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Research Article

RESEARCH ARTICLE

Paraburkholderia nodosa is the main N_2-fixing species trapped by promiscuous common bean (*Phaseolus vulgaris* L.) in the Brazilian 'Cerradão'

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One sentence summary: Study of the diversity of nitrogen-fixing symbionts from a 'hot spot area', the Brazilian 'Cerradão', reveals that *Paraburkholderia nodosa* is the main symbiont trapped by promiscuous common bean (*Phaseolus vulgaris* L.).

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ABSTRACT

The bacterial genus *Burkholderia* comprises species occupying several habitats, including a group of symbionts of leguminous plants—also called beta-rhizobia—that has been recently ascribed to the new genus *Paraburkholderia*. We used common bean (*Phaseolus vulgaris* L.) plants to trap rhizobia from an undisturbed soil of the Brazilian Cerrado under the vegetation type 'Cerradão'. Genetic characterization started with the analyses of 181 isolates by BOX-PCR, where the majority revealed unique profiles, indicating high inter- and intra-species diversity. Restriction fragment length polymorphism-PCR of the 16S rRNA of representative strains of the BOX-PCR groups indicated two main clusters, and gene-sequencing analysis identified the minority (27%) as *Rhizobium* and the majority (73%) as *Paraburkholderia*. Phylogenetic analyses of the 16S rRNA and housekeeping (*recA* and *gyrB*) genes positioned all strains of the second cluster in the species *P. nodosa*, and the phylogeny of a symbiotic gene—*nodC*—was in agreement with the conserved genes. All isolates were stable vis-à-vis nodulating common bean, but, in general, with a low capacity for fixing N_2, although some effective strains were identified. The predominance of *P. nodosa* might be associated with the edaphic properties of the Cerrado biome, and might represent an important role in terms of maintenance of the ecosystem, which is characterized by acid soils with high saturation of aluminum and low N_2 content.

Keywords: beta-rhizobia; phylogeny of prokaryotes; biological N_2 fixation; Cerrado

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INTRODUCTION

Burkholderia is one of the most ubiquitous and numerous bacterial gender, being capable of colonizing a great variety of niches, including aquatic, edaphic, rhizospheric and clinical environments. It encompasses species of human-health concern—such as the *B. cepacia* complex and *B. pseudomallei*—and plant-pathogenic species, whereas others can promote plant growth, degrade xenobiotics and participate in important environmental processes (Coenye and Vandamme 2003; Gyaneshwar *et al.* 2011; Vial *et al.* 2011; Suarez-Moreno *et al.* 2012; Zuleta *et al.* 2014).

Several *Burkholderia* species have been discovered in the last few years, including symbionts of the mimosoid and papilionoid legume families (de Meyer *et al.* 2013; Sheu *et al.* 2013, 2015; Steenkamp *et al.* 2015), as well as environmental species such as *B. ferrariae* and *B. insulsa* (Valverde *et al.* 2006a; Rusch *et al.* 2015), indicating an expanding understanding of the diversity of this genus. However, the great majority of the studies performed so far are of clinical species, with less information about environmental non-pathogenic strains.

In the last few years, an increasing number of studies—based on analyses of 16S-rRNA (Bontemps *et al.* 2010; Gyaneshwar *et al.* 2011) and of other housekeeping genes (Estrada-de Los Santos *et al.* 2013), as well as genomic approaches (Sawana, Adeolu and Gupta 2014; Zuleta *et al.* 2014)—have indicated clustering of environmental non-pathogenic species in a separate phylogenetic clade. The creation of a new genus comprising non-pathogenic *Burkholderia* could have profound impacts on the use of these bacteria in agriculture and for environmental purposes (Estrada-de los Santos *et al.* 2015). Recently, the proposal of splitting the species into two new genera, *Paraburkholderia* and *Caballeronia* has been taxonomically accepted (Oren and Garrity 2015; Dobritsa and Samadpour 2016). The new genera include environmental species, but it is worth mentioning that some are opportunistic human pathogens.

The Brazilian Cerrado is an important savannah biome distributed across 16 states and comprising 24% of the country's territory (Hungria, Vargas and Araujo 1997). It is characterized by poorly fertile, acidic soils with high aluminum saturation (Adámoli *et al.* 1986; Goedert 1989), leading to a very specific vegetation composed of small trees with gnarled branches, irregularly distributed over a gramineous carpet (Vargas and Hungria 1997). With improvement of fertility, the Cerrado's soils have been increasingly used for agriculture and livestock since the 1960s, and they currently contribute almost 50% of the national production of grains and meat (Oliveira 2013).

One typical property of the Cerrado's soils is their low content of nitrogen (N); therefore, biological nitrogen (N_2) fixation is a key component for biome sustainability (Hungria, Vargas and Araujo 1997). However, despite this agronomic importance, studies of microbial diversity of symbiotic diazotrophic bacteria in undisturbed areas of the biome are few. Some information suggest that rhizobial species, such as *Rhizobium tropici* and *Rhizobium leucaenae,* are abundant (Mostasso *et al.* 2002; Pinto, Hungria and Mercante 2007; Ribeiro *et al.* 2012), that bradyrhizobia are symbionts of several native legumes (Menna *et al.* 2006; Fonseca *et al.* 2012), and that *Paraburkholderia* species are the predominant symbionts of *Mimosa* spp. (Bontemps *et al.* 2010; Gyaneshwar *et al.* 2011). To improve our understanding of the diversity of indigenous symbiotic bacteria, we performed a study in an undisturbed area under the vegetation type of 'Cerradão' vegetation type. To trap bacteria, we used common bean (*Phaseolus vulgaris* L.), a legume known for its high promiscuity in nodulating with a variety of rhizobial species (Velázquez *et al.* 2001; Valverde *et al.* 2006b; Ribeiro *et al.* 2015). As our preliminary results showed that most strains fall into the *Paraburkholderia* genus, we proceeded with a more detailed molecular characterization of strains in this group.

MATERIALS AND METHODS

Site description

Soil samples were collected at the Ecological Reserves of Embrapa Cerrados, a preserved area of 700 ha located in Planaltina, Federal District, Brazil (15° 35′ 30′S/ 47° 42′ 30′ W, at 1175 m of altitude), in the heart of the biome Cerrado. The reserve has all vegetation types of this biome, as shown in Table S1 (Supporting Information) and Fig. S1 (Supporting Information). Our collection was obtained from the undisturbed area of 'Cerradão', covering 37 ha.

The 'Cerradão' vegetation type is a forest formation with xeromorphic aspects (dense and thick leaf cuticles and trunk barks that allow water conservation and therefore withstand drought conditions). Several of the 'Cerradão' tree species lose their leaves during the dry season. Despite the forest-like vegetation type, the floristic composition of 'Cerradão' is similar to the Cerrado *sensu stricto*. The tree coverage ranges from 50% to 90%, and the average tree height ranges from 8 to 15 m (Mendes *et al.* 2012). Information about the legume species found in the 'Cerradão' does not include *Mimosa* spp. (Aquino *et al.* 2009), but other legumes capable of nodulating with *Paraburkholderia* as *Dalbergia* and *Machaerium*.

The regional climate is Cwa (according to the Köppen classification), which is a typical savanna climate with a mean annual precipitation of 1500 mm and two well-defined seasons: dry (from May to September) and rainy (from October to April). Maximum and minimum average temperatures are 26.4°C and 15.9°C, respectively (Baptista 1998; Mendes *et al.* 2012)

In the native area, soil samples were randomly collected because there was no evidence of lack of uniformity at the sampling site. We considered 50 soil samplings that were randomly collected in the rainy season, from the 0 to 20 cm layer, spatially covering the whole 'Cerradão' area of 37 ha. We collected bulk soil. The soil is classified as a clay loan Dystrophic-Red-Latossol (Brazilian classification) or a Rhodic Haplustox (American classification). Subsamples were homogenized to represent one soil sample. Chemical properties and granulometry were determined according to Hungria *et al.* (2006) and are displayed in Table S2 (Supporting Information).

Isolation of strains

The 'Cerradão' soil was used to fill 50 pots with 2 kg per pot. Common bean (*P. vulgaris* L.) cultivar Pérola (colored seeds) seeds were surface-sterilized as described before (Hungria and Araujo 1994) and two seeds were sown in each of the 50 pots. Plants were grown receiving N-free nutrient solution (Hungria and Araujo 1994) for 30 days and then were harvested. A total of 10 nodules were randomly collected per pot, representing about one-third of the nodules of each plant; internal nodule color ranged from light to dark pink. Nodules were surface-sterilized as described before (Vincent 1970), followed by streaking each nodule on a modified YMA (Yeast-Mannitol-Agar) medium (Vincent 1970; Menna *et al.* 2006). All isolates with confirmed purity were characterized in relation to morpho-physiological properties [colony morphology (form, elevation, borders, surface, consistency, optical details and color), mucus production

and chromogenesis (acid/alkaline reaction) in YMA modified medium with bromothymol blue or Congo red] (Vincent 1970; Hungria and Araujo 1994).

Strains are deposited at the 'Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja' (WFCC Collection No. 1213, WDCM Collection No. 1054). Stock cultures were maintained on YMA at 4°C, while long-term preservation was performed by cryopreservation with 30% glycerol at −80°C and −150°C, and by lyophilization. Routinely, unless otherwise indicated, strains were grown in modified YMA medium at 28°C.

rep-PCR (BOX-A1R) genomic fingerprinting

DNA was extracted with AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Biosciences), following the manufacturer's instructions. rep-PCR profiles were obtained after DNA amplification with BOX-AIR primer (Versalovic *et al.* 1994; Koeuth, Versalovic and Lupski 1995; Velázquez *et al.* 2001), proceeding as described before, which included the addition of a molecular marker (1 kb Plus DNA Ladder, Invitrogen®) on the left, right and in the center of each gel (Fernandes, Fernandes and Hungria 2003; Kaschuk *et al.* 2006a). Primers used and amplification conditions are listed in Table S3 (Supporting Information).

Restriction fragment length polymorphism-PCR of the 16S rRNA region

Restriction fragment length polymorphism (RFLP) analyses of the 16SrRNA gene were performed as described before (Germano *et al.* 2006; Pinto, Hungria and Mercante 2007). Basically, the 16S DNA gene was amplified with primers fD1 and rD1 (Table S3, Supporting Information) and 6 μL of the PCR product individually digested with 10 U of endonucleases HpaII (5′-C/CGG-3′; 3′-CGC/C-5′), RsaI (5′-GT/AC-3′; 3′-CA/TG-5′) and HaeIII (5′-GG/CG-3′; 3′-CC/GG-5′) (Invitrogen) at 37°C for 2 h, and then submitted to electrophoresis on a 3% agarose gel. A molecular marker (1 kb Plus DNA Ladder, Invitrogen) was always applied on the left, right and center of each gel.

Sequencing of the 16S rRNA, *recA*, *gyrB* and *nodC* genes

For taxonomic characterization, the 16S rRNA and two housekeeping gene markers—*recA* and *gyrB*—were chosen for PCR amplification and gene fragment sequencing. Primers and amplification conditions are shown in Table S3 (Supporting Information). To infer nodulation genes phylogeny, a fragment of *nodC* was also amplified and sequenced, and primers and PCR conditions are listed in Table S3 (Supporting Information).

All PCR products were purified with Purelink kit (Invitrogen), following the manufacturer's instructions and sequenced on an ABI 3500xL (Applied Biosystems®) capillary sequencer analyzer.

A total of 58 16S rRNA, 45 *recA*, 45 *gyrB* and 45 *nodC* gene sequences were obtained and deposited at the GenBank database. Accession numbers are given in Table S4 (Supporting Information).

Molecular data analyses

BOX-PCR and RFLP-PCR fingerprints

A dendrogram was built with the BOX-PCR profiles with the software Bionumerics (Applied Mathematics, Kortrijk, Belgium, v. 7.0), applying the Unweighted Pair-Group Method with Arithmetic mean (UPGMA) algorithm and Jaccard coefficient with 3% of tolerance.

For the RFLP-PCR profiles, the analyses were performed using the Bionumerics software with the parameters as the BOX-PCR, first with each restriction enzyme and then with the combined profiles obtained with all restriction enzymes, as described before (Germano *et al.* 2006; Pinto, Hungria and Mercante 2007).

Phylogenetic analyses

Sequences were corrected with Bionumerics (Applied Mathematics, Kortrijk, Belgium, v. 7.0). The DNA sequence alignment and the phylogenetic trees were constructed with MEGA (Tokyo, Hachiaji, Japan) software version 6.0 (Tamura *et al.* 2013), using maximum likelihood (ML) algorithm (Felsenstein 1981), Tamura-Nei Model (Tamura and Nei 1993) and a statistical support of 1000 re-samplings (Felsenstein 1985; Hedges 1992). For the Multilocus sequencing analysis (MLSA), multiple alignments were performed for each gene and a common fragment for all strains was obtained; the size of each fragment is shown in Table S3 (Supporting Information). After, that, genes were concatenated and analyzed with the same parameters. Nucleotide sequence identity was calculated with Bioedit v. 7.2.5 (Carlsbad, California, USA).

Nodulation and biological nitrogen fixation efficiency

Representative strains identified as belonging to the genus *Paraburkholderia* were selected to represent the main groups found in the phylogeny analysis, based on their *recA* and *gyrB* phylogenies and tested for their ability to nodulate and fix N_2 with common bean. The experiment consisted of 10 treatments, comprising 2 controls (non-inoculated with and without N-fertilizer, with the application of 30 mg of N plant^{-1} week^{-1}); 2 elite strains used in commercial inoculants for the common bean crop in Brazil [*R. tropici* CIAT 899 (=SEMIA 4077) and *R. freirei* PRF 81 (=SEMIA 4080)] and 6 representative *Paraburkholderia* isolates representing the main groups of *Paraburkholderia* identified in the phylogeny. Strains were grown in YMA medium and concentration adjusted to 10^9 cells mL^{-1}.

Common bean seeds of cultivar Pérola (colored seeds) were surface-sterilized as described in item 2.2 and transferred to modified Leonard Jars (Vincent 1970) containing sterile substrate, consisting of mixture of sand and pulverized coal (1:1, v/v). Four seeds were sown per jar, and each received 0.5 mL of inoculant with adjusted concentration. Plants were thinned to two seedlings per jar six days after emergence. The experiment was conducted with a completely randomized block design with three replicates. The experiment was performed under greenhouse conditions, with 28°–30°C day/23°–25°C night, for 30 days and plants received sterile N-free nutrient solution (Hungria and Araujo 1994) every week.

Plants were harvested 30 days after emergence, roots and shoots were split and nodules were harvested from roots. Shoots and nodules dry weights were obtained after drying at 65°C until constant weight (~72 h). Nodules were counted and total N was determined by Kjeldahl's digestion method followed by the indophenol-blue colorimetric assay (Feije and Anger 1972).

RESULTS

BOX-PCR profiles of isolates from the 'Cerradão'

A total of 181 isolates were obtained and their purity and stable morpho-physiological properties were confirmed after three consecutive replicates (data not shown). Their DNAs were successfully amplified with the BOX-A1R primer and the

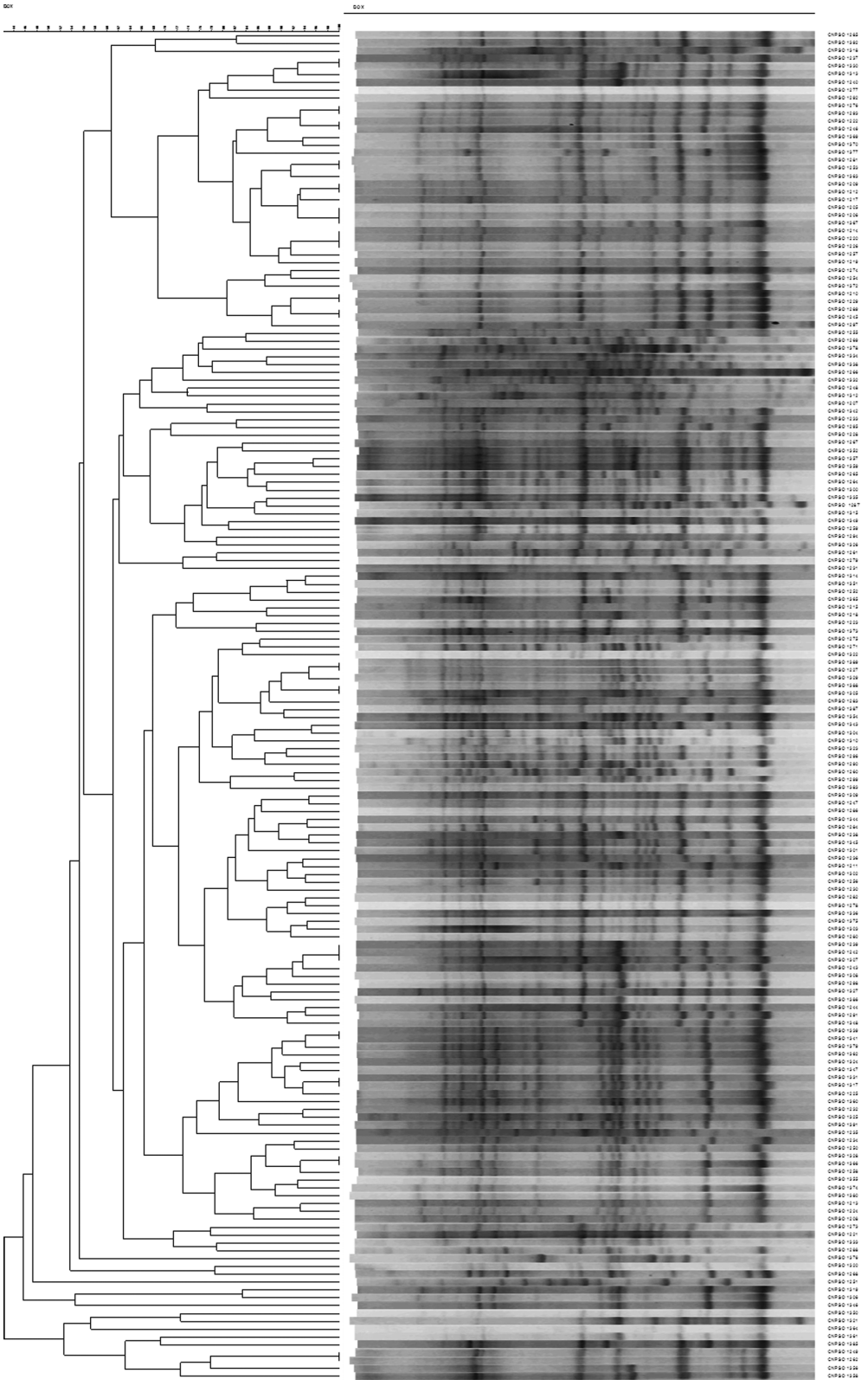

Figure 1. BOX-PCR profiles of 181 isolates trapped by common bean from an undisturbed Brazilian 'Cerradão' soil. Dendrograms built with the Bionumerics software using the UPGMA algorithm and the Jaccard coefficient with 3% of tolerance.

results obtained indicated a very high level of genetic diversity (Fig. 1). Considering a cut-off value of 70% similarity to delineate the BOX-PCR groups, as suggested in previous studies (Coenye *et al.* 2002; Grange and Hungria 2004; Kaschuk *et al.* 2006a,b; Menna *et al.* 2009), almost 100 different profiles were observed. The dendrogram obtained indicates several major clusters, in addition to some isolates occupying isolated positions, and all strains were joined at a very low final level of similarity, of 42% (Fig. 1).

RFLP-PCR analysis of 16S rRNA region

From the BOX-PCR dendrogram, 84 representative isolates were selected and submitted to the RFLP-PCR analysis of the 16S rRNA region with three endonucleases. The patterns obtained are shown in Fig. 2, and the combined analysis with the results obtained with the three enzymes indicated two major groups. The smaller group included 19 isolates distributed in two main subgroups that included 9 (G.R1) and 8 (G.R2) isolates, and 2 isolates joined the subgroups, all clustered at a final level of similarity of 40%. The larger cluster was composed by 65 isolates distributed in six subgroups, with high similarity among the isolates within each subgroup, and joined at a final level of similarity of 80% (Fig. 2).

Sequencing analysis of the 16S rRNA of the 'Cerradão' isolates

Rhizobium isolates

From the results obtained with the RFLP-PCR analysis, a new set of 62 representative isolates was chosen to proceed with the sequence analysis of the 16S rRNA gene. Sequencing analysis identified that 13 isolates belonged to the genus *Rhizobium*, 45 to the *Paraburkholderia* and 5 to endophytes that were not included in the subsequent analyses. From this identification, the isolates were named as strains.

Phylogeny was built separately for the alpha and beta-rhizobia. The 16S rRNA phylogeny of the *Rhizobium* strains showed that five sequences clustered with *R. pusense*, five with *R. miluonense* and one with *R. leucaenae*, while strain CNPSo 1251 occupied an isolated position, possibly indicating a new species; the position of CNPSo 1318 was not well defined (Fig. S4, Supporting Information).

With the taxonomic definition of the genus for each strain, the results of BOX-PCR and RFLP-PCR were re-analyzed. The position of the *Rhizobium* strains in the smaller group in the RFLP-PCR dendrogram was confirmed (Fig. 2). In the BOX-PCR dendrogram, each *Rhizobium* strain showed a unique profile, and they were grouped at a final level of similarity of 50% (Fig. S2, Supporting Information). The two strains that were not grouped with known species in the 16S rRNA sequencing analysis, CNPSo 1251 and CNPSo 1318 (Fig. S4, Supporting Information) showed the most distinct BOX-PCR (Fig. S2, Supporting Information) and RFLP (Fig. 2) profiles.

Paraburkholderia isolates

About two-third of the strains were classified as *Paraburkholderia* in the sequencing analysis of the 16S rRNA gene, and they all fit into the *P. nodosa* group (Fig. 3), showing 98.4%–98.7% of nucleotide identity with the *P. nodosa* type strain (Table 1). Although some intra-species diversity in the 16S rRNA genes was observed, in general the strains showed high similarity, and CNPSo 1376 was the most divergent one (Fig. 3).

We have also re-analyzed the BOX-PCR and RFLP-PCR profiles of the strains classified as *Paraburkholderia*. They all fit into the great group of the RFLP-PCR (Fig. 2). A new analysis of BOX-PCR profiles considering exclusively the *Paraburkholderia* strains confirmed a remarkable intra-specific diversity, with none of the strains showing identical profiles and with several groups sharing <70% similarity among each other (Fig. S3, Supporting Information).

MLSA of the *Paraburkholderia* strains

As the *Paraburkholderia* represented the most abundant symbionts of the 'Cerradão' trapped by common bean, we proceeded with a deeper characterization of this group of strains. Two housekeeping genes were sequenced and analyzed, *recA* (Fig. S5, Supporting Information) and *gyrB* (Fig. S6, Supporting Information). The group of the Brazilian strains showed 55% bootstrap support for the *recA* gene (85%, if we do not include strains CNPSo 1385 and CNPSo 1376) and 92% for the *gyrB* gene (Figs S5 and S6, Supporting Information) and from 99.5% to 100% (*recA*)/ 97.3% to 100% (*gyrB*) of nucleotide identity among the strains from our study, and from 98.3% to 98.5% (*recA*)/ 96.8% to 97.3% (*gyrB*) between our strains and the type strain of *P. nodosa* (Table 1).

A concatenated phylogenetic tree with *recA* and *gyrB* genes was built and resulted in four subclusters; again, CNPSo 1376 occupied an isolated position (Fig. 4). The similarity of the strains was higher with the *P. nodosa* type strain, and a larger cluster included *P. silvatlantica*, *P. mimosarum* and *P. sacchari*. Nucleotide identity between the strains from the 'Cerradão' and the type strain of *P. nodosa* were in the range of 97.4%–97.6% (Table 1).

nodC phylogeny of *Paraburkholderia* strains

The *Paraburkholderia* isolates from the 'Cerradão' also showed high similarity of the *nodC* gene with *P. nodosa* (Fig. 5). It is worth mentioning that *P. silvatlantica* was not included in the analysis because it carries no *nodC* and it does not nodulate. The *nodC* sequence of *P. nodosa* type strain was not available, but we used as comparison strain BR 3470, that belongs to the same species and was isolated from *Mimosa bimucronata* (Chen *et al.* 2007). Nucleotide identity ranged from 94.6% to 100% among our strains and from 93.0% to 100% in the comparison with *P. nodosa* BR 3470; the lowest value (93.0%) referred to CNPSo 1376 strain (Table 1).

Nodulation and nitrogen fixation capacity of *Paraburkholderia* from the 'Cerradão'

Strains were tested for the ability to nodulate effectively *M. caesalpiniifolia*, and all strains classified as *Paraburkholderia* were positive, while the *Rhizobium* strains were not.

Strains of *Paraburkholderia* from the 'Cerradão' were then verified for their capacity of nodulating and fixing N_2 with common bean, and compared to elite *Rhizobium* strains used as commercial inoculants for this legume in Brazil. First, *Paraburkholderia* strains were evaluated to confirm their ability of nodulating common bean, but the majority was either non-effective or showed lower effectiveness in fixing N_2 (data not shown). However, two of them—CNPSo 1258 and CNPSo 1294—exhibited shoot dry weight and N concentration in shoots similar to *R. freirei* PRF 81, while CNPSo 1341 showed low shoot dry weight but high concentration of N in shoots (Table 2). When compared with *R. freirei* PRF 81, strains CNPSo 1258 and CNPSo 1341 showed high total N accumulated in shoots.

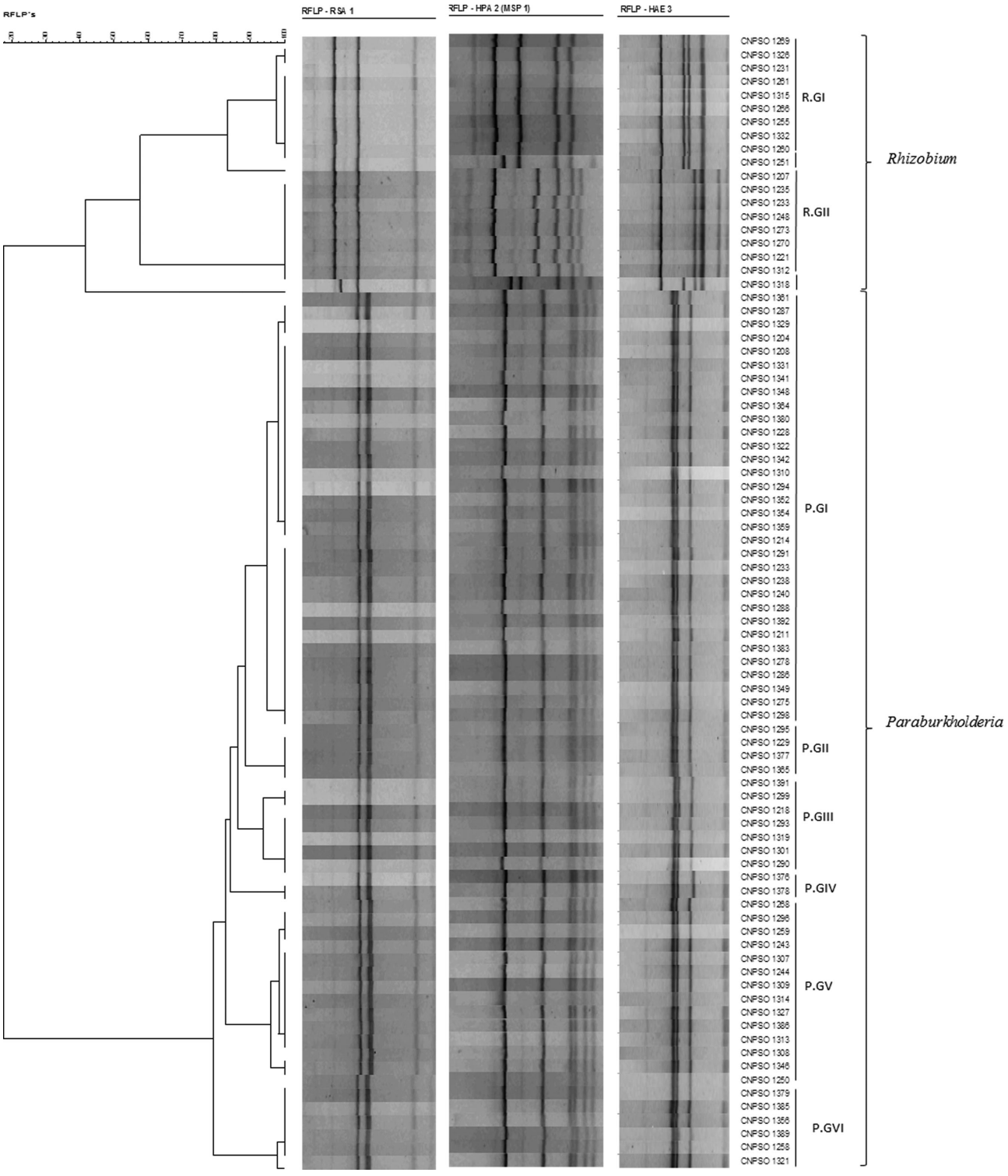

Figure 2. Polyphasic cluster analysis of the PCR products of isolates trapped by common bean from a 'Cerradão' soil by RFLP of the 16S rRNA region digested with three restriction enzymes. Dendrogram built with the Bionumerics software with the UPGMA algorithm, and 3% of tolerance. RGI and RGII = *Rhizobium* Group I and II; PGI, PGII, PGIII, PGIV, PGV and PGVI = *Paraburkholderia* Group I–VI.

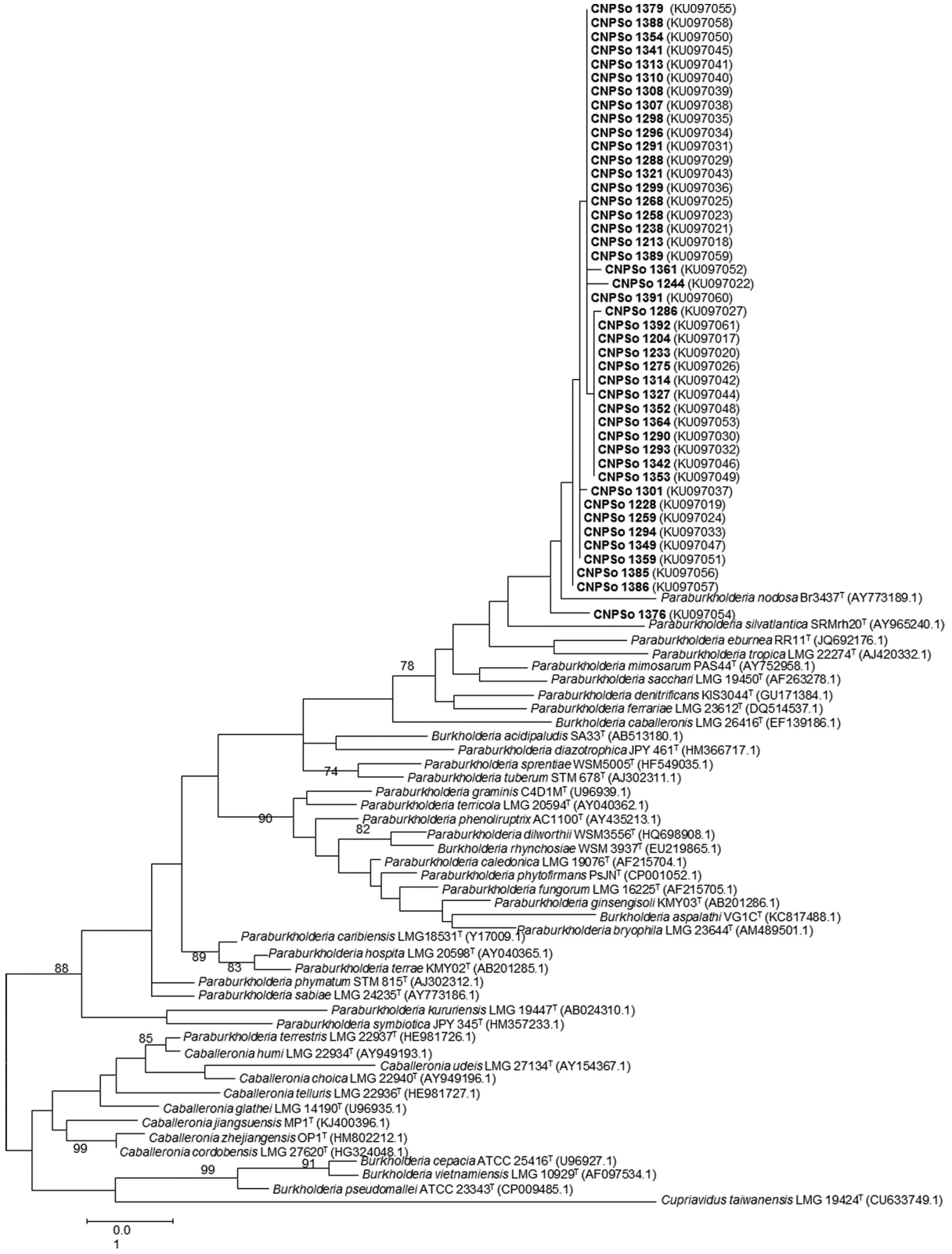

Figure 3. Phylogenetic tree of 16S rDNA gene based on a 1259 bp alignment of *Paraburkholderia* strains trapped by common bean from a 'Cerradão' soil and of reference strains. Phylogeny was built with MEGA v.6 using the ML statistical method with Tamura Nei model and 1000 bootstrap replicates. Support values are shown when ≥70%. Bar indicates the percentage of nucleotide substitutions.

Table 1. Nucleotide identity among and between strains trapped by common bean from a 'Cerradão' soil and *Paraburkholderia nodosa* type strain BR 3437^T.

	Nucleotide sequence identity	
Gene	Among strains	Between strains and *P. nodosa*
16SrRNA	98.9%–100%	98.4%–98.7%
gyrB	97.3%–100%	96.8%–97.3%
recA	99.5%–100%	98.3%–98.5%
Concatenated	99.1%–100%	97.4%–97.6%
nodC	94.6%–100%	93.0%–100%

DISCUSSION

Common bean is a promiscuous legume and, as such, its capacity of establishing root-nodule symbioses with different rhizobial species has been reported in several studies (Michiels *et al.* 1998; Grange and Hungria 2004; Alberton, Kaschuk and Hungria 2006; Kaschuk *et al.* 2006b; Ribeiro *et al.* 2009, 2015; Oliveira *et al.* 2011; Cardoso, Hungria and Andrade 2012) as one of the most suitable plants to trap rhizobia for diversity studies. Michiels *et al.* (1998) stated that the ability of common bean to accept a variety of symbionts might be due to a capacity to recognize many molecular signals (Nod factors). Recently, del Cerro *et al.* (2015a,b) suggested that contributions both from the bacteria, in producing a variety of Nod factors, and from the host plant in perceiving diverse sets of these molecules explain the common bean-rhizobia promiscuity. In our study, with common bean, we were able to trap 181 rhizobia from an undisturbed area of 37 ha, under the vegetation type of 'Cerradão', in the Brazilian Cerrado, and we found that *Rhizobium* spp. represented a small proportion of the strains, whereas the majority fit into the genus *Paraburkholderia*.

Rhizobium encompasses several species and strains of high agronomic importance—such as *R. tropici* CIAT 899 and *R. freirei* PRF 81, which are currently and successfully used as inoculants for application to common bean crops in Brazil—in addition to many others carrying important biotechnological properties. Several rhizobial symbionts of common bean have been described, including those that form both effective (*R. leguminosarum* sv. phaseoli, *R. phaseoli*, *R. tropici*, *R. etli*, *R. leucaenae*, *R. giardinii* sv. phaseoli, *R. gallicum*, *R. lusitanum*, *R. pisi*, *R. freirei*, *R. mesoamericanum*, *R. paranaense*, *R. ecuadorense*) and ineffective (*R. giardinii* sv. *giardinii*, *R. miluonense*) symbioses (Ribeiro *et al.* 2015). The preliminary resulted 16S-rRNA analysis of 12 *Rhizobium* strains isolated from the 'Cerradão' indicated 5 different strains that deserve further investigation, with an emphasis on CNPSo 1251, which might represent a new species. Therefore, our results highlight both the intriguing promiscuity of common bean and the diversity of microsymbionts of this legume.

As *Paraburkholderia* was the main genus found in our survey, we focused on these beta-rhizobia, which also represent another expanding group of symbionts of interest to microbiologists, because of their ecological importance and biotechnological properties. *Burkholderia* has been known as a versatile genus, being adaptable to multiple environments and representing an important component of soil microbial communities (Dalmastri *et al.* 1999). Many new species of this genus have been described or reclassified in recent years; in addition, two new genera encompassing environmental species—*Paraburkholderia* and *Caballeronia*—have been created (Sawana, Adeolu and Gupta 2014; Oren and Garrity 2015; Dobritsa and Samadpour 2016). Nevertheless, information about their ecology, distribution and function is still required.

Despite the biotechnological advances that may be achieved with the accommodation of environmental strains in the genera *Paraburkholderia* and *Caballeronia* (Sawana, Adeolu and Gupta 2014; Oren and Garrity 2015; Dobritsa and Samadpour 2016), concerns continue over the possibility of these strains carrying pathogenic genes. Indeed, studies show that some N_2-fixing species can act as opportunistic pathogens, such *P. vietnamiensis* (Mahenthiralingam, Baldwin and Dowson 2008). However, other studies report differences in the virulence of clinical and environmental species of *Burkholderia*. Angus *et al.* (2014) used bioinformatics tools to search for virulence determinants in many representative species of mammalian and plant pathogens, opportunistic pathogens, environmental and legume-nodulating *Burkholderia* and *Paraburkholderia*. They found that the proteins responsible for virulence in the pathogenic group (T3SS-3 and T6SS-5 proteins, connected to the type-3 and type-4 secretion system, respectfully) were absent in most environmental species. Moreover, inoculation of *Caenorhabditis elegans* confirmed that the symbiotic species were unable to cause pathogenicity in this nematode, and were sensitive to most of the antibiotics tested (Angus *et al.* 2014). In another study, Chen *et al.* (2014) verified that environmental *Burkholderia* (now reclassified as *Paraburkholderia*) carrying pathogenic proteins were lacking the *bsaN* gene, required for cell invasion.

The N_2-fixing group of *Paraburkholderia* species is particularly interesting, for their potential applications in agriculture. Diazotrophic *Paraburkholderia* include strains with both free-living (e.g. *P. tropica* and *P. unamae*) (Caballero-Mellado *et al.* 2004; Reis *et al.* 2004) and symbiotic (e.g. *P. mimosarum* and *P. nodosa*) (Chen *et al.* 2006, 2007) styles, some of them with both styles, as *P. tuberum*, *P. phymatum*, *P. caballeronis*.

In our study, based on the analysis of the 16S rRNA, *recA* and *gyrB* genes, we demonstrated that in an undisturbed 'Cerradão' soil, *P. nodosa* was the predominant species when common bean was used as trapping host. According to Konstantinidis, Ramette and Tiedje (2006), a percentage of nucleotide identity higher than 94% in single housekeeping gene phylogenies and higher than 96% in concatenated phylogenies indicates that the strains belong to the same species, and the strains from our study fit within *P. nodosa*.

The *Paraburkholderia* strains isolated in our study were also capable of nodulating *M. caesalpiniifolia*. Interestingly, the relationship between *Mimosa* and common bean microsymbionts has been noted in another center of *Mimosa* diversity, central Mexico, which also happens to be a center of *Phaseolus* diversity. However, in the case of Mexico, the symbionts of both legume genera are mainly alphaproteobacteria (Bontemps *et al.* 2016), whereas in the undisturbed 'Cerradão', they were *Paraburkholderia*. *Paraburkholderia nodosa* has also been isolated from other *Mimosa* species, such as *M. scabrella* and *M. bimucronata* (Chen *et al.* 2005).

Our results confirm previous surveys carried out in the Cerrado biome by Bontemps *et al.* (2010) and dos Reis Jr *et al.* (2010) with isolates trapped by *Mimosa* spp. In these studies, *P. nodosa* and *P. tuberum* were the predominant species; *P. nodosa* was found at altitudes above 800 m. Mishra *et al.* (2012) analyzed several soil properties, including pH, texture, and phosphate and $CaCO_3$ contents, and verified that these parameters contributed to the predominance of *P. tuberum* nodulating *M. pudica* in French Guiana. Other studies suggested that soil properties such as pH, and geographic conditions such as altitude contribute to the predominance of *Burkholderia/Paraburkholderia*

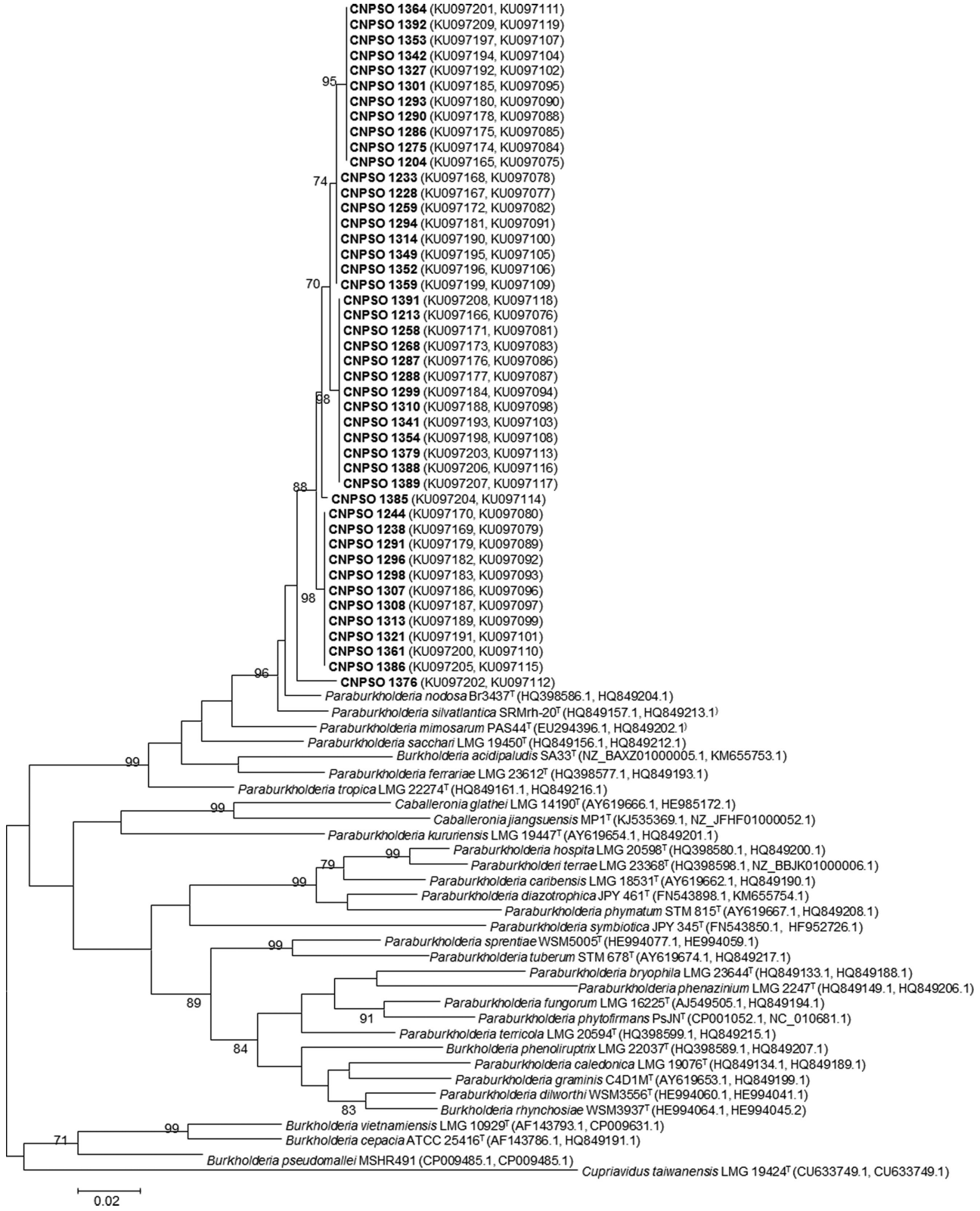

Figure 4. Concatenated phylogenetic tree of *recA* and *gyrB* genes, based on 1000 bp alignment of *Paraburkholderia* strains trapped by common bean from a Cerradão soil and of reference strains. Phylogeny was built with MEGA v.6 using the ML statistical method with Tamura Nei model and 1000 bootstrap replicates. Support values are shown when ≥70%. Bar indicates the percentage of nucleotide substitutions.

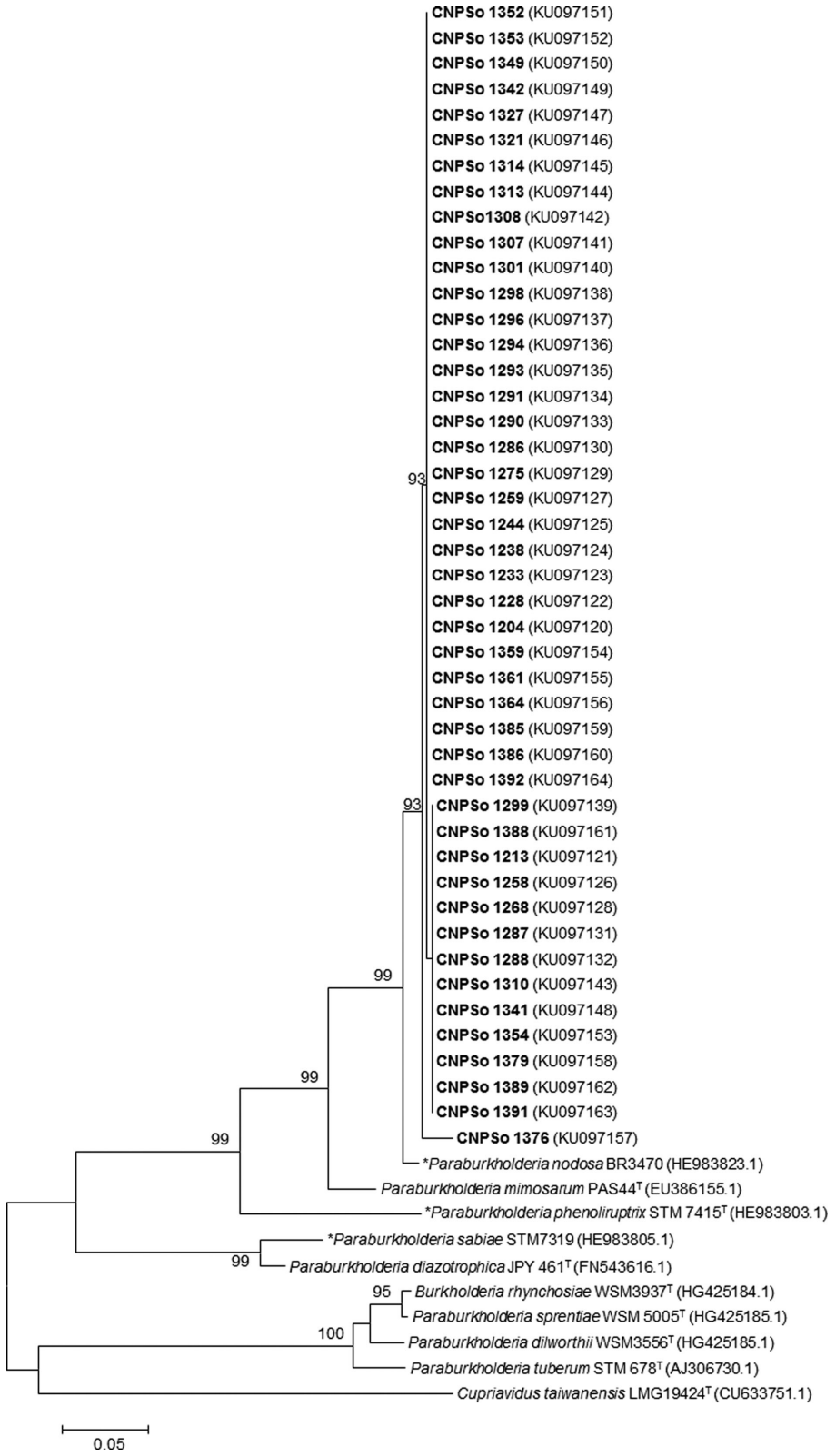

Figure 5. Phylogenetic tree of *nodC* gene based on a 358 bp alignment of representative isolates and reference strains. Phylogeny was built with MEGA v.6 using the ML statistical method with Tamura Nei model and 1000 bootstrap replicates. Support values are shown when ≥70%. Bar indicates the percentage of nucleotide substitutions. Asterisk indicates not type strains.

Table 2. Comparison of nodulation and N_2-fixation capacity of *Paraburkholderia* strains trapped with common bean in a 'Cerradão' soil in this study and two *Rhizobium* elite strain used in commercial inoculants in Brazil.

CNPSo	Number of nodules (n° plant^{-1})	Nodule dry weight (mg plant^{-1})	Shoot dry weight (g plant^{-1})	Nitrogen concentration (g kg^{-1})	Total nitrogen (g kg^{-1})
1258	365 ± 140*	500 ± 70	2.04 ± 0.32	50.8 ± 2.55	104.29 ± 21.33
1294	478 ± 71	428 ± 107	1.39 ± 0.39	52.9 ± 0.85	37.56 ± 4.67
1307	332 ± 70	187 ± 10	0.72 ± 0.01	12.5 ± 0.85	8.98 ± 2.11
1309	299 ± 25	179 ± 50	0.66 ± 0.26	15.9 ± 1.98	33.12 ± 1.71
1322	235 ± 47	157 ± 49	0.76 ± 0.04	17.5 ± 0.50	18..31 ± 0.44
1341	447 ± 62	310 ± 50	0.94 ± 0.06	40.0 ± 7.64	117.03 ± 19.77
R. freirei PRF 81^T	222 ± 71	374 ± 50	1.78 ± 0.02	42.4 ± 10.11	75.88 ± 18.95
R. tropici CIAT 899^T	249 ± 86.3	740 ± 225	6.59 ± 1.22	38.7 ± 2.12	253.74 ± 33.09
Control +N	0	0	3.775 ± 0.813	20.5 ± 0.707	77.28 ± 14
Control –N	0	0	0.62 ± 0.099	13.15 ± 0.45	8.15 ± 0.99

*Values indicate the average between replicates and standard deviation.

(Elliott *et al.* 2009; Garau *et al.* 2009; Bontemps *et al.* 2010; dos Reis Jr *et al.* 2010; Liu *et al.* 2012, 2014; Mishra *et al.* 2012; Lemaire *et al.* 2015). Accordingly, we might conclude that soil properties such as low pH, high concentrations of Al and low fertility (Table 2), as well as geographic conditions such as high altitude (1175 m) contributed to the predominance of *P. nodosa* in our study. In relation to soil pH, it is also worth mentioning that Stopnisek *et al.* (2014) emphasized that *Burkholderia/Paraburkholderia* might not have an actual preference for acidic conditions, but rather be tolerant of them. Interestingly, still in Brazil, at Campos do Jordão, in a biome transitional between the Atlantic and the Araucaria Forests, with subzero temperatures during winter months (contrary to the Cerrados), and high altitudes (1628 m), *Paraburkholderia* spp. was also the predominant symbiont of *Mimosa* spp. (Lammel *et al.* 2015). In contrast, Gehlot *et al.* (2013) have shown that an Indian *Mimosa* sp. (*M. himalayana*) nodulates with *Sinorhizobium* in Cerrado soils, a genus that we have not found in our study; however, the soils used in their study were disturbed and received lime (information not included in the paper), which certainly would affect the rhizobial population. In conclusion, we think that it is simplistic to attribute the predominance of one species to a few edaphoclimatic properties, as an intricate mixture of biotic and abiotic properties compose each biome.

In relation to other legume hosts of *P. nodosa*, in Mata Atlântica (Brazilian Atlantic Forest), another important Brazilian biome, *P. nodosa* also appears as one of the main rhizobial species, along with *P. sabiae* (Bournaud *et al.* 2013), in species comprising the '*Piptadenia* group'. Our *P. nodosa* strains were trapped by *P. vulgaris*, tribe Phaseoleae. Similarly, *P. phymatum* (Talbi *et al.* 2010, 2013) and *P. tuberum* (Elliott *et al.* 2007) have been reported to nodulate common bean in other studies. Indeed, *Paraburkholderia* are common symbionts of legumes in the tribe Phaseoleae that are native to South Africa (Garau *et al.* 2009; Liu *et al.* 2014; Lemaire *et al.* 2015), but with rare reports from South America.

Although *P. nodosa* was the predominant species in our study, we detected a remarkably high intra-diversity. By analyzing BOX elements, we found that each isolate showed a unique profile. Furthermore, within the *P. nodosa* cluster, some strains showed variability in the housekeeping phylogenies, e.g. strain CNPSo 1376.

The *nodC* phylogeny of *Paraburkholderia* from our study was congruent with the phylogenies of the 16S-rRNA and housekeeping genes, suggesting co-evolution. The diversity of *nodC* genes was low, grouping all isolates in the same cluster with 93% bootstrap and 94.6%–100% nucleotide identity, with the lowest value attributed to strain CNPSo 1376. Indeed, according to Bontemps *et al.* (2010), nodulation is an ancient and stable trait among *Burkholderia/Paraburkholderia* lineages and the diversity found in symbiotic genes among beta-rhizobia strains is lower than those found in well-established groups of alpha-rhizobia.

Although the great majority of the *Paraburkholderia* strains from our study maintained the capacity to nodulate common bean, the N_2-fixation capacity was low. However, some strains, such as CNPSo 1258 and CNPSo 1341, were as efficient as one *Rhizobium* strain used as a commercial inoculant in Brazil. In relation to the other strains with low capacity for N_2-fixation, it is important to consider that, in a natural ecosystem in equilibrium, the strains possibly do not need to supply large amounts of N to their original host legumes, in contrast to the needs in areas of intensive agriculture where high-yielding legume genotypes are grown.

Despite the limitations inherent in attributing the abundance of microbial species to single soil properties, *P. nodosa* seems to carry important functions in soils of low nutrient content, such as the area chosen for our study. Since the Cerrado biome is an important region for Brazilian agriculture and livestock, and also one of the 'hottest hotspots' for biological conservation (Myers *et al.* 2000), it is important to keep in mind that the more information we have about its natural microbiota, the more we can help maintain soil microbial diversity. Therefore, identifying diazotrophic strains in this natural environment very poor on soil N may contribute to improving our understanding of ecosystem functioning.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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