AMANDA AZARIAS GUIMARÃES

GENOTYPIC, PHENOTYPIC AND SYMBIOTIC CHARACTERIZATION OF *BRADYRHIZOBIUM* STRAINS ISOLATED FROM AMAZONIA AND MINAS GERAIS SOILS

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Orientadora

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GENOTYPIC, PHENOTYPIC AND SYMBIOTIC CHARACTERIZATION OF *BRADYRHIZOBIUM* STRAINS ISOLATED FROM AMAZONIA AND MINAS GERAIS SOILS (CARACTERIZAÇÃO GENÉTICA, FENOTÍPICA E SIMBIÓTICA DE ESTIRPES DE *Bradyrhizobium* ISOLADAS DE SOLOS DA AMAZÔNIA E MINAS GERAIS)

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Biologia, Microbiologia e Processos Biológicos do Solo, para a obtenção do título de Doutor.

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LAVRAS – MG

2013

A Deus, por me iluminar e permitir que eu concluísse mais uma etapa da minha vida.

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RESUMO GERAL

O gênero *Bradyrhizobium* se destaca entre os gêneros de bactérias fixadoras de nitrogênio que nodulam leguminosas por predominar entre os microssimbiontes eficientes de espécies florestais, forrageiras e de adubação verde, além de importantes espécies de grãos, como a soja, caupi e amendoim. Devido a essa importância, o objetivo do trabalho foi caracterizar simbiótica, fenotípica e geneticamente estirpes de *Bradyrhizobium* isoladas de diferentes ecossistemas brasileiros. Para isso, realizou-se teste de nodulação em soja, testes de resistência a diferentes antibióticos e tolerância a diferentes níveis de salinidade e análise filogenéticas de cinco genes *housekeeping* (*atp*D, *dna*K, *gyr*B, *rec*A e *rpo*B) com 50 estirpes isoladas de solos da Amazônia e Minas Gerais. A partir dos resultados obtidos na análise filogenética, um grupo de cinco estirpes (GII) foi selecionado para a realização de novos estudos com o intuito de identificar a posição taxonômica dessas estirpes. Nessa segunda parte do trabalho foram realizados testes de tolerância a diferentes temperaturas e valores de pH, teste de solubilização de fosfatos inorgânicos e hibridização DNA:DNA. Em relação às 50 estirpes estudadas, verificou-se que onze estirpes foram capazes de nodular soja e que, no geral, as estirpes de *Bradyrhizobium* foram resistentes a maioria dos antibióticos testados. No teste de salinidade observou-se crescimento bacteriano de todas as estirpes até a concentração de 0,5% NaCl e apenas as estirpes UFLA03-142, UFLA03-143, UFLA03-145 e UFLA03-146 cresceram à 1% de NaCl. A análise filogenética destacou grupos de estirpes com potencial de serem novas espécies de *Bradyrhizobium*. A análise filogenética das sequências concatenada dos genes housekeeping *atpD, recA, dnaK* e *gyrB* gerou um grupo formado pelas cinco estirpes estudadas diferente das espécies de *Bradyrhizobium* já descritas, confirmou o resultado encontrado na primeira parte do trabalho, sendo a espécie de *B. liaoningense* a mais próxima do grupo com 96,12% de similaridade. A delimitação da espécie foi realizada pelo teste de hibridização DNA:DNA que apresentou resultado abaixo de 70%. As estirpes desse grupo (GII) crescem em valores de pH de 4 a 9, nas temperaturas 15 e 28ºC, salinidade igual a 0,5% NaCl e variaram quanto à resitência aos antibióticos. As estirpes não apresentam a capacidade de solubilizar fosfatos inorgânicos, não nodulam soja e nodulam caupi. Baseado nas características genéticas, fenotípicas e simbióticas demonstradas nesse trabalho, verificou-se a existência de estirpes com alto potencial de serem novas espécies de *Bradyrhizobium* e foi proposto que as estirpes pertencentes ao GII sejam incluídas em uma nova espécie denominada *Bradyrhizobium amazonense* sp. nov. com UFLA03-150^{T} como estirpe tipo.

Palavras-chave: Bactérias fixadoras de nitrogênio. Taxonomia. Hibridização DNA:DNA. *Housekeeping* genes.

GENERAL ABSTRACT

The genus Bradyrhizobium stands out among the genus of legume-nodulating nitrogen-fixing bacteria because it predominates among efficient microsymbionts of forest species, forage and green manure, as well as grain species. Due to this importance, the aim of this study was to characterize symbiotic, phenotypic and genetically *Bradyrhizobium* strains isolated from different soils of Brazilian ecosystems. For it was performed nodulation test in soybean, test of resistance to different antibiotics and tolerance to different levels of salinity and phylogenetic analysis of five housekeeping genes (*atpD, dnaK, gyrB, recA,* and *rpoB*) with 50 strains isolated from soils of Amazon region and Minas Gerais state. From the results obtained in the phylogenetic analysis, a group of five strains (GII) was selected for further studies in order to identify the taxonomic position of these strains. In this second part of the work it was performed tests of tolerance at different temperatures and pH values, solubilization of inorganic phosphates and DNA:DNA hybridization. Regarding the 50 strains analyzed, eleven strains were able to nodulate soybean and, in general, the strains were resistant to most of the tested antibiotics. In the salinity test bacterial growth was observed for all strains up to a concentration of 0,5% NaCl and only the strains UFLA03-142, UFLA03-143, UFLA03-145 and UFLA03-146 grew at 1% NaCl. Phylogenetic analysis highlighted strains with the potential to be new species of bacteria *Bradyrhizobium*. Phylogenetic analysis of concatenated sequences of four housekeeping genes *atpD, recA, dnaK* and *gyrB* generated a group formed by the five strains that were different from *Bradyrhizobium* species already described, confirming the results found in the first part of the work. The type strain of the specie *B. liaoningense* was closest to the group with 96.12% similarity. The delimitation of the species was performed by DNA:DNA hybridization test, of which results were shown below 70%. The strains of this group (GII) grow at pH 4 to 9, at temperatures 15 and 28ºC, salinity equal to 0,5% NaCl and shown variable behavior in regarding resistance to antibiotics. The strains do not have the capacity to solubilize inorganic phosphates, not nodulate soybean, and nodulate cowpea. Based on genetic, phenotypic and symbiotic analyses demonstrated in this work, we verified the existence of strains with high potential to be new species of *Bradyrhizobium*. It was proposed that the strains belonging to GII should be included in a new species called *Bradyrhizobium amazonense* sp. nov., with UFLA03-150^{T} as the type strain.

Keywords: Nitrogen fixing bacteria. Taxonomy. DNA:DNA hybridization. *housekeeping* genes.

SUMMARY

FIRST PART

1 GENERAL INTRODUCTION

Atmospheric nitrogen fixation generally occurs through industrial, electrical, or biological means. Biological nitrogen fixation (BNF) is mediated by some prokaryote species known as diazotrophic microrganism that possess the enzyme nitrogenase, which are able to reducing N_2 to NH_3 (assimilable form by plants). Among the diazotrophic microorganisms, the legume-nodulating nitrogen-fixing bacteria (LNNFB) are the most important because of the high efficiency of the process and are extremely important for agricultural systems, both economically and environmentally.

In addition to BNF, LNNFB influence other processes that are associated with plant growth, such as solubilization of inorganic phosphates. Phosphorus (P) is a limiting nutrient for plant development; although it can be found in high concentrations in Brazilian soils, it occurs in a non-labile form that plants are unable to directly utilize. The use of phosphate-solubilizing bacteria in soil, thus, facilitates its assimilation in plants. This approach has resulted in a significant reduction in the agricultural production costs.

Approximately, 15 LNNFB genera have currently been described, representing approximately 150 species, of which 16 belong to the *Bradyrhizobium* genus. This genus has been recognized as the most efficient microsymbionts of legumes, thriving in forest, forage, and green manure species, as well as in grains, such as soybean, cowpea, and peanut (MOREIRA, 2006).

Studies about the LNNFB diversity in the Amazon and Minas Gerais, which were conducted by the Sector of Biology, Microbiology and Biological Soil Processes of the Federal University of Lavras (SBMPBS/UFLA), have

shown the predominance of the genus *Bradyrhizobium* (GUIMARÃES et al., 2012; MELLONI et al., 2006; MOREIRA et al., 1993; MOREIRA; HAUK; YOUNG, 1998; RUFINI et al., 2013) among microsymbionts of cowpea, siratro and forest species. However, these investigations did not identify the taxonomic position of the isolated strains. Because LNNFBs play an important role in agriculture and forestry, additional studies on their characterization are imperative.

The aim of this study was to characterize symbiotic, phenotypic, and genetically strains of *Bradyrhizobium* isolated from Amazon and Minas Gerais to generate information about these specific microorganisms.

2 THEORETICAL BACKGROUND

2.1 Biological nitrogen fixation and solubilization of calcium phosphate, aluminum, and iron

Nitrogen is one of the most abundant essential elements in living beings. This element commonly exists as N_2, which is often inaccessible to most prokaryotes and eukaryotes, including plants. Plants obtain nitrogen from nitrogen fertilizers, electrical discharges, or through BNF. BNF is an important biological process involving diazotrophic bacteria, which may be free living, endophytic, or form symbiotic association with some legume species (MOREIRA, 2006).

Agricultural sustainability is strongly influenced by various economic, social, and environmental factors. BNF plays an important role in environmental and economical sustainability because it reduces gas emissions, contamination of rivers, lakes, and water tables, as well as production costs of agricultural crops. Approximately 48 grain and 50 forest legume species had inoculant strains approved by the Brazilian Ministry of Agriculture as inoculants. Of these, soybean is recognized as a plant system in which *Bradyrhizobium* inoculants supply 100% of its nitrogen requirement, thus, saving Brazil billions of dollars each year (MOREIRA, 2010).

Nitrogen-fixing bacteria also increase the availability of nutrients in the soil, including P, thus, enhancing plant growth. Although tropical soil contains high concentrations of P, plants are incapable of directly assimilating this nutrient (MALAVOLTA, 1980); thus, the application of high doses of phosphate fertilizers is necessary. The inability of absorbing P directly from soil is attributable to the strong interaction between P and calcium ions (in alkaline soil), aluminum, and iron (in acidic soil), forming insoluble inorganic

phosphates. One alternative to minimize this effect and thereby reduce production costs involves the use microorganisms that are capable of solubilizing calcium, aluminum, and iron phosphates, as well as performing nitrogen fixation (MARRA et al., 2011, 2012).

2.2 Legume-nodulating nitrogen-fixing bacteria (LNNFB)

The diazotrophic bacterias possess the enzyme nitrogenase, which are able to reducing N_2 to NH_3. Among the diazotrophic bacterias the legume-nodulating nitrogen-fixing bacteria (LNNFB) are the most important because of the high efficiency of the process. They are capable of forming nodules on the roots of most plants and stems of species of the Leguminosae (Fabaceae) family. Approximately 15 genera of LNNFB have been described: *Rhizobium* (FRANK, 1889), *Bradyrhizobium* (JORDAN, 1982), *Azorhizobium* (DREYFUS; GARCIA; GILLIS, 1988), *Sinorhizobium (Ensifer)* (CHEN; YAN; LI, 1988), *Mesorhizobium* (JARVIS et al., 1997), *Allorhizobium* (LAJUDIE et al., 1998), *Burkholderia* (MOULIN et al., 2001), *Methylobacterium* (SY et al., 2001), *Devosia* (RIVAS et al., 2002), *Cupriavidus* (CHEN et al., 2001), *Ochrobactrum* (TRUJILLO et al., 2005), *Phyllobacterium* (VALVERDE et al., 2005), *Achromobacter* (BENATA et al., 2008; GUIMARÃES et al., 2012), *Microvirga* (ARDLEY et al., 2011), and *Aminobacter* (MAYNAUD et al., 2012).

In 1984, only four LNNFB species had been identified; to date, approximately 150 species are known and are distributed across these 17 genera (RHIZOBIAL…, 2013). The taxonomy of LNNFBs was initially based on strains isolated from grain-producing species from temperate climates. Currently, taxonomic classification of these bacteria is also based on plant hosts and sites of tropical areas.

The advances in molecular taxonomy have resulted in the reclassification of strains as new species or genera. For example, *Blastobacter denitrificans* (HIRSCH; MÜLLER, 1985, 1986) has been reclassified as *Bradyrhizobium denitrificans* (BERKUM; LEIBOLD; EARDLY, 2006, 2011), whereas *Agromonas oligotrophica* (OHTA; HATTORI, 1983, 1985) is now recognized as *Bradyrhizobium oligotrophicum* (RAMIREZ-BAHEMA et al., 2012).

It has been estimated that there are around 20,000 legume species thriving across the globe (LEWIS et al., 2005); Brazil supports approximately 3.000 legume species, of which 2,000 thrive in the Amazon (SILVA; SOUZA; CARREIRA, 2004). Although LNNFBs have been extensively studied over the past few years, information on their symbiotic relationships with most tropical plant species as well as their taxonomic classification is limited. This knowledge may also open new avenues in identifying LNNFBs of biotechnological importance.

2.3 *Bradyrhizobium* spp. genus

The genus *Bradyrhizobium* was established in 1982 by Jordan, who showed that these slow-growing bacteria were capable of medium alkalinization; this feature was then used to distinguish these from fast-growing acidifying bacteria. As a result, *Rhizobium japonicum* was then reclassified as *Bradyrhizobium japonicum.*

Approximately 16 species are currently classified under genus *Bradyrhizobium*: *B. japonicum* (JORDAN, 1982), *B. elkanii* (KUYKENDALL et al., 1992), *B. liaoningense* (XU et al., 1995), *B. yuanmingense* (YAO et al., 2002), *B. betae* (RIVAS et al., 2004), *B. canariense* (VINUESA et al., 2008), *B. denitrificans* (BERKUM; LEIBOLD; EARDLY, 2006, 2011), *B. pachyrhizi*, *B. jicamae* (RAMIREZ-BAHEMA et al., 2009), *B. iriomotense* (ISLAM et al.,

2008), *B. cytisi* (CHAHBOUNE et al., 2011), *B. lablabi* (CHANG et al., 2011), *B. daqingense* (WANG et al., 2012), *B. huanghuaihaiense* (ZHANG et al., 2012), *B. oligotrophicum (*RAMIREZ-BAHEMA et al., 2012), and *B. rifense* (CHAHBOUNE et al., 2012).

Although various LNNFBs thrive in Brazilian soils, the *Bradyrhizobium* genus is regarded as the most important because it has been isolated from all studied genera of Caesalpinioideae and from 84 and 80% of the classified genera of forest Mimosoideae and Papilionoideae, respectively (MOREIRA, 2008).

Studies on LNNFB diversity in soils of the Amazon and Minas Gerais, which were conducted by the sector of Biology, Microbiology and Biological Processes of the Soil, Federal University of Lavras (SBMPBS/UFLA), have shown the predominance of the *Bradyrhizobium* genus (GUIMARÃES et al., 2012; MELLONI et al., 2006; MOREIRA; HAUK; YOUNG, 1998; RUFINI et al., 2013). However, these studies did not conduct any taxonomic classification of these isolates at the species level. Considering that this bacterial genus plays an important role in agriculture, better characterization studies are thus imperative.

2.4 Phenotypic and genotypic methods for the identification and characterization of *Bradyrhizobium*

In 1982, *Rhizobium* was the only recognized LNNFB and this was divided into two separate genera, namely *Rhizobium* and *Bradyrhizobium*. Slow-growing bacteria that showed a capacity to alkalinize YMA medium and had G+C content between 62 and 66% were classified as belonging to the genus *Bradyrhizobium*, and strains belonging to the species *Rhizobium japonicum* were thus reclassified as *B. japonicum* (JORDAN, 1982). However, due to the high diversity within this new genus, additional genetic and phenotypic analyses were

conducted (KUYKENDALL et al., 1988, 1992), resulting in the identification of a second species, *B. elkanii*. The main differentiating features between *B. japonicum* and *B. elkanii* involved fatty acid content and resistance to different antibiotics (KUYKENDALL et al., 1992). In addition, *B. elkanii* showed <60% hybridization DNA:DNA with the other strains of *B. japonicum*.

To define the taxonomic classification of a bacterial species, genetic and phenotypic characterization, including chemotaxonomic tests, are performed, following the recommendations of the International Committee for the Systematics of Prokaryotes (GRAHAM et al., 1991; STACKEBRANDT et al., 2002; WAYNE et al., 1987). DNA:DNA hybridization and sequencing of the 16S rRNA gene are amongst the suggested methods to be used as minimum standards for the identification of a new species.

The identification and classification of members of genus *Bradyrhizobium* using sequence analysis of the 16S rRNA gene is generally more difficult because of its high degree of conservation (VINUESA et al., 2005b, 2008; WILLEMS; COOPMAN; GILLIS, 2001). Internal fragment sequencing of housekeeping genes has thus been proposed for greater discrimination among strains and species and for the selection of strains that require DNA:DNA hybridization analysis (RIVAS et al., 2009; STEPKOWSKI et al., 2005, 2007, 2012; VINUESA et al., 2005a, 2005b). This pre-selection has been considered as a cost-effective way of performing molecular characterization analyses on bacterial isolates.

Housekeeping genes, which generally play a major role in metabolism and are constantly expressed (MAIDEN, 2006), have been recognized for their ability in discriminating bacteria for taxonomic classification. The *atpD, recA, dnaK, gyrB, glnII,* and *rpoB* genes have been extensively used in the taxonomic classification of *Bradyrhizobium* species. The *atpD* gene encodes for the β subunit of the ATP synthase membrane, which plays an essential role in energy

production. The *recA* gene encodes for recombinase A, which is involved in the recombination of complementary DNA sequences. The *dnaK* gene encodes Hsp70, which is a protein belonging to the chaperone class. The *gyrB* gene encodes for topoisomerase II; glnII encodes for the β subunit of DNA gyrase; and rpoB encodes for the β subunit of RNA polymerase.

Major advances in molecular biology have occurred in the past decade, facilitating the genetic characterization and taxonomic classification of *Bradyrhizobium*. Phenotypic characterization has also continued to play a critical role in species identification. In addition to studies on its propagation in culture and morphology, tolerance tests at different temperatures, pH values, and NaCl concentrations, antibiotic resistance and assimilation of different carbon and nitrogen sources must be performed. Chemotaxonomic characterization has also been widely used, as well as analysis of bacterial G+C content (mol%) and fatty acid composition (GRAHAM et al., 1991; STACKEBRANDT et al., 2002; WAYNE et al., 1987).

Tables 1 and 2 list the major tests used in the genetic and phenotypic characterization and identification of *Bradyrhizobium* strains. Phylogenetic analysis of the 16S rRNA, *atpD, recA, glnII, nodC,* and *nifH* genes and 16S–23S rRNA internal transcribed spacer (ITS), DNA:DNA hybridization, G+C content (mol%), morphology, assimilation of different sources of carbon and nitrogen, tolerance to different temperatures, pH values, salinity, antibiotic resistance, and nodulation, are the most frequently used characterization assays.

Their symbiotic relationships with legumes also play an important role in the characterization of LNNFBs. Table 3 lists the leguminous species that are symbiotically associated with type strains of *Bradyrhizobium* spp. Nodulation tests have been widely used to identify the symbiotic relationship between a bacterial strain and a leguminous plant. Host plants commonly used in nodulation tests are those described as promiscuous (i.e., capable of undergoing

symbiosis with different bacterial genera and species) and the original host (i.e. the host from which the strain was isolated) (GRAHAM et al., 1991).

Table 1 Main molecular techniques used for characterization and identification of the type strains of *Bradyrhizobium*: 1 (16S r RNA); 2 (23S rRNA); 3 (ITS 16S-23S rDNA); 4 (*nod*C); 5 (*nif*H); 6 (*vir*A); 7 (*puf*M); 8 (*atp*D); 9 (*gln*II); 10 (*rec*A); 11 (*gyr*B); 12 (RFLP: *Restriction Fragment Length Polymorfism*); 13 (RAPD: *Random Amplification of Polymorphic DNA);* 14 (rep-PCR); 15 (AFLP: *Amplified Fragment Length Polymorfism));* 16 (DNA-DNA hybridization).

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Bradyrhizobium japonicum LMG 6138^T	*v															v
Bradyrhizobium elkanii LMG 6134^T	v											v				v
Bradyrhizobium liaoningense LMG 18230^T	v															v
Bradyrhizobium yuanmingense LMG 21827^T	v											v				v
Bradyrhizobium betae LMG 21987^T	v		v	v	v	v							v			v
Bradyrhizobium canariense LMG 22265^T	v		v	v	v			v	v	v		v		v		v
Bradyrhizobium denitrificans LMG 8443^T	v	v	v				v								v	
Bradyrhizobium iriomotense EK05^T	v		v	v	v			v	v	v	v					v
Bradyrhizobium jicamae PAC68^T	v		v	v	v			v	v				v			v
Bradyrhizobium pachyrhizi PAC48^T	v		v	v	v			v	v				v			v
Bradyrhizobium lablabi CCBAU 23086^T	v		v	v	v			v	v	v		v		v		v
Bradyrhizobium cytisi CTAW11^T	v			v	v			v	v	v			v			v
Bradyrhizobium huanghuaihaiense CCBAU23303^T	v		v	v	v			v	v	v		v		v		v
Bradyrhizobium daqingense CCBAU 15774^T	v		v	v	v			v	v	v				v		v
Bradyrhizobium oligotrophicum LMG 10732^T	v							v	v	v						v
Bradyrhizobium rifense CTAW71^T	v							v	v	v						v

*v: Tests performed on the work description of each species

Table 2 Main phenotypic and chemotaxonomic tests used in the characterization and identification of the type strains of *Bradyrhizobium*: 1 – DNA G-C content (mol%); 2 - Fatty acids; 3 - Polar lipids; 4 – Respiratory quinones; 5 - Cell morphology; 6 - SDS-PAGE; 7 - Nitrate reduction; 8 – Serology; 9 - Litmus Milk; 10 - Nitrogen source; 11 - Carbon source; 12 - Voges-Proskauter; 13 – EPS; 14 – Temperature test; 15 – Salinity test; 16 - Tolerance to dyes; 17 – pH test; 18 - Sensitivity to antibiotics

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Bradyrhizobium japonicum LMG 6138^T	v				v				v	v	v			v	v		v	v
Bradyrhizobium elkanii LMG 6134^T		v			v								v					v
Bradyrhizobium liaoningense LMG 18230^T	v				v		v	v	v	v	v			v	v	v	v	v
Bradyrhizobium yuanmingense LMG 21827^T	v				v	v	v			v	v			v	v	v	v	v
Bradyrhizobium betae LMG 21987^T	v				v		v			v	v			v	v		v	v
Bradyrhizobium canariense LMG 22265^T	v				v					v	v			v	v		v	
Bradyrhizobium denitrificans LMG 8443^T					v					v	v			v				
Bradyrhizobium iriomotense EK05^T	v	v			v		v			v	v			v	v		v	v
Bradyrhizobium jicamae PAC68^T	v				v		v			v	v			v	v		v	v
Bradyrhizobium pachyrhizi PAC48^T	v				v		v			v	v			v	v		v	v
Bradyrhizobium lablabi CCBAU 23086^T	v	v			v				v	v	v			v	v		v	v
Bradyrhizobium cytisi CTAW11^T	v				v		v			v	v			v	v		v	v
Bradyrhizobium huanghuaihaiense CCBAU23303^T	v	v	v		v					v	v	v		v	v		v	v
Bradyrhizobium daqingense CCBAU 15774^T	v	v	v	v	v					v	v			v	v		v	
Bradyrhizobium oligotrophicum LMG 10732^T	v	v		v	v		v			v	v			v	v			
Bradyrhizobium rifense CTAW71^T	v				v		v			v	v			v	v		v	v

*v: Tests performed on the work description of each species

Table 3 Legumes that establish symbiosis with the type strains of *Bradyrhizobium*

Species	**Test for nodulation**	
	Positive	**Negative**
Bradyrhizobium japonicum LMG 6138^{T}	*Glycine* sp., *Macroptilium atropurpureum*	
Bradyrhizobium elkanii LMG 6134^{T}	*Glycine* sp.	
Bradyrhizobium liaoningense LMG 18230^{T}	*Glycine max*	*Pisum sativum, Lotus* sp., *Astragalus sinicus, Melilotus* sp.
Bradyrhizobium yuanmingense LMG 21827^{T}	*Lespedeza* sp., *Vigna unguiculata, Glycyrrhiza uralensis*	*Glycine max, Phaseolus vulgaris, Pisum sativum, Galega officinalis, Trifolium repens, Leucaena leucocephala*
Bradyrhizobium betae LMG 21987^{T}		*Glycine max, Pachyrrihizus ahipa*
Bradyrhizobium canariense LMG 22265^{T}	*Lupinus* spp., *Adenocarpus* spp., *Chamaecytisus proliferus, Spatocytisus supranubius, Teline* spp.	*Glycine max*
Bradyrhizobium denitrificans LMG 8443^{T}	*Aeschynomene indica*	
Bradyrhizobium iriomotense EK05T Continued...	*Macroptilium atropurpureum*	
Bradyrhizobium jicamae PAC68^{T}	*Pachyrhizus erosus, Lespedeza* sp.	*Glycine max*
Bradyrhizobium pachyrhizi PAC48^{T}	*Pachyrhizus erosus*	*Lespedeza* sp., *Glycine max*
Bradyrhizobium lablabi CCBAU 23086^{T}	*Lablab purpureus, Arachis hypogaea, Vigna unguiculata*	*Glycine max, Trifolium repens, Lotus corniculatus, Vigna radiata, Pisum sativum, Medicago sativa*
Bradyrhizobium cytisi CTAW11^{T}	*Cytisus villosus*	*Glycine max*

Table 3, conclusion

Species	**Test for nodulation**	
	Positive	**Negative**
Bradyrhizobium huanghuaihaiense CCBAU23303^{T}	*Glycine max, Vigna unguiculata, Medicago sativa*	*Trifolium repens, Lotus corniculatus, Phaseolus vulgaris, Pisum sativum*
Bradyrhizobium daqingense CCBAU 15774^{T}	*Glycine max, Vigna unguiculata, Medicago sativa*	*Trifolium repens, Lotus corniculatus, Phaseolus vulgaris, Pisum sativum*
Bradyrhizobium oligotrophicum LMG 10732^{T}		
Bradyrhizobium rifense CTAW71^{T}	*Cytisus villosus*	*Glycine max*

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SECOND PART - PAPERS

PAPER 1

SYMBIOTIC, PHENOTYPIC, AND PHYLOGENETIC CHARACTERISATION OF *Bradyrhizobium* STRAINS FROM DIFFERENT BRAZILIAN ECOSYSTEMS.

Running title: *Bradyrhizobium* strains from different Brazilian ecosystems

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ABSTRACT

The genus *Bradyrhizobium* stands out among the genera of nitrogen-fixing legume-nodulating bacteria because it is predominant among the efficient microsymbionts of forest, forage, and green manure legume species, including important species of grain legumes, such as soybean, cowpea, and peanut. Due to this prominence, our goal was to characterise symbiotically, phenotypically, and genetically *Bradyrhizobium* strains that were isolated from different Brazilian ecosystems. Therefore, we performed nodulation tests using soybean plants, tests that measured the resistance of the strains to different antibiotics, and the tolerance of the strains to different levels of salinity, as well as phylogenetic analyses of five housekeeping genes (*atp*D, *dna*K, *gyr*B, *rec*A, and *rpo*B). We found that only some of the strains were able to nodulate soybean and that the *Bradyrhizobium* strains were generally resistant to antibiotics. The salinity tests showed the bacterial growth of all of the strains in salinity levels of up to 0,5% NaCl, and only the UFLA03-142, UFLA03-143, UFLA03-145, and UFLA03-146 strains grew in 1% mM NaCl conditions. The phylogenetic trees revealed two clusters of strains, GI and GII, which were separated from the species that have been described to date. In conclusion, our results show that some strains were able to nodulate soybean. Moreover, the *Bradyrhizobium* strains tested showed a high ability to overcome the antagonism that can be exerted by other soil microorganisms and are able to grow on soils that are considered saline. The individual phylogenetic analyses of the *gyr*B, *dna*K, *atp*D, *rec*A, and *rpo*B housekeeping genes indicated that some strains isolated from the Amazon are potentially novel species.

Keywords: soil biology, legume-nodulating bacteria, taxonomy, biological nitrogen fixation, *housekeeping* genes

1. Introduction

Biological nitrogen fixation is one of the most important processes that are performed by soil microorganisms, which include a group of prokaryotes that are known as diazotrophic bacteria. These organisms can live independently in diverse habitats, endophytically or in symbiosis with some plant families, especially legumes.

Currently, 15 genera of legume-symbiotic bacteria have been described. Among these genera, the genus *Bradyrhizobium* is considered predominant among the efficient microsymbionts that nodulate leguminous plants. *Bradyrhizobium* strains have been isolated from all of the studied genera of Caesalpinioideae and from 84% and 80% of the studied genera of forest-dwelling Mimosoideae and Papilionoideae, respectively (Moreira, 2006).

Currently, approximately 150 species of legume-nodulating nitrogen-fixing bacteria (LNNFB) have been identified. Of these species, 16 belong to the genus *Bradyrhizobium*: *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1993), *B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), *B. betae* (Rivas et al., 2004), *B. canariense* (Vinuesa et al., 2005a), *B. denitrificans* (van Berkum et al., 2006), *B. pachyrhizi* (Ramirez-Bahema et al., 2009), *B. jicamae* (Ramirez-Bahema et al., 2009), *B. iriomotense* (Islam et al., 2008), *B. cytisi* (Chahboune et al., 2011), *B. lablabi* (Chang et al., 2011), *B. daqingense* (Wang et al., 2012), *B. huanghuaihaiense* (Zhang et al,.2012), and *B. oligotrophicum (*Ramirez-Bahema et al., 2012), *B. rifense* (Chahboune et al., 2012).

The sequence analysis of the 16S rRNA gene has been used for over 20 years as the standard in taxonomy studies for the identification of most of the nitrogen-fixing LNB species (Graham et al., 1991). However, the identification and classification of *Bradyrhizobium* species through the analysis of the 16S rRNA gene sequence is limited due to the high level of conservation of this gene

among these species (Willems et al., 2001; Vinuesa et al., 2005b, 2008). Thus, the sequencing of housekeeping genes, such as *atp*D, *rec*A, *dna*K, *gyr*B, and *rpo*B, has been proposed as an alternative approach for the taxonomic study of *Bradyrhizobium* strains (Rivas et al., 2009; Stepkowski et al., 2005, 2007, 2012; Vinuesa et al., 2005a, b).

Previous studies (e.g., Moreira et al.1998; Jaramillo et al., (submitted); Guimarães et al., 2012; Rufini et al., (submitted); Melloni et al., 2006) conducted in the Sector of Biology, Microbiology and Biological Processes of Soil (Setor de Biologia, Microbiologia e Processos Biológicos do Solo - SBMPBS) at Federal University of Lavras (Universidade Federal de Lavras - UFLA) analysed the 16S rRNA gene of several strains and classified them as members of the genus *Bradyrhizobium*. However, in line with similar observations by other authors (Willems et al., 2001; Vinuesa et al., 2005b, 2008; Fonseca et al., 2012), it was not possible to obtain a satisfactory differentiation between the strains.

Thus, the aim of the present study was to characterise symbiotically, phenotypically, and genetically 48 *Bradyrhizobium* strains that were isolated from different Brazilian ecosystems. Consequently, nodulation tests using soybean (*Glycine max*) as the host plant, tests of the sensitivity of the strains to different antibiotics and the tolerance of the strains to different levels of salinity, as well as phylogenetic analyses of the housekeeping genes (i.e., *atp*D, *gyr*B, *dna*K, *rec*A, and *rpo*B) were performed.

2. Materials and methods

2.1. Strains

Forty-eight strains of the genus *Bradyrhizobium* from the SBMPBS/UFLA collection (code: UFLA and INPA) and two strains from the collection at the National Centre of Agrobiological Research at EMBRAPA

(Centro Nacional de Pesquisa de Agrobiologia – EMBRAPA; code: BR) were analysed in the present study. Of these strains, four are recommended as inoculants of legumes in Brazil: UFLA03-84 and INPA03-11b for *Vigna unguiculata* (Soares et al., 2006) and BR2801 and BR2003 for *Cajanus cajan*. Two other stains (UFLA03-153 and UFLA03-164) are undergoing selection tests to determine their effectiveness as inoculants for *Vigna unguiculata* (Melloni et al., 2006). The symbiotic efficiencies of all of the strains with the original host and with other plants were tested (Table 1).

Table 1 *Bradyrhizobium* strains used in this study

	Strain	Geographic Origin	Land Use Systems	Host Plant or Source Isolation	Symbiotic Efficiency[1]	Source and Reference[2]
1	UFLA03-173	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Guimarães et al., 2012
2	UFLA03-197	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
3	UFLA03-270	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
4	UFLA03-148	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Guimarães et al., 2012
5	UFLA03-146	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
6	UFLA03-174	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
7	UFLA03-280	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
8	UFLA03-150	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
9	UFLA03-139	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Guimarães et al., 2012
10	UFLA03-268	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
11	UFLA03-144	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
12	UFLA03-305	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
13	UFLA03-147	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
14	UFLA03-145	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
15	UFLA03-214	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012

Table 1, continue

	Strain	Geographic Origin	Land Use Systems	Host Plant or Source Isolation	Symbiotic Efficiency[1]	Source and Reference[2]
16	UFLA03-315	Amazônia	Forestry	*Vigna unguiculata*	*Vigna unguiculata* (N)	SBMPBS/UFLA
17	UFLA03-316	Amazônia	Forestry	*Vigna unguiculata*	*Vigna unguiculata* (N)	SBMPBS/UFLA
18	UFLA03-290	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
19	UFLA03-319	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
20	UFLA03-322	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
21	UFLA03-323	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
22	UFLA03-324	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
23	UFLA03-326	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
24	UFLA03-286	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
25	UFLA03-149	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Guimarães et al., 2012
26	UFLA03-143	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
27	UFLA03-153	Minas Gerais	Bauxite Mining	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Melloni et al., 2006
28	UFLA03-164	Minas Gerais	Bauxite Mining	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Melloni et al., 2006
29	UFLA03-313	Amazônia	Forestry	*Vigna unguiculata*	*Vigna unguiculata* (N)	SBMPBS/UFLA
30	UFLA03-314	Amazônia	Forestry	*Vigna unguiculata*	*Vigna unguiculata* (N)	SBMPBS/UFLA
31	UFLA03-227	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)

Table 1, continue

	Strain	Geographic Origin	Land Use Systems	Host Plant or Source Isolation	Symbiotic Efficiency[1]	Source and Reference[2]
32	UFLA03-239	Amazônia	Agroforestry	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA; Jaramillo et al., (submitted)
33	UFLA03-320	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
34	UFLA03-321	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
35	UFLA03-325	Minas Gerais	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (E)	SBMPBS/UFLA; Rufini et al., (submitted)
36	UFLA04-212	Amazônia	Agriculture	*Macroptilium atropurpureum*	*Macroptilium atropurpureum* (E)	SBMPBS/UFLA
37	UFLA03-318	Amazônia	Pasture	*Vigna unguiculata*	*Vigna unguiculata* (I)	SBMPBS/UFLA
38	UFLA03-182	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
39	UFLA03-142	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
40	UFLA03-140	Amazônia	Agriculture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA; Guimarães et al., 2012
41	UFLA03-317	Amazônia	Pasture	*Vigna unguiculata*	*Vigna unguiculata* (e)	SBMPBS/UFLA
42	INPA 10A	Amazônia	Forestry	*Samanea saman* (Jaq.) Merr.	*Macroptilium atropurpureum* (N)	SBMPBS/UFLA;
43	INPA 54B	Amazônia	Forestry	*Inga* sp	*Macroptilium atropurpureum* (N)	SBMPBS/UFLA; Moreira et al., 1998
44	INPA 86A	Amazônia	Forestry	*Swartzia* sp	*Macroptilium atropurpureum* (N)	SBMPBS/UFLA
45	INPA 104A	Amazônia	Forestry	*Campsiandra surinamensis* Benth.	*Macroptilium atropurpureum* (N)	SBMPBS/UFLA;
46	INPA 237B	Amazônia	Forestry	*Pterocarpus*	*Macroptilium atropurpureum* (N)	SBMPBS/UFLA;

Table 1, conclusion

	Strain	Geographic Origin	Land Use Systems	Host Plant or Source Isolation	Symbiotic Efficiency[1]	Source and Reference[2]
47	INPA 03-11b	Amazônia	Forestry	*Centrosema sp*.	*Vigna unguiculata* (R)	SBMPBS/UFLA; Soares et al., 2006
48	UFLA 03-84	Rondônia	Pasture	*Vigna unguiculata*	*Vigna unguiculata* (R)	SBMPBS/UFLA; Soares et al., 2006
49	BR2801	-	-	*Crotalaria* spp	*Cajanus cajan* (R)	EMBRAPA Agrobiologia
50	BR2003	-	-	*Stylosantes* spp.	*Cajanus cajan* (R)	EMBRAPA Agrobiologia

[1]**E**=Efficient (Dry matter of shoots of the treatment inoculated with strain tested = to uninoculated controls/ with nitrogen supplementation); **e**=Efficient (Dry matter of shoots of the treatment inoculated with strain tested < to uninoculated controls/ with nitrogen supplementation, and > to uninoculated controls /low nitrogen concentration; **I**=Inefficient= Dry matter of shoots of the treatment inoculated with strain tested = to uninoculated controls / low nitrogen concentration; **R**= approved as *Vigna unguiculata* inoculants (UFLA03-84, INPA03-11b) and *Cajanus cajan* inoculants (BR2801, BR2003) by the Ministry of Agriculture; **N=** Strain authenticated in *Macroptilium atropurpureum* (INPA 86A; INPA 104A; INPA 237B; INPA 10A; INPA 54B) and in *Vigna unguiculata* (UFLA03-313; UFLA 03-314; UFLA 03-315; UFLA 03-316)

[2]SBMPBS/UFLA= Collection of bacteria from Sector of Biology, Microbiology and Biological Processes of Soil at Federal University of Lavras (Setor de Biologia, Microbiologia e Processos Biológicos do Solo - SBMPBS da Universidade Federal de Lavras)

2.2. Nodulation tests on soybeans

The 48 strains from SBMPBS/UFLA and two strains from the collection at the National Centre of Agrobiological Research at EMBRAPA (Table 1) were tested for their ability to establish a symbiosis with *Glycine max* (soybean). The experiments were conducted over a period of 30 days in a greenhouse, and 28 and 22 strains were analyzed in the first and second experiments, respectively. The first experiment was started on May 8th, 2012 and the second was started on May 22nd, 2012. The two experiments were conducted under the same conditions. During this period, the daily temperature registered varied from 15 to 35°C and the relative air humidity varied from 30 to 90%. The soybeans (5G 830 RR) were grown under axenic conditions in bottles with a nutrient solution, as previously described by Guimarães et al. (2012). To prepare the treatments, liquid 79 medium (Fred and Waksman, 1928) was inoculated with bacterial cells that were previously grown on solid 79 medium through the use of a platinum needle. The cells were incubated at 28°C with constant agitation for five days. At planting, each seed was inoculated with 1 mL of the prepared bacterial culture, which corresponded to approximately 10^9 bacterial cells. The study was completely randomised and performed in triplicate. Three positive controls were inoculated with the soybean reference strains CNPAB-29W (BR29), CPAC15 (BR85), CPAC7 (BR86), and SEMIA 587 (BR96), and the *Bradyrhizobium japonicum* strain LMG 6138^T. The three negative controls included the plants inoculated with the *Bradyrhizobium canariense* strain LMG 22265T and two uninoculated controls with low and high nitrogen content (Guimarães et al., 2012). After 30 days, the presence or absence of nodules on the soybeans roots was assessed.

2.3. Sensitivity to antibiotics

Ten antibiotics were tested at the following concentrations: 10 μg mL^{-1} ampicillin (AMP), 30 μg mL^{-1} cefuroxime (CRX), 5 μg mL^{-1} ciprofloxacin (CIP), 30 μg mL^{-1} chloramphenicol (CLO), 30 μg mL^{-1} doxycycline (DOX), 15 μg mL^{-1} erythromycin (ERI), 10 μg mL^{-1} gentamicin (GEN), 30 μg mL^{-1} kanamycin (KAN), 30 μg mL^{-1} neomycin (NEO), and 10 μg mL^{-1} penicillin G (PEN). The choice of these antibiotics was based on previous work that described new species of *Bradyrhizobium* (Xu et al., 1995; Yao et al.; 2002; Rivas et al., 2004; Vinuesa et al. 2005a; Islam et al., 2008; Ramirez-Bahema et al.; 2009).

The strains were grown in liquid 79 medium under constant agitation for five days, which is a sufficient period of time for the cells to reach logarithmic growth phase. Subsequently, the cells were rinsed with 0.85% saline solution to remove any residual culture medium from the inoculum, which could result in false positive effect on growth. Aliquots of 1.0 mL of each bacterial culture, which corresponded to approximately 10^9 cells, were transferred to 1.5-mL sterile microtubes and centrifuged at 12,768 x g and 4°C for 10 minutes. The supernatants were discarded, and the cells were resuspended in 1.0 mL of 0.85% sterile saline solution and re-centrifuged. This process was repeated three times. Then, 0.1 mL aliquots of the bacterial cell suspensions were inoculated and streaked using a Drigalski spatula in Petri dishes with 79 medium. For each strain, ten antibiotics were tested in different dishes. On each dish, three disks of the same antibiotic were inserted, which comprised three biological replicates. The *Bradyrhizobium japonicum* strain LMG 6138^T, the *B. elkanii* strain LMG 6134^T, and the *B. canariense* strain LMG 22265^T were used as controls and compared with the 50 strains under study. All of the treatments were incubated at 28ºC for seven days. The treatments were subsequently assessed to determine

the presence or absence of bacterial growth, and measuring the halo diameter formed.

2.4. Salinity tolerance

The cell suspensions were prepared as described in the previous section. The suspensions were incubated and streaked using a Drigalski spatula in Petri dishes with 79 medium (Fred and Waksman, 1928), which was modified with different saline concentrations (%): 0,25; 0,5; 0,75, and 1 sodium chloride (NaCl). Medium 79 without modifications (0,01% NaCl) was used as the control treatment. In addition to the 50 strains that were studied, the *Bradyrhizobium japonicum* strain LMG 6138^{T}, the *B. elkanii* strain LMG 6134^{T}, and the *B. canariense* strain LMG 22265^{T} were used as controls. All of the treatments were performed in triplicate and incubated at 28ºC for seven days. The treatments were then assessed to determine the presence or absence of bacterial growth.

2.5. DNA extraction

The strains were grown in duplicate on liquid 79 medium under constant agitation for five days. The genomic DNA was obtained using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research Corp., CA, US) according to the manufacturer's instructions. Subsequently, the quality and concentration of the extracted DNA were verified using a NanoDrop device.

2.6. Amplification and sequencing of housekeeping genes (gyrB, dnaK, atpD, recA, and rpoB).

Five housekeeping genes (*gyr*B, *dna*K, *atp*D, *rec*A, and *rpo*B) were amplified and sequenced as described previously. Table 2 shows all of the primers and conditions that were used during the procedure. The PCR reaction was performed on a Bio-Rad Thermo Cycler using a 25-µL reaction volume that

contained 2.5 µL of DNA, 2.5 µL of 2 mM deoxyribonucleotide triphosphates (dNTP), 2.5 µL of 10X PCR buffer, 0.25 µL of each primer (Table 2), and 0.5 µL of 1 U/µL Taq. The quantity and concentration of the PCR products were verified on a 1% agarose gel that was stained with ethidium bromide. The products were then purified using the NucleoFast® 96 PCR clean-up kit under vacuum, rinsed, and resuspended in ultrapure water. They were sequenced using the dideoxynucleotide chain termination method with fluorescent ddNTPs (AppliedBiosystems) on an ABI Prism 3130xl capillary sequencer according to the manufacturer's instructions (Applied Biosystems). Prior to sequencing, the PCR products were purified again using 45 µL SAM™ and 10 µL BigDye XTerminator™ in a MicroAmp™ optical 96-well reaction plate.

Table 2 Oligonucleotide primers used and PCR cycling conditions

Primer	Sequence 5'-3'	Position	PCR cycling	References
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	8-32	5'95ºC,	Stepkowski et al.,
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	594-620	32x(45''94ºC,1'60º,	2005
			1'30''74ºC),	Stepkowski et al.,
			5'72ºC	2005
gyrB343F	TTCGACCAGAAYTCCTAYAAGG	343-364	5'95ºC, 5x(2'94ºC, 2'57ºC,	Martens et al., 2007
			1'30''72ºC),	
gyrB1043R	AGCTTGTCCTTSGTCTGCG	1043-1061	28x(30''94ºC,	Martens et al., 2007
			1'57º,1'30''72ºC), 5'72ºC	
dnaK1466F	AAGGARCANCAGATCCGCATCCA	1466-1488	5'94ºC, 35x(1'94ºC, 1'62º,	Stepkowski et al.,
dnaK1777R	TASATSGCCTSRCCRAGCTTCAT	1777-1799	40''72ºC),	2003
			5'72º	Stepkowski et al.,
				2003
atpD352F	GGCCGCATCATSAACGTSATC	352-372	5'95ºC, 2x(2'94ºC,	Modified from
atpD871R	AGMGCCGACACTTCMGARCC	890-871	1'64.3ºC, 1'72ºC),	Guant et al., 2001
			30 x (30''94ºC, 1'72ºC),	and Martens et al.,
			5'72ºC	2007
rpoB83F	CCTSATCGAGGTTCACAGAAGGC	83-103	5'95ºC,3x(2'94ºC,	Martens et al., 2007
rpoB1540R	AGCTGCGAGGAACCGAAG	1557-1540	2'58.2ºC, 1'72ºC),	
			30x(30''94ºC,	
			1'58.2ºC,172ºC), 5'72ºC	
rpoB83F	CCTSATCGAGGTTCACAGAAGGC	83-103		
rpoB1061R	AGCGTGTTGCGGATATAGGCG	1081-1061		

2.7. Phylogenetic analysis

The 48 strains from SBMPBS/UFLA and two strains from the collection at the National Centre of Agrobiological Research at EMBRAPA (Table 1) belong to the genus *Bradyrhizobium*. They were identified via partial sequencing of the 16S rRNA gene by Moreira et al.1998; Jaramillo et al., (submitted); Guimarães et al., 2012; Rufini et al., (submitted); Melloni et al., 2006. Of the 50 strains, 41 where used to build the phylogenetic tree using their 16S rRNA gene partial sequences.

The quality of the 16S rRNA gene sequences was verified through their Phred quality scores, and the sequences of the housekeeping genes (*atp*D, *gyr*B, *dna*K, *rec*A, and *rpo*B) were visually inspected. ClustalW was used for the sequence alignment. Subsequently, the phylogenetic tree was constructed using the neighbour-joining method for the 16S rRNA gene and the maximum likelihood method for the *atp*D, *gyr*B, *dna*K, *rec*A, and *rpo*B genes. The construction of the phylogenetic trees was performed using the Kimura 2 Parameter model (Kimura, 1980) in the Molecular Evolutionary Genetic Analysis software (MEGA, version 5) (Tamura et al., 2011). For comparison, the alignment included the sequences of the *Bradyrhizobium* species that are available in GenBank (National Center for Biotechnology Information, NCBI). A bootstrap confidence analysis with 1000 iterations was performed.

3. Results

3.1. Nodulation test in soybeans

A total of 50 strains were assessed through a nodulation test in which soybean was used as the host plant. All of the plants that were inoculated with three of the strains (UFLA03-290, UFLA03-325, and UFLA03-326) died before the evaluation. Of the remaining 47 strains that were studied, we found that only 11 were able to establish a symbiosis with soybean (BR2003, UFLA03-321,

UFLA03-147, UFLA03-322, UFLA03-319, INPA03-11B, UFLA03-286, UFLA03-323, UFLA03-153, UFLA03-320, and UFLA04-212). No nodules were found on the control plants, which included uninoculated plants with supplementary nitrogen, uninoculated plants with low nitrogen content, and plants inoculated with the LMG 22265^{T} type strain, which is not able to establish symbiosis with the soybean plant (Vinuesa et al., 2005a). Moreover, we found that the plants inoculated with the strains that are recommended as soybean inoculants [CNPAB-29W (BR29), CPAC15 (BR85), CPAC7 (BR86), as well as those with SEMIA 587 (BR96)] and with *Bradyrhizobium elkanii* LMG 6134^{T}, all nodulated soybean, which demonstrates the reliability of the results obtained in the present study.

3.2. Sensitivity to antibiotics

Of the 10 antibiotics tested, four (AMP, CRX, CIP, and DOX) are semisynthetic or synthetic, and six (CLO, ERI, GEN, KAN, NEO, and PEN) are natural products and may occur in soil. As in the phylogenetic analysis, the analysis of the antibiotic resistance patterns revealed a high diversity of strains. In general, the KAN antibiotic inhibited the growth of the most strains, and only strains UFLA 03-144, UFLA 03-143, UFLA 03-314, UFLA 03-84, and INPA 237B grew in the presence of this antibiotic. The antibiotic CLO was the only antibiotic that did not inhibit the growth of any of the tested strains. Of all of the strains tested, UFLA 03-143 and UFLA 03-84 were resistant to all antibiotics (Table 3).

Table 3 Resistance of the 50 strains analyzed plus three controls to the 10 antibiotics tested: Ampicillin - AMP (10); Cefuroxime – CRX (30); Ciprofloxacin – CIP (5); Chloramphenicol – CLO (30); Doxycycline – DOX (30); Erythromycin – ERI (15); Gentamicin – GEN (10); Kanamycin – KAN (30); Neomycin – NEO (30); Penicillin G - PEN (10)

	Strain	**AMP**	**DOX**	**KAN**	**NEO**	**CIP**	**PEN**	**GEN**	**CRX**	**ERI**	**CLO**
1	UFLA03-173	*R	R	16,07	R	R	13,51	R	26,04	R	R
2	UFLA03-197	14,79	9,7	24,92	8,18	R	R	11,41	21,20	R	R
3	UFLA03-270	12,12	R	R	10,69	R	8,63	7,24	16,36	R	R
4	UFLA03-148	R	R	30,20	6,27	R	7,0	12,79	17,04	R	R
5	UFLA03-146	R	R	17,62	R	R	R	9,37	R	R	R
6	UFLA03-174	13,73	R	16,21	R	R	10,27	R	R	R	R
7	UFLA03-280	R	R	28,67	10,17	R	R	19,28	R	R	R
8	UFLA03-150	R	R	23,9	9,35	R	13,51	10,83	19,3	6,65	R
9	UFLA03-139	R	R	12,30	12,82	R	R	13,79	R	R	R
10	UFLA03-268	R	R	40,95	10,57	R	R	R	R	7,05	R
11	UFLA03-144	R	R	R	21,87	R	R	R	R	R	R
12	UFLA03-305	R	R	22,22	10,22	R	R	R	R	R	R
13	UFLA03-147	12,40	R	19,27	12,78	R	11,51	9,17	18,49	R	R
14	UFLA03-145	13,43	R	13,35	R	R	12,20	R	24,65	R	R
15	UFLA03-214	13,11	R	20,56	20,46	R	R	R	R	R	R
16	UFLA03-315	R	R	31,40	R	R	R	9,57	R	R	R
17	UFLA03-316	R	R	12,68	R	R	R	R	R	R	R
18	UFLA03-290	R	R	15,45	R	R	R	R	R	R	R
19	UFLA03-319	R	R	12,82	R	R	R	R	R	R	R
20	UFLA03-322	R	R	14,99	7,15	R	R	R	R	R	R

Table 3, continue

	Strain	AMP	DOX	KAN	NEO	CIP	PEN	GEN	CRX	ERI	CLO
21	UFLA03-323	R	R	16,34	R	R	R	R	R	R	R
22	UFLA03-324	R	R	14,99	6,93	R	R	R	R	R	R
23	UFLA03-326	R	R	17,73	R	R	R	R	R	R	R
24	UFLA03-286	R	R	17,57	R	R	R	R	R	R	R
25	UFLA03-149	R	R	22,97	9,75	R	R	R	R	R	R
26	UFLA03-143	R	R	R	R	R	R	R	R	R	R
27	UFLA03-153	R	R	30,20	R	7,61	R	R	R	R	R
28	UFLA03-164	R	R	R	15,55	R	R	R	R	R	R
29	UFLA03-313	R	R	23,55	R	R	R	R	R	R	R
30	UFLA03-314	R	-	12,14	R	R	R	R	R	R	R
31	UFLA03-227	R	R	19,58	R	R	R	R	R	R	R
32	UFLA03-239	11,62	R	16,33	R	R	R	R	24,42	R	R
33	UFLA03-320	R	R	18,06	R	R	R	R	R	R	R
34	UFLA03-321	R	R	21,10	R	R	R	R	R	R	R
35	UFLA03-325	7,72	R	33,61	7,61	R	6,87	R	R	R	R
36	UFLA04-212	R	R	28,20	R	R	R	R	R	R	R
37	UFLA03-318	R	R	12,08	R	R	R	R	R	R	R
38	UFLA03-182	R	R	35,41	15,37	R	R	R	R	R	R
39	UFLA03-142	R	R	25,36	14,04	R	R	R	R	R	R
40	UFLA03-140	R	R	12,83	R	R	R	R	R	R	R
41	UFLA03-317	R	R	24,18	R	R	R	R	R	R	R
42	INPA 10A	R	R	20,0	R	R	R	R	R	R	R

Table 3, conclusion

	Strain	AMP	DOX	KAN	NEO	CIP	PEN	GEN	CRX	ERI	CLO
43	INPA 54B	23,68	R	17,12	7,89	R	11,66	9,50	28,06	31,70	R
44	INPA 86A	21,50	R	22,03	6,53	R	12,07	8,98	35,03	R	R
45	INPA 104A	R	R	16,30	R	R	R	R	R	R	R
46	INPA 237B	R	R	R	7,88	R	R	R	R	R	R
47	INPA 03-11b	R	R	17,34	R	R	R	R	R	R	R
48	UFLA 03-84	R	R	R	R	R	R	R	R	R	R
49	BR2801	R	R	18,95	R	R	R	R	R	R	R
50	BR2003	R	R	25,14	R	R	R	7,95	R	R	R
51	CNPAB-29W	R	R	14,15	R	R	R	R	R	R	R
52	LMG 22265^{T}	R	R	27,28	16,68	R	R	11,45	13,95	R	R
53	LMG 6138^{T}	R	R	18,11	7,48	R	R	R	18,86	R	R
54	LMG 6134^{T}	R	R	25,34	7,19	R	R	7,82	R	R	R

*R: Strain resistant to the antibiotic

3.3. Salinity tolerance

The tolerance of the strains to different levels of salinity (0,01; 0,25; 0,5; 0,75, and 1% NaCl) was also tested. All of the strains were found to be tolerant of 0,5% NaCl, and 36 strains, including CNPAB-29W (BR29), which is used as a soybean inoculant, and the *Bradyrhizobium elkanii* LMG 6134^{T} strain, were tolerant of salinity levels up to 0,75% NaCl. The UFLA03-146, UFLA03-145, UFLA03-143, and UFLA03-142 strains were the only strains that were able to tolerate 1% NaCl in the culture medium (Table S1 - supplementary material).

*3.4. Phylogenetic analyses of the 16S rRNA and other housekeeping genes (gyr*B, *dna*K, *atp*D, *rec*A, and *rpo*B*)*

Figure 1 shows the phylogenetic analysis of the 16S rRNA gene partial sequences of 41 of the strains, which were sequenced by Moreira et al.1998; Jaramillo et al., (submitted); Guimarães et al., 2012; Rufini et al., (submitted); Melloni et al., 2006. It is evident that the sequencing of this gene alone does not allow differentiation between most of the *Bradyrhizobium* strains.

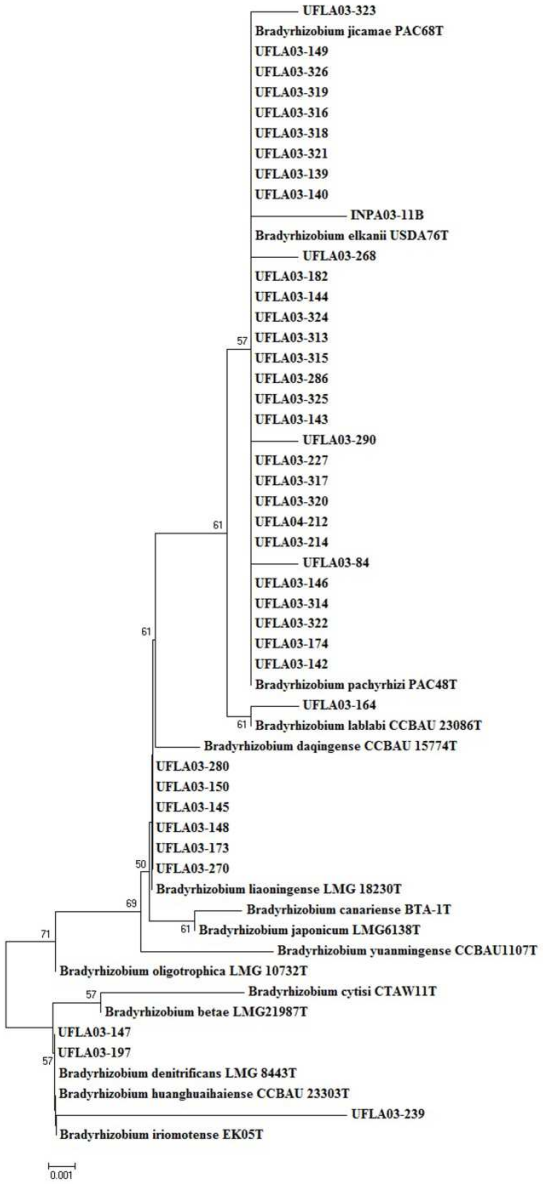

Figure 1 Phylogenetic relationships of 41 *Bradyrhizobium* strains based on the 16S rRNA gene partial sequence. Phylogeny was determined by the neighbor joining method. Bootstrap values were based on 1,000 trials. Phylogenetic analyses were conducted in Mega5

Therefore, in order to better genetically characterise the studied strains, we sequenced the internal fragments of five housekeeping genes (*gyr*B, *atp*D, *dna*K, *rec*A, and *rpo*B), which resulted in the partial sequencing of 641 base pairs (bp) of *gyr*B, 280 bp of *dna*K, 510 bp of *atp*D, 561 bp of *rec*A, and 978 bp of *rpo*B. Fewer sequences were obtained for the latter two genes due to difficulties in their amplification. Thus, the phylogenetic analyses of these genes were performed individually using 43, 44, 38, 24, and 22 sequences of the *gyr*B, *dna*K, *atp*D, *rec*A, and *rpo*B genes, respectively (Figures 2, 3, 4, 5, and 6). There was high genetic diversity in all five genes between the different *Bradyrhizobium* strains, and the *dna*K gene showed the least difference among the strains that were studied. Nevertheless, although it exhibited less diversity, the phylogenetic tree of the *dna*K gene differentiated the strains into two groups (GI and GII). The GI group was identifiable in the phylogenetic trees of all of the genes. With the exception of the *rpo*B gene, the same result was found for the GII group. This result is most likely observed because it has not been possible to obtain the sequences of the *rpo*B gene in the strains that belong to the GII group. In addition to these two clear groups, further groups were observed in some, but not all of the trees; other strains also stood out with a separate position in the trees of some of the genes. These included BR2003, UFLA03-164 and UFLA03-147 in the *atp*D tree, UFLA03-147 in the *rec*A tree, BR2003, BR2801, UFLA03-318, and UFLA03-153 in the *gyr*B tree, and INPA10A, INPA86A, and UFLA03-147 in the *dna*K tree. In future work, these strains will be studied to determine their taxonomic position.

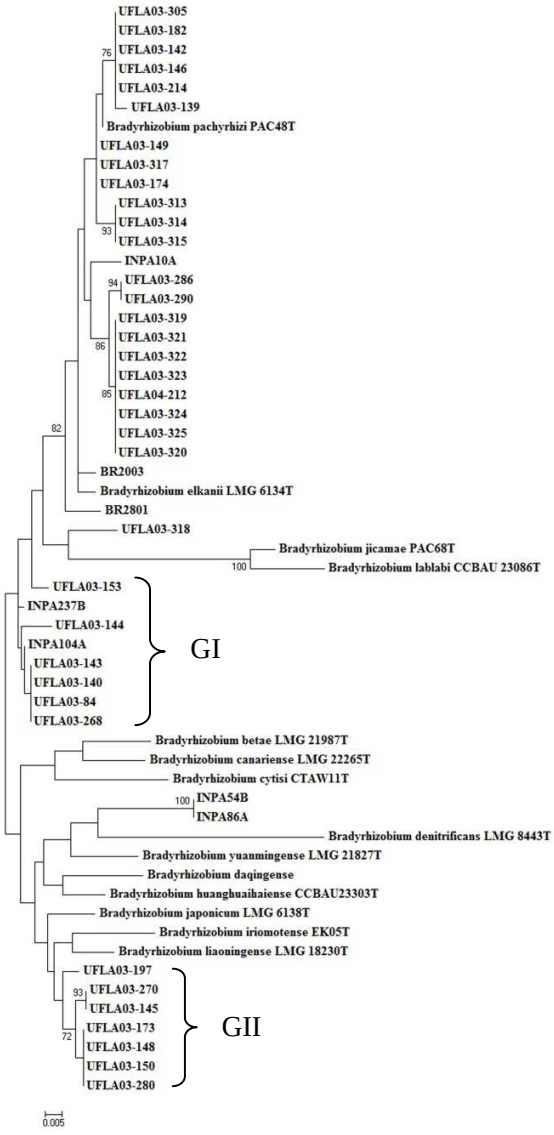

Figure 2 Phylogenetic relationships of 43 *Bradyrhizobium* strains based on the *gyrB* gene. Phylogeny was determined by the maximum likelihood method. Bootstrap values were based on 1.000 trials. Phylogenetic analyses were conducted in Mega5

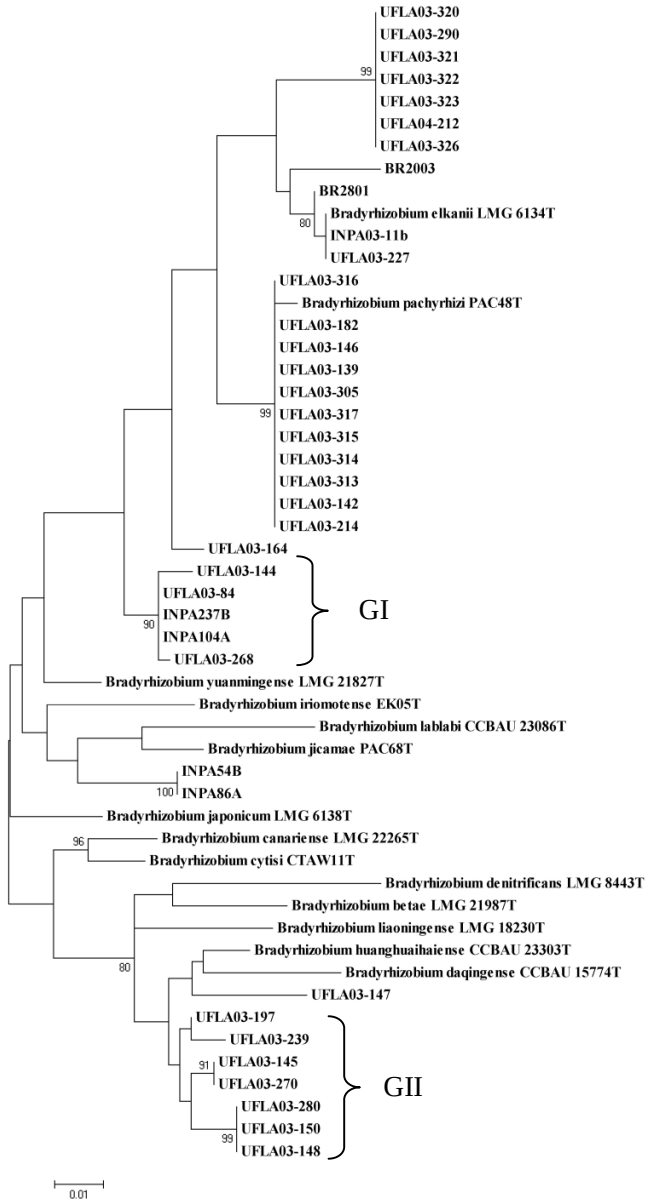

Figure 3 Phylogenetic relationships of 38 *Bradyrhizobium* strains based on the *atpD* gene. Phylogeny was determined by the maximum likelihood method. Bootstrap values were based on 1,000 trials. Phylogenetic analyses were conducted in Mega5

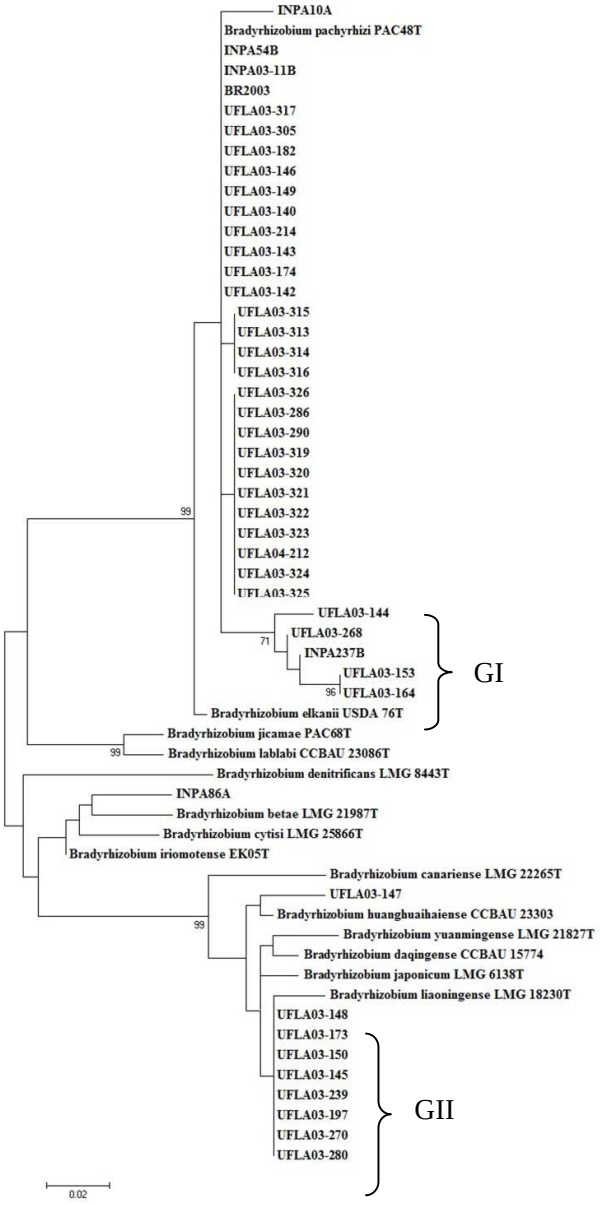

Figure 4 Phylogenetic relationships of 44 *Bradyrhizobium* strains based on the *dnaK* gene. Phylogeny was determined by the maximum likelihood method. Bootstrap values were based on 1,000 trials. Phylogenetic analyses were conducted in Mega5

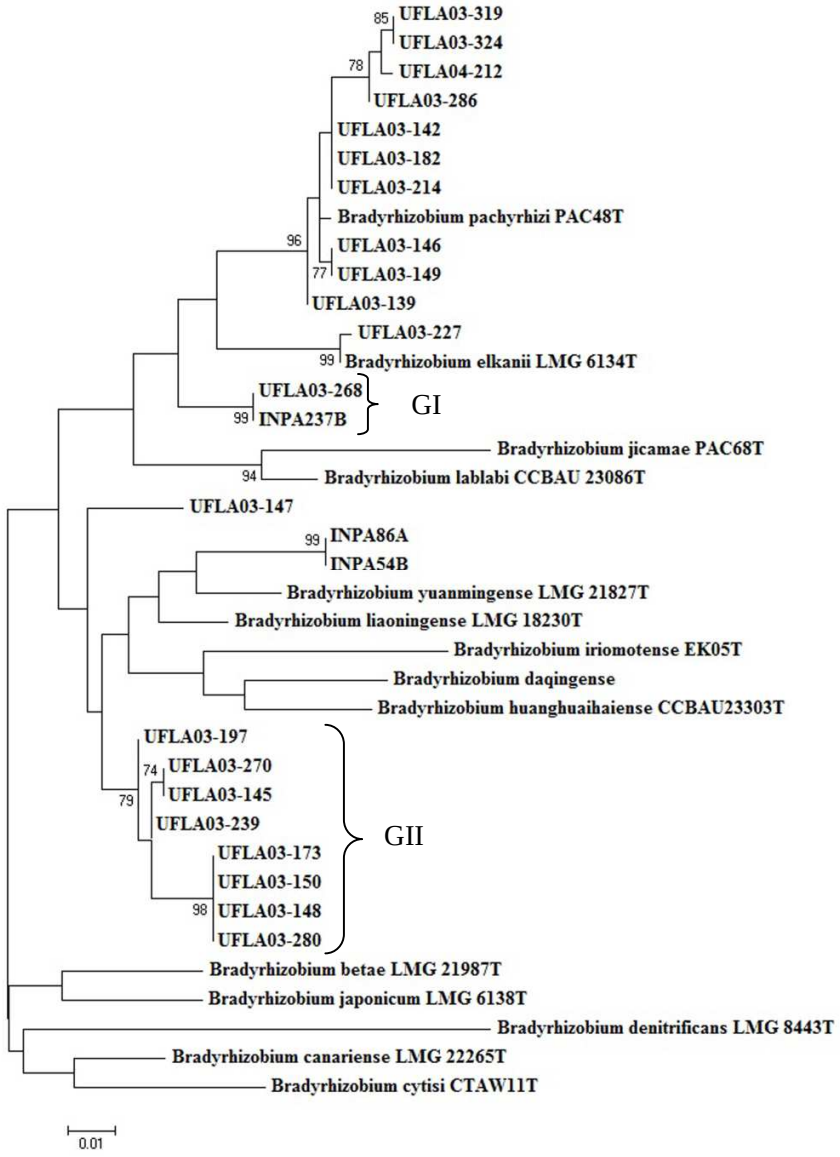

Figure 5 Phylogenetic relationships of 24 *Bradyrhizobium* strains based on the *rec*A gene. Phylogeny was determined by the maximum likelihood method. Bootstrap values were based on 1.000 trials. Phylogenetic analyses were conducted in Mega5

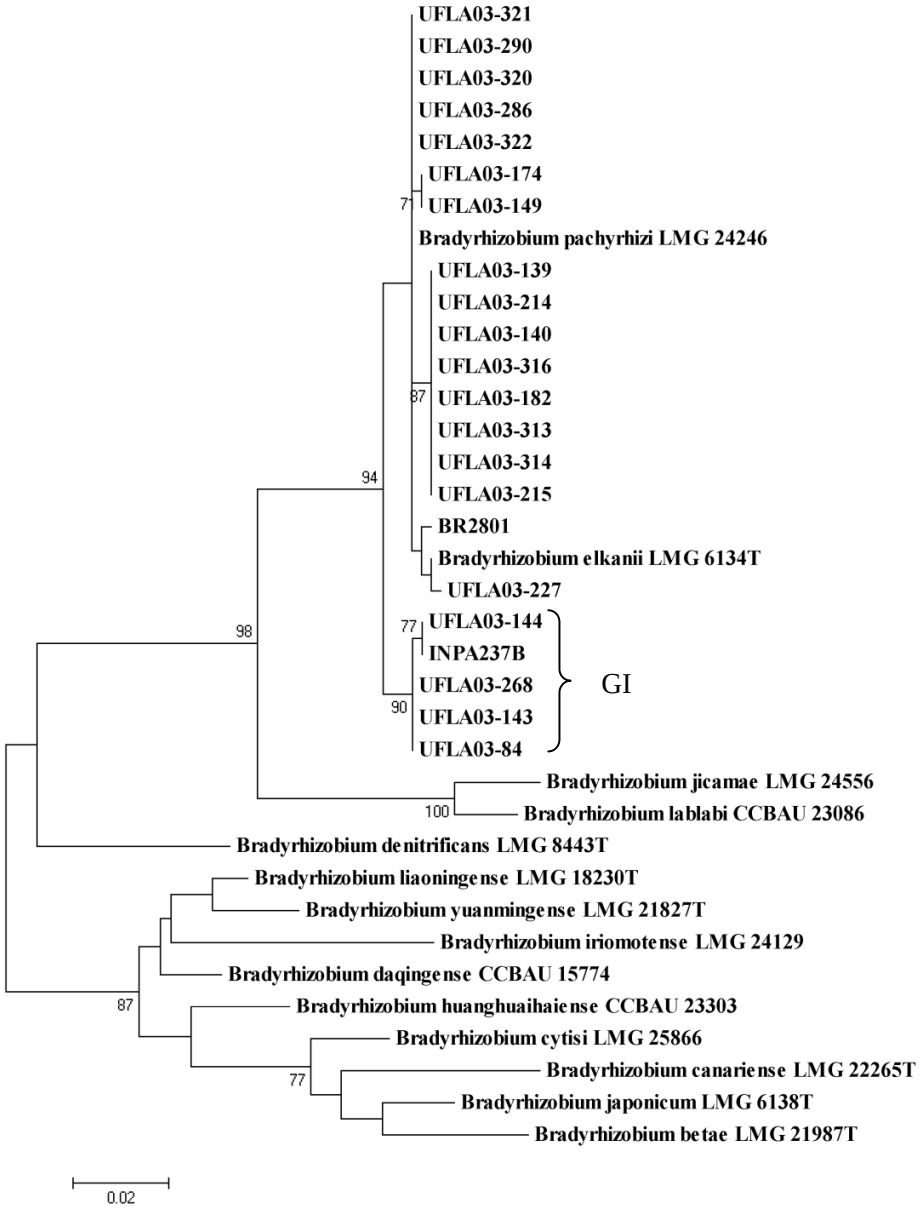

Figure 6 Phylogenetic relationships of 22 *Bradyrhizobium* strains based on the *rpoB* gene. Phylogeny was determined by the maximum likelihood method. Bootstrap values were based on 1,000 trials. Phylogenetic analyses were conducted in Mega5

4. Discussion

4.1. Symbiotic characteristics

We chose to use soybean as the host plant in the symbiosis test because it is the most common plant that is used in the nodulation tests that have been performed in previous works that have described new species of *Bradyrhizobium* (Jordan, 1982; Kuykendall et al., 1993; Xu et al., 1995; Yao et al., 2002; Rivas et al., 2004; Vinuesa et al., 2005a; van Berkum et al., 2006; Ramirez-Bahema et al., 2009; Chahboune, et al., 2011; Chang et al., 2011; Wang et al., 2012; Zhang et al.,2012*;* Ramirez-Bahema et al., 2012); the frequent use of this plant is most likely due to its economic interest. The authors of the abovementioned studies have shown that most of the species described to date do not nodulate soybean. Of the 50 strains that were tested in this study, we found that 11 were able to nodulate soybean, including INPA03-11b, which exhibited 100% sequence similarity with the *B. elkanii* strain in the analysis of its *atp*D gene. The results of the present study are consistent with those of Kuykendall et al. (1993), who isolated *B. elkanii* from soybean nodules.

4.2. Phenotypic characterization

We performed the characterisation of the strains by assessing their sensitivity to 10 different antibiotics. The INPA03-11b strain was resistant to all antibiotics except to KAN, it is in contradiction with the results obtained by Florentino et al. (2010). The UFLA03-84 strain proved to be extremely tolerant to different types of antibiotics because it was not sensitive to any of the antibiotics tested; this finding corroborates the results obtained by Florentino et al. (2010). The latter authors tested 15 different antibiotics, 10 of which were different from those tested in the present study. The controls gave different results to those in the literature (Kuykendall et al., 1993; Vinuesa et al., 2005a), most likely due to differences in the antibiotic concentrations that were used. In

general, we found that most strains showed resistant to most of the antibiotics that occur in soil, which is a desirable trait for nitrogen-fixing LNBs, as they need to compete with and overcome antagonism from other soil microorganisms. The diversity in the antibiotic resistance that was found in the present study is not related to genetic diversity.

The assessment of the tolerance of the strains to salinity showed that the *B. elkanii* strain was able to grow in 0,75% mM NaCl and that the *B. canariense* and *B. japonicum* strains were able to grow in 0,75% NaCl, which corroborates the results obtained by Islam et al. (2008). The strains that are recommended as cowpea inoculants (UFLA03-84 and INPA03-11b) tolerated salinity levels of up to 0,75% NaCl, which complements the results reported by Florentino et al. (2010). The latter authors found that these strains grew in 0,75% NaCl but not in 1% NaCl; however, they did not test the intermediate concentrations.

4.3. Genetic characterization

Phylogenetic analysis of the parcial sequences of the 16S rRNA genes of 41 *Bradyrhizobium* strains revealed that the sequencing of only this gene does not allow for precise differentiation and identification of the strains, and results in the grouping of the tested strains with previously described species. This finding corroborates the results found in the literature (Chang et al., 2011; Ramírez-Bahena et al., 2012; Willems et al., 2001). Thus, the phylogenetic analysis of housekeeping genes has been suggested by several authors as a tool to characterise and identify species belonging to genus *Bradyrhizobium* (Gaunt et al., 2001; Vinuesa et al., 2005b; Rivas et al., 2009). For example, it is evident that even the previously described species *Bradyrhizobium jicamae*, *B. pachyrhizi*, *B. elkanii*, *B. iriomotense*, *B. denitrificans*, and *B. huanghuaihaiense* are in the same 16S rRNA group, although phylogenetic analyses of the

housekeeping genes of these species indicates that they belong to separate groups.

The selection of the five housekeeping genes was based on the study performed by Rivas et al. (2009) and was aimed at characterising genetically 50 strains of *Bradyrhizobium*. The phylogenetic analyses were conducted individually for each gene. These different analyses showed greater differentiation between the strains compared with the analysis of the 16S rRNA gene partial sequences, and resulted in the formation of different groups of *Bradyrhizobium* strains, which corroborates the results found in the literature (Vinuesa et al., 2005a, b; Ramírez-Bahena et al., 2009 and 2012; Rivas et al., 2009; Chahboune et al., 2011; Fonseca et al., 2012).

The results from the analyses of the housekeeping genes clearly show the probability of finding novel species of *Bradyrhizobium* in the Brazilian Amazon region; however, DNA:DNA hybridization analyses must be performed in order to define any new species.

The strains selected for the present study were isolated from four different regions and from four different land-use systems. Nevertheless, no relationship was found between the groups identified through the phylogenetic analysis and the region of origin and/or the land-use system from which the strains were isolated. However, we did find that certain strains that were isolated in the same location and from the same plant but from different nodules were found to be in the same phylogenetic group. This observation was made for the strains UFLA03-313, UFLA03-314, and UFLA03-315, which exhibited 100% similarity in the phylogenetic analysis of their *gyr*B, *dna*K, *atp*D, and *rpo*B genes.

In conclusion, we found that some of the strains that were studied were able to nodulate soybean. In addition, *Bradyrhizobium* strains have a high ability to overcome antagonism from other soil microorganisms and are able to grow on

soils that are considered saline. The individual phylogenetic analysis of the *gyr*B, *dna*K, *atp*D, *rec*A, and *rpo*B housekeeping genes showed that many of the strains isolated in the Brazilian Amazon potentially represent new species.

Acknowledgements

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Supplementary material

Table S1. Salinity tolerance: (+) Growth;(-) No growth.

	Strain	NaCl (%)				
		0,01	**0,25**	**0,5**	**0,75**	**1**
1	UFLA03-173	+	+	+	-	-
2	UFLA03-197	+	+	+	-	-
3	UFLA03-270	+	+	+	-	-
4	UFLA03-148	+	+	+	-	-
5	UFLA03-146	+	+	+	+	+
6	UFLA03-174	+	+	+	+	-
7	UFLA03-280	+	+	+	-	-
8	UFLA03-150	+	+	+	-	-
9	UFLA03-139	+	+	+	+	-
10	UFLA03-268	+	+	+	-	-
11	UFLA03-144	+	+	+	+	-
12	UFLA03-305	+	+	+	-	-
13	UFLA03-147	+	+	+	-	-
14	UFLA03-145	+	+	+	+	+
15	UFLA03-214	+	+	+	+	-
16	UFLA03-315	+	+	+	+	-
17	UFLA03-316	+	+	+	+	-
18	UFLA03-290	+	+	+	-	-
19	UFLA03-319	+	+	+	+	-
20	UFLA03-322	+	+	+	+	-
21	UFLA03-323	+	+	+	+	-

Table S1, continue

	Strain	NaCl (%)				
		0,01	0,25	0,5	0,75	1
22	UFLA03-324	+	+	+	+	-
23	UFLA03-326	+	+	+	+	-
24	UFLA03-286	+	+	+	-	-
25	UFLA03-149	+	+	+	+	-
26	UFLA03-143	+	+	+	+	+
27	UFLA03-153	+	+	+	+	-
28	UFLA03-164	+	+	+	-	-
29	UFLA03-313	+	+	+	-	-
30	UFLA03-314	+	+	+	+	-
31	UFLA03-227	+	+	+	-	-
32	UFLA03-239	+	+	+	-	-
33	UFLA03-320	+	+	+	+	-
34	UFLA03-321	+	+	+	+	-
35	UFLA03-325	+	+	+	+	-
36	UFLA04-212	+	+	+	+	-
37	UFLA03-318	+	+	+	+	-
38	UFLA03-182	+	+	+	+	-
39	UFLA03-142	+	+	+	+	+
40	UFLA03-140	+	+	+	+	-
41	UFLA03-317	+	+	+	+	-
42	INPA 10A	+	+	+	+	-
43	INPA 54B	+	+	+	+	-
44	INPA 86A	+	+	+	-	-

Table S1, conclusion

	Strain	NaCl (%)				
		0,01	**0,25**	**0,5**	**0,75**	**1**
45	INPA 104A	+	+	+	+	-
46	INPA 237B	+	+	+	+	-
47	INPA 03-11b	+	+	+	+	-
48	UFLA 03-84	+	+	+	+	-
49	BR2801	+	+	+	+	-
50	BR2003	+	+	+	+	-
51	CNPAB-29W	+	+	+	+	-
52	LMG 22265^{T}	+	+	+	-	-
53	LMG 6138^{T}	+	+	+	-	-
54	LMG 6134^{T}	+	+	+	+	-

PAPER 2

Bradyrhizobium amazonense sp. nov. isolated from soils of the Western Amazon

Normas da revista: Systematic and Applied Microbiology (Versão preliminar)

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Running title: *Bradyrhizobium amazonense* sp. nov.

Abstract:

Five strains (UFLA03-150^{T}, UFLA03-197, UFLA03-270, UFLA03-148, and UFLA03-280) isolated from the soils of the western Amazon, Brazil, were analyzed using genotypic, phenotypic, and symbiotic methods. Phylogenetic analysis of partial sequences in the 16S rRNA gene had previously identified the five strains as belonging to the *Bradyrhizobium* genus. Phylogenetic analysis of concatenated sequences of the housekeeping genes *atpD*, *recA*, *dnaK*, and *gyrB* previously revealed that these five were distinct from described *Bradyrhizobium* species, with *B. liaoningense* being the closest to the group with 96.12% similarity. Species delimitation was assessed using the DNA:DNA hybridization, which showed a result below 70% with other *Bradyrhizobium* species. Strains from that group grew at the following pH values: 4, 5, 6, 6.8, 8, and 9, at temperatures of 15°C–28°C, and 0.5% NaCl salinity; their resistance to antibiotics varied. These strains can not solubilize inorganic phosphates, do not nodulate soybean plants, but do nodulate cowpea. On the basis of the genetic, phenotypic, and symbiotic traits demonstrated in this study, we propose that these strains are included in a new species named *Bradyrhizobium amazonense* sp. nov. with UFLA03-150^{T} as the type strain of the species.

To date, species of the genus *Bradyrhizobium*, have been described from several different geographic origins: three were isolated in Japan [*B. japonicum* (Jordan, 1982), *B. iriomotense* (Islam et al., 2008), and *B. oligotrophicum* (Ramirez-Bahema et al., 2012)]; five in China [*B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), *B. lablabi* (Chang et al., 2011), *B. daqingense* (Wang et al., 2012), and *B. huanghuaihaiense* (Zhang et al., 2012)]; two in Morocco [*B. cytisi* (Chahboune, et al., 2011) and *B. rifense* (Chahboune, et al., 2012)]; and one in each of the following countries: United States, Spain, Canary Islands, Honduras, and Costa Rica, *B. elkanii* (Kuykendall et al., 1993), *B. betae* (Rivas et al., 2004), *B. canariense* (Vinuesa et al., 2005a), *B. jicamae*, and *B. pachyrhizi* (Ramirez-Bahema et al., 2009), respectively.

The territorial extension of the Amazon rainforest is approximately 5.5 million km^2, with 4.2 million km^2 belonging to Brazil; thus, the Amazon rainforest represents nearly 50% of the national territory of Brazil (www.ibge.gov.br). Considered to be the largest on the planet, the Amazon rainforest shows high biodiversity both above and below ground.

Several studies have revealed a high diversity of legume-nodulating N_2-fixing bacteria in soils of six land use systems (LUS) of the western Amazon region: agricultural forest, pasture, primary forest, agricultural, and secondary forest in the advanced stage of regeneration, and primary forest in the initial stage of regeneration (Lima et al., 2005, 2009; Jesus et al, 2005; Moreira et al., 1993, 1998; Jaramillo et al., submitted; Guimarães et al., 2012).

Recent studies have shown that most strains isolated in the agricultural and agricultural forest LUS, using cowpea as a trap plant, were identified as belonging to *Bradyrhizobium*, following phylogenetic analysis of their 16S rRNA gene partial sequences (Jaramillo et al., submitted; Guimarães et al., 2012).

The aim of this study was to complete the characterization of five strains (UFLA03-197, UFLA03-270, UFLA03-148, UFLA03-150^T, and UFLA03-280) isolated from soils of the western Amazon region in order to verify their taxonomic positions. We demonstrated that these strains represent a new species, with the proposed name "*Bradyrhizobium amazonense* sp. nov.," and UFLA03-150^T was designated the type strain.

These five strains were collected in areas of agriculture and agricultural forests, and were inoculated onto cowpea plants that were grown under controlled conditions.

In previous studies, genetic characterization was performed by sequencing of the 16S rRNA gene (Jaramillo et al., submitted; Guimarães et al., 2012) and the housekeeping genes *dnaK, atpD, recA,* and *gyrB* (Guimarães et al., submitted).

The phylogenetic trees based on 16S rRNA partial sequences that were constructed using the ML (maximum likelihood) (Fig. 1) and NJ (neighbor joining) (Fig. 2) methods were similar in the groups. The 16S rRNA gene partial sequences of the type strain UFLA03-150^T and of strains UFLA03-148, UFLA03-270, and UFLA03-280 were identical to each other and showed 99.41% similarity to strain UFLA03-197. Among the *Bradyrhizobium* species described to date, only *B. liaoningense* displayed 100% similarity to strains from the group containing the proposed new species. With regard to the other species of *Bradyrhizobium*, the similarity varied between 98.8% and 99.85%. This high similarity was also observed among the previously described group of strains, including *B. huanghuaihaiense* and *B. iriomotense* (99.7% similarity) (Zhang et al., 2012). The type strains of *B. pachyrhizi* and *B. jicamae* showed 99.8% and 99.4% similarity, respectively, to *B. elkanii*. The difficulty in identifying and classifying *Bradyrhizobium* species by only analyzing partial sequences of the 16S rRNA gene, due to the high degree of conservation between species, has

been noted by several authors (Willems et al., 2001b; Vinuesa et al., 2005a, 2005b, 2008). Therefore, other housekeeping genes have been used owing to their higher discriminating power of *Bradyrhizobium* species.

The phylogenetic trees constructed from concatenated partial sequences of *atpD, recA, dnaK,* and *gyrB* genes by using the ML (Fig 3) and NJ (Fig 4) methods generated similar groups. The strains of *B. amazonense* formed a different group from all other species of the genus. The type strain of *B. amazonense* UFLA03-150^{T} was 100% similar to UFLA03-280 and UFLA03-148, and it showed 99.05% and 98.86% similarity with strains UFLA03-270 and UFLA03-197, respectively. As in the phylogenetic analysis of the 16S rRNA gene, the type strain of the *B. liaoningense* species showed the highest similarity (96.12%) when compared to the type strain of the *B. amazonense* species. The type strain of *B. japonicum* with 94.60% similarity to UFLA03-150^{T} was selected, along with the type strain of *B. liaoningense*, for comparison in the DNA:DNA hybridization test. This analysis confirmed that the strains under study form an isolated group relative to currently described *Bradyrhizobium* species. Thus, analysis of multiple housekeeping gene sequences strongly contributes to species delimitation, and together with the DNA:DNA hybridization test, corroborates previous studies (Vinuesa et al., 2005a; Zhang et al., 2012; Chahboune et al., 2011, 2012)

The DNA-DNA hybridizations were performed for species delimitation purposes (Wayne et al., 1987) among the five strains (UFLA03-150^{T}, UFLA03-197, UFLA03-270, UFLA03-148, and UFLA03-280), as well as with the type strains of *B. liaoningense* and *B. japonicum*. Hybridizations were performaded using a microplate method and following the protocol described previously (Willems et al., 2001b). The DNA:DNA similarity among the UFLA03-150^{T}, UFLA03-197, UFLA03-270, UFLA03-148, and UFLA03-280 strains varied between 72.7% and 84.8%, indicating that they belong to the same species. The

similarity of UFLA03-150^{T} with the type strains of *B. liaoningense* and *B. japonicum* was 39.8% and 42.3%, respectively; these values are below the limit (70% of DNA:DNA similarity) proposed for delimitation of a species (Wayne et al., 1987).

Regarding phenotypic characterization, the strains were previously evaluated for their tolerance to salinity in 79 medium (Fred & Waksman, 1928) containing different concentrations of NaCl (0,01%, 0.25%, 0.5%, 0.75%, and 1% [w/v]). Resistance of the strains to 10 different antibiotics at the following concentrations ($\mu g.ml^{-1}$) was also verified: ampicillin (10), cefuroxime (30), ciprofloxacin (5), chloramphenicol (30), doxycycline (30), erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), and penicillin G (10) (Guimarães et al., submitted). In this study, all five strains were tested for their ability to grow at different temperatures and pH values. As such, the strains were inoculated in 79 medium and incubated at 4°C, 15°C, 28°C, 37°C, and 45°C, and inoculated in the same medium at pH values adjusted to 4, 5, 6, 6.8, 8, and 9. Type strains of the *Bradyrhizobium japonicum*, *B. elkanii*, and *B. canariense* were included in all tests for comparison. The five strains grew in 0.5% NaCl at all tested pH values, rendering the growth medium alkaline, and under temperatures of 15°C and 28°C, with 28°C being the optimal growth temperature for these strains. Strain UFLA03-150^{T} was resistant to the following antibiotics: doxycycline, neomycin, ciprofloxacin, penicillin G, gentamicin, erythromycin, and chloramphenicol. The strain was not resistant to ampicillin, cefuroxime, doxycycline, and kanamycin. All other strains showed variable resistance to the antibiotics.

As an additional test were performed to verify their ability to solubilize inorganic phosphate in the forms of calcium phosphate, aluminum phosphate, and iron phosphate. The NBRIP medium was used (Nautiyal, 1999) for calcium phosphate, GES medium (Sylvestes-Bradley, 1982) for aluminum phosphate,

and GELP medium (Sylvestes-Bradley, 1982) for iron phosphate. The strains were inoculated in 79 medium until isolated colonies were obtained, which were suspended in a tube containing 20 mL sterile saline (0.85% NaCl), until turbidity equivalent to Nº 2 on the McFarland scale (6×10^8 cells) was reached. Twenty microliters of the cell suspensions were inoculated at 4 equidistant points on the plates containing the different growth media, with 8 replicate plates. The plates were incubated for 15 days, with the solubilization halo determined every three days via a digital pachymeter. The solubilization indexes (SI) of the strains were obtained and classified according to their solubilization ability: low (SI < 2 mm), medium (2 < SI < 4 mm) and high (SI > 4 mm) (Berraquero et al., 1976). In this test, the type strains of the *B. japonicum* LMG6138^T, *B. elkanii* LMG 6134^T, and *B. canariense* LMG 22265^T were also included. The type strain of *B. elkanii* LMG6134^T was the only strain that could solubilize calcium phosphate. As for aluminum and iron phosphates, they were not solubilized by any of the tested strains (Table 1).

In previous studies of their symbiotic ability, all five strains were found to be able to form nodules on cowpeas, but only strains UFLA03-197 and UFLA03-150^T were efficient in their biological N_2 fixation ability (Jaramillo et al., submitted; Guimarães et al., 2012). Guimarães et al. (submitted) observed that none of the strains under study showed the ability to establish a symbiosis with soybean (*Glycine max*).

Comparisons of the phenotypic and symbiotic characteristics of the type strains of *B. amazonense*, *B. japonicum*, *B. elkanii*, *B. canariense*, and *B. liaoningense* and their ability to solubilize inorganic phosphates are shown in Table 1.

On the basis of the genetic, phenotypic, and symbiotic characteristics demonstrated in this study, we propose that strain UFLA03-150^T, isolated in the west Amazon region, should be included as a new species named

Bradyrhizobium amazonense sp. nov., with the strains UFLA03-197, UFLA03-270, UFLA03-148, and UFLA03-280 belonging to the same species.

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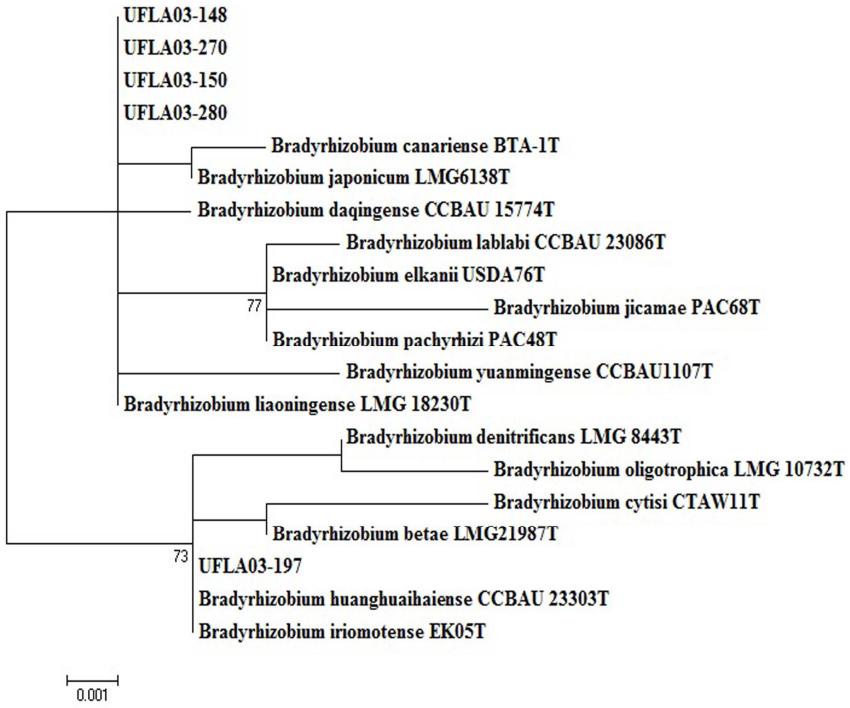

Figure 1 ML phylogenetic tree based on partial sequences of the 16S rRNA gene of *B. amazonense* strains, the type strains of the remaining species of genus *Bradyrhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets

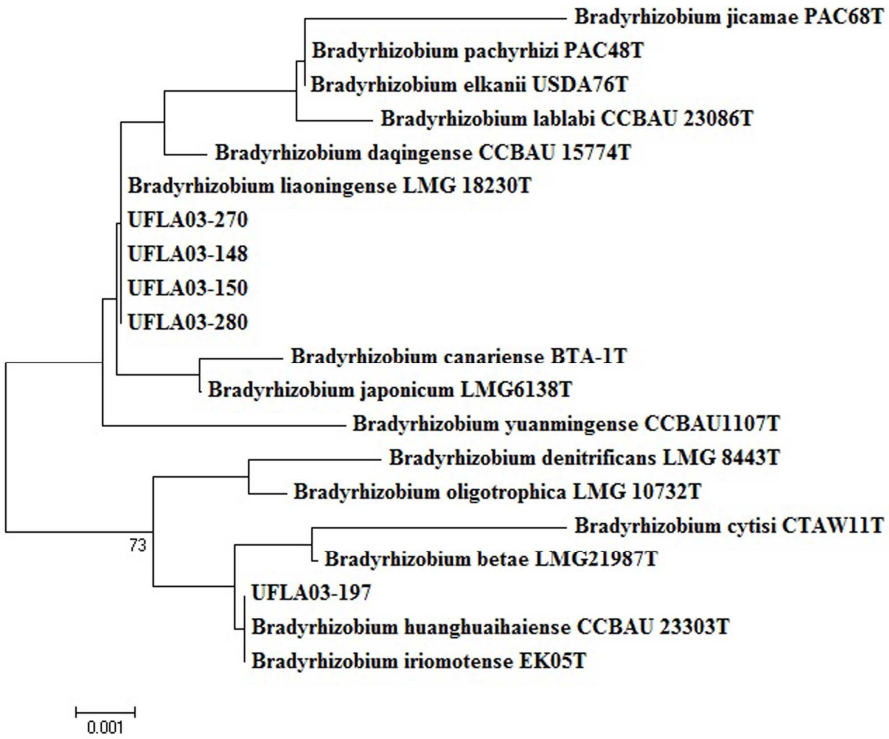

Figure 2 NJ phylogenetic tree based on partial sequences of the16S rRNA gene of *B. amazonense* strains, the type strains of the remaining species of genus *Bradyrhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets

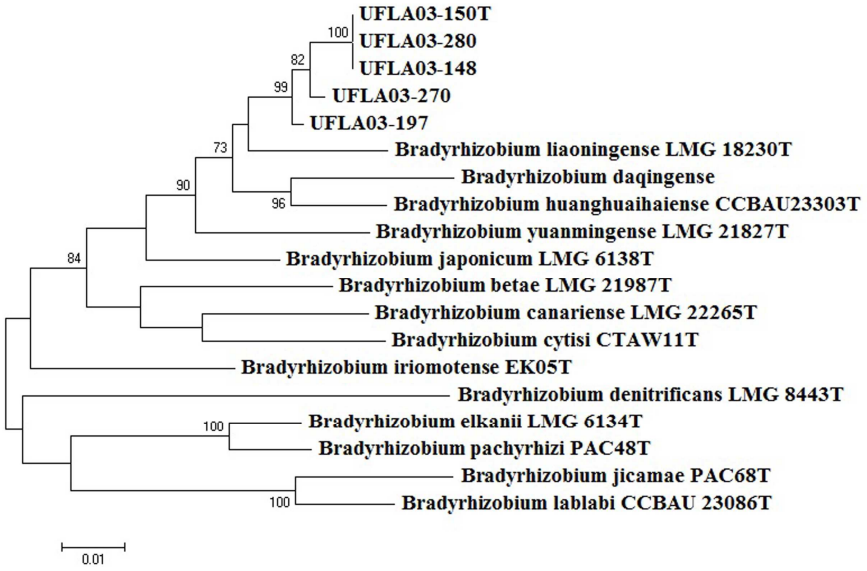

Figure 3 ML phylogenetic tree based on partial concatenated sequences of the *atpD*, *dnaK*, *gyrB*, and *recA* genes of *B. amazonense* strains, the type strains of the remaining species of genus *Bradyrhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets

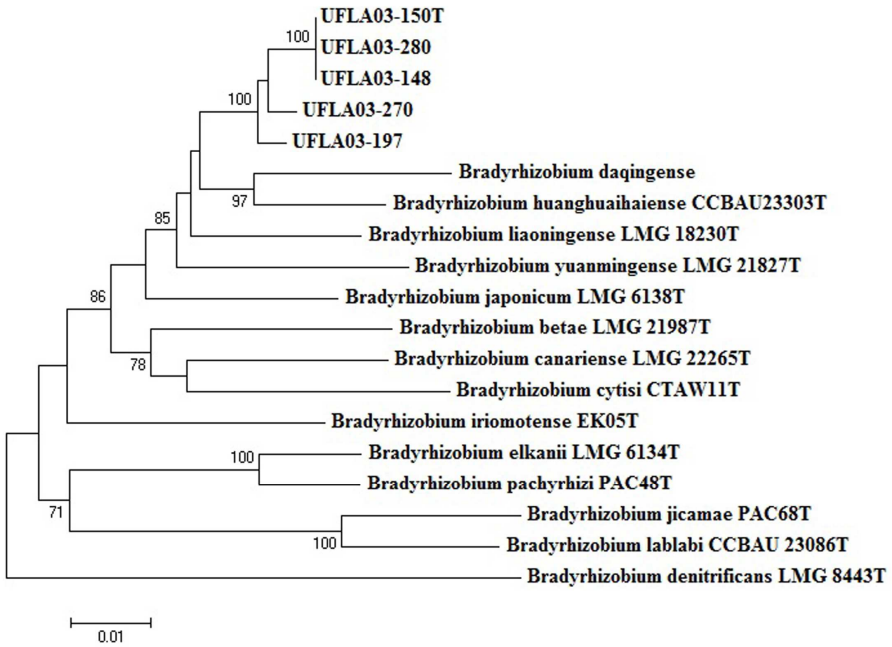

Figure 4 NJ phylogenetic tree based on partial concatenated sequences of the *atpD, dnaK, gyrB,* and *recA* genes of *B. amazonense* strains, the type strains of the remaining species of genus *Bradyrhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets

Table 1 Phosphate solubilization, phenotypic and symbiotic features of *Bradyrhizobium amazonense* and reference of *B. japonicum*, *B. elkanii*, *B. canariense*, and *B. lioningense*

1, *B. amazonense* UFLA03-150^{T}; 2, *B. japonicum* LMG6138^{T}; 3, *B. elkanii* LMG6134^{T}; 4, *B. liaoningense* LMG 18230^{T}; 5, *B. canariense* LMG 22265^{T}. NR: No Reported. ND: Not Done. GNS: Grew and did not solubilize. NG: No Grow. LS: Low Solubilization (solubilization index < 2)

Features	**1** [a, b]	**2** [a, b]	**3** [a, b]	**4**[c]	**5** [a, b]
pH	4/9	4/9	4/9	5/9	4/9
NaCl (1%)	-	-	-	-	-
Temp. (°C)					
15ºC	+	+	+	NR	+
28ºC	+	+	+	+	+
37ºC	-	-	-	-	-
Resistance to antibiotics[b]					
Ampicillin (10 µg mL^{-1})	-	+	+	NR	+
Penicillin G (10 µg mL^{-1})	+	+	+	NR	+
Doxycycline (30 µg mL^{-1})	+	+	+	NR	+
Neomycin (30 µg mL^{-1})	+	-	-	+	-
Kanamycin (30 µg mL^{-1})	-	-	-	NR	-
Cefuroxime (30 µg mL^{-1})	-	-	+	NR	-
Erythromycin (15 µg mL^{-1})	+	+	+	NR	+
Chloramphenicol (30 µg mL^{-1})	+	+	+	+	+
Ciprofloxacin (5 µg mL^{-1})	+	+	+	NR	+
Gentamicin (10 µg mL^{-1})	+	+	-	+	-
Test of nodulation (Soybean)	-	+	+	+	-
Phosphate solubilization					
Calcium phosphate	GNS	GNS	LS	ND	GNS
Aluminum phosphate	NG	NG	NG	ND	NG
Iron phosphate	GNS	GNS	GNS	ND	GNS

[a]Results obtained in this work
[b] Guimarães et al.(submitted)
[c]Xu et al., 199