ELAINE MARTINS DA COSTA

NEW *BRADYRHIZOBIUM* SPECIES FROM SOILS OF DIFFERENT BRAZILIAN REGIONS: TAXONOMY AND SYMBIOTIC EFFICIENCY

LAVRAS – MG

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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 Doutora.

Orientadora

Dra. Fatima Maria de Souza Moreira

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Orientadora

LAVRAS - MG

2016

A Deus, que sempre ilumina o meu caminho, dando-me forças e sabedoria para superar grandes desafios.

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RESUMO GERAL

O gênero *Bradyrhizobium* representa um importante grupo de bactérias fixadoras de nitrogênio que nodulam leguminosas. Em ecossistemas brasileiros, esse gênero tem se destacado devido a sua predominância como microssimbionte eficiente de importantes espécies leguminosas de grãos, florestais e forrageiras, e por apresentar alta diversidade. O objetivo deste trabalho foi definir a posição taxonômica e avaliar a eficiência simbiótica de estirpes de *Bradyrhizobium* oriundas de solos de diferentes regiões brasileiras (Nordeste, Norte e Sudeste). As estirpes utilizadas nesse estudo são representantes de diferentes grupos filogenéticos do gênero *Bradyrhizobium*, indicados em estudos prévios, com base no sequenciamento de genes housekeeping. A caracterização fenotípica, incluindo testes de temperatura, pH, salinidade, resistência à antibióticos, assimilação de diferentes fontes de carbono e nitrogênio e a análise de MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry), permitiu diferenciar as estirpes de cada grupo das estirpes tipo das espécies de *Bradyrhizobium* filogeneticamente próximas. Os resultados das análises de hibridização DNA-DNA confirmaram a existência de quatro novas espécies, para as quais os nomes *Bradyrhizobium brasilense*, *Bradyrhizobium forestalis*, *Bradyrhizobium piauiense* e *Bradyrhizobium neoglycine* foram propostos, com as estirpes UFLA 03-321^{T}, INPA 54B^{T}, UFLA 06-13^{T} e UFLA 06-10^{T}, designadas como estirpes tipo, respectivamente. A avaliação da eficiência simbiótica foi realizada usando feijão-fava, mucuna-preta e acácia como espécies hospedeiras. As estirpes INPA 54B e INPA 86A se destacaram na produção de matéria seca da parte aérea (MSPA) de feijão-fava. As estirpes UFLA 03-144 e INPA 104A superaram o controle com alta concentração de nitrogênio mineral e a estirpe inoculante BR 2811 na produção de MSPA da mucuna-preta. A estirpe UFLA 03-268 foi a mais eficiente em simbiose com acácia, inclusive foi superior à estirpe inoculante BR 3617. Essas estirpes apresentam grande potencial para serem utilizadas como inoculantes nas respectivas espécies hospedeiras com as quais formaram simbiose eficiente. Os resultados apresentados nesse estudo ressaltam a alta diversidade fenotípica, genotípica e simbiótica de estirpes de *Bradyrhizobium* nativas de solos brasileiros.

Palavras-chave: Bactérias fixadoras de nitrogênio. Hibridização DNA-DNA. Taxonomia polifásica. MALDI-TOF MS. Simbioses.

GENERAL ABSTRACT

The *Bradyrhizobium* genus is an important group of nitrogen-fixing bacteria that nodulate legumes. In Brazilian ecosystems, this genus stands out because it predominates as efficient microsymbionts of important legumes, including grains, forest and forage species, and because it shows high diversity. The aim of this study was to determine the taxonomic position and evaluate the symbiotic efficiency of *Bradyrhizobium* strains from soils of different Brazilian regions (Northeast, North and Southeast). The strains used in this study are representatives from different phylogenetic groups of *Bradyrhizobium* genus, indicated in previous studies, based on sequencing of housekeeping genes. Phenotypic characterization, including tests for temperature, pH, salinity, resistance to antibiotics, assimilation of different carbon and nitrogen sources, and analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles allowed differentiating the strains of each group from type strains of *Bradyrhizobium* species phylogenetically close. The results of DNA-DNA hybridization analysis confirmed the existence of four new species, for which the names *Bradyrhizobium brasilense*, *Bradyrhizobium forestalis*, *Bradyrhizobium piauiense* and *Bradyrhizobium neoglycine* have been proposed, with the strains UFLA 03-321^{T}, INPA 54B^{T}, UFLA 06- 13^{T} and UFLA 06-10^{T} designated as type strains, respectively. The evaluation of symbiotic efficiency was performed using lima bean, velvet bean and acacia as host species. Strains INPA 54B and INPA 86A stood out in the production of shoot dry matter (SDM) of lima bean. Strains UFLA 03-144 and INPA 104A were more efficient than the control with high mineral N concentration and the inoculant strain BR 2811 in the production of SDM of velvet bean. Strain UFLA 03-268 was the most efficient in symbiosis with acacia, inclusive it was more efficient than the inoculant strain BR 3617. These strains exhibit potential for use as inoculants in their respective host species in which they have established efficient symbiosis. The results presented in this study emphasize the high phenotypic, genotypic and symbiotic diversity of native *Bradyrhizobium* strains from Brazilian soils.

Keywords: Nitrogen-fixing bacteria. DNA-DNA hybridization. Polyphasic taxonomy. MALDI-TOF MS. Symbiosis.

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PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

A fixação biológica de nitrogênio (FBN) constitui um dos principais processos para a manutenção da produtividade dos ecossistemas terrestres. Esse processo é mediado por bactérias capazes de converter o N_2 à forma inorgânica combinada (NH_3), e assim, torná-lo disponível às plantas e a outros organismos. Dentre os organismos diazotróficos, as bactérias fixadoras de nitrogênio que nodulam leguminosas (BFNNL) compreendem um grupo que apresenta grande contribuição na sustentabilidade agrícola e ambiental, e têm sido bastante investigadas quanto à ocorrência em diferentes ambientes, à diversidade e à taxonomia.

Entre os gêneros de BFNNL atualmente descritos, o gênero *Bradyrhizobium* destaca-se visto a sua ampla distribuição geográfica e gama de hospedeiros, incluindo diversas espécies de leguminosas de importância socioeconômica e ambiental. Atualmente, existem 37 espécies de *Bradyrhizobium* descritas, as quais são oriundas de solos de diferentes regiões geográficas. Ressalta-se que 18 espécies foram descritas recentemente, entre os anos de 2014 e 2015, e cinco dessas espécies são oriundas de solos brasileiros: *B. viridifuturi*, *B. tropiciagri*, *B. manausense*, *B. ingae* e *B. neotropicale* (DELAMUTA et al., 2015; HELENE et al., 2015; SILVA et al., 2014a, 2014b; ZILLI et al., 2014). Esse elevado número de espécies descritas nos últimos anos deve-se, principalmente, à utilização de novas técnicas da biologia molecular, e também ao estudo de novas estirpes isoladas de diferentes espécies hospedeiras e regiões geográficas.

Em estudos de taxonomia bacteriana, o sequenciamento do gene 16S rRNA tem sido amplamente empregado. No entanto, a baixa divergência genética entre as sequências desse gene torna-o limitado para classificação taxonômica ao nível de espécie (VINUESA et al., 2005b; WILLEMS; COOPMAN; GILLIS, 2001a). Para o gênero *Bradyrhizobium*, diversos

estudos têm proposto, além do gene 16S rRNA, o sequenciamento de genes housekeeping, como *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* e *rpoB*, que permite uma melhor discriminação entre espécies intimamente relacionadas (DURÁN et al., 2014a, 2014b; GUIMARÃES et al., 2015; MENNA et al., 2009; RIBEIRO et al., 2015; RIVAS et al., 2009; VINUESA et al., 2005a). O sequenciamento desses genes tem permitido a seleção de estirpes potencialmente representativas de novas espécies para serem avaliadas através da análise de hibridização DNA-DNA, que é uma técnica usada como critério padrão na descrição de novas espécies (WAYNE et al., 1987), contudo é muito trabalhosa e apresenta alto custo.

Em ecossistemas brasileiros, o gênero *Bradyrhizobium* além de compor o microssimbionte mais abundante identificado em nódulos de leguminosas (GUIMARÃES et al., 2012, 2015; JARAMILLO et al., 2013; LIMA et al., 2009; MOREIRA, 1991; MOREIRA et al., 1993; MOREIRA; HAUKKA; YOUNG, 1998; PERRINEAU et al., 2011), forma simbiose eficiente com uma ampla gama de hospedeiros de importância agrícola, florestal e pastoril. No Brasil, a maioria das estirpes de BFNNL aprovadas pelo Ministério da Agricultura Pecuária e Abastecimento (MAPA) para inoculação em espécies leguminosas pertence ao gênero *Bradyrhizobium* (BRASIL, 2011).

Recentemente, estudos desenvolvidos pelo nosso grupo de trabalho demonstraram, através do sequenciamento de genes housekeeping (*atpD*, *dnaK*, *gyrB*, *recA* e *rpoB*), alta diversidade entre estirpes de *Bradyrhizobium* isoladas de nódulos de espécies leguminosas em solos de diferentes ecossistemas/regiões brasileiras, e indicaram novos grupos filogenéticos dentro desse gênero, que possivelmente representavam novas espécies (GUIMARÃES et al., 2015; RIBEIRO et al., 2015). No entanto, seriam necessários mais estudos para definir a posição taxonômica desses grupos.

Em estudos de descrição de novas espécies de *Bradyrhizobium*, além da caracterização genética e fenotípica, a caracterização simbiótica por meio de testes de nodulação em diferentes espécies leguminosas tem sido

amplamente empregada (CHANG et al., 2011; DURÁN et al., 2014a; VINUESA et al., 2005a, XU et al., 1995; YAO et al., 2002; ZHANG et al., 2012). Além disso, para utilização de espécies nativas de *Bradyrhizobium* como inoculantes em culturas agrícolas, forrageiras e/ou florestais exploradas no país são necessários testes de eficiência simbiótica.

O objetivo deste trabalho foi definir a posição taxonômica e avaliar a eficiência simbiótica de estirpes de *Bradyrhizobium* oriundas de solos de diferentes regiões brasileiras (Norte, Nordeste e Sudeste).

2 REFERENCIAL TEÓRICO

2.1 Bactérias fixadoras de nitrogênio nodulíferas em leguminosas

O nitrogênio (N) é um dos nutrientes de maior importância para manutenção e funcionamento dos ecossistemas. É um macronutriente requerido em grande quantidade pela maioria dos vegetais, pois atua como componente estrutural de macromoléculas, como proteínas, aminoácidos, ácidos nucléicos, entre outras. No ar atmosférico, o N é o elemento mais abundante (compreende cerca de 78%) e encontra-se na forma N_2. A transformação do N_2 a formas inorgânicas combinadas pode ser realizada através de três processos: fixação atmosférica, fixação industrial e fixação biológica (FBN). Apesar da contribuição dos processos industriais para o fornecimento de N aos sistemas agrícolas e florestais, a FBN é o processo que contribui com a maior parte do N fixado anualmente no planeta, cerca de 65% do total (MOREIRA; SIQUEIRA, 2006).

A FBN é mediada por um grupo restrito de procariotos que possuem o complexo enzimático nitrogenase, tornando-os capazes de quebrar a tripla ligação da molécula do N_2 para obter a forma inorgânica combinada NH_3 que pode, assim, tornar-se disponível às plantas e a outros organismos. Os organismos fixadores de N_2 apresentam alta diversidade morfológica, fisiológica, genética e filogenética, garantindo a ocorrência da FBN nos mais diversos tipos de ecossistemas. Eles podem ser de vida livre, formar associações e/ou simbioses com outros organismos. Dentre esses organismos, aqueles que formam simbiose com leguminosas, denominados de bactérias fixadoras de nitrogênio nodulíferas em leguminosas (BFNNL), destacam-se visto à ampla diversidade, distribuição geográfica e utilização das plantas dessa família e a maior eficiência do processo devido à formação de estruturas especializadas nas raízes e/ou no caule, chamadas nódulos (MOREIRA; SIQUEIRA, 2006).

As BFNNL têm sido investigadas extensivamente quanto à ocorrência em diversos ecossistemas cultivados e naturais, além da diversidade e taxonomia. Atualmente, são conhecidos 18 gêneros de BFNNL. Dentre esses, a maioria pertence à subclasse α-proteobactéria: *Rhizobium* (FRANK, 1889), *Bradyrhizobium* (JORDAN, 1982), *Azorhizobium* (DREYFUS; GARCIA; GILLIS, 1988), *Sinorhizobium* (*Ensifer*) (CHEN; YAN; LI, 1988), *Mesorhizobium* (JARVIS et al., 1997), *Allorhizobium* (DE LAJUDIE et al., 1998), *Methylobacterium* (SY et al., 2001), *Devosia* (RIVAS et al., 2002), *Ochrobactrum* (TRUJILLO et al., 2005), *Phyllobacterium* (VALVERDE et al., 2005), *Shinella* (LIN et al., 2008), *Agrobacterium* (COMMINGS et al., 2009), *Microvirga* (ARDLEY et al., 2012) e *Aminobacter* (MAYNAUD et al., 2012), *Neorhizobium* (MOUSAVI et al., 2014) e *Pararhizobium* (MOUSAVI et al., 2015). Apenas dois gêneros pertencem à subclasse β-proteobactéria: *Burkholderia* (MOULIN et al., 2001) e *Cupriavidus* (CHEN et al., 2001).

Espécies leguminosas e estirpes de BFNNL podem variar de altamente específicas até altamente promíscuas. Algumas espécies, como o siratro (*Macroptilium atropurpureum*) e o feijão-caupi (*Vigna unguiculata*), podem ser infectadas por diferentes gêneros de BFNNL (COSTA et al., 2013; GUIMARÃES et al., 2012; JARAMILLO et al., 2013; LIMA et al., 2009; MOREIRA, 2010); enquanto outras espécies, como a *Sesbania virgata*, são extremamente específicas (MOREIRA et al., 2006). A inoculação de espécies leguminosas com estirpes de BFNNL eficientes representa uma técnica alternativa indispensável para a sustentabilidade dos agroecossistemas, devido à economia no uso de fertilizantes nitrogenados industrializados, além da redução dos impactos ambientais decorrentes do manejo inadequado desses fertilizantes.

Adicionalmente ao processo de FBN, algumas estirpes de BFNNL podem também beneficiar o hospedeiro atuando em outros processos promotores do crescimento vegetal, tais como: produção de ácido-3-indol acético (AIA), produção de sideróforos e solubilização de fosfatos

inorgânicos insolúveis (CARSON et al., 1992; MARRA et al., 2011; COSTA et al., 2013; OLIVEIRA-LONGATTI et al., 2013; COSTA et al., 2015), entre outros.

2.2 O gênero *Bradyrhizobium*

O gênero *Bradyrhizobium* engloba bactérias que vivem em vida livre, em associação com plantas, e principalmente em simbiose com espécies leguminosas. Esse gênero apresenta alta diversidade fenotípica, genotípica e simbiótica e ampla distribuição geográfica, e é bastante versátil, incluindo representantes com capacidade para solubilizar fosfatos inorgânicos insolúveis (MARRA et al., 2011) e produzir substâncias promotoras do crescimento vegetal (OLIVEIRA-LONGATTI et al., 2013), embora a sua maior contribuição, relatada em diversos estudos, seja na aquisição de nitrogênio para as plantas através da simbiose com espécies leguminosas. A origem de *Bradyrhizobium* ainda não está esclarecida, mas foi sugerido que esse gênero, provavelmente, é originário dos trópicos (NORRIS, 1965), onde há uma grande diversidade de espécies leguminosas.

Na primeira classificação de BFNNL, todas as estirpes foram agrupadas no gênero *Rhizobium* (FRANK, 1889) e a taxonomia das espécies tinham como base, principalmente, a especificidade das estirpes com as espécies leguminosas hospedeiras que fossem capazes de formar nódulos e fixar nitrogênio. Posteriormente, esse critério deixou de ser relevante, pois foi verificada a promiscuidade simbiótica entre estirpes de BFNNL, e entre espécies leguminosas.

Com base em características morfológicas e fisiológicas, foi proposto por Jordan (1982) que estirpes, até então agrupadas no gênero *Rhizobium*, de crescimento lento e com capacidade de alcalinizar o meio de cultura 79 (FRED; WAKSMAN, 1928), utilizando manitol como fonte de carbono, deveria formar um novo gênero, *Bradyrhizobium*, o qual difere do gênero *Rhizobium*, formado por bactérias de crescimento rápido e que acidificam no meio de cultura 79. Assim a espécie *Rhizobium japonicum*,

simbionte da soja, foi reclassificada como *Bradyrhizobium japonicum*. Posteriormente, análises fenotípicas e genotípicas detectaram alta diversidade entre estirpes de *Bradyrhizobium japonicum*, identificando-se a segunda espécie, *Bradyrhizobium elkanii* (KUYKENDALL et al., 1992). A terceira espécie, *Bradyrhizobium liaoningense*, também microssimbionte da soja, foi descrita por Xu et al. (1995). Desde então, o estudo de estirpes de diferentes regiões geográficas e espécies hospedeiras e os constantes avanços nas técnicas de biologia molecular têm permitido a reclassificação e identificação de novas espécies de *Bradyrhizobium.* Atualmente, o gênero possui 37 espécies descritas, com representantes de diferentes regiões geográficas, plantas hospedeiras e capacidade simbiótica (Tabela 1), mas certamente novas espécies serão descritas nos próximos anos, considerando-se o grande interesse pelo estudo desse gênero em várias regiões do mundo.

2.3 Métodos empregados na caracterização fenotípica e genotípica de estirpes do gênero *Bradyrhizobium*

Vários métodos são aplicados no estudo da diversidade e taxonomia bacteriana. Segundo recomendação do Comitê Internacional para Sistemática de Procariotos (International Committee for the Systematics of Prokaryotes), para definição da posição taxonômica de bactérias é necessário realizar tanto a caracterização genotípica, quanto fenotípica, para obter uma descrição precisa da nova espécie (WAYNE et al., 1987; GRAHAM et al., 1991).

Os métodos fenotípicos compreendem caracterização cultural, morfológica, fisiológica e bioquímica. Dentre os métodos fenotípicos estão também as técnicas quimiotaxonômicas, que são aquelas que permitem a geração de informações sobre vários constituintes da célula, através de métodos analíticos (VANDAMME et al., 1996). No estudo de BFNNL, a caracterização fenotípica, com base em características morfofisiológicas, tem

permitido o agrupamento de um grande número de estirpes para posterior seleção de representantes que serão analisados quanto a outras características fenotípicas mais complexas e/ou quanto à diversidade genética e posicionamento filogenético (GUIMARÃES et al., 2012, 2015; JARAMILLO et al., 2013; LIMA et al., 2009; MOREIRA, 1991; MOREIRA et al., 1993; MOREIRA; HAUKKA; YOUNG, 1998; SILVA et al., 2012; SILVA et al., 2014a).

Tabela 1 Origem geográfica, hospedeiro de origem e testes de nodulação na espécie de origem e/ou em outras espécies leguminosas hospedeiras

Espécie/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. japonicum LMG 6138^T	Japão	*Glycine max*	*Glycine* sp. e *Macroptiliurn Atropurpureum*	ND	Jordan (1982)
B. elkanii LMG 6134^T	Estados Unidos (Maryland)	*Glycine max*	*Glycine* sp.	ND	Kuykendall et al. (1992)
B. liaoningense LMG 18230^T	China(Província Heilongjiang)	*Glycine max*	*Glycine max*	*Pisurn sativum, Lotus* sp., *Astragalus sinicus* e *Melilotus* sp.	Xu et al. (1995)
B. yuanmingense LMG 21827^T	China (Província Beijing)	*Lespedeza cuneata*	*Lespedeza* sp., *Vigna unguiculata* e *Glycyrrhiza uralensis*	*Glycine max, Phaseolus vulgaris, Pisum sativum, Galega officinalis, Trifolium repens* e *Leucaena leucocephala*	Yao et al. (2002)
B. betae LMG 21987^T	Espanha	*Beta vulgaris*	ND	*Glycine max* e *Pachyrrihizus ahipa*	Rivas et al. (2004)
B. canariense LMG 22265^T	Espanha (Ilha das Canárias)	*Chamaecytisus proliferus*	*Lupinus* spp., *Adenocarpus* spp., *Chamaecytisus proliferus, Spatocytisus supranubius, Teline* spp. e *Ornithopus* spp.	*Glycine max* e *Glycine soja*	Vinuesa et al. (2005a)
B. denitrificans LMG 8443^T	Alemanha	*	*Aeschynomene indica*	ND	Van Berkum et al. (2006)
B. iriomotense EK05^T	Japão (Ilha Iriomote)	*Entada koshunensis*	*Macroptilium Atropurpureum*	ND	Islam et al. (2008)

"Tabela 1, continua"

Espécies/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. jicamae PAC68^T	Honduras (Copan)	*Pachyrhizus erosus*	*Pachyrhizus erosus* e *Lespedeza* sp.	*Glycine Max*	Ramirez-Bahena et al. (2009)
B. pachyrhizi PAC48^T	Costa Rica (Guanacaste)	*Pachyrhizus erosus*	*Pachyrhizus erosus*	*Glycine max* e *Lespedeza* sp.	Ramírez-Bahena et al. (2009)
B. lablabi CCBAU 23086^T	China (Provincia Anhui)	*Lablab purpureus*	*Lablab purpureus, Arachis hypogaea* e *Vigna unguiculata*	*Glycine max, Trifolium repens, Lotus corniculatus, Vigna radiata, Pisum sativum* e *Medicago sativa*	Chang et al. (2011)
B. cytisi CTAW11^T	Moroccos (Montanhas de Rif)	*Cytisus villosus*	*Cytisus villosus*	*Glycine max*	Chahboune et al. (2011)
B. huanghuaihaiense CCBAU23303^T	China (Província Huang-Huai-Hai)	*Glycine max*	*Glycine max, Glycine soja e Vigna unguiculata*	*Lotus corniculatus, Trifolium repens, Medicago sativa, Pisum sativum, Phaseolus vulgaris, Leucaena leucocephala* e *Melilotus albus.*	Zhang et al. (2012)
B. daqingense CCBAU 15774^T	China (Provincia Heilongjiang)	*Glycine max*	*Glycine max, Vigna unguiculata* e *Medicago sativa*	*Trifolium repens, Lotus corniculatus, Phaseolus vulgaris* e *Pisum sativum*	Wang et al. (2012)
B. oligotrophicum LMG 10732^T	Japão (Kashimadai)	**	ND	ND	Ramírez-Bahena et al. (2012)

"Tabela 1, continua"

Espécies/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. rifense CTAW71^T	Moroccos (Montanhas de Rif)	*Cytisus villosus*	*Cytisus villosus*	*Glycine max*	Chahboune et al.(2012)
B. retamae Ro19^T	Moroccos (Ras el Ma)	*Retama monosperma*	*Retama sphaerocarpa* e *Retama monosperma*	*Glycine max*	Guerrouj et al. (2013)
B. arachidis CCBAU 051107^T	China (Provincia Hebei)	*Arachis hypogaea*	*Arachis hypogaea* e *Lablab purpureus*	*Medicago sativa, Melilotus officinalis, Trifolium repens, Phaseolus vulgaris e G. max*	Wang et al. (2013)
B. diazoefficiens USDA 110^T	Estados Unidos (Florida)	*Glycine max*	*Glycine max*	ND	Delamuta et al. (2013)
B. paxllaeri LMTR 21^T	Peru (Ica)	*Phaseolus lunatus*	*Phaseolus lunatus, Vigna unguiculata*	*Glycine max, Pisum Sativum, Medicago sativa, Lupinus mariaejosephae*	Durán et al. (2014a)
B. icense LMTR 13^T	Peru (Ica)	*Phaseolus lunatus*	*Phaseolus lunatus, Vigna unguiculata*	*Glycine max, Pisum Sativum, Medicago sativa, Lupinus mariaejosephae*	Durán et al. (2014a)
B. ganzhouense RITF806^T	China (Jiangxi Province)	*Acacia melanoxylon*	*Acacia melanoxylon, Acacia aneura, Acacia victoriae, Acacia implexa*	*Medicago sativa, Pisum sativum, Trifolium albus, Vigna unguiculata*	Lu et al. (2014)
B. manausense BR 3351^T	Brasil (Amazônia)	*Vigna unguiculata*	*V. unguiculata, Cajanus cajan, M. atropurpureum, Vigna angularis, P. vulgaris, Vigna radiata*	*Arachis hypogaea, Acacia ligulata, Crotalaria junceae, G. max, Lupinus angustifolius, Ornithopus compressus, P.sativum, Vicia faba*	Silva et al. (2014a)

Tabela 1, continua”

Espécies/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. ottawaense OO99^{T}	Canadá (Ottawa)	*Glycine max*	*Glycine max*, *Glycine soja*, *Macroptilium atropurpureum*, *Desmodium canadense*, *Vigna unguiculata*, *Amphicarpaea bracteata*, *Phaseolus vulgaris*	*Leucaena leucocephala*, *Desmodium glutinosum*	Yu et al. (2014)
B. valentinum LmjM3^{T}	Espanha (Valencia)	*Lupinus mariae-josephae*	*Lupinus mariae-josephae*, *Retama raetam*, *Retama sphaerocarpa*	*Lupinus angustifolius*, *Lupinus cosentinii*, *Lupinus luteus*, *Lupinus micranthus*	Durán et al. (2014b)
B. ingae BR 10250^{T}	Brasil (Amazônia)	*Inga laurina*	*Inga edulis*, *Arachis hypogaea*, *Macroptillium atropurpureum*, *Vigna radiata*, *Vigna unguiculata*, *Glycine max*	*Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Lupinus angustifolius*, *Ornithopus compressus*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia faba*, *Vigna angularis*	Silva et al. (2014b)
B. neotropicale BR 10247^{T}	Brasil (Amazônia)	*Centrolobium paraense*	*Centrolobium paraense*, *Arachis hypogaea*, *Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Macroptillium atropurpureum*, *Vigna unguiculata*, *Vigna angularis*, *Vigna radiata*, *Ornithopus compressus*, *Phaseolus Vulgaris*	*Glycine max*, *Lupinus angustifolius*, *Pisum sativum*, *Vicia faba*	Zilli et al. (2014)

"Tabela 1, continua"

Espécies/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. erythrophlei CCBAU 53325^{T}	China	*Erythrophleum fordii*	*Erythrophleum fordii, Arachis hypogaea, Lablab purpureus and Glycine max*	ND	Yao et al. (2015)
B. ferriligni CCBAU 51502^{T}	China	*Erythrophleum fordii*	*Erythrophleum fordii, Arachis* hypogaea, *Lablab purpureus Glycine max*	ND	Yao et al. (2015)
B. subterraneum 58 2-1^{T}	Namíbia	*Arachis hypogaea*	*Arachis hypogaea, Vigna unguiculata, Vigna subterranea*	ND	Gronemeyer et al. (2015a)
B. tropiciagri CNPSo 1112^{T}	Brasil (São Paulo)	*Neonotonia wightii*	*Neonotonia wightii, Phaseolus vulgaris, Macroptilium atropurpureum*	*Glycine max*	Delamuta et al. (2015)
B. embrapense CNPSo 2833^{T}	Colombia	*Desmodium heterocarpon*	*Desmodium heterocarpon, Phaseolus vulgaris, Macroptilium atropurpureum*	*Glycine max, Neonotonia wightii*	Delamuta et al. (2015)
B. viridifuturi SEMIA 690^{T}	Brasil (Rio de Janeiro)	*Centrosema pubescens*	*Centrosema pubescens*	ND	Helene et al. (2015)
B. guangdongense CCBAU 51649^{T}	China	*Arachis hypogaea*	*Arachis hypogaea, Lablab purpureus, Aeschynomene indica*	ND	Li et al. (2015)
B. guangxiense CCBAU 53363^{T}	China	*Arachis hypogaea*	*Arachis hypogaea, Lablab purpureus, Aeschynomene indica*	ND	Li et al. (2015)
B. kavangense 14-3^{T}	Namíbia	*Vigna unguiculata*	*Vigna subterranea, Vigna unguiculata, Arachis hypogaea, Lablab purpureus.*	ND	Grönemeyer et al. (2015b)

"Tabela 1, conclusão"

Espécies/Estirpe tipo	Origem geográfica	Hospedeiro de origem	Nodulação		Referências
			Positiva	Negativa	
B. vignae 7-2^{T}	Namíbia	*Vigna unguiculata*	*Vigna subterranea, Vigna unguiculata, Arachis hypogaea, Lablab purpureus*	ND	Grönemeyer et al. (2015c)

*_B. denitrificans_ foi isolada de água superficial de um lago, ** _B. oligotrophicum_ foi isolada diretamente de solo sob plantação de arroz, ND - Não determinado.

Em estudos de descrição de novas espécies de *Bradyrhizobium*, a caracterização fenotípica é baseada em vários testes. Os principais são: tolerância a diferentes valores de pH, temperatura e concentrações de NaCl, resistência a antibióticos, utilização de diferentes fontes de carbono e nitrogênio, além da caracterização morfológica celular (Tabela 2). Jordan (1982) foi o primeiro a aplicar esses testes na descrição de *B. japonicum*. Testes quimiotaxonômicos como: determinação da composição de ácidos graxos e, principalmente, do teor de guanina (G) + citosina (C) (mol%) também são bastante utilizados. Este último faz parte da recomendação para descrição padrão na taxonomia bacteriana (STACKEBRANDT et al., 2002). Estirpes com diferenças em mais de 5% no teor de G+C não devem ser considerados da mesma espécie, e aqueles com mais de 10% de diferença não devem ser classificados dentro do mesmo gênero (BULL; GOODFELLOW; SLATER, 1992).

Recentemente, novas técnicas de caracterização fenotípica de estirpes de *Bradyrhizobium*, como a análise de ionização/dessorção a laser assistida por matriz acoplada à espectrometria de massa por tempo de voo (MALDI-TOF MS - Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry) tem sido sugerida (DURÁN et al., 2014b; SÁNCHEZ-JUANES et al., 2013). Essa análise permite uma rápida e confiável caracterização dos perfis proteicos característicos de cada estirpe bacteriana, permitindo, assim uma boa discriminação entre espécies, e também entre estipes de *Bradyrhizobium* (SÁNCHEZ-JUANES et al., 2013; DURÁN et al., 2014b).

De uma maneira geral, a caracterização fenotípica, isoladamente, tem aplicabilidade limitada na definição da posição taxonômica. No entanto, continua até hoje sendo de extrema importância, pois fornece dados complementares aos dados genotípicos na descrição de novas espécies.

Os métodos genotípicos compreendem várias análises direcionadas ao estudo das moléculas de RNA ou DNA. Técnicas da biologia molecular utilizadas na caracterização genotípica de BFNNL tornam possível

determinar objetivamente as diferenças inter e intra gêneros e/ou espécies, as quais não eram possíveis de determinar utilizando-se apenas métodos fenotípicos.

Tabela 2 Testes fenotípicos e bioquímicos usados na caracterização e identificação de estirpes tipo de *Bradyrhizobium*. Tolerância a diferentes condições de temperatura (1), salinidade (2) e pH (3); sensibilidade a antibióticos (4); assimilação de fontes de carbono (5); assimilação de nitrogênio (6); redução de nitrato (7); atividade da urease (8); morfologia da célula (9); conteúdo de guanina + citosina (G+C) no DNA (10), perfil de ácidos graxos (11); perfil de lipídeo polar (12); quinonas respiratórias (13); sorologia (14), reação Litmus Milk (15); reação Voges-Proskauter (16); Tolerância à corantes (17); EPS (18); SDS-PAGE (19), MALDI TOF MS (20). Testes realizados nos trabalhos de descrição de cada espécie

Espécies/Estirpes tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
B. japonicum LMG 6138^T	x	x	x	x	x	x	x		x	x				x	x					
B. elkanii LMG 6134^T				x							x							x		
B. liaoningense LMG 18230^T	x	x	x	x	x	x	x	x	x	x				x	x		x			
B. yuanmingense LMG 21827^T	x	x	x	x	x	x	x		x	x							x		x	
B. betae LMG 21987^T	x	x	x	x	x		x	x	x	x										
B. canariense LMG 22265^T	x	x	x		x	x			x	x										
B. denitrificans LMG 8443^T	x				x	x	x	x	x											
B. iriomotense EK05^T	x	x	x	x	x	x	x		x	x	x									
B. jicamae PAC68^T	x	x	x	x	x	x	x	x	x	x										
B. pachyrhizi PAC48^T	x	x	x	x	x	x	x	x	x	x										
B. lablabi CCBAU 23086^T	x	x	x	x	x	x	x	x	x	x	x				x					
B. cytisi CTAW11^T	x	x	x	x	x	x	x	x	x	x										
B. huanghuaihaiense CCBAU23303^T	x	x	x	x	x	x			x	x	x	x				x				
B. daqingense CCBAU 15774^T	x	x	x		x	x			x	x	x	x	x							
B. oligotrophicum LMG 10732^T	x	x			x	x	x	x	x	x	x									
B. rifense CTAW71^T	x	x	x	x	x	x	x	x	x	x										

"Tabela 2, continua"

Espécies/Estirpes tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
B. retamae Ro19 ^{T}	x	x	x	x	x	x	x	x	x	x										
B. arachidis CCBAU 051107 ^{T}	x	x	x	x	x	x	x	x	x	x	x	x	x		x					
B. diazoefficiens USDA 110 ^{T}	x	x	x	x	x	x	x	x	x	x	x									
B. paxllaeri LMTR 21^{T}	x	x	x	x	x	x	x	x	x	x	x									
B. icense LMTR 13 ^{T}	x	x	x	x	x	x		x	x	x	x									
B. ganzhouense RITF806 ^{T}	x	x	x	x	x		x	x	x	x	x	x					x			
B. manausense BR 3351 ^{T}	x	x	x	x	x		x	x	x	x	x									
B. ottawaense OO99 ^{T}	x	x	x	x	x	x	x	x	x	x	x									
B. valentinum LmjM3 ^{T}	x	x	x	x	x	x			x	x	x									x
B. ingae BR 10250 ^{T}	x	x	x	x	x		x	x	x	x	x									
B. neotropicale BR 10247 ^{T}	x	x	x	x	x		x	x	x	x	x									
B. erythrophlei CCBAU 53325 ^{T}	x	x	x		x				x	x	x									
B. ferriligni CCBAU 51502^{T}	x	x	x		x				x	x	x									
B. subterraneum 58 2-1T	x	x	x	x	x				x	x										
B. tropiciagri CNPSo 1112 ^{T}	x	x	x	x	x			x	x	x	x									
B. embrapense CNPSo 2833 ^{T}	x	x	x	x	x			x	x	x	x									
B. viridifuturi SEMIA 690 ^{T}	x	x	x	x	x				x	x	x									
B. guangdongense CCBAU 51649 ^{T}	x	x	x	x	x				x	x	x									
B. guangxiense CCBAU 53363 ^{T}	x	x	x	x	x				x	x	x									
B. kavangense 14-3 ^{T}	x	x	x	x	x				x	x	x									

"Tabela 2, conclusão"

Espécies/Estirpes tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
B. vignae 7-2^{T}	x	x	x	x	x				x	x	x									

Várias técnicas vêm sendo empregadas na caracterização genética de espécies de *Bradyrhizobium*, como: RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorfism), RFLP (Restriction Fragment Length Polymorphis) da região do DNA que codifica o gene 16S rRNA e da região intergênica entre os genes 16S e 23S rRNA, PCR de sequências repetitivas de DNA (rep-PCR - repetitive DNA elements) com *primers* específicos, como o BOX e REP (Tabela 3). A aplicação dessas técnicas permite indicar diversidade entre estirpes de *Bradyrhizobium* pertencentes a diferentes grupos e/ou pertencentes a um mesmo grupo, contudo, não permite determinar a sua posição taxonômica.

Em estudos de taxonomia bacteriana, o sequenciamento do gene 16S rRNA tem sido amplamente empregado, pois esse gene constitui um excelente marcador molecular (WOESE; KANDLER; WHEELIS, 1990). No entanto, a variação genética entre as sequências desse gene é geralmente baixa, não tendo, portanto, boa resolução para diferenciação a nível de espécie. Para o gênero *Bradyrhizobium*, diversos estudos têm proposto, além do gene 16S rRNA, o sequenciamento de genes housekeeping (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* e *rpoB*), ou regiões ribossomais, como o gene 23S rRNA e o espaço transcrito intergênico entre os genes 16S e 23S rRNA (ITS) para obtenção de uma melhor resolução a nível de espécie (GUIMARÃES et al., 2015; MENNA et al., 2009; RIBEIRO et al., 2015; RIVAS et al., 2009; SILVA et al., 2014b; STEPKOWSKI et al., 2005; VINUESA et al., 2005a; WILLEMS; COOPMAN; GILLIS, 2001b). Essas análises representam, juntamente com a análise de hibridização DNA-DNA, os principais métodos genotípicos aplicados na caracterização e identificação das estirpes tipo de *Bradyrhizobium* já descritas (Tabela 3).

Os genes housekeeping são genes responsáveis por funções metabólicas fundamentais para o funcionamento celular, encontram-se amplamente distribuídos no genoma bacteriano e são sempre expressos (STACKEBRANDT et al., 2002; ZEIGLER., 2003). Em estudos relacionados à diversidade de estirpes do gênero *Bradyrhizobium*, os genes

housekeeping *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* e *rpoB* têm sido os mais utilizados (DELAMUTA et al., 2012; GUIMARÃES et al., 2015; ISLAM et al., 2008; MENNA et al., 2009; VINUESA et al., 2005a; VINUESA et al. 2008; RIBEIRO et al., 2015; RIVAS et al. 2009). Todos esses genes são codificadores de importantes proteínas: o *atpD* codifica para a subunidade β da membrana da ATP sintase, *o dnaK* codifica para proteína Hsp70 da classe chaperone, o *glnII* codifica para a subunidade β da proteína DNA girase, o *gyrB* codifica para topoisomerase II, o *recA* codifica para a recombinase A, envolvida na recombinação de sequências complementares de DNA e o *rpoB* codifica para a subunidade β da proteína RNA polimerase.

Tabela 3 Análises moleculares usadas na caracterização e identificação de estirpes tipo de *Bradyrhizobium*: 1 (16S rRNA); 2 (23S rRNA); 3 (ITS 16S-23S rRNA); 4 (IGS 16S-23S rRNA); 5 (nodA); 6 (nodC); 7 (nodD); 8 (nifH); 9 (virA); 10 (pufM); 11 (atpD); 12 (glnII); 13 (recA); 14 (dnaK); 15 (rpoB); 16 (gyrB); 17 (Restriction Fragment Length Polymorfism - RFLP); 18 (Random Amplification of Polymorphic DNA - RAPD); 19 (rep-PCR); 20 (BOX-PCR); 21 (Amplified Fragment Length Polymorfism - AFLP); 22 (Multilocus Enzyme Electrophoresis MLEE); 23 (DNA-DNA hybridization), 24 (Average Nucleotide Identity - ANI). Testes realizados nos trabalhos de descrição de cada espécie

Espécies/ Estirpe tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
B. japonicum LMG 6138^{T}																							x	
B. elkanii LMG 6134^{T}	x																x						x	
B. liaoningense LMG 18230^{T}	x																						x	
B. yuanmingense LMG 21827^{T}	x																x						x	
B. betae LMG 21987^{T}	x		x				x	x	x									x					x	
B. canariense LMG 22265^{T}	x		x			x		x			x	x	x						x			x	x	
B. denitrificans LMG 8443^{T}	x	x	x							x											x			
B. iriomotense EK05^{T}	x		x		x	x		x			x	x	x			x							x	
B. jicamae PAC68^{T}	x		x			x		x			x	x						x					x	
B. pachyrhizi PAC48^{T}	x		x			x		x			x	x						x					x	
B. lablabi CCBAU 23086^{T}	x			x		x		x			x	x	x				x			x			x	
B. cytisi CTAW11^{T}	x					x		x			x	x	x					x					x	
B. huanghuaihaiense CCBAU23303^{T}	x			x		x		x			x	x	x				x			x			x	
B. daqingense CCBAU 15774^{T}	x			x		x		x			x	x	x							x			x	
B. oligotrophicum LMG 10732^{T}	x										x	x	x										x	

"Tabela 3, continua"

Espécies/ Estirpe tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
B. rifense CTAW71^{T}	x										x	x	x										x	
B. retamae Ro19^{T}	x					x					x	x	x					x					x	
B. arachidis CCBAU 051107^{T}	x			x		x		x			x	x	x	x	x		x						x	
B. diazoefficiens USDA 110^{T}	x		x								x	x	x	x	x	x								x
B. paxllaeri LMTR 21^{T}	x					x		x			x	x	x	x		x			x					x
B. icense LMTR 13^{T}	x					x		x			x	x	x	x		x			x					x
B. ganzhouense RITF806^{T}	x					x		x			x	x	x										x	
B. manausense BR 3351^{T}	x					x		x				x	x	x	x	x				x			x	
B. ottawaense OO99^{T}	x					x		x			x	x	x	x	x	x		x					x	
B. valentinum LmjM3^{T}	x				x	x		x			x	x	x											x
B. ingae BR 10250^{T}	x					x		x				x	x	x	x	x				x			x	
B. neotropicale BR 10247^{T}	x		x			x		x				x	x	x	x	x							x	
B. erythrophlei CCBAU 53325^{T}	x			x		x		x				x	x			x				x			x	
B. ferriligni CCBAU 51502^{T}	x			x		x		x				x	x			x				x			x	
B. subterraneum 58 2-1^{T}	x		x					x				x	x	x	x					x			x	
B. tropiciagri CNPSo 1112^{T}	x		x			x		x			x	x	x	x		x				x				x
B. embrapense CNPSo 2833^{T}	x		x			x		x			x	x	x	x		x				x				x
B. viridifuturi SEMIA 690^{T}	x							x				x	x	x		x				x				x
B. guangdongense CCBAU 51649^{T}	x				x			x				x	x	x		x				x			x	
B. guangxiense CCBAU 53363^{T}	x				x			x				x	x	x		x				x			x	

"Tabela 3, conclusão"

Espécies/ Estirpe tipo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
B. kavangense 14-3^{T}	x		x			x		x				x	x	x	x					x			x	
B. vignae 7-2^{T}	x		x			x		x				x	x	x	x					x			x	

De maneira geral, no gênero *Bradyrhizobium*, as sequências dos genes housekeeping mencionados acima apresentam uma divergência genética bem maior em relação às sequências do gene 16S rRNA, mas conservam-se o suficiente para manter a informação genética, e por isso são considerados marcadores filogenéticos alternativos para uma melhor discriminação entre estirpes (VINUESA et al. 2008; RIVAS et al. 2009). A combinação de sequências de três ou mais genes housekeeping em uma análise concatenada, ou seja, uma análise de sequências multilocus (MLSA), tem gerado informações precisas sobre as relações filogenéticas dentro do gênero *Bradyrhizobium*, e indicado grupos com estirpes representativas de novas espécies (DELAMUTA et al, 2012; GUIMARÃES et al., 2015; RIBEIRO et al., 2015; RIVAS et al., 2009; STEPKOWSKI et al., 2005; VINUESA et al., 2005a), que deverão ser avaliadas pela análise de hibridização DNA-DNA para confirmação.

A hibridização DNA-DNA é uma técnica baseada em comparações das sequências do genoma de diferentes organismos, a fim de calcular as suas semelhanças genéticas em geral (STACKEBRANDT; GOEBEL, 1994). Essa técnica é recomendada como um critério padrão na descrição de uma nova espécie de bactéria e valores de hibridização DNA-DNA inferiores a 70% são indicativos de novas espécies (WAYNE et al., 1987). No entanto, é uma técnica que requer uma grande quantidade de DNA de alta qualidade, o que muitas vezes não é facilmente obtido a partir de estirpes do gênero *Bradyrhizobium* (WILLEMS; COOPMAN; GILLIS, 2001a). Além disso, é muito trabalhosa tecnicamente e realizada em poucos laboratórios do mundo. Considerando essas limitações, é recomendável que seja feita, previamente, uma seleção, através da análise de ITS (16S-23S rRNA) e/ou de genes housekeeping em uma abordagem multilocus, de estirpes potencialmente representativas de novas espécies, buscando-se diminuir o número de experimentos de hibridização DNA:DNA necessários para identificação taxonômica de uma nova espécie (MARTENS et al., 2008; WILLEMS; COOPMAN; GILLIS, 2001b).

A análise de ANI (Average Nucleotide Identity – Identidade Média de Nucleotídeos) das sequências do genoma bacteriano foi proposta como uma alternativa para substituir a análise de hibridização DNA-DNA na taxonomia bacteriana (GORIS et al., 2007), e tem sido utilizada, recentemente, na descrição de novas espécies do gênero *Bradyrhizobium* (DELAMUTA et al., 2013; DURÁN et al., 2014a, 2014b; HELENE et al., 2015).

2.4 Ocorrência e diversidade do gênero *Bradyrhizobium* em solos de ecossistemas brasileiros

Em ecossistemas brasileiros, o gênero *Bradyrhizobium* destaca-se, pois além de compor um grupo de BFNNL identificado em nódulos radiculares de várias espécies de leguminosas e apresentar ampla distribuição geográfica, forma simbiose eficiente com diversos hospedeiros nativos e exóticos. Estudos desenvolvidos em solos de diferentes ecossistemas e/ou regiões brasileiras têm mostrado predominância do gênero *Bradyrhizobium* entre os gêneros de BFNNL isolados (ARAÚJO et al., 2015; GRANADA et al., 2015; GUIMARÃES et al., 2012; JARAMILLO et al,. 2013; LIMA et al., 2009; MOREIRA et al., 1993; MOREIRA; HAUKKA; YOUNG, 1998; RIBEIRO et al., 2015; RUFINI et al., 2013; SILVA et al., 2012).

Cerca de 800 estirpes isoladas de nódulos de várias espécies de leguminosas florestais das três subfamílias: Caesalpinioideae, Mimosoideae e Papilionoideae na região Amazônica e Mata Atlântica do Brasil foram caracterizadas culturalmente e a maioria delas apresentou crescimento lento ou muito lento e capacidade de alcalinizar o meio de cultura (MOREIRA, 1991; MOREIRA et al., 1993). Posteriormente, 171 dessas estirpes, representantes culturais de diferentes grupos de divergência de Leguminosae, foram estudadas quanto à diversidade em relação ao perfil de bandas de proteínas totais obtidas por SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) e comparadas ao banco de dados da

Universidade de Ghent, na Bélgica. Dentre essas, 120 foram agrupadas dentro do gênero *Bradyrhizobium* (MOREIRA et al., 1993). A posição taxonômica de 44 estirpes representantes dos grupos formados a partir da análise de SDS-PAGE foram estudadas por Moreira, Haukka e Young (1998) através do sequenciamento parcial do gene 16S rRNA, e esses autores confirmaram a validade de identificação por SDS-PAGE e a predominância de estirpes do gênero *Bradyrhizobium* entre as estirpes estudadas.

A partir da análise do padrão de bandas obtido por SDS-PAGE (LIMA; PEREIRA; MOREIRA, 2005) e da análise filogenética do gene 16S rRNA (LIMA et al., 2009) de estirpes de isoladas de nódulos de siratro em solos da região Amazônica Ocidental, verificou-se predominância do gênero *Bradyrhizobium* e alta diversidade entre as estirpes desse gênero. Guimarães et al. (2012) e Jaramillo et al. (2013), trabalhando com estirpes também isoladas dessa região, utilizado feijão-caupi como planta isca, verificaram predominância do gênero *Bradyrhizobium*, através do sequenciamento parcial do gene 16S rRNA, e alta diversidade entre estirpes, através da análise rep-PCR (BOX).

A caracterização morfofisiológica de 417 estirpes isoladas de soja em cinco áreas sob uso frequente de inoculante no Rio Grande do Sul foi realizada por Giongo et al. (2008). Dessa coleção, 100 estirpes que apresentavam crescimento lento e reação alcalina em meio 79 foram avaliadas quanto à diversidade genética, através da análise de rep-PCR (ERIC e BOX) e AFLP e comparadas com as quatro estirpes inoculantes: SEMIA 587, SEMIA 5019 (*B. elkanii*), SEMIA 5079 (*B. japonicum*) e SEMIA 5080 (*B. diazoefficiens*). Estes autores verificaram alta diversidade entre as estirpes estudadas, o que indica que além das estirpes utilizadas como inoculante havia presença de estirpes nativas de *Bradyrhizobium*. Bizarro et al. (2011), também estudando a variabilidade genética pela análise de rep-PCR (ERIC, REP e BOX) de 75 estirpes de *Bradyrhizobium* isoladas de soja em solos inoculados do Rio Grande do Sul verificaram alta

diversidade genética entre as estirpes, e apenas 25% mostraram alta similaridade com estirpes inoculantes da soja. Através da análise de rep-PCR (BOX), Vargas et al. (2007) constataram a formação de dez grupos distintos de estirpes de *Bradyrhizobium* isoladas de nódulos de acácia-negra, em cinco regiões no estado do Rio Grande do Sul.

A partir de uma coleção de 188 estirpes de nódulos de feijão-caupi cultivado com solos da Amazônia sob diferentes sistemas de uso, incluindo floresta nativa, áreas em recuperação e monocultura, 50 estirpes representativas de diferentes áreas foram estudadas quanto à diversidade genética e ao posicionamento filogenético, através do sequenciamento parcial do gene 16S rRNA (SILVA et al., 2012). Entre essas estirpes, 46 foram identificadas como pertencentes ao gênero *Bradyrhizobium*. Posteriormente, a análise filogenética dos genes housekeeping *glnII*, *recA*, *rpoB*, *dnaK*, *gyrB* indicou que 6 estirpes desse grupo pertenciam a um novo grupo dentro do *Bradyrhizobium*, e a análise de hibridização de DNA–DNA confirmou essa indicação, sendo assim, proposta uma nova espécie (*Bradyrhizobium manausense*) (SILVA et al. 2014a). Outras duas espécies de *Bradyrhizobium*, oriundas de solos da Amazônia, foram recentemente descritas: *B. ingae*, isolada de nódulo de *Inga laurina* (SILVA et al., 2014b) e *B. neotropicale*, isolada de nódulo de *Centrolobium paraense* (ZILLI et al., 2014).

Além das três espécies oriundas de solos da Amazônia, existem mais duas espécies de *Bradyrhizobium* atualmente descritas, oriundas de solos brasileiros: *B. tropiciagri*, isolada de nódulo de *Neonotonia wightii* em solo do estado de São Paulo (DELAMUTA et al., 2015), e *B. viridifuturi*, isolada de nódulo de *Centrosema pubescens* em solo do estado do Rio de Janeiro (HELENE et al., 2015). Esse impulso na taxonomia de espécies do gênero *Bradyrhizobium* isoladas de solos brasileiros nos últimos anos deve-se aos avanços nas técnicas da biologia molecular, especialmente o sequenciamento de genes housekeeping, que tem permitido selecionar grupos representativos de novas espécies.

Alta variabilidade genética foi detectada por Menna et al. (2009) ao realizarem o sequenciamento parcial de genes housekeeping (*atpD, dnaK, glnII, e recA*) de 40 estirpes (23 de origem brasileira) isoladas de diferentes espécies leguminosas. Nesse estudo, foram identificados vários grupos filogeneticamente distantes das estirpes tipo das espécies de *Bradyrhizobium*. Delamuta et al. (2012) também detectaram, através do sequenciamento de genes housekeeping (*recA, atpD, glnII, gyrB* e *rpoB*), alta diversidade genética entre 12 estirpes de *Bradyrhizobium* (6 de origem brasileira) isoladas de diferentes espécies leguminosas. Posteriormente uma dessas estirpes (SEMIA 6148) foi descrita como uma nova espécie (*Bradyrhizobium tropiciagri*) (DELAMUTA et al., 2015).

Em trabalho conduzido por Perrineau et al. (2011), em solos dos estados de São Paulo e Rio de Janeiro, em áreas não inoculadas e inoculadas com *B. elkanii*, foram obtidas 79 estirpes de *Bradyrhizobium* de nódulos de *Acacia mangium*. Através do sequenciamento de três genes housekeeping (*glnII, dnaK e recA*) verificou-se que a maioria das estirpes foi agrupada com a espécie *B. elkanni*, o que indica uma preferencialidade de *A. mangium* por esse microssimbionte. Ressalta-se que 39 dessas estirpes foram oriundas de solos que nunca haviam sido inoculados, o que indica a ocorrência natural dessa espécie de *Bradyrhizobium* em ecossistemas brasileiros. Granada et al. (2015), ao avaliarem 74 estirpes de *Bradyrhizobium* simbiontes de *Lupinus albescens* em solos da região sul do Brasil, constataram alta diversidade genética, através do sequenciamento dos genes housekeeping *dnaK*, *atpD*, *recA*, *glnII*, *rpoB* e *gyrB*. Nesse estudo verificou-se a formação de novos grupos que possivelmente representam novas espécies de *Bradyrhizobium*.

Um total de 50 estirpes de *Bradyrhizobium* isoladas de espécies leguminosas em diferentes ecossistemas brasileiros, em solos da Amazônia e Minas Gerais, identificadas em estudo pelo nosso grupo de trabalho prévio (GUIMARÃES et al., 2012; JARAMILLO et al,. 2013; MOREIRA et al, 1993; MOREIRA, HAUKKA; YOUNG, 1998; RUFINI et al., 2013) foram analisadas quanto à diversidade genética através do sequenciamento de

genes housekeeping (*atpD, dnaK, gyrB* e *recA*) por Guimarães et al. (2015). As análises filogenéticas desse estudo mostraram alta diversidade genética entre as estirpes, e indicaram vários grupos com estirpes potencialmente representativas de novas espécies. A posição taxonômica de dois desses grupos está incluída nos objetivos deste trabalho.

Em outro estudo recente desenvolvido pelo nosso grupo de trabalho, 46 estirpes de bactérias fixadoras de nitrogênio isoladas de nódulos de soja inoculada com solos de diferentes regiões brasileiras (Nordeste, Centro Oeste, Sudeste e Sul), com histórico de inoculação com as estirpes SEMIA 587, SEMIA 5019 (*B. elkanii*), SEMIA 5079 (*B. japonicum*) e SEMIA 5080 (*B. diazoefficiens*), foram identificadas como *Bradyrhizobium* sp., por meio do sequenciamento parcial do gene 16S rRNA e caracterizadas com base na análise de sequência multilocus (MLSA) de genes housekeeping (*atpD, dnaK, gyrB, recA* e *rpoB*) (RIBEIRO et al., 2015). Essa análise indicou dois novos grupos, os quais também são objetivos desse trabalho.

De maneira geral, ainda são poucos os estudos que avaliam a ocorrência e a diversidade genética do gênero *Bradyrhizobium* no Brasil, especialmente no Centro Oeste e Nordeste. No entanto, as informações já disponíveis na literatura são suficientes para afirmar que esse gênero ocorre naturalmente em ecossistemas brasileiros, pois em vários trabalhos aqui apresentados, realizados em diferentes regiões geográficas, as estirpes de *Bradyrhizobium* foram isoladas em florestas nativas ou áreas agrícolas sem histórico de uso de inoculantes. Mesmo em alguns trabalhos em que as bactérias foram isoladas de áreas inoculadas, foi detectada alta diversidade genética, o que indica a ocorrência de estirpes nativas também nessas áreas. A ocorrência natural de *Bradyrhizobium* em ecossistemas brasileiros conflita com relatos de alguns trabalhos (LOPES; GIARDINI; KIIHL, 1976; MARTÍNEZ-ROMERO; CABALLERO-MELLADO, 1996) indicando que os solos brasileiros eram originalmente desprovidos de bactérias do gênero *Bradyrhizobium*, e estas foram introduzidas a partir do uso na cultura da soja de inoculantes com estirpes exóticas, que se disseminaram pelo país.

2.5 Eficiência de *Bradyrhizobium* em simbiose com espécies leguminosas em ecossistemas brasileiros

A família Leguminosae (Fabaceae), de maneira geral, encontra-se bem representada em ecossistemas brasileiros, na qual estão incluídas diversas espécies de porte herbáceo, arbustivo ou arbóreo que são capazes de estabelecer simbiose com BFNNL. A inoculação de espécies leguminosas com estirpes de BFNNL selecionadas, além de proporcionar economia no uso de fertilizantes nitrogenados, reduz os impactos provocados pelo manejo inadequado desses fertilizantes. A seleção tem por objetivo a obtenção de estirpes eficientes na FBN, adaptadas a diversas condições edafoclimáticas, competitivas frente às populações nativas de BFNNL, com capacidade de estabelecer simbiose com diferentes hospedeiros e que apresentem alta estabilidade genética.

Dentre os gêneros de BFNNL que ocorrem no Brasil, o *Bradyrhizobium* tem grande destaque visto a sua capacidade de estabelecer simbiose eficiente com diversas espécies de leguminosas de importância agrícola, pastoril e florestal. Uma grande vantagem desse gênero é que a maioria dos seus genes de nodulação e fixação de nitrogênio localiza-se no cromossomo, e com isso são mais estáveis, ou seja, não são perdidos facilmente em condições adversas (MOREIRA; SIQUEIRA, 2006). Essa característica é extremamente importante para que a estirpe selecionada não perca a eficiência simbiótica ao longo dos anos.

No Brasil, estirpes de BFNNL já foram selecionadas e autorizadas pelo MAPA para produção de inoculantes de 83 espécies leguminosas (BRASIL, 2011). Dentre essas, 25 espécies herbáceas (produtoras de grãos, adubação verde e forrageiras) e 27 espécies arbóreas possuem estirpes inoculantes pertencentes ao gênero *Bradyrhizobium*. Entre as espécies leguminosas produtoras de grãos que possuem estirpes inoculantes desse gênero tem-se a soja (*Glycine max*) e o feijão-caupi (*Vigna unguiculata*). O melhor exemplo de contribuição do gênero *Bradyrhizobium* em

agroecossistemas brasileiros é para a cultura da soja, em que a inoculação com estirpes desse gênero substitui totalmente a adubação química nitrogenada, o que representou, em 2006, uma economia para o país de cerca de US$ 3,3 bilhões, considerando uma área de 21 milhões de hectares, onde se produziu 57 milhões de toneladas de grãos (MOREIRA, 2010).

Para o feijão-caupi, diversos trabalhos têm mostrado a contribuição da inoculação com estirpes autorizadas pelo MAPA para essa cultura (INPA 03-11B, UFLA 03-84 e BR 3267), na substituição total da adubação nitrogenada, em diferentes regiões brasileiras (COSTA et al., 2011; LACERDA et al., 2004; MARINHO et al., 2014; SOARES et al., 2006; ZILLI et al., 2009). O fornecimento de nitrogênio para essa cultura via inoculação com estirpes eficientes representa uma importante estratégia na redução dos custos de produção, uma vez que é cultivada principalmente por pequenos e médios produtores. No entanto, a utilização de inoculantes não é uma prática comum no cultivo dessa e de outras importantes espécies leguminosas de grãos, adubação verde, forrageiras e arbóreas que já possuem estirpes selecionadas.

Com exceção da soja, a recomendação das estirpes inoculantes para as demais espécies leguminosas foi baseada em poucos experimentos e em condições edafoclimáticas particulares. Assim, existe a necessidade de mais estudos que visem testar a eficiência simbiótica das estirpes já selecionadas em diferentes ecossistemas, e também a seleção de novas estirpes eficientes para culturas de interesse. Como exemplo de uma leguminosa de grãos de interesse agrícola, principalmente para a região Nordeste, pode-se citar o feijão-fava (*Phaseolus lunatus* L.), uma cultura capaz de estabelecer simbiose eficiente com *Bradyrhizobium* e outros gêneros de BFNNL em solos brasileiros (SANTOS et al., 2011; ARAÚJO et al., 2015), contudo, ainda não há estirpes selecionadas para otimizar o processo de FBN nessa cultura.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - *Bradyrhizobium brasilense* sp. nov., a symbiotic nitrogen-fixing bacterium isolated from cowpea in tropical ecosystems

Artigo submetido para a revista International Journal of Systematic and Evolutionary Microbiology (Versão preliminar)

Bradyrhizobium brasilense sp. nov., a symbiotic nitrogen-fixing bacterium isolated from cowpea in tropical ecosystems

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Running title: *Bradyrhizobium brasilense* sp. nov.

New sequences: *recA* of *B. brasilense* UFLA 03-321^T (KT793142), UFLA 03-320 (KT793140), UFLA 03-290 (KT793141); *nodC* of *B. brasilense* UFLA 03-321^T (KT793173), UFLA 03-320 (KT793171), UFLA 03-290 (KT793172); *nifH* of *B. brasilense* UFLA 03-321^T (KT825890), UFLA 03-320 (KT825889), UFLA 03-290 (KT793147).

Abstract

Three strains of nitrogen-fixing bacteria isolated from cowpea (*Vigna unguiculata* L.) nodules inoculated with soil of the Minas Gerais (UFLA 03-321^T and UFLA 03-320) and Amazonas (UFLA 03-290) states, Brazil, were previously identified and reported as a new group within the genus *Bradyrhizobium*. To determine their taxonomic position, these strains were characterized in this study using a polyphasic approach. The phylogenetic analysis of the 16S rRNA gene grouped the three strains with *Bradyrhizobium elkanii* LMG 6134^T and *Bradyrhizobium pachyrhizi* LMG 24246^T, with similarity above 99.8%. However, the concatenated sequence analysis of the housekeeping genes *atpD*, *dnaK*, *gyrB* and *recA* indicated that these three strains represent a novel species of *Bradyrhizobium*, which is closely related to *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T. The status of the novel species was confirmed by DNA-DNA hybridization analysis, which showed homology of 20.6 and 22.6%, respectively, between UFLA 03-321^T and *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T. Analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles and some phenotypic characteristics allowed the differentiation of the novel species from its two neighboring species. In phylogenetic analysis of *nodC* and *nifH* genes, UFLA 03-321^T presented maximum similarity with *B. tropiciagri* CNPSo 1112^T (94.47 and 99.47%, respectively). Based on the data presented, it is suggested that these strains represent a novel species, for which the name *Bradyrhizobium brasilense* sp. nov. is proposed, with UFLA 03-321^T (= LMG 29353^T) as type strain. G+C content in the DNA of UFLA 03-321^T is 63.7 mol%.

Keywords: *Bradyrhizobium*, *Vigna unguiculata* L., polyphasic taxonomy, MALDI-TOF MS

Cowpea [*Vigna unguiculata* (L.) Walp.] is a legume species, cultivated in tropical and subtropical regions of the world, and is one of the major sources of vegetable protein for the low income population. This species can greatly benefit from biological nitrogen fixation in symbiosis with nitrogen-fixing legume nodulating bacteria (NFLNB) (Soares *et al*., 2006; Costa *et al*., 2014; Soares *et al*., 2014).

Studies carried out in soils of different Brazilian ecosystems, using cowpea as trap plant to evaluate the diversity of NFLNB have pointed out *Bradyrhizobium* genus as the major symbiont of this legume. In addition, high phenotypic and/or genotypic diversity of *Bradyrhizobium* strains isolated from cowpea nodules were observed (Florentino *et al*., 2010; Guimarães *et al*., 2012; 2015; Silva *et al*., 2012; Jaramillo *et al*., 2013; Rufini *et al*., 2014). Recently, six strains isolated from cowpea nodules in the Brazilian Amazon soil (Silva *et al*., 2012) were classified as a novel species of *Bradyrhizobium*, *B. manausense*) (Silva *et al*., 2014a).

In a previous study, 50 *Bradyrhizobium* strains isolated from different legume species, mainly cowpea, in Brazilian soils, were characterized by phylogenetic analyses of the housekeeping genes *atpD, dnaK, gyrB* and *recA,* which indicated five clusters with strains that are potential representatives of novel species (Guimarães *et al*., 2015). In this study, one of the clusters, which included three strains (UFLA 03-321^{T}, UFLA 03-320 and UFLA 03-290), was selected for further analysis, by molecular and phenotypic methods. Results obtained indicate that the three strains represent a single novel species, for which the name *Bradyrhizobium brasilense* sp. nov is proposed.

Strains UFLA 03-321^{T} and UFLA 03-320 were isolated from soil under agriculture, in Lavras, Minas Gerais (Rufini *et al*., 2014), and UFLA 03-290 was isolated from soil under agroforestry, in Benjamin Constant, Amazonas (Jaramillo *et al*., 2013), in Brazil, using cowpea as trap plant. Medium 79 (Fred & Waksman, 1928), also known as YMA (Vincent, 1970), was used for isolation and characterization of strains. These strains are

deposited in the culture collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras, Brazil, and the type strain was also deposited in the culture collection (BCCM/LMG) of the Ghent University, Belgium.

DNA extraction from the strains was carried out by the alkaline lysis method (Niemann *et al*., 1997). Sequences of 16S rRNA (1179 to 1301 pb) and *recA* (483 to 501 pb) genes of the three strains were obtained using the same primers and cycles of amplification and sequencing used by Ribeiro *et al*. (2015). The *atpD*, *dnaK* and *gyrB* genes have been sequenced in our previous study (Guimarães *et al*., 2015). The sequences of type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI), were also included. The alignment of the sequences of each gene was carried out using the ClustalW multiple alignment algorithm in BioEdit. Phylogenetic trees were constructed by the neighbor joining method (NJ) (Saitou & Nei, 1987) and by the maximum likelihood (ML) (Felsenstein, 1981), using the Kimura 2 parameter model (Kimura, 1980). The MEGA 5 software package (Tamura *et al*., 2011) was used in the construction of trees, with bootstrap values based on 1000 replications.

Phylogenetic trees of the 16S rRNA gene, using the NJ (Fig. 1) and ML (data not shown) methods, were very similar. Strain UFLA 03-321^{T} shared more than 99.5% sequence similarity of 16S rRNA gene with nine *Bradyrhizobium* species (Table S1), and its closest neighbors were *B. pachyrhizi* LMG 24246^{T} and *B. elkanii* LMG 6134^{T} (Fig. 1). Strains UFLA 03-320, UFLA 03-290 and *B. pachyrhizi* LMG 24246^{T} presented 100% similarity among them and shared 99.91% similarity with UFLA 03-321^{T}. Similarity of *B. elkanii* LMG 6134^{T} with UFLA 03-321^{T} was 99.83% (Table S1). The present results confirm the high degree of conservation of nucleotide sequences of the 16S rRNA gene between members of the *Bradyrhizobium* genus, corroborating previous reports (Willems *et al*., 2001; Ramírez-Bahena *et al*., 2009; Duran *et al*., 2014; Guimarães *et al*., 2015;

Ribeiro *et al*., 2015), and demonstrating the need for additional analyses, such as analysis of housekeeping genes.

Phylogenetic analysis of the sequences of housekeeping genes *atpD, dnaK, glnII, gyrB, recA* and *rpoB* have been successfully used in addition to the analysis of 16S rRNA gene for discrimination between species of the *Bradyrhizobium* genus (Vinuesa *et al*., 2005; Ramírez-Bahena *et al*., 2009; Wang *et al*., 2012;. Durán *et al*., 2014; Zilli *et al*., 2014). In the present study, the results of the concatenated sequence analysis of the *atpD* (429 pb), *dnaK* (222 pb), *gyrB* (561 pb) and *recA* (381 pb) genes were similar when using the NJ (Fig. 2) and ML (data not shown) methods, and indicated that the studied strains form a separate cluster, supported by high bootstrap value (100%) (Fig. 2). The similarity between UFLA 03-321^{T} and strains UFLA 03-320 and UFLA 03-290 was 100 and 99.69%, respectively, in the concatenated analysis (Table S1). *Bradyrhizobium pachyrhizi* LMG 24246^{T} and *B. elkanii* LMG 6134^{T} were the most similar species to UFLA 03-321^{T}, both in the concatenated analysis (Fig. 2) and in individual analysis of the studied housekeeping genes (Table S1). In concatenated analysis, similarity values between UFLA 03-321^{T} and *B. elkanii* LMG 6134^{T} and *B. pachyrhizi* LMG 24246^{T} were 97.78 and 97.28%, respectively (Table S1). These similarity values are close to those found between different *Bradyrhizobium* species (Chahboune *et al*., 2011; Silva *et al*., 2014b), suggesting that the studied strains belong to a novel species within this genus.

Characterization the three strains and the type strains of two closely related species (*B. elkanii* LMG 6134^{T} and *B. pachyrhizi* LMG 24246^{T}) was carried out by the MALDI-TOF MS analysis (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry). For this analysis, cultures of the third generation were grown in medium YMA (Vincent, 1970). Sample preparation and data analyses were carried out as previously described (Wieme *et al*., 2014). Results of this analysis were in accordance with those of phylogenetic analysis of housekeeping genes, confirming that the studied strains form a separate cluster of *B.elkanii* LMG 6134^{T} and *B.*

pachyrhizi LMG 24246^T, and that UFLA 03-290 is the most peripheral of the three strains (Fig. 3). In recent studies, this analysis allowed good discrimination between species and between strains of the same species within the *Bradyrhizobium* genus (Sánchez-Juanes *et al*., 2013; Duran *et al*., 2014), corroborating the present results.

In previous study, the ability of UFLA 03-321^T, UFLA 03-320 and UFLA 03-290 to grow in medium 79 (Fred & Waksman, 1928) was evaluated under different NaCl concentrations (w/v) (0.01, 0.25, 0.5, 0.75 and 1%) (Guimarães *et al*., 2015), as well as their resistance to the following antibiotics: ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), doxycycline (30 μg mL^{-1}), erythromycin (15 μg mL^{-1}), gentamicin (10 μg mL^{-1}), kanamycin (30 μg mL^{-1}) and neomycin (30 μg mL^{-1}) (Guimarães *et al*., 2015). To allow comparison, in the present study we evaluated the growth of *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T in 79 medium under the same conditions of NaCl, as well as their resistance to the nine antibiotics cited, using the same methods applied in the characterization of UFLA strains.

This study additionally evaluated the ability of the three new strains and of *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T to grow in medium 79 (Fred & Waksman, 1928) under different conditions of pH (4, 5.5, 6.8, 8, 9 and 10) and temperature (5, 15, 20, 28, 34, 37 and 40°C), according to Florentino *et al*. (2010), and the ability to assimilate 16 carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, maltose, mannitol, L-methionine, sodium lactate and sucrose), and 8 nitrogen sources (L-arginine, L-asparagine, casein hydrolisate, L-cysteine, glycine, L-glutamic acid, L-methionine and tryptophan). The assimilation of carbon sources was evaluated in modified medium 79: 10 g carbon source; 0,5g K_2HPO_4; 0,5 g KNO_3; 0,2 g $MgSO_4.7H_2O$; 0,1 g NaCl; 0,5 g $CaCO_3$; 4 mL Fe-EDTA (1,64%); 2 mL of micronutrients solution (2.86 mg H_3BO_3 $liter^{-1}$; 2.03 mg $MnSO_4.4H_2O$ $liter^{-1}$; 0.22 mg $ZnSO_4 \cdot 7H_2O$ $liter^{-1}$; 0.08 mg

$CuSO_4 \cdot 5H_2O$ liter^{-1} and 0.09 mg $Na_2MoO_4 \cdot H_2O$ liter^{-1}); 5 mL of bromothymol blue solution (0.5% in KOH at 0,2N); 15 g agar, and pH 6.8. The composition of medium 79 to evaluate the assimilation of nitrogen sources is the same as described above, substituting KNO_3 by one of the cited sources, and using mannitol as carbon source. Besides these tests, strain UFLA 03-321^T was characterized using the API 20NE (bioMérieux), according to the manufacturer's instructions, with five days incubation. Differential phenotypic characteristics between the three strains and *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T are shown in Table 1. The detailed phenotypic characterization is presented in the description of the novel species.

As standard method for defining novel species (Wayne *et al*., 1987), DNA-DNA hybridization experiments were carried out, according to methodology previously described (Ezaki *et al*., 1989; Willems *et al*., 2001). DNA-DNA binding between UFLA 03-321^T and *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T was 20.6 and 22.6%, respectively. These results allows the clustering of the studied strains as a novel species within the *Bradyrhizobium* genus, since DNA-DNA hybridization values below to 70% are indicative of novel species (Wayne *et al*., 1987). The G+C content of the DNA of UFLA 03-321^T, determined by HPLC (Mesbah *et al*., 1989), is 63.7 mol%. This value is within the range reported for *Bradyrhizobium* species (59 to 65.1 mol%) (Chahboune *et al*., 2011; Ramírez-Bahena *et al*., 2012).

In the present study, additionally the sequences of *nodC* and *nifH* genes of the three strains were analyzed. DNA extraction was carried out as described above. The primers used and the amplification and sequencing of the *nodC* gene were carried out according to Sarita *et al*. (2005), modified by De Meyer *et al*. (2011). For *nifH* gene, the analysis was carried out according to Gaby and Buckley (2012). Alignment of sequences and construction of phylogenetic trees were carried out as described above. Results of the phylogenetic analysis of these genes are presented in the

supplementary material (Fig. S1 and S2). UFLA 03-321^T and UFLA 03-320 presented sequences identical *nodC* and *nifH* genes. Sequence analysis of the *nifH* gene clustered these two strains with *B. tropiciagri* CNPSo 1112^T, with 99.47% similarity (Table S1). In the sequence analysis of *nodC* gene, strains UFLA 03-321^T and UFLA 03-320 formed a new cluster, close to *B. tropiciagri* CNPSo 1112^T and *B. embrapense* CNPSo 2833^T, with similarity of 94.47 and 93.42%, respectively (Table S1). On the other hand, strain UFLA 03-290 grouped separately from UFLA 03-321^T in the phylogenetic tree of both genes (Fig. S1 and S2), and the similarity with this strain was only 78.30 and 87.47% for *nodC* and *nifH* genes, respectively (Table S1). Probably, the evolutionary history of *nodC* and *nifH* genes of UFLA 03-290 and the strains UFLA 03-321^T and UFLA 03-320 is different, which may be associated with the region and ecosystem of origin.

In previous studies, the three strains were evaluated for the ability of nodulation and nitrogen fixation in symbiosis with *Vigna unguiculata*, *Phaseolus lunatus*, *Stizolobium aterrimum* and *Acacia mangium* (Jaramillo *et al*., 2013; Rufini *et al*., 2014; Costa *et al*. unpublished data). Strains UFLA 03-321^T and UFLA 03-320 efficiently nodulate and fix nitrogen with *V. unguiculata* and *S. aterrimum*. On the other hand, UFLA 03-290 nodulates these species, but presents low symbiotic efficiency. None of the strains nodulates *A. mangium*. With regard to *P. lunatus*, strains UFLA 03-321^T and UFLA 03-320 inefficiently nodulate, and strain UFLA 03-290 does not nodulate this legume. UFLA 03-321^T also nodulates *Glycine max* (Guimarães *et al*., 2015). These results confirm symbiotic differences between strains UFLA 03-321^T and UFLA 03-320 and strain UFLA 03-290, as reflected in the phylogeny of *nodC* and *nifH* genes.

Based on the phenotypic, genotypic and symbiotic characteristics presented in this study, it is suggested to classify the studied strains within a novel species, for which the name *Bradyrhizobium brasilense* sp. nov. is proposed, with UFLA 03-321^T as the type strain.

Description of *Bradyrhizobium brasilense* sp. nov.

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

Cells are gram-negative, aerobic, non-spore-forming rods (Fig. S3). Colonies present a diameter of 1 mm in medium 79, after five days incubation at 28°C, and are cream-colored. The three strains show alkaline reaction in medium 79 using mannitol as carbon source and bromothymol blue as indicator. They grow in pH from 4 to 10, and temperature from 15 to 37°C, with optimal growth at 28°C. Salinity tolerance varies between strains. UFLA 03-321^T tolerates up to 0.75% NaCl. They are resistant to ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), doxycycline (30 μg mL^{-1}) erythromycin (15 μg mL^{-1}), gentamicin (10 μg mL^{-1}) and neomycin (30 μg mL^{-1}), and are sensitive to kanamycin (30 μg mL^{-1}). They are positive for the use of D-arabinose, glycerol, L-glutamic acid and mannitol, but do not use citric acid, glycine, malic acid, maltose, L-methionine, sodium lactate nor sucrose as carbon source. They barely use L-asparagine, D-glucose. The use of D-fructose, L-glutamine, and lactose as carbon source varies among strains. L-asparagine, casein hydrolysate, and L-glutamic acid are used as nitrogen source, whereas L-cysteine, glycine, L-methionine and tryptophan are not used as nitrogen source. The use of L-arginine as nitrogen source varies between strains. UFLA 03-321T shows negative reaction for reduction of nitrate, tryptophan deaminase activity, glucose fermentation, arginine dihydrolase, and esculin hydrolysis; and positive reaction for urease and hydrolysis of gelatin.

The type strain UFLA 03-321^T (= LMG 29353^T) was isolated from effective nodules of cowpea inoculated with soil collected in Lavras, Minas Gerais, Brazil. G+C content of DNA of UFLA 03-321^T is 63.7 mol%.

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Table 1 Differential phenotypic of *Bradyrhizobium brasilense* (UFLA 03-321^T, UFLA 03-320 and UFLA 03-290) and the most closely related type strains (*Bradyrhizobium elkanii* LMG 6134^T and *Bradyrhizobium pachyrhizi* LMG 24246^T). Data represent the means of three biological replicates.+, growth; −, no growth; w, weakly positive.

Characteristic	**UFLA 03-321^T**	**UFLA 03-320**	**UFLA 03-290**	**LMG 6134^T**	**LMG 24246^T**
Growth at					
40 °C	-	-	-	w	w
0.75% NaCl	+	+	-	+	+
Carbon source assimilation					
L-asparagine	w	w	w	+	+
D-fructose	+	+	w	+	+
D-glucose	w	w	w	+	+
L-Glutamine	w	w	w	+	+
Lactose	-	w	w	w	+
Malic acid	-	-	-	w	w
L-Methionine	-	-	-	w	w
Sodium lactate	-	-	-	+	+
Sucrose	-	-	-	+	+
Nitrogen source assimilation					
L-Arginine	w	+	w	+	+
Casein hydrolysate	+	+	+	w	w
L-Cysteine	-	-	-	+	+
L-Methionine	-	-	-	w	w
Resistance to antibiotics (µg mL^{-1})					
Gentamycin (10)	+	+	+	w	-
Neomycin (30)	+	+	+	-	-

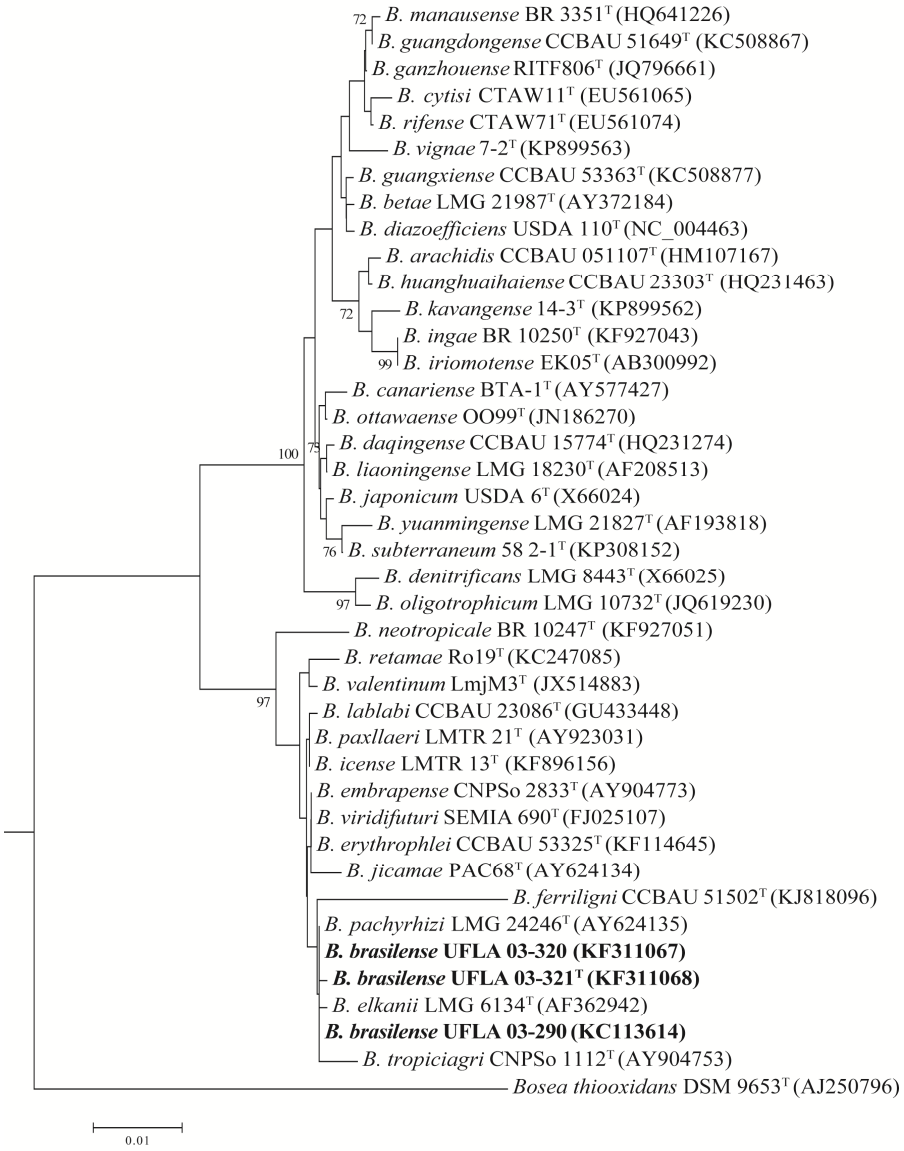

Fig. 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences (1176 pb) showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. The 16S rRNA sequence of *Bosea thiooxidans* DSM9653^T was used as outgroup. Gene accession numbers for each strain are given in parentheses.

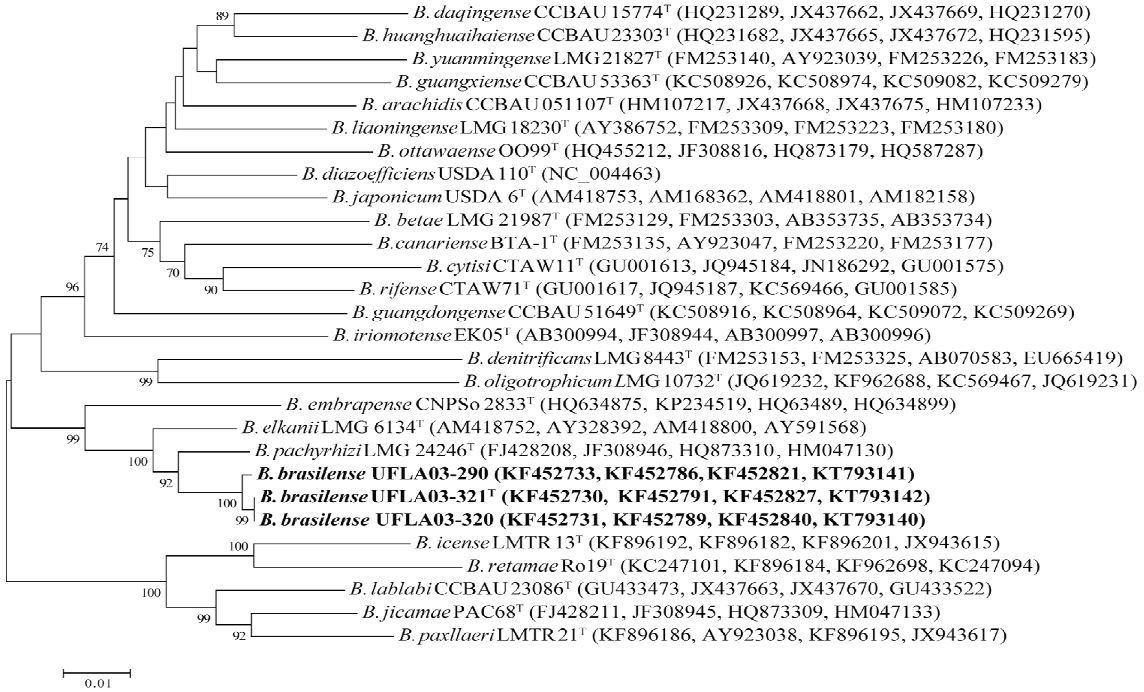

Fig. 2. Neighbour-joining phylogeny based on partial concatenated sequences (1593 pb) of *atpD, dnaK, gyrB* and *recA* genes showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. Gene accession numbers for each strain are given in parentheses.

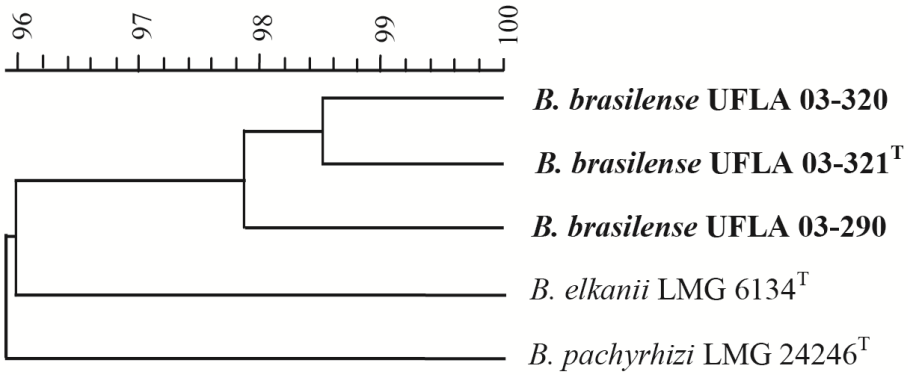

Fig. 3. Cluster analysis of MALDI-TOF MS profiles of *Bradyrizobium brasilense* and type strains of the closest species (*B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T). The dendrogram was constructed by applying UPGMA method using arithmetic averages with correlation levels expressed.

Supplementary Material

Table S1 Similarity within *Bradyrhizobium brasilense* and between *B. brasilense* UFLA 03-321^{T} and other type strains of *Bradyrhizobium* species in the 16S rRNA, housekeeping and symbiotic genes.

Strains	Similarity with UFLA 03-321^{T} (%)							
	16S rRNA	***atpD***	***dnaK***	***gyrB***	***recA***	**Concatenated***	***nodC***	***nifH***
***B. brasilense* UFLA 03-320**	99.91	100	100	100	100	100	100	100
***B. brasilense* UFLA 03-290**	99.91	100	100	99.46	99.46	99.69	78.30	87.47
B. arachidis CCBAU 051107^{T}	96.50	92.12	85.22	92.42	91.84	91.23	79.82	87.14
B. betae LMG 21987^{T}	96.84	92.30	89.28	90.76	91.20	91.20	-	-
B. canariense BTA-1^{T}	96.93	93.34	84.99	91.10	91.00	91.38	66.25	80.70
B. cytisi CTAW11^{T}	96.40	93.33	88.79	90.40	90.04	90.48	68.80	80.58
B. daqingense CCBAU 15774^{T}	97.08	91.24	86.71	92.14	90.35	90.41	75.28	82.87
B. denitrificans LMG 8443^{T}	96.54	90.80	88.58	89.69	85.38	89.68	-	81.38
B. diazoefficiens USDA 110^{T}	96.84	93.31	86.66	93.21	89.91	92.16	75.28	82.87
B. elkanii LMG 6134^{T}	99.83	96.91	98.58	98.36	95.12	97.28	78.64	89.07
B. embrapense CNPSo 2833^{T}	99.74	95.39	92.94	95.88	93.80	94.62	93.42	98.92
B. erythrophlei CCBAU 53325^{T}	99.74	-	-	91.19	92.89	-	71.11	87.13
B. ferriligni CCBAU 51502^{T}	97.75	-	-	92.57	95.56	-	78.88	88.04
B. ganzhouense RITF 806^{T}	96.68	91.80	-	91.75	89.89	-	62.56	81.87
B. guangdongense CCBAU51649^{T}	96.61	92.00	86.96	92.67	89.88	-	-	75.69
B. guangxiense CCBAU53363^{T}	96.84	91.46	84.57	91.96	91.51	-	-	85.54
B. huanghuaihaiense CCBAU23303^{T}	96.61	93.08	86.54	92.42	90.71	91.36	75.28	82.87
B. icense LMTR 13^{T}	99.74	91.07	89.41	87.94	91.58	90.31	71.28	85.78
B. ingae BR 10250^{T}	96.28	-	89.54	90.96	88.88		64.82	83.37

Continuation....

Strains	Similarity with UFLA 03-321^T (%)							
	16S rRNA	***atpD***	***dnaK***	***gyrB***	***recA***	**Concatenated***	***nodC***	***nifH***
B. iriomotense EK05^T	96.28	93.73	90.88	92.07	89.25	91.72	64.19	78.74
B. japonicum USDA 6^T	97.08	94.37	86.43	93.01	90.54	91.81	75.28	82.87
B. jicamae PAC68^T	99.40	92.62	90.32	89.94	89.95	90.97	70.46	83.38
B. kavangense 14-3^T	96.32	-	81.01	-	90.99	-	78.93	88.66
B. lablabi CCBAU 23086^T	99.65	91.71	90.27	88.87	92.68	91.16	71.10	85.72
B. liaoningense LMG 18230^T	97.16	91.59	86.35	93.02	92.86	91.71	61.95	82.87
B. manausense BR 3351^T	96.52	-	88.68	90.67	91.14	-	63.61	79.87
B. neotropicale BR 10247^T	98.61	-	88.59	92.47	91.64	-	67.07	82.44
B. oligotrophicum LMG 10732^T	96.65	90.29	87.30	89.68	87.08	89.80	-	-
B. ottawaense OO99^T	97.16	91.71	85.35	91.29	92.13	91.01	75.28	82.87
B. pachyrhizi LMG 24246^T	99.91	96.03	99.58	98.45	98.29	97.83	79.20	89.63
B. paxllaeri LMTR 21^T	99.74	90.87	90.43	89.10	90.32	90.45	70.94	85.72
B. retamae Ro19^T	99.25	89.78	86.83	87.41	91.51	89.58	71.34	85.63
B. rifense CTAW71^T	96.61	92.94	89.60	91.19	91.11	91.37	67.84	
B. subterraneum 58 2-1^T	96.98	-	89.97	-	90.23	-		88.68
B. tropiciagri CNPSo 1112^T	99.49	-	95.40	96.34	94.86	-	94.47	99.47
B. valentinum Lmj M3^T	99.51	90.00	-	-	92.44	-	71.47	85.93
B. vignae 7-2^T	96.45	-	86.16		90.87	-	79.12	89.67
B. viridifuturi SEMIA 690^T	99.74	-	96.66	96.31	94.43	-		98.92
B. yuanmingense LMG 21827^T	96.64	95.07	85.09	91.50	91.80	90.55	75.67	85.50

*Concatenated sequences with four genes (*atpD*, *dnaK*, *gyrB* and *recA*).

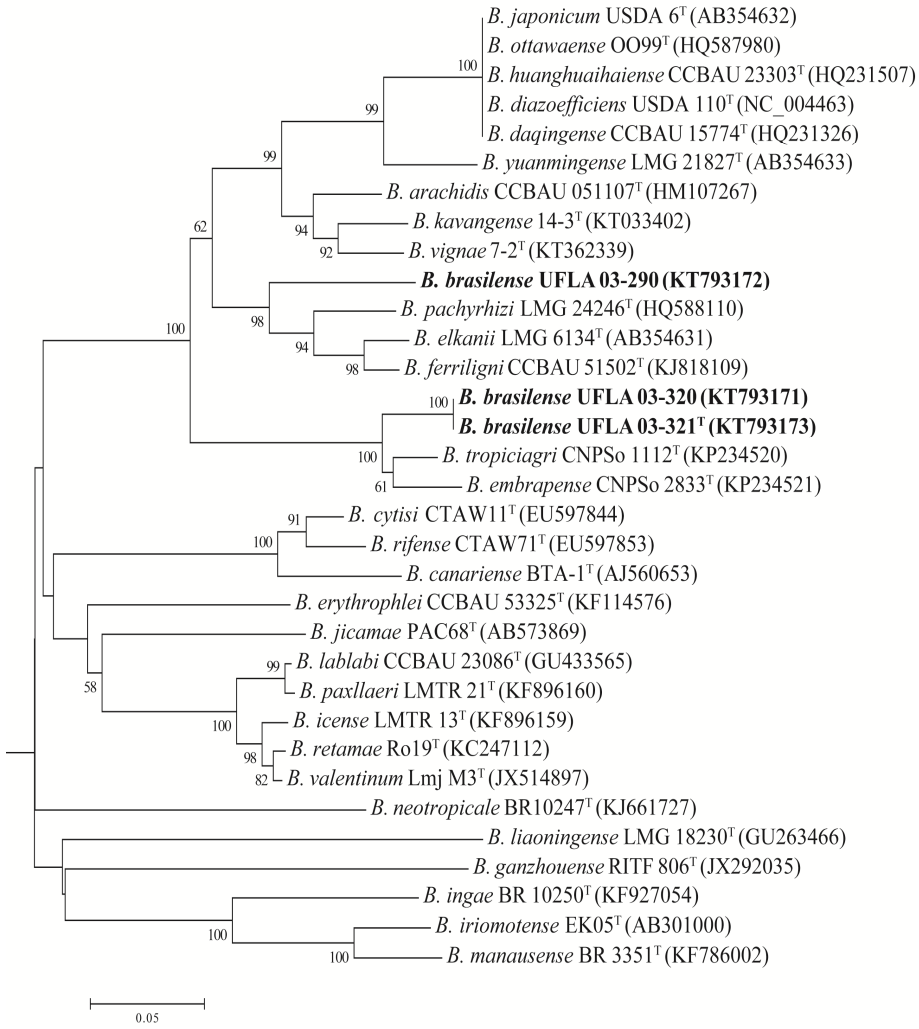

Fig. S1. Neighbour-joining phylogeny based on partial sequences (441 pb) of *nodC* gene showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene accession numbers for each strain are given in parentheses.

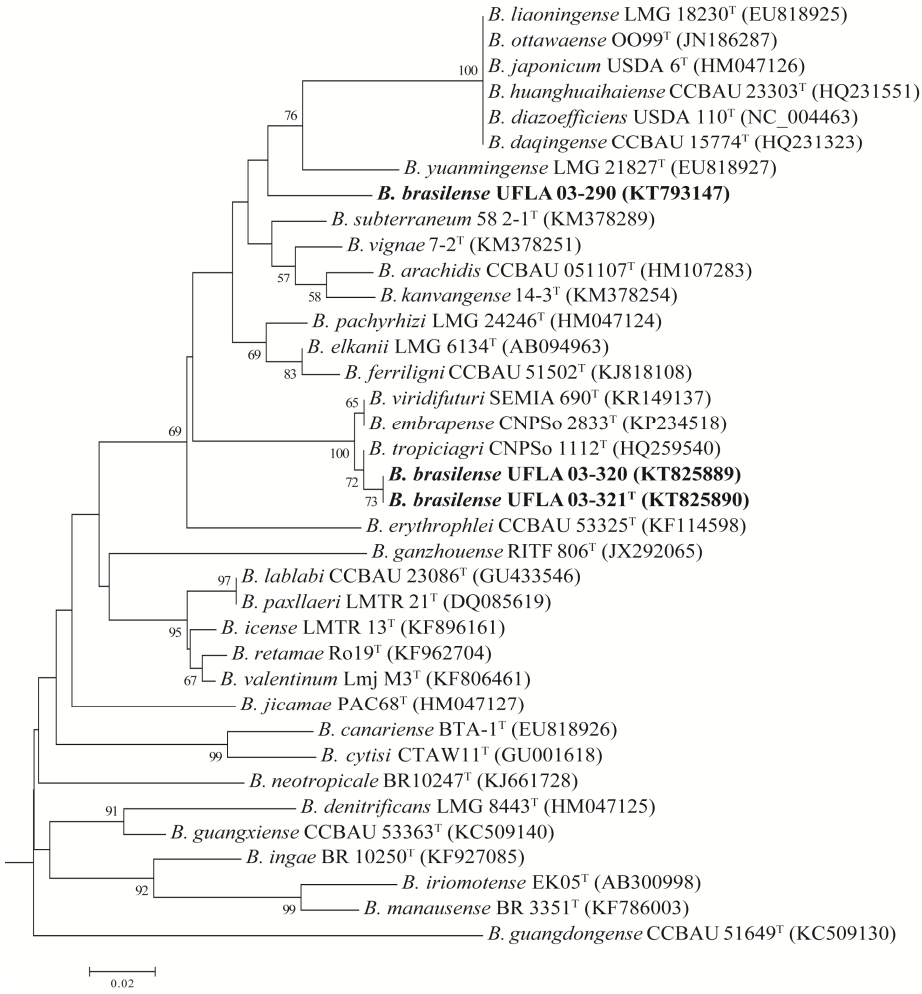

Fig. S2. Neighbour-joining phylogeny based on partial sequence (177 pb) of *nifH* gene showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 50% are indicated at nodes. Gene accession numbers for each strain are given in parentheses.

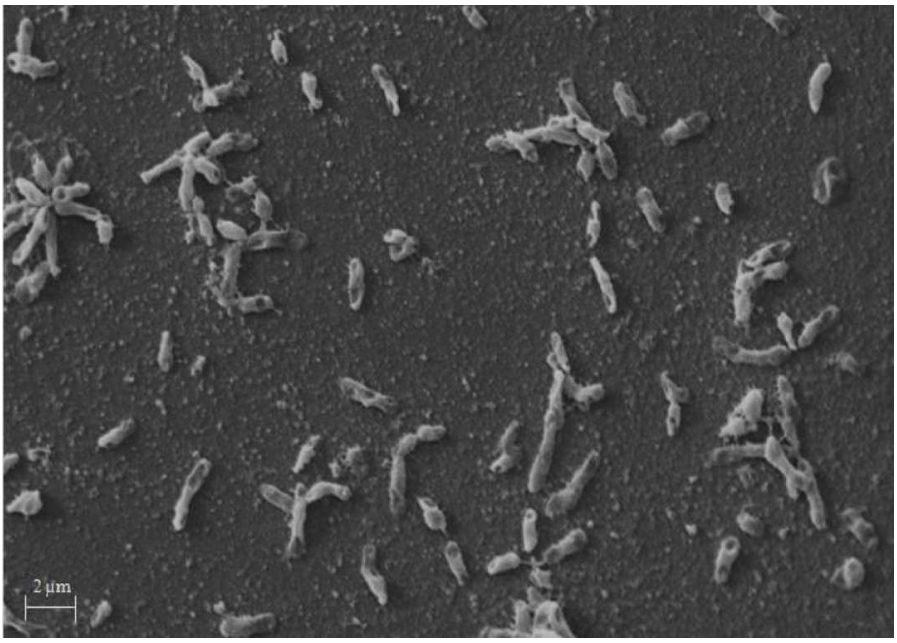

Fig. S3. Image of strain UFLA 03-321^{T} obtained by scanning electron microscopy.

ARTIGO 2 - *Bradyrhizobium forestalis* sp. nov., an efficient nitrogen-fixing bacterium isolated from nodules of forest legume species in the Amazon

Artigo de acordo com as normas da revista International Journal of Systematic and Evolutionary Microbiology (Versão preliminar)

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

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Running title: *Bradyrhizobium forestalis* sp. nov.

New sequences: The accession numbers for the 16S rRNA , *atpD*, *gyrB*, *recA*, *nodC* and *nifH* sequences of strain INPA 01-91A are KU230296, KT793129, KT793136, KT793139, KT825895 and KT793162, respectively. The accession numbers for *nodC* and *nifH* sequences of strain INPA 54B^T are KT793177 and KT793160, respectively. The accession numbers *nodC* and *nifH* sequences of strain INPA 86A are KT793178 and KT793161, respectively.

Abstract

Three strains of nitrogen-fixing bacteria isolated from nodules of *Inga* sp. (INPA 54B^T) and *Swartzia* sp. (INPA 86A and INPA 01-91A) in soils under native forest in the Brazilian Amazon were previously identified as belonging to the *Bradyrhizobium* genus and grouped into a single group. In this study, these strains were characterized using a polyphasic approach in order to establish their taxonomic position. The three strains shared more than 99.50% sequence similarity of 16S rRNA gene with five *Bradyrhizobium* species (*B. japonicum* USDA 6^T, *B. liaoningense* LMG 18230^T, *B. ottawaense* OO99^T, *B. subterraneum* 58 2-1^T and *B. yuanmingense* LMG 21827^T). However, concatenated sequence analysis of the housekeeping genese (*atpD, gyrB* and *recA*) indicated that these three strains represent a new *Bradyrhizobium* species, and the most closely related species is *B. yuanmingense* LMG 21827^T, with 94.63% similarity. DNA-DNA relatedness between INPA 54B^T and *B. yuanmingense* LMG 21827^T was only 38.2%. Phenotypic characterization, including tests of temperature, pH and salinity range, resistance to antibiotics and assimilation of different carbon and nitrogen sources also allowed the differentiation of the novel species from *B. yuanmingense* LMG 21827^T. In the phylogenetic analysis of the symbiotic genes *nodC* and *nifH*, the three strains showed similar sequences that were divergent from those of type strains of all *Bradyrhizobium* species. Based on the data presented, we concluded that these strains represent a novel species, for which the name *Bradyrhizobium forestalis* is proposed, with INPA 54B^T (= LMG 10044^T) as type strain. The G+C content in the DNA of INPA 54B^T is 63.7 mol%.

Keywords: *Bradyrhizobium*, biological nitrogen fixation, polyphasic taxonomy, symbiotic genes

Nitrogen-fixing legume nodulating bacteria (NFLNB) are of great socioeconomic and environmental importance. Among the NFLNB genera currently described, *Bradyrhizobium* has stood out due to its wide geographic distribution and host range, besides forming efficient symbiosis with important legume species.

In the Amazon biome, which occupies approximately 49% of the Brazilian territory, studies carried out in soils under different land use systems have indicated predominance of *Bradyrhizobium* among the NFLNB genera isolated from different legume species and high phenotypic and genotypic diversity of *Bradyrhizobium* strains (Moreira *et al*., 1993; 1998; Guimarães *et al*., 2012; 2015; Silva *et al*., 2012; Jaramillo *et al*., 2013; Baraúna *et al*., 2014). Recently, three *Bradyrhizobium* species from this biome have been described: *B. manausense*, isolated from *Vigna unguiculata* (Silva *et al*., 2014a); *B. ingae*, isolated from *Inga laurina* (Silva *et al*., 2014b) and *B. neotropicale*, isolated from *Centrolobium paraense* (Zilli *et al*., 2014).

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

Guimarães *et al*. (2015) carried out sequencing of housekeeping genes of 50 *Bradyrhizobium* strains isolated in different Brazilian ecosystems, including six strains from the Amazon biome, which had been previously characterized by Moreira *et al*. (1993). Among these six strains, two (INPA

54B^T and INPA 86A) formed a separate group different from the other strains studied and from *Bradyrhizobium* species currently described.

In this study, INPA 54B^T (LMG 10044^T) and INPA 86A (LMG 10053) were selected for further analysis, by molecular and phenotypic methods. Strain INPA 01-91A (LMG 10054), which is grouped with INPA 54B^T and INPA 86 by the SDS-PAGE (Moreira *et al.*, 1993), was also included in the analyses. Results obtained in this study indicate that the three strains represent a single novel species, for which the name *Bradyrhizobium forestalis* sp. nov. is proposed.

The three strains are derived from soil under native forest of the Brazilian Amazon region. Strain INPA 54B^T was isolated from *Inga* sp. (Subfamily: Mimosoideae) nodules, and strains INPA 86A and INPA 01-91A (=INPA 91A) were isolated from *Swartzia* sp. (Subfamily: Papilionoideae) nodules (Moreira et al., 1993). Isolation and characterization of strains were carried out in culture medium 79 (Fred and Waksman, 1928), also known as YMA (Vincent, 1970). The three strains are deposited in the culture collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras, Brazil, and in the culture collection (BCCM/LMG) of the Ghent University, Belgium. INPA 54B^T = LMG 10044^T, INPA 86A = LMG 10053, INPA 01-91A = LMG 10054.

Alkaline lysis method was used for DNA extraction from the strains (Niemann et al., 1997).

Sequences of 16S rRNA gene (1284 to 1290 bp) of the three strains, and partial sequences of the genes *atpD* (453 bp), *gyrB* (594) and *recA* (510 bp) of INPA 01-91A were obtained using the same primers and amplification and sequencing cycles used by Ribeiro *et al.* (2015). The sequences of the genes *atpD*, *gyrB* and *recA* of INPA 54B^T and INPA 86A were obtained by Guimarães *et al.* (2015). For each gene, the sequences of all type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI) were included in the

alignment. The alignment of the sequences was carried out using the ClustalW Multiple Alignment algorithm in BioEdit. Distances were calculated according to the Kimura 2 Parameter method (Kimura, 1980). Phylogenetic trees were constructed by the neighbor joining (NJ) (Saitou e Nei, 1987) and maximum likelihood (ML) (Felsenstein, 1981) methods using the MEGA 5 software package (Tamura *et al*., 2011), with bootstrap values based on 1000 replications.

Results of the phylogenetic analysis of the 16S rRNA gene were similar when using the ML (data not shown) and NJ (Fig. 1) methods. The three INPA strains showed identical 16S rRNA gene sequences (Fig. 1). Strain INPA 54B^{T} shared more than 99.50% similarity with five *Bradyrhizobium* species (*B. japonicum* USDA 6^{T}, *B. liaoningense* LMG 18230^{T}, *B. ottawaense* OO99^{T}, *B. subterraneum* 58 2-1^{T} and *B. yuanmingense* LMG 21827^{T}) (Table S1). High similarity between 16S rRNA gene sequences of different *Bradyrhizobium* species have been reported previously (Willems *et al*., 2001; Wang *et al*., 2013; Durán *et al*., 2014; Silva *et al*., 2014b), which reflects the high conservation degree of this gene.

For better discrimination between members of the *Bradyrhizobium* genus, analysis of multilocus sequences of housekeeping genes have been pointed out as a reliable method (Vinuesa *et al*., 2005; Islam *et al*., 2008; Ramírez-Bahena *et al*., 2009; Durán *et al*., 2014; Guimarães *et al*., 2015; Ribeiro *et al*., 2015). In the present study, three housekeeping genes, *atpD*, *gyrB* and *recA*, were chosen for evaluation because these genes have shown high differentiation potential between *Bradyrhizobium* species (Vinuesa *et al*., 2005; Islam *et al*., 2008; Wang *et al*., 2013; Durán *et al*., 2014). Phylogenetic trees based on concatenated sequences of *atpD* (429 bp), *gyrB* (552 bp) and *recA* (374 bp) genes showed similar results when using ML (data not shown) and NJ (Fig. 2) methods. This analysis clearly showed that the INPA strains form a new group, supported with high bootstrap value (100%), separate from all *Bradyrhizobium* species described (Fig. 2). *B. yuanmingense* LMG 21827^{T} was the species that most shared similarity with

INPA 54B^T, both in the individual analysis of *atpD* (95,23%), *gyrB* (93,35%) and *recA* (95,22%) genes, and in the analysis of concatenated sequences (94.63%) (Table S1). These data suggest that INPA strains belong to a novel species within *Bradyrhizobium*, since these similarity values are similar to those found between different *Bradyrhizobium* species (Chahboune *et al*., 2011; Durán *et al*., 2014; Silva *et al*., 2014b).

Several phenotypic characteristics were evaluated to compare INPA 54B^T, INPA 86A and INPA 01-91A with *B. yuanmingense* LMG 21827^T. Strains INPA 54B^T and INPA 86A had been evaluated, in previous studies, regarding the ability to grow in culture medium 79 (Fred and Waksman, 1928) under different temperature condition and NaCl concentrations (w/v) (0.01, 0.25, 0.5, 0.75 and 1%) (Guimarães *et al*., 2015), and regarding their resistance to the following antibiotic: ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 ug μg mL^{-1}), doxycycline (30 μg mL^{-1}), erythromycin (15 μg mL^{-1}), gentamicin (10 μg mL^{-1}), kanamycin (30 μg mL^{-1}), and neomycin (30 μg mL^{-1}) (Guimarães *et al*., 2015). This data set was supplemented with test to establish the pH (pH 4, 5.5, 6.8, 8, 9 and 10) and temperatures (5, 15, 20, 28, 34, 37 and 40°C) range for growth. To allow comparison, in the present study we evaluated the growth of INPA 01-91A and *B. yuanmingense* LMG 21827^T in culture medium 79 under the same conditions of temperature, pH and NaCl, and their resistance to the nine antibiotics cited, following the same protocols used to INPA 54B^T and INPA 86A.

The three INPA strains and *B. yuanmingense* LMG 21827^T were also evaluated, in this study, regarding the ability to assimilate 16 carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, maltose, mannitol, L-methionine, sodium lactate and sucrose) and 8 nitrogen sources (L-arginine, L-asparagine, hydrolyzed casein, L-cysteine, glycine, L glutamic-acid, L-methionine and tryptophan). To evaluate the assimilation of carbon sources, modified culture medium 79 was used containing, per liter,

10 g carbon source; 0.5 g K_2HPO_4; 0.5 g KNO_3; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.5 g $CaCO_3$; 4 mL Fe-EDTA (1.64%); 2 mL micronutrient solution (2.86 mg H_3BO_3 $liter^{-1}$; 2.03 mg $MnSO_4.4H_2O$ $liter^{-1}$; 0.22 mg $ZnSO_4 \cdot 7H_2O$ $liter^{-1}$; 0.08 mg $CuSO_4 \cdot 5H_2O$ $liter^{-1}$ and 0.09 mg $Na_2MoO_4 \cdot H_2O$ $liter^{-1}$); 5 mL bromothymol blue solution (0.5% in KOH at 0,2N); 15 g agar; and pH 6.8. To evaluate the assimilation of nitrogen sources, the same protocol was used as described above, replacing KNO_3 by one of the cited sources, using mannitol as carbon source. Strain INPA $54B^T$ was also characterized using the API 20NE kit (bioMérieux), according to the manufacturer's instructions, with five days incubation.

The main differential phenotypic characteristics between the strains of the new group and *B. yuanmingense* LMG 21827^T are shown in Table 1. In the description of the new species, the phenotypic characterization is detailed. For some characteristics, different behaviors between INPA $54B^T$ and strains INPA 86A and INPA 01-91A were observed, indicating phenotypic diversity within the novel species (Table 1).

The strains were also compared by analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles. For this analysis, third generation cultures grown in YMA medium (Vincent, 1970) were used. Sample preparation and data analysis were carried out as previously described (Wieme *et al*., 2014). Results of MALDI-TOF MS analysis showed that INPA $54B^T$ has protein profile slightly different from INPA 86A and INPA 01-91A, however, all three strains showed very similar profiles (Fig. 3).

For confirmation of the novel species, DNA-DNA hybridization experiments were carried out, according to methodology previously described (Ezaki *et al*., 1989; Willems *et al*., 2001). First, DNA-DNA hybridization experiment was carried out with strain INPA $54B^T$ (proposed as type strain), which was isolated from *Inga* sp., and the strain INPA 86A, isolated from *Swartzia* sp. DNA-DNA relatedness between these two strains was high (83%), confirming that they belong to the same species (Wayne *et*

al., 1987). Subsequently, strains INPA 54B^T and INPA 86A were hybridized with *B. yuanmingense* LMG 21827^T. DNA-DNA relatedness between them was only 38.2 and 33.6%, respectively. Since this value is far below the limit value (70%) indicated for delineation of new species (Wayne *et al*., 1987), we can confirm that INPA strains represent a novel species within the *Bradyrhizobium* genus. The G+C content in the DNA of strain INPA 54B^T, determined by HPLC (Mesbah *et al*.1989), was 63.7 mol%.

Genes involved in nodulation and nitrogen fixation, such as *nodC* and *nifH*, respectively, are generally evaluated in symbiotic characterization of novel species of NFLNB. In this study, sequences of *nodC* and *nifH* genes of the three INPA strains were compared with those of type stains of *Bradyrhizobium* species available in GenBank (National Center for Biotechnology Information, NCBI). DNA extraction was carried out as described above. Primers used, amplification and sequencing of *nodC* gene were carried out according to Sarita *et al*. (2005), modified by De Meyer *et al*. (2011). The analysis of *nifH* gene was carried out according to Gaby and Buckley (2012). Sequences alignment and construction of phylogenetic trees were carried out as described above. In the analyses of both genes (*nodC* and *nifH*), INPA strains showed identical sequences and formed a new phylogenetic line (Fig. S1 and S2). The closest species to INPA 54B^T was *B. arachidis* CCBAU 051107^T, with 97.30 and 97.44% similarity, in the analysis of the sequences of *nodC* and *nifH* genes, respectively (Table S1).

Nodulation ability of INPA 54B^T, INPA 86A and INPA 01-91A was confirmed by inoculation tests with three legume species: *Macroptilium atropurpureum*, *Vigna unguiculata* and *Phaseolus lunatus*. Strain INPA 54B^T was also evaluated regarding its ability to nodulate other legume species, which indicated that this strain does not nodulate *Glycine max* and *Acacia mangium*, but it does nodulate *Stizolobium aterrimum*.

Results of genotypic, phenotypic and symbiotic analyses presented in this study indicate that INPA strains should be classified as a novel species

within the *Bradyrhizobium* genus. The name *Bradyrhizobium forestalis* sp. nov is proposed for the new taxon, with INPA 54B^T as type strain.

Description of *Bradyrhizobium forestalis* sp. nov.

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

Cells are gram-negative, aerobic, non-spore-forming rods (Fig. S3). The three strains form cream-colored colonies with diameter > 1 mm. They produce an alkaline reaction with culture medium 79 using mannitol as carbon source and bromothymol blue as indicator, five days after incubation, at 28 ºC. All strains grow in pH from 4 to 10, and at temperature from 15 to 37 ºC, with optimal growth at 28°C, but do not grow at 5°C. INPA 86A and INPA 01-91A show weak growth at 40°C, but INPA 54B^T does not grow at this temperature. Salinity tolerance varies among strains. INPA 54B^T tolerates up to 0.75% NaCl, while INPA 86A and INPA 01-91A tolerate only up to 0.50% NaCl. The three strains are resistant to ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}) and doxycycline (30 μg mL^{-1}); but they are sensitive to ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), kanamycin (30 μg mL^{-1}), neomycin (30 μg mL^{-1}) and gentamicin (10 μg mL^{-1}). Resistance to erythromycin (15 mL^{-1}) varies among strains. They can assimilate D-arabinose, D-fructose, L-glutamic acid and mannitol, but they do not use citric acid, malic acid, glycine, lactose, L-methionine and sodium lactate, as carbon source. They weakly use L-asparagine, D-glucose and sucrose. The use of glycerol and L-glutamine as carbon source varies among strains. The use of L-asparagine and L-glutamic acid as nitrogen source is positive, but the use of casein hydrolyzate, L-cysteine, glycine, L-methionine and tryptophan is negative. The use of L-arginine as nitrogen source varies among the strains. Strain INPA 54B^T is positive for urease, esculin hydrolysis and gelatin hydrolysis, and negative for nitrate reduction,

tryptophan deaminase activity, glucose fermentation and arginine dihydrolase.

The type strain INPA 54B^T (= LMG 10044^T) was isolated from effective nodules of *Inga* sp. in soil under native forest in the Amazon, Brazil. The G+C content in the DNA of INPA 54B^T is 63.7 mol%.

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Table 1 Differential phenotypic characteristics between strains of *Bradyrhizobium forestalis* (INPA 54B^T, INPA 86A and INPA 01-91A) and the most closely related type strain (*Bradyrhizobium yuanmingense* LMG 21827^T).

Characteristic	INPA 54B^T	INPA 86A	INPA 01-91A	LMG 21827^T
Growth at				
40 ºC	-	w	w	+
0,75% NaCl	+	-	-	-
Assimilation of carbon source				
D-fructose	+	+	+	w
Glycerol	w	+	+	+
D-glucose	w	w	w	+
Lactose	-	-	-	+
Methionine	-	-	-	w
Sodium lactate	-	-	-	+
Sucrose	w	w	w	+
Assimilation of nitrogen source				
Arginine	w	w	w	-
Cysteine	-	-	-	w
Resistance to antibiotics (µg mL^{-1})				
Erythromycin (15)	-	+	+	+
Gentamycin (10)	-	-	-	+
Kanamycin (30)	-	-	-	+
Neomycin (30)	-	-	-	+

Data represent the means of three biological replicates.+, growth; −, no growth; w, weakly positive.

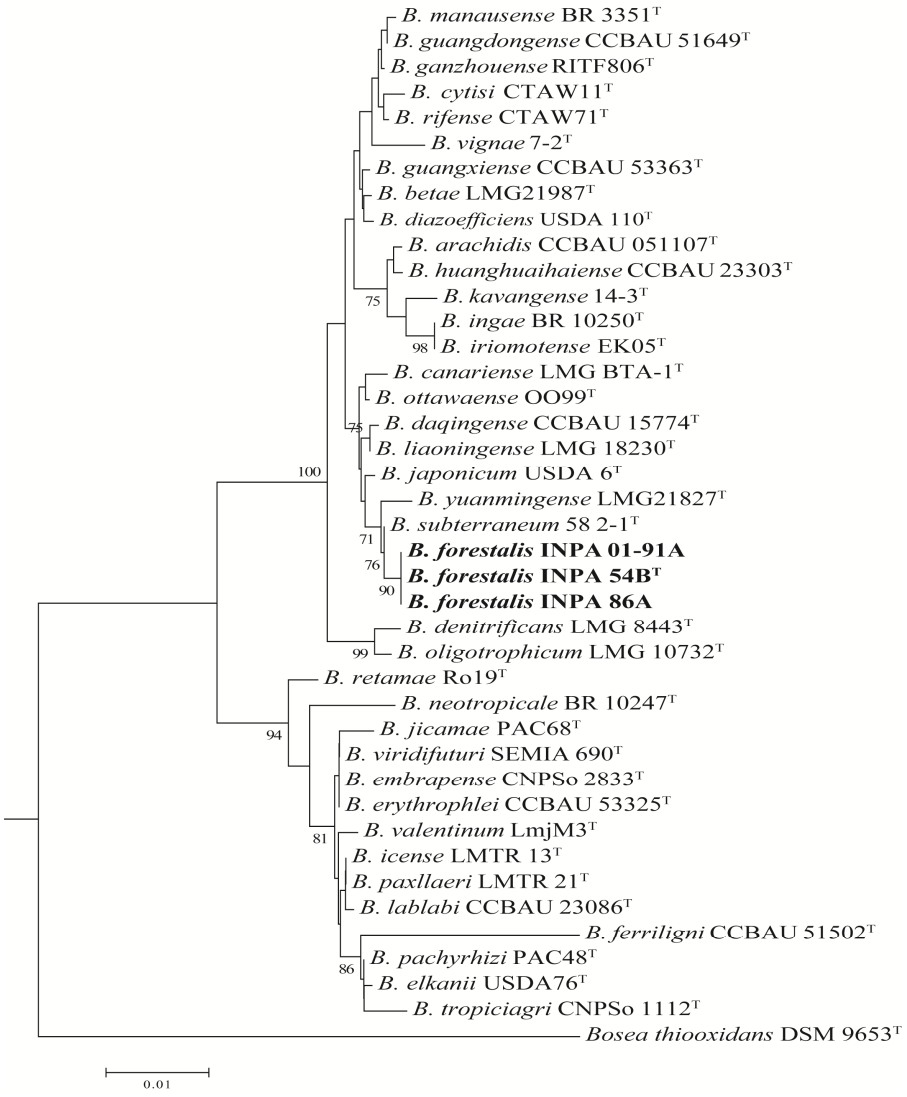

Fig. 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences (1228 bp) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. The 16S rRNA sequence of *Bosea thiooxidans* DSM9653^T was used as outgroup. GenBank accession numbers for each strain are given in Table S2.

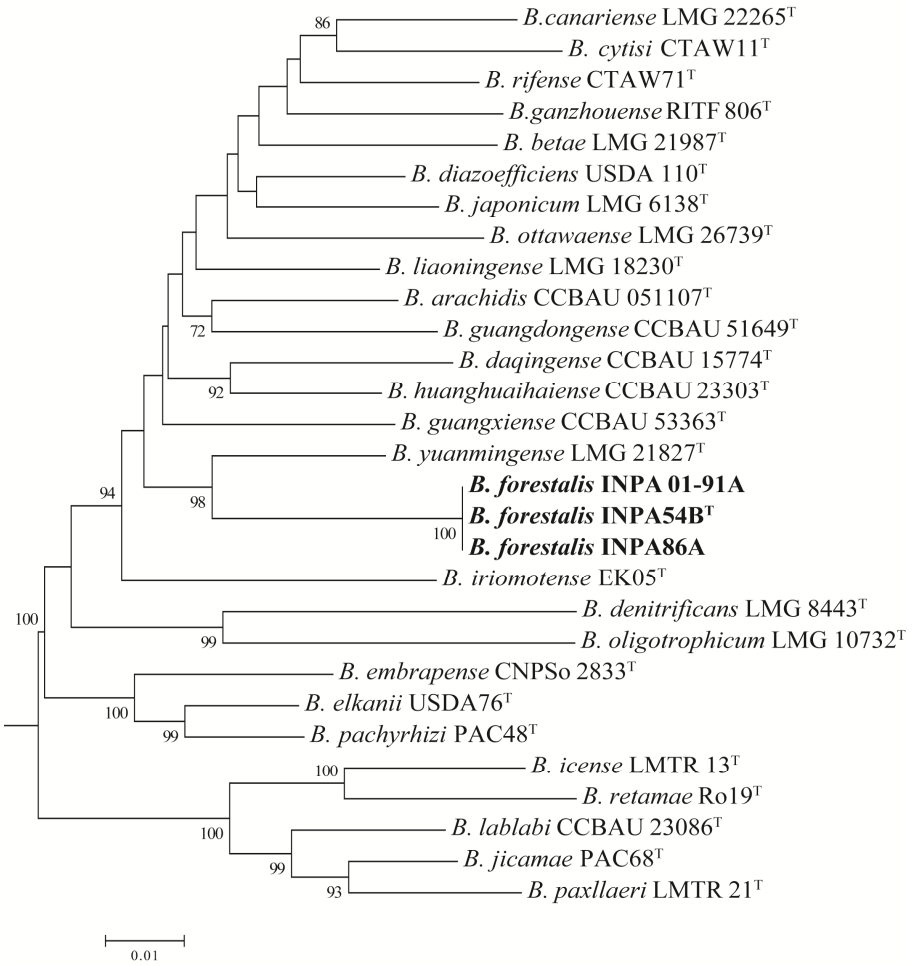

Fig. 2. Neighbour-joining phylogeny based on partial concatenated sequences (1355 bp) of housekeeping genes (*atpD*, *gyrB* and *recA*) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers of the genes sequences for each strain are given in Table S2.

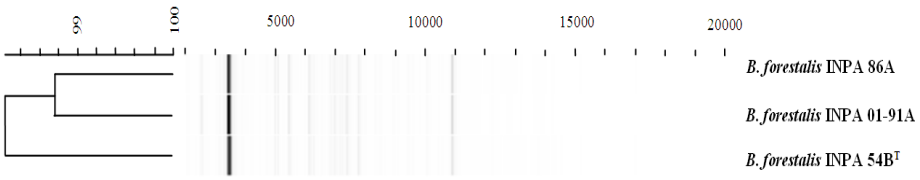

Fig. 3. Cluster analysis of MALDI-TOF MS profiles of *Bradyrizobium forestalis*. The dendrogram was constructed by applying UPGMA method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient. Profiles are shown as band patterns, units are m/z values

Supplementary Material

Table S1 Similarity within *Bradyrhizobium forestalis* and between *B. forestalis* INPA 54B^{T} and other type strains of *Bradyrhizobium* species in the 16S rRNA, housekeeping and symbiotic genes.

Strains	**Similarity with INPA 54B^{T} (%)**						
	16S rRNA	***atpD***	***gyrB***	***recA***	**Concatenated***	***nodC***	***nifH***
***B. forestalis* INPA 86[a]**	100.00	100.00	100.00	100.00	100.00	100.00	100.00
***B. forestalis* INPA 01-91[a]**	100.00	100.00	100.00	100.00	100.00	100.00	100.00
B. arachidis CCBAU 051107^{T}	98.91	92.74	91.56	93.42	92.74	97.30	97.44
B. betae LMG 21987^{T}	99.21	92.94	89.84	92.45	91.48	-	-
B. canariense BTA-1^{T}	99.32	93.90	90.25	92.05	91.24	70.34	83.65
B. cytisi CTAW11^{T}	98.88	93.89	89.31	90.85	91.24	72.86	83.39
B. daqingense CCBAU 15774^{T}	99.45	91.76	92.40	91.47	92.04	87.66	88.01
B. denitrificans LMG 8443^{T}	98.57	90.90	86.39	87.53	88.63	-	83.66
B. diazoefficiens USDA 110^{T}	99.19	94.13	92.14	92.27	92.65	87.66	88.01
B. elkanii USDA76^{T}	96.70	93.72	90.25	92.70	91.48	83.72	92.77
B. embrapense CNPSo 2833^{T}	97.02	93.70	89.61	91.96	91.04	78.94	88.21
B. erythrophlei CCBAU 53325^{T}	97.02	-	87.71	91.92	-	76.33	88.74
B. ferriligni CCBAU 51502^{T}	94.69	-	86.48	92.58	-	84.52	91.77
B. ganzhouense RITF 806^{T}	99.08	92.71	90.70	92.15	91.41	66.18	83.13
B. guangdongense CCBAU 51649^{T}	99.05	92.74	91.59	91.54	92.24	-	78.22
B. guangxiense CCBAU 53363^{T}	99.22	92.23	91.81	93.19	92.77	-	87.30
B. huanghuaihaiense CCBAU23303^{T}	98.90	93.63	92.77	91.80	92.95	87.66	88.01
B. icense LMTR 13^{T}	96.96	92.25	84.63	91.42	88.45	72.60	86.82
B. ingae BR 10250^{T}	98.59	-	89.89	89.81	-	65.74	84.20
B. iriomotense EK05^{T}	98.59	93.92	90.87	90.16	91.69	64.74	80.05
B. japonicum USDA 6^{T}	99.56	94.85	92.16	92.86	92.22	87.66	88.01

Continuation...

Strains	**Similarity with INPA 54B^T (%)**						
	16S rRNA	***atpD***	***gyrB***	***recA***	**Concatenated***	***nodC***	***nifH***
B. jicamae PAC68^T	96.69	94.16	86.54	89.54	89.30	71.57	86.85
B. kavangense 14-3^T	98.57	-	-	92.49	-	93.60	96.88
B. lablabi CCBAU 23086^T	96.88	93.20	85.68	91.95	89.46	72.41	86.21
B. liaoningense LMG 18230^T	99.53	92.01	92.08	95.02	92.98	60.86	88.01
B. manausense BR 3351^T	98.97	-	91.15	92.28	-	64.21	81.06
B. neotropicale BR 10247^T	96.48	-	91.41	93.15	-	67.89	84.31
B. oligotrophicum LMG 10732^T	98.66	91.52	86.40	89.05	88.65	-	-
B. ottawaense OO99^T	99.51	92.15	90.28	92.07	91.65	87.66	88.01
B. pachyrhizi PAC48^T	96.79	93.59	90.34	91.68	91.41	83.40	94.75
B. paxllaeri LMTR 21^T	96.96	92.19	85.79	89.68	88.49	72.18	86.21
B. retamae Ro19^T	97.23	91.00	83.95	91.46	87.80	73.07	86.50
B. rifense CTAW71^T	99.04	93.53	90.18	92.11	91.72	71.45	-
B. subterraneum 58 2-1T	99.84	-	-	94.11	-	-	94.47
B. tropiciagri CNPSo 1112T	96.36	-	90.28	92.59	-	80.93	88.18
B. valentinum Lmj M3^T	96.84	91.46	-	92.06	-	72.87	86.83
B. viridifuturi SEMIA 690^T	97.02	-	90.30	92.24	-	-	88.21
B. vignae 7-2^T	98.68	-	-	92.23	-	93.65	96.13
B. yuanmingense LMG 21827^T	99.67	95.23	93.35	95.22	94.63	86.62	90.60

*Concatenated sequences with three genes (*atpD*, *gyrB* and *recA*).

Table S2 GenBank accession numbers of the sequences used used in this study. Sequences obtained in this study are shown in bold.

Strains	Genome	16S rRNA	*atpD*	*gyrB*	*recA*	*nodC*	*nifH*
***B. forestalis* INPA 54B^T**		KR779520	KF452722	KF452831	KF452867	**KT793177**	**KT793160**
***B. forestalis* INPA 86^a**		KR779521	KF452725	KF452832	KF452865	**KT793178**	**KT793161**
***B. forestalis* INPA 01-91A**		**KU230296**	**KT793129**	**KT793136**	**KT793139**	**KT825895**	**KT793162**
B. arachidis CCBAU 051107^T		HM107167	HM107217	JX437675	HM107233	HM107267	HM107283
B. betae LMG 21987^T		AY372184	FM253129	AB353735	AB353734	-	-
B. canariense BTA-1^T		AY577427	FM253135	FM253220	FM253177	AJ560653	EU818926
B. cytisi CTAW11^T		EU561065	GU001613	JN186292	GU001575	EU597844	GU001618
B. daqingense CCBAU 15774^T		HQ231274	HQ231289	JX437669	HQ231270	HQ231326	HQ231323
B. denitrificans LMG 8443^T		X66025	FM253153	AB070583	EU665419	-	HM047125
B. diazoefficiens USDA 110^T	NC 004463						
B. elkanii USDA76^T		AF362942	AM418752	AM418800	AY591568	AB354631	AB094963
B. embrapense CNPSo 2833^T		AY904773	HQ634875	HQ63489	HQ634899	KP234521	KP234518
B. erythrophlei CCBAU 53325^T		KF114645	-	KF114717	KF114669	KF114576	KF114598
B. ferriligni CCBAU 51502^T		KJ818096	-	KJ818102	KJ818112	KJ818109	KJ818108
B. ganzhouense RITF 806^T		JQ796661	JX277182	KP420022	JX277144	JX292035	JX292065
B. guangdongense CCBAU 51649^T		KC508867	KC508916	KC509072	KC509269	-	KC509130
B. guangxiense CCBAU 53363^T		KC508877	KC508926	KC509082	KC509279	-	KC509140
B. huanghuaihaiense CCBAU23303^T		HQ231463	HQ231682	JX437672	HQ231595	HQ231507	HQ231551

Continuation...

Strains	**16S rRNA**	***atpD***	***gyrB***	***recA***	***nodC***	***nifH***
B. icense LMTR 13^{T}	KF896156	KF896192	KF896201	JX943615	KF896159	KF896161
B. ingae BR 10250^{T}	KF927043	-	KF927079	KF927061	KF927054	KF927085
B. iriomotense EK05^{T}	AB300992	AB300994	AB300997	AB300996	AB301000	AB300998
B. japonicum USDA 6^{T}	X66024	AM418753	AM418801	AM182158	AB354632	HM047126
B. jicamae PAC68^{T}	AY624134	FJ428211	HQ873309	HM047133	AB573869	HM047127
B. kavangense 14-3^{T}	KP899562	-	-	KM378399	KT033402	KM378254
B. lablabi CCBAU 23086^{T}	GU433448	GU433473	JX437670	GU433522	GU433565	GU433546
B. liaoningense LMG 18230^{T}	AF208513	AY386752	FM253223	FM253180	GU263466	EU818925
B. manausense BR 3351^{T}	HQ641226	-	KF786000	KF785992	KF786002	KF786003
B. neotropicale BR 10247^{T}	KF927051	-	KJ661707	KJ661714	KJ661727	KJ661728
B. oligotrophicum LMG 10732^{T}	JQ619230	JQ619232	KC569467	JQ619231	-	-
B. ottawaense OO99^{T}	JN186270	HQ455212	HQ873179	HQ587287	HQ587980	JN186287
B. pachyrhizi PAC48^{T}	AY624135	FJ428208	HQ873310	HM047130	HQ588110	HM047124
B. paxllaeri LMTR 21^{T}	AY923031	KF896186	KF896195	JX943617	KF896160	DQ085619
B. retamae Ro19^{T}	KC247085	KC247101	KF962698	KC247094	KC247112	KF962704
B. rifense CTAW71^{T}	EU561074	GU001617	KC569466	GU001585	EU597853	-
B. subterraneum 58 2-1^{T}	KP308152	-	-	KM378397	-	KM378289
B. tropiciagri CNPSo 1112^{T}	AY904753	-	HQ634890	FJ391168	KP234520	HQ259540
B. valentinum Lmj M3^{T}	JX514883	JX518561	-	JX518589	JX514897	KF806461
B. vignae 7-2^{T}	KP899563	-	-	KM378374	KT362339	KM378251
B. viridifuturi SEMIA 690^{T}	FJ025107	-	KR149134	KR149140	-	KR149137
B. yuanmingense LMG 21827^{T}	AF193818	FM253140	FM253226	FM253183	AB354633	EU818927

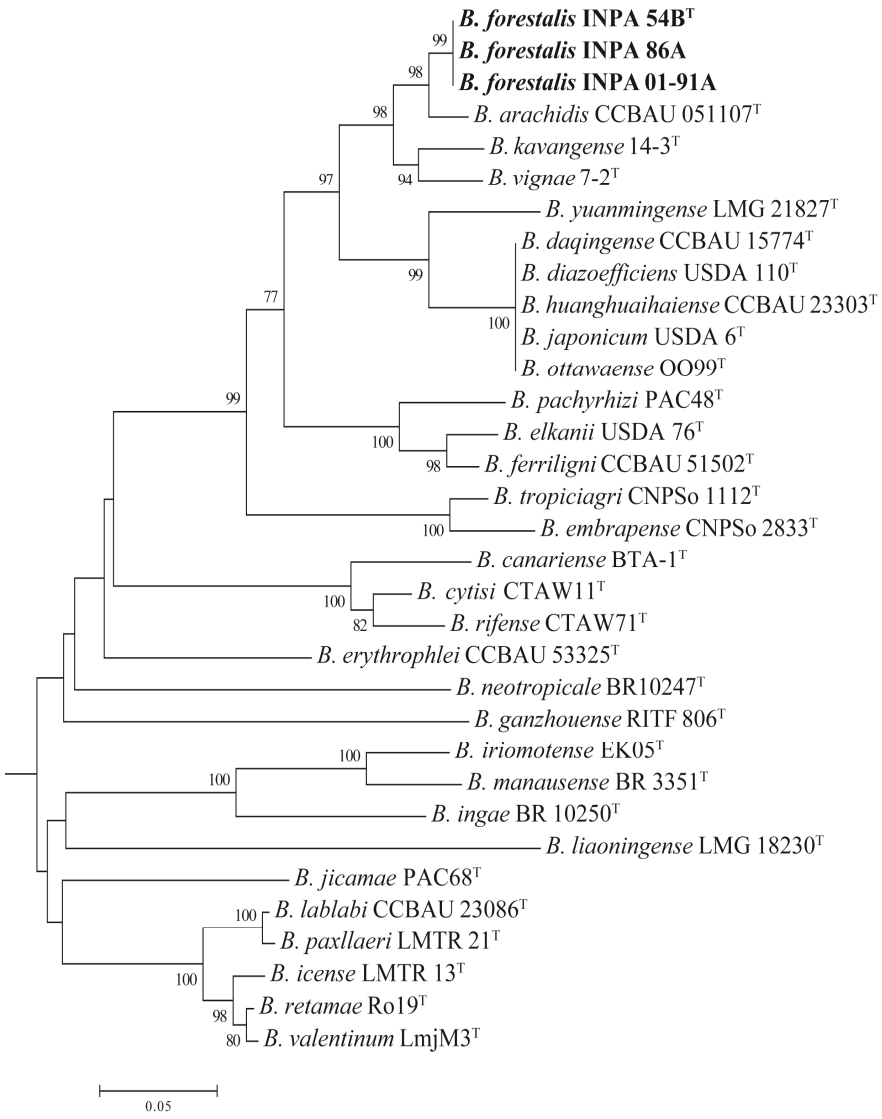

Fig. S1. Neighbour-joining phylogeny based on partial sequences (378 bp) of *nodC* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

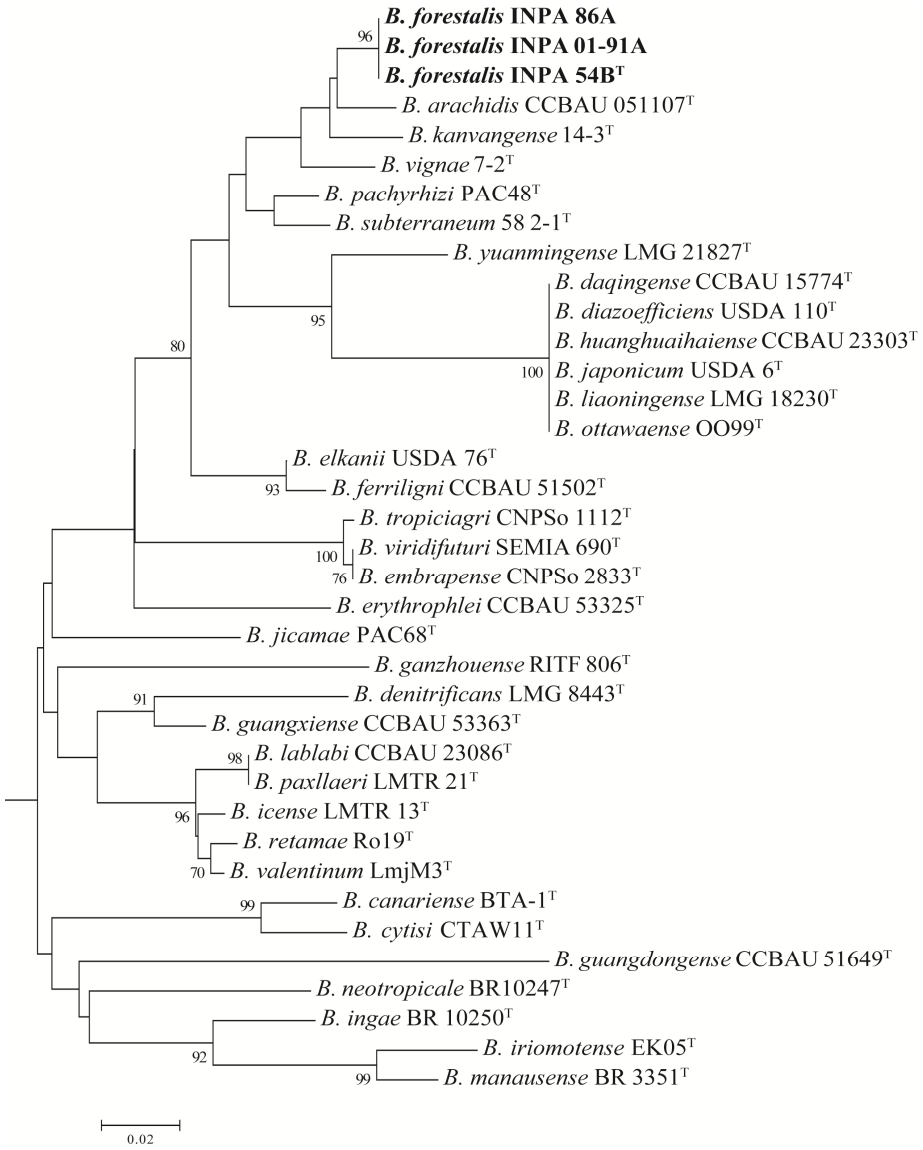

Fig. S2. Neighbour-joining phylogeny based on partial sequence (201 bp) of *nifH* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

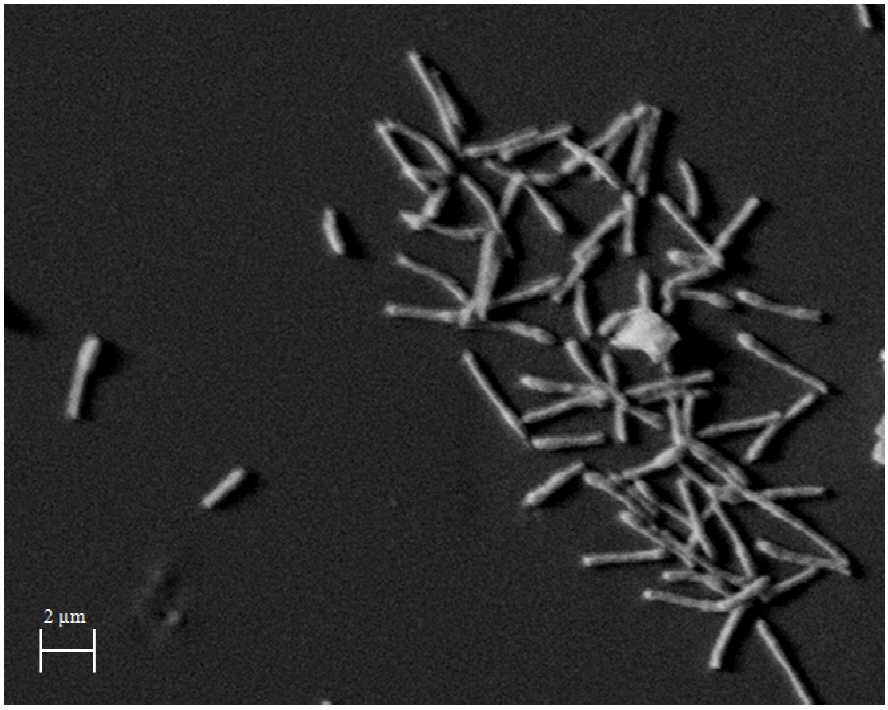

Fig. S3. Image of strain INPA 54B^{T} (*Bradyrhizobium forestalis*) obtained by scanning electron microscopy.

ARTIGO 3 - *Bradyrhizobium piauiense* sp. nov., an efficient symbiotic bacterium isolated from soybean nodules in the Brazilian Northeast

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Bradyrhizobium piauiense sp. nov., an efficient symbiotic bacterium isolated from soybean nodules in the Brazilian Northeast

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Running title: *Bradyrhizobium piauiense* sp. nov.

New sequences: *nodC* of *B. piauiense* UFLA 06-13^T (KT793167), UFLA 06-15 (KT793168), UFLA 06-19 (KT793169) and UFLA 06-22 (KT793170); *nifH* of *B. piauiense* UFLA 06-13^T (KT793152), UFLA 06-15 (KT793148), UFLA 06-21 (KT793153) and UFLA 06-22 (KT793154).

Abstract

In previous study, five nitrogen-fixing bacteria strains (UFLA 06-13^T, UFLA 06-15, UFLA 06-19, UFLA 06-21 and UFLA 06-22), isolated from soybean (*Glycine max* L.) nodules inoculated with soil from the state of Piauí, Northeast Brazil, were identified and indicated as a new group within the *Bradyrhizobium* genus. The taxonomic status of these strains was evaluated in this study using a polyphasic approach. Phylogenetic analysis of the 16S rRNA gene grouped the five strains with *Bradyrhizobium elkanii* LMG 6134^T and *Bradyrhizobium pachyrhizi* LMG 24246^T, with similarity of 99.92 and 100%, respectively. However, the concatenated sequence analysis of the housekeeping genes *atpD, dnaK, gyrB, recA* and *rpoB* revealed that the five strains represent a novel *Bradyrhizobium* species, which is closely related to *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T. DNA-DNA relatedness between UFLA 06-13^T and two closely related species (*B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T) was only 18.8 and 21.6%, respectively. Analysis of MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) profiles and some phenotypic characteristics, including tests for temperature, salinity, resistance to antibiotics and assimilation of different carbon and nitrogen sources allowed differentiating the five strains from the two neighboring species (*B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T). Based on the data presented, we suggest that the five strains represent a novel species, for which the name *Bradyrhizobium piauiense* sp. nov. is proposed, with UFLA 06-13^T (LMG 29354^T) as type strain.

Keywords: *Bradyrhizobium*, *Glycine max* L., housekeeping genes, polyphasic taxonomy

Soybean (*Glycine max* L.) is an important protein source used worldwide. In Brazil, which is the second largest world soybean producer,

this crop occupies about 57% of the planted area (CONAB, 2014), playing important economic and social role. One factor that decisively contributes to the success of soybean production in Brazil and to its market competitiveness is the exploitation of biological nitrogen fixation (BNF) through its inoculation with strains of the *Bradyrhizobium* genus: SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*).

Soybean plant has been the main host species from which species of the *Bradyrhizobium* genus were isolated. Currently, there are seven species of this genus isolated from soybean plants: *B. japonicum* USDA 6^T, from Japan (Jordan, 1982); *B. elkanii* LMG 6134^T and *B. diazoefficiens* USDA 110^T, from the United States (Kuykendall *et al*., 1992; Delamuta *et al*., 2013); *B. liaoningense* LMG 18230^T, *B. huanghuaihaiense* CCBAU23303^T and *B. daqingense* CCBAU 15774^T, from China (Xu *et al*., 1995; Zhang *et al*., 2011; Wang *et al*., 2012) and *B. ottawaense*, from Canada (Yu *et al*., 2014). Despite the importance of soybean in Brazil, there is no described *Bradyrhizobium* species from nodules of this crop in Brazilian soil.

In recent study, 46 strains isolated from soybean nodules inoculated with soils from different Brazilian regions (Midwest, Northeast, Southeast and South) were classified within the *Bradyrhizobium* genus based on the partial sequencing of the 16S rRNA gene (Ribeiro *et al*., 2015). The concatenated sequence analysis of the five housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA* and *rpoB*) of these strains indicated two groups with potentially representative strains of the novel species (Ribeiro *et al*., 2015). In the present study, five strains of one of these groups (UFLA 06-13^T, UFLA 06-15, UFLA 06-19, UFLA 06-21 and UFLA 06-22), which are efficient in N_2 fixation in symbiosis with soybean, were selected for further analysis, by molecular and phenotypic methods. Based on these results, a novel species within the genus *Bradyrhizobium* is proposed, whose name is *Bradyrhizobium piauiense* sp. nov, with UFLA 06-13^T as type strain.

The five strains were isolated from effective nodules of soybean plants inoculated with soil from the Northeast, collected in Bom Jesus (9º19‟21‟ S and 44º48‟55‟ W), in the state of Piauí. The inoculum soil had previous inoculation with the following strains inoculant of soybean: SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*) and/or SEMIA 5019 (*B. elkanii*). Isolation was carried out on plates with culture 79 medium (Fred and Walkmam, 1928), also known as YMA (Vincent, 1970). These strains are currently deposited in the collection of the Soil Biology, Microbiology and Biological Processes Department of the Federal University of Lavras, Brazil, and the type strain was also deposited in the culture collection (BCCM/LMG) of the Ghent University, Belgium.

Sequences of 16S rRNA (1288 to 1331 bp), *atpD* (510 pb), *dnaK* (280 pb), *gyrB* (669 pb), *recA* (474 to 559 pb) and *rpoB* (903 to 957 pb) genes of the five UFLA strains were obtained in a previous study (Ribeiro *et al*., 2015). Sequences of each gene were aligned using the ClustalW Multiple Alignment algorithm in the BioEdit software. For comparison, the alignment included the sequences of type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI). The sequences of four strains currently used as soybean inoculants in Brazil (SEMIA 5079 *B. japonicum*, SEMIA 5080 *B. diazoefficiens*, SEMIA 587 *B. elkanii* and SEMIA 5019 *B. elkanii*) were also included in the alignment of 16S rRNA, *dnaK* and *recA* genes. However, it was possible to include only the sequences of SEMIA 5079 and SEMIA 5080 strains in the alignment of *atpD* and *rpoB* genes, since the sequences of these two genes are not available for SEMIA 587 and SEMIA 5019 strains. Sequences of *gyrB* gene of SEMIA 5079 and SEMIA 5080 are available in the GenBank; however, it was not possible to include them in the alignment, since they do not seem to have good quality. Phylogenetic trees were constructed by the neighbor joining (NJ) (Saitou and Nei, 1987) and by the maximum likelihood (ML) (Felsenstein, 1981) methods, using the Kimura 2 parameter model (Kimura,

1980). The MEGA 5 software package (Tamura *et al*., 2011) was used in the construction of trees, with bootstrap values based on 1000 replications.

Results of phylogenetic analysis of the 16S rRNA gene were similar when both methods were used: neighbor-joining (NJ) (Fig. 1) and maximum likelihood (ML) (data not shown). The five UFLA strains showed 16S rRNA gene sequence similar to the two inoculant strains, SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*) and to *B. pachyrhizi* LMG 24246^T, and shared 99.91% similarity with *B. elkanii* LMG 6134^T (Table S1). These data confirm that the 16S rRNA gene has low discriminatory power between members of the *Bradyrhizobium* genus, corroborating previous studies (Willems *et al*., 2001; Vinuesa *et al*., 2005; Rivas *et al*., 2009; Durán *et al*., 2014; Guimarães *et al*., 2015).

Analysis of multilocus sequences of housekeeping genes have been successfully employed for better discrimination between closely related species within the *Bradyrhizobium*, genus (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) (Vinuesa *et al*., 2005; Ramírez-Bahena *et al*., 2009; Zhang *et al*., 2012; Delamuta *et al*., 2013; Silva *et al*., 2014; Yao *et al*., 2015). In this study, it was firstly carried out a concatenated analysis of the sequences of the *dnaK* and *recA* genes, in order to include the four soybean inoculant strains. In this analysis, the five UFLA strains presented similar sequences between each other and formed a separate group (Fig. S1), which shared similarity of 97.89% with *B. elkanii* LMG 6134^T and of 97.76% with SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*) (Table S1). Subsequently, it was carried out concatenated sequence analysis of the five genes, *atpD* (429 bp), *dnaK* (223 bp), *gyrB* (561 bp), *recA* (381 bp) and *rpoB* (485 bp), which confirmed that the five UFLA strains form a monophyletic group supported by high bootstrap value (100%) (Fig. 2). Strain UFLA 06-13^T shared similarity of 97.87% with *B. elkanii* LMG 6134^T and of 97.85% with *B. pachyrhizi* LMG 24246^T (Table S1). Among the studied genes, *atpD* showed the best discriminative power between UFLA 06-13^T and the two closely related species (*B. elkanii* LMG 6134^T and *B pachyrhizi* LMG

24246^T). For this gene, similarity of UFLA 06-13^T with *B. elkanii* LMG 6134^T was 96.48%, and with *B. pachyrhizi* LMG 24246^T, similarity was 96.31% (Table S1). These similarity values are close to those found between different *Bradyrhizobium* species (Chahboune *et al*., 2011; Lu *et al*., 2014; Yu *et al*., 2014).

The five UFLA strains and the two neighboring species (*B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T) were characterized by the MALDI-TOF MS (Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry) analysis. For this analysis, it was used third generation cultures grown in YMA 79 medium (Vincent, 1970). Sample preparation and data analysis was carried out according to a protocol used by Wieme *et al*. (2014). Results of this analysis was consistent with those of concatenated sequence analysis of housekeeping genes, confirming that the five UFLA strains form a separate group of *B.elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T (data not shown). Sánchez-Juanes *et al*. (2013) have recently demonstrated that such analysis provides good discrimination among *Bradyrhizobium* species.

Phenotypic characterization of the five strains was carried out based on parameters previously used to differentiate *Bradyrhizobium* species. *B. elkanii* LMG 6134^T, *B. pachyrhizi* LMG 24246^T and the two inoculant strains classified as *Bradyrhizobium elkanii* (SEMIA 587 and SEMIA 5019) were also included in the analyzes. The growth of these strains in 79 medium (Fred and Waksman, 1928) was evaluated under different conditions of pH (4, 5,5, 6,8, 8, 9 and 10), NaCl (w/v) (0,01, 0,25, 0,5, 0,75 and 1%) and temperature (5, 15, 20, 28, 34, 37 and 40°C), according to the methodology previously described (Florentino *et al*., 2012). Ability to assimilate different carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, maltose, mannitol, L-methionine, sodium lactate and sucrose) was evaluated in modified 79 medium: 10 g carbon source; 5g K_2HPO_4; 0.5 g KNO_3; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.5 g $CaCO_3$; 4 mL Fe-EDTA (1,64%); 2 mL

micronutrients solution (2.86 mg H_3BO_3 liter^{-1}; 2.03 mg $MnSO_4.4H_2O$ liter^{-1}; 0.22 mg $ZnSO_4 \cdot 7H_2O$ liter^{-1}; 0.08 mg $CuSO_4 \cdot 5H_2O$ liter^{-1} and 0.09 mg $Na_2MoO_4 \cdot H_2O$ liter^{-1}); 5 mL bromothymol blue solution (0.5% in 0.2 N KOH); 15 g Agar; and pH 6.8. Composition of 79 medium for evaluation of assimilation of different nitrogen sources (L-arginine, L-asparagine, casein hydrolyzed, L-cysteine, glycine, L-glutamic acid, and L-methionine and tryptophan) was the same as described above, substituting KNO_3 by one of the mentioned sources, and using mannitol as carbon source. Antibiotic resistance was also tested in 79 medium on plates with bio-discs containing the following antibiotics: ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), doxycycline (30 μg mL^{-1}), erythromycin (15 μg mL^{-1}), gentamicin (10 μg mL^{-1}), kanamycin (30m μg mL^{-1}) and neomycin (30 μg mL^{-1}). Besides these tests, strain UFLA 06-13^T was characterized based on the API 20NE (bioMérieux), according to the manufacturer's instructions, with five days incubation. Table 1 shows the differential phenotypic characteristics between UFLA strains, *B. elkanii* LMG 6134^T, *B. pachyrhizi* LMG 24246^T and inoculant strains (SEMIA 587 and SEMIA 5019). Detailed phenotypic characterization is presented in the description of the novel species.

To confirm the novel species, DNA-DNA hybridization experiments were carried out between strain UFLA 06-13^T and the closely related species (*B. elkanii* LMG 6134^T e *B. pachyrhizi* LMG 24246^T), according to the methodology previously described (Ezaki *et al*., 1989; Willems *et al*., 2001). DNA-DNA relatedness between UFLA 06-13^T and *B. elkanii* LMG 6134^T and *B. pachyrhizi* LMG 24246^T was only 18.8 and 21.6%, respectively. These data confirm that UFLA strains belong to a novel species within the *Bradyrhizobium* genus, since the recommended limit for delimitation of novel species is below 70% (Wayne *et al*., 1987).

Phylogeny of symbiotic genes (*nodC* and *nifH*) of UFLA strains were also investigated in this study. DNA extraction from strains was carried out by the alkaline lysis method (Niemann *et al*., 1997). For amplification and

sequencing of *nodC*, the protocol of Sarita *et al*. (2005), modified by De Meyer *et al*. (2011) was used. On the other hand, the amplification and sequencing of *nifH* was carried out according to Gaby and Buckley (2012). For strains UFLA 06-21 and UFLA 06-19, amplification of *nodC* and *nifH* genes, respectively, was not possible. In the alignment of sequences *nifH* gene, it was included the type strains of *Bradyrhizobium* species available in the GenBank and the four soybean inoculant strains (SEMIA 5079 *B. japonicum*, SEMIA 5080 *B. diazoefficiens*, SEMIA 587 *B. elkanii* e SEMIA 5019 *B. elkanii*). However, it was not possible to include SEMIA 587 and SEMIA 5019 strains in the alignment of *nodC* gene, since the sequences of this gene are not available in the GenBank. Phylogenetic trees were constructed as previously described. Results of phylogenetic analyses of *nodC* and *nifH* are shown in the supplementary material (Fig. S2 and S3). The strains UFLA presented *nodC* gene sequences identical to *B. elkanii* LMG 6134^{T} (Fig. S2). However, in the analysis of *nifH* gene, strains UFLA showed phylogenetic differences in relation to *B. elkanii* LMG 6134^{T} and to the inoculant strains SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*) (Fig. S3).

Ability of the five strains to effectively nodulate and fix nitrogen with their original host (*Glycine max*) was confirmed in a previous study (Ribeiro *et al*., 2015). Strain UFLA 06-13^{T} was also evaluated for nodulation capacity in other hosts (*Vigna unguiculata, Phaseolus lunatus, Stizolobium aterrimum* and *Acacia mangium*). This strain formed nodules in *V. unguiculata* and *S.aterrimum*, but did not nodulate *P. lunatus* and *A. mangium*.

Genotypic, phenotypic and symbiotic data presented in this study demonstrate that the five strains isolated from nodules of *Glycine max* inoculated with soil from the Brazilian Northeast is a novel species, for which the name *Bradyrhizobium piauiense* sp. nov is proposed, with UFLA 06-13^{T} as type strain.

Description of *Bradyrhizobium piauiense* sp. nov.

Bradyrhizobium piauiense (pi.au.i.en'se. N.L. neut. adj. *piauiense*, referring to the fact that strains were isolated from soils of the state of Piauí, Brazil).

Cells are aerobic gram-negative, non-spore-forming rods (Fig. S4). The strains present cream-colored colonies with 1mm diameter and alkalize in 79 medium within 5–7 days of incubation at 28 ºC. The pH and temperature range for growth on 79 medium is pH 4.0–10.0 and between 15 to 37°C, with optimal growth at 28°C. They do not grow above 0.75% (w/v) NaCl concentration. They are resistant to ampicillin, cefuroxime and ciprofloxacin, but sensitive to kanamycin, gentamycin and neomycin. Resistance to chloramphenicol, doxycycline and erythromycin varies between strains. They are positive for the use of D-arabinose, D-fructose, glycerol, D-glucose and mannitol, but they use weakly L-asparagine and L-glutamic acid as a carbon source. The use of citric acid, malic acid, glycine, maltose, L-methionine, lactate and sucrose as carbon source, is negative. The use of L-glutamine and lactose as carbon source varies between strains. The use of L-asparagine, casein hydrolysate and L-glutamic acid, as nitrogen source, is positive; however, the use of L-arginine, L-cysteine, glycine, L-methionine and tryptophan is negative. Strain UFLA 06-13^{T} presents negative reaction to reduce nitrate, tryptophan deaminase activity, glucose fermentation and arginine dihidrolase; and positive reaction for urease, esculin hydrolysis and gelatin hydrolysis. All strains efficiently nodulate *Glycine max*.

The type strain UFLA 06-13^{T} (LMG 29354^{T}) was isolated from effective soybean nodule inoculated with soil from the state of Piauí, northeast Brazil.

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Table 1 Differential phenotypic characteristics among *Bradyrhizobium piauiense* sp. nov. and and phylogenetically related to *Bradyrhizobium* species.

Strains: 1, *B. piauiense* UFLA 06-13^{T}; 2, *B. piauiense* UFLA 06-15; 3, *B. piauiense* UFLA 06-19; 4, *B. piauiense* UFLA 06-21; 5, *B. piauiense* UFLA 06-22; 6, *B. pachyrhizi* LMG 24246^{T}; 7, *B. elkanii* LMG 6134^{T}; 8, *B .elkanii* SEMIA 5019 ; 9, *B. elkanii* SEMIA 587. Data represent the means of three biological replicates. +, growth; −, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at									
40 °C	-	-	-	-	-	w	w	-	w
0.75 % NaCl	w	w	w	w	w	+	+	+	+
Carbon source assimilation									
L-asparagine	w	w	w	w	w	+	+	+	+
D-fructose	+	+	+	+	+	+	+	w	w
L-Glutamine	+	+	+	+	w	+	+	+	+
Glutamic acid	w	w	w	w	w	+	+	+	+
Lactose	w	-	-	w	w	+	w	+	+
Malic acid	-	-	-	-	-	w	w	+	w
L-Methionine	-	-	-	-	-	w	w	w	w
Sodium lactate	-	-	-	-	-	+	+	+	+
Sucrose	-	-	-	-	-	+	+	+	+
Nitrogen source assimilation									
L-Arginine	-	-	-	-	-	+	+	+	+
Casein hydrolysate	+	+	+	+	+	w	+	+	+

Continuation...

Characteristic	1	2	3	4	5	6	7	8	9
L-Cysteine	-	-	-	-	-	+	+	+	+
L-Methionine	-	-	-	-	-	w	w	-	-
Tryptophan	-	-	-	-	-	-	-	w	w
Resistance to antibiotics (µg mL^{-1})									
Erythromycin (15)	+	w	+	+	+	+	+	+	+
Gentamycin (10)	-	-	-	-	-	-	+	+	+
Neomycin (30)	-	-	-	-	-	-	w	w	w

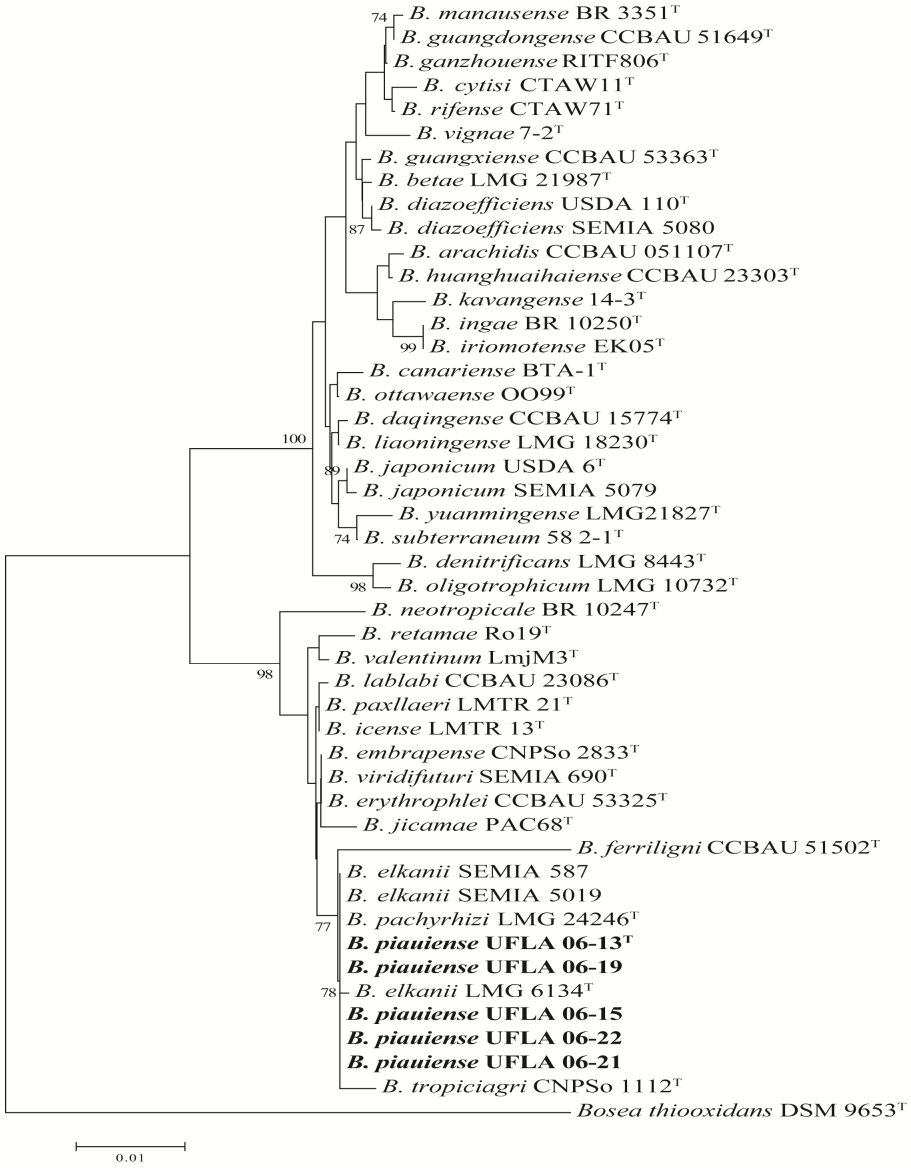

Fig 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences (1228 pb) showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. The 16S rRNA gene sequence of *Bosea thiooxidans* DSM9653^T was used as outgroup. GenBank accession numbers for each strain are given in Table S2.

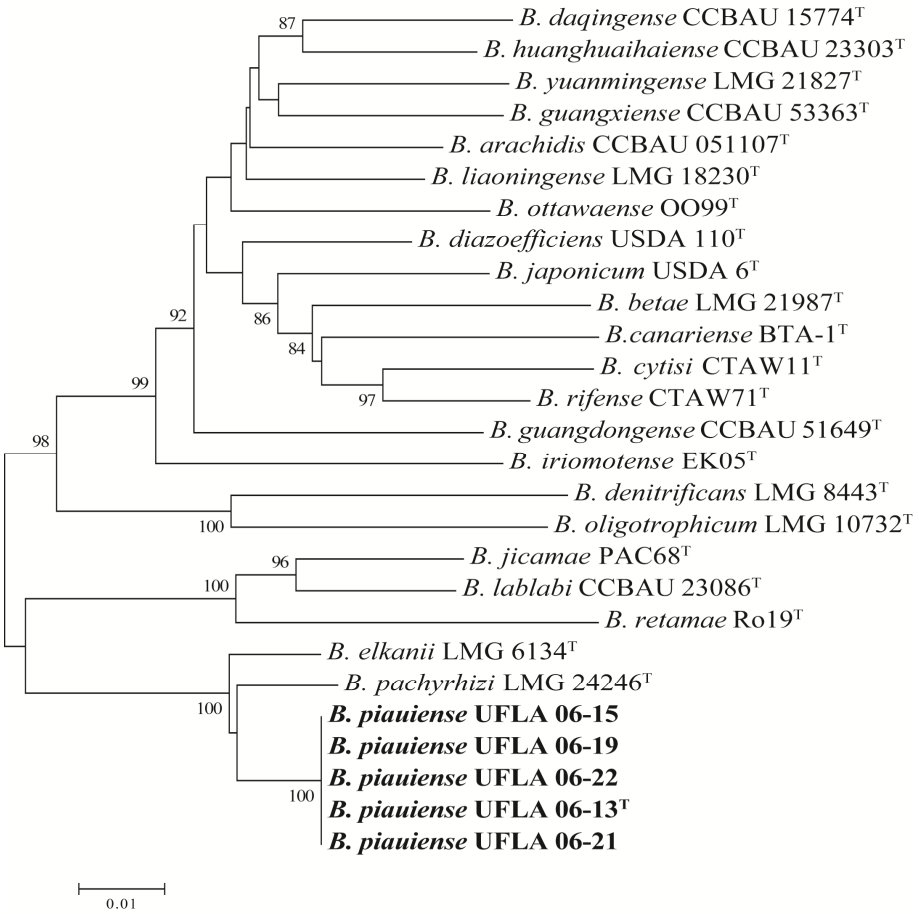

Fig 2. Neighbour-joining phylogeny based on partial concatenated sequences (2079 pb) of housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA* and *rpoB*) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers of the genes sequences for each strain are given in Table S2.

Supplementary Material

Table S1. Similarity within *Bradyrhizobium piauiense* and between *B. piauiense* UFLA 06-13^T and other type strains of *Bradyrhizobium* species in the 16S rRNA. housekeeping and symbiotic genes.

Strains	**Similarity with UFLA 06-13^T (%)**									
	16S rRNA	***atpD***	***dnaK***	***gyrB***	***recA***	***rpoB***	**Concatenated (Two genes*)**	**Concatenated (Five genes*)**	***nodC***	***nifH***
***B. piauiense* UFLA 06-15**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
***B. piauiense* UFLA 06-19**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	-
***B. piauiense* UFLA 06-21**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	-	100.00
***B. piauiense* UFLA 06-22**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
B. arachidis CCBAU 051107^T	96.65	92.64	82.27	92.67	93.55	89.04	90.54	91.20	83.28	92.66
B. betae LMG 21987^T	96.95	92.84	85.53	91.30	93.03	84.94	91.63	89.48	-	-
B. canariense BTA-1^T	97.03	93.79	81.47	91.70	92.58	84.49	88.86	89.39	67.88	84.06
B. cytisi CTAW11^T	96.53	93.74	85.91	90.77	91.44	87.44	90.39	89.45	70.64	84.04
B. daqingense CCBAU 15774^T	97.17	91.62	84.22	92.60	91.62	88.24	90.42	90.39	80.06	86.34
B. denitrificans LMG 8443^T	96.68	91.30	84.77	89.80	87.57	89.12	88.09	89.75	-	85.49
B. diazoefficiens USDA 110^T	96.95	94.02	83.51	93.62	92.78	88.91	90.00	91.56	80.06	86.34
B. elkanii LMG 6134^T	99.92	96.48	98.72	98.49	97.23	98.17	97.89	97.87	100.00	99.50
B. embrapense CNPSo 2833^T	99.75	95.66	89.26	96.09	96.02	-	94.80	-	77.14	89.82
B. erythrophlei CCBAU 53325^T	99.75	-	-	91.21	94.26	-	-	-	73.73	90.86
B. ferriligni CCBAU 51502^T	97.83	-	-	92.70	96.13	-	-	-	96.47	98.18
B. ganzhouense RITF 806^T	96.81	92.37	-	92.27	92.79	-	-	-	63.14	85.45
B. guangdongense CCBAU51649^T	96.74	92.68	83.16	92.78	91.30	88.48	89.94	90.73	-	80.16
B. guangxiense CCBAU53363^T	96.95	92.14	81.82	92.08	92.95	87.48	90.27	90.50	-	88.98
B. huanghuaihaiense CCBAU23303^T	96.75	93.46	83.46	92.80	92.00	88.76	90.55	90.81	80.06	86.34
B. icense LMTR 13^T	99.75	91.34	88.72	88.00	93.92		91.95	-	70.17	88.18

Continuation...

Strains	**16S rRNA**	***atpD***	***dnaK***	***gyrB***	***recA***	***rpoB***	**Concatenated (Two genes*)**	**Concatenated (Five genes*)**	***nodC***	***nifH***
B. ingae BR 10250^T	96.47	-	86.33	90.29	89.97	82.62	89.75	-	62.91	86.95
B. iriomotense EK05^T	96.47	94.25	87.61	91.30	90.22	84.66	90.40	90.50	61.65	82.39
B. japonicum USDA 6^T	97.17	94.94	83.43	93.39	93.47	86.63	90.29	90.66	80.06	86.34
B. jicamae PAC68^T	99.42	93.19	87.04	90.03	92.07	91.90	91.18	91.45	68.69	86.34
B. kavangense 14-3^T	96.45	-	83.44	-	93.15	89.59	90.01	-	82.58	92.30
B. lablabi CCBAU 23086^T	99.67	92.26	87.61	88.97	94.45	91.78	92.65	91.54	69.82	88.08
B. liaoningense LMG 18230^T	97.25	91.96	83.94	93.04	94.19	88.92	91.43	91.41	58.56	86.34
B. manausense BR 3351^T	96.66	-	85.09	91.14	92.93	86.79	90.90	-	61.38	83.76
B. neotropicale BR 10247^T	98.66	-	84.21	91.83	93.30	84.67	91.41	-	66.18	86.18
B. oligotrophicum LMG 10732^T	96.77	90.61	83.26	89.78	89.03	89.93	89.22	89.99	-	-
B. ottawaense OO99^T	97.25	92.07	82.13	91.74	92.73	88.81	90.09	90.65	80.06	86.34
B. pachyrhizi LMG 24246^T	100.00	96.31	98.82	98.57	96.23	98.79	97.10	97.85	91.18	95.90
B. paxllaeri LMTR 21^T	99.75	91.31	89.17	89.20	92.17	-	91.44	-	69.68	88.08
B. retamae Ro19^T	99.27	90.13	86.58	87.47	93.97	89.92	90.83	89.88	70.48	88.12
B. rifense CTAW71^T	96.74	93.19	85.91	91.58	92.72	87.24	91.19	90.19	68.86	-
B. subterraneum 58 2-1^T	97.08	-	87.05	-	90.94	88.37	90.36	-	-	94.35
B. tropiciagri CNPSo 1112^T	99.67	-	96.13	96.47	96.65	-	96.50	-	78.99	89.98
B. valentinum Lmj M3^T	99.51	90.56	-	-	94.59	-	-	-	70.34	88.48
B. viridifuturi SEMIA 690^T	99.75	-	95.35	96.48	96.31	-	96.63	-	-	89.82
B. vignae 7-2^T	96.59	-	86.05	-	92.38	88.84	87.38	-	82.31	93.02
B. yuanmingense LMG 21827^T	96.76	100.00	81.41	92.05	92.80	88.33	90.13	90.43	80.06	89.19
B. diazoefficiens SEMIA 5080	96.86	94.02	83.51	-	93.39	88.42	90.40	-	80.06	86.34
B. japonicum SEMIA 5079	97.09	95.48	83.43	-	93.78	87.03	90.49	-	80.06	86.34
B. elkanii SEMIA 5019	100.00	-	98.82	-	97.23	-	97.76	-	-	98.47
B. elkanii SEMIA 587	100.00	-	98.82	-	97.23	-	97.76	-	-	98.47

* Concatenated sequences with two genes (*dnaK* and *recA*), **Concatenated sequences with five genes (*atpD, dnaK, gyrB recA* and *rpoB*).

Table S2. GenBank nucleotide accession numbers of genes used in this study. Sequences obtained in this study are shown in bold.

Strains	**16S rRNA**	***atp*D**	***dna*K**	***gyr*B**	***rec*A**	***rpo*B**	***nod*C**	***nif*H**
***B. piauiense* UFLA 06-13^T**	KJ739898	KJ739964	KJ740010	KJ740050	KJ740091	KJ740133	**KT793167**	**KT793152**
***B. piauiense* UFLA 06-15**	KJ739900	KJ739972	KJ739976	KJ740046	KJ740099	KJ740142	**KT793168**	**KT793148**
***B. piauiense* UFLA 06-19**	KJ739904	KJ739966	KJ740012	KJ740058	KJ740093	KJ740127	**KT793169**	**-**
***B. piauiense* UFLA 06-21**	KJ739906	KJ739955	KJ739991	KJ740040	KJ740082	KJ740139	**-**	**KT793153**
***B. piauiense* UFLA 06-22**	KJ739907	KJ739960	KJ739993	KJ740058	KJ740087	KJ740131	**KT793170**	**KT793154**
B. arachidis CCBAU 051107^T	HM107167	HM107217	JX437668	JX437675	HM107233	JX437682	HM107267	HM107283
B. betae LMG 21987^T	AY372184	FM253129	FM253303	AB353735	AB353734	FM253260	-	-
B. canariense BTA-1^T	AY577427	FM253135	FM253306	FM253220	FM253177	FM253263	AJ560653	EU818926
B. cytisi CTAW11^T	EU561065	GU001613	JN186290	JN186292	GU001575	JN186288	EU597844	GU001618
B. daqingense CCBAU 15774^T	HQ231274	HQ231289	JX437662	JX437669	HQ231270	JX437676	HQ231326	HQ231323
B. denitrificans LMG 8443^T	X66025	FM253153	FJ347273	AB070583	EU665419	FM253282	-	HM047125
B. diazoefficiens USDA 110^T	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463
B. elkanii LMG 6134^T	AF362942	AM418752	AM168363	AM418800	AY591568	AM295348	AB354631	AB094963
B. embrapense CNPSo 2833^T	AY904773	HQ634875	KP234519	HQ63489	HQ634899	-	KP234521	KP234518
B. erythrophlei CCBAU 53325^T	KF114645	-	-	KF114717	KF114669	-	KF114576	KF114598
B. ferriligni CCBAU 51502^T	KJ818096	-	-	KJ818102	KJ818112	-	KJ818109	KJ818108
B. ganzhouense RITF 806^T	JQ796661	JX277182	-	KP420022	JX277144	-	JX292035	JX292065
B. guangdongense CCBAU 51649^T	KC508867	KC508916	KC508964	KC509072	KC509269	KC509318	-	KC509130
B. guangxiense CCBAU 53363^T	KC508877	KC508926	KC508974	KC509082	KC509279	KC509328	-	KC509140
B. huanghuaihaiense CCBAU23303^T	HQ231463	HQ231682	JX437665	JX437672	HQ231595	JX437679	HQ231507	HQ231551
B. icense LMTR 13^T	KF896156	KF896192	KF896182	KF896201	JX943615	-	KF896159	KF896161
B. ingae BR 10250^T	KF927043	-	KF927055	KF927079	KF927061	KF927073	KF927054	KF927085
B. iriomotense EK05^T	AB300992	AB300994	JF308944	AB300997	AB300996	HQ587646	AB301000	AB300998
B. japonicum USDA 6^T	X66024	AM418753	AM182120	AM418801	AM182158	AM295349	AB354632	HM047126
B. jicamae PAC68^T	AY624134	FJ428211	JF308945	HQ873309	HM047133	HQ587647	AB573869	HM047127
B. kavangense 14-3^T	KP899562	-	KR259949	-	KM378399	KM378311	KT033402	KM378254

Continuation...

Strains	**16S rRNA**	***atp*D**	***dna*K**	***gyr*B**	***rec*A**	***rpo*B**	***nod*C**	***nif*H**
B. lablabi CCBAU 23086^T	GU433448	GU433473	JX437663	JX437670	GU433522	JX437677	GU433565	GU433546
B. liaoningense LMG 18230^T	AF208513	AY386752	FM253309	FM253223	FM253180	FM253267	GU263466	EU818925
B. manausense BR 3351^T	HQ641226	-	KF786001	KF786000	KF785992	KF783998	KF786002	KF786003
B. neotropicale BR 10247^T	KF927051	-	KJ661693	KJ661707	KJ661714	KF983829	KJ661727	KJ661728
B. oligotrophicum LMG 10732^T	JQ619230	JQ619232	KF962688	KC569467	JQ619231	KC569469	-	-
B. ottawaense OO99^T	JN186270	HQ455212	JF308816	HQ873179	HQ587287	HQ587518	HQ587980	JN186287
B. pachyrhizi LMG 24246^T	AY624135	FJ428208	JF308946	HQ873310	HM047130	HQ587648	HQ588110	HM047124
B. paxllaeri LMTR 21^T	AY923031	KF896186	AY923038	KF896195	JX943617	-	KF896160	DQ085619
B. retamae Ro19^T	KC247085	KC247101	KJ560555	KF962698	KC247094	KF962714	KC247112	KF962704
B. rifense CTAW71^T	EU561074	GU001617	JQ945187	KC569466	GU001585	KC569468	EU597853	-
B. subterraneum 58 2-1^T	KP308152	-	KP308157	-	KM378397	KM378349	-	KM378289
B. tropiciagri CNPSo 1112^T	AY904753	-	FJ391008	HQ634890	FJ391168	-	KP234520	HQ259540
B. valentinum Lmj M3^T	JX514883	JX518561	-	-	JX518589	-	JX514897	-
B. vignae 7-2^T	KP899563	-	-	-	KM378374	KM378308	KT362339	KM378251
B. viridifuturi SEMIA 690^T	FJ025107	-	-	KR149134	KR149140	-	-	KR149137
B. yuanmingense LMG 21827^T	AF193818	FM253140	FM253312	FM253226	FM253183	FM253269	AB354633	EU818927
B. diazoefficiens SEMIA 5080	AF234889	FJ390957	FJ390997	-	FJ391157	JX867243	DQ376592	HQ259555
B. japonicum SEMIA 5079	AF234888	FJ390956	FJ390996	-	FJ391156	CP007569	DQ376594	HQ259534
B. elkanii SEMIA 5019	AF237422	-	FJ390990	-	FJ391150	-	-	HQ259531
B. elkanii SEMIA 587	AF234890	-	FJ390985	-	FJ391145	-	-	HQ259549

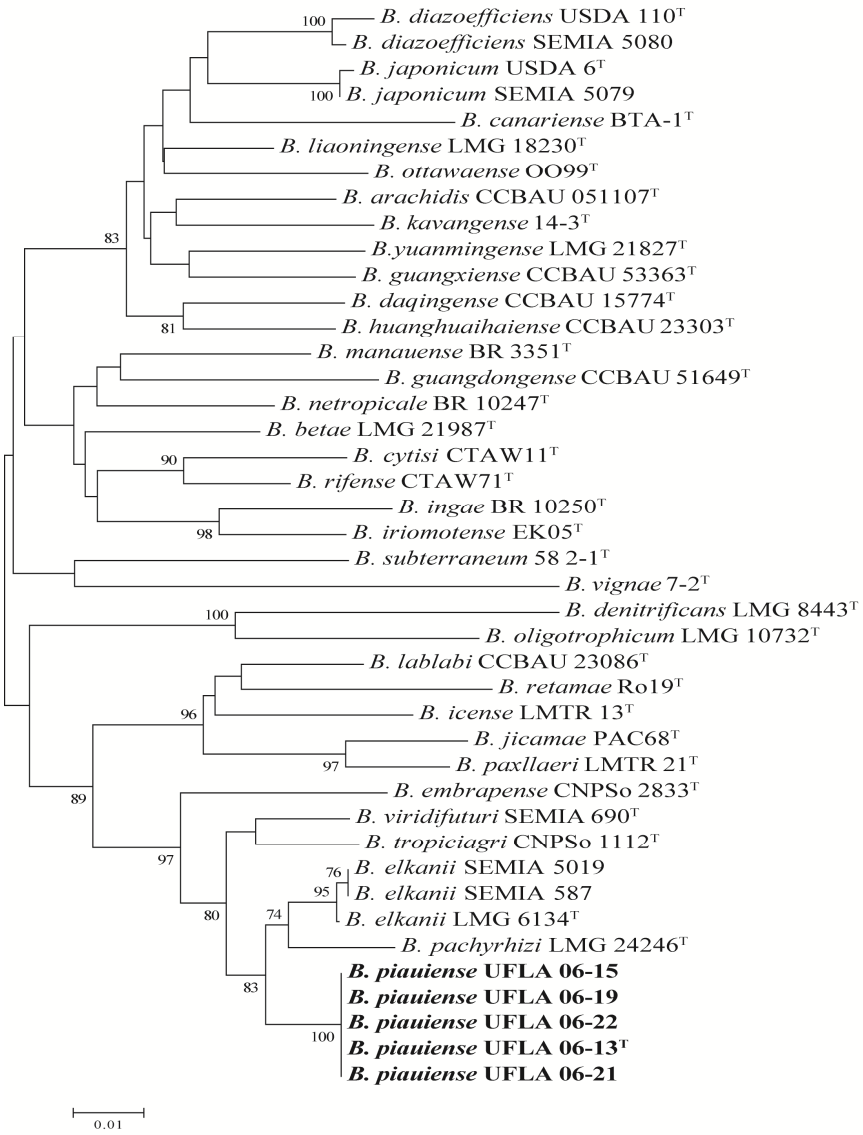

Fig. S1. Neighbour-joining phylogeny based on partial concatenated sequences (536 pb) of housekeeping genes (*dnaK* and *recA*) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers of the genes sequences for each strain are given in Table S2.

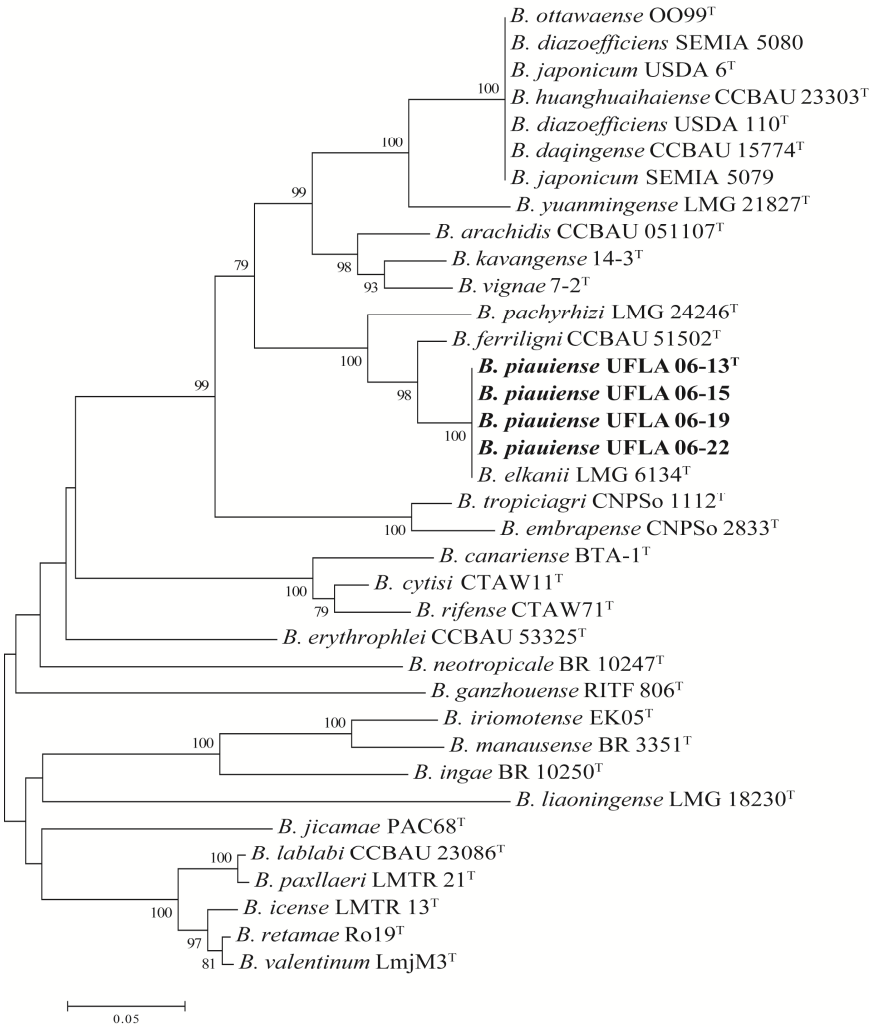

Fig. S2. Neighbour-joining phylogeny based on partial sequences (378 pb) of *nodC* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

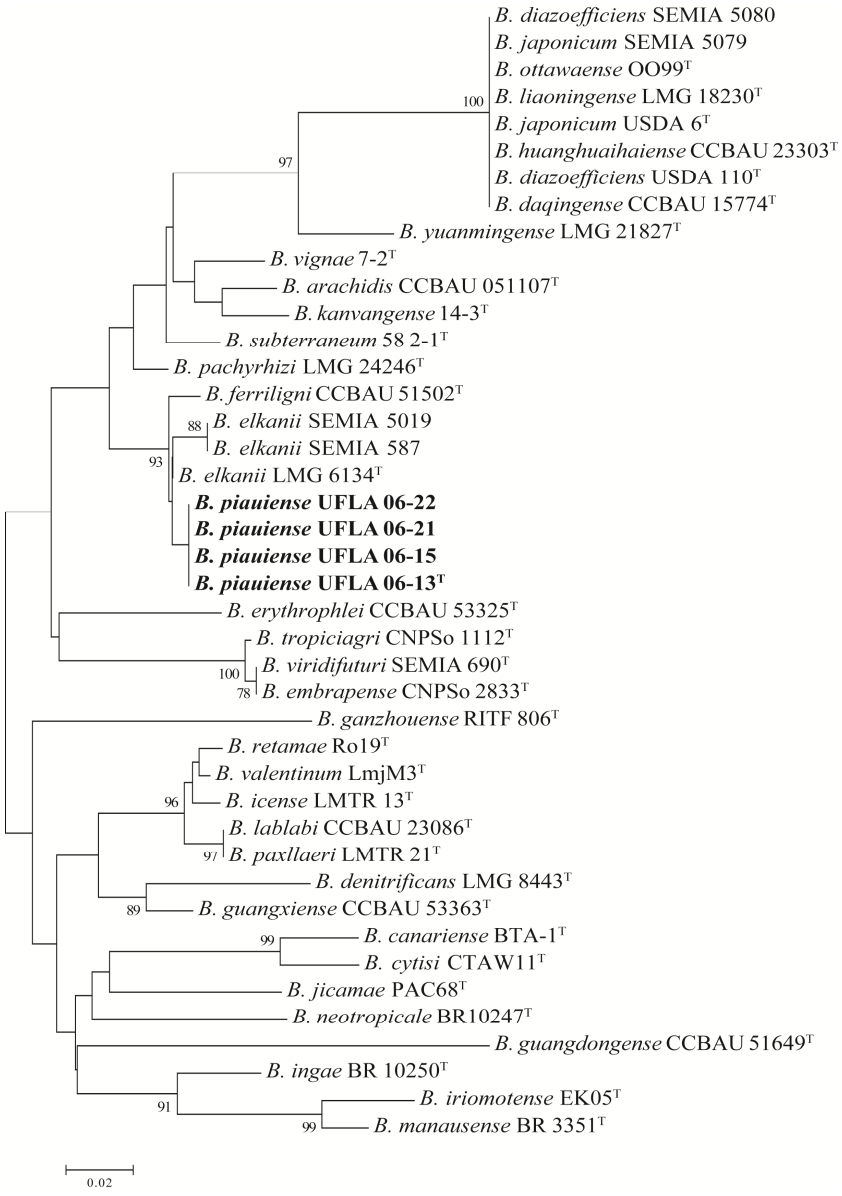

Fig. S3. Neighbour-joining phylogeny based on partial sequence (201 pb) of *nifH* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

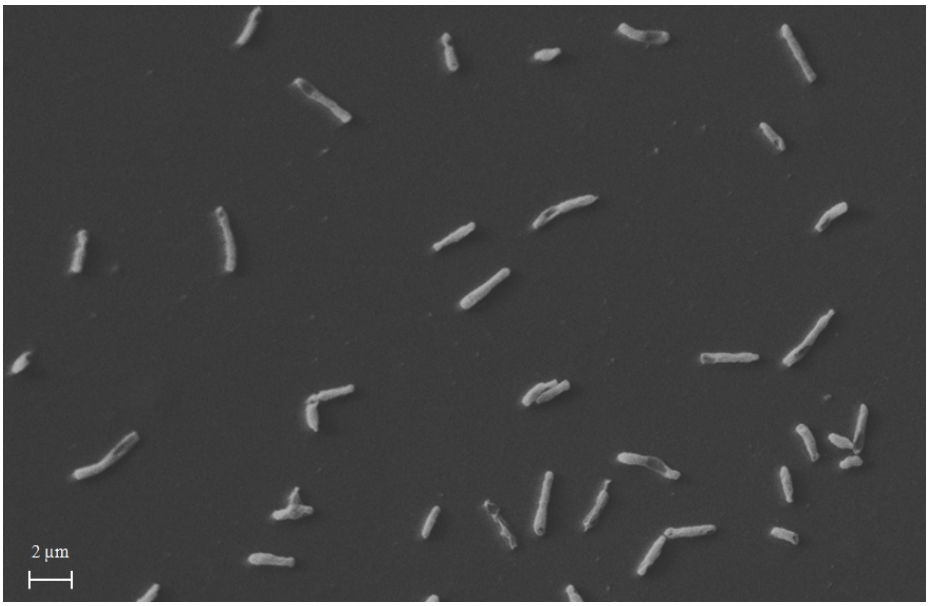

Fig. S4. Image of *Bradyrizobium piauiense* strain UFLA 06-13^{T} obtained by scanning electron microscopy.

ARTIGO 4 - *Bradyrhizobium neoglycine* an nitrogen-fixing bacterium isolated from soybean nodules in the Brazilian Southeast

Artigo de acordo com as normas da revista International Journal of Systematic and Evolutionary Microbiology (Versão preliminar)

Bradyrhizobium neoglycine sp. nov., a nitrogen-fixing bacterium isolated from soybean nodules in Brazilian Southeast

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Running title: *Bradyrhizobium neoglycine* sp. nov.

New sequences: *nodC* of *B. neoglycine* UFLA 06-10^T (KT793166), UFLA 06-05 (KT793164) and UFLA 06-06 (KT793165); *nifH* of *B. neoglycine* UFLA 06-10^T (KT793151), UFLA 06-05 (KT793149) and UFLA 06-06 (KT793150).

Abstract

In previous study, three nitrogen-fixing bacteria strains (UFLA 06-10^T, UFLA 06-05 and UFLA 06-06) isolated from soybean (*Glycine max* L.) nodules inoculated with soil from the state of Minas Gerais, southeastern Brazil, were identified and indicated as a new group within the *Bradyrhizobium* genus. These strains were characterized in this study using a polyphasic approach, in order to define their taxonomic position. The three strains presented 16S rRNA gene sequences identical to *Bradyrhizobium pachyrhizi* LMG 24246^T and *Bradyrhizobium piauiense*. However, the concatenated sequence analysis of the housekeeping genes *atpD, dnaK* and *recA* indicated that all the three strains represent a new species within the *Bradyrhizobium* genus, and it is phylogenetically close to *B. elkanii* LMG 6134^T (97.03 %), *B. piauiense* UFLA 06-13^T (96.86 %), *B. pachyrhizi* LMG 24246^T (96.42 %) and *B. brasilense* UFLA 03-321^T (96.34%). DNA-DNA relatedness between UFLA 06-10^T and the four neighboring species confirmed that this is a novel species. MALDI-TOF MS (Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) analysis and some phenotypic characteristics also allow distinguishing the three strains from the four phylogenetically related species. In the phylogenetic analysis of *nodC* and *nifH* genes, the three strains were grouped with *B. elkanii* LMG 6134^T and *B. piauiense* UFLA 06-13^T. Based on the data presented in this study, it is concluded that the three strains represent a novel species, for which the name *Bradyrhizobium neoglycine* sp. nov is proposed, with UFLA 06-10^T (LMG 29355) as the type strain.

Keywords: *Bradyrhizobium*, *Glycine max* L., MLSA, taxonomy, symbiotic genes

Bradyrhizobium genus, which includes slow-growing bacteria that produce alkaline reaction in culture medium with mannitol as carbon source,

was proposed by Jordan (1982). In Brazilian ecosystems, this genus has broad occurrence and has been indicated as the most abundant microsymbiont in root nodules of various legume species (Moreira et al., 1993; 1998; Giongo et al., 2008; Lima et al., 2009; Perrineau et al., 2011; Guimarães et al., 2012; Ribeiro et al., 2015).

Soybean [*Glycine max* (L.) Merrill] is an economically important legume species that form symbiosis with nitrogen-fixing bacteria of the *Bradyrhizobium* genus. In Brazil, soybean inoculation with the *Bradyrhizobium* strains SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*) completely replaces nitrogen chemical fertilizers, resulting in huge savings for the country.

Soybean is currently the main legume species from which *Bradyrhizobium* species have been isolated: *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992), *B. liaoningense* (Xu et al., 1995), *B. huanghuaihaiense* (Zhang et al., 2011), *B. daqingense* (Wang et al., 2012), *B. diazoefficiens* (Delamuta et al., 2013), *B. ottawaense* (Yu et al., 2014), and *B. piauiense* (Costa et al., submitted). Only the latter (*B. piauiense*) was isolated from soybean nodules in Brazilian soil.

In a previous study, a group of 46 nitrogen-fixing bacteria strains isolated from soybean nodules inoculated with soils from different Brazilian regions (Northeast, Midwest, Southeast and South) were identified as *Bradyrhizobium* sp. through the partial sequencing of the 16S rRNA gene and characterized based on multilocus sequence analysis (MLSA) of housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA* and *rpoB*) (Ribeiro et al., 2015). This analysis indicated two new groups (G-I and G-II) within the *Bradyrhizobium* genus (Ribeiro et al., 2015). Strains from G-II group have been recently classified as *B. piauiense* (Costa et al., submitted). In this study, the other group (G-I) was selected, represented by three strains (UFLA 06-10^{T}, UFLA 06-05, and UFLA 06-06), for further analysis, by molecular and phenotypic methods. Based on these results, a novel species is

proposed, whose name is *Bradyrhizobium neoglycine* sp. nov., with UFLA 06-10^T as type strain.

The three strains were isolated from effective soybean nodules inoculated with soil collected in Ijaci (21º12'17"S and 44º58'49"W), in the state of Minas Gerais, Southeast Brazilian. The soil had previous inoculation with soybean inoculant strains: SEMIA 5079 (*B. japonicum*), SEMIA 5080 (*B. diazoefficiens*), SEMIA 587 (*B. elkanii*), and/or SEMIA 5019 (*B. elkanii*). Isolation of strains was carried out in petri dishes containing 79 culture medium (Fred and Walkmam, 1928), also known as YMA (Vincent, 1970). These strains are currently deposited in the collection of the Department of Soil Biology, Microbiology and Biological Processes of the Federal University of Lavras, Brazil. The type strain UFLA 06-10^T (LMG 29355) is also deposited in the culture collection of the University of Ghent (BCCM / LMG), Belgium.

16S rRNA (1267 to 1347 pb), *atpD* (510 pb), *dnaK* (280 pb) and *recA* (545 à 561 pb) gene sequences of the three strains were obtained in a previous study (Ribeiro et al., 2015). Sequences of each gene were aligned using the ClustalW Multiple Alignment algorithm in the BioEdit software. For comparison, sequences of type strains of *Bradyrhizobium* species available in the GenBank (National Center for Biotechnology Information, NCBI) were included in the alignment. In the alignment of 16S rRNA, *dnaK* and *recA* genes, it was also included sequences of the four strains used as soybean inoculants in Brazil (SEMIA 587 *B. elkanii*, SEMIA 5019 *B. elkanii*, SEMIA 5079 *B. japonicum*, and SEMIA 5080 *B. diazoefficiens*). However, in the alignment of the *atpD* gene, it was only possible to include sequences of SEMIA 5079 and SEMIA 5080 strains, since the sequences of this gene are not available for SEMIA 587 and SEMIA 5019. Phylogenetic trees were constructed by the neighbor-joining (NJ) (Saitou and Nei, 1987) and maximum likelihood (ML) (Felsenstein, 1981) methods, using the Kimura 2 Parameter model (Kimura 1980). The MEGA 5 software package

(Tamura et al., 2011) was used in the construction of trees with bootstrap values based on 1000 replications.

Phylogenetic analysis of the 16S rRNA gene showed very similar results using neighbor- joining (NJ) (Fig. 1) and maximum likelihood (ML) (data not shown) methods. The three strains presented 16S rRNA gene sequences identical to SEMIA 587 (*B. elkanii*), SEMIA 5019 (*B. elkanii*), LMG 24246^T (*B. pachyrhizi*), and UFLA 06-13^T (*B. piauiense*), and shared 99.91% similarity with *B. elkanii* LMG 6134^T (Table S1). High similarity between sequences of the 16S rRNA gene of *Bradyrhizobium* species have been observed in several studies, indicating the limitation of this gene for discrimination between species this genus (Willems et al., 2001; Vinuesa et al., 2005; Rivas et al., 2009; Wang et al., 2013; Silva et al., 2014; Guimarães et al., 2015, Ribeiro et al., 2015).

In addition to the analysis of the 16S rRNA gene, analysis of multilocus sequences of housekeeping genes, such as *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*, has been successfully used for better discrimination between *Bradyrhizobium* species (Vinuesa et al., 2005; Ramírez-Bahena et al., 2009; Wang et al., 2013; Silva et al., 2014; Yu et al., 2014). Initially, it was carried out concatenated sequence analysis of the *dnaK* and *recA* genes, in order to enable including the four soybean inoculant strains (Fig. S1). In this analysis, the three strains formed a separate group, which shared 98.80% similarity with *B. elkanii* LMG 6134^T, SEMIA 587 and SEMIA 5019, and of 98.13% similarity with *B. piauiense* UFLA 06-13^T. Subsequently, it was carried out concatenated sequence analysis of the *atpD*, *dnaK* and *recA* genes, in which the three strains formed a monophyletic group supported by the high bootstrap value (100%) (Fig. 2). In this analysis, the *Bradyrhizobium* species with the greatest similarity with UFLA 06-10^T were *B. elkanii* LMG 6134^T (97.03%), *B. piauiense* UFLA 06-13^T (96.86%), *B. pachyrhizi* LMG 24246^T (96.42%) and *B. brasilense* UFLA 03-321^T (96.34%) (Table S1). These similarity values corroborate previous studies with different *Bradyrhizobium* species (Chahboune et al., 2011; Yu et al.,

2014, Silva et al., 2014; Durán et al., 2014), and indicates that the present strains belong to a novel species within this genus.

It was carried out characterization of the three strains and their four neighboring species (*B. elkanii* LMG 6134^{T}, *B. piauiense* UFLA 06-13^{T}, *B. pachyrhizi* LMG 24246^{T} and *B. brasilense* UFLA 03-321^{T}) by the MALDI-TOF MS (Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry) analysis. In this analysis, it was used cultures of the third generation, grown in YMA (Vincent, 1970). Sample preparation and data analysis were carried out according to Wieme et al. (2014). MALDI-TOF MS analysis enabled differentiating the three strains from *B. elkanii* LMG 6134^{T}, *B. piauiense* UFLA 06-13^{T}, *B. pachyrhizi* LMG 24246^{T} and *B. brasilense* UFLA 03-321^{T} (data not shown). This analysis has provided good discrimination between species of the *Bradyrhizobium* genus (Sánchez-Juanes et al., 2013; Durán et al., 2014).

Several phenotypic characteristics were evaluated to differentiate UFLA 06-10^{T}, UFLA 06-05 and UFLA 06-06 from the closest *Bradyrhizobium* species (*B. elkanii* LMG 6134^{T}, *B. piauiense* UFLA 06-13^{T}, *B. pachyrhizi* LMG 24246^{T} and *B. brasilense* UFLA 03-321^{T}). The two inoculant strains classified as *B. elkanii* (SEMIA 587 and SEMIA 5019) were also included in the analysis. It was evaluated the growth of these strains in 79 medium under different conditions of temperature (5, 15, 20, 28, 34, 37 and 40 °C), pH (4, 5,5, 6,8, 8, 9 and 10) and NaCl (w/v) (0.01, 0.25, 0.5, 0.75 and 1%) and resistance to the following antibiotics: ampicillin (10 μg mL^{-1}), cefuroxime (30 μg mL^{-1}), ciprofloxacin (5 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), doxycycline (30 μg mL^{-1}), erythromycin (15 μg mL^{-1}), gentamicin (10 μg mL^{-1}), kanamycin (30 μg mL^{-1}), and neomycin (30 μg mL^{-1}), following the methodology used by Florentino et al. (2012). It was also evaluated the ability of these strains to assimilate different carbon sources (D-arabinose, L-asparagine, citric acid, D-fructose, glycerol, glycine, D-glucose, L-glutamine, L-glutamic acid, lactose, malic acid, maltose, mannitol, L-methionine, sodium lactate and sucrose), and nitrogen sources

(L-arginine, L-asparagine, casein hydrolyzate, L-cysteine, glycine, L-glutamic acid, L-methionine and tryptophan). For the evaluation of the carbon and nitrogen sources, it was used modified 79 culture medium, according to Costa et al. (Submitted). UFLA 06-10^{T} was further characterized based on the Kit API 20NE (bioMérieux), according to the manufacturer's instructions, five days after incubation. Table 1 shows the main differential phenotypic characteristics between the evaluated strains.

For the delimitation of novel bacterial species, DNA-DNA hybridization analysis is recommended as a standard method (Wayne et al., 1987). For confirmation of the novel species, DNA-DNA hybridization experiments were carried out between UFLA 06-10^{T} and closely related species (*B. elkanii* LMG 6134^{T}, *B. piauiense* UFLA 06-13^{T}, *B. pachyrhizi* LMG 24246^{T}, and *B. brasilense* UFLA 03-321^{T}), following the described methodology (Ezaki et al., 1989; Willems et al., 2001). DNA-DNA relatedness between UFLA 06-10^{T} and the four neighboring species was low: *B elkanii* LMG 6134^{T} (23.5%), *B. piauiense* UFLA 06-13^{T} (26.7%), *B. pachyrhizi* LMG 24246^{T} (20.3%), and *B. brasilense* UFLA 03-321^{T} (24.6%). These results confirm a novel species within the Bradyrhizobium genus.

The *nodC* and *nifH* genes involved in nodulation and nitrogen fixation, respectively, are often evaluated in symbiotic characterization of novel species of *Bradyrhizobium*. In this study, the phylogeny of *nodC* and *nifH* genes of UFLA 06-10^{T}, UFLA 06-05 and UFLA 06-06 was investigated. DNA extraction from strains was carried out by the alkaline lysis method (Niemann et al., 1997). Amplification and sequencing of *nodC* was carried out according to the protocol of Sarita et al. (2005), modified by De Meyer et al. (2011). Amplification and sequencing of *nifH* gene was carried out according to Gaby and Buckley (2012). In the alignment of *nifH* gene sequences, it was included the four soybean inoculant strains (SEMIA 5079 *B. japonicum*, SEMIA 5080 *B. diazoefficiens*, SEMIA 587 *B. elkanii* and SEMIA 5019 *B. elkanii*) and the type strains of *Bradyrhizobium* species available in the GenBank. In the alignment of *nodC* gene, it was not possible

to include the strains SEMIA 587 and SEMIA 5019, since the sequences of this gene are not available in the GenBank. Phylogenetic trees were constructed as previously described. The three strains presented *nodC* gene sequences identical to *B. elkanii* LMG 6134^T and *B. piauiense* UFLA 06-13^T (Fig. S2). In the analysis of the *nifH* gene, the three strains showed sequences identical to the inoculant strains SEMIA 587 (*B. elkanii*) and SEMIA 5019 (*B. elkanii*), and shared 99.43% similarity with *B. elkanii* LMG 6134^T, and of 98.85% with *B. piauiense* UFLA 06-13^T (Table S1).

Ability of the three strains to effectively nodulate and fix nitrogen with their original host (*Glycine max*) was confirmed in a previous study by Ribeiro et al. (2015). Nodulation ability of UFLA 06-10^T strain was also evaluated in other hosts (*Vigna unguiculata*, *Stizolobium aterrimum* e *Acacia mangium*). This strain formed nodules in *V. unguiculata* and *S. aterrimum*, but it did not nodulate *A. mangium*.

Based on the results of genotypic, phenotypic and symbiotic analyses presented in this study, it is proposed the classification of the three strains isolated from *Glycine max* nodules inoculated with soil from the Brazilian Southeast into a novel species, for which the name *Bradyrhizobium neoglycine* sp. nov is proposed, with UFLA 06-10^T as the type strain.

Description of *Bradyrhizobium neoglycine* sp. nov.

Bradyrhizobium neoglycine (ne.o.gly.ci'ne. Gr. adj. neos new; N.L. gen. glycine, de *Glycine*, referring to the fact that the strains represent a novel group of bacteria isolated from root nodules of *Glycine max* L.).

Cells are aerobic gram-negative, non-spore-forming rods (Fig. S4). The three strains present cream-colored colonies with diameter > 1 mm, and alkaline reaction in 79 culture medium 79, using mannitol as carbon source and bromothymol blue as an indicator, from 5 to 7 days after incubation, at 28 °C. Strains grow in a pH range from 4 to 10, and in a temperature range from 20 to 37 °C, with optimal growth at 28 °C. Strains do not grow in NaCl

concentration above 0.5%. They are resistant to ampicillin, cefuroxime, ciprofloxacin, chloramphenicol, doxycycline and erythromycin, and sensitive to kanamycin, gentamicin and neomycin. They are positive for assimilation of D-fructose, L-glutamic acid and mannitol, and negative for assimilation of citric acid, malic acid, glycine, maltose, L-methionine, and sodium lactate as carbon source. They use weakly D-arabinose, D-glucose, glutamine, lactose and sucrose; however, the use of L-asparagine and glycerol as carbon source varies among strains. The use of L-glutamic acid as nitrogen source is positive, while the use of L-asparagine and casein hydrolyzate is weak. None of the strains used L-arginine, L-cysteine, glycine, L-methionine and tryptophan as a nitrogen source. UFLA 06-10^T presents positive reaction to urease, esculin hydrolysis, gelatin hydrolysis, and β-galactosidase. However, it shows negative reaction to nitrate reduction, tryptophan deaminase activity, glucose fermentation and arginine dihydrolase.

The type strain UFLA 06-10^T (LMG 29355) was isolated from effective soybean nodule inoculated with soil of the state of Minas Gerais, Southeast Brazilian.

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Zhang, Y. M., Li Jr, Y., Chen, W. F., Wang, E. T., Sui, X. H., Li, Q.Q., Zhang, Y. Z., Zhou, Y. G., Chen, W. X. (2012). *Bradyrhizobium huanghuaihaiense* sp. nov., an effective symbiotic bacterium isolated from soybean (*Glycine max* L.) nodules. *Int J Syst Evol Microbiol* **62**, 1951-1957.

Table 1. Differential phenotypic characteristics among *Bradyrhizobium neoglycine* sp. nov. and phylogenetically related to *Bradyrhizobium* species.

Strains: 1, *B. neoglycine* UFLA 06-10^T; 2, *B. neoglycine* UFLA 06-05; 3, *B. neoglycine* UFLA 06-06; 4, *B. brasilense* UFLA 03-321^T; 5, *B. piauiense* UFLA 06-13^T; 6, *B. pachyrhizi* PAC48^T; 7, *B. elkanii* LMG 6134^T; 8, *B. elkanii* SEMIA 5019 ; 9, *B. elkanii* SEMIA 587. Data represent the means of three biological replicates. +, growth; −, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at									
40 ºC	-	-	-	-	-	w	w	-	w
0.75 % NaCl	-	-	-	+	w	+	+	+	+
Carbon source assimilation									
D-arabinose	w	w	w	+	+	+	+	+	+
L-asparagine	w	+	w	w	w	+	+	+	+
D-fructose	+	+	+	+	+	+	+	w	w
Glycerol	+	w	w	+	+	+	+	+	+
D-glucose	w	w	w	w	+	+	+	+	+
L-Glutamine	w	w	w	+	+	+	+	+	+
Glutamic acid	+	+	+	+	w	+	+	+	+
Lactose	w	w	w	-	w	+	w	+	+
Malic acid	-	-	-	-	-	w	w	+	w
L-Methionine	-	-	-	-	-	w	w	w	w
Sodium lactate	-	-	-	-	-	+	+	+	+
Sucrose	w	w	w	-	-	+	+	+	+

Continuation...

Characteristic	1	2	3	4	5	6	7	8	9
Nitrogen source assimilation									
L-Arginine	-	-	-	-	w	+	+	+	+
L-Asparagine	w	w	w	+	+	+	+	+	+
Casein hydrolysate	w	w	w	+	+	w	+	+	+
L-Cysteine	-	-	-	-	-	+	+	+	+
L- Metionine	-	-	-	-	-	w	w	-	-
Tryptophan	-	-	-	-	-	-	-	w	w
Resistance to antibiotics (µg mL^{-1})									
Gentamycin (10)	-	-	-	+	-	-	+	+	+
Neomycin (30)	-	-	-	+	-	-	w	w	w

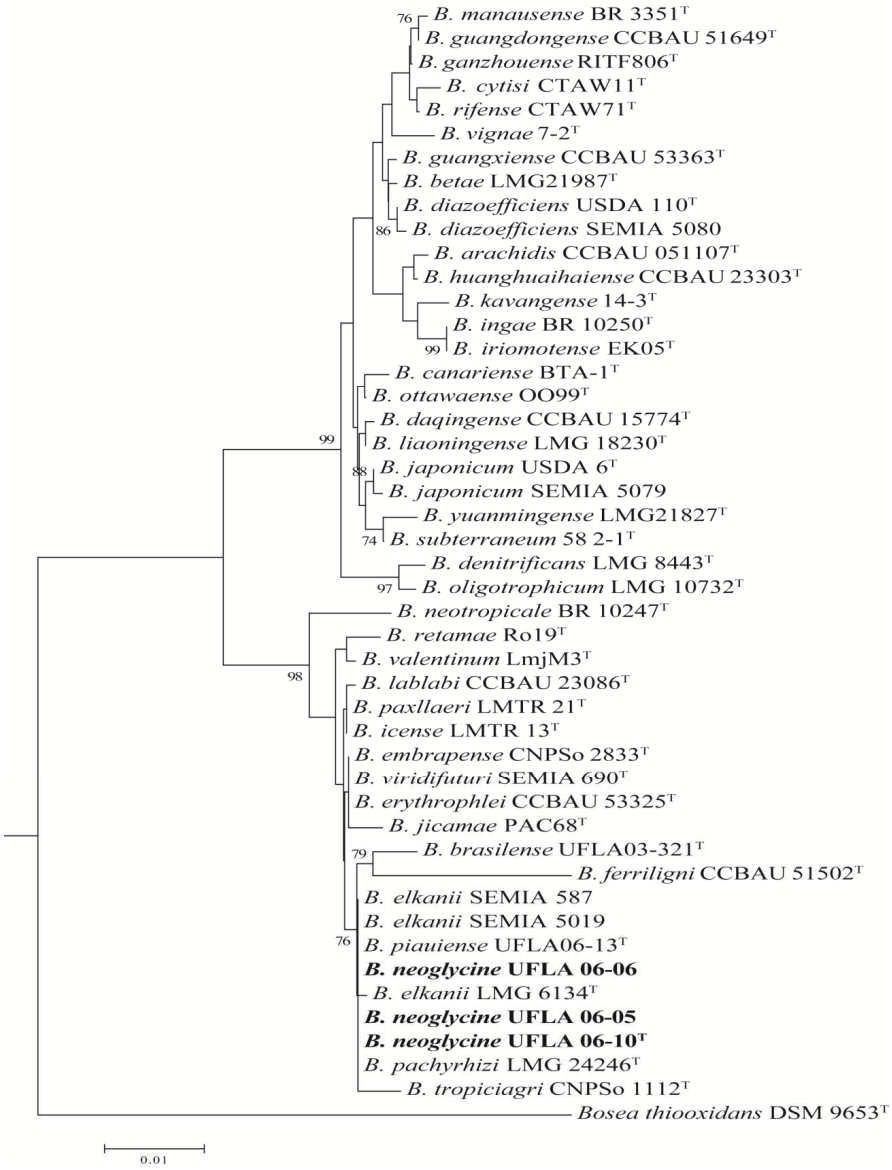

Fig. 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences (1176 pb) showing the relationships between strains of the novel species (shown in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. The 16S rRNA gene sequence of *Bosea thiooxidans* DSM9653^T was used as outgroup. GenBank accession numbers for each strain are given in Table S2.

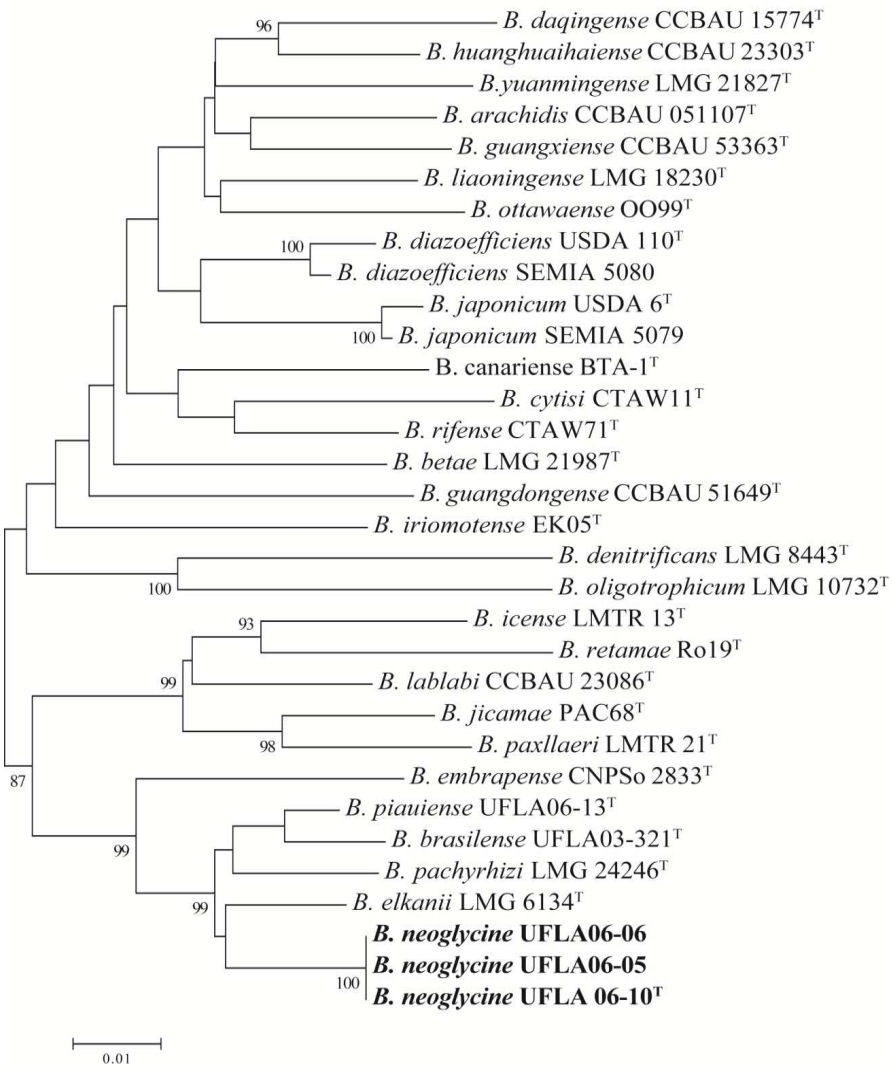

Fig. 2. Neighbour-joining phylogeny based on partial concatenated sequences (1033 pb) of housekeeping genes (*atpD*, *dnaK* and *recA*) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers of the genes sequences for each strain are given in Table S2.

Supplementary Material

Table S1. Similarity between *B. neoglycine* UFLA 06-10^T and other type strains of *Bradyrhizobium* species in the 16S rRNA, housekeeping and symbiotic genes.

Strains	Similarity with UFLA 06-10^T (%)							
	16S rRNA	***atpD***	***dnaK***	***recA***	**Concatenated (Two genes*)**	**Concatenated (Three genes*)**	***nodC***	***nifH***
***B. neoglycine* UFLA 06-05**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
***B. neoglycine* UFLA 06-06**	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
B. arachidis CCBAU 051107^T	96.59	91.99	82.49	93.36	90.36	90.97	83.62	92.74
B. betae LMG 21987^T	96.90	92.20	85.44	92.85	91.45	91.54	-	-
B. brasilense UFLA 03-321^T	99.39	95.24	99.23	95.68	97.14	96.34	78.77	89.76
B. canariense BTA-1^T	96.98	93.15	81.61	92.40	88.68	91.06	67.83	83.41
B. cytisi CTAW11^T	96.47	93.10	85.83	91.26	90.22	90.32	70.65	83.31
B. daqingense CCBAU 15774^T	97.13	90.97	84.34	91.43	90.24	90.29	80.18	87.31
B. denitrificans LMG 8443^T	96.62	90.66	83.73	87.42	87.92	89.66	-	84.97
B. diazoefficiens USDA 110^T	96.90	93.37	84.68	92.60	89.82	91.67	80.18	87.31
B. elkanii LMG 6134^T	99.91	95.32	99.28	97.21	98.80	97.03	100.00	99.43
B. embrapense CNPSo 2833^T	99.83	96.16	89.37	94.75	94.60	94.34	77.73	90.21
B. erythrophlei CCBAU 53325^T	99.83	-	-	94.06	-	-	74.36	90.78
B. ferriligni CCBAU 51502^T	97.84	-	-	94.56	-	-	96.72	98.26
B. ganzhouense RITF 806^T	96.75	91.72	-	92.61	-	-	63.66	85.78
B. guangdongense CCBAU51649^T	96.69	92.03	83.07	91.12	89.76	91.24	-	78.50
B. guangxiense CCBAU53363^T	96.91	91.48	82.03	92.77	90.09	90.80	-	88.99
B. huanghuaihaiense CCBAU23303^T	96.70	92.81	83.68	91.81	90.37	91.16	80.18	87.31
B. icense LMTR 13^T	99.83	90.73	88.79	93.72	91.77	91.26	70.35	88.26

Continuation...

Strains	**16S rRNA**	***atpD***	***dnaK***	***recA***	**Concatenated (Two genes*)**	**Concatenated (Three genes*)**	***nodC***	***nifH***
B. ingae BR 10250^T	96.40	-	86.24	89.76	89.55	-	62.90	86.31
B. iriomotense EK05^T	96.40	93.76	87.53	90.05	90.25	91.76	61.97	80.70
B. japonicum USDA 6^T	97.14	94.29	83.56	93.29	90.11	91.13	80.18	87.31
B. jicamae PAC68^T	99.49	92.55	86.94	91.90	91.00	91.63	69.25	85.89
B. kavangense 14-3^T	96.38	-	83.66	92.97	89.83	-	82.93	92.30
B. lablabi CCBAU 23086^T	99.74	91.62	87.51	94.29	92.47	92.34	70.00	88.15
B. liaoningense LMG 18230^T	97.22	91.30	84.07	94.00	91.25	91.19	59.17	87.31
B. manausense BR 3351^T	96.60	-	85.00	92.75	90.72	-	61.71	82.26
B. neotropicale BR 10247^T	98.69	-	84.22	93.11	91.24	-	66.49	85.13
B. oligotrophicum LMG 10732^T	96.71	89.97	83.17	88.81	89.02	89.66	-	-
B. ottawaense OO99^T	97.21	91.42	82.35	92.55	89.91	90.65	80.18	87.31
B. pachyrhizi LMG 24246^T	100.00	95.13	99.93	96.73	97.45	96.42	91.41	96.54
B. paxllaeri LMTR 21^T	99.83	90.65	89.10	92.01	91.25	91.21	69.86	88.15
B. piauiense UFLA 06-13^T	100.00	95.72	99.23	98.42	98.13	96.86	100.00	98.85
B. retamae Ro19^T	99.33	89.46	86.48	93.77	90.65	90.29	70.66	88.19
B. rifense CTAW71^T	96.68	92.54	85.83	92.54	91.01	91.41	68.89	-
B. subterraneum 58 2-1^T	97.04	-	86.97	90.75	90.19	-	-	94.50
B. tropiciagri CNPSo 1112^T	99.57	-	96.25	96.37	96.31	-	79.56	90.37
B. valentinum Lmj M3^T	99.58	89.92	-	94.41	-	-	70.54	88.60
B. viridifuturi SEMIA 690^T	99.83	-	95.47	96.03	96.44	-	-	90.21
B. vignae 7-2^T	96.53	-	85.96	92.19	87.21	-	82.67	93.17
B. yuanmingense LMG 21827^T	96.70	94.21	81.55	92.61	89.95	90.56	79.93	89.79
B. diazoefficiens SEMIA 5080	96.81	93.37	83.73	93.21	90.22	92.18	80.18	87.31
B. japonicum SEMIA 5079	97.05	94.83	83.56	93.60	90.31	91.47	80.18	87.31

Continuation...

Strains	**16S rRNA**	***atpD***	***dnaK***	***recA***	**Concatenated (Two genes*)**	**Concatenated (Three genes*)**	***nodC***	***nifH***
B. elkanii SEMIA 5019	100.00	-	99.28	97.21	98.80	-	-	100.00
B. elkanii SEMIA 587	100.00	-	99.28	97.21	98.80	-	-	100.00

* Concatenated sequences with two genes (*dnaK* and *recA*). **Concatenated sequences with three genes (*atpD*, *dnaK* and *recA*).

Table S2 GenBank accession numbers of the sequences used used in this study. Sequences obtained in this study are shown in bold

Strains	**16S rRNA**	***atp*D**	***dna*K**	***rec*A**	***nod*C**	***nif*H**
***B. neoglycine* UFLA 06-10^{T}**	KJ739895	KJ739962	KJ740004	KJ740089	**KT793166**	**KT793151**
***B. neoglycine* UFLA 06-05**	KJ739890	KJ739961	KJ740009	KJ740088	**KT793164**	**KT793149**
***B. neoglycine* UFLA 06-06**	KJ739891	KJ739936	KJ740008	KJ740063	**KT793165**	**KT793150**
B. arachidis CCBAU 051107^{T}	HM107167	HM107217	JX437668	HM107233	HM107267	HM107283
B. betae LMG 21987^{T}	AY372184	FM253129	FM253303	AB353734	-	-
B. brasilense UFLA 03-321^{T}	KF311068	KF452730	KF452791	KT793142	KT793173	KT825890
B. canariense BTA-1^{T}	AY577427	FM253135	FM253306	FM253177	AJ560653	EU818926
B. cytisi CTAW11^{T}	EU561065	GU001613	JN186290	GU001575	EU597844	GU001618
B. daqingense CCBAU 15774^{T}	HQ231274	HQ231289	JX437662	HQ231270	HQ231326	HQ231323
B. denitrificans LMG 8443^{T}	X66025	FM253153	FJ347273	EU665419	-	HM047125
B. diazoefficiens USDA 110^{T}	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463	NC 004463
B. elkanii LMG 6134^{T}	AF362942	AM418752	AM168363	AY591568	AB354631	AB094963
B. embrapense CNPSo 2833^{T}	AY904773	HQ634875	KP234519	HQ634899	KP234521	KP234518
B. erythrophlei CCBAU 53325^{T}	KF114645	-	-	KF114669	KF114576	KF114598
B. ferriligni CCBAU 51502^{T}	KJ818096	-	-	KJ818112	KJ818109	KJ818108
B. ganzhouense RITF 806^{T}	JQ796661	JX277182	-	JX277144	JX292035	JX292065
B. guangdongense CCBAU 51649^{T}	KC508867	KC508916	KC508964	KC509269	-	KC509130
B. guangxiense CCBAU 53363^{T}	KC508877	KC508926	KC508974	KC509279	-	KC509140
B. huanghuaihaiense CCBAU23303^{T}	HQ231463	HQ231682	JX437665	HQ231595	HQ231507	HQ231551
B. icense LMTR 13^{T}	KF896156	KF896192	KF896182	JX943615	KF896159	KF896161
B. ingae BR 10250^{T}	KF927043	-	KF927055	KF927061	KF927054	KF927085
B. iriomotense EK05^{T}	AB300992	AB300994	JF308944	AB300996	AB301000	AB300998
B. japonicum USDA 6^{T}	X66024	AM418753	AM182120	AM182158	AB354632	HM047126
B. jicamae PAC68^{T}	AY624134	FJ428211	JF308945	HM047133	AB573869	HM047127
B. kavangense 14-3^{T}	KP899562	-	KR259949	KM378399	KT033402	KM378254

Continuation...

Strains	**16S rRNA**	***atp*D**	***dna*K**	***rec*A**	***nod*C**	***nif*H**
B. oligotrophicum LMG 10732^T	JQ619230	JQ619232	KF962688	JQ619231	-	-
B. liaoningense LMG 18230^T	AF208513	AY386752	FM253309	FM253180	GU263466	EU818925
B. manausense BR 3351^T	HQ641226	-	KF786001	KF785992	KF786002	KF786003
B. neotropicale BR 10247^T	KF927051	-	KJ661693	KJ661714	KJ661727	KJ661728
B. ottawaense OO99^T	JN186270	HQ455212	JF308816	HQ587287	HQ587980	JN186287
B. pachyrhizi LMG 24246^T	AY624135	FJ428208	JF308946	HM047130	HQ588110	HM047124
B. paxllaeri LMTR 21^T	AY923031	KF896186	AY923038	JX943617	KF896160	DQ085619
B. piauiense UFLA 06-13^T	KJ739898	KJ739964	KJ740010	KJ740091	KT793167	KT793152
B. retamae Ro19^T	KC247085	KC247101	KJ560555	KC247094	KC247112	KF962704
B. rifense CTAW71^T	EU561074	GU001617	JQ945187	GU001585	EU597853	-
B. subterraneum 58 2-1^T	KP308152	-	KP308157	KM378397	-	KM378289
B. tropiciagri CNPSo 1112^T	AY904753	-	FJ391008	FJ391168	KP234520	HQ259540
B. valentinum Lmj M3^T	JX514883	JX518561	-	JX518589	JX514897	-
B. vignae 7-2^T	KP899563	-	-	KM378374	KT362339	KM378251
B. viridifuturi SEMIA 690^T	FJ025107	-	-	KR149140	-	KR149137
B. yuanmingense LMG 21827^T	AF193818	FM253140	FM253312	FM253183	AB354633	EU818927
B. diazoefficiens SEMIA 5080	AF234889	FJ390957	FJ390997	FJ391157	DQ376592	HQ259555
B. japonicum SEMIA 5079	AF234888	FJ390956	FJ390996	FJ391156	DQ376594	HQ259534
B. elkanii SEMIA 5019	AF237422	-	FJ390990	FJ391150	-	HQ259531
B. elkanii SEMIA 587	AF234890	-	FJ390985	FJ391145	-	HQ259549

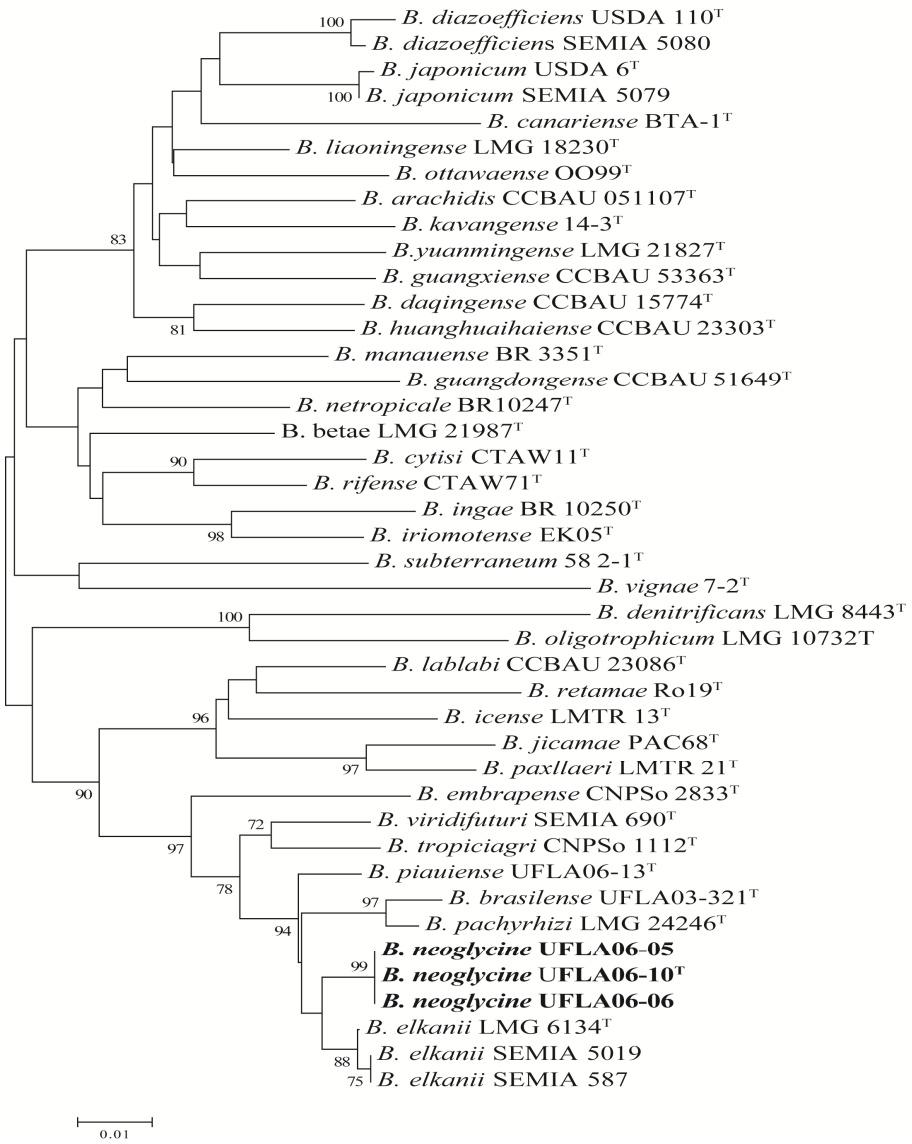

Fig. S1. Neighbour-joining phylogeny based on partial concatenated sequences (551 pb) of housekeeping genes (*dnaK* and *recA*) showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers of the genes sequences for each strain are given in Table S2.

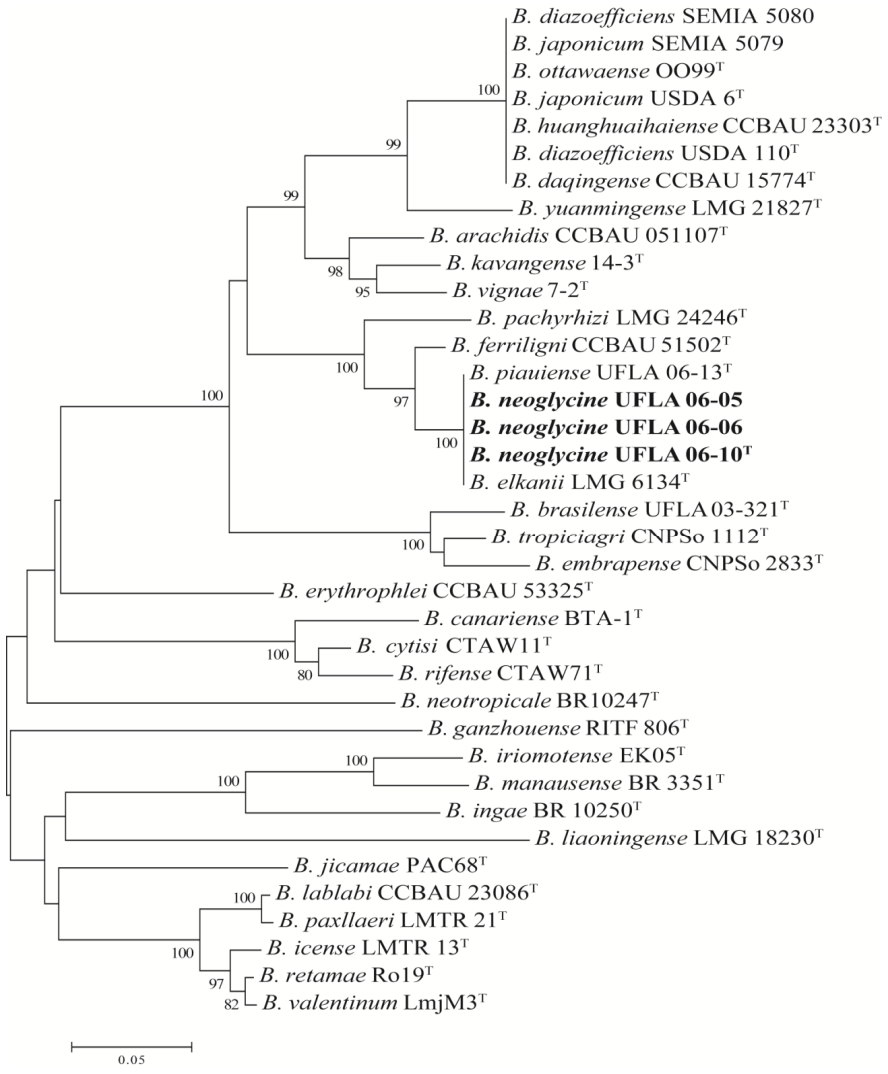

Fig. S2. Neighbour-joining phylogeny based on partial sequences (375 pb) of *nodC* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

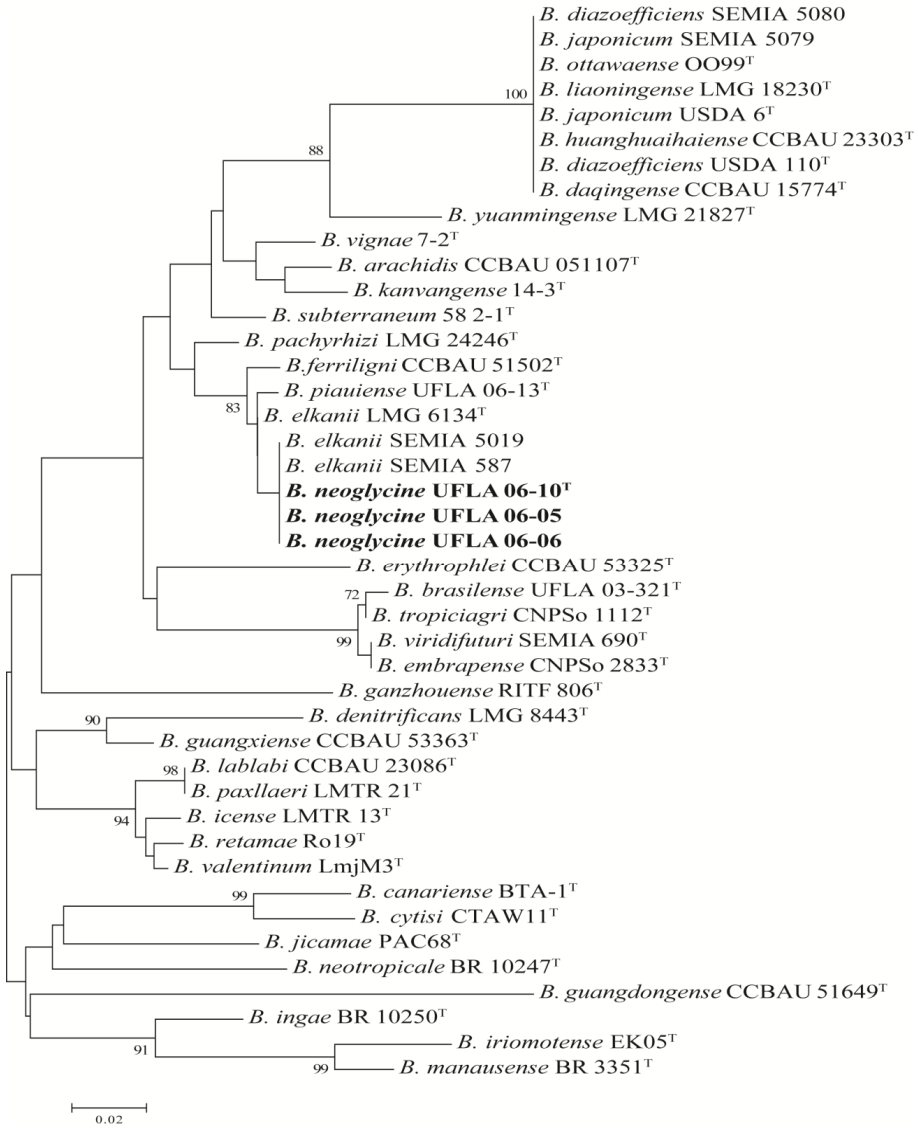

Fig. S3. Neighbour-joining phylogeny based on partial sequence (177 pb) of *nifH* gene showing the relationships between strains of the novel species (in bold) and type strains of the *Bradyrhizobium* species. Bootstrap values greater than 70% are indicated at nodes. GenBank accession numbers for each strain are given in Table S2.

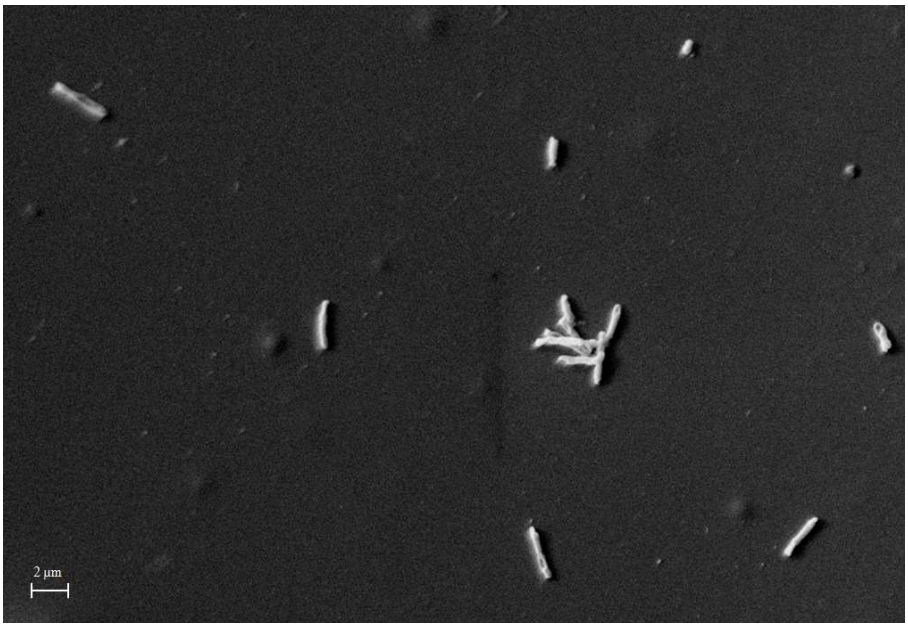

Fig. S4. Image of *Bradyrizobium neoglycine* strain UFLA 06-10^{T} obtained by scanning electron microscopy.

ARTIGO 5 - *Bradyrhizobium* strains from Amazonian soils promote lima bean growth in an Oxisol

Artigo de acordo com as normas da revista Annals of Microbiology
(Versão preliminar)

Bradyrhizobium **strains from Amazonian soils promote lima bean growth in an Oxisol**

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Abstract Lima bean (*Phaseolus lunatus* L.) is an important legume species that establishes symbiosis with legume nodulating nitrogen-fixing bacteria (LNNFB), mainly of the *Bradyrhizobium* genus. However, there are still no strains selected as inoculants for this crop in Brazil. This study aimed to evaluate the efficiency of LNNFB of the genus *Bradyrhizobium* from different ecosystems in symbiosis with lima bean, in both Leonard jars and pots with a dystrophic Yellow Latosol (Oxisol). In the experiment in Leonard jars, 17 strains (11 from Amazonian soils and 6 from soils of other regions) and two uninoculated controls, one with a low (5.25 mg L^{-1}) and another with a high (52.5 mg L^{-1}) concentration of mineral nitrogen (N) were evaluated. The six strains (UFLA 03-144, UFLA 03-84, INPA 104A, INPA 54B, INPA 86A and UFLA 03-150) that exhibited the highest efficiency in Leonard jars, all isolated from Amazonian soils, were compared to two uninoculated controls, one without and another with 300 mg N dm^{-3} (NH_4NO_3) applied to the pots with samples of a Oxisol in the presence and absence of liming. In this experiment, liming did not affect nodulation and plant growth; the INPA 54B and INPA 86A strains stood out in terms of shoot dry matter production and provided increases of approximately 48% in shoot N accumulation compared to the native LNNFB populations, findings that indicate the potential of these strains to be used as lima bean inoculants.

Keywords *Phaseolus lunatus* L . biological nitrogen fixation . inoculant . liming

Introduction

Lima bean (*Phaseolus lunatus* L.) is ranked second most economically important crop among the four commercially exploited species of genus *Phaseolus* in the worldwide (Fofana et al. 1999). Although its use is relatively lower than common bean (*P. vulgaris*), lima bean is one of the main legume species cultivated in tropical regions and represents an

important alternative as protein source for human consumption (Maquet et al. 1999). In Brazil, the Northeastern region accounts for approximately 95% of Brazil's national lima bean production, with a planted area of 41,318 ha, a production of 17,078 tons of beans and grain yield of approximately 420 kg ha^{-1} for the year 2009 (available at http://www.sidra.ibge.gov.br). This low grain yield may be attributed to the fact that most of the production is from small-scale farmers who adopted no agricultural technology.

Like other species of the genus *Phaseolus*, lima bean is able to establish symbiosis with legume nodulating nitrogen-fixing bacteria (LNNFB) (Thies et al. 1991; Ormeño-Orrillo et al. 2006; Antunes et al. 2011; Matsubara and Zúñiga-Dávila 2015) and the genus *Bradyrhizobium* has been highlighted as its predominant symbiont (Ormeño-Orrillo et al. 2006; López-López et al. 2013; Matsubara and Zúñiga-Dávila 2015). However, information about the agronomic implications of this symbiosis is scarce, especially in Brazil, where there is only one study evaluating the symbiotic efficiency of LNNFB strains in lima bean, which was performed under axenic conditions (Antunes et al. 2011). Thus, there is a great need to expand studies of symbiotic efficiency in lima bean to select and recommend strains efficient in biological nitrogen fixation (BNF) for use as lima bean inoculants, aiming to increase the grain yield of this crop.

Among the LNNFB genera, *Bradyrhizobium* has stood out in Brazilian ecosystems because it has broad geographic distribution and establishes efficient symbiosis with many legume species of agricultural, pastoral and forestry importance. Natural and altered ecosystems within the Amazon region house a large diversity of *Bradyrhizobium* strains (Moreira et al. 1993; Moreira et al. 1998; Lima et al. 2009; Guimarães et al. 2012; Jaramillo et al. 2013) which may be considered important sources of genetic resources for biotechnological application. Two *Bradyrhizobium* strains isolated from soils within this region are currently authorized by the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento - MAPA) (available at http://www.agricultura.gov.br) for

inoculation in cowpea and have been successfully used in different Brazilian regions (Soares et al. 2006; Ferreira et al. 2013).

In Brazil, LNNFB strains have been selected and authorized by the MAPA for the production of inoculants to 83 legume species, and among these, 62% have inoculant strains of the genus *Bradyrhizobium* (available at http://www.agricultura.gov.br). A major advantage of this genus is that most of its nodulation and N_2 fixation genes are located on the chromosome, which allows them to be genetically stable. This characteristic is extremely important for the selected strain does not lose its symbiotic efficiency over the years. In addition to the strain's symbiotic efficiency and its genetic stability, the ability to nodulate and fix nitrogen in symbiosis with different legumes species and competitiveness with native LNNFB populations should also be considered during the processes of selecting and recommending new LNNFB strains for a certain legume species. The competitiveness with the native LNNFB populations is one of the main factors determining the magnitude of responses of the legume species to inoculation (Singleton and Tavares 1986; Thies et al. 1991). Other factors, such as genetic traits of both the macro and microsymbiont, soil acidity and nutrient availability can also affect nodulation and the BNF process (Hartwig 1998; Bonilla and Bolaños 2009; Moreira et al. 2010; Rufini et al. 2011).

This study aimed to evaluate the efficiency of nitrogen-fixing bacteria of the genus *Bradyrhizobium* from different ecosystems in symbiosis with lima bean under axenic conditions and in a dystrophic Yellow Latosol (Oxisol).

Materials and methods

Strains evaluated

In this study, 17 *Bradyrhizobium* strains from collection of the Sector of Biology, Microbiology and Biological Processes of the Soil (Setor de Biologia, Microbiologia e Processos Biológicos do Solo - SBMPBS), Federal University of Lavras (Universidade Federal de Lavras - UFLA) were

used. These strains have recently been characterized by housekeeping genes sequencing (Guimarães et al. 2015; Ribeiro et al. 2015), which indicated that they belong to different phylogenetic groups (Table 1). The origin and efficiency of these strains in symbiosis with other legume species: cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*) or soybean (*Glycine max*) is shows in Table 1. The ability of the LNNFB strains to fix nitrogen in symbiosis with different legumes species is a desirable characteristic, once it facilitates the commercialization by companies producing inoculants.

Strains efficiency under axenic conditions

The experiment was conducted at the SBMPBS (UFLA) from April to May 2014. The treatments consisted of individual inoculations of the 17 *Bradyrhizobium* strains and two uninoculated negative controls, one with a low (5.25 mg L^{-1}) and the other with a high (52.5 mg L^{-1}) concentration of mineral nitrogen (N). The experiment followed a completely randomized design, with three replicates.

A 1:2 mixture of sand (150 cm^3) and vermiculite (300 cm^3) was added to the tops of the Leonard jars. Hoagland nutrient solution (Hoagland and Arnon 1950) modified as described by Guimarães et al. (2012) was added to the bottoms of the jars. The same N concentration used in the nutrient solution for the control with low N concentration was used for the inoculated treatments. After preparing the jars and nutrient solution, they were autoclaved for one hour at 1.5 kg cm^{-2} at 121°C.

One accession of lima bean “criolo” (white seed), which is one of the accessions often used by farmers in Northeastern Brazil, was used. Before sowing, the seeds were surface-sterilized using 98% ethyl alcohol (30 seconds) and 2% sodium hypochlorite (2 minutes). Seeds were subjected to successive washing in sterile distilled water and after pre-germinated in sterile petri dishes containing filter paper and wet cotton, where they were kept for 48 hours in a growth chamber at 28ºC. Four seeds were sowed in

each Leonard jar and seven days after emergence, thinning was performed, leaving one plant per jar.

The bacterial strains were cultured in liquid culture medium 79 (Fred and Waksman 1928) under stirring of 110 rpm at 28ºC for five days. In each inoculated treatment, 1 mL of the inoculant at the concentration of $1x10^8$ bacterial cells mL^{-1} was added to each seedling. After inoculation, a layer of paraffin sand (10 kg of sand, 1 L of chloroform and 10 g of paraffin) was added to each pot to avoid possible contamination. The nutrient solution was prepared, autoclaved and reapplied to the pots periodically throughout the experiment.

At 45 days after sowing (onset of flowering) the SPAD (Soil Plant Analysis Development) index, which represents an indirect measurement of leaf chlorophyll content, was determined. A total of 15 readings were taken in the last fully developed trifoliate leaf for each plant, using a Minolta SPAD-502 chlorophyll meter. After the readings, the plants were collected to evaluate the following variables: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), efficiency compared to the control with high mineral N concentration (EFNC) and shoot nitrogen accumulation (SNA). The nodules, shoots and roots were placed in paper bags and dried in a forced air oven at 60°C to constant weight to determine the NDM, SDM and RDM. The EFNC was calculated using the following formula: EFNC = (treatment SDM * 100) / (SDM of the treatment with high mineral N concentration). Shoot N content was determined using the semi-micro Kjedahl method. The SNA was calculated by multiplying the SDM (mg) by the N content (%) / 100.

According to the Shapiro-Wilk test the experimental data exhibited normal distribution and were subjected to analysis of variance using the statistical analysis program SISVAR, version 5.3 (Ferreira 2011). Mean values were grouped by the Scott-Knott test at 5% probability. The NN and NDM data were transformed into the square root of Y + 0.5. Pearson's correlation coefficients were estimated at 1% and 5% probability levels.

Strains efficiency in pots with soil

The experiment was conducted from October to November 2014 in a greenhouse at the SBMPBS (UFLA). A typical dystrophic Yellow Latosol (Oxisol) collected in the municipality of São Luís-MA in a site at the Federal Institute of Education, Science and Technology of Maranhão (Instituto Federal de Educação, Ciência e Tecnologia do Maranhão) (altitude 27m, 2°36'37"S and 4°16'18"W) was used. Maranhão state is one of the main lima bean producers of Northeastern Brazil. Additionally, this soil class is predominant within the state and throughout Brazil. The collection site of the samples has a history of annual corn (*Zea mays* L.) cultivation under a conventional planting system. Every three years, liming and N-P-K fertilizing (100-80-90) is performed and there is no history of any type of inoculant that has been previously applied. The last liming was performed at this site two years before this study.

The soil samples were collected at a depth of 0-20 cm and then crushed, homogenized, sieved through a 4-mm sieve and placed in pots (3.5 dm^3 capacity). Before implementing the experiment, the soil presented the following characteristics: pH in H_2O 6.0; P (Mehlich 1) 2.3 mg dm^{-3}; K^+ 20 mg dm^{-3}; Ca^2 1.60 $cmol_c$ dm^{-3}; Mg^{2+} 0.60 $cmol_c$ dm^{-3}; Al^{3+} 0.00 $cmol_c$ dm^{-3}; H + Al 2.32 $cmol_c$ dm^{-3}; sum of exchangeable bases 2.25 $cmol_c$ dm^{-3}; cation exchange capacity 2.25 $cmol_c$ dm^{-3}; cation exchange capacity at pH 7.0 4.57 $cmol_c$ dm^{-3}; aluminum saturation 0.00%; base saturation 49.26% organic matter 1.87 dag kg^{-1}; clay 13 dag kg^{-1}; silt 5 dag kg^{-1} and sand 82 dag kg^{-1}.

The experiment followed a randomized block design with four replicates in an 8 x 2 factorial arrangement [8 N sources and 2 liming levels (with and without liming)]. The following N sources were used: individual inoculation of the 6 *Bradyrhizobium* strains that exhibited highest efficiency in the experiment in Leonard jars (UFLA 03-84, UFLA 03-144, INPA 104 A, INPA 54B, INPA 86A and UFLA 03-150) and 2 uninoculated controls, one without and another with mineral N fertilizer (300 mg dm^{-3}). The lime dose

in the liming treatments was calculated according to the base saturation method to raise the saturation to 60% using calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$), respectively, at a 4:1 ratio. The soil was kept moist and was incubated for 30 days before planting. In all plots, the following fertilization was carried out: 300, 300, 40, 5.0, 1.5, 3.6, 0.8 and 0.15 mg dm^{-3} of K, P, S, Zn, Cu, Mn, B and Mo, respectively. For the control with mineral N, NH_4NO_3 (300 mg N dm^{-3}) was provided into three applications.

The lima bean seeds and the method for disinfecting them were the same as were used in the previous experiment. Six seeds were sowed per pot, and each inoculated treatment received 1 mL of the inoculant at 1 x 10^8 bacterial cells mL^{-1} on each seed. Eight days after emergence, the plants were thinned to two seedlings per pot.

The plants were collected at 45 days after sowing (during flowering) to evaluate the following variables: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), efficiency compared to the control fertilized with mineral N (EFNC) and shoot nitrogen accumulation (SNA). These variables were obtained by the procedure described in the previous experiment. According to the Shapiro-Wilk test, the experimental data exhibited normal distribution and were subjected to analysis of variance using the statistical analysis program SISVAR, version 5.3 (Ferreira 2011). Mean values were grouped by the Scott-Knott test at 5% probability. The NN and NDM data were transformed into the square root of Y + 0.5. Pearson's correlation coefficients were estimated at 1% and 5% probability levels.

Results

Strains efficiency under axenic conditions

The treatments had effects ($p<0.05$) on all variables evaluated (Table 2). There was no nodulation in the uninoculated controls, indicating that there

was no contamination in the experiments. Only four strains did not nodulate lima bean (UFLA 03-268, UFLA 03-290, UFLA 06-24 and UFLA 06-13). Of the strains that nodulated, the mean NN values per plant ranged from 15 to 506 for the treatments inoculated with the UFLA 03-153 and UFLA 03-144 strains, respectively. The UFLA 03-84, UFLA 03-144, INPA 104A, INPA 54B, UFLA 03-150, UFLA 03-197 and UFLA 03-164 strains promoted higher NN ($p<0.05$) than the other strains. The highest ($p<0.05$) NDM values were obtained in the treatments inoculated with the UFLA 03-144 and INPA 104A strains. The UFLA 03-84, INPA 54B, INPA 86A, UFLA 03-150 and UFLA 03-197 strains formed a second group that also stood out ($p<0.05$) in terms of NDM production.

Of the 17 strains evaluated, the highest ($p<0.05$) SDM, TDM, EFNC, SPAD index and SNA values were obtained with the seven strains that also stood out in terms of NDM production (UFLA 03-84, UFLA 03-144, INPA 104A, INPA 54B, INPA 86A, UFLA 03-150 and UFLA 03-197) (Table 2). Positive correlations ($p<0.01$) were detected between NDM and the other variables, except for RDM. There was also high correlation ($p<0.01$) between the SPAD index and SNA (Table 3).

The INPA 54B strain promoted higher ($p<0.05$) SDM (7.29 g $plant^{-1}$), TDM (8.77 g $plant^{-1}$), EFNC (164%) and SNA (260.76 mg $plant^{-1}$) values than the other strains and the control with high N concentration (Table 2). The UFLA 03-144 strain was the second most efficient for these variables, promoting higher ($p<0.05$) values than the control with high N concentration and the other strains. The treatments inoculated with the UFLA 03-84, INPA 104A and INPA 86A strains formed a third group with higher ($p<0.05$) SDM, TDM, EFNC and SNA values than the control with high N concentration. The treatments inoculated with the UFLA 03-150 and UFLA 03-197 strains exhibited similar and lower ($p<0.05$) values of these variables, respectively, compared to the control with high N concentration. The other strains were inefficient in BNF, promoting SDM, EFNC and SNA values similar ($p<0.05$) to the control with low N concentration.

For the RDM, 82% of the strains evaluated exhibited higher ($p<0.05$) values than the control with low N concentration (Table 2). The INPA 54B strain stood out ($p<0.05$) compared to the other strains and behaved similarly ($p<0.05$) to the control with high N concentration. The UFLA 03-144, INPA 237B, INPA 104A and UFLA 03-153 nodulating strains, together with three strains that did not nodulate lima bean (UFLA 03-290, UFLA 06-24 and UFLA 06-13), formed a second group with higher ($p<0.05$) RDM values than the other strains and the control with low N concentration, though these values were lower ($p<0.05$) than that of the control with high N concentration.

Strains efficiency in pots with soil

There was effects ($p<0.05$) only of nitrogen sources on the NN, NDM, SDM, TDM and SNA variables (Table 4). The highest ($p<0.05$) NN values were measured in the treatments inoculated with the UFLA 03-144 and INPA 86A strains; however, for NDM, there was no difference between the inoculated treatments and the uninoculated control without mineral N. The plants fertilized with mineral N did not nodulate.

All strains, except UFLA 03-150, promoted higher SDM and TDM production ($p<0.05$) than the uninoculated control without mineral N (Table 4). However, no strain was similar or superior ($p<0.05$) to the uninoculated control with mineral N. The INPA 54B and INPA 86A strains stood out ($p<0.05$) from the others in SDM production and promoted 34 and 36% increases compared to the production obtained in the uninoculated control without mineral N, respectively. The treatments inoculated with the UFLA 03-84, UFLA 03-144 and INPA 104A strains promoted similar ($p<0.05$) SDM production.

The highest ($p<0.05$) SNA value was obtained in the uninoculated control with mineral N (Table 4). The INPA 54B and INPA 86A strains were most efficient in N_2 fixation, promoting higher ($p<0.05$) SNA than the other

strains and the uninoculated control without mineral N. The UFLA 03-84 and UFLA 03-144 strains also promoted higher ($p<0.05$) SNA than the uninoculated control without mineral N. The treatments inoculated with the INPA 104A and UFLA 03-150 strains exhibited similar and lower ($p<0.05$) SNA than the uninoculated control without N, respectively. Positive correlations ($p<0.05$) were observed between the NN, NDM and SNA (Table 3).

For EFNC, there was an individual effect ($p<0.05$) of two factors (N sources and liming levels) (Table 4). Among the inoculated treatments, the INPA 54B and INPA 86A strains exhibited higher ($p<0.05$) efficiency than the others and the uninoculated control without mineral N. The UFLA 03-84, UFLA 03-144 and INPA 104A strains exhibited similar efficiencies that were higher ($p<0.05$) than the efficiency of the uninoculated control without mineral N. Between the liming levels, EFNC was higher ($p<0.05$) with liming application.

Regarding RDM, there was an interaction ($p<0.05$) between the factors liming and N source (Table 5). The UFLA 03-144 and UFLA 03-150 strains induced higher ($p<0.05$) RDM production in the presence of liming, whereas the INPA 104A strain promoted higher ($p<0.05$) RDM production in the absence of liming. Among the N sources, in the absence of liming, the treatments inoculated with the UFLA 03-84 and INPA 104A strains exhibited higher ($p<0.05$) RDM production than the other inoculated treatments and the controls with and without mineral N application. In the presence of liming, the inoculated treatments, except for the one inoculated with the INPA 104A strain, promoted higher ($p<0.05$) RDM production than the controls with and without mineral N application.

Discussion

Selecting LNNFB strains efficient in BNF in symbiosis with lima bean is an important strategy to economically and sustainably increase the grain yield of this crop. The six strains that exhibited the highest efficiency in N_2

fixation in symbiosis with lima bean in Leonard jars were all isolated from Amazonian soils under different land-use systems: pasture (UFLA 03-84), agriculture (UFLA 03-144 and UFLA 03-150) and forest (INPA 104A, INPA 54B and INPA 86A). These strains were also efficient in symbiosis with cowpea (UFLA 03-144, UFLA 03-84 and UFLA 03-150) and siratro (INPA 104A, INPA 54B and INPA 86A) in previous studies (Table 1), indicating the potential of the Amazon as a source of genetic resources for biotechnological applications.

The UFLA 03-84 strain is currently authorized by the MAPA as a cowpea inoculant (available at http://www.agricultura.gov.br) and its symbiotic efficiency was also observed in pigeon pea (*Cajanus cajan* L.) in Leonard jars (Rufini et al. 2014). In contrast, the UFLA 03-153, UFLA 03-164, UFLA 03-320 and UFLA 03-321 strains, which are efficient in symbiosis with cowpea (Rufini et al. 2013; Soares et al. 2014), and the INPA 237B and UFLA 04-0212 strains, which form efficient symbiosis with siratro (Table 1), were inefficient in symbiosis with lima bean in this study, indicating that BNF is affected by the intrinsic characteristics of the symbionts, as reported by Hartwig (1998).

High symbiotic efficiency of isolates (genetically unidentified) of lima bean nodules inoculated in this crop was reported by Antunes et al. (2011) in an experiment conducted in Leonard jars. These authors also detected positive correlations ($p<0.05$) between NDM and SDM production and SNA, corroborating the results obtained in the present study. Similar correlation results were reported by Ferreira et al. (2012) when working with common bean (*Phaseolus vulgaris* L.) inoculated with LNNFB strains.

The SPAD index, which represents an indirect measurement of the leaf chlorophyll content, has been positively correlated with shoot N accumulation and/or grain yield and/or shoot dry matter in some legume species, such as common bean, soybean and cowpea (Fritschi and Ray 2007; Remans et al. 2008; Jaramillo et al. 2013). However, for lima bean, our study is the first to report this index and its excellent correlation with both

SNA and SDM production. Thus, we suggest evaluating the SPAD index during the process of selecting LNNFB strains for this crop, especially at the first selection steps, in which large numbers of strains are typically evaluated, making N analysis very expensive.

The INPA 237B, UFLA 03-153 and UFLA 03-320 strains that were inefficient in BNF and the four strains that did not nodulate lima bean (UFLA 03-268, UFLA 03-290, UFLA 06-24 and UFLA 06-13) in Leonard jars most likely increased ($p<0.05$) the RDM production by acting in other biological processes, different from BNF. Although the main function of genus *Bradyrhizobium* has already been reported as BNF, this genus is quite versatile and may also act in other plant growth-promoting processes, such as inorganic phosphate solubilization and phytohormone production (Boiero et al. 2007; Marra et al. 2011; Oliveira-Longatti et al. 2014).

In the experiment in pots with soil, the absence of an effect of liming on lima bean nodulation and shoot growth differs from the results obtained by Rufini et al. (2011), who worked with common bean and found positive responses ($p<0.05$) to liming on nodulation, plant growth, and N_2 fixation; however, these results were observed in a Oxisol with an initial pH (H_2O) of 5.1 and a base saturation of 21%. Liming is an important practice for two reasons: it raises soil pH, reducing acidity, and increases the base saturation, supplying calcium and magnesium, which are important nutrients for plant and diazotrophic bacterial development and, consequently, for symbiosis establishment (Norris 1958; Lodeiro et al. 1995). In the present study, the low acidity (pH 6.0) and Ca^{+2} (1.60 $cmol_c\ dm^{-3}$) and Mg^{2+} (0.60 $cmol_c\ dm^{-3}$) levels present in the soil were sufficient for symbiosis establishment and shoot growth and may have been the reason for the lack of response to liming. However, two strains (UFLA 03-144 and UFLA 03-150) increased ($p<0.05$) the RDM in response to liming.

The lack of correlation ($p>0.05$) between NDM and SDM and the lower correlation ($p<0.05$) between NDM and SNA in the experiment in pots with soil differs from the results obtained in Leonard jars and may be due mainly

to the effects of edaphic factors. The absence of nodulation in the uninoculated control with mineral N in pots with soil evidences the mitigating role of nitrogen in lima bean nodulation, which has also been observed in other legume species, such as cowpea (Costa et al. 2014), common bean (Rufini et al. 2011) and pigeon pea (Rufini et al. 2014). In turn, the similarity in NDM between the uninoculated control without mineral N and the inoculated treatments indicates the good nodulating capacity of the native LNNFB populations present in the soil under study. Despite this similarity, four strains (UFLA 03-144, UFLA 03-84, INPA 54B and INPA 86A) exhibited good competition and promoted increased ($p<0.05$) SDM and TDM production, EFNC and SNA, indicating the potential of these strains for use as lima bean inoculants, especially INPA 54B and INPA 86A, which were the most efficient in pots with soil.

The rapid plant response to mineral N explains the fact that no strain is equal to the control fertilized with high NH_4NO_3 concentration (300 mg N dm^{-3}), as the establishment and function of symbiosis is more time consuming. However, once symbiosis is established, the performance of the inoculated treatments can equal that of the mineral N treatment, which occurs during the grain production phase for other crops, such as cowpea (Soares et al. 2006; Ferreira et al. 2013). There is still no data available about this effect for lima bean.

Our study is the first to report the symbiotic efficiency of LNNFB strains with lima bean in an experiment conducted in soil and constitutes the first step to recommend strains for lima bean inoculation, aiming to at least partially meet their N demand. In future experiments, the INPA 54B and INPA 86A strains will be evaluated under field conditions and under other edaphoclimatic conditions to confirm its symbiotic efficiency as lima bean inoculants.

Conclusions

Strains isolated from Amazonian soils (UFLA 03-144, UFLA 03-84, INPA 54B and INPA 86A) are efficient in symbiosis with lima bean under axenic conditions and in pots with soil. Liming does not affect lima bean nodulation and shoot growth in the soil evaluated. The INPA 54B and INPA 86A strains promote higher shoot dry matter production and shoot nitrogen accumulation compared to the other strains and the native rhizobial populations, exhibiting potential for use as lima bean inoculants.

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Table 1 Origin, symbiotic efficiency in other hosts and phylogenetic affiliation of the *Bradyrhizobium* strains used in this study.

Strains	Origin			Symbiotic efficiency in axenic conditions[a] and/or in soil[b]	Phylogenetic groups [c,d]	Phylogenetic Affiliation	References
	Region/State	Land use systems	Host plant				
UFLA 03-84	Amazonian /RO	Pasture	*Vigna unguiculata*	*V. unguiculata* (AI) [a, b]	I [c]	*Bradyrhizobium* sp.	Soares et al. (2006)
UFLA 03-144	Amazonian/AM	Agriculture	*V. unguiculata*	*V. unguiculata* (IE) [a]	I [c]	*Bradyrhizobium* sp.	Guimarães et al. (2012)
UFLA 03-268	Amazonian/AM	Agroforestry	*V. unguiculata*	*V. unguiculata* (IE) [a]	I [c]	*Bradyrhizobium* sp.	Jaramillo et al. (2013)
INPA 237B	Amazonian/AM	Forestry	*Pterocarpus* sp.	*M. atropurpureum* (E)[a]	I [c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
INPA 104A	Amazonian/AM	Forestry	*Campsiandra surinamensis*	*M. atropurpureum* (E) [a]	I [c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
UFLA 03-290	Amazonian/AM	Agroforestry	*V. unguiculata*	*V. unguiculata* (I) [a]	IV [c]	*Bradyrhizobium* sp.	Jaramillo et al. (2013)
UFLA 04-0212	Amazonian/AM	Agriculture	*Macroptilium atropurpureum*	*M. atropurpureum* (E) [a]	IV [c]	*Bradyrhizobium* sp.	Florentino et al. (2009)
INPA 54B	Amazonian/AM	Forestry	*Inga* sp.	*M. atropurpureum* (E) [a]	V [c]	*Bradyrhizobium* sp.	Moreira et al. (1998)
INPA 86A	Amazonian/AM	Forestry	*Swartzia* sp.	*M. atropurpureum* (E) [a]	V [c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
UFLA 03-150	Amazonian/AM	Agriculture	*V. unguiculata*	*V. unguiculata* (E) [a]	II [c]	*Bradyrhizobium* sp.	Guimarães et al. (2012)
UFLA 03-197	Amazonian/AM	Agriculture	*V. unguiculata*	*V. unguiculata* (E) [a]	II [c]	*Bradyrhizobium* sp.	Guimarães et al. 2012)
UFLA 03-153	MG	Bauxite Mining	*V. unguiculata*	*V. unguiculata* (E) [a, b]	I [c]	*Bradyrhizobium* sp.	Soares et al. (2014)
UFLA 03-164	MG	Bauxite Mining	*V. unguiculata*	*V. unguiculata* (E) [a, b]	I [c]	*Bradyrhizobium* sp.	Soares et al. (2014)

Continuation...

Strains	Origin			Symbiotic efficiency in axenic conditions[a] and/or in soil[b]	Phylogenetic groups [c,d]	Phylogenetic Affiliation	References
	Region/State	Land use systems	Host plant				
UFLA 03-320	MG	Agriculture	*V. unguiculata*	*V. unguiculata* (E) [a]	IV [c]	*Bradyrhizobium* sp.	Rufini et al. (2013)
UFLA 03-321	MG	Agriculture	*V. unguiculata*	*V. unguiculata* (E) [a]	IV [c]	*Bradyrhizobium* sp.	Rufini et al. (2013)
UFLA 06-24	Cerrado/PI	Agriculture	*Glycine max*	*G. max* (E) [a]	Single [d]	*Bradyrhizobium* sp.	Ribeiro et al. (2015)
UFLA 06-13	Cerrado/PI	Agriculture	*G. max*	*G. max* (E) [a]	G-II [d]	*Bradyrhizobium* sp.	Ribeiro et al. (2015)

RO Rondôna, *AM* Amazonas, *MG* Minas Gerais, *PI* Piauí

AI approved as inoculant by the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura Pecuária e Abastecimento)

IE intermediate efficiency (Shoot dry matter of the treatment inoculated with the tested strain < to that of the uninoculated control with nitrogen supplementation, and > to that of the uninoculated control with low nitrogen concentration)

I inefficient (Shoot dry matter of the treatment inoculated with the tested strain = to that of the uninoculated control with low nitrogen concentration)

E efficient = (Shoot dry matter of the treatment inoculated with the tested strain = to that of the uninoculated control with nitrogen supplementation)

[c] Grouping according to the phylogenetic analysis of the housekeeping genes by Guimarães *et al*. (2015)

[d] Grouping according to the phylogenetic analysis of the housekeeping genes by Ribeiro *et al*. (2015).

Table 2 Number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), efficiency compared to the control with high nitrogen concentration (EFNC), SPAD index (SPAD) and shoot nitrogen accumulation (SNA) obtained in lima bean plants in Leonard jars with different nitrogen sources at 45 days after sowing.

N sources	NN	NDM	SDM	RDM	TDM	EFNC	SPAD	SNA
	-		---------- g plant^{-1} ----------			%	-	mg plant^{-1}
Without I + High N (52.5 mg L^{-1}) [a]	0 d	0 e	4.45 d	1.60 a	6.04 c	100 d	27.23 c	88.33 f
Without I + Low N (5.25 mg L^{-1}) [a]	0 d	0 e	1.49 f	0.83 d	2.27 g	33 f	14.06 e	17.08 g
UFLA 03-84 + Low N	352 a	0.343 b	5.03 c	1.06 c	6.09 c	113 c	30.53 b	197.31 c
UFL 03-144 + Low N	506 a	0.568 a	6.57 b	1.24 b	7.82 b	148 b	32.43 b	226.86 b
UFLA 03-268 + Low N	0 d	0 e	1.65 f	0.98 c	2.63 g	37 f	17.30 d	22.63 g
INPA 237B + Low N	51 c	0.080 e	1.59 f	1.14 b	2.73 g	36 f	15.16 e	19.03 g
INPA 104A + Low N	464 a	0.511 a	5.35 c	1.21 b	6.57 c	121 c	27.53 c	190.19 c
UFLA 03-290 + Low N	0 d	0 e	1.59 f	1.17 b	2.75 g	36 f	17.60 d	21.41 g
UFLA 04-2012 + Low N	207 b	0.125 d	1.91 f	0.79 d	2.70 g	43 f	18.46 d	24.29 g
INPA 54B + Low N	435 a	0.415 b	7.29 a	1.48 a	8.77 a	164 a	37.73 a	260.76 a
INPA 86A + Low N	220 b	0.335 b	5.24 c	1.07 c	6.31 c	118 c	33.26 b	196.68 c
UFLA 03-150 + Low N	471 a	0.358 b	4.04 d	1.08 c	5.12 d	91 d	31.03 b	150.30 d
UFLA 03-197 + Low N	336 a	0.350 b	3.48 e	0.97 c	4.45 e	79 e	31.26 b	113.17 e
UFLA 03-153 + Low N	15 c	0.001 e	2.34 f	1.15 b	3.50 f	53 f	14.00 e	28.60 g
UFLA 03-164 + Low N	362 a	0.210 c	1.83 f	0.82 d	2.65 g	40 f	13.16 e	19.95 g
UFLA 03-320 + Low N	283 b	0.051 e	1.92 f	1.01 c	2.93 g	43 f	15.20 e	22.23 g
UFLA 03-321 + Low N	274 b	0.124 d	1.93 f	0.91 d	2.84 g	43 f	19.13 d	26.86 g
UFLA 06-24 + Low N	0 d	0 e	2.01 f	1.34 b	3.35 f	45 f	17.26 d	27.32 g
UFLA 06-13 + Low N	0 d	0 e	1.80 f	1.23 b	3.04 g	41 f	17.73 d	21.17 g
CV (%)	18.66	3.40	9.35	12.09	8.27	8.85	11.20	10.33

[a] Nitrogen sources used in the nutrient solution: $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3 $NH_4H_2PO_4$. In each column, means followed by the same letter are not statistically different and belong to the same group, according to the Scott–Knott test at $p<0.05$.

Table 3 Pearson's correlation coefficients between the parameters studied in the experiments in Leonard jars and in pots with non sterilized soil (Oxisol)

	NDM	SDM	RDM	TDM	EFNC	SNA	SPAD
Leonard jars (axenic conditions)							
NN	0.85**	0.59**	-0.091^{ns}	0.55**	0.59**	0.66**	0.58**
NDM		0.80**	0.062^{ns}	0.76**	0.81**	0.85**	0.74**
SDM			0.46*	0.99**	0.99**	0.96**	0.88**
RDM				0.55**	0.47**	0.35*	0.37*
TDM					0.98**	0.95**	0.87**
EFNC						0.96**	0.88**
SNA							0.91**
Pots with non sterilized soil (Oxisol)							
NN	0.76**	0.15^{ns}	-0.09^{ns}	0.13^{ns}	0.21^{ns}	0.32*	-
NDM		0.23^{ns}	0.06^{ns}	0.25^{ns}	0.30*	0.36*	-
SDM			0.17^{ns}	0.95^{ns}	0.90**	0.84**	-
RDM				0.43**	0.21^{ns}	0.11^{ns}	-
TDM					0.89**	0.80**	-
EFNC						0.74**	-
SNA							-

**p<0.01; *p<0.05; ^{ns}not significant.

Table 4 Number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), efficiency compared to the control with high nitrogen concentration (EFNC) and shoot nitrogen accumulation (SNA) obtained in lima bean plants in pots with samples of a Oxisol according to the nitrogen sources and with or without liming at 45 days after sowing

Factors	NN	NDM	SDM	TDM	EFNC	SNA
	Nº pot^{-1}	mg pot^{-1}	-------- g pot^{-1} -------		-- % --	mg pot^{-1}
			N Sources			
UFLA 03-84	169 b	292.25 a	3.93 c	5.47 b	58 c	120.67 c
UFLA 03-144	250 a	337.87 a	3.94 c	5.31 b	59 c	127.70 c
INPA 104A	178 b	288.37 a	3.97 c	5.86 b	59 c	104.58 d
INPA 54B	202 b	338.12 a	4.47 b	5.64 b	67 b	153.24 b
INPA 86A	309 a	340.62 a	4.53 b	5.77 b	68 b	153.32 b
UFLA 03-150	184 b	257.62 a	3.27 d	4.51 c	49 d	65.10 e
Without N without I	218 b	308.37 a	3.34 d	4.47 c	50 d	103.54 d
With N (300 mg dm^{-3})	0 c	0 b	6.69 a	7.91 a	100 a	347.33 a
			Liming			
Without liming	187 a	0.269 a	4.23 a	5.52 a	61 b	150.82 a
With liming	190 a	0.271 a	4.29 a	5.63 a	67 a	143.05 a
CV (%)	23.15	5.27	12.23	9.54	10.55	16.52

N nitrogen, *I* inoculation

Means followed by the same letters within the columns are not significantly different from each other by the Scott-Knott test at $p<0.05$.

Table 5 Root dry matter (RDM) of lima bean plants in pots with a Oxisol according to different nitrogen sources with or without liming

N sources	RDM (g pot^{-1})	
	Without liming	With liming
UFLA 03-84	1.54 aA	1.55 aA
UFLA 03-144	1.17 bB	1.51 aA
INPA 104A	1.48 aA	1.13 bB
INPA 54B	1.29 bA	1.49 aA
INPA 86A	1.14 bA	1.37 aA
UFLA 03-150	1.12 bB	1.37 aA
Without N without I	1.13 bA	1.14 bA
With N (300 mg dm^{-3})	1.27 bA	1.15 bA
Means	1.27 A	1.34 A
CV (%)	12.90	

N nitrogen, *I* inoculation

Means followed by the same letters, lowercase letters in columns and uppercase in the lines are not significantly different from each other by the Scott-Knott test at $p<0.05$.

ARTIGO 6 - *Bradyrhizobium* strains from Brazilian soils are tolerant to acidity and high temperatures and promote *Acacia mangium* and *Stizolobium aterrimum* growth

Artigo de acordo com as normas da revista Ecological Engineering
(Versão preliminar)

Bradyrhizobium strains from Brazilian soils tolerant to acidity and high temperatures promote *Acacia mangium* and *Stizolobium aterrimum* growth

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Abstract In selecting legume nodulating nitrogen-fixing bacteria it is important to consider a strain's tolerance to adverse conditions, in addition to its symbiotic efficiency. The capacity to perform other plant growth-promoting processes also confers desirable multifunctionality to inoculant strains. In this study, we evaluated *Bradyrhizobium* strains from soils of the Brazilian ecosystems regarding tolerance to different pH and temperature conditions, inorganic calcium phosphate solubilisation capacity and symbiotic efficiency when inoculated in acacia (*Acacia mangium*) and velvet bean (*Stizolobium aterrimum*) in Leonard jars. In each experiment of symbiotic efficiency, the 18 strains were compared to two uninoculated controls, one with low (5.25 mg L^{-1}) and another with high (52.5 mg L^{-1}) concentrations of mineral nitrogen (N) and one inoculant strain (BR 3617 in the acacia experiment and BR 2811 in the velvet bean experiment). All strains grew at the different tested pH values (4 to 10), and most tolerated a wide temperature range (15 to 37 °C) and solubilised inorganic calcium phosphate. The UFLA 03-268 strain was the most efficient in symbiosis with acacia and promoted twice the shoot dry matter (SDM) obtained with the BR 3617 strain. In the velvet bean experiment, the INPA 104A strain was more efficient in shoot nitrogen accumulation, which promoted 101 and 241% increases, respectively, compared to the values obtained in the control with high mineral N concentration and with the BR 2811 strain. The UFLA 03-144 and INPA 104A strains outperformed the other treatments in SDM production of velvet bean. In conclusion, the UFLA 03-268, UFLA 03-144 and INPA 104A strains exhibit potential for use as inoculants in these species under tropical soil conditions.

Keywords: Biological nitrogen fixation, symbiotic efficiency, inoculant, inorganic phosphate solubilisation

1. Introduction

Biological nitrogen fixation (BNF) is one of the main processes that ensures sustainable terrestrial ecosystems. This process is mediated by free-living, associative and legume nodulating nitrogen-fixing bacteria (LNNFB). Among the genera of LNNFB that occur in Brazilian tropical ecosystems, *Bradyrhizobium* is highlighted. In addition to being the most common microsymbiont in legume nodules (Moreira et al., 1993; 1998; Lima et al., 2009; Perrineau et al., 2011; Guimarães et al., 2012; Ribeiro et al., 2015), it establishes efficient symbiosis with a wide range of socioeconomically and environmentally important hosts. In Brazil, the most LNNFB strains approved by the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura Pecuária e Abastecimento - MAPA) for inoculation in legume species belonging to this genus (available at http://www.agricultura.gov.br).

Although LNNFB strains authorised by MAPA currently exist for 83 legume species in Brazil, recommendations for most of these species were based on few experiments and under particular edaphoclimatic conditions. Additionally, there are few or no results published for some species that prove symbiotic efficiency of the inoculant strains. Among these species, we can state that acacia (*Acacia mangium*) has few published results regarding the symbiotic efficiency of its bacterial strains approved as inoculants (BR 3609 and BR 3617) (Franco and Faria, 1997; Trannin et al., 2001; Schiavo and Martins, 2003), and for velvet bean (*Stizolobium aterrimum*) still does not have consistent published data regarding efficiency of its inoculant strain (BR 2811) and of new LNNFB strains. It is noteworthy that the inoculant strains for these species belong to the genus *Bradyrhizobium* (Moreira and Siqueira, 2006).

Acacia and velvet bean are promising legume species for the revegetation of degraded soils, as they are tolerant to heavy metals and low-fertility soils and are able to fix N_2 in symbiosis with LNNFB, especially the genus

Bradyrhizobium (Trannin et al., 2001; Galiana et al., 1998; Okito et al., 2004). Velvet bean is also considered one of the best alternatives for green manure (Matos et al., 2011) and acacia is one of the main arboreal species used in reforestation programs and agroforestry systems (Galiana et al., 1998). Considering the ecological and economic importance of these species, new studies based on results of consistent research are necessary, aiming to select new strains with potential use as inoculants.

During the process of selecting new LNNFB strains, it is important to consider the ability of the strain to nodulate and fix nitrogen in symbiosis with wide range of hosts and its tolerance to the adverse conditions predominant in tropical soils, such as high temperature and acidity, in addition to the strain's symbiotic efficiency. Additionally, it is desirable to select strains that can also act in other plant growth-promoting processes other than BNF, such as insoluble inorganic phosphate solubilisation (Marra et al., 2011). In this study, we evaluated tolerance to different pH and temperature conditions, inorganic calcium phosphate solubilisation capacity and efficiency of *Bradyrhizobium* strains in symbiosis with acacia and velvet bean.

2. Materials and Methods

2.1. Strains evaluated

The *Bradyrhizobium* strains used in this study are belonging to the collection of the Sector of Biology, Microbiology and Biological Processes of the Soil (Setor de Biologia, Microbiologia e Processos Biológicos do Solo - SBMPBS) at the Federal University of Lavras (Universidade Federal de Lavras - UFLA). These strains come from different Brazilian ecosystems and they have recently been characterized by housekeeping genes sequencing (Guimarães et al., 2015; Ribeiro et al., 2015), which indicated that they belong to different phylogenetic groups (Table 1). Their symbiotic efficiencies were already evaluated in previous studies with legume species different from those evaluated in this study (Table 1). Characteristics of the

strains approved as inoculants for acacia (BR 3617) and velvet bean (BR 2811) are also shown in Table 1. The ability of the LNNFB strains to fix nitrogen in symbiosis with different legumes species is a desirable characteristic, once it facilitates the commercialization by companies producing inoculants.

2.2. Tolerance of the strains to different pH and temperature conditions

For both tests (pH and temperature), the 11 strains (UFLA 03-84, UFLA 03-144, UFLA 03-268, INPA 237B, INPA 104A, UFLA 03-153, UFLA 03-164, UFLA 03-150, UFLA 03-197, UFLA 04-0212 and UFLA 06-24) were cultured in liquid 79 culture medium (Fred and Waksman, 1928) under constant agitation at 110 rpm at 28 °C for five days. One-millilitre aliquots of each bacterial culture were transferred to 1.5 mL sterile microtubes and centrifuged at 10,000 rpm for ten minutes. The supernatant was discarded, and the cells were resuspended in 1.0 mL of sterile saline solution (0.85%) and then centrifuged. The process of discarding the supernatant, resuspending the cells, and centrifugation was repeated three times to remove the residue of the inoculum culture medium, which would result in false-positive growth. Next, 0.1 mL aliquots of these bacterial suspensions were inoculated and spread onto Petri dishes containing 79 culture medium using a Drigalski spatula. In the pH test, the pH values for the 79 culture medium were adjusted to 4.0, 5.5, 6.8, 8.0, 9.0 and 10, and the Petri dishes were incubated at 28 °C. In the tolerance test at different temperatures, the pH of the 79 culture medium was adjusted to 6.8, and the Petri dishes were incubated at 15, 20, 28, 34, 37 and 40 °C. The treatments were randomly distributed, with three replicates. To test the tolerance of the 18 strains at these pH and temperatures values, the presence (+) or absence (-) of growth in the 79 culture medium was evaluated after seven days of incubation. The strains that exhibited lower growth under the extreme pH and temperature conditions compared to the optimal conditions (pH 6.8 and 28 °C) were considered as having weak growth (w).

The other 7 strains (UFLA 03-320, UFLA 03-321, UFLA 03-290, INPA 54B, INPA 86 A, UFLA 06-10 and UFLA 06-13) were evaluated, in previous studies (Costa et al., unpublished data), in 79 medium under the same conditions of pH and temperature cited, using the same methods applied above.

2.3. Inorganic calcium phosphate solubilisation

The 18 strains were evaluated for their capacity to solubilise inorganic calcium phosphate ($CaHPO_4$) in modified solid NBRIP culture medium (Nautiyal, 1999), where arabinose was used as the carbon source, replacing glucose. This substitution was performed because in previous studies, most evaluated strains grew better using arabinose as the carbon source than glucose in 79 culture medium.

Initially, the strains were cultured in liquid 79 culture medium, under agitation at 110 rpm at 28 °C. Next, the optical density was adjusted (0.5) by adding saline solution (0.85%), and 20 μL of suspended cells were applied to four equidistant points on the Petri dishes (9.5 cm wide) containing solid NBRIP culture medium. Each strain was evaluated with two replicates (two Petri dishes). At 18 days after inoculation, the diameter of the solubilisation zone (translucent area surrounding the colony) and the colony corresponding to the zone were measured. Based on these measurements, the solubilisation indices (SIs) were obtained = Ø zone (mm) / Ø colony (mm) (Berraquero et al., 1976). Based on the SIs, the strains were classified as having either low (SI < 2 mm), medium (2 ≥ SI < 4.0 mm) or high (IS > 4 mm) solubilisation capacity.

2.4. Experiments with acacia and velvet bean in Leonard jars

To evaluate nodulating capacity and efficiency of the 18 *Bradyrhizobium* strains in symbiosis with acacia and velvet bean, two experiments were conducted in Leonard jars at the SBMPBS of the UFLA, under a completely randomised design, with three replicates. The first experiment was

conducted with acacia, from May to August 2014, and the second with velvet bean, from June to August 2014. The respective mean maximum and minimum temperatures recorded in the greenhouse were 13 and 38 °C during the experimental period.

For each experiment, the treatments consisted of individual inoculations of the 18 *Bradyrhizobium* strains, two uninoculated negative controls, one with low (5.25 mg L^{-1}) and another with high (52.5 mg L^{-1}) mineral nitrogen (N) concentration, and one positive control inoculated with the BR 3617 strain (*Bradyrhizobium japonicum*) in acacia and with the BR 2811 strain (*Bradyrhizobium elkanii*) in velvet bean, both approved by MAPA as inoculants for these legume species (available at http://www.agricultura.gov.br).

A 1:2 mixture of sand (150 cm^3) and vermiculite (300 cm^3) was added to the top of the Leonard jars and Hoagland nutrient solution (Hoagland and Arnon, 1950) modified (Guimarães et al., 2012) was added to the bottom of the jars. In the inoculated treatments, nutrient solution with the same N concentration (5.25 mg L^{-1}) as the control with low N concentration was used. After preparing the jars and nutrient solution, they were autoclaved for one hour at a pressure of 1.5 kg cm^{-2} at 121 °C.

Before sowing, the velvet bean seeds were subjected to break in dormancy using concentrated H_2SO_4 for 45 minutes. Next, they were washed with sterile distilled water. Dormancy was broken in the acacia seeds by immersing them in boiling water for one minute. To disinfect the surface of the acacia seeds, 98% ethanol (30 seconds) and 2% sodium hypochlorite (2 minutes) were used, followed by successive washing in sterile distilled water. The seeds of each species were pre-germinated in sterile Petri dishes containing filter paper and wet cotton, where they were kept for 48 hours in a growth chamber at 28 °C. Four seeds were sowed in each jar for each species, and the plants were thinned after eight days, leaving one plant per jar in the velvet bean experiment and two plants per jar in the acacia experiment.

The bacterial strains were cultured in liquid 79 culture medium under agitation at 110 rpm at 28 °C for five days. In each inoculation treatment, 1 mL of the inoculant was added to each plant at a concentration of $1x10^8$ bacterial cells mL^{-1}. After inoculation, a layer of paraffin sand (10 kg of sand, 1 L of chloroform and 10 g of paraffin) was added to each jar to avoid possible contamination. The nutrient solution was prepared, autoclaved, and reapplied to the jars periodically throughout the experiment. Nutrient solution (1/4 ionic strength) was added to the jars until 45 days after planting the species. Starting from this period, the ionic strength of the nutrient solution was increased to 1/3.

The acacia and velvet bean plants were collected at 90 and 60 days after sowing, respectively. The following variables were evaluated in each experiment: nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM) and efficiency compared to the control with high mineral N concentration (EFNC). For the velvet bean experiment, shoot nitrogen accumulation (SNA) was also evaluated. In the acacia experiment, it was not possible to determine SNA, as the quantity of dry matter produced in the shoots was not sufficient for N content analysis in some of the treatments.

The nodules, shoots and roots were placed in paper bags and dried in an air-circulated oven at 60 °C until reaching constant weight to determine the NDM, SDM and RDM. EFNC was calculated using the following formula: EFNC = (treatment SDM * 100) / (SDM of the treatment with high mineral N concentration). SNA in the velvet bean plants was calculated by multiplying SDM (mg) by N content (%) / 100. N content in the shoots of the velvet bean plants was determined using the semi-microkjedahl method. After applying the Shapiro-Wilk test to verify normality of the data, the data were subjected to analysis of variance using the SISVAR statistical analysis program, version 5.3 (Ferreira, 2011). The NN and NDM data were transformed into the square root of Y + 0.5. Effects of the treatments were compared using the Scott-Knott test at 5% probability.

3. Results

3.1. Tolerance of the strains to different pH and temperature conditions

All the strains grew well in 79 culture medium with the pH adjusted to the different evaluated values (4.0, 5.5, 6.8, 8.0, 9.0 and 10) and at temperatures of 20, 28, 34 and 37 °C (Table 2). At 40 °C, only the UFLA 06-24 strain grew well; the UFLA 03-150, UFLA 03-197 and INPA 86A strains exhibited weak growth and the other strains had completely inhibited growth.

3.2. Inorganic calcium phosphate solubilisation

Most strains (55%) were able to solubilise inorganic calcium phosphate (Table 2). Of the strains that solubilised, UFLA 06-24 stood out by exhibiting a solubilisation index of 2.1, which characterises it as a strain with medium solubilisation capacity. The other strains exhibited solubilisation indices lower than 2.0 and were classified as having low inorganic calcium phosphate solubilisation capacity.

3.3. Efficiency of the strains in promoting acacia growth

There were treatment effects ($p<0.05$) on all variables evaluated (Table 3). Only seven strains (UFLA 03-84, UFLA 03-144, UFLA 03-150, UFLA 03-197, UFLA 03-268, INPA 104A and UFLA 03-153) and the inoculant strain (BR 3617) nodulated. The treatments inoculated with the UFLA 03-150, UFLA 03-197 and INPA 104A strains exhibited mean NN values similar ($p>0.05$) to BR 3617 and higher ($p<0.05$) than the other strains. The UFLA 03-144, UFLA 03-150, UFLA 03-197, UFLA 03-268 and INPA 104A strains provided NDM similar ($p>0.05$) to that of BR 3617 and higher ($p<0.05$) than that of the UFLA 03-84 and UFLA 03-153 strains. There was

no correlation ($p>0.05$) between NN or NDM with the other evaluated variables.

For SDM, RDM, TDM and EFNC, no strain, including BR 3617, had higher values ($p<0.05$) than the control with high N concentration (Table 3). Of the strains evaluated, 83% had increased ($p<0.05$) SDM, TDM and EFNC compared to the control with low N concentration. Regarding SDM and EFNC, the nodulating UFLA 03-268 strain stood out ($p<0.05$) from the others evaluated (including the BR 3267) and the control with low mineral N concentration. This strain provided 236% higher SDM compared to that obtained in the control with low mineral N concentration. Another four nodulating strains (UFLA 03-150, UFLA 03-197, INPA 104A and UFLA 03-153) and five non-nodulating strains of acacia (UFLA 04-0212, UFLA 03-290, UFLA 03-164, UFLA 03-320 and UFLA 03-321) provided higher ($p<0.05$) SDM and EFNC than those of the BR 3617 inoculant strain and those of the control with low N concentration.

Of the evaluated strains, 61% of the evaluated strains achieved higher RDM values ($p<0.05$) than that of the control with low N concentration (Table 3). Among the inoculated treatments, the highest RDM ($p<0.05$) was obtained with the UFLA 03-320 strain. The treatments inoculated with the UFLA 04-0212, UFLA 03-150, UFLA 03-268, UFLA 03-290, UFLA 03-153, UFLA 03-320, UFLA 03-321 and UFLA 06-10 strains exhibited higher RDM values ($p<0.05$) than that of the treatment inoculated with the BR 3617 strain. The UFLA 03-84, INPA 237B and INPA 54B strains were inefficient at promoting acacia growth, behaving similarly ($p>0.05$) to the control with low N concentration for SDM, RDM, TDM and EFNC.

3.4. Efficiency of the strains in promoting velvet bean growth

There were treatment effects ($p<0.05$) on all variables evaluated (Table 4). Of the 18 evaluated strains, only one (UFLA 06-24) did not nodulate velvet bean. The mean NN values ranged from 3 to 70 nodules per plant, which were obtained in the treatments inoculated with the UFLA 03-290 and

INPA 86A strains, respectively. The INPA 54B, INPA 86A and UFLA 03-321 strains provided higher NN values ($p<0.05$) than the other strains and clustered with the BR 2811 inoculant strain. The highest NDM values were obtained with the UFLA 03-144 (0.491 g plant^{-1}) and INPA 104A strains (0.539 g plant^{-1}), which stood out ($p<0.05$) from the other tested strains, including the BR 2811. The UFLA 03-84, UFLA 03-150, INPA 86A, UFLA 03-268, UFLA 03-153, UFLA 03-320 and UFLA 03-321 strains formed a second group with higher NDM ($p<0.05$) than that obtained with the BR 2811 strain. The UFLA 04-0212, UFLA 03-197, INPA 54B and UFLA 06-10 strains formed a third group with NDM values similar ($p>0.05$) to that of BR 2811.

NN did not correlate ($p>0.01$) with the other variables evaluated. However, NDM was positively correlated ($p<0.01$) with SDM, RDM, TDM, EFNC and SNA (Table 5). The two strains (UFLA 03-144 and INPA 104A) that are highlighted for NDM provided higher SDM and EFNC ($p<0.05$) values than those of the other strains and the control with high N concentration (Table 4). The treatment inoculated with the UFLA 03-321 strain also exhibited higher SDM and EFNC values than those of BR 2811 and the control with high N concentration. The UFLA 03-84, UFLA 04-0212, UFLA 03-150, UFLA 03-268, INPA 54B, INPA 86A, UFLA 03-153 and UFLA 03-320 strains behaved similarly ($p>0.05$) to the control with high N concentration and provided higher results ($p<0.05$) than the BR 2811 strain for these two variables.

No strain had RDM values similar to or higher than ($p<0.05$) those of the control with high N concentration (Table 4). The INPA 104A strain stood out ($p<0.05$) from the others, and 11 strains (UFLA 03-84, UFLA 04-0212, UFLA 03-144, UFLA 03-150, UFLA 03-268, UFLA 03-290, INPA 237B, UFLA 03-320, UFLA 03-321, UFLA 06-13 and UFLA 06-10) exhibited RDM values similar ($p>0.05$) to that obtained with the BR 2811 strain and higher values ($p<0.05$) than the control with low N concentration.

The highest TDM (5.575 g plant^{-1}) and SNA values (127.76 mg plant^{-1}) were obtained in the treatment inoculated with the INPA 104A strain, which provided higher results ($p<0.05$) than the other treatments (Table 4). This strain promoted twice the SNA obtained in the control with high N concentration and more than triple the SNA obtained in the treatment inoculated with the BR 2811 strain. The UFLA 03-84, UFLA 03-144, UFLA 03-150, UFLA 03-268, INPA 86A, UFLA 03-320 and UFLA 03-321 strains were also efficient in SNA, promoting higher values ($p<0.05$) than the control with high N concentration. Of the strains evaluated, ten strains (UFLA 03-84, UFLA 04-0212, UFLA 03-144, UFLA 03-150, UFLA 03-268, INPA 104A, INPA 86A, UFLA 03-153, UFLA 03-320 and UFLA 03-321) exhibited higher SNA values than that of the BR 2811 inoculant strain.

Only four strains (INPA 237B, UFLA 03-164, UFLA 06-24 and UFLA 06-13) were inefficient at promoting velvet bean growth, behaving similarly ($p>0.05$) to the control with low N concentration for SDM, RDM, TDM, EFNC and SNA (Table 5).

4. Discussion

Tolerance to wide ranges of pH (4.0 to 10) and temperatures (15 to 37 °C) of the *Bradyrhizobium* strains evaluated in the present study corroborates the results of other studies with strains of this genus isolated from Brazilian tropical ecosystems (Florentino et al., 2010; Silva et al., 2014; Zilli et al., 2014). Tolerance to different pH and temperature conditions is an important characteristic in selecting LNNFB strains, as inoculant strains are authorised for use in all Brazilian regions, which in turn exhibit vastly diverse climate and soil conditions. However, strains tolerant to acidity and high temperatures are more desirable because these conditions are predominant in Brazilian soils. In the present study, we highlighted the UFLA 03-150, UFLA 03-197, INPA 86A and UFLA 06-24 strains, which were able to tolerate temperatures up to 40 °C.

Insoluble inorganic phosphate solubilisation capacity is another important characteristic to be evaluated in selecting LNNFB strains, using them not only for supplying nitrogen but also to increase phosphorus availability for the plants. For *Bradyrhizobium* strains that solubilise inorganic calcium phosphate in NBRIP culture medium, the solubilisation indices usually range from low to medium (Marra et al., 2011; Costa et al., 2013), corroborating the results obtained in the present study. The UFLA 06-24 strain, which exhibited a medium solubilisation index, despite no symbiosis forming with acacia and velvet bean, can be evaluated in the future regarding its capacity to solubilise inorganic calcium phosphate in soil and to provide phosphorus, in addition to nitrogen, to their host of origin (*Glicine max*) with which this strain forms efficient symbiosis (Table 1).

The wide functional variability in symbiotic efficiency of the strains evaluated in both studied species (acacia and velvet bean) demonstrates the high symbiotic diversity of the genus *Bradyrhizobium*. We emphasize that these strains belong to different phylogenetic groups, characterized by housekeeping genes sequencing (Guimarães et al., 2012; Ribeiro et al., 2015). Besides, it was verified variability in symbiotic efficiency even among strains of the same phylogenetic group. High symbiotic diversity of *Bradyrhizobium* strains has been also observed in other studies with different hosts (Thies et al., 1991; Guimarães et al., 2012; Jaramillo et al., 2013; Rufini et al., 2014).

The lower growth in acacia plants inoculated with strains that nodulated (BR 3617, UFLA 03-84, UFLA 03-144, UFLA 03-150, UFLA 03-197, UFLA 03-268, INPA 104A and UFLA 03-153) compared to the plants with high mineral N concentration indicates the rapid plant response to mineral N, which was readily available since the onset of the experiment. Established symbiosis and N supply via BNF takes longer; therefore, it is likely that none of these strains were able to meet all of the N quantity required for this species' growth during the experimental period. This response has already been reported in an experiment with acacia conducted in soil for 100 days

(Trannin et al., 2001) and also for other arboreal legume species inoculated with LNNFB strains (Trannin et al., 2001; Florentino et al., 2009; Ferreira et al., 2012a).

Although symbiotic efficiency of the BR 3617 strain with acacia was reported by Franco and Faria (1997), shoot dry matter data from the inoculated treatments and from the controls with and without mineral nitrogen were not presented. In the present study, the BR 3617 inoculant strain was less efficient compared to five (UFLA 03-150, UFLA 03-197, UFLA 03-268, INPA 104A and UFLA 03-153) of the seven strains that nodulated acacia, indicating the need for selecting new LNNFB strains for this legume species. Of these five, we highlighted UFLA 03-268, which reached twice the efficiency of BR 3617, demonstrating its potential for use as an inoculant in acacia, aiming to at least partially meet its N demand during the seedling production phase.

The fact that most (82%) of the non-nodulating strains of acacia promoted growth plants confirms the good capacity of the genus *Bradyrhizobium* to promote plant growth by acting in other biological processes, as already observed for some plant species (Antoun et al., 1998; Machado et al., 2013). However, our study is the first that reports *Bradyrhizobium* strains promoting growth in this acacia species.

In the velvet bean experiment, the high correlation between NDM and quantity of biologically fixed N corroborates results in the scientific literature for other legume species (Dobereiner et al., 1966; Ferreira et al., 2012b). The lower efficiency of the BR 2811 inoculant strain compared to most (61%) of the strains evaluated and the control with high mineral N concentration most likely suggests that this strain should be replaced by new LNNFB strains efficient in symbiosis with this species. Eight of the strains evaluated in the present study (UFLA 03-84, UFLA 03-144, UFLA 03-150, UFLA 03-268, INPA 104A, INPA 86A, UFLA 03-320 and UFLA 03-321) outperformed ($p<0.05$) the control with high mineral N concentration in SNA, thus demonstrating high efficiency in N_2 fixation in symbiosis with

velvet bean. These strains, especially UFLA 03-144 and INPA 104A, which were the most efficient in producing SDM, exhibit high potential for use as inoculants in velvet bean.

We highlight that the strains most efficient in symbiosis with acacia (UFLA 03-268) and velvet bean (UFLA 03-144 and INPA 104A) were isolated from Amazonian soils (Table 1), which have been highlighted as sources of LNNFB with high potential for use as inoculants in legume species (Soares et al., 2006; Ferreira et al., 2012b). Our study is the first to indicate new *Bradyrhizobium* strains isolated from Amazonian soils, which, in addition to being tolerant to acidity and high temperatures, exhibit higher symbiotic efficiency than the inoculant strains of the acacia (BR 3267) and velvet bean (BR 2811) and can be used as future inoculants in these species.

5. Conclusions

All strains evaluated are tolerant to acidity and high temperatures and most solubilise inorganic calcium phosphate. The UFLA 03-268 strain is the most efficient in symbiosis with acacia and promotes twice the shoot dry matter obtained with the BR 3617 inoculant strain. The UFLA 03-144 and INPA 104A strains have high potential for use as inoculant in velvet bean because they exhibit higher symbiotic efficiency than the BR 2811 inoculant strain and the control with high mineral nitrogen concentration.

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Table 1

Origin, symbiotic efficiency in other hosts and phylogenetic affiliation of the *Bradyrhizobium* strains used in this study.

Strains	Brazilian ecosystems – state	Host plant / Symbiotic efficiency in axenic conditions[a] and/or in soil[b]	Phylogenetic groups[c,d]	Phylogenetic affiliation	References
UFLA 03-84	Pasture – RO	*V. unguiculata* (AI)[a,b]	I[c]	*Bradyrhizobium* sp.	Soares et al. (2006)
UFLA 03-144	Agriculture – AM	*V. unguiculata* (IE)[a]	I[c]	*Bradyrhizobium* sp.	Guimarães et al. (2012)
UFLA 03-268	Agroforestry – AM	*V. unguiculata* (IN)[a]	I[c]	*Bradyrhizobium* sp.	Jaramillo et al. (2013)
INPA 237B	Forestry – AM	*Pterocarpus* sp. / *Macroptilium atropurpureum* (E) [a]	I[c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
INPA 104A	Forestry – AM	*Campsiandra surinamensis* /*M. atropurpureum* (E)[a]	I[c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
UFLA 03-153	Bauxite Mining – MG	*V. unguiculata* (E)[a,b]	I[c]	*Bradyrhizobium* sp.	Soares et al. (2014)
UFLA 03-164	Bauxite Mining – MG	*V. unguiculata* (E)[a,b]	I[c]	*Bradyrhizobium* sp.	Soares et al. (2014)
UFLA 03-150	Agriculture – AM	*V. unguiculata* (E)[a]	II[c]	*Bradyrhizobium* sp.	Guimarães et al. (2012)
UFLA 03-197	Agriculture – AM	*V. unguiculata* (E)[a]	II[c]	*Bradyrhizobium* sp.	Guimarães et al. (2012)
UFLA 03-290	Agroforestry – AM	*V. unguiculata* (IN)[a]	IV[c]	*Bradyrhizobium* sp.	Jaramillo et al. (2013)
UFLA 04-0212	Agriculture – AM	*M. atropurpureum* (E)[a]	IV[c]	*Bradyrhizobium* sp.	Florentino et al. (2009)
UFLA 03-320	Agriculture – MG	*V. unguiculata* (E)[a,b]	IV[c]	*Bradyrhizobium* sp.	Rufini et al. (2014)

Continuation...

Strains	Brazilian ecosystems – state	Host plant / Symbiotic efficiency in axenic conditions[a] and/or in soil[b]	Phylogenetic groups[c,d]	Phylogenetic affiliation	References
UFLA 03-321	Agriculture – MG	*V. unguiculata* (E)[a,b]	IV[c]	*Bradyrhizobium* sp.	Rufini et al. (2014)
INPA 86[a]	Forestry – AM	*Swartzia* sp./*M. atropurpureum* (E)[a]	V[c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
INPA 54B	Floresta – AM	*Inga* sp. /*M. atropurpureum* (E)[a]	V[c]	*Bradyrhizobium* sp.	Guimarães et al. (2015)
UFLA 06-10	Agriculture – PI	*G. max* (E)[a]	G-I[d]	*Bradyrhizobium* sp.	Ribeiro et al. (2015)
UFLA 06-13	Agriculture – PI	*G. max* (E)[a]	G-II[d]	*Bradyrhizobium* sp.	Ribeiro et al. (2015)
UFLA 06-24	Agriculture – PI	*Glicine max* (E)[a]	Single[d]	*Bradyrhizobium* sp.	Ribeiro et al. (2015)
BR 2811	-	*Crotalaria spectabilis/ Stizolobium aterrimum* (AI)[a,b]	-	*B. elkanii*	Menna et al. (2006)
BR 3617	-	*Acacia mangium* (AI) [a,b]	-	*B. elkanii*	Moreira et al. (1998)

RO, Rondôna; AM, Amazonas; MG, Minas Gerais; PI, Piauí; AI, Approved as inoculant by the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura Pecuária e Abastecimento); IE, Intermediate efficiency (Shoot dry matter of the treatment inoculated with the tested strain < to that of the uninoculated control with nitrogen supplementation, and > to that of the uninoculated control with low nitrogen concentration); IN, Inefficient (Shoot dry matter of the treatment inoculated with the tested strain = to that of the uninoculated control with low nitrogen concentration); E, Efficient = (Shoot dry matter of the treatment inoculated with the tested strain = to that of the uninoculated control with nitrogen supplementation).

[c]Data from Guimarães et al. (2015), [d]Data from Ribeiro et al. (2015).

Table 2

Growth in 79 medium under different temperature and pH range and solubilisation of $CaHPO_4$ in modified NBRIP culture medium by *Bradyrhizobium* strains.

Strains	Temperatures (ºC) /pH						Solubilisation of $CaHPO_4$	
	15/4.0	20/5.5	28/6.8	34/8.0	37/9.0	40/10	SIs	SC
UFLA 03-84	w / +	+	+	+	+	- / +	1.2	Low
UFLA 03-144	w / +	+	+	+	+	- / +	1.0	Low
UFLA 03-268	w / +	+	+	+	+	- / +	1.0	Low
INPA 237B	w / +	+	+	+	+	- / +	1.0	Low
INPA 104A	w / +	+	+	+	+	- / +	1.1	Low
UFLA 03-153	w / +	+	+	+	+	- / +	GDS	-
UFLA 03-164	w / +	+	+	+	+	- / +	GDS	-
UFLA 03-150	w / +	+	+	+	+	w / +	GDS	-
UFLA 03-197	w / +	+	+	+	+	w / +	1.0	Low
UFLA 03-290	w / +	+	+	+	+	- / +	1.1	Low
UFLA 04-0212	w / +	+	+	+	+	- / +	1.0	Low
UFLA 03-320	w / +	+	+	+	+	- / +	1.0	Low
UFLA 03-321	w / +	+	+	+	+	- / +	GDS	-
INPA 54B	w / +	+	+	+	+	- / +	GDS	-
INPA 86A	w / +	+	+	+	+	w / +	GDS	-
UFLA 06-10	- / +	+	+	+	+	- / +	GDS	-
UFLA 06-13	w / +	+	+	+	+	- / +	GDS	-
UFLA 06-24	w / +	+	+	+	+	- / +	2.1	Medium

SIs, Final solubilisation indices (18 days after inoculation); SC, Solubilisation capacity; GDS, Grow and does not solubilise.

Table 3

Nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM) and efficiency compared to control with high nitrogen concentration (EFNC) obtained in acacia plants inoculated with *Bradyrhizobium* strains in Leonard jars at 90 days after sowing.

Treatments	NN	NDM	SDM	RDM	TDM	EFNC
	-	---------- mg plant^{-1} ----------				%
Without I + High N (52.5 mg L^{-1})[a]	0 c	0 d	768 a	361 a	1129 a	100 a
Without I + Low N (5.25 mg L^{-1})[a]	0 c	0 d	104 f	62 e	166 f	13 f
UFLA 03-84 + Low N	12 b	4 b	78 f	39 e	117 f	10 f
UFLA 03-144 + Low N	12 b	8 a	153 e	59 e	212 e	20 e
UFLA 03-268 + Low N	6 b	8 a	354 b	119 c	473 b	46 b
INPA 237B + Low N	0 c	0 d	82 f	38 e	121 f	11 f
INPA 104A + Low N	23 a	9 a	220 d	65 e	285 e	29 d
UFLA 03-153 + Low N	3 c	4 b	226 d	138 c	364 d	30 d
UFLA 03-164 + Low N	0 c	0 d	210 d	77 d	287 e	27 d
UFLA 03-150 + Low N	24 a	10 a	262 d	147 c	409 c	34 c
UFLA 03-197 + Low N	28 a	11 a	210 d	56 e	266 e	27 d
UFLA 03-290 + Low N	0 c	0 d	214 d	138 c	352 d	28 d
UFLA 04-0212 + Low N	0 c	0 d	198 d	132 c	330 d	26 d
UFLA 03-320 + Low N	0 c	0 d	304 c	168 b	472 b	40 c
UFLA 03-321 + Low N	0 c	0 d	224 d	178 b	402 c	29 d
INPA 86A + Low N	0 c	0 d	170 e	85 d	255 e	22 e
INPA 54B + Low N	0 c	0 d	74 f	45 e	119 f	10 f

Continuation...

Treatments	NN	NDM	SDM	RDM	TDM	EFNC
	-	---------- mg plant^{-1} ----------				%
UFLA 06-10 + Low N	0 c	0 d	174 e	134 c	309 d	23 e
UFLA 06-13 + Low N	0 c	0 d	153 e	60 e	213 e	20 e
UFLA 06-24 + Low N	0 c	0 d	152 e	79 d	232 e	20 e
BR 3617 + Low N	33 a	12 a	175 e	79 d	254 e	23 e
CV (%)	44.51	46.12	12.48	14.76	10.83	12.02

N, nitrogen; I, inoculation

In each column, means followed by the same letter are not statistically different and belong to the same group, according to the Scott–Knott test at $P<0.05$.

[a] Nitrogen sources used in the nutrient solution: $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3, $NH_4H_2PO_4$

Table 4

Nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), efficiency compared to the control with high nitrogen concentration (EFNC) and shoot nitrogen accumulation (SNA) obtained in velvet bean plants inoculated with *Bradyrhizobium* strains in Leonard jars at 60 days after sowing.

Treatments	NN	NDM	SDM	RDM	TDM	EFNC	SNA
	-	---------- mg plant^{-1} ----------				%	mg plant^{-1}
Without I + High N (52.5 mg L^{-1})[a]	0 d	0 e	2554 c	2112 a	4666 b	100 c	63 e
Without I + Low N (5.25 mg L^{-1})[a]	0 d	0 e	1096 e	767 d	1863 f	43 e	15 g
UFLA 03-84 + Low N	10 c	311 b	2579 c	902 c	3481 d	102 c	83 d
UFLA 03-144 + Low N	27 b	491 a	3728 a	1142 c	4870 b	147 a	93 c
UFLA 03-268 + Low N	15 c	287 b	2847 c	1077 c	3924 c	112 c	89 d
INPA 237B + Low N	4 d	80 d	1278 e	894 c	2173 f	50 e	21 g
INPA 104A + Low N	13 c	539 a	4132 a	1443 b	5575 a	163 a	128 a
UFLA 03-153 + Low N	12 c	293 b	2380 c	785 d	3165 e	94 c	73 e
UFLA 03-164 + Low N	27 b	64 d	1447 e	702 d	2149 f	57 e	19 g
UFLA 03-150 + Low N	33 b	274 b	2805 c	1032 c	3837 c	110 c	94 c
UFLA 03-197 + Low N	63 a	205 c	1653 d	636 d	2289 f	65 d	37 f
UFLA 03-290 + Low N	3 d	35 d	1721 d	1059 c	2780 e	68 d	37 f
UFLA 04-0212 + Low N	18 c	234 c	2603 c	943 c	3546 d	102 c	68 e
UFLA 03-320 + Low N	17 c	307 b	2790 c	970 c	3760 c	110 c	103 b
UFLA 03-321 + Low N	44 a	321 b	3309 b	1007 c	4316 c	130 b	109 b
INPA 86A + Low N	70 a	291 b	2467 c	750 d	3217 e	97 c	79 d

Continuation...

Treatments	NN	NDM	SDM	RDM	TDM	EFNC	SNA
	-	---------- mg plant^{-1} ----------				%	mg plant^{-1}
INPA 54B + Low N	58 a	252 c	2563 c	852 d	3416 d	101 c	47 f
UFLA 06-10 + Low N	5 d	218 c	2011 d	977 c	2988 e	80 d	30 f
UFLA 06-13 + Low N	19 c	66 d	1413 e	1005 c	2418 f	55 e	22 g
UFLA 06-24 + Low N	0 d	0 e	1070 e	727 d	1797 f	41 e	15 g
BR 2811 + Low N	62 a	205 c	1989 d	923 c	2913 e	79 d	37 f
CV (%)	28.38	3.40	10.70	12.13	9.32	10.60	13.13

N, nitrogen; I, inoculation

In each column, means followed by the same letter are not statistically different and belong to the same group, according to the Scott–Knott test at $p<0.05$

[a] Nitrogen sources used in the nutrient solution: $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3, $NH_4H_2PO_4$.

Table 5

Pearson correlation coefficients between nodule number (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), relative efficiency compared to the control with high mineral nitrogen concentration (EFNC) and shoot nitrogen accumulation (SNA) obtained in velvet bean plants inoculated with *Bradyrhizobium* strains in Leonard jars at 60 days after sowing.

	NDM	SDM	RDM	TDM	EFNC	SNA
NN	0.28^{ns}	0.17^{ns}	-0.16^{ns}	0.11^{ns}	0.17^{ns}	0.14^{ns}
NDM	-	0.92^{**}	0.56^{**}	0.90^{**}	0.91^{**}	0.85^{**}
SDM		-	0.65^{**}	0.98^{**}	0.96^{**}	0.91^{**}
RDM			-	0.76^{**}	0.63^{**}	0.55^{**}
TDM				-	0.95^{**}	0.89^{**}
EFNC					-	0.90^{**}

$^{**}P<0.01$; $^{*}P<0.05$; ^{ns}not significant