the novel species (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

This analysis clearly showed that the INPA strains form a new group, closely related to *B. yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T, supported with high bootstrap value. The similarities between INPA54B^T and *B. yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T were 95.7–94.4 and 96.7–95.0%, respectively, in the analysis of two (*glnII* and *recA*) and three (*atpD, gyrB*, and *recA*) (Table S1). These data suggest that INPA strains belong to a novel species within *Bradyrhizobium*, since these similarity values are similar to those found between different *Bradyrhizobium* species (Chahboune et al. 2011; Silva et al. 2014b; Costa et al. 2017a).

DNA–DNA hybridization (DDH) and G+C content

DNA–DNA relatedness between INPA54B^T and INPA86A was high (83%), confirming that they belong to the same species. DNA–DNA relatedness between strain INPA54B^T and *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T were only 41.5 and 30.9%, respectively. Since this value is far below the limit value (70%) indicated for delineation of new species (Wayne et al. 1987), we can confirm that the three INPA strains represent a novel species within *Bradyrhizobium*. The G+C content in the DNA of strain INPA54B^T was 63.7 mol%, which is within the range reported for *Bradyrhizobium* species (Xu et al. 1995; Chahboune et al. 2011; Ramírez-Bahena et al. 2012).

Average nucleotide identity (ANI)

The ANI values between INPA54B^T and *B. yuanmingense* LMG 21827^T was 89.76%, which indicates that they represent genomically distinct species. The recommended cut-off value of 70% genomic relatedness based on 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).

Phenotypic characterization

The main differential phenotypic characteristics between the strains of the new group and *B. yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T are shown in Table 1. In the description of the new species, the phenotypic characterization is detailed. For some characteristics, differences between INPA54B^T and strains INPA86A and INPA01-91A were observed, indicating phenotypic diversity within the novel species (Table 1). Results of MALDI-TOF MS analysis showed that INPA54B^T has protein profile slightly different from INPA86A and INPA01-91A, indicating that the three representative strains are not clones (Fig. S2).

Phylogeny of *nodC* and *nifH* genes

Genes involved in nodulation and nitrogen fixation, such as *nodC* and *nifH*, respectively, are generally evaluated in symbiotic characterization of novel species of LNB. In this study, sequences of *nodC* and *nifH* genes of the three INPA strains were compared with those of type stains of *Bradyrhizobium* species available in GenBank (National Center for Biotechnology Information, NCBI). In the analyses of both genes (*nodC* and *nifH*), the three INPA strains showed identical sequences that were divergent from those of type strains of all *Bradyrhizobium* species (Fig. 3 and Fig. S3). Phylogenetic analysis of *nodC* (Fig. 3) placed the INPA strains into a cluster of *Bradyrhizobium cajani* AMBPC1010^T (98.6% similarity), isolated from nodules of *Cajanus cajan* L. in Dominican Republic (Araújo et al. 2017), *Bradyrhizobium arachidis* CCBAU 051107^T (97.3% similarity), isolated from nodules of *Arachis hypogeaea* in China (Wang et al. 2013), *B. kavangense* 14-3^T (93.6% similarity) and *B. vignae* 7-2^T (93.6% similarity), isolated from nodules of members of the genus *Vigna* in in Africa (Grönemeyer et al. 2015b, 2016). In the phylogenetic tree of *nifH* (Fig. S3), the INPA strains were placed into a cluster grouping with the same *Bradyrhizobium* species is in the *nodC* phylogeny. The highest similarity (97.4%) was observed with *B. arachidis* CCBAU 051107^T (Table S1).

Nodulation ability of INPA54B^T, INPA86A and INPA01-91A was confirmed by inoculation tests with *Macroptilium atropurpureum* (Moreira et al. unpublished data). Strain INPA54B^T and INPA86A were evaluated for nodulation and nitrogen fixation ability in symbiosis with *Phaseolus lunatus* (Costa et al. 2017b). Both strains promoted higher shoot dry matter production and shoot nitrogen accumulation, exhibiting potential for use as *Phaseolus lunatus* inoculants (Costa et al. 2017b). Strain INPA54B^T was also evaluated regarding its ability to nodulate other legume species, which indicated that this strain does not nodulate *Glycine max* and *Acacia mangium*, but it does nodulate *Stizolobium aterrimum* (Guimarães et al. 2015; Costa et al. unpublished data).

Results of genotypic, phenotypic and symbiotic analyses presented in this study indicate that the three INPA strains should be classified as a novel species within *Bradyrhizobium*. The name *Bradyrhizobium forestalis* sp. nov. is proposed for the new taxon, with INPA54B^T as type strain.

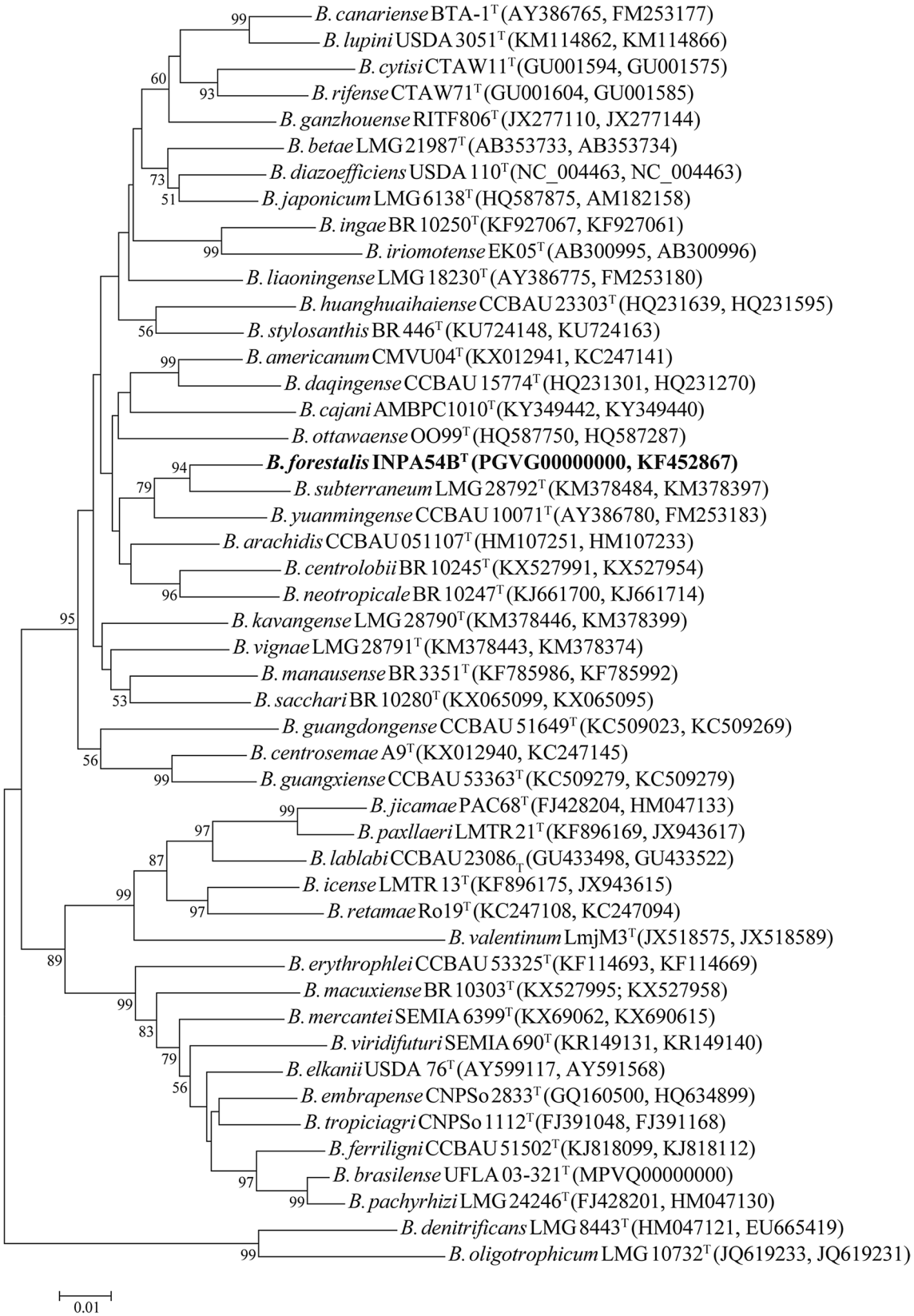


Fig. 2 Neighbour-joining phylogeny based on partial concatenated sequences (798 bp) of the *recA* and *glnII* genes showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene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

Table 1 Differential phenotypic characteristics between strains of *Bradyrhizobium forestalis* (INPA54B^T, INPA86A and INPA01-91A) and the most closely related types strains (*Bradyrhizobium yuanmingense* LMG 21827^T and *B. subterraneum* 58 2-1^T)

Characteristic	INPA 54B^T	INPA 86A	INPA 01-91A	LMG 21827^T	58 2-1Ta
Growth at					
40 °C	−	w	w	+	−
0.75% NaCl	+	−	−	−	nd
Assimilation of carbon source					
L-Asparagine	w	w	w	w	−
D-Fructose	+	+	+	w	−
Citric acid	−	−	−	−	+
Glycerol	w	+	+	+	+
D-Glucose	w	w	w	+	+
Lactose	−	−	−	+	nd
Methionine	−	−	−	w	nd
Lactate	−	−	−	+	+
Sucrose	w	w	w	+	nd
Assimilation of nitrogen source					
Arginine	w	w	w	−	nd
Cysteine	−	−	−	w	nd
Resistance to antibiotics (μg mL^{-1})					
Erythromycin (15)	−	+	+	+	w
Gentamycin (10)	−	−	−	+	nd
Kanamycin (30)	−	−	−	+	w
Neomycin (30)	−	−	−	+	nd

Data represent the means of three biological replicates

+ growth, − no growth, *w* weakly positive, *nd* not determined

Data are from this study except for those for *B. subterraneum* 58 2-1^T that are from Grönemeyer et al. (2014, 2015a)

[a]Data obtained from Grönemeyer et al. (2014, 2015a)

Description of *Bradyrhizobium forestalis* sp. nov.

Bradyrhizobium forestalis (fo.res.ta'lis. N.L. neut. adj. *forestalis* of forest, referring to the fact that these strains were isolated from nodules of forest legume species).

Cells are Gram-negative rods, aerobic and do not form spores. The three strains form cream-colored colonies with diameter > 1 mm. They produce an alkaline reaction in culture medium 79 using mannitol as carbon source and bromothymol blue as indicator, five days after incubation, at 28 °C. All strains grow at pH from 4 to 10, and at temperature from 15 to 37 °C, with optimal growth at 28 °C, but do not grow at 5 °C. INPA86A and INPA01-91A show weak growth at 40 °C, but INPA54B^T does not grow at this temperature. Salinity tolerance varies among strains. INPA54B^T tolerates up to 0.75% NaCl, while INPA86A and INPA01-91A tolerate only up to 0.50% NaCl. The three strains are resistant to ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}) and doxycycline (30 μg mL^{-1}); but they are sensitive to ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), kanamycin (30 μg mL^{-1}), neomycin (30 μg mL^{-1}) and gentamicin (10 μg mL^{-1}). Resistance to erythromycin (15 mL^{-1}) varies among strains. They can assimilate D-arabinose, D-fructose, L-glutamic acid and mannitol, but they do not use citric acid, malic acid, glycine, lactose, L-methionine and sodium lactate, as carbon source. They weakly use L-asparagine, D-glucose and sucrose. The use of glycerol and L-glutamine as carbon source varies among strains. The use of L-asparagine and L-glutamic acid as nitrogen source is positive, but the use of casein hydrolysate, L-cysteine, glycine, L-methionine and tryptophan is negative. The use of L-arginine as nitrogen source varies among the strains. Strain INPA54B^T is positive for urease, esculin hydrolysis and gelatin hydrolysis, and negative for nitrate reduction, tryptophan deaminase activity, glucose fermentation and arginine dihydrolase.

The type strain INPA54B^T (=LMG 10044^T) was isolated from effective nodules of *Inga* sp. in soil under native forest in the Amazon, Brazil. The G+C content in the DNA of INPA54B^T is 63.7 mol%.

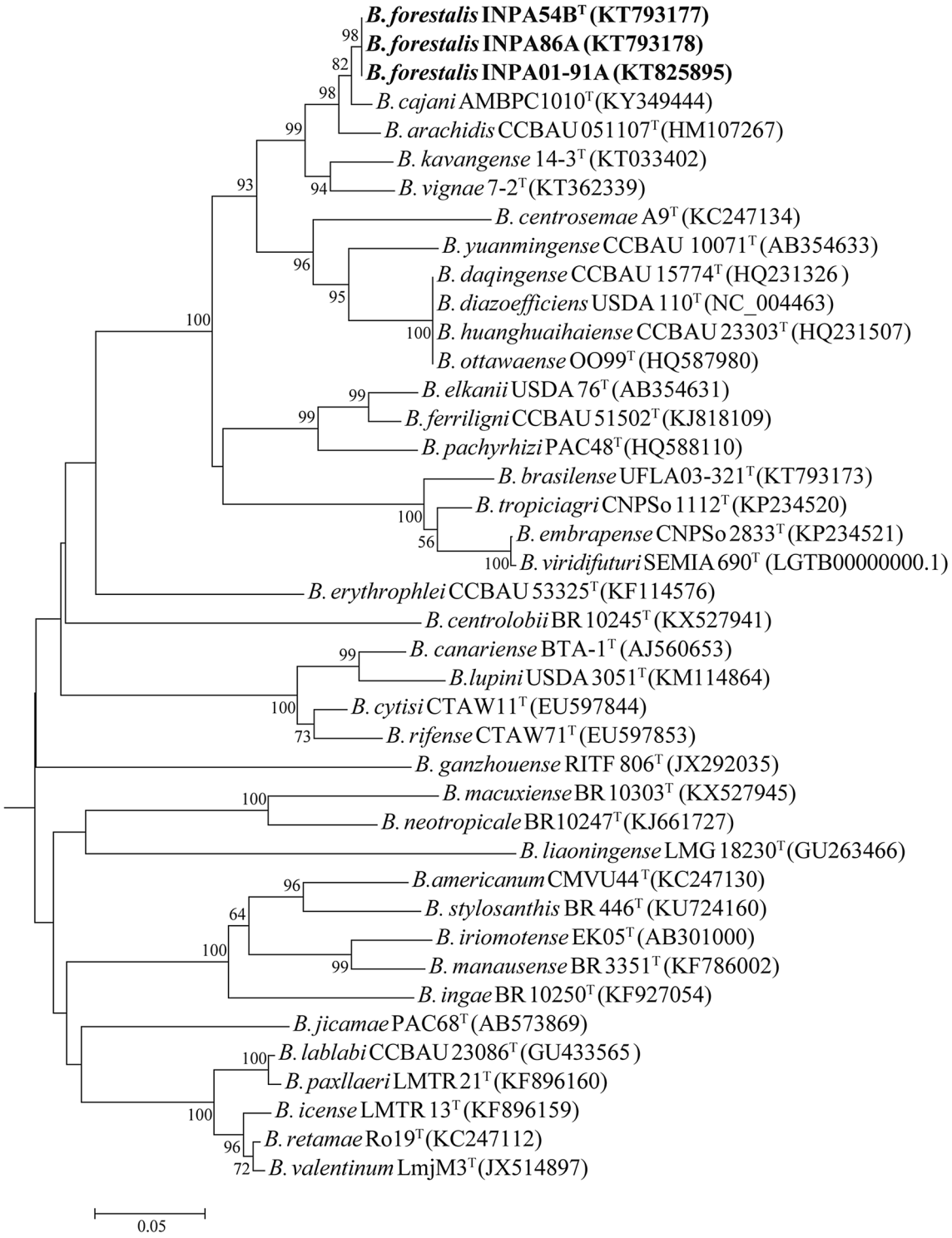


Fig. 3 Neighbor-joining phylogeny based on partial sequences (363 bp) of the *nodC* gene showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene accession numbers for each strain

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