FERNANDA TEREZINHA MOURA

DIVERSIDADE E TAXONOMIA DE ESTIRPES ISOLADAS DE NÓDULOS DE DUAS ESPÉCIES DE FEIJÃO CULTIVADAS EM SOLOS DE BIOMAS DO MATO GROSSO DO SUL, BRASIL

Londrina
2025

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutora em Biotecnologia.

Orientadora: Dra. Mariangela Hungria
Coorientadora: Dra. Milena Serenato Klepa

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FERNANDA TEREZINHA MOURA

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Londrina, 11 de Março de 2025

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“Nunca deixe que lhe digam que não vale a pena acreditar no sonho que se tem, ou que seus planos nunca vão dar certo, ou que você nunca vai ser alguém.”
(Mais uma vez – Legião Urbana)

MOURA, Fernanda Terezinha. **Diversidade e taxonomia de estirpes isoladas de nódulos de duas espécies de feijão cultivadas em solos de biomas do Mato Grosso do Sul, Brasil**. 2025. (160 p.) Tese (Doutorado em Biotecnologia) – Centro de Ciências Exatas, Universidade Estadual de Londrina, Londrina, 2025.

RESUMO

As bactérias diazotróficas, classificadas em associativas, endofíticas e simbióticas (rizóbios), se associam com diferentes espécies vegetais e possuem a capacidade de realizar a Fixação Biológica do Nitrogênio (FBN), processo enzimático no qual o nitrogênio atmosférico (N_2) é convertido em amônia (NH_3). Os rizóbios, em particular, são amplamente estudados devido à sua heterogeneidade em gêneros e espécies e à sua capacidade de estabelecer simbiose com plantas leguminosas, resultando na formação de nódulos, principalmente nas raízes das plantas, onde ocorre a FBN. Contudo, grande parte da diversidade microbiana, incluindo os rizóbios, ainda é pouco conhecida, especialmente no Hemisfério Sul. Neste trabalho, dois estudos empregaram uma abordagem polifásica de análises para avaliar a diversidade e caracterizar taxonomicamente grupos de estirpes isoladas de nódulos de duas espécies de leguminosas. O Estudo I avaliou a diversidade de 89 estirpes isoladas de nódulos de feijão-caupi (*Vigna unguiculata*) inoculados com solos de seis aldeias indígenas do estado do Mato Grosso do Sul (MS), abrangendo os biomas Cerrado e Pantanal. Foram realizadas a caracterização morfofisiológica, análise de perfil genético por BOX-PCR e o sequenciamento e análise filogenética do gene 16S RNAr. Na análise de BOX-PCR foi verificada alta diversidade genética entre as estirpes, com a formação de 20 grupos e 23 estirpes ocupando posições únicas. Na análise do gene 16S RNAr, as estirpes foram classificadas em dez gêneros (*Agrobacterium* [47], *Ancylobacter* [2], *Burkholderia* [12], *Ensifer* [1], *Enterobacter* [1], *Mesorhizobium* [1], *Microbacterium* [1], *Paraburkholderia* [1], *Rhizobium* [22] e *Stenotrophomonas* [1]), distribuídos em quatro classes bacterianas. No Estudo II foi realizada uma análise polifásica com 13 estirpes de *Rhizobium* isoladas de nódulos de feijoeiro-comum (*Phaseolus vulgaris* L.) provenientes de solos de três biomas (Cerrado, Mata Atlântica e Pantanal) do MS. Com base na filogenia do gene 16S RNAr, as 13 estirpes foram divididas em dois grandes clados, sete em *Rhizobium etli* e seis em *Rhizobium tropici.* A análise de sequência *multilocus* (*Multilocus Sequence Analysis* - MLSA) utilizando quatro genes *housekeeping* (*glnII*, *gyrB*, *recA* e *rpoA*) corroborou com a alocação filogenética das estirpes na árvore do 16S RNAr. A identidade média de nucleotídeos (*Average Nucleotide Identity* - ANI) e a hibridização DNA-DNA digital (HDDd) apresentaram valores abaixo do limiar de delimitação de espécies quando comparados com as estirpes tipo das espécies descritas do gênero, variando de 81,84 a 92,50% e 24,0 a 50,7%, respectivamente. Além disso, outras características fenotípicas, genotípicas e simbióticas foram avaliadas e, com os resultados obtidos, foi possível descrever cinco novas espécies, "*Rhizobium atlanticum",* "*Rhizobium aureum*", "Rhizobium *centroccidentale"*, *"Rhizobium cerradonense"* e "*Rhizobium pantanalense"*. Algumas estirpes do Estudo II ficaram próximas de estirpes tipo descritas, mas sem genoma disponível, o que possibilitou a publicação do anúncio de genoma de *Rhizobium calliandrae* CCGE524^T e *Rhizobium mayense* CCGE526^T. Os

resultados encontrados contribuem para o avanço no conhecimento sobre a diversidade dos rizóbios em solos brasileiros, destacando o país como uma região de elevada diversidade bacteriana. Além disso, ressaltam a importância de investigações contínuas para explorar a diversidade microbiana em outros biomas brasileiros, visando tanto a preservação da biodiversidade, quanto o aprimoramento de práticas agrícolas sustentáveis.

Palavras-chave: Bactérias diazotróficas; Fixação Biológica de Nitrogênio; Abordagem polifásica; *Vigna unguiculata*; *Phaseolus vulgaris*

MOURA, Fernanda Terezinha. **Diversity and taxonomy of strains isolated from nodules of two species of beans grown in soils of biomes of Mato Grosso do Sul State, Brazil**. 2025. (160 p.) Thesis (Doctor of Philosophy – PhD in Biotechnology) – Center of Exact Sciences, State University of Londrina, Londrina, 2025.

ABSTRACT

Diazotrophic bacteria, classified as associative, endophytic and symbiotic (rhizobia), associate with different plant species and have the ability to perform Biological Nitrogen Fixation (BNF), an enzymatic process in which atmospheric nitrogen (N_2) is converted into ammonia (NH_3). Rhizobia, in particular, are widely studied due to their diversity in genera and species and their ability to establish symbiotic association with legumes plants, resulting in the formation of nodules, mainly in plant roots, where BNF occurs. However, most of the microbial diversity, including rhizobia, is still poorly understood, especially in the South Hemisphere. Here, two studies applied a polyphasic analysis approach to assess the diversity and taxonomically characterize groups of strains isolated from nodules of two legume species. Study I aimed to evaluate the diversity of 89 strains isolated from cowpea (*Vigna unguiculata*) nodules inoculated with soils from six indigenous villages in Mato Grosso do Sul (MS) State, covering the Cerrado and Pantanal biomes. Morphophysiological characterization, genetic profile analysis by BOX-PCR, sequencing, and phylogenetic analysis of the 16S rRNA gene were performed. The BOX-PCR analysis revealed high genetic diversity among the strains, there were positioned in 20 groups and 23 strains occupying unique positions. When analyzing the 16S rRNA gene, the strains were classified into ten genera (*Agrobacterium* [47], *Ancylobacter* [2], *Burkholderia* [12], *Ensifer* [1], *Enterobacter* [1], *Mesorhizobium* [1], *Microbacterium* [1], *Paraburkholderia* [1], *Rhizobium* [22] and *Stenotrophomonas* [1]), distributed in four bacterial classes. In Study II, a polyphasic analysis was conducted with 13 *Rhizobium* strains isolated from nodules of common bean (*Phaseolus vulgaris* L.) inoculated with soils from MS, encompassing three biomes (Cerrado, Atlantic Forest and Pantanal). Based on the 16S rRNA gene phylogeny, the 13 strains were divided into two major clades, seven in *Rhizobium etli* and six in *Rhizobium tropici*. Multilocus Sequence Analysis (MLSA) using four housekeeping genes (*glnII*, *gyrB*, *recA* and *rpoA*) corroborated with the 16S rRNA phylogeny. The Average Nucleotide Identity (ANI) and digital DNA-DNA hybridization (dDDH) presented values below the species delimitation threshold when compared with the described species of the genus, with values ranging from 81.84 to 92.50% and 24.0 to 50.7%, respectively. In addition, other phenotypic, genotypic and symbiotic characteristics were also evaluated and with the results obtained it was possible to describe five new species, *“Rhizobium atlanticum”, “Rhizobium aureum”, “Rhizobium centroccidentale”, “Rhizobium cerradonense”* and *“Rhizobium pantanalense”*. Some strains from Study II were close to described type strains, but with no genome available; therefore, there was a genome announcement of *Rhizobium calliandrae* CCGE524^T and *Rhizobium mayense* CCGE526^T. The results contribute to the advancing knowledge about the diversity of rhizobia in Brazilian soils, highlighting the country as a region of high bacterial diversity. They also emphasize the importance of ongoing investigations

to explore microbial diversity in other Brazilian biomes, aiming for both biodiversity preservation and improvement of sustainable agricultural practices.

Keywords: Diazotrophic bacteria; Biological Nitrogen Fixation; Polyphasic approach; *Vigna unguiculata*; *Phaseolus vulgaris*

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LISTA DE ABREVIATURAS E SIGLAS

ANI: *Average Nucleotide Identity*

ATP: Adenosina Trifosfato

CBD: *Convention on Biological Diversity*

dDDH: *digital DNA-DNA Hybridization*

DNA: Ácido Desoxirribonucleico

FBN: Fixação Biológica de Nitrogênio

IBGE: Instituto Brasileiro de Geografia e Estatística

INFOSANBAS: Informações Do Saneamento Básico

LPSN: *List of Prokaryotic Names with Standing in Nomenclature*

MLSA: *Multilocus Sequence Analisys*

MS: Mato Grosso do Sul

N: Nitrogênio

N_2: Nitrogênio Atmosférico

NAD: Nicotinamida Adenina Dinucleotídeo

NH_3: Amônia

NH_4^+: Íons Amônio

PCR: *Polymerase Chain Reaction*

RNA: Ácido Ribonucleico

SIBCs: Sistema Brasileiro de Classificação de Solos

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1. INTRODUÇÃO

A Fixação Biológica do Nitrogênio (FBN) é um processo enzimático no qual o nitrogênio atmosférico (N_2) é reduzido em amônia (NH_3) (Liu-Xu *et al*., 2024; Wannicke *et al*., 2024). Esse processo é realizado por microrganismos denominados diazotróficos, principalmente por bactérias diazotróficas, também conhecidas como bactérias fixadoras de N_2, que podem ser classificadas em três grupos, com variação na sua interação com as espécies vegetais: associativas, endofíticas e simbióticas (Kaschuk; Hungria, 2017; Thiebaut *et al*., 2022).

Dentre esses grupos, destacam-se as bactérias que estabelecem simbiose com diversas espécies de leguminosas, popularmente conhecidas como rizóbios, promovendo a formação de nódulos, principalmente nas raízes das plantas, onde ocorre a FBN (Basu; Kumar, 2020). A especificidade dessa associação varia entre espécies de rizóbios, com algumas espécies capazes de estabelecer simbiose com diferentes cultivares de leguminosas, enquanto outras interagem de forma mais restrita (Moura *et al*., 2020; Goyal; Mattoo; Schmidt, 2024). Essa variação na compatibilidade entre o rizóbio e a planta hospedeira também afeta na eficiência da fixação da FBN, podendo resultar em um processo menos eficiente (Mendoza-Suárez *et al*., 2021).

A simbiose rizóbios-leguminosas, por meio da FBN, proporciona uma fonte sustentável de nitrogênio (N), reduzindo a necessidade de fertilizantes nitrogenados e mitigando impactos ambientais, além de contribuir para melhorias na fertilidade do solo e ciclagem de nutrientes, tornando-se essencial para a sustentabilidade agrícola e a saúde dos ecossistemas (Kaschuk; Hungria, 2017; Mahmud *et al*., 2020; Abd-Alla; Al-Amri; El-Enany, 2023; Liu-Xu *et al*., 2024).

A heterogeneidade de gêneros, como *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, e *Microvirga*, bem como de espécies de rizóbios nesses gêneros, além da eficiência variável na capacidade de FBN, despertam grande interesse científico (Moura *et al*., 2020; Helene; Klepa; Hungria, 2022). A caracterização desses microrganismos é essencial para compreender sua diversidade e explorar seu potencial agrobiotecnológico. No entanto, grande parte dos microrganismos do solo, incluindo os rizóbios, ainda permanece desconhecida, assim como o conhecimento sobre seu potencial (Vitorino; Bessa,

2018). Estimativas indicam que cerca de 10^{12} espécies microbianas existem na Terra, mas que apenas cerca de 1% delas sejam conhecidas (Locey; Lennon, 2016). No Brasil, essa questão é particularmente relevante, dada a riqueza biológica dos biomas (Ellwanger; Nobre; Chies, 2022).

Diante dessa enorme diversidade bacteriana, a taxonomia é essencial para a sua compreensão. A taxonomia, definida por Paterlini (2007, p. 814) como "um sistema de classificação para o mundo natural que padroniza a nomenclatura de espécies e as organiza de acordo com suas características e relações", foi inicialmente proposta por Carl Linnaeus no século XVIII. Em seu estudo sobre plantas, Linnaeus desenvolveu o sistema binomial que categorizava os organismos em diferentes níveis hierárquicos, baseando-se em critérios sistemáticos (Paterlini, 2007; Sentausa; Fournier, 2013; Pallen; Telatin; Oren, 2021). Ele propôs a classificação dos organismos em dois níveis principais: gênero e espécie, um princípio que se mantém como base da taxonomia até hoje (Hugenholtz *et al.*, 2021). Embora Linnaeus tenha trabalhado principalmente com organismos macroscópicos (visíveis), seu sistema binomial abriu caminho para a classificação de organismos microscópicos, especialmente com o desenvolvimento da microscopia por Antonie van Leeuwenhoek e da microbiologia por Louis Pasteur e Robert Koch (Lunn; Winder; Shaw, 2023; Gaouar, 2024). Além disso, avanços em tecnologias moleculares como a amplificação e o sequenciamento do gene que codifica o RNA ribossomal (RNAr) localizado na subunidade menor do ribossomo (16S em procariotos, 18S em eucariotos), revolucionaram a taxonomia bacteriana, permitindo classificações mais precisas e melhor entendimento da diversidade microbiana nos ecossistemas (Kämpfer; Glaeser, 2011; Kumari; Rai, 2020; Bartoš; Chmel; Swierczková, 2024; Gaouar, 2024).

Atualmente, a taxonomia bacteriana segue uma abordagem polifásica, que combina análises fenotípicas, genotípicas e filogenéticas para classificar, identificar e nomear procariotos (Hugenholtz *et al.*, 2021; Helene; Klepa; Hungria, 2022). Essa abordagem tem se mostrado essencial para os estudos sobre rizóbios, especialmente no Brasil, impulsionada pelos avanços em ferramentas genômicas, como o sequenciamento de nova geração (*Next-Generation Sequencing* - NGS) e de bioinformática. Entre as técnicas moleculares empregadas estão a análise de perfis genéticos por BOX-PCR, análise da

sequência do gene 16S RNAr, análise de sequências *multilocus* (*Multilocus Sequence Analysis* - MLSA) com genes *housekeeping*, cálculos de identidade média de nucleotídeos (*Average Nucleotide Identity* - ANI) por comparações de genomas, hibridização DNA-DNA digital (HDDd), e a análise do conteúdo de guaninas e citosinas no DNA (G+C mol%) (Raina *et al*., 2019; Hugenholtz *et al*., 2021; Helene; Klepa; Hungria, 2022). O uso combinado dessas técnicas possibilita não apenas a caracterização e descrição de novas espécies, mas também contribui para o aprofundamento do conhecimento sobre a diversidade bacteriana (Das *et al*., 2014; Pascoal; Costa; Magalhães, 2021; Helene; Klepa; Hungria, 2022).

O Brasil é mundialmente reconhecido como um dos 17 países mais megadiversos, abrigando a maior diversidade biológica (fauna e flora) (Myers, 2000; Abranches, 2020). Com base no clima, vegetação, fauna, flora e solo, o Instituto Brasileiro de Geografia e Estatística (IBGE) define seis grandes biomas continentais terrestres no Brasil: Amazônia (Floresta Amazônica), Caatinga, Cerrado, Mata Atlântica, Pampa e Pantanal (IBGE, 2019). Em cada bioma ocorre adaptação e especiação de microrganismos, incluindo os rizóbios, resultando em riqueza de diversidade microbiana (Caballero; Ruhoff; Biggs, 2022; Ellwanger; Nobre; Chies, 2023).

Até o momento, os rizóbios (capazes ou não de fixar nitrogênio) registrados nos solos dos biomas brasileiros, conforme o site LPSN (*List of Prokaryotic Names with Standing in Nomenclature*), pertencem predominantemente à classe alfaproteobacteria, nos gêneros *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microvirga* e *Rhizobium*; e em betaproteobacteria, nos gêneros *Paraburkholderia* e *Trinickia* (Parte *et al*., 2020).

Por isso, a compreensão da diversidade e da eficiência de FBN das bactérias diazotróficas, com destaque aos rizóbios, é imprescindível para o avanço das práticas agrícolas sustentáveis, particularmente em países como o Brasil, que apresentam uma vasta biodiversidade biológica. A caracterização taxonômica e genômica dos rizóbios, por meio do sequenciamento de nova geração e de ferramentas de bioinformática, permite aprofundar o entendimento sobre as interações rizóbios-leguminosa e a FBN. Portanto, o objetivo geral deste trabalho foi ampliar o conhecimento sobre a diversidade microbiana no Brasil, empregando análises polifásicas para classificar e identificar rizóbios nos

biomas brasileiros. Com os resultados obtidos, espera-se contribuir para o desenvolvimento de soluções mais eficazes e sustentáveis para a agricultura, promovendo a preservação dos recursos naturais e gerando benefícios tanto para o meio ambiente, quanto para a produção agrícola.

2. OBJETIVOS

2.1 Objetivo Geral

Estudar bactérias isoladas de nódulos de duas espécies de feijão (*Vigna unguiculata* (L.) Walp.) e (*Phaseolus vulgaris* L.) cultivadas em biomas do Mato Grosso do Sul, com foco na avaliação da diversidade e na caracterização fenotípica, genotípica e filogenética.

2.2 Objetivos Específicos

- Analisar a diversidade intraespecífica dos isolados por meio da técnica de BOX-PCR (Estudo I);

- Identificar os isolados em nível de gênero, utilizando a relação filogenética por meio do gene 16S RNAr (Estudo I);

- Inferir relações evolutivas das estirpes por meio da análise filogenética do gene 16S RNAr e dos genes *housekeeping* (*glnII*, *gyrB*, *recA* e *rpoA*) por meio da técnica de MLSA (Estudo II);

- Determinar a posição taxonômica das estirpes de *Rhizobium* estudadas utilizando as análises de ANI e HDDd com dados genômicos (Estudo II);

- Por meio de análise polifásica de características morfofisiológicas, genéticas, filogenéticas e simbióticas, definir novas espécies de rizóbios (Estudo II).

3. REFERENCIAL TEÓRICO

3.1. Fixação Biológica do Nitrogênio e a Enzima Nitrogenase

O N é um elemento essencial para todos os organismos vivos, sendo um componente fundamental de compostos como aminoácidos, proteínas, ácidos nucléicos (DNA e RNA), clorofila e moléculas bioenergéticas como a adenosina trifosfato (ATP) e a nicotinamida adenina dinucleotídeo (NAD). Ele também desempenha um papel crucial para os vegetais, atuando como limitante na produção agrícola (Cherkasov; Ibhadon; Fitzpatrick, 2015; Javed *et al*., 2022; Robertson; Groffman, 2024).

Embora o N na forma de N_2 (dinitrogênio) corresponda a 78% dos gases atmosféricos, as plantas não conseguem utilizá-lo diretamente, devido à sua grande estabilidade, causada pela forte tripla ligação entre dois átomos de nitrogênio (N≡N) (Das; Prasanna; Saxena, 2017; de Mello Prado, 2021; Wekesa *et al*., 2022). Para ser assimilado pelas plantas, o N_2 precisa ser transformado em formas reativas, como nitrato (NO_3^-) ou íons amônio (NH_4^+) (Burén; Rubio, 2018; Lindström; Mousavi, 2020). Essa conversão é fundamental para que o N possa se combinar com outros átomos e formar compostos orgânicos essenciais (Bottomley; Myrold, 2007; Cherkasov; Ibhadon; Fitzpatrick, 2015).

Embora o NH_4^+ seja facilmente absorvido pelas plantas, em altas concentrações se torna tóxico para as células vegetais (Bittsánszky *et al*., 2015; Raza *et al*., 2020). No entanto, as bactérias diazotróficas possuem mecanismos que asseguram sua rápida exportação e assimilação pelas plantas, convertendo-o em formas orgânicas como aminoácidos, ureídos e amidas (Hungria; Campo; Mendes, 2001; Mus *et al*., 2016; Imran *et al*., 2021; Bhatla; Lal, 2023).

A disponibilização do N para as plantas pode ocorrer a partir de quatro fontes: diretamente do solo (mineralização da matéria orgânica), pela ação de fenômenos naturais como raios, combustão e vulcanismo (chamado de fixação não biológica), pela adição de fertilizantes nitrogenados ou pela fixação biológica do nitrogênio (FBN) (Wang, 2019a; Ladha *et al*., 2022; Hungria; Nogueira, 2023; Robertson; Groffman, 2024).

A disponibilidade de N proveniente da matéria orgânica do solo é limitada, podendo se esgotar rapidamente após alguns cultivos. Igualmente ocorre com a fixação não biológica, resultante de fenômenos naturais, que contribui com apenas cerca de 10% do N necessário para as plantas (Hungria; Campo; Mendes, 2001; Hungria; Mendes; Mercante, 2013; Hungria; Nogueira, 2023).

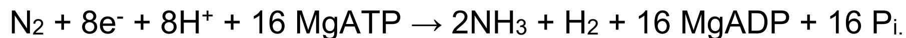
A produção de fertilizantes nitrogenados, majoritariamente pelo processo Haber-Bosch, é um processo químico industrial que necessita combustíveis fósseis, altas temperaturas (~ 600 – 750 °C) e altas pressões (~ 100 atm) para a produção de amônia (NH_3), combinando N_2 com hidrogênio (H_2) (Vojvodic *et al*., 2014; Cherkasov; Ibhadon; Fitzpatrick, 2015; Soumare *et al*., 2020).

O uso de fertilizantes nitrogenados, embora facilite a assimilação de N pelas plantas, apresenta um custo financeiro elevado e baixa eficiência (30% - 70%), o que leva a perdas significativas. Essas perdas não apenas aumentam as emissões de gases do efeito estufa, mas também causam problemas como a desnitrificação, volatilização e lixiviação, resultando na poluição do solo, da água e do ar (Stewart; Lal, 2017; Rashmi *et al*., 2020; Hungria; Nogueira, 2023).

A FBN é uma fonte natural e eficiente de incorporação de N à biosfera. Considerada o processo biológico mais importante depois da fotossíntese, a FBN desempenha um papel essencial para a sustentabilidade agrícola (Soumare *et al*., 2020; Ladha *et al*., 2022). É um processo realizado exclusivamente por procariotos dos domínios Bacteria e Archaea, comumente chamados de diazotróficos (di = dois, azoto = nitrogênio; trófico = relativo á alimentação), que possuem a enzima nitrogenase, responsável por quebrar a tripla ligação do N_2, reduzindo-o em NH_3 (Hungria; Nogueira, 2023).

A FBN é extremamente vantajosa por mitigar impactos ambientais, otimizar o aproveitamento do N pelas plantas e contribuir para a fertilidade do solo e a sustentabilidade dos ecossistemas, porém, exige uma grande quantidade de energia (Hungria; Nogueira, 2023; Abd-Alla; Al-Amri; El-Enany, 2023). Para que a nitrogenase converta uma molécula de N_2, são necessários 8 elétrons e a hidrólise de 16 ATP. Embora a conversão de N_2 em NH_3 necessite

da transferência de 6 elétrons (3 elétrons para cada molécula de NH_3), o processo completo envolve oito eventos de transferência, já que 2 elétrons adicionais são necessários para reduzir H^+ a H_2 (Burén; Rubio, 2018; Gu; Milton, 2020; Einsle; Rees, 2020), conforme descrito na estequiometria da reação:



$$N_2 + 8e^- + 8H^+ + 16\ MgATP \rightarrow 2NH_3 + H_2 + 16\ MgADP + 16\ P_i.$$

A nitrogenase é um complexo enzimático composto por duas metaloproteínas, dinitrogenase e dinitrogenase redutase (também chamadas de componente I e II), onde ambas contêm aglomerados FeS (sulfeto de ferro). As três formas conhecidas de nitrogenase são geneticamente distintas, porém, estruturalmente semelhantes: Mo-nitrogenase (dependente de molibdênio), V-nitrogenase (dependente de vanádio) e Fe-nitrogenase (dependente de ferro), cada uma codificada por genes fixadores de nitrogênio específicos (*nifHDK*, *vnfHDK* e *anfHDK*, respectivamente) (Burén; Rubio, 2018; Harris *et al*., 2019; Gu; Milton, 2020; Pi *et al*., 2022).

A Mo-nitrogenase é a mais estudada, e é composta pelas metaloproteínas molibdênio-ferro proteína (MoFe-proteína ou dinitrogenase) e pela ferro-proteína (Fe-proteína ou dinitrogenase redutase). A MoFe-proteína, codificada pelos genes *nifD* e *nifK,* contém o sítio ativo para a ligação e redução do N_2, enquanto a Fe-proteína, codificado pelo gene *nifH,* fornece os elétrons necessários para esse processo (Imran *et al*., 2021; Pi *et al*., 2022). Além disso, entre 10 e 20 genes adicionais são necessários para a formação de um complexo nitrogenase completamente funcional; a quantidade de genes necessários varia entre espécies (Mus *et al*., 2016; Burén; Rubio, 2018; Bellenger *et al*., 2020).

A reação de redução também envolve uma terceira molécula, a ferredoxina ou flavodoxina, que são pequenas proteínas de transferência de elétrons, que doam elétrons para a Fe-proteína, possibilitando a redução da MoFe-proteína (Alleman; Peters 2023).

3.2. Bactérias Diazotróficas e Leguminosas

As bactérias diazotróficas ou bactérias fixadoras de nitrogênio, são morfológica, fisiológica, genética e filogeneticamente diversas. Elas podem ser encontradas no solo, vivendo de forma livre e fixando nitrogênio para seu próprio uso, ou estabelecendo associações/interações com diferentes

espécies vegetais, variando em graus de especificidade (Santi; Bogusz; Franche, 2013; Carvalho *et al*., 2014; Thiebaut *et al*., 2022; Xu; Wang, 2023; Giller *et al*., 2024).

As associações com plantas podem ocorrer de forma associativa, endofítica e/ou simbiótica. As bactérias diazotróficas associativas ou rizosféricas colonizam e fixam N_2 na rizosfera das plantas (Carvalho *et al*., 2014; Imran *et al*., 2021; Klepa; Helene; Hungria, 2024). As bactérias diazotróficas endofíticas colonizam os tecidos internos vegetais, sem efeitos nocivos à planta hospedeira (Kandel; Joubert; Doty, 2017; Kaschuk; Hungria, 2017; Chaudhary *et al*., 2022; Klepa; Helene; Hungria, 2024). As bactérias diazotróficas simbióticas (popularmente conhecidas como rizóbios) promovem alterações funcionais e estruturais na leguminosa e em suas próprias células, resultando na formação de nódulos em raízes e, caules, onde ocorre a FBN (Sachs; Quides; Wendlandt, 2018; Kirova; Kocheva, 2021; Goyal; Mattoo; Schmidt, 2024).

Essas alterações têm como objetivo principal transferir fontes de carbono da planta para a bactéria em troca do N fixado biologicamente, garantindo uma relação de mutualismo eficaz (Mus *et al*., 2016; Kirova; Kocheva, 2021). Para que a FBN seja viabilizada, é necessário o estabelecimento de uma simbiose eficaz entre bactéria e planta hospedeira (Mus *et al*. 2016; Goyal; Mattoo; Schmidt, 2021).

Segundo Maunoury *et al*. (2008), três eventos principais são necessários para a formação de uma relação simbiótica eficiente: (*i*) infecção intercelular do microssimbionte na célula hospedeira; (*ii*) organogênese do nódulo, ou seja, a formação dos nódulos; e (*iii*) o processo de FBN. A infecção e a organogênese ocorrem simultaneamente, enquanto a FBN acontece após a completa formação dos nódulos e somente se a infecção bacteriana for bem-sucedida (Mahmud *et al*., 2020).

A simbiose começa com a sinalização molecular pela planta, geralmente flavonoides ou isoflavonoides, moléculas que atuam como quimioatraentes para as bactérias (Wilkinson *et al*., 2023). Após a sinalização inicial, as bactérias respondem com a síntese de lipo-quito-oligossacarídeos (LCOs), ou fatores Nod, que ativam genes de nodulação na bactéria (*nod*, *nol* e *noe*), e desencadeiam a divisão celular da planta, induzindo à modificações

específicas (Jamil *et al*., 2022; Wilkinson *et al*., 2023).

Essas modificações incluem o encurvamento do pelo radicular ao redor das bactérias e a formação do cordão de infecção, uma invaginação que transporta as bactérias até o córtex da raiz, onde se localiza o primórdio do nódulo (Hassen; Lamprecht; Bopape, 2020; Compton *et al*., 2020). Neste local, as bactérias são liberadas, diferenciam-se em bacteroides fixadores de nitrogênio, e ficam contidas no simbiossoma, uma organela delimitada por uma membrana de origem vegetal, responsável por regular a troca de nutrientes entre a planta e a bactéria (Mus *et al*., 2016; Wang; Liu; Zhu, 2018; Ledermann; Schulte; Poole, 2021).

Além disso, para que a conversão enzimática seja viável dentro dos nódulos, é necessária a produção de leghemoglobina, uma hemeproteína responsável por proteger a enzima nitrogenase da desnaturação, uma vez que as plantas precisam operar em baixo nível de O_2, pois a nitrogenase é altamente sensível ao O_2, e sua presença inativa irreversivelmente o complexo; contudo, ao mesmo tempo, a leghemoglobina fornece uma quantidade suficiente de oxigênio para os bacteroides respirarem e gerarem ATP (Singh; Varma, 2017; Schwember *et al*., 2019; Wekesa *et al*., 2022).

A expressão da leghemoglobina altera a coloração interna dos nódulos, sendo um indício de efetividade da FBN. Quando o interior dos nódulos apresenta coloração rosa (avermelhada), indica funcionalidade da proteína leghemoglobina; a coloração branca indica ausência da proteína, enquanto a coloração verde indica nódulos senescentes, com perda da funcionalidade da leghemoglobina (Du *et al*., 2020; Ledermann; Schulte; Poole, 2021; Berrabah *et al*., 2024).

Em geral, os genes simbióticos responsáveis pelo processo de fixação fazem parte do genoma acessório dos rizóbios e estão localizados em elementos genéticos móveis, como plasmídeos ou ilhas simbióticas (Black *et al*., 2012). Esses elementos genéticos móveis transportam genes que desempenham um papel importante na adaptação do hospedeiro ao ambiente, influenciando características como resistência a antibióticos, simbiose, vias metabólicas, entre outras (Dobrindt *et al*., 2004; Rankin; Rocha; Brown, 2011; Bellanger *et al*., 2014; Weisberg; Chang, 2023).

Embora as bactérias diazotróficas associativas/rizosféricas e

endofíticas não sejam rizóbios, elas também desempenham um papel significativo no crescimento vegetal e são denominadas como bactérias promotoras do crescimento de plantas (BPCP) (Guimarães *et al*., 2022; Chauhan *et al*., 2023). Elas atuam por mecanismos diretos, como FBN, produção de fitohormônios e solubilização de fosfato, e por mecanismos indiretos, como, indução de resistência a estresses bióticos e abióticos e proteção contra patógenos (Olanrewaju; Glick; Babalola, 2017; Hakim *et al*., 2021; Prisa; Fresco; Spagnuolo, 2023). Essas bactérias são frequentemente associadas a gramíneas de importância agrícola, tais como arroz (*Oryza sativa*), milho (*Zea mays*), cana-de-açúcar (*Saccharum* spp.) e trigo (*Triticum aestivum*) (dos Santos *et al*., 2020; Thiebaut *et al*., 2022; Guo *et al*., 2023).

Por muito tempo acreditou-se que os nódulos radiculares de leguminosas eram habitados exclusivamente por rizóbios. No entanto, estudos passaram a demostrar que nódulos, frequentemente, abrigam outras bactérias, consideradas não rizóbios endofíticos (Dudeja *et al*., 2012; Primieri *et al*., 2016; Zhao; Xu; Lai, 2018; Muindi *et al*., 2021; Rahal; Chekireb, 2021). Embora essas bactérias não induzam a formação de nódulos, elas podem colonizar seu interior ao serem transportadas junto com os rizóbios pelos cordões de infecção (Pandya; Naresh Kumar; Rajkumar, 2013; Hnini; Aurag, 2024). A maioria dos não rizóbios endofíticos são considerados não patogênicos e, ao colonizar os nódulos, recebem abrigo e suprimento de nutrientes da planta hospedeira, protegendo-os contra diversos estresses abióticos. Em contrapartida, sua presença pode beneficiar a planta por meio de diferentes mecanismos diretos e indiretos, como já mencionado (Martínez-Hidalgo; Hirsch, 2017; Deng *et al*., 2020).

Entretanto, a associação simbiótica com espécies da família Fabaceae (=Leguminosae) é a mais estudada e eficiente entre as bactérias diazotróficas, proporcionando o maior aporte de N para os ecossistemas terrestres (Ladha *et al*., 2022; Klepa; Helene; Hungria, 2024). Exemplos notáveis de leguminosas incluem soja (*Glycine max*), feijão (*Phaseolus vulgaris*), alfafa (*Medicago sativa*) e amendoim (*Arachis hypogaea*) (Guo *et al*., 2023).

Fabaceae ou Leguminosae, é a terceira maior família de plantas, composta por cerca de 751 gêneros e 19.000 espécies conhecidas (Christenhusz; Byng, 2016; Bahadur *et al*., 2023; Estrada-Castillón *et al*., 2024).

Amplamente distribuídas em todos os biomas terrestres, com maior diversidade em regiões tropicais e subtropicais, essas plantas desempenham papéis cruciais nos sistemas naturais e agrícolas devido à sua importância nutritiva, econômica, social e ecológica (Sprent; Ardley; James, 2017; Mrunalini *et al*.; 2022; Skrzypkowski *et al*., 2023; Telles; Nogueira; Hungria, 2023; Estrada-Castillón *et al*., 2024). A família Fabaceae é atualmente subdividida em seis subfamílias, das quais apenas Caesalpinioideae e Papilionoideae possuem espécies de rizóbios descritas isoladas de nódulos radiculares (Azani *et al*., 2017; Ardley; Sprent, 2021; Longhi *et al*., 2024).

O feijão comum (*Phaseolus vulgaris* L.) é amplamente cultivado no Brasil, destacando-se por sua excelente adaptação climática, desempenhando um papel essencial na alimentação humana, representando uma rica fonte de proteína e tendo grande relevância socioeconômica (Myers; Kmiecik, 2017; Shamseldin; Velázquez, 2020; Pias *et al.*, 2022; Lisciani *et al*., 2024). Já o feijão-caupi (*Vigna unguiculata* L. Walp), fundamental para as populações do Norte e Nordeste do Brasil, é um componente essencial na dieta alimentar local. Sua adaptabilidade às condições tropicais tem impulsionado a expansão de cultivo para outras regiões, como o Centro-Oeste (Freire Filho *et al*., 2011; de Andrade *et al*., 2021).

Ambas as espécies são consideradas leguminosas promíscuas, capazes de estabelecer associações com uma ampla variedade de espécies bacterianas (Lira Jr; Nascimento; Fracetto, 2015; Shamseldin; Velázquez, 2020; Mendoza-Suárez *et al*., 2021; Xavier *et al*., 2023). No entanto, a interação com essas espécies ocorre em diferentes níveis de eficiência, resultando, em alguns casos, em baixa eficiência de FBN (Reinprecht *et al*., 2020; Mendoza-Suárez *et al*., 2021; Xavier *et al*., 2023). Nesse contexto, a diversidade de solos e biomas brasileiros, com sua rica variedade de ecossistemas, oferece um ambiente propício para investigar essas interações, o que pode contribuir para a melhoria da eficiência da FBN e, consequentemente, para a saúde dos ecossistemas e a sustentabilidade agrícola.

3.3. Diversidade de Solos e Biomas Brasileiros

O Brasil é reconhecido como um dos países mais megadiversos do mundo, estimando-se que abrigue aproximadamente 20% de toda a diversidade biológica terrestre (fauna e flora) (Myers, 2000; CBD, 2024). Essa diversidade biológica reflete a heterogeneidade de climas e solos do país, que, por sua vez, contribuem para a formação de diferentes biomas, cada um com suas particularidades (Pylro *et al*., 2014; Abranches, 2020; Caballero; Ruhoff; Biggs, 2022; Ellwanger; Nobre; Chies, 2022; Oliveira Filho, 2024).

Biomas (bio = vida, oma = grupo, conjunto, massa) são regiões caracterizadas por um conjunto de vida vegetal e animal, com tipos de vegetação semelhantes e condições geológicas e climáticas similares, que passaram pelos mesmos processos de formação da paisagem, resultando em uma diversidade regional única de fauna e flora (IBGE, 2019). No Brasil, o IBGE define seis grandes biomas terrestres, Amazônia, Caatinga, Cerrado, Mata Atlântica, Pantanal e Pampa, baseando-se no clima, vegetação, fauna, flora e solo (IBGE, 2019; Caballero; Ruhoff; Biggs, 2022).

Cada um desses biomas é acompanhado por uma rica diversidade de solos, que se desenvolveram por meio do intemperismo - desintegração ou decomposição das rochas por meio de propriedades físicas, químicas e biológicas. Os solos são formados por uma combinação de minerais (por exemplo, areia, silte e argila), gases, água, matéria orgânica e organismos (Goudie, 1999; Kalev; Toor, 2018; Adewara *et al*., 2024). Existem 13 diferentes classes (ordens) de solos determinadas pelo Sistema Brasileiro de Classificação de Solos (SiBCS). As classes incluem: argissolos, cambissolos, chernossolos, espodossolos, gleissolos, latossolos, luvissolos, neossolos, nitossolos, organossolos, planossolos, plintossolos e vertissolos (dos Santos *et al*., 2018).

Abaixo, são descritas as principais características dos solos associados aos biomas brasileiros, destacando sua diversidade e relevância para a formação dos ecossistemas.

Os argissolos, constituídos por material mineral, apresentam um horizonte B caracterizado pelo acúmulo de argila, cuja capacidade de retenção de nutrientes e água varia conforme a atividade da argila (alta ou baixa), influenciando diretamente na fertilidade do solo. Os espodossolos são solos

ácidos e de baixa fertilidade, caracterizados por um horizonte B com acúmulo de matéria orgânica, óxidos de ferro e alumínio. Os latossolos, são altamente intemperizados, profundos, bem drenados, e de baixa fertilidade. Os luvissolos, por sua vez, possuem um horizonte B enriquecido com argila, conferindo-lhes maior fertilidade natural. Neossolos são solos minerais jovens, poucos desenvolvidos, com pouca ou nenhuma formação do horizonte B, enquanto planossolos são caracterizados por drenagem limitada e um horizonte B com acúmulo de argila. Por fim, os plintossolos, comuns em áreas com dificuldade de percolação (penetração) de água, apresentam excesso temporário de umidade e um horizonte B com plintita, material argiloso rico em ferro (Matos *et al*., 2017; dos Santos *et al*., 2018).

A variedade de climas e solos nos biomas permite o desenvolvimento de uma vasta gama de formas de vida, incluindo microrganismos como os rizóbios (Xavier *et al*., 2010; Bruce *et al*., 2012; Arruda *et al*., 2017; Guayasamin *et al*., 2024). Contudo, a perda da biodiversidade causada, principalmente, pelo desmatamento continua sendo uma ameaça significativa (Procópio; Barreto, 2021). Nesse contexto, estudos que avaliem a diversidade microbiana são fundamentais não apenas para compreender as interações entre os organismos e os solos, mas também para promover estratégias eficazes na preservação dos biomas e seus ecossistemas (de Castro Pires *et al*., 2018; Chauhan *et al*., 2023; Jayaramaiah *et al*., 2025).

3.3.1. Amazônia

O bioma Amazônia, também conhecido como floresta amazônica, é a maior floresta tropical do mundo, representando o maior reservatório de biodiversidade vegetal e animal do planeta. Está distribuída em nove países da América do Sul: Brasil (64,3%), Bolívia (6,2%), Colômbia (6,2%), Equador (1,5%), Guiana (2,8%), Peru (10%), Suriname (2,1%), Venezuela (5,8%) e a Guiana Francesa (1,1%), ocupando uma extensão territorial de 7,8 milhões de km^2. No Brasil, o bioma abrange toda a região Norte (Acre – AC, Amapá - AP, Amazonas - AM, Pará - PA, Rondônia - RO, Roraima - RR e Tocantins - TO), partes das regiões Centro-Oeste (Mato Grosso - MT) e Nordeste (Maranhão - MA) (Figura 1), cobrindo uma área de 4.212.742 km^2, o que corresponde a cerca

de 49,5% do território brasileiro, sendo considerado o maior bioma do Brasil (Bieski *et al*., 2015; IBGE, 2019; Viana, 2020; Fernandes; Marcovitch; Pinto, 2024).

A Amazônia é caracterizada por um clima úmido e alta precipitação, com temperaturas anuais variando de mínimas e máximas entre 25°C e 30°C. Entretanto, variações de temperatura podem ocorrer, e a umidade relativa do ar é elevada (Bueno *et al*., 2019). No que diz respeito aos solos, todas as 13 classes de solos classificadas pelo SiBCS estão presentes no bioma, destacando-se os argissolos e latossolos como as classes predominantes (Schaefer *et al*., 2023).

3.3.2. Caatinga

A Caatinga é uma das maiores e mais biodiversas terras secas tropicais do mundo, com uma flora e fauna adaptadas a longos períodos de seca, sendo um bioma exclusivo do Brasil. Cobre, predominantemente, a porção Nordeste do país (Alagoas - AL, Bahia - BA, Ceará - CE, Paraíba - PB, Pernambuco - PE, Piauí - PI, Rio Grande do Norte – RN e Sergipe - SE), e uma pequena extensão na Região Sudeste (Minais Gerais - MG) (Figura 1), ocupando uma área de aproximadamente 862.818 km², o que corresponde a cerca de 10,1% do território brasileiro (Santos *et al*., 2014; IBGE, 2019; de Magalhães *et al*., 2023; de Araújo Filho *et al*., 2023).

O bioma Caatinga apresenta um clima semiárido, com temperaturas anuais de mínimas e máximas entre 25°C e 30°C, entretanto, variações de temperatura podem ocorrer. Os períodos secos são prolongados, e as chuvas ocorrem de forma irregular, concentrando-se em um curto período, com grandes variações anuais e secas recorrentes sendo frequentes (Moro *et al*., 2016; Silva *et al*., 2017; de Barros Corrêa *et al*., 2019; de Magalhães *et al*., 2023). Em termos de solos, a Caatinga abriga diversidade, destacando-se os latossolos, neossolos, argissolos e luvissolos. Os organossolos, por sua vez, não são encontrados no bioma Caatinga ou têm uma ocorrência muito reduzida (Pedone-Bonfim *et al*., 2017; de Araújo Filho *et al*., 2023).

3.3.3. Cerrado

O Cerrado, localizado principalmente na parte central do Brasil, cobre os estados de Distrito Federal - DF, Goiás - GO, Mato Grosso - MT, Mato Grosso do Sul - MS (Centro-Oeste), Bahia - BA, Ceará - CE, Maranhão - MA, Piauí - PI (Nordeste), Rondônia - RO, Tocantins - TO (Norte), e Minas Gerais - MG (Sudeste), pequenas áreas em Amapá - AP, Pará – PA, Roraima - RR (Norte), São Paulo - SP (Sudeste) e Paraná - PR (Sul) (Figura 1), e partes do Paraguai e Bolívia; cobre aproximadamente 1.983.017 km², o que representa 23,3% do território brasileiro (Silva Oliveira *et al*., 2018; IBGE, 2019; de Oliveira *et al*., 2023). É o segundo maior bioma do país e é considerado um dos mais biodiversos do mundo, com a flora mais rica entre as savanas. Reconhecido como um *hotspot* de biodiversidade, com um elevado número de espécies endêmicas - ou seja, espécies que só existem nesta região, mas que está sofrendo perdas significativas de habitat, o que o torna uma área de grande preocupação em termos de conservação (Myers, 2000; Marchese, 2015; Colli; Vieira; Dianese, 2020; Lambers *et al*., 2020).

O cerrado tem um clima sazonal, com duas estações bem definidas: verão chuvoso (de outubro a abril) e inverno seco (de maio a setembro). As temperaturas variam de mínimas e máximas entre 20°C a 30°C no verão, e entre 12°C a 14°C no inverno (Salgado *et al*., 2019; de Oliveira *et al*., 2023). Entretanto, variações de temperatura podem ocorrer. Os principais solos do bioma Cerrado incluem os latossolos, neossolos e argissolos. Vale ressaltar que, das 13 classes de solos, os espodossolos são os únicos que não são encontrados ou têm uma ocorrência muito reduzida no bioma Cerrado (de Freitas *et al*., 2013; Lepsch, 2016; de Oliveira *et al*., 2023).

3.3.4. Mata Atlântica

A Mata Atlântica é um dos biomas mais ricos em biodiversidade do mundo, abrigando uma vasta variedade de espécies, muitas das quais são endêmicas. Além disso, é uma das florestas mais ameaçadas, o que a torna um dos principais *hotspots* de biodiversidade do planeta (Peres *et al*., 2020; Grelle; Rajão; Marques, 2021; Marques *et al*., 2021; Almeida; Souza, 2023). Embora

sua biodiversidade seja significativa, restam apenas de 11% a 16% da cobertura vegetal original, o que a coloca em risco de degradação irreversível (Ribeiro *et al.*, 2009; Scarano; Ceotto, 2015; Almeida; Souza, 2023). Sua distribuição geográfica abrange principalmente a costa do oceano Atlântico, da qual deriva seu nome, e se estende por diversas regiões do Brasil como o Sudeste (Espírito Santo - ES, Minais Gerais - MG, Rio de Janeiro - RJ, São Paulo - SP), Sul (Santa Catarina - SC, Paraná - PR, Rio Grande do Sul - RS), Nordeste (Alagoas - AL, Bahia - BA, Ceará - CE, Paraíba - PB, Pernambuco - PE, Piauí - PI, Rio Grande do Norte - RN, Sergipe - SE), e Centro-Oeste (Goiás - GO, Mato Grosso do Sul - MS) (Figura 1), além de partes da Argentina e do Paraguai. Com uma área total de aproximadamente 1.107.419 km^2, que representa cerca de 13% do território brasileiro (Ribeiro *et al.*, 2009; IBGE, 2019; Fontana *et al.*, 2023).

O clima do bioma Mata Atlântica é, predominantemente, tropical úmido, caracterizado por alta umidade relativa, chuvas abundantes e bem distribuídas ao longo do ano. As temperaturas anuais variam de mínimas e máximas entre 19°C a 26°C, embora possam ocorrer variações regionais (Kamino *et al.*, 2019; Francisquini *et al.*, 2020). Quanto aos solos, as principais classes encontradas são latossolos e argissolos. Contudo, das 13 classes de solos, apenas espodossolos e plintossolos são ausentes ou apresentam ocorrência muito reduzida nesse bioma (Kamino *et al.*, 2019; Fontana *et al.*, 2023).

3.3.5. Pampa

O bioma Pampa, localizado em todo o Uruguai, parte da Argentina, e na região sul do Brasil, exclusivamente no estado do RS (Figura 1), correspondendo a 63% do estado, ocupa uma área de 193.836 km^2, o que representa cerca de 2,3% do território brasileiro (IBGE, 2019; Verdum *et al.*, 2019). Caracterizado por uma grande diversidade de ecossistemas, o Pampa abriga uma variedade de hábitats que sustentam diferentes espécies adaptadas ao clima subtropical úmido, tornando-se uma região de grande importância para a conservação da fauna e flora no sul do continente (Marchi *et al.*, 2018; Silva *et al.*, 2020).

O clima do Pampa é subtropical úmido, com verões amenos e invernos frios, e precipitações bem distribuídas ao longo do ano. A temperatura anual varia de mínimas e máximas entre 17°C e 20°C, mas variações de temperatura

podem ocorrer (Verdum *et al*., 2019; de Almeida *et al*., 2023). Quanto aos solos, o Pampa apresenta uma diversidade de classes, com 11 delas sendo encontradas, destacando-se os neossolos como predominantes. Já os espodossolos e organossolos têm ocorrência muito reduzida ou não são encontrados no bioma (de Almeida *et al*., 2023).

3.3.6. Pantanal

O Pantanal está localizado principalmente na região Centro-Oeste do Brasil (MT e MS) (Figura 1), estendendo-se também pela Bolívia e Paraguai, cobrindo aproximadamente 150.988 km^2, o que representa 1,8% do território brasileiro. Embora seja o menor bioma do país, é considerado a maior planície tropical de inundação do mundo. Além disso, foi reconhecido como Patrimônio Nacional pela Constituição Brasileira de 1988 e, desde o ano de 2000, é também Patrimônio da Humanidade e Reserva da Biosfera, conforme reconhecimento da Organização das Nações Unidas para a Educação, a Ciência e a Cultura (UNESCO) (Boin *et al*., 2019; IBGE, 2019; Santos *et al*., 2021).

O clima do Pantanal é tropical, com uma estação seca de maio a setembro e uma estação chuvosa de outubro a abril. As temperaturas anuais variam de mínimas e máximas entre 25°C e 30°C, embora ocorram variações de temperatura. Durante a estação chuvosa, o nível dos rios e lagos aumenta, provocando inundações prolongadas, um fenômeno característico do Pantanal (Junk; Nunes da Cunha, 2016; Boin *et al*., 2019).

Os solos do Pantanal são predominantemente úmidos e apresentam uma grande variação. As principais classes de solo encontradas são os planossolos, plintossolos e espodossolos, mas também podem ser encontrados argissolos, cambissolos, chernossolos, gleissolos, latossolos, neossolos e vertissolos (Boin *et al*., 2019; Couto *et al*., 2023).

Figura 1 - Mapa do Brasil com a localização dos municípios e a delimitação dos biomas brasileiros.

Fonte: a própria autora

3.4. Taxonomia Bacteriana

A taxonomia é a ciência responsável por organizar e classificar os organismos vivos com base em suas características e relações evolutivas (Oren, 2010; Sentausa; Fournier, 2013; Helene; Klepa; Hungria, 2022; Jiao *et al*., 2024). Abrangendo três áreas inter-relacionadas: classificação; nomenclatura; e identificação. A classificação organiza os organismos em grupos taxonômicos hierárquicos; a nomenclatura atribui nomes a esses grupos taxonômicos, seguindo as regras estabelecidas pelo Código Internacional de Nomenclatura de Procariotos (*Internacional Code of Nomenclature of Prokaryotes* - ICNP);

e a identificação consiste em determinar a qual grupo taxonômico uma estirpe pertence ou se representa um novo táxon (Oren, 2010; Sentausa; Fournier, 2013; Helene; Klepa; Hungria, 2022).

O sistema binomial, proposto por Carl Linnaeus atribui a cada organismo um nome único composto de duas partes, sempre em latim (bi = dois, nomial = nome). A primeira refere-se ao gênero, que agrupa espécies intimamente relacionadas, com a primeira letra sempre escrita em formatação maiúscula; enquanto a segunda, chamada de epíteto específico, diferencia uma espécie das demais dentro do mesmo gênero, e não é escrita com letra maiúscula (Oren, 2010; Briški; Vuković Domanovac, 2017; Lunn; Winder; Shaw, 2023).

Inicialmente, o sistema de classificação de microrganismos baseava-se apenas em características fenotípicas, como a morfologia das colônias, coloração, tempo de crescimento e potencial patogênico (Schleifer, 2009; Briški; Vuković Domanovac, 2017; Lunn; Winder; Shaw, 2023). No entanto, essa abordagem apresenta limitações, pois algumas características fenotípicas, podem variar dentro de uma mesma espécie ou se repetir entre espécies diferentes. Essas variações podem ocorrer por meio da transferência horizontal de genes, um processo no qual a informação genética é transferida em ilhas simbióticas ou plasmídeos, entre genomas de uma espécie para outra (Heuer; Smalla, 2007; Ormeño-Orrillo; Martínez-Romero, 2013; Sutcliffe, 2015; Franco-Duarte *et al.*, 2019; Okoń, 2024).

No entanto, mudanças na classificação bacteriana foram impulsionadas pelos avanços das técnicas moleculares, como o sequenciamento de DNA e genomas, que revolucionaram a taxonomia bacteriana (Das *et al.*, 2014; Riesco; Trujillo, 2024; Gaouar, 2024). Atualmente, essa ciência adota a abordagem polifásica, que integra informações fenotípicas, genotípicas e filogenéticas para inferir taxonomia bacteriana (Helene; Klepa; Hungria, 2022).

As características fenotípicas resultam da expressão dos genes, mas que podem ser influenciadas por fatores ambientais, como temperatura e pH (Moore *et al.*, 2010; Kämpfer; Glaeser, 2011; Narsing Rao; Li, 2024). Essas características podem ser avaliadas por meio de diversas abordagens, incluindo análises morfológicas, fisiológicas e bioquímicas, fornecendo dados sobre a morfologia das colônias e células, condições de crescimento em diferentes faixas de pH, temperatura, tolerância à salinidade e a diferentes antibióticos,

metabolização de compostos (como fontes de carbono e nitrogênio), atividade enzimática da urease, entre outras propriedades (Kämpfer; Glaeser, 2011; Suneja *et al*., 2017; Moura *et al*., 2020; Helene; Klepa; Hungria, 2022; Narsing Rao; Li, 2024).

As características genotípicas são determinadas pelo material genético, o genoma dos microrganismos. Estas características podem ser analisadas por diversas técnicas moleculares, como amplificação por PCR (*Polymerase Chain Reaction* - Reação em Cadeia da Polimerase) de vários genes, análise de perfis genéticos por BOX-PCR, sequenciamento do gene 16S RNAr, análise de MLSA com genes *housekeeping*, ANI, HDDd, e análise do conteúdo de G+C no DNA (Kämpfer; Glaeser, 2011; Suneja *et al*., 2017; Raina *et al*., 2019; Kumari; Rai, 2020; Helene; Klepa; Hungria, 2022). Por fim, a filogenia estuda as relações evolutivas entre os organismos, utilizando dados moleculares para inferir sua história evolutiva (Helene; Klepa; Hungria, 2022).

Focando em algumas análises genotípicas, o BOX-PCR se destaca como uma técnica bastante utilizada em estudos iniciais de caracterização genética devido à sua confiabilidade, rapidez, alto poder discriminatório e reprodutibilidade (Das *et al*., 2014; Wang, 2019b). O BOX-PCR é baseado na amplificação de sequências repetitivas de DNA, conhecidas como elementos BOX, que estão localizadas nas regiões intergênicas do genoma bacteriano. Esses elementos BOX são compostos por três subunidades, denominadas boxA, boxB e boxC, com comprimentos de 59, 45 e 50 nucleotídeos, respectivamente. A técnica de BOX-PCR envolve a amplificação desses elementos utilizando um único primer. Após a amplificação, os produtos gerados são analisados por eletroforese em gel de agarose, resultando em um perfil único de fragmentos para cada isolado e permitindo, assim, identificar diversidade dentro do grupo de estirpes em estudo (diversidade inter e intra específica), além de distinguir clones bacterianos (Das *et al*., 2014; Bilung *et al*., 2018; Gohil *et al*., 2019; Wang, 2019b).

O gene 16S RNAr, presente em todos os procariotos, se manteve altamente conservado ao longo do processo evolutivo, mas possui regiões variáveis suficientes para inferir relações filogenéticas entre microrganismos, sendo considerado um excelente marcador filogenético para identificação bacteriana em nível de gênero (Glaeser; Kämpfer, 2015; Gohil *et al*., 2019;

Helene; Klepa; Hungria, 2022; Bartoš; Chmel; Swierczková, 2024). A pequena subunidade ribossômica procariótica possui uma molécula de RNA com aproximadamente 1.500 pares de bases (pb), o que facilita sua amplificação por PCR utilizando *primers* universais específicos direcionados às regiões conservadas na molécula, que posteriormente podem ser sequenciadas e analisadas. Embora o sequenciamento do gene 16S RNAr seja de grande importância devido à sua utilidade e confiabilidade, ele apresenta limitações. Isso ocorre porque sua sequência conservada normalmente não consegue distinguir espécies intimamente relacionadas (Gohil *et al*., 2019; Hugenholtz *et al*., 2021; Helene; Klepa; Hungria, 2022; Bartoš; Chmel; Swierczková, 2024).

Consequentemente, uma abordagem alternativa para superar as limitações do sequenciamento do gene 16S RNAr é o MLSA, que integra informações evolutivas concatenadas de múltiplos genes *housekeeping* (Gevers *et al*., 2005; Azevedo *et al*., 2015; Helene; Klepa; Hungria, 2022). Esses genes codificadores de proteínas, responsáveis por papéis essenciais no metabolismo celular são conservados, mas apresentam taxas de evolução mais rápidas que o gene 16S RNAr, permitindo uma análise mais detalhada e precisa das relações evolutivas, contribuindo para a identificação em nível de espécie (Gevers *et al*., 2005; Glaeser; Kämpfer, 2015; López-Hermoso *et al*., 2017; Helene; Klepa; Hungria, 2022). O MLSA analisa genes *housekeeping* que devem estar presentes e distribuídos pelo genoma de todos os organismos em estudo, em uma única cópia, além de apresentarem um tamanho suficiente para a análise (Gevers *et al*., 2005; De vos *et al*., 2017; Helene; Klepa; Hungria, 2022). Alguns dos genes mais utilizados no MLSA são a subunidade *β* da ATP sintase (*atpD*), a proteína chaperona (*dnaK*), a glutamina sintetase II (*glnII*), a subunidade *β* da DNA girase (*gyrB*), a recombinase A (*recA*) e a subunidade *β* da RNA polimerase (*rpoB*) (Das *et al*., 2014; Helene; Klepa; Hungria, 2022).

Com o avanço do sequenciamento genômico, diversas ferramentas de bioinformática passaram a ser utilizadas para estimar a relação genética entre microrganismos intimamente relacionadas. Essas técnicas calculam os índices de similaridade entre duas sequências genômicas *in silico,* como o *Overall Genome Relatedness Index* - OGRI. Dentre as abordagens que calculam OGRIs, destacam-se, a técnica de ANI e o HDDd, ferramentas comuns para comparar genomas, permitindo sua alocação nos respectivos grupos taxonômicos ou,

ainda, a descrição de novos táxons (Chun; Rainey, 2014; Helene; Klepa; Hungria, 2022; Narsing Rao; Thamchaipenet, 2024). O ANI mede a identidade genética, analisando as sequências nucleotídicas das regiões codificantes por meio de comparação pareada, utilizando 95-96% como valor de corte. Já o HDDd calcula a similaridade geral entre o genoma de uma estirpe a ser identificada e o genoma de uma estirpe tipo, integrando a análise de similaridade da sequência do 16S RNAr, utilizando 70% como valor de corte (Chun *et al*., 2018; Cortimiglia *et al*., 2024).

Conforme Das e colaboradores (2014), a adoção da abordagem polifásica, que integra informações fenotípicas, genotípicas e filogenéticas é essencial para compreender melhor a diversidade microbiana. No caso dos rizóbios presentes nos solos brasileiros, esse aprofundamento permite entender como esses microrganismos se adaptam às condições específicas de cada bioma, proporcionando uma visão mais detalhada de suas interações e capacidades.

3.5. Diversidade de Rizóbios em Solos de Biomas Brasileiros

Até o momento da finalização desta tese, 36 espécies de rizóbios foram descritas provenientes de solos de biomas brasileiros, incluindo algumas com nomes ainda não validados, conforme especificado na Tabela 1. Essas espécies estão distribuídas nos gêneros *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microvirga*, *Paraburkholderia*, *Rhizobium* e *Trinickia* (Parte *et al*., 2020).

Dessas, 20 espécies de rizóbios foram isoladas no bioma Mata Atlântica, evidenciando a riqueza microbiana dessa região. Entre elas, destaca-se o primeiro rizóbio descrito a partir de solos brasileiros, *Azorhizobium doebereinerae* (estirpe UFLA1-100^{T}), isolado de nódulos radiculares de *Sesbania virgata*, em Seropédica, Rio de Janeiro (de Souza Moreira *et al*., 2006). Outras espécies incluem “*Bradyrhizobium uaiense*” UFLA03-164^{T}, isolado de nódulos efetivos de feijão-caupi (*Vigna unguiculata*), em Poços de Caldas, Minas Gerais (Cabral Michel *et al*., 2020a); *Mesorhizobium atlanticum* CNPSo 3140^{T}, isolado de nódulos de *Phaseolus vulgaris*, também em Seropédica, Rio de Janeiro (Helene *et al*., 2019); *Paraburkholderia guartelaensis* CNPSo 3008^{T}, isolado de nódulos de *Mimosa gymnas*, em Tibagi, Paraná (Paulitsch *et al*.,

2019b); e “*Rhizobium aureum*” estirpe CNPSo 3968[T], isolado de nódulos de *P. vulgaris*, em Dourados, Mato Grosso do Sul (Moura *et al*., 2023). As 15 outras espécies do bioma estão descritas na Tabela 1.

Em um estudo de revisão sobre a diversidade da Mata Atlântica, Peres *et al*. (2020) destacaram que as áreas topograficamente complexas dos estados de São Paulo e Rio de Janeiro apresentam uma maior riqueza de espécies. Das 20 espécies de rizóbios identificadas no bioma, 10 foram isoladas no Rio de Janeiro. As demais espécies foram encontradas nos estados do Paraná (quatro espécies), Mato Grosso do Sul (três espécies), Minas Gerais (duas espécies) e São Paulo (uma espécie). Além disso, Peres *et al*. (2020) enfatizaram que a diversidade de condições ambientais da Mata Atlântica, com seus diferentes nichos ecológicos, contribui para a diversificação biológica em outras regiões onde o bioma está presente, o que reflete na distribuição geográfica das espécies descritas.

Outro aspecto relevante é que algumas dessas espécies foram isoladas em áreas de transição entre a Mata Atlântica e o Cerrado, conhecidas como ecótonos. Nessas áreas, as condições ecológicas de ambos os biomas podem influenciar a composição microbiana. Por exemplo, a espécie *Bradyrhizobium tropiciagri* foi isolada em Campinas, São Paulo, município onde 97% do território é inserido no bioma Mata Atlântica e 3% no Cerrado. “*Rhizobium aureum*”, isolada em Dourados, Mato Grosso do Sul, ocorre em uma região onde 54% do seu território é inserido na Mata Atlântica e 46% no Cerrado. Já *Paraburkholderia guartelaensis*, isolada em Tibagi, Paraná, está presente em um município com 76% do território na Mata Atlântica e 24% no Cerrado (Infosanbas, 2025).

Além da diversidade de rizóbios, a variedade de leguminosas também é notável; os 20 rizóbios foram isolados de 11 diferentes espécies de leguminosas, *Centrosema pubescens, Mimosa caesalpiniifolia*, *M. gymnas*, *M. scabrella*, *M. pudica*, *Neonotonia wightii*, *P. vulgaris*, *Piptadenia gonoacantha*, *S. virgata, Stylosanthes guianensis* e *V. unguiculata*.

Ressalta-se que o termo “rizóbio” engloba todas as bactérias simbióticas associadas a nódulos de plantas leguminosas, ou seja, bactérias capazes de induzir a formação de nódulos em raízes e/ou caules das hospedeiras (Wang, 2019a). Neste contexto, “*Bradyrhizobium sacchari*” (estirpe BR 10280[T]), isolado de raízes de cana-de-açúcar, em Seropédica, Rio de Janeiro, é classificado

como rizóbio, pois, conforme demonstrado por De Matos *et al*., (2017), a espécie apresentou capacidade de nodular efetivamente as leguminosas: *Cajanus cajan, Macroptilium atropurpureum* e *V*. *unguiculata*.

Além disso, pode-se considerar, também, todos os rizóbios isolados de solos brasileiros, incluindo aqueles que perderam a capacidade de nodulação e fixação (por exemplo, devido à perda dos genes *nodC* e *nifH*). A inclusão desses microrganismos se deve ao fato de terem sido originalmente isolados de nódulos efetivos, mesmo que atualmente não sejam capazes de reinfectar a plantas hospedeiras ou demais leguminosas.

No bioma Amazônia, seis espécies de rizóbios foram descritas, "*Bradyrhizobium campsiandrae*" UFLA 01-1174^{T}, isolado de nódulos radiculares de *Campsiandra laurilifolia* Benth, em Manacapuru, Amazonas (Cabral Michel *et al*., 2020b); "*Bradyrhizobium centrolobii*" BR 10245^{T} e "*Bradyrhizobium macuxiense*" BR 10303^{T}, isolados de nódulos de *Centrolobium paraense*, em Bonfim, Roraima (Cabral Michel *et al*., 2017); *Bradyrhizobium ingae* BR 10250^{T}, isolado de nódulos de *Inga laurina* (Sw.) Willd, em Boa Vista, Roraima (da Silva *et al*., 2014); *Bradyrhizobium manauense* BR 3351^{T}, isolado de nódulos de *V*. *unguiculata*, no Amazonas (Silva *et al*., 2014); e *Bradyrhizobium neotropicale* BR 10247^{T}, isolado de nódulos de *C*. *paraense*, em Mucajaí, Roraima (Zilli *et al*., 2014) (Tabela 1).

Guayasamin *et al*. (2024) ressaltam que a Amazônia, com suas biotas diversas e rica ecologia, favorece processos de diversificação, coexistência e coevolução de espécies, culminando em um extraordinário acúmulo de biodiversidade. Ainda assim, a diversidade microbiana amazônica permanece pouco conhecida. Das seis espécies de rizóbios identificadas no bioma, quatro foram isoladas no estado de Roraima e duas no Amazonas, ambos localizados na região Norte do Brasil. É importante mencionar que o bioma Amazônia se estende também por partes das regiões Centro-Oeste e Nordeste.

Além disso, todas as seis espécies descritas pertencem à classe Alfaproteobacteria, ao gênero *Bradyrhizobium*, e foram isoladas de quatro diferentes espécies de leguminosas, *Campsiandra laurilifolia* Benth, *Centrolobium paraense*, *Inga laurina*, e *V. unguiculata*. Esses dados destacam a alta diversidade vegetal da Amazônia, evidenciada mesmo pelo pequeno número de espécies bacterianas identificadas até o momento.

No Cerrado, quatro espécies de rizóbios foram descritas, *Rhizobium altiplani* BR 10423[T], isolado de nódulos eficientes de *M. pudica*, em Brasília, Distrito Federal (Baraúna *et al*., 2016); "*Rhizobium cerradonense*" CNPSo 3464[T], isolado de nódulos radiculares de *P. vulgaris*, em Bataguassu, Mato Grosso do Sul (Moura *et al*., 2023); *Burkholderia diazotrophica* JPY461[T], posteriormente reclassificado como *Paraburkholderia diazotrophica*, isolado de nódulos de *Mimosa candolleina*, na Chapada dos Veadeiros, Goiás (Sheu *et al*., 2013; Sawana *et al*., 2014); e *Paraburkholderia youngii* JPY169[T], isolado de nódulos de *Mimosa xanthocentra*, na Chapada dos Guimarães, no Mato Grosso (Bontemps *et al*., 2009; Mavima *et al*., 2021) (Tabela 1).

O Cerrado, localizado na região central do Brasil, é reconhecido por sua riqueza em fauna e flora. Compartilha áreas de transição com biomas adjacentes, como a Amazônia, a Mata Atlântica, o Pantanal e a Caatinga (Catão *et al*., 2014; Paulitsch; dos Reis Jr; Hungria, 2021; de Oliveira *et al*., 2023). Cerca de 70% do Cerrado é composto por vegetação típica de savana, enquanto as áreas de transições apresentam características de outros tipos de vegetação, como florestas, áreas úmidas e matagais semiáridos (Catão *et al*., 2014; Colli; Vieira; Dianese, 2020; de Oliveira *et al*., 2023). Essa integração de paisagens confere ao bioma uma biodiversidade rica e única, combinando espécies características do Cerrado com outras que coexistem devido à proximidade com os biomas vizinhos, além de incluir espécies endêmicas das áreas de transição (Colli; Vieira; Dianese, 2020; Ortiz-Colin; Hulshof, 2024).

Apesar da impressionante riqueza biológica, os estudos voltados para a diversidade microbiana no Cerrado, especialmente nas áreas de transição, ainda são limitados. Até o momento da redação desta tese foram descritas apenas quatro espécies de rizóbios, das quais somente "*Rhizobium centroccidentale*" e "*Rhizobium cerradonense*" foram isoladas em zonas ecotonais. Segundo o Infosanbas - Informações do Saneamento Básico (2025), o município de Rio Brilhante, onde foi isolado "*R. centroccidentale*", tem 60% do seu território inserido no bioma Cerrado e 40% na Mata Atlântica. De forma semelhante, o município de Bataguassu possui 69% de sua área no Cerrado e 31% na Mata Atlântica.

Ortiz-Colin e Hulshof (2024) descrevem os ecótonos como sistemas hiperdiversos e dinâmicos, que controlam o fluxo de energia e a movimentação

de organismos entre os ecossistemas vizinhos. A localização desses ecótonos é determinada pelos limites fisiológicos das espécies, o que pode resultar em um alta diversidade de rizóbios, uma vez que proporcionam condições ambientais variadas, favorecendo a coexistência de diferentes bactérias adaptadas a distintos nichos ecológicos. Além disso, os ecótonos apresentam atributos naturais únicos, desempenhando um papel essencial na conservação da biodiversidade e na compreensão dos processos ecológicos que conectam diferentes paisagens (Souza *et al*., 2020).

Além disso, estudos prévios de Dos Reis Jr. *et al*. (2010); Simon *et al*. (2011); Baraúna *et al*. (2016); e Soares Neto *et al*. (2022) destacam que as maiores concentrações de diversidade e endemismo de espécies do gênero *Mimosa* estão localizadas no Brasil central. Três das quatro espécies descritas de rizóbios foram isoladas de diferentes espécies de *Mimosa*: *M*. *candolei*, *M*. *pudica* e *M*. *xanthocentra*. É interessante ressaltar que, no Brasil, esse gênero vegetal é frequentemente associado a rizóbios do gênero *Paraburkholderia*.

Na Caatinga, duas espécies de rizóbios foram descritas, *Microvirga vignae* BR 3299^{T}, isolado de nódulos radiculares de *V*. *unguiculata*, em Canindé de São Francisco, Sergipe (Radl *et al*., 2014); e *Burkholderia symbiotica* JPY-345^{T}, posteriormente reclassificado como *Trinickia symbiotica*, isolado de nódulos efetivos de *Mimosa cordistipula*, no Morro do Chapéu, Bahia (Sheu *et al*., 2012; Estrada-de Los Santos *et al*., 2018) (Tabela 1).

O termo Caatinga refere-se a uma vasta área geográfica, caracterizada por diferentes tipos de vegetação, adaptados ao clima tropical seco. Apesar de ser uma das regiões semiáridas tropicais mais populosas e biologicamente diversas do mundo, o bioma ainda é subvalorizado (Santos *et al*., 2014; Lacerda Júnior *et al*., 2017; de Araujo *et al*., 2022; Souza; Córdula; Cavalcanti, 2024).

Vigna unguiculata é uma leguminosa promíscua, adaptada ao clima semiárido, e capaz de formar associações simbióticas com diversos gêneros de rizóbios, como *Bradyrhizobium*, *Ensifer*, *Microviga*, *Rhizobium* e *Paraburkholderia* (Castro *et al*., 2017; Sena *et al*., 2020; Barros *et al*., 2021; Moura *et al*., 2025). Por outro lado, *M*. *cordistipula*, endêmica da Caatinga, destaca-se pela adaptação às condições edafoclimáticas do bioma (Dos Reis Jr *et al*., 2010; Sheu *et al*., 2012; Estrada-de Los Santos *et al*., 2018), evidenciando a necessidade de mais estudos para explorar a diversidade de rizóbios na região,

principalmente em associação com *M. cordistipula* e outras plantas nativas e endêmicas adaptadas ao bioma.

No Pantanal, foi descrita uma espécie de rizóbio, da classe alfaproteobacteria, "*Rhizobium pantanalense*" CNPSo 4039^{T}, isolado de nódulos de *P*. *vulgaris* em Aquidauana, no Mato Grosso do Sul (Moura *et al*., 2023) (Tabela 1). O município, que possui 79% de seu território no bioma Pantanal e 21% no Cerrado (Infosanbas, 2025), exemplifica as zonas ecotonais características do estado, que podem favorecer interações únicas entre rizóbios e leguminosas adaptadas a diferentes condições ambientais.

Considerado o bioma mais preservado do Brasil, o Pantanal é conhecido por sua notável biodiversidade e ciclos sazonais de cheias e secas (Guerreiro *et al*., 2019; Couto *et al*., 2023). O Pantanal é influenciado diretamente pela interação com outros três biomas brasileiros, Amazônia, Cerrado e Mata Atlântica; além do *Gran Chaco* boliviano e paraguaio (Boin *et al*., 2019). Apesar da sua importância ecológica, os estudos sobre a diversidade microbiana no Pantanal ainda são escassos.

A ocorrência de "*Rhizobium pantanalense*" em áreas de transição ilustra a adaptabilidade de rizóbios às condições edafoclimáticas do bioma. Além disso, *P*. *vulgaris* é reconhecida como uma leguminosa promíscua, capaz de se estabelecer simbiose com uma grande variedade de rizóbios, e crescer sob diferentes condições ambientais (Shamseldin; Velázquez, 2020). Essas características fazem com que a utilização de *P*. *vulgaris* como planta isca em estudos no Pantanal possa auxiliar na identificação de novas espécies bacterianas, ampliando o conhecimento sobre a diversidade microbiana do bioma.

Por fim, vale destacar que para "*Bradyrhizobium forestalis*" INPA54B^{T}, isolada de nódulos radiculares de *Inga* sp. em floresta nativa da Amazônia (Martins da Costa *et al*., 2018); *Burkholderia nodosa* Br3437^{T}, posteriormente reclassificada como *Paraburkholderia nodosa*, isolada de nódulos efetivos de *Mimosa scabrella*, em Curitiba, Paraná, (Chen *et al*., 2007; Sawana *et al*., 2014); e *Burkholderia sabiae* Br3407^{T}, reclassificada como *Paraburkholderia sabiae*, isolada de nódulos efetivos de *Mimosa caesalpiniifolia*, em Seropédica, no Rio de Janeiro (Chen *et al*., 2008; Sawana *et al*., 2014), as informações sobre os

locais de isolamento foram de acordo com o AleloMicro da Embrapa (https://am.cenargen.embrapa.br/amconsulta/localizacao?id=3281).

Bradyrhizobium mercantei SEMIA 6399^T, isolada de nódulos efetivos de *Deguelia costata* (Helene *et al*., 2017), contém apenas a indicação de ter sido isolada da região Sudeste; abrangendo os estados de Espírito Santo, Minas Gerais, Rio de Janeiro e São Paulo, mas sem informações mais precisas sobre o local de coleta. Quanto a *Rhizobium leucaenae* CFN 299^T, isolada de nódulos efetivos de *P*. *vulgaris* (Ribeiro *et al*., 2012) não há informações detalhadas sobre o bioma, cidade ou região de isolamento, sendo registrada apenas como provenientes do Brasil.

Concluindo, até março de 2025, há 36 espécies de rizóbios descritas, isoladas de solos da Amazônia (6), Caatinga (2), Cerrado (4), Mata Atlântica (20), e Pantanal (1), e (3) não possuem informações sobre o bioma. Esses biomas se mostram ricos não apenas em biodiversidade de flora e fauna, mas também de microrganismos, evidenciando a complexidade e a adaptação dos rizóbios aos diferentes ambientes. Ainda, não há relatos de espécies de rizóbios provenientes do bioma Pampa brasileiro, o que destaca uma lacuna importante na pesquisa. Vale ressaltar que as 36 espécies descritas foram isoladas de solos de biomas brasileiros, com nomes válidos e não validamente publicados na nomenclatura (LPSN), independentemente de sua capacidade de fixar nitrogênio.

Embora existam inúmeros estudos que avaliam e caracterizam a diversidade bacteriana no Brasil (como de Grange; Hungria, 2004; Cardoso; Hungria; Andrade, 2012; Azarias Guimarães *et al*., 2012; Azarias Guimarães *et al*., 2015; Baraúna *et al*., 2014; Granada *et al*., 2014; Dall'Agnol *et al*., 2017; dos Santos *et al*., 2017; Rodrigues *et al*., 2018; Paulitsch *et al*., 2019a; De Oliveira *et al*., 2019; Chibeba *et al*., 2020; Banasiewicz *et al*., 2021; Moura *et al*., 2025), muitas estirpes são classificadas apenas em nível de gênero ou apresentadas como intimamente relacionadas às espécies já descritas. Esses dados reforçam a necessidade de mais estudos que explorem a verdadeira diversidade de rizóbios nos biomas brasileiros. O entendimento da diversidade e a identificação de novos rizóbios podem beneficiar tanto a agricultura como a conservação ambiental, ao promover práticas agrícolas mais sustentáveis e adequadas a diferentes ecossistemas.

Finalmente, cabe salientar um ponto muito importante para justificar porque algumas espécies de rizóbios não foram ainda validadas e encontram-se “em aspas” e porque, com certeza, outras não foram descritas. De acordo com as regras do *International Committee on Systematics of Prokaryotes, Subcommittee on the taxonomy of Rhizobia and Agrobacteria,* as estirpes tipo têm que estar plenamente disponíveis para qualquer pessoa em pelo menos duas coleções de culturas internacionais reconhecida. Contudo, de acordo com a legislação brasileira vigente, as estirpes podem ser disponibilizadas, mas com a assinatura de um MTA (*Material Transfer Agreement*) (artigo 12 da Lei 13.123/2015), o que não é aceito pelo subcomitê (Mousavi; Young, 2024). Esforços vêm sendo feitos nesse sentido, mas o problema perdura, impedindo a descrição de novas espécies de rizóbios da biodiversidade brasileira pela comunidade científica.

Tabela 1 - Espécies de rizóbios isoladas de biomas brasileiros, incluindo espécies não validadas de acordo com o site LPSN (Parte *et al*., 2020) até março de 2025.

Espécies	Fonte de isolamento	Planta hospedeira	Região	Estirpe tipo	Referência
α-Proteobacteria					
Bioma Mata Atlântica					
Azorhizobium doebereinerae	nódulos efetivos	*Sesbania virgata*	Seropédica, Rio de Janeiro	UFLA1-100^T	de Souza Moreira *et al.*, 2006
“Bradyrhizobium brasilense”	nódulos efetivos	*Vigna unguiculata*	Lavras, Minas Gerais	UFLA03-321^T	Martins da Costa *et al.*, 2017
"Bradyrhizobium sacchari"	raízes	cana-de-açúcar	Seropédica, Rio de Janeiro	BR 10280^T	De Matos *et al.*, 2017
Bradyrhizobium stylosanthis	nódulos efetivos	*Stylosanthes guianensis*	Itaguaí, Rio de Janeiro	BR 446^T	Delamuta *et al.*, 2016
Bradyrhizobium tropiciagri	nódulos efetivos	*Neonotonia wightii*	Campinas, São Paulo	CNPSo 1112^T	Delamuta *et al.*, 2015
“Bradyrhizobium uaiense”	nódulos efetivos	*Vigna unguiculata*	Poços de Caldas, Minas Gerais	UFLA03-164^T	Cabral Michel *et al.*, 2020a
Bradyrhizobium viridifuturi	nódulos efetivos	*Centrosema pubescens*	Itaguaí, Rio de Janeiro	SEMIA 690^T	Helene *et al.*, 2015
Mesorhizobium atlanticum	nódulos efetivos	*Phaseolus vulgaris*	Seropédica, Rio de Janeiro	CNPSo 3140^T	Helene *et al.*, 2019
“Rhizobium atlanticum”	nódulos efetivos	*Phaseolus vulgaris*	Itaquiraí, Mato Grosso do Sul	CNPSo 3490^T	Moura *et al.*, 2023
“Rhizobium aureum”	nódulos efetivos	*Phaseolus vulgaris*	Dourados, Mato Grosso do Sul	CNPSo 3968^T	Moura *et al.*, 2023
“Rhizobium centroccidentale”	nódulos efetivos	*Phaseolus vulgaris*	Dourados, Mato Grosso do Sul	CNPSo 4062^T	Moura *et al.*, 2023
Rhizobium freirei	nódulos efetivos	*Phaseolus vulgaris*	Irati, Paraná	PRF 81^T	Dall'Agnol *et al.*, 2013
Rhizobium paranaense	nódulos efetivos	*Phaseolus vulgaris*	Pitanga, Paraná	PRF 35^T	Dall'Agnol *et al.*, 2014
Bioma Amazônia					
"Bradyrhizobium campsiandrae"	nódulos efetivos	*Campsiandra laurilifolia* Benth	Manacapuru, Amazonas	UFLA 01-1174^T	Cabral Michel *et al.*, 2020b

Espécies	Fonte de isolamento	Planta hospedeira	Região	Estirpe tipo	Referência
"Bradyrhizobium centrolobii"	nódulos efetivos	*Centrolobium paraense*	Bonfim, Roraima	BR 10245^T	Cabral Michel *et al.*,2017
Bradyrhizobium ingae	nódulos efetivos	*Inga laurina*	Boa Vista, Roraima	BR 10250^T	Da Silva *et al.*, 2014
"Bradyrhizobium macuxiense"	nódulos efetivos	*Centrolobium paraense*	Bonfim, Roraima	BR 10303^T	Cabral Michel *et al.*, 2017
Bradyrhizobium manausense	nódulos efetivos	*Vigna unguiculata*	Manaus, Amazonas	BR 3351^T	Silva *et al.*, 2014
Bradyrhizobium neotropicale	nódulos efetivos	*Centrolobium paraense*	Mucajaí, Roraima	BR 10247^T	Zilli *et al.*, 2014
Bioma Cerrado					
Rhizobium altiplani	nódulos efetivos	*Mimosa pudica*	Brasília, Distrito Federal	BR 10423^T	Baraúna *et al.*, 2016
“Rhizobium cerradonense”	nódulos efetivos	*Phaseolus vulgaris*	Bataguassu, Mato Grosso do Sul	CNPSo 3464^T	Moura *et al.*, 2023
Bioma Caatinga					
Microvirga vignae	nódulos efetivos	*Vigna unguiculata*	Canindé de São Francisco, Sergipe	BR 3299^T	Radl *et al.*, 2014
Bioma Pantanal					
“Rhizobium pantanalense”	nódulos efetivos	*Phaseolus vulgaris*	Aquidauana, Mato Grosso do Sul	CNPSo 4039^T	Moura *et al.*, 2023
β-Proteobacteria					
Bioma Mata Atlântica					
“Paraburkholderia atlantica”	nódulos efetivos	*Mimosa pudica*	Búzios, Rio de Janeiro	CNPSo 3155^T	Paulitsch *et al.*, 2020
Paraburkholderia franconis	nódulos efetivos	*Mimosa pudica*	Búzios, Rio de Janeiro	3157^T	Paulitsch *et al.*, 2020
Paraburkholderia guartelaensis	nódulos efetivos	*Mimosa gymnas*	Tibagi, Paraná	CNPSo 3008^T	Paulitsch *et al.*, 2019b
Paraburkholderia nodosa	nódulos efetivos	*Mimosa scabrella*	Curitiba, Paraná	Br3437^T	Chen *et al.*, 2007; Sawana *et al.*, 2014
Paraburkholderia piptadeniae	nódulos efetivos	*Piptadenia gonoacantha*	Cabo Frio, Rio de Janeiro	STM 7183^T	Bournaud *et al.*, 2017

Espécies	Fonte de isolamento	Planta hospedeira	Região	Estirpe tipo	Referência
Paraburkholderia ribeironis	nódulos efetivos	*Piptadenia gonoacantha*	Cabo Frio, Rio de Janeiro	STM 7296^T	Bournaud *et al.*, 2017
Paraburkholderia sabiae	nódulos efetivos	*Mimosa caesalpiniifolia*	Seropédica, Rio de Janeiro	Br3407^T	Chen *et al.*, 2008; Sawana *et al.*, 2014
Bioma Cerrado					
Paraburkholderia diazotrophica	nódulos efetivos	*Mimosa candollei*	Chapada dos Veadeiros, Goiás	JPY461^T	Sheu *et al.*, 2013; Sawana *et al.*, 2014
Paraburkholderia youngii	nódulos efetivos	*Mimosa xanthocentra*	Chapada dos Guimarães, Mato Grosso	JPY169T	Mavima *et al.*, 2021
Bioma Caatinga					
Trinickia symbiotica	nódulos efetivos	*Mimosa cordistipula*	Morro do Chapéu, Bahia	JPY-345^T	Sheu *et al.*, 2012; Estrada-de Los Santos *et al.*, 2018
Bioma não informado					
"Bradyrhizobium forestalis"	nódulos efetivos	*Inga* sp.	Local específico não disponível	INPA54B^T	Martins da Costa *et al.*, 2018
Bradyrhizobium mercantei	nódulos efetivos	*Deguelia costata*	Região Sudeste (local específico não disponível)	SEMIA 6399^T	Helene *et al.*, 2017
Rhizobium leucaenae	nódulos efetivos	*Phaseolus vulgaris*	Local específico não disponível	CFN 299^T	Ribeiro *et al.*, 2012

Fonte: Elaborado pela própria autora

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5. CAPÍTULO I

Desvendando a notável diversidade bacteriana capturada por nódulos de feijão-caupi (*Vigna unguiculata* (L.) Walp.) inoculado com solos de terras indígenas da Região Centro-Oeste do Brasil

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RESUMO

O feijão-caupi (*Vigna unguiculata* (L.) Walp) é reconhecido como uma leguminosa promíscua em sua relação simbiótica com rizóbios, capaz de formar associações com uma ampla gama de espécies bacterianas. Nosso estudo se concentrou em avaliar a diversidade de estirpes bacterianas presentes em nódulos de feijão-caupi quando inoculado com solos de seis terras indígenas do estado do Mato Grosso do Sul, Região Centro-Oeste do Brasil, compreendendo os biomas Cerrado e Pantanal, que são conhecidos por sua rica diversidade. Os perfis de DNA (BOX-PCR) de 89 estirpes indicaram grande diversidade genética, com 20 grupos e 23 estirpes ocupando posições únicas, e todas as estirpes agrupadas em um nível de similaridade final de apenas 25%. A caracterização adicional usando sequenciamento do gene 16S RNAr revelou uma gama diversificada de gêneros bacterianos associados aos nódulos de feijão-caupi. As estirpes foram classificadas em dez gêneros: *Agrobacterium* (47), *Ancylobacter* (2), *Burkholderia* (12), *Ensifer* (1), *Enterobacter* (1), *Mesorhizobium* (1), *Microbacterium* (1), *Paraburkholderia* (1), *Rhizobium* (22) e *Stenotrophomonas* (1), divididos em quatro classes diferentes. É interessante ressaltar que, apenas *Ensifer*, *Mesorhizobium*, *Rhizobium* e *Paraburkholderia* são classificados como rizóbios. A análise filogenética foi conduzida com base nas classes dos gêneros identificados e nas estirpes-tipo das espécies mais próximas. Nossas análises integradas, combinando abordagens fenotípicas, genotípicas e filogenéticas, destacaram a promiscuidade significativa do feijão-caupi na associação com uma gama diversificada de bactérias dentro dos nódulos, evidenciando os solos brasileiros como uma área de elevada diversidade bacteriana.

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SBM SOCIEDADE BRASILEIRA DE MICROBIOLOGIA

SOIL AND AGRICULTURE - RESEARCH PAPER

Unveiling remarkable bacterial diversity trapped by cowpea (*Vigna unguiculata*) nodules inoculated with soils from indigenous lands in Central-Western Brazil

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Abstract

Cowpea (*Vigna unguiculata*) is recognized as a promiscuous legume in its symbiotic relationships with rhizobia, capable of forming associations with a wide range of bacterial species. Our study focused on assessing the diversity of bacterial strains present in cowpea nodules when inoculated with soils from six indigenous lands of Mato Grosso do Sul state, Central-Western Brazil, comprising the Cerrado and the Pantanal biomes, which are known for their rich diversity. The DNA profiles (BOX-PCR) of 89 strains indicated great genetic diversity, with 20 groups and 23 strains occupying single positions, and all strains grouped at a final similarity level of only 25%. Further characterization using 16S rRNA gene sequencing revealed a diverse array of bacterial genera associated with the cowpea nodules. The strains (number in parenthesis) were classified into ten genera: *Agrobacterium* (47), *Ancylobacter* (2), *Burkholderia* (12), *Ensifer* (1), *Enterobacter* (1), *Mesorhizobium* (1), *Microbacterium* (1), *Paraburkholderia* (1), *Rhizobium* (22), and *Stenotrophomonas* (1), split into four different classes. Notably, only *Ensifer*, *Mesorhizobium*, *Rhizobium*, and *Paraburkholderia* are classified as rhizobia. Phylogenetic analysis was conducted based on the classes of the identified genera and the type strains of the closest species. Our integrated analyses, combining phenotypic, genotypic, and phylogenetic approaches, highlighted the significant promiscuity of cowpea in associating with a diverse array of bacteria within nodules, showcasing the Brazilian soils as a hotspot of bacterial diversity.

Keywords Biological nitrogen fixation · Rhizobia · Diversity · BOX-PCR · 16S rRNA

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Introduction

Brazil is a megadiverse country with enriched fauna, flora, and microbial diversity, although the latter is relatively unexplored [1]. A hotspot of this diversity lies in the state of Mato Grosso do Sul (MS), which contains three out of the six Brazilian biomes (Cerrado, Mata Atlântica, and Pantanal). Brazil also boasts a diverse population enriched in social and cultural diversity, mainly composed of European, African, Asian, and Indigenous populations [2–5]. According to the last demographic census in Brazil [6], Mato Grosso do Sul is the third largest state in the indigenous population, with about 116,400 people comprising 11 ethnicities [7].

Cowpea (*Vigna unguiculata* (L.) Walp.) is a legume of great economic and social importance due to its high nutritional value. It is an excellent source of protein with low-fat content [8, 9]. Human consumption includes dry and green seeds, green pods, and leaves, and the legume can also be used as feed, forage, hay, and silage for animals [9, 10].

As with many legume species, cowpea can establish symbiotic interactions with diazotrophic bacteria, collectively known as rhizobia, forming specialized structures on the roots and occasionally on the stems, called nodules, where the biological nitrogen fixation process takes place, contributing to plant nitrogen nutrition [11–13]. However, with the advancement of molecular tools applied to biodiversity studies, it has been increasingly shown that nodule occupants are not restricted to the association with rhizobia (alpha- and beta-rhizobia). Several other bacterial species occupy the nodules, whose role is still not well understood [14, 15]. Considering that a variety of microorganisms inhabiting the rhizosphere and plant tissues may promote plant growth through processes other than biological nitrogen fixation, including phytohormone synthesis and biocontrol of pests and diseases [15–17], we can hypothesize that they perform similar roles inside the nodules. In studies conducted with cowpea in Brazil, species isolated from nodules include both rhizobia (*Bradyrhizobium*, *Cupriavidus*, *Rhizobium*) and non-rhizobia (*Agrobacterium*, *Bacillus*, *Burkholderia*, *Chryseobacterium*, *Enterobacter*, *Sphingobacterium*, among others) [18–21].

Despite its great importance, microbial diversity in indigenous lands located in Brazilian reserves is practically unknown. Therefore, this study aimed to investigate the diversity of bacteria trapped in cowpea nodules inoculated with soils of six indigenous reserves of Mato Grosso do Sul, Central-Western Brazil. We employed a polyphasic approach, analyzing phenotypic, genotypic, and phylogenetic properties, and we report here an outstanding diversity.

Materials and methods

Bacterial isolation

Eighty-nine isolates from soils of six indigenous reserves of Mato Grosso do Sul, Central-Western Brazil (Table 1), comprising the Cerrado and Pantanal biomes, were previously obtained by Dr. Fabio Martins Mercante (1963–2016), at Embrapa Agropecuária Oeste (Dourados, Mato Grosso do Sul). Soil samples from these reserves were collected and used as a substrate for the cultivation of cowpea (*Vigna unguiculata* (L.) Walp), a legume used as a trapping host, as described before [22]. Nodules were collected, surface-sterilized, individually crushed in sterile water, and streaked into a modified yeast extract-mannitol (YM) culture medium, as described by Hungria et al. [23]. Whenever necessary, different colonies were streaked until single colonies were obtained, and pure cultures of each bacterial isolate were maintained in a modified-YM medium with agar (YMA)

Table 1 Municipalities and biomes from where soil samples were taken in the state of Mato Grosso do Sul and the isolates

Indigenous lands	Municipalities	Biomes	Identification of Isolates (CNPSo)
1. Água Branca	Nioaque (Nio)	Cerrado	3652/3660/3662/3666/3669/3670/3673/3677/3678/3681/3682/3683/3686/3687/3691/3694
2. Buriti	Dois Irmãos do Buriti (Dib)	Cerrado	3885/3887/3888/3889/3890/3891/3892/3893/3894/3895/3896/3897/3898/3899/3900/3901/3902/3903/3909/3910/3911/3912/3950/3951/3952/3954
3. Cachoeirinha	Miranda (Mir)	Cerrado	3785/3794/3795/3800/3814/3827/3839/3843/3857/3860/3874/3880/3940/3955
4. Lagoinha	Sidrolândia (Sdn)	Cerrado	3913/3916/3918/3919/3920/3921/3922/3923/3924/3925/3926/3927/3928
5. Lalima	Miranda (Mir)	Cerrado	3957/3958/3959/3960/3961/3964/3965
6. Limão Verde	Aquidauana (Ada)	Pantanal	3719/3721/3722/3732/3738/3740/3742/3744/3753/3755/3769/3777/3779

and Congo red as an indicator [23] at 4°C as culture sources, and for long-term storage were cryopreserved at −80°C and − 150°C in modified-YM with 30% glycerol (v/v) and also lyophilized [24]. For the analyses, the isolates were grown in modified-YMA with Congo red and incubated at 28°C for three days, or according to the growth rate of each isolate.

The isolates were deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil.

DNA extraction

The 89 isolates were inoculated in flasks containing approximately 10 mL of modified-YM medium and incubated at 28°C under shaking at 100 rpm for three days, or according to the growth rate of each strain. The genomic DNAs of the 89 isolates were extracted using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions, and DNA quality was verified by electrophoresis at 70 volts (V) for 30 minutes on agarose gels (1%), then stained with ethidium bromide and visualized under UV light.

BOX-PCR fingerprinting

To estimate the intraspecific diversity of the isolates, BOX-PCR fingerprinting fragments were obtained by PCR reactions using the BOX-A1R primer (5'-CTACGGCAAGGC GACGCTGACG-3') [25]. Each reaction contained 2.5 µL buffer 10x (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen®); 5.0 µL dNTPs (1,5 mM of each nucleotide); 1.5 µL $MgCl_2$ (50 mM) (Invitrogen®); 1.0 µL BOX-A1R primer (50 pmol/µL); 0.2 µL Taq DNA polymerase (5 U/µL) (Invitrogen®); 1.0 µL DNA (50 ng/µL), and sterile water to reach the final volume of 25 µL. The reactions were carried out in a ProFlex PCR System Thermocycler (Applied Biosystems), with an initial cycle of 95°C for 7 minutes; followed by 35 cycles of 94°C for 1 minute, 53°C for 1 minute, 65°C for 8 minutes, and a final cycle of 65°C for 16 minutes [26]. The amplified fragments were separated by electrophoresis on a 1.5% agarose gel at 120V for 5 hours, and a molecular marker (1kb Plus Ladder, Invitrogen®) was placed at both ends of each gel.

The fingerprinting profiles were used to construct a dendrogram of similarity using the Bionumerics software (Applied Mathematics, Kortrijk, Belgium, version 7.6.3), applying the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) algorithm [27], and the Jaccard coefficient [28] with 2% tolerance, considering a level of similarity of 70% as a cutoff to consider isolates as belonging to the same group [26].

Amplification and sequencing of 16S rRNA gene

The 16S rRNA gene of each of the 89 isolates was amplified using the pair of primers fD1 (5'-AGAGTTTGATCCTGG CTCAG-3') and rD1 (5'-CTTAAGGAGGTGATC CAGCC-3') [29]. Each reaction contained 5.0 µL buffer 10x (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen®); 3.0 µL dNTPs (1,5 mM of each nucleotide); 1.5 µL $MgCl_2$ (50 mM) (Invitrogen®); 1.5 µL each primer (10 pmol/µL); 0.2 µL Taq DNA polymerase (5 U/µL) (Invitrogen®); 2.0 µL DNA (from 10 to 20 ng/µL), and sterile water to reach the final volume of 50 µL. PCR cycles included an initial denaturation cycle of 95°C for 2 minutes; 30 cycles of denaturation at 94°C for 15 seconds and 93°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 2 minutes, and a final extension cycle of 72°C for 5 minutes [30, 31]. The PCR products were purified with the PureLink kit (Invitrogen®), following the manufacturer's recommendations.

For sequencing, the purified PCR products of the 16S rRNA gene were subjected to a new amplification. The reaction was prepared with 5.0 µL of purified PCR product, 1.5 µL of BigDye, 1.1 µL of each primer (3.2 pmol/µL), 3.0 µL of 5x buffer (BigDye), and 9.4 µL of sterile ultrapure water, totaling a volume of 20 µL per reaction distributed in 96-well plates [32]. The PCR reaction was carried out in a thermocycler following an initial cycle of 96ºC for 1 minute and 35 cycles of 96ºC for 15 seconds, 50ºC for 15 seconds, and 60ºC for 4 minutes. After amplification, the samples were purified again and each well received 2.0 µL of sterile ammonium acetate (7.5 M) and 65 µL of 99.5% ethanol at room temperature. The plate was sealed, homogenized, and subjected to centrifugation at 4000 rpm for 45 minutes [30]. The supernatant was discarded, and the plate was inverted on absorbent paper to dry. Subsequently, 150 µL of freshly prepared 70% ethanol was added; the plate was sealed again, homogenized, and centrifuged at 4000 rpm for 20 minutes. After discarding the supernatant, a new centrifugation was performed at 300 rpm for approximately 25 seconds to remove residues. The plate was incubated at 28ºC for approximately 30 minutes to dry the material and eliminate any possible odor.

After drying, the samples were denatured by resuspension in 10 µL of formamide, followed by heating in a thermocycler at 95ºC for 5 minutes and cooling on ice (thermal shock). Sequencing was performed on an ABI 3500XL Genetic Analyzer (Applied Biosystems), using the primers 362f (5'-CTCCTACGGGAGGGCAGCAGTGGGG-3') and 786r (5'-CGAAAGCGTGGGGAGCAAACAGG-3') [30].

Nucleotide sequences obtained were assembled and corrected manually using the Bionumerics software (version 7.6.3). To minimize the impact of sequence anomalies formed during PCR amplification, such as chimeras, defined as sequences containing DNA from multiple template origins

[33], the 16S rRNA sequences of the 89 strains were subjected to chimera analysis using the –uchime denovo algorithm implemented in the VSEARCH software (v2.21.1) [34]. All sequences were found to be chimera-free. The sequences were compared with sequences deposited in the Genbank database of the NCBI (National Center for Biotechnology Information) using the Basic Local Alignment Search Tool (Blastn tool) [35]. Specifically, the searches were performed with the 'highly similar sequences (megablast)' filter to identify the genus of each isolate. To summarize the results, the sequence with the highest percentage of identity for each isolate was included, as shown in Table 2. Sequences obtained in this study were deposited in the NCBI GenBank database, and the access numbers are shown in the phylogenetic trees and in Table 2.

Phylogenetic analysis

The isolates from this study were analyzed along with the type strains of the closest species of the genus, whose sequences were retrieved from the GenBank database. Multiple sequence alignment was obtained with MUSCLE [36] in the MEGA software (Molecular Evolutionary Genetics Analysis, version 7.0) [37]. Bayesian phylogenetic trees were constructed using MrBayes v3.2.7a [38] in the CIPRES portal [39], performing two runs simultaneously for random trees for 10 million generations each. Phylogenetic trees were edited with the iTOL tool [40]. *Acinetobacter baumannii* DSM30007^T, *Cupriavidus taiwanensis* LMG 19424^T, *Bradyrhizobium japonicum* USDA 6^T, and *Frankia asymbiotica* NRRL B-16386^T were used as outgroups. The evolutionary model used to construct the trees is described in the figure captions. For the determination of Nucleotide Identity (NI), sequences were aligned in the Bioedit Sequence Alignment Editor software (version 7.2.5) [41]. The heatmaps were created using the Seaborn [42] and Matplotlib libraries [43].

Morphophysiological characterization

The 89 isolates were grown on modified-YMA medium containing Congo red and pH ranging from 6.8 to 7.0 [23]. The isolates were also grown on modified-YMA medium with bromothymol blue used as an indicator to assess the acid/alkaline reactions. Growth was verified after two to four days of incubation at 28°C. Tests were performed in triplicate. The following morphological properties were evaluated: a) growth rate; b) colony diameter (mm); c) mucus production; d) colony shape; e) colony border; f) colony surface; g) colony elevation; h) consistency; i) optical details; j) acid or alkaline reaction; k) chromogenesis in media with bromothymol blue and Congo Red indicators [23, 44].

Results

BOX-fingerprinting

Fragments ranging from 300 to 3,000 bp (base pairs) were chosen to construct a dendrogram of similarity (Fig. 1). Using a 70% similarity threshold for groupings, as suggested by Kaschuk et al. [26], the dendrogram generated 43 distinct groups or single positions. Among these, 66 strains were distributed across 20 groups, while the remaining 23 occupied single positions. The overall grouping of all strains showed a final similarity level of only 25.07%. Abbreviations within parentheses in the dendrogram (Fig. 1) denote the municipalities where the respective strains originated (Fig. 1, Table 1), highlighting significant heterogeneity regarding strain groups and their geographical origins.

Each of the largest groups (4 and 8) comprised seven strains from the Cerrado biome. Group 3 included six strains from the Cerrado, while groups 10 and 29 contained five strains each from the Cerrado and Pantanal biomes. Other groups consisted of four (group 27), three (groups 5, 9, 22, and 23), and two strains (groups 1, 7, 13, 15, 17, 18, 19, 24, 28, 29, and 41), with strains isolated from both biomes. Additionally, four groups exhibited strains with 100% similarity, comprising strains CNPSo 3889 and 3897 (group 3), CNPSo 3921, 3922, 3927, and 3928 (group 8), CNPSo 3902 and 3903 (group 27), and CNPSo 3721 and 3961 (group 29).

16S rRNA gene analysis

First, the 16S rRNA gene sequences of the 89 strains were subjected to analysis using the Blastn tool comparing with 'highly similar sequences (megablast)' for genus-level identification, and the results are summarized in Table 2. The strains were classified into four classes, encompassing ten genera, indicating high diversity among the strains. Within the Alphaproteobacteria class, the identified genera included *Agrobacterium* (47 strains), *Rhizobium* (22), *Ancylobacter* (= *Starkeya*) (2), *Ensifer* (1), and *Mesorhizobium* (1). The Betaproteobacteria class comprised the genera *Burkholderia* (12) and *Paraburkholderia* (1). The Gammaproteobacteria class included the genera *Enterobacter* (1) and *Stenotrophomonas* (1), while the Actinobacteria class included the genus *Microbacterium* (1).

Phylogenetic trees were constructed based on the classes of the ten identified genera, resulting in four distinct phylogenetic trees.

The first phylogenetic tree was built with the 73 strains of the Alphaproteobacteria class and 74 type strains representing the genera. To distinguish between genera, representative colors were employed (Fig. 2). The first group, depicted in green color, comprised 40 strains identified as

Table 2 Summary of BLASTn results for the 89 16S rRNA gene sequences obtained in this study. The table displays the highest percentage of identity for each isolate, including the genus, query coverage, and accession number

Strains (CNPSo)	Genera	Query cover	Per. Ident	Acession
Class Alphaproteobacteria				
3652 (PP657162)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3660 (PP657163)	*Agrobacterium* sp.	100%	100.00%	OL347557.1
3662 (PP657164)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3666 (PP657165)	*Agrobacterium* sp.	100%	100.00%	PQ340219.1
3669 (PP657166)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3673 (PP657168)	*Agrobacterium* sp.	99%	100.00%	PP104400.1
3677 (PP657169)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3678 (PP657170)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3681 (PP657171)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3682 (PP657172)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3687 (PP657175)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3719 (PP657178)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3740 (PP657183)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3742 (PP657184)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3744 (PP657185)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3755 (PP657187)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3779 (PP657190)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3785 (PP657191)	*Agrobacterium* sp.	100%	99.91%	CP119417.1
3814 (PP657195)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3827 (PP657196)	*Agrobacterium* sp.	100%	100.00%	CP039922.1
3839 (PP657197)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3843 (PP657198)	*Agrobacterium* sp.	100%	100.00%	CP039922.1
3857 (PP657199)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3860 (PP657200)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3880 (PP657202)	*Agrobacterium* sp.	100%	100.00%	PP104400.1
3889 (PP657206)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3897 (PP657214)	*Agrobacterium* sp.	99%	100.00%	PP104400.1
3898 (PP657215)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3909 (PP657221)	*Agrobacterium* sp.	100%	100.00%	CP039922.1
3910 (PP657222)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3911 (PP657223)	*Agrobacterium* sp.	100%	99.91%	CP039922.1
3912 (PP657224)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3916 (PP657226)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3921 (PP657230)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3922 (PP657231)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3923 (PP657232)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3924 (PP657233)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3925 (PP657234)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3926 (PP657235)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3927 (PP657236)	*Agrobacterium* sp.	100%	99.91%	CP119417.1
3928 (PP657237)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3940 (PP657238)	*Agrobacterium* sp.	100%	99.91%	CP119417.1
3950 (PP657239)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3951 (PP657240)	*Agrobacterium* sp.	99%	100.00%	CP119417.1
3955 (PP657243)	*Agrobacterium* sp.	100%	100.00%	CP119417.1
3957 (PP657244)	*Agrobacterium* sp.	100%	99.90%	CP119417.1
3958 (PP657245)	*Agrobacterium* sp.	100%	100.00%	PQ380216.1
3670 (PP657167)	*Rhizobium* sp.	100%	100.00%	KF638354.1
3694 (PP657177)	*Rhizobium* sp.	99%	99.91%	LC483383.1
3722 (PP657180)	*Rhizobium* sp.	99%	100.00%	KY426377.1

Table 2 (continued)

Strains (CNPSo)	Genera	Query cover	Per. Ident	Acession
Class Alphaproteobacteria				
3732 (PP657181)	*Rhizobium* sp.	100%	100.00%	PP554517.1
3794 (PP657192)	*Rhizobium* sp.	100%	99.47%	KY064114.1
3795 (PP657193)	*Rhizobium* sp.	100%	100.00%	NR_179313.1
3888 (PP657205)	*Rhizobium* sp.	100%	99.91%	NR_179313.1
3890 (PP657207)	*Rhizobium* sp.	99%	100.00%	NR_179313.1
3891 (PP657208)	*Rhizobium* sp.	100%	99.91%	NR_179313.1
3893 (PP657210)	*Rhizobium* sp.	100%	99.91%	LC483383.1
3894 (PP657211)	*Rhizobium* sp.	100%	100.00%	NR_179313.1
3899 (PP657216)	*Rhizobium* sp.	100%	100.00%	LC483383.1
3900 (PP657217)	*Rhizobium* sp.	100%	99.91%	LC483383.1
3901 (PP657218)	*Rhizobium* sp.	100%	99.81%	MK249695.1
3902 (PP657219)	*Rhizobium* sp.	100%	100.00%	KY292478.1
3903 (PP657220)	*Rhizobium* sp.	100%	99.91%	LC483383.1
3918 (PP657227)	*Rhizobium* sp.	100%	100.00%	ON677772.1
3919 (PP657228)	*Rhizobium* sp.	100%	99.79%	MK249695.1
3920 (PP657229)	*Rhizobium* sp.	99%	98.56%	KF441696.1
3959 (PP657246)	*Rhizobium* sp.	100%	100.00%	GQ181135.1
3960 (PP657247)	*Rhizobium* sp.	100%	100.00%	GQ181135.1
3964 (PP657249)	*Rhizobium* sp.	99%	100.00%	MW960246.1
3887 (PP657204)	*Ancylobacter* sp.	100%	100.00%	CP002026.1
3896 (PP657213)	*Ancylobacter* sp.	100%	100.00%	JN606070.1
3874 (PP657201)	*Ensifer* sp.	100%	100.00%	KY992904.1
3954 (PP657242)	*Mesorhizobium* sp.	100%	100.00%	CP171975.1
Class Betaproteobacteria				
3686 (PP657174)	*Burkholderia sp.*	100%	99.91%	PP554516.1
3691 (PP657176)	*Burkholderia sp.*	100%	100.00%	EU418711.1
3721 (PP657179)	*Burkholderia sp.*	100%	100.00%	MW383543.1
3738 (PP657182)	*Burkholderia sp.*	99%	100.00%	PP554516.1
3753 (PP657186)	*Burkholderia sp.*	99%	100.00%	MT994999.1
3769 (PP657188)	*Burkholderia sp.*	100%	100.00%	PP554516.1
3885 (PP657203)	*Burkholderia sp.*	100%	100.00%	AP029053.1
3892 (PP657209)	*Burkholderia sp.*	100%	100.00%	CP045994.1
3895 (PP657212)	*Burkholderia sp.*	100%	99.91%	CP022210.1
3913 (PP657225)	*Burkholderia sp.*	100%	100.00%	MW383543.1
3952 (PP657241)	*Burkholderia sp.*	100%	100.00%	MW980774.1
3961 (PP657248)	*Burkholderia sp.*	100%	99.91%	MW383543.1
3777 (PP657189)	*Paraburkholderia sp.*	99%	99.38%	OK445525.1
Class Gammaproteobacteria				
3683 (PP657173)	*Enterobacter* sp.	99%	100.00%	KC951920.1
3800 (PP657194)	*Stenotrophomonas* sp.	100%	99.90%	MT649753.1
Class Actinobacteria				
3965 (PP657250)	*Microbacterium* sp.	100%	99.69%	HQ901776.1

Agrobacterium together with the species *Agrobacterium pusense* NRCPB10^T and *Agrobacterium salinitolerans* YIC 5082^T, with a statistical support of 100%, and sharing Nucleotide Identity (NI) ranging from 99.7 to 100% (Fig. S1). *Agrobacterium* sp. CNPSo 3958 occupied an isolated position, closely related to the species *Agrobacterium larrymoorei* ATCC 51759^T and *Agrobacterium cavarae* RZME10^T, with statistical support of 100%, and NI values of 99.7 and 100%, respectively. Additionally, six other strains of *Agrobacterium* showed the highest NI with the species *A. shirazense* OT33^T (100%). The second group, depicted in yellow color (Fig. 2), comprised strain CNPSo

3874, identified as *Ensifer*, alongside with *Ensifer sesbaniae* CCBAU 65729^{T} and *Ensifer adhaerens* NBRC 100388^{T}. This grouping showed 100% statistical support and exhibited NI of 99.6 and 99.7% with the two type strains, respectively (Fig. S3).

In the third group, represented by the color blue (Fig. 2), strain CNPSo 3722, identified as belonging to the genus *Rhizobium*, occupied an isolated position, grouping with four type strains of *Rhizobium*, sharing NI values ranging from 99.3 to 99.8% (Fig. S2) and 96% of statistical support. Four strains from this study (CNPSo 3732, 3959, 3960, and 3964) exhibited NI ranging from 99.8 to 100% with each other and were grouped with the species *R. tropici* CIAT 899^{T}, sharing 99.8% of NI and 90% of statistical support. Strain CNPSo 3670 occupied an isolated position, presenting higher NI values with *Rhizobium brockwellii* CC275e^{T} (99.6%) and *Rhizobium johnstonii* 3841^{T} (99.7%). Eleven other strains from this study were grouped with the type strain *Rhizobium dioscoreae* S-93^{T}, with 100% of statistical support, sharing NI values ranging from 99.7 to 100%. The strains CNPSo 3901, CNPSo 3918, and CNPSo 3919, identified as belonging to the genus *Rhizobium*, supported by 100% statistical support, and exhibiting 100% of NI among each other, shared 97.1% of NI with the closest species *Rhizobium phenanthrenilyticum* F11^{T} (Fig. S2). Strains CNPSo 3794 and CNPSo 3920 displayed 100% of NI with each other; however, they were were not grouped type strains in the phylogenetic tree, showing the highest NI value (97.8%) with *Rhizobium hedysari* 5- 1–2^{T,} .

In the fourth group, represented by the color pink (Fig. 2), the strain CNPSo 3954, classified as *Mesorhizobium*, occupied an isolated position. It shared NI of 98.5% with the species *Mesorhizobium huakuii* CCBAU 2609^{T}, *Mesorhizobium carmichaelinearum* ICMP 18942^{T}, *Mesorhizobium erdmanii* USDA 3471^{T}, *Mesorhizobium japonicum* MAFF303099^{T}, and *Mesorhizobium jarvisii* ATCC 33669^{T} (Fig. S3).

Continuing with the Alphaproteobacteria phylogenetic tree (Fig. 2), the fifth group, depicted in gray, included strains CNPSo 3887 and CNPSo 3896, identified as *Ancylobacter*. They share 98.6% of NI with each other, and from 97.8 to 99.5% with the species *Ancylobacter novellus* IAM 12100^{T} and *Ancylobacter koreensis* NBRC 100963^{T} (Fig. S4).

The second phylogenetic tree was constructed with 13 strains classified in the Betaproteobacteria class, in addition to 70 type strains (Fig. 3). The strain CNPSo 3777, identified as *Paraburkholderia*, occupied an isolated position, presenting a higher NI value of 98.8% with the type strain *Paraburkholderia panacihumi* DCY115^{T} (Fig. 3; Fig. S5). Strains CNPSo 3895 and CNPSo 3952, identified as *Burkholderia*, exhibited 100% NI (Fig. S6) with each other, and 100% with the closest species *Burkholderia gladioli* CFBP 2427^{T} with 99% of statistical support. Strains CNPSo 3913 and CNPSo 3961 presented 100% NI with each other and the highest NI value (99.8%) with the type strain *Burkholderia contaminans* LMG 23361^{T}. The strain CNPSo 3686 occupied an isolated position, presenting a higher NI value of 99.8% with *B. contaminans* LMG 23361^{T}. Seven other strains from this study identified as *Burkholderia* showing the highest NI with the species *B. contaminans* LMG 23361^{T}, sharing NI values ranging from 99.6 to 100% (Fig. 3).

In the Gammaproteobacteria phylogenetic tree (Fig. 4), the genera *Enterobacter* and *Stenotrophomonas* formed two distinct clades. Strain CNPSo 3683 of *Enterobacter* shared NI of 99.8% with *Enterobacter sichuanensis* WCHECL1597^{T}, *Enterobacter quasiroggenkampii* WCHECL1060^{T}, and *Enterobacter roggenkampii* DSM 16690^{T} (Fig. S7). Strain CNPSo 3800 of *Stenotrophomonas* clustered with the species *Stenotrophomonas geniculata* ATCC 19374^{T}, *Stenotrophomonas maltophilia* IAM 12423^{T}, and *Stenotrophomonas pavanii* ICB 89^{T}, sharing NI values of 99.3, 99.4 and 99. 2%, respectively (Fig. S8).

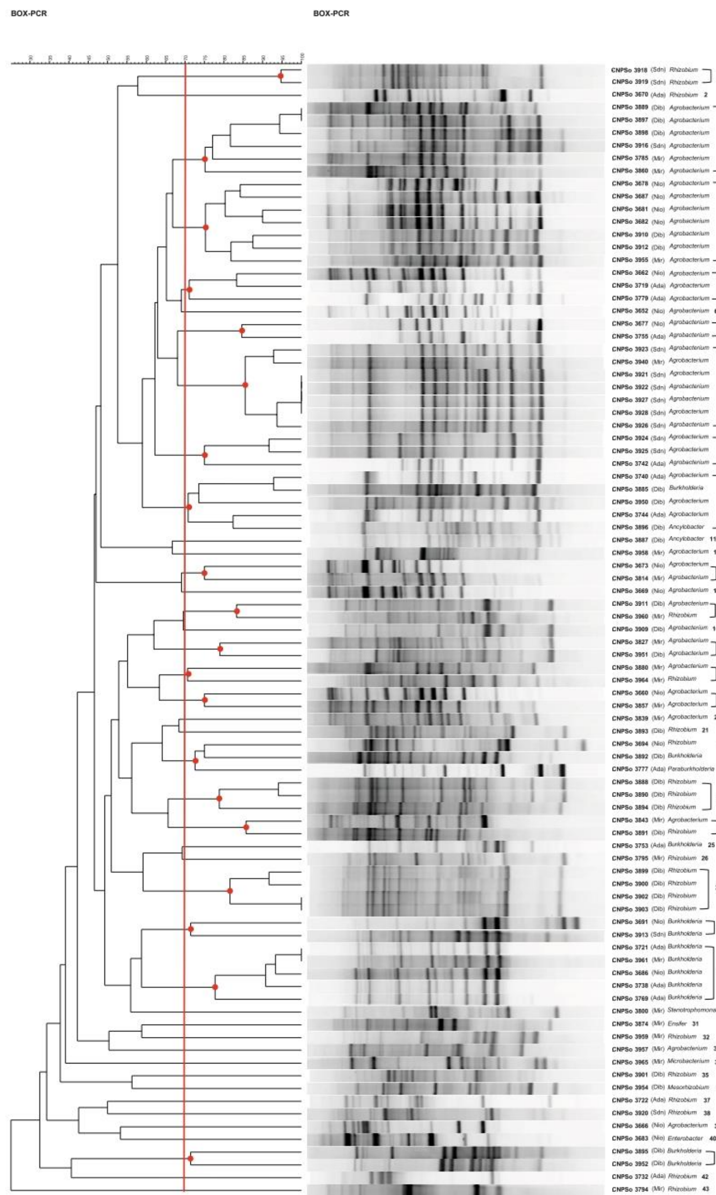
Finally, in the Actinobacteria phylogenetic tree (Fig. 5), strain CNPSo 3965 of *Microbacterium* clustered with *Microbacterium aquimaris* JCM 15625^{T} and *Microbacterium kyungheense* THG C26^{T}, sharing NI values of 98.9 and 99.6%, respectively. Moreover, the species *Microbacterium resistens* DMMZ 1710^{T}, *Microbacterium testaceum* DSM 20166^{T}, and *Microbacterium azadirachtae* AI-S262^{T} also exhibited high NI values with the strain, of 99.4%, 99.1% and 99.2%, respectively (Fig. S9).

Morphophysiological characterization

The results of the *in vitro* morphophysiological characterization performed with the 89 strains are listed in Supplementary Table S1 and Supplementary Figure S10. Among the 89 strains, 80 showed fast (three days), and nine intermediate (four days) growth rates. Regarding the acid/alkaline reaction in modified-YMA medium containing bromothymol blue as a pH indicator, 47 strains showed neutral, 35 acid, and seven alkaline reactions.

Regarding the chromogenesis of the colonies in modified-YMA medium with Congo red, colonies of 52 strains were pink, 19 red, and 18 white. In the presence of bromothymol blue as a pH indicator, 59 strains resulted in colonies with yellow color, and 30 presented blue color.

In the evaluation of the properties of border, surface, shape, and elevation of the colonies, all strains were similar, with smooth border and surface, circular colonies, and convex elevation, except for strains CNPSo 3683, CNPSo 3874, and CNPSo 3887, showing an irregular shape. The diameter of the colonies ranged from 1.2 to 3.2 mm, and


BOX-PCR
BOX-PCR
CNPSo 3918 (Sdn) Rhizobium
CNPSo 3919 (Sdn) Rhizobium
1
CNPSo 3670 (Ada) Rhizobium 2
CNPSo 3889 (Dib) Agrobacterium
CNPSo 3897 (Dib) Agrobacterium
CNPSo 3898 (Dib) Agrobacterium
CNPSo 3916 (Sdn) Agrobacterium
CNPSo 3785 (Mir) Agrobacterium
CNPSo 3860 (Mir) Agrobacterium
3
CNPSo 3678 (Nio) Agrobacterium
CNPSo 3687 (Nio) Agrobacterium
CNPSo 3681 (Nio) Agrobacterium
CNPSo 3682 (Nio) Agrobacterium
CNPSo 3910 (Dib) Agrobacterium
CNPSo 3912 (Dib) Agrobacterium
CNPSo 3955 (Mir) Agrobacterium
4
CNPSo 3662 (Nio) Agrobacterium
CNPSo 3719 (Ada) Agrobacterium
CNPSo 3779 (Ada) Agrobacterium
5
CNPSo 3652 (Nio) Agrobacterium 6
CNPSo 3677 (Nio) Agrobacterium
CNPSo 3755 (Ada) Agrobacterium
7
CNPSo 3923 (Sdn) Agrobacterium
CNPSo 3940 (Mir) Agrobacterium
CNPSo 3921 (Sdn) Agrobacterium
CNPSo 3922 (Sdn) Agrobacterium
CNPSo 3927 (Sdn) Agrobacterium
CNPSo 3928 (Sdn) Agrobacterium
CNPSo 3926 (Sdn) Agrobacterium
8
CNPSo 3924 (Sdn) Agrobacterium
CNPSo 3925 (Sdn) Agrobacterium
CNPSo 3742 (Ada) Agrobacterium
9
CNPSo 3740 (Ada) Agrobacterium
CNPSo 3885 (Dib) Burkholderia
CNPSo 3950 (Dib) Agrobacterium
CNPSo 3744 (Ada) Agrobacterium
CNPSo 3896 (Dib) Ancylobacter
10
CNPSo 3887 (Dib) Ancylobacter 11
CNPSo 3958 (Mir) Agrobacterium 12
CNPSo 3673 (Nio) Agrobacterium
CNPSo 3814 (Mir) Agrobacterium
13
CNPSo 3669 (Nio) Agrobacterium 14
CNPSo 3911 (Dib) Agrobacterium
CNPSo 3960 (Mir) Rhizobium
15
CNPSo 3909 (Dib) Agrobacterium 16
CNPSo 3827 (Mir) Agrobacterium
CNPSo 3951 (Dib) Agrobacterium
17
CNPSo 3880 (Mir) Agrobacterium
CNPSo 3964 (Mir) Rhizobium
18
CNPSo 3660 (Nio) Agrobacterium
CNPSo 3857 (Mir) Agrobacterium
19
CNPSo 3839 (Mir) Agrobacterium 20
CNPSo 3893 (Dib) Rhizobium 21
CNPSo 3694 (Nio) Rhizobium
CNPSo 3892 (Dib) Burkholderia
CNPSo 3777 (Ada) Paraburkholderia
22
CNPSo 3888 (Dib) Rhizobium
CNPSo 3890 (Dib) Rhizobium
CNPSo 3894 (Dib) Rhizobium
23
CNPSo 3843 (Mir) Agrobacterium
CNPSo 3891 (Dib) Rhizobium
24
CNPSo 3753 (Ada) Burkholderia 25
CNPSo 3795 (Mir) Rhizobium 26
CNPSo 3899 (Dib) Rhizobium
CNPSo 3900 (Dib) Rhizobium
CNPSo 3902 (Dib) Rhizobium
CNPSo 3903 (Dib) Rhizobium
27
CNPSo 3691 (Nio) Burkholderia
CNPSo 3913 (Sdn) Burkholderia
28
CNPSo 3721 (Ada) Burkholderia
CNPSo 3961 (Mir) Burkholderia
CNPSo 3686 (Nio) Burkholderia
CNPSo 3738 (Ada) Burkholderia
CNPSo 3769 (Ada) Burkholderia
29
CNPSo 3800 (Mir) Stenotrophomonas 30
CNPSo 3874 (Mir) Ensifer 31
CNPSo 3959 (Mir) Rhizobium 32
CNPSo 3957 (Mir) Agrobacterium 33
CNPSo 3965 (Mir) Microbacterium 34
CNPSo 3901 (Dib) Rhizobium 35
CNPSo 3954 (Dib) Mesorhizobium 36
CNPSo 3722 (Ada) Rhizobium 37
CNPSo 3920 (Sdn) Rhizobium 38
CNPSo 3666 (Nio) Agrobacterium 39
CNPSo 3683 (Nio) Enterobacter 40
CNPSo 3895 (Dib) Burkholderia
CNPSo 3952 (Dib) Burkholderia
41
CNPSo 3732 (Ada) Rhizobium 42
CNPSo 3794 (Mir) Rhizobium 43

◂**Fig. 1** Fingerprinting dendrogram of similarity based on the BOX-PCR profiles of the isolates of this study, using the UPGMA algorithm and the Jaccard coefficient with 2% tolerance (software Bionumerics v. 7.6.3). The abbreviations in parentheses indicate the municipalities in the state of Mato Grosso do Sul, Brazil, from which each isolate was obtained, according to Table 1: Nioaque (Nio), Dois Irmãos do Buriti (Dib), Miranda (Mir), Sidrolândia (Sdn), and Aquidauana (Ada)

the mucus production was considered low for 45 strains, and moderate for 44 strains. For the optical details, 47 strains were opaque and 42 translucid. The consistency of the growth mass was considered gummy for all strains tested, except for strains CNPSo 3683, CNPSo 3800, and CNPSo 3887, which showed aqueous consistency.

Discussion

Brazil stands out as a megadiverse nation, with Mato Grosso do Sul state emerging as a prominent hotspot of this biological richness. However, exploring soil microbial diversity in the country, especially within this state, remains relatively unknown. Previous investigations in Mato Grosso do Sul, focusing on rhizobial diversity using common bean (*Phaseolus vulgaris* L.) as a promiscuous trapping host, indicated high diversity [22, 45]. These two studies identified *Rhizobium* and *Agrobacterium* as primary genera inhabiting common bean nodules, alongside *Bradyrhizobium* and *Mesorhizobium*.

Similar to the common bean, cowpea is very interesting for the detection of bacterial diversity due to its promiscuous nature, enabling associations with a diverse array of bacterial species [18, 46]. Our investigation corroborates this perspective, highlighted when employing a 70% similarity cutoff in the analysis of BOX-PCR profiles, as proposed by Kaschuk et al. [26]. Notably, our analysis revealed remarkable diversity, with clustering occurring at a final level of similarity of only 25%. The exceptional microbial diversity observed was further confirmed with the 16S rRNA phylogenetic analysis.

For decades, nodules were primarily recognized as habitats for rhizobia. In our investigation, we not only confirmed the presence of classic nodulating genera known as alpha-rhizobia (*Ensifer*, *Mesorhizobium, Rhizobium*), and beta-rhizobia (*Paraburkholderia*), but also identified several non-rhizobia genera, including *Agrobacterium*, *Ancylobacter*, *Burkholderia*, *Enterobacter*, *Microbacterium*, and *Stenotrophomonas*. Indeed, an increasing number of studies report the presence of non-rhizobial endophytes coinhabiting the nodules [47–49]. These endophytes have been proposed to contribute to plant growth by several beneficial properties, including phosphate solubilization, synthesis of phytohormones and siderophores, protection against abiotic stresses such as salinity, drought, and biocontrol of pathogens [15, 50–53]. On the other hand, some endophytes can be merely considered opportunistic occupants of the nodule nitrogen-rich niche [54–56]. Since efficient next-generation sequencing strategies have become accessible, plant microbiome studies have been showing that nodules may present a wide diversity of bacterial occupants. For example, Bender et al. [15] reported that although *Bradyrhizobium* and *Rhizobium* were predominant in the nodules of soybean (*Glycine max* L. Merr.) and common bean, respectively, there was a large diversity of other bacterial genera. Mayhood and Mirza [57] detected a prevalence of *Bradyrhizobium japonicum* in root nodules of soybean; however, other non-rhizobial such as *Nitrobacter, Tardiphaga, Bacillus, Pseudomonas, Flavobacterium*, and *Variovorax* were also identified.

The presence of both rhizobia and non-rhizobia endophytes in cowpea nodules has been often documented. Muindi et al. [58] identified 20 isolates closely related to the genera *Enterobacter* (9), *Rhizobium* (8), *Paraburkholderia* (1), *Pseudomonas* (1), and *Stenotrophomonas* (1) from semi-arid areas of lower Eastern Kenya, Africa. In Brazil, Castro et al. [20] sequenced 156 isolates from mining areas in Minas Gerais state, southeast Brazil, and identified strains belonging to the genera *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Burkholderia* (higher predominance), *Bradyrhizobium*, *Brevibacillus*, *Chitinophaga*, *Cupriavidus*, *Dyella*, *Enterobacter*, *Herbaspirillum*, *Novosphingobium*, *Paenebacillus*, *Pseudomonas*, *Rhizobium*, and *Terriglobus*. In another study in the Amazonas state, Brazil, Azarias Guimarães and collaborators [18] classified 23 isolates as *Bradyrhizobium* (16), *Achromobacter* (4), *Rhizobium* (2) and *Burkholderia* (1).

Although cowpea is commonly recognized as a promiscuous legume, *Bradyrhizobium* has been consistently identified as its primary symbiont in Africa, Brazil, and Japan [11, 21, 59–62]. Intriguingly, in our study, we were unable to detect *Bradyrhizobium*. However, this finding is consistent with the results reported by Muindi et al. [58] in Africa, whereas in the study of Castro et al. [20] in Brazil the predominant genus was *Burkholderia*, but *Bradyrhizobium* was detected in a small number.

In our study, from the 89 strains identified at the genus level, 72% were composed of non-rhizobia, belonging to the genera *Agrobacterium*, *Burkholderia*, *Enterobacter*, *Microbacterium*, *Ancylobacter*, and *Stenotrophomonas*, and 28% of rhizobia of the genera *Rhizobium, Ensifer, Mesorhizobium*, and *Paraburkholderia*.

There are reports of *Agrobacterium* being isolated from root nodules. Delamuta et al. [63] described a new species named *Agrobacterium fabacearum* isolated from healthy nodules of common bean plants in Ecuador; however, the species was not able to re-nodulate the original host, suggesting an endophytic lifestyle. Cavalcanti and

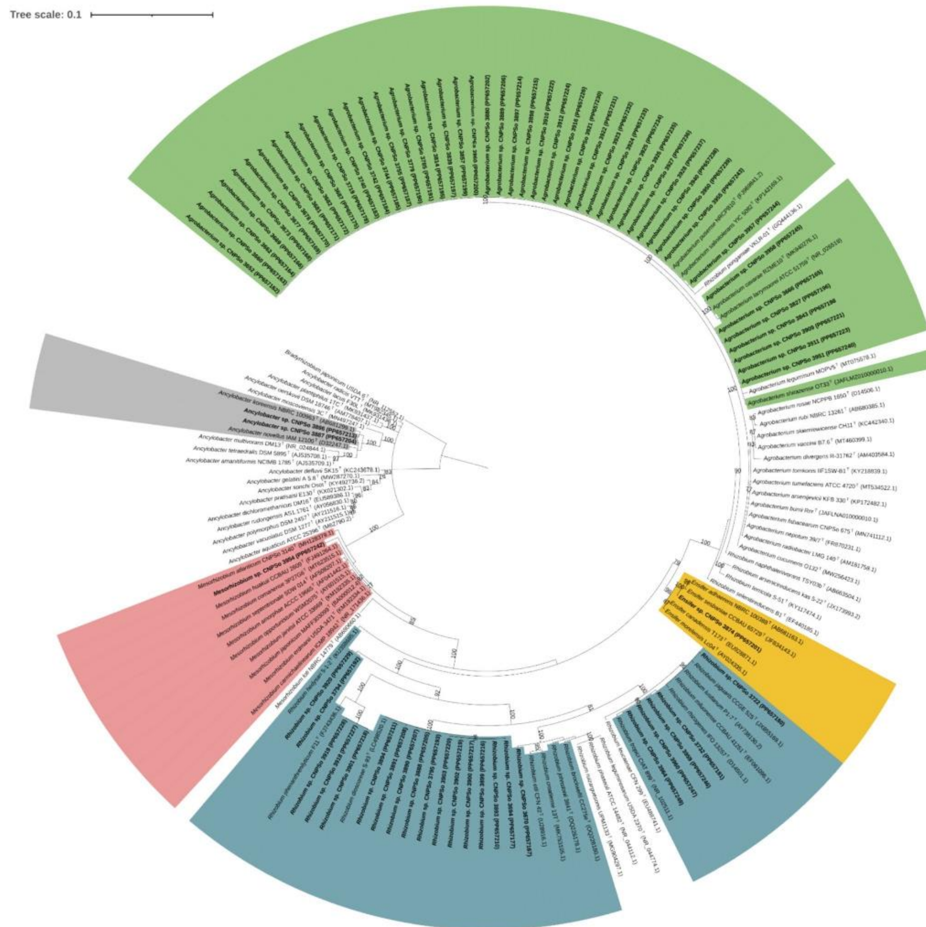


Fig. 2 Bayesian phylogeny tree based on the 16S rRNA alignment for the genera *Agrobacterium*, *Rhizobium*, *Ensifer*, *Mesorhizobium,* and *Ancylobacter* of the class Alphaproteobacteria (846 bp), using General Time Reversible model (GTR). Accession numbers are indicated in parentheses. Strains from this study are shown in bold. Representative colors were used for the genera: green is for *Agrobacterium*, blue is for *Rhizobium*, yellow is for *Ensifer*, pink is for *Mesorhizobium* and gray is for *Ancylobacter*. Bootstrap values >70% are indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^T was used as an outgroup. Bar indicates the number of substitutions per site

collaborators [64] trapped endophytic bacteria from maize (*Zea mays* L.) that were identified as cowpea-noculating rhizobia, more specifically, *Agrobacterium, Bradyrhizobium*, and *Rhizobium* by the 16S rRNA gene sequencing. Putative roles assigned to the *Agrobacterium* endophytes were the synthesis of phytohormones such as indole-3-acetic acid (IAA), which can promote plant growth, and the capacity to confer tolerance to various abiotic stresses, such as in zinc-contaminated soils [63, 65]. In this study, we detected 47 strains isolated from six indigenous lands (Água Branca, Buriti, Cachoeirinha, Lagoinha, Lalima, and Limão Verde), comprising the Cerrado and Pantanal biomes, that were identified as members of the genus *Agrobacterium*. However, it is important to mention that the

16S rRNA gene often lacks the resolution to differentiate closely related species, as is the case of species within the genera *Agrobacterium* and *Rhizobium*. This might be the case in our study, where a great diversity of species was detected in the BOX-PCR analysis, resulting in the positioning of strains within distinct clusters (Fig. 1), but relatively restricted in the 16S rRNA phylogenetic tree (Fig. 2).

The presence of Beta-proteobacteria in nodules was detected only after the 2000s, when Moulin et al. [66] identified a *Burkholderia* strain nodulating *Aspalathus carnosa*. Years later, phylogenetic studies maintained the clinically important strains in the *Burkholderia* genus and reclassified the strains involved with environmental processes to a new genus called *Parabukholderia* [67–69]. Since then, several studies have isolated *Paraburkholdeira* from leguminous nodules [70]. However, *Burkholderia* strains are consistently isolated from root nodules [15, 20, 71]. We detected 12 *Burkholderia* strains from five indigenous lands (Água Branca, Buriti, Lagoinha, Lalima, and Limão Verde), comprising the Cerrado and Pantanal biomes (Fig. 3), and each strain exhibited a distinct profile in the BOX-PCR analysis (Fig. 1). The presence of endophytic *Burkholderia* in root nodules of leguminous plants may represent a promising application in sustainable agriculture if proved the non-pathogenicity of the strains, since the genus is recognized by the high capacity of phosphate solubilization [72–74].

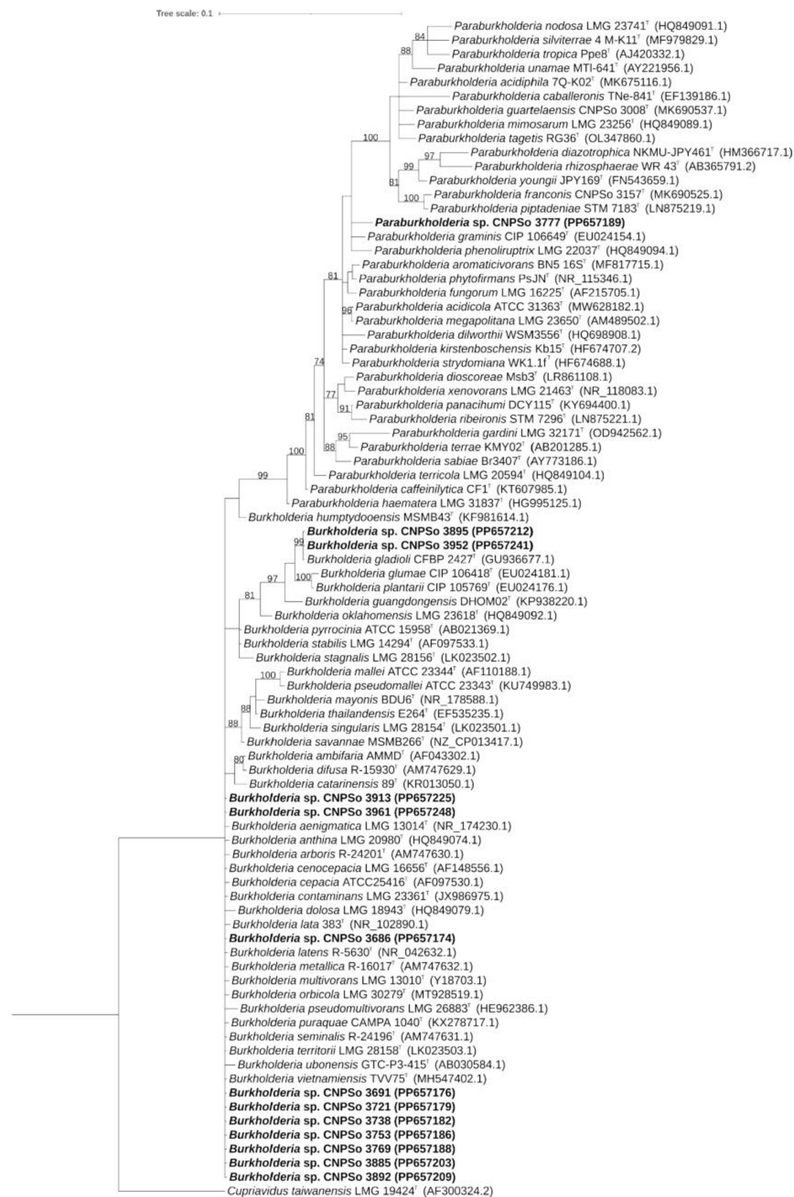
Even though *Enterobacter* is part of the normal intestinal microbiota of humans and animals and encompasses pathogenic and phytopathogenic strains [75, 76], the genus has been frequently isolated from plant endosphere, including root nodules [58, 71, 77]. Interestingly, Ludueña et al. [78] pointed out the presence of genes involved in phosphate solubilization and mineralization, siderophore production, synthesis of IAA, and acetoin synthesis in the genome of *Enterobacter* sp. J49 isolated from peanut (*Arachis hypogaea* L.) root nodules, grown in a production area rotated with maize in Cordoba, Argentina. In the BOX-PCR analysis (Fig. 1), strain CNPSo 3683 was positioned in group 40 of the dendrogram, and the 16S rRNA gene sequence supports its classification as *Enterobacter* (Fig. 4). However, the strain exhibited high NI values (99.8%) with three type strains of the genus, and further analysis is needed to clarify its proper phylogenetic position.

Concerning the *Microbacterium* genus, we identified a unique strain, CNPSo 3965, isolated in Lalima indigenous land, in the Cerrado biome. *Microbacterium* genus comprises a diverse and versatile collection of pathogenic and endophytic species [79, 80], including various strains known for their beneficial traits such as auxin production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, which contributes to plant growth promotion [81]. Interestingly, Zakhia et al. [82] isolated 34 strains from root nodules of spontaneous legumes in Tunisia belonging to several genera of non-rhizobial endophytes; however, although the strains were not able to nodulate siratro (*Macroptilium atropurpureum* (DC.) Urb.), some of them, including those identified as *Microbacterium, Agromyces, Ancylobacter*, and *Phyllobacterium*, presented *nifH* sequences with higher similarity to those of *Sinorhizobium meliloti*.

We detected two strains belonging to the *Ancylobacter* genus, CNPSo 3887 and CNPSo 3896, isolated from the Buriti indigenous land in the Cerrado biome, exhibiting 98.6% NI of the 16S rRNA to each other (Fig. 2). Strain CNPSo 3887 displayed the highest similarity with *Ancylobacter novellus* IAM 12100^T (99.1%), while CNPSo 3896 showed the highest similarity with *Ancylobacter koreensis* NBRC 100963^T (99.5%). There are very few studies reporting the genus in legume nodules. As mentioned before, Zakhia et al. [82] isolated strains of *Ancylobacter* from *Retama raetam* nodules with *nifH* sequences sharing 90% similarity with *S. meliloti;* however, they were unable to nodulate the promiscuous siratro. The study performed by Valdez-Nuñez et al. [83] was the first one to detect *Ancylobacter* in cowpea nodules and, interestingly, the strain 5740O had the highest capacity of solubilizing tricalcium phosphate (> 300%). In addition, *Ancylobacter* genus was also isolated from root nodules of *Lotus* plants in Egypt [84].

The *Stenotrophomonas* genus is widely known for presenting plant growth-promoting strains as well as pathogenic species [85]. In our study, we identified the strain CNPSo 3800 as *Stenotrophomonas*, from Cachoeirinha indigenous land, in the Cerrado biome. A notable plant-growth promotion has been reported for *Stenotrophomonas*, both solely and along with rhizobial strains. Aeron et al. [86] characterized *S. maltophilia* strain RMC6 isolated from *Mucuna utilis* root nodules as a strong antagonist against the plant pathogenic fungus *Fusarium udum*, in addition to several direct and indirect plant growth-promoting features such as IAA production, phosphate solubilization, presence of *nifH* gene, and ACC deaminase and siderophore production. Another relevant study was performed by Egamberdieva et al. [87], which showed a positive effect of *S. Stenotrophomonas rhizophila* ep-17 on soybean growth under salt stress when co-inoculated with *Bradyrhizobium japonicum* BDYD1. The first study reporting *Stenotrophomonas* isolated from cowpea nodules was described by Abd-Alla et al. [88], which isolated good exopolysaccharide producers belonging to *S. maltophilia* and *Brevibacillus parabrevis* from cowpea and *Cicer arietinum*. Later, Muindi et al. [58] also described the presence of endophytic *Stenotrophomonas* in cowpea nodules.

Concerning the rhizobia genera, we isolated 25 strains belonging to this group. However, the absence of positive nodulation tests and *nodC* and *nifH* amplification in these strains (data not shown), may indicate the loss of nodulation ability and the assumption of an endophytic lifestyle in cowpea nodules, along with the other non-rhizobia strains


Tree scale: 0.1
Paraburkholderia nodosa LMG 23741^{T} (HQ849091.1)
Paraburkholderia silviterrae 4 M-K11^{T} (MF979829.1)
Paraburkholderia tropica Ppe8^{T} (AJ420332.1)
Paraburkholderia unamae MTI-641^{T} (AY221956.1)
Paraburkholderia acidiphila 7Q-K02^{T} (MK675116.1)
Paraburkholderia caballeronis TNe-841^{T} (EF139186.1)
Paraburkholderia guartelaensis CNPSo 3008^{T} (MK690537.1)
Paraburkholderia mimosarum LMG 23256^{T} (HQ849089.1)
Paraburkholderia tagetis RG36^{T} (OL347860.1)
Paraburkholderia diazotrophica NKMU-JPY461^{T} (HM366717.1)
Paraburkholderia rhizosphaerae WR 43^{T} (AB365791.2)
Paraburkholderia youngii JPY169^{T} (FN543659.1)
Paraburkholderia franconis CNPSo 3157^{T} (MK690525.1)
Paraburkholderia piptadeniae STM 7183^{T} (LN875219.1)
Paraburkholderia sp. CNPSo 3777 (PP657189)
Paraburkholderia graminis CIP 106649^{T} (EU024154.1)
Paraburkholderia phenoliruptrix LMG 22037^{T} (HQ849094.1)
Paraburkholderia aromaticivorans BN5 16S^{T} (MF817715.1)
Paraburkholderia phytofirmans PsJN^{T} (NR_115346.1)
Paraburkholderia fungorum LMG 16225^{T} (AF215705.1)
Paraburkholderia acidicola ATCC 31363^{T} (MW628182.1)
Paraburkholderia megapolitana LMG 23650^{T} (AM489502.1)
Paraburkholderia dilworthii WSM3556^{T} (HQ698908.1)
Paraburkholderia kirstenboschensis Kb15^{T} (HF674707.2)
Paraburkholderia strydomiana WK1.1f^{T} (HF674688.1)
Paraburkholderia dioscoreae Msb3^{T} (LR861108.1)
Paraburkholderia xenovorans LMG 21463^{T} (NR_118083.1)
Paraburkholderia panacihumi DCY115^{T} (KY694400.1)
Paraburkholderia ribeironis STM 7296^{T} (LN875221.1)
Paraburkholderia gardini LMG 32171^{T} (OD942562.1)
Paraburkholderia terrae KMY02^{T} (AB201285.1)
Paraburkholderia sabiae Br3407^{T} (AY773186.1)
Paraburkholderia terricola LMG 20594^{T} (HQ849104.1)
Paraburkholderia caffeinilytica CF1^{T} (KT607985.1)
Paraburkholderia haematera LMG 31837^{T} (HG995125.1)
Burkholderia humptydooensis MSMB43^{T} (KF981614.1)
Burkholderia sp. CNPSo 3895 (PP657212)
Burkholderia sp. CNPSo 3952 (PP657241)
Burkholderia gladioli CFBP 2427^{T} (GU936677.1)
Burkholderia glumae CIP 106418^{T} (EU024181.1)
Burkholderia plantarii CIP 105769^{T} (EU024176.1)
Burkholderia guangdongensis DHOM02^{T} (KP938220.1)
Burkholderia oklahomensis LMG 23618^{T} (HQ849092.1)
Burkholderia pyrrocinia ATCC 15958^{T} (AB021369.1)
Burkholderia stabilis LMG 14294^{T} (AF097533.1)
Burkholderia stagnalis LMG 28156^{T} (LK023502.1)
Burkholderia mallei ATCC 23344^{T} (AF110188.1)
Burkholderia pseudomallei ATCC 23343^{T} (KU749983.1)
Burkholderia mayonis BDU6^{T} (NR_178588.1)
Burkholderia thailandensis E264^{T} (EF535235.1)
Burkholderia singularis LMG 28154^{T} (LK023501.1)
Burkholderia savannae MSMB266^{T} (NZ_CP013417.1)
Burkholderia ambifaria AMMD^{T} (AF043302.1)
Burkholderia difusa R-15930^{T} (AM747629.1)
Burkholderia catarinensis 89^{T} (KR013050.1)
Burkholderia sp. CNPSo 3913 (PP657225)
Burkholderia sp. CNPSo 3961 (PP657248)
Burkholderia aenigmatica LMG 13014^{T} (NR_174230.1)
Burkholderia anthina LMG 20980^{T} (HQ849074.1)
Burkholderia arboris R-24201^{T} (AM747630.1)
Burkholderia cenocepacia LMG 16656^{T} (AF148556.1)
Burkholderia cepacia ATCC25416^{T} (AF097530.1)
Burkholderia contaminans LMG 23361^{T} (JX986975.1)
Burkholderia dolosa LMG 18943^{T} (HQ849079.1)
Burkholderia lata 383^{T} (NR_102890.1)
Burkholderia sp. CNPSo 3686 (PP657174)
Burkholderia latens R-5630^{T} (NR_042632.1)
Burkholderia metallica R-16017^{T} (AM747632.1)
Burkholderia multivorans LMG 13010^{T} (Y18703.1)
Burkholderia orbicola LMG 30279^{T} (MT928519.1)
Burkholderia pseudomultivorans LMG 26883^{T} (HE962386.1)
Burkholderia puraquae CAMPA 1040^{T} (KX278717.1)
Burkholderia seminalis R-24196^{T} (AM747631.1)
Burkholderia territorii LMG 28158^{T} (LK023503.1)
Burkholderia ubonensis GTC-P3-415^{T} (AB030584.1)
Burkholderia vietnamiensis TVV75^{T} (MH547402.1)
Burkholderia sp. CNPSo 3691 (PP657176)
Burkholderia sp. CNPSo 3721 (PP657179)
Burkholderia sp. CNPSo 3738 (PP657182)
Burkholderia sp. CNPSo 3753 (PP657186)
Burkholderia sp. CNPSo 3769 (PP657188)
Burkholderia sp. CNPSo 3885 (PP657203)
Burkholderia sp. CNPSo 3892 (PP657209)
Cupriavidus taiwanensis LMG 19424^{T} (AF300324.2)

◂**Fig. 3** Bayesian phylogeny tree based on the 16S rRNA alignment of the genera *Burkholderia*and *Paraburkholderia* of the class Betaproteobacteria (772 bp), using General Time Reversible model (GTR). Accession numbers are indicated in parentheses. Strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. *Cupriavidus taiwanensis* LMG 19424^T was used as an outgroup. Bar indicates the number of substitutions per site

detected here. Interestingly, Valdez-Nuñez et al. [83] detected members of *Rhizobium, Mesorhizobium, Bradyrhizobium*, and *Ensifer* unable to reinfect cowpea, their host plant, in Peru. Therefore, the authors considered these isolates as non-nodulating bacterial endophytes, along with other isolates belonging to *Agrobacterium, Phyllobacterium, Bosea,*

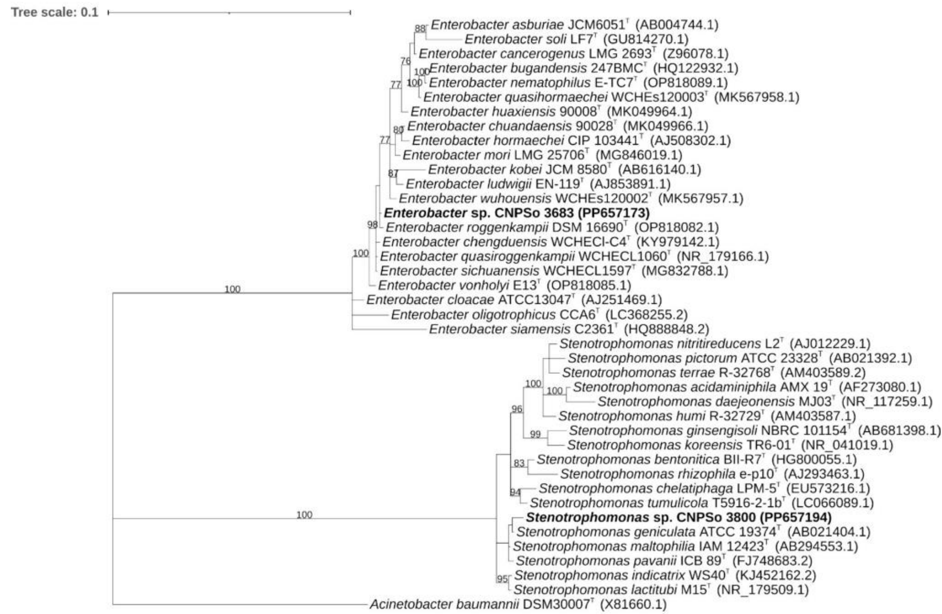


Fig. 4 Bayesian phylogeny tree based on the 16S rRNA alignment of the genera *Enterobacter* and *Stenotrophomonas* of the class Gammaproteobacteria (1003 bp), using General Time Reversible model (GTR). Accession numbers are indicated in parentheses. Strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. *Acinetobacter baumannii* DSM30007^T was used as an outgroup. Bar indicates the number of substitutions per site

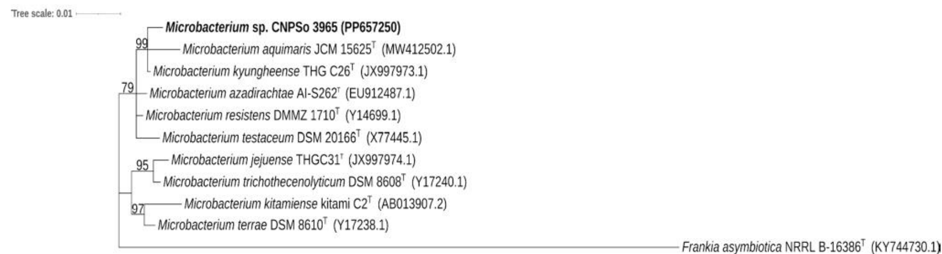


Fig. 5 Bayesian phylogeny tree based on the 16S rRNA alignment of the genus *Microbacterium* of the class Actinobacteria (956 bp), using General Time Reversible model (GTR). Accession numbers are indicated in parentheses. Strains from this study are shown in bold. Bootstrap values >70% are indicated at the nodes. *Frankia asymbiotica* NRRL B-16386^T was used as an outgroup. Bar indicates the number of substitutions per site

Ochrobactrum, Labrys, Starkeya, Nordella, and *Mycobacterium*. Indeed, some studies point out that rhizobial species, e.g. belonging to the genera *Ensifer, Mesorhizobium, Paraburkholderia*, and *Rhizobium*, may harbor strains capable of adopting an endophytic lifestyle, e.g. *E. adhaerens* [89], *Mesorhizobium ephedrae* [90], *Paraburkholderia phytofirmans* [91], and *Rhizobium hidalgonense* [92]. Therefore, our understanding of the true contribution of this diverse array of bacterial species inhabiting nodules remains limited.

Here we isolated the strain CNPSo 3874 from the Cachoeirinha indigenous land in the Cerrado biome, exhibiting highest similarity with *E. sesbaniae* CCBAU 65729^T, from United States soils and *E. adhaerens* NBRC 100388^T, from Chinese *Sesbania cannabina* root nodules (Fig. 2). Interestingly, the strain *Ensifer* 5440N, also unable to reinfect cowpea, presented closeness to the same species *E. sesbaniae* and *E. adhaerens* in 16S rRNA phylogeny [83].

We identified the strain CNPSo 3954 belonging to *Mesorhizobium* from Buriti indigenous land, in the Cerrado biome. The strain occupied an isolated position in 16S rRNA phylogeny, which may indicate a novel lineage (Fig. 2). Interestingly, another study performed on Cerrado soils of Mato Grosso do Sul state isolated a strain of *Mesorhizobium* from *P. vulgaris* nodules sharing high similarity with the type strains *Mesorhizobium acaciae* RITF741^T, *Mesorhizobium atlanticum* CNPSo 3140^T, and *Mesorhizobium plurifarium* LMG 11892^T in 16S rRNA phylogeny [22].

As mentioned before, the genus *Paraburkholderia* includes rhizobia species of Beta-proteobacteria. The strain CNPSo 3777, isolated from Limão Verde indigenous land in the Pantanal biome, occupied an isolated position, presenting higher NI values (98.8%) with *Paraburkholderia panacihumi* DCY115^T. Although limited studies exist on *Paraburkholderia* in cowpea nodules, Muindi et al. [58] also identified a strain of this genus when assessing rhizobial genetic diversity in semi-arid regions of lower eastern Kenya. However, the *Paraburkholderia* isolates of Muindi et al. [58] were able to reinfect cowpea plants.

Finally, the last genus described here, *Rhizobium* with 22 strains isolated from six indigenous lands (Água Branca, Buriti, Cachoeirinha, Lagoinha, Lalima, and Limão Verde), comprising the Cerrado and Pantanal biomes, comprises one of the few reports exploring the relationship between the genus *Rhizobium* and cowpea in Brazil, along with Azarias Guimarães et al. [18] that studied the genetic diversity of strains from cowpea nodules in agricultural soils of the state of Amazonas and Castro et al. [20], which isolated *Rhizobium* strains from cowpea nodules in Minas Gerais state.

Conclusion

Our results highlight a remarkable genetic diversity of bacteria isolated from cowpea nodules cultivated in soils across six indigenous lands in Mato Grosso do Sul, Central-Western Brazil, including ten different genera. Further studies are needed to elucidate the prevalence and specific contributions of non-rhizobial species within cowpea nodules. Additionally, our results confirm the high promiscuity of cowpea in forming associations with a diverse array of bacterial species, highlighting the complexity and potential ecological significance of these interactions.

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Data availability All data supporting this study's findings are available within the paper and its Supplementary Information. Sequences obtained in this study were deposited in the NCBI GenBank database, and the access numbers are shown in the phylogenetic trees and Table 2.

Declarations

Conflicts of interests The authors declare that they have no competing or conflicting interests, or ethical conflicts.

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1 **SUPPLEMENTARY MATERIAL**

2

3 **Table S1** Morphophysiological characteristics of the strains trapped in nodules of
4 cowpea plants (Vigna unguiculata) cultivated in soils of six indigenous lands in
5 Mato Grosso do Sul, Central-West Region of Brazil.

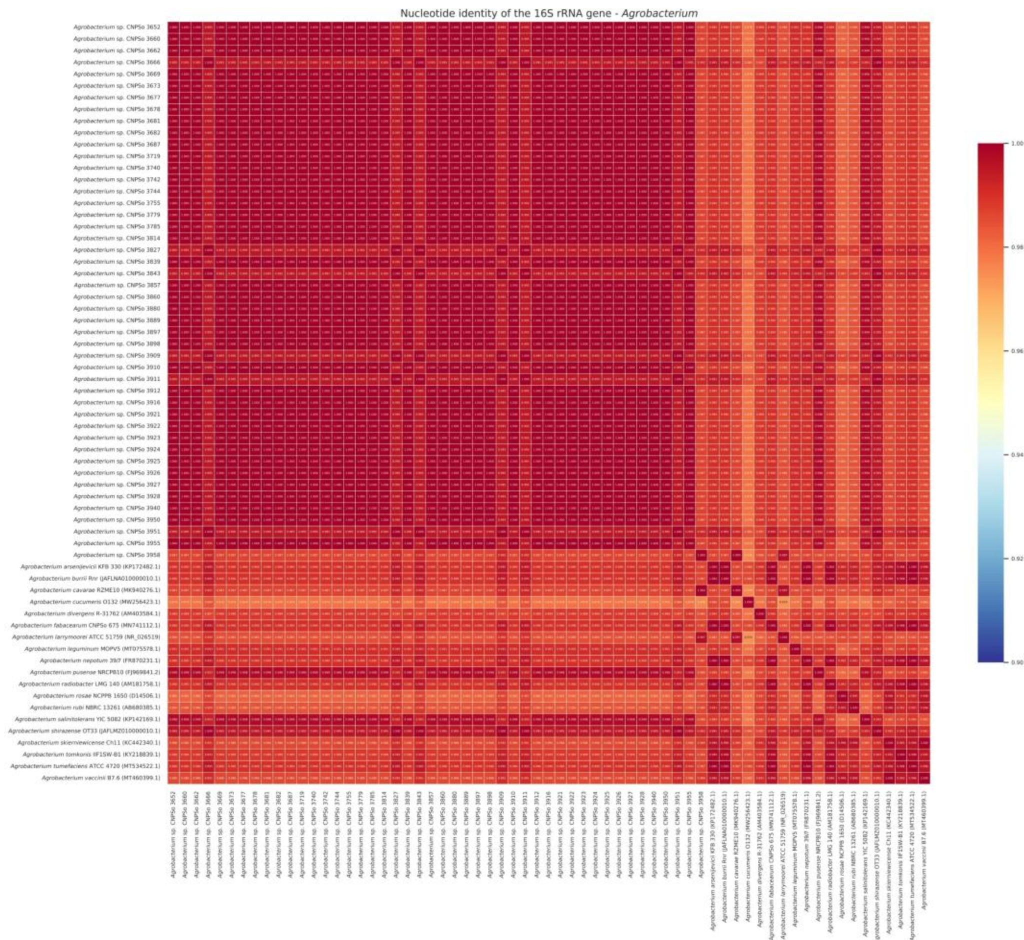
6

Strains	Genera	Colony diameter (mm)	alkali/acidic reaction	Optical Details	Chromogenesis in Bromothymol blue	Chromogenesis in Congo red
3652	*Agrobacterium* sp.	3,0	Acid	Translucent	Yellow	Red
3660	*Agrobacterium* sp.	1,2	Neutral	Opaque	Yellow	Pink
3662	*Agrobacterium* sp.	2,2	Acid	Opaque	Yellow	Pink
3666	*Agrobacterium* sp.	2,0	Neutral	Opaque	Yellow	Pink
3669	*Agrobacterium* sp.	1,7	Neutral	Translucent	Yellow	Pink
3670	*Rhizobium* sp.	1,6	Acid	Opaque	Yellow	White
3673	*Agrobacterium* sp.	2,2	Neutral	Translucent	Yellow	Red
3677	*Agrobacterium* sp.	2,0	Acid	Opaque	Yellow	Red
3678	*Agrobacterium* sp.	2,2	Neutral	Translucent	Yellow	Pink
3681	*Agrobacterium* sp.	2,4	Neutral	Translucent	Yellow	Pink
3682	*Agrobacterium* sp.	2,2	Neutral	Translucent	Yellow	Pink
3683	*Enterobacter* sp.	2,7	Neutral	Translucent	Yellow	Red
3686	*Burkholderia sp.*	1,4	Neutral	Opaque	Blue	Pink
3687	*Agrobacterium* sp.	2,7	Neutral	Translucent	Yellow	Pink
3691	*Burkholderia sp.*	1,5	Neutral	Opaque	Blue	Pink
3694	*Rhizobium* sp.	1,2	Acid	Opaque	Blue	White
3719	*Agrobacterium* sp.	2,5	Neutral	Translucent	Yellow	Pink
3721	*Burkholderia sp.*	1,7	Neutral	Opaque	Blue	Pink
3722	*Rhizobium* sp.	2,0	Acid	Opaque	Yellow	Pink
3732	*Rhizobium* sp.	1,3	Acid	Opaque	Yellow	Pink
3738	*Burkholderia sp.*	1,6	Neutral	Opaque	Blue	White
3740	*Agrobacterium* sp.	2,2	Neutral	Opaque	Yellow	Pink
3742	*Agrobacterium* sp.	1,2	Neutral	Opaque	Blue	White
3744	*Agrobacterium* sp.	1,5	Neutral	Translucent	Yellow	White
3753	*Burkholderia sp.*	1,4	Neutral	Opaque	Blue	Pink
3755	*Agrobacterium* sp.	2,1	Acid	Opaque	Blue	Red
3769	*Burkholderia sp.*	1,4	Neutral	Opaque	Yellow	Pink
3777	*Paraburkholderia sp.*	1,5	Alkali	Opaque	Blue	White
3779	*Agrobacterium* sp.	2,2	Neutral	Translucent	Blue	White
3785	*Agrobacterium* sp.	2,5	Neutral	Translucent	Blue	Pink
3794	*Rhizobium* sp.	1,8	Neutral	Translucent	Yellow	Pink
3795	*Rhizobium* sp.	1,6	Acid	Opaque	Blue	White
3800	*Stenotrophomonas* sp.	1,5	Acid	Opaque	Yellow	White

3814	*Agrobacterium* sp.	2,2	Neutral	Opaque	Yellow	Pink
3827	*Agrobacterium* sp.	1,6	Neutral	Translucent	Yellow	White
3839	*Agrobacterium* sp.	2,2	Neutral	Translucent	Blue	Pink
3843	*Agrobacterium* sp.	2,3	Neutral	Opaque	Blue	Pink
3857	*Agrobacterium* sp.	2,6	Acid	Translucent	Yellow	Pink
3860	*Agrobacterium* sp.	2,4	Alkali	Opaque	Blue	Pink
3874	*Ensifer* sp.	1,9	Alkali	Translucent	Blue	Pink
3880	*Agrobacterium* sp.	3,1	Neutral	Translucent	Yellow	Pink
3885	*Burkholderia sp.*	1,5	Alkali	Opaque	Blue	White
3887	*Ancylobacter* sp.	1,9	Neutral	Translucent	Yellow	Pink
3888	*Rhizobium* sp.	2,1	Acid	Opaque	Yellow	Pink
3889	*Agrobacterium* sp.	2,5	Neutro	Opaque	Blue	Pink
3890	*Rhizobium* sp.	1,2	Alkali	Opaque	Blue	Pink
3891	*Rhizobium* sp.	1,8	Acid	Translucent	Yellow	Red
3892	*Burkholderia sp.*	1,4	Acid	Opaque	Yellow	White
3893	*Rhizobium* sp.	1,9	Acid	Opaque	Yellow	Red
3894	*Rhizobium* sp.	1,8	Acid	Translucent	Yellow	Red
3895	*Burkholderia sp.*	2,4	Acid	Opaque	Yellow	Red
3896	*Ancylobacter* sp.	1,4	Neutral	Translucent	Yellow	Pink
3897	*Agrobacterium* sp.	2,4	Neutral	Translucent	Blue	Pink
3898	*Agrobacterium* sp.	2,3	Neutral	Opaque	Blue	Pink
3899	*Rhizobium* sp.	2,1	Acid	Translucent	Yellow	Red
3900	*Rhizobium* sp.	1,8	Acid	Opaque	Yellow	Pink
3901	*Rhizobium* sp.	1,7	Neutral	Opaque	Yellow	Red
3902	*Rhizobium* sp.	2,2	Acid	Translucent	Yellow	Red
3903	*Rhizobium* sp.	1,8	Neutral	Opaque	Blue	White
3909	*Agrobacterium* sp.	1,3	Acid	Translucent	Yellow	Pink
3910	*Agrobacterium* sp.	1,7	Neutral	Opaque	Yellow	White
3911	*Agrobacterium* sp.	2,0	Neutral	Translucent	Blue	Pink
3912	*Agrobacterium* sp.	2,1	Acid	Translucent	Yellow	Pink
3913	*Burkholderia sp.*	1,7	Acid	Opaque	Yellow	White
3916	*Agrobacterium* sp.	2,9	Acid	Opaque	Yellow	Pink
3918	*Rhizobium* sp.	1,2	Acid	Translucent	Yellow	Pink
3919	*Rhizobium* sp.	1,6	Neutral	Translucent	Yellow	Pink
3920	*Rhizobium* sp.	1,9	Acid	Translucent	Yellow	Pink
3921	*Agrobacterium* sp.	2,6	Acid	Opaque	Yellow	Pink
3922	*Agrobacterium* sp.	3,0	Acid	Translucent	Yellow	Pink
3923	*Agrobacterium* sp.	3,2	Acid	Translucent	Yellow	Red
3924	*Agrobacterium* sp.	1,8	Neutral	Translucent	Yellow	Pink
3925	*Agrobacterium* sp.	2,7	Neutral	Translucent	Yellow	Red
3926	*Agrobacterium* sp.	2,9	Neutral	Translucent	Blue	Pink
3927	*Agrobacterium* sp.	2,6	Neutral	Translucent	Yellow	Red
3928	*Agrobacterium* sp.	2,4	Neutral	Translucent	Blue	Pink
3940	*Agrobacterium* sp.	2,0	Neutral	Opaque	Yellow	Pink
3950	*Agrobacterium* sp.	2,0	Neutral	Opaque	Blue	Red
3951	*Agrobacterium* sp.	2,3	Neutral	Translucent	Yellow	Pink

3952	*Burkholderia sp.*	2,1	Acid	Opaque	Yellow	Red
3954	*Mesorhizobium* sp.	1,2	Acid	Opaque	Blue	Red
3955	*Agrobacterium* sp.	1,3	Alkali	Opaque	Yellow	White
3957	*Agrobacterium* sp.	1,9	Acid	Translucent	Yellow	Pink
3958	*Agrobacterium* sp.	1,6	Neutral	Opaque	Blue	Red
3959	*Rhizobium* sp.	1,4	Acid	Translucent	Yellow	Pink
3960	*Rhizobium* sp.	1,9	Acid	Opaque	Blue	Pink
3961	*Burkholderia sp.*	1,7	Alkali	Opaque	Blue	White
3964	*Rhizobium* sp.	1,5	Acid	Opaque	Yellow	Pink
3965	*Microbacterium* sp.	1,5	Neutral	Translucent	Yellow	White

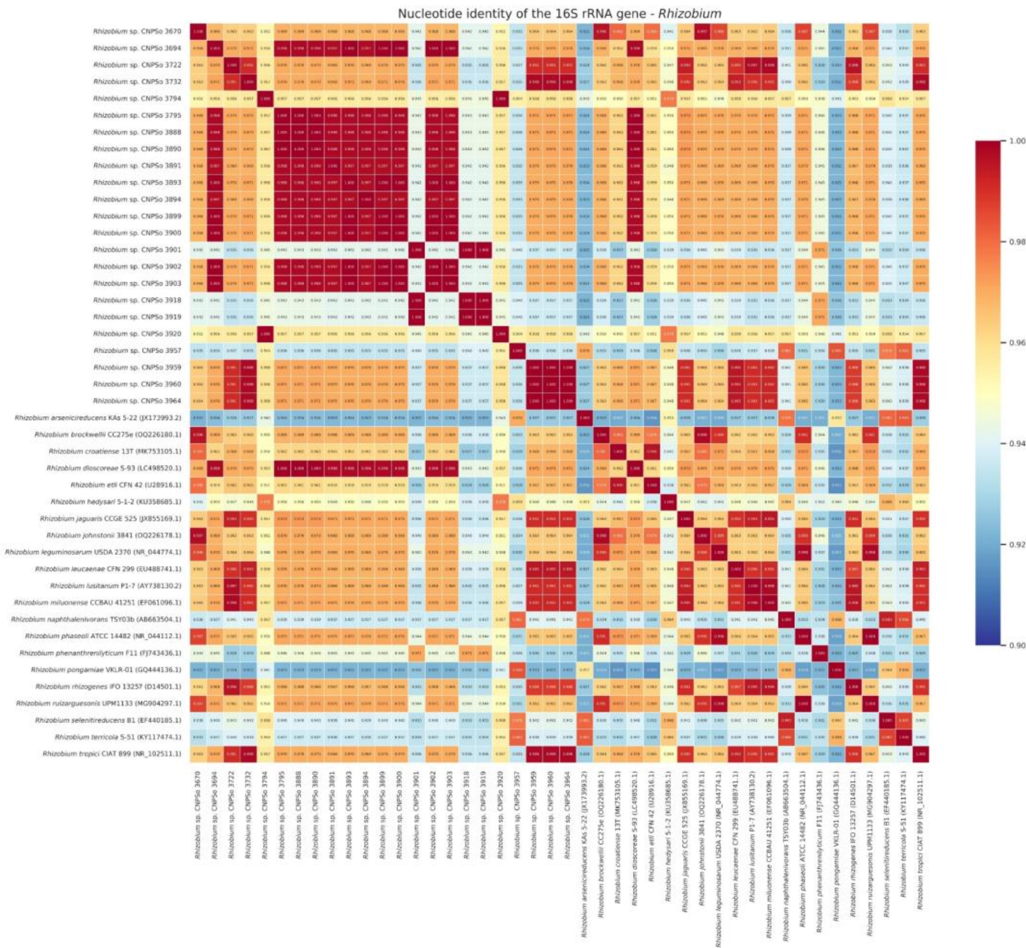
7



8

9 **Fig. S1** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
10 the genus *Agrobacterium* obtained through a similarity matrix built in Bioedit
11 version (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn
12 libraries. NI values above 90% are represented by different shades of blue, yellow
13 and red, with blue being the values closest to 0.9 and red being the values closest
14 to 1.

15



16

17 **Fig. S2** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
18 the genus *Rhizobium* obtained through a similarity matrix built in Bioedit version
19 (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn libraries.
20 NI values above 90% are represented by different shades of blue, yellow and red,
21 with blue being the values closest to 0.9 and red being the values closest to 1.

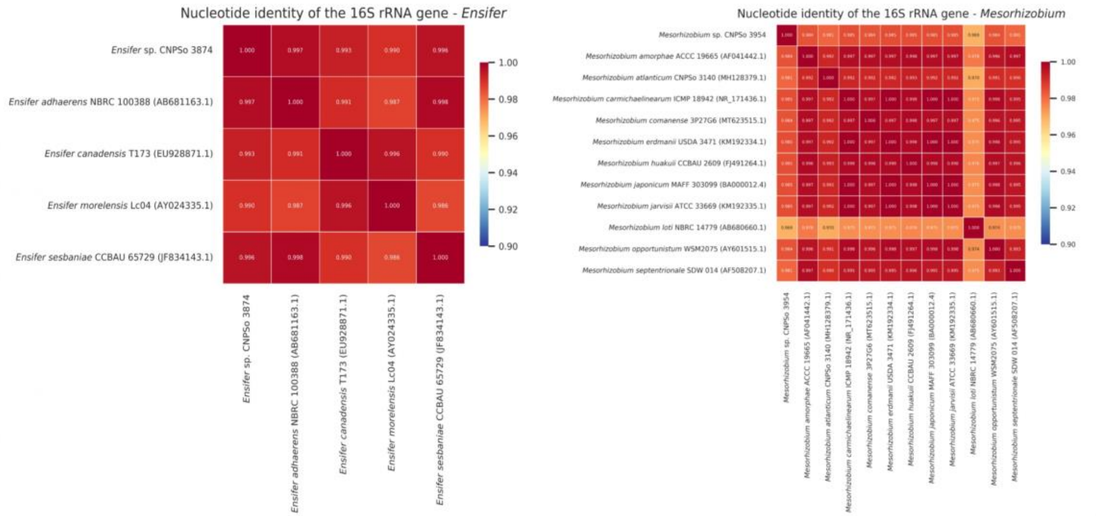
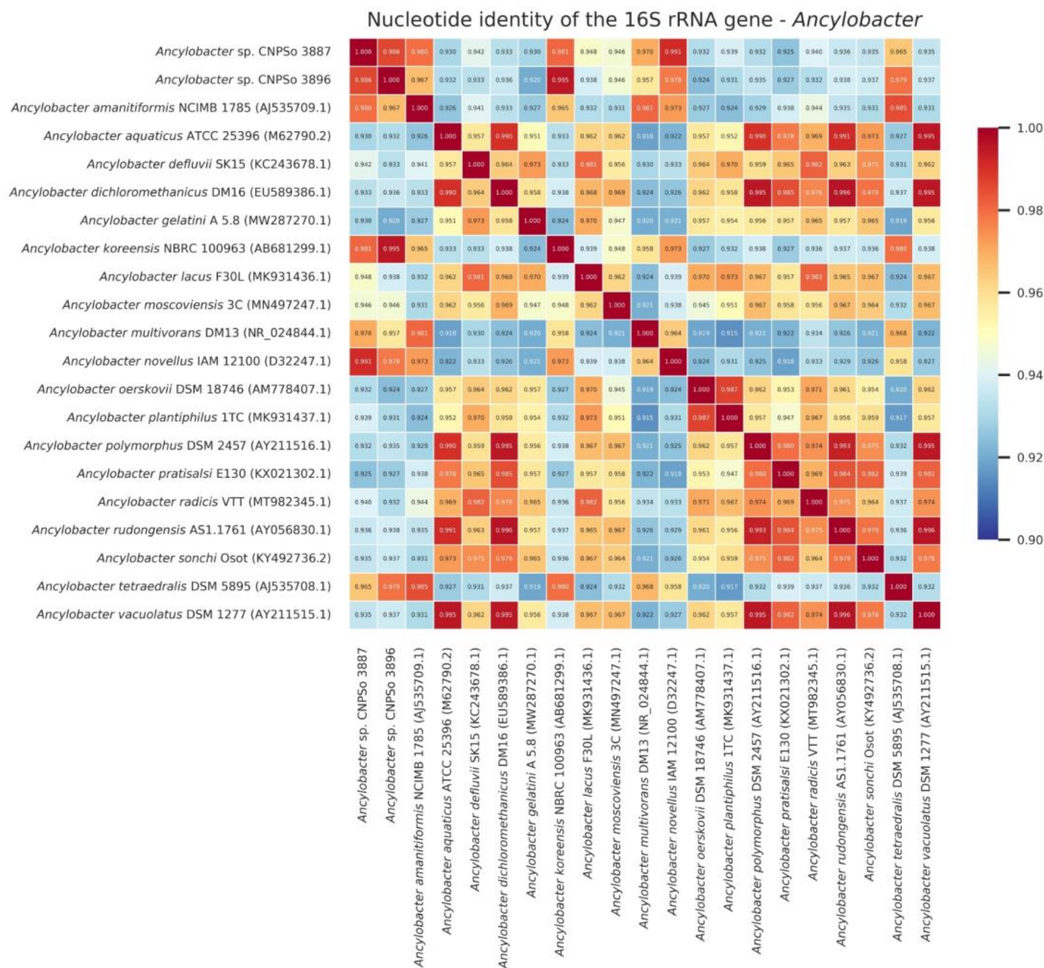
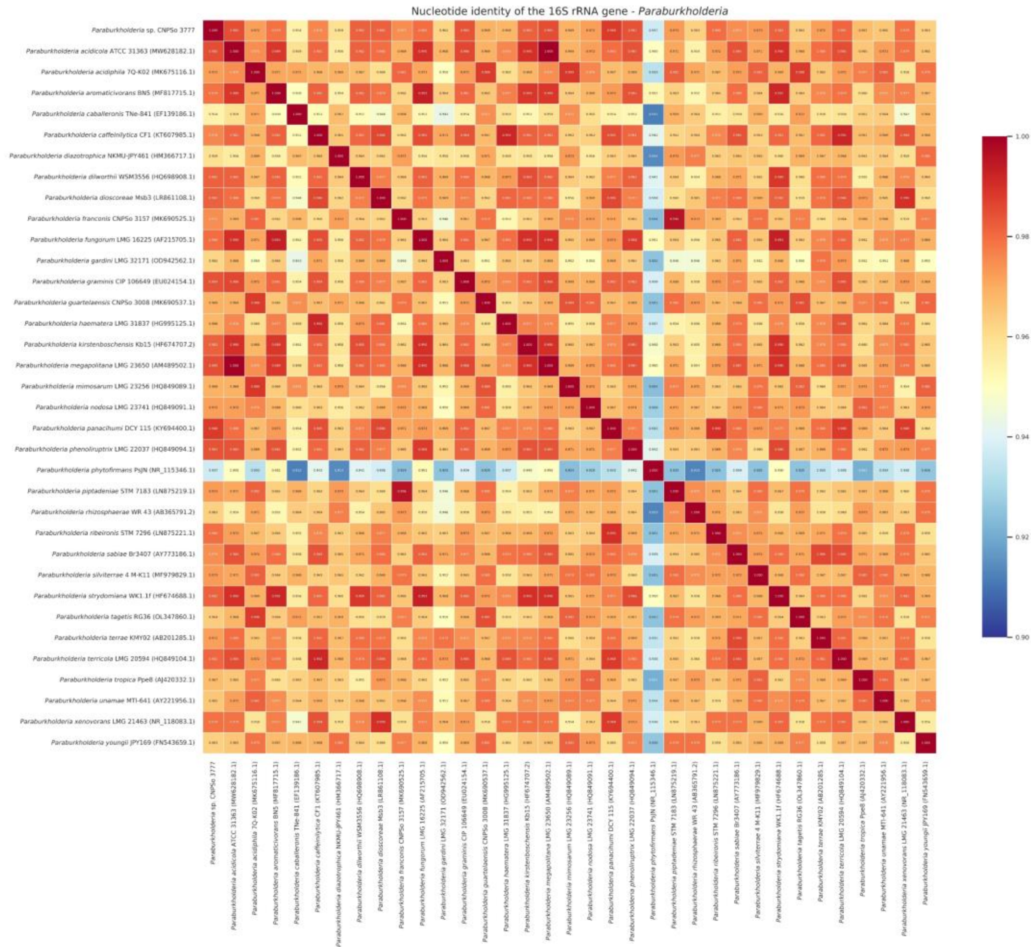


Fig. S3 Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of the genus *Ensifer* and *Mesorhizobium* obtained through a similarity matrix built in Bioedit version (v. 7.2.5). The heatmaps were generated using the Matplotlib and Seaborn libraries. NI values above 90% are represented by different shades of blue, yellow and red, with blue being the values closest to 0.9 and red being the values closest to 1.



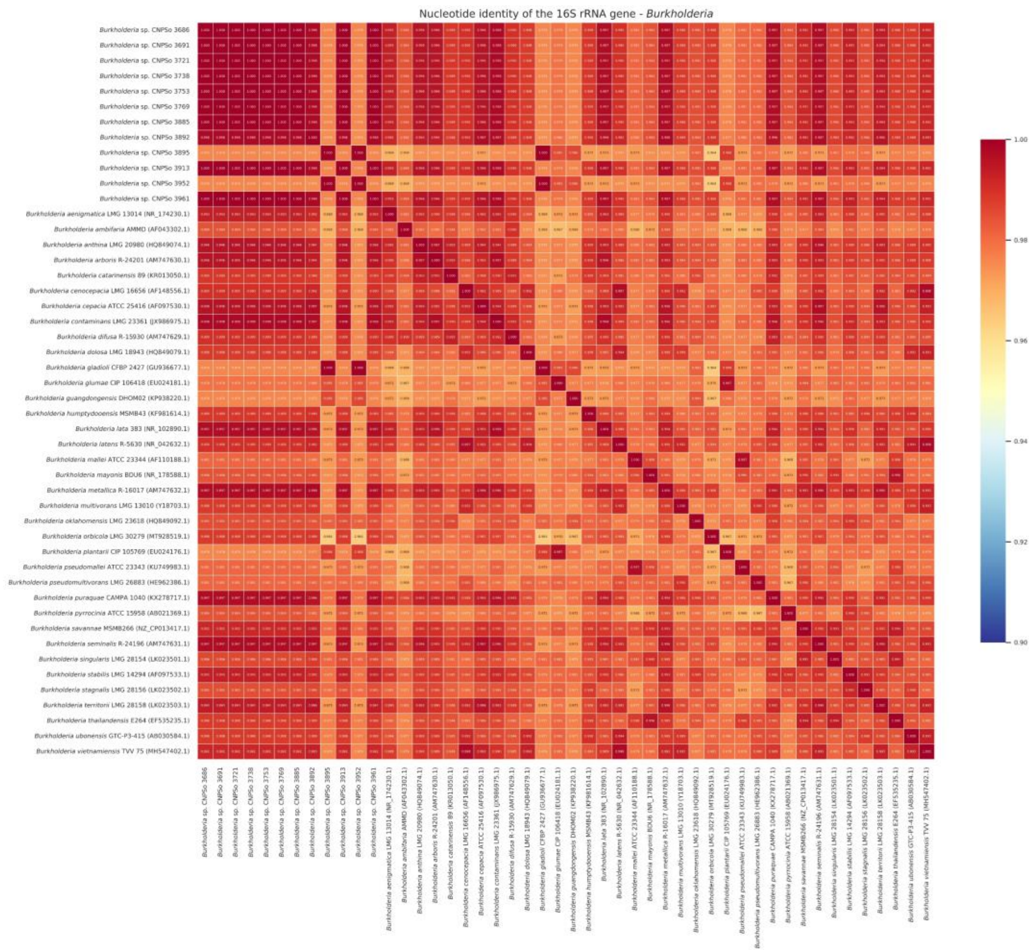
29

30 **Fig. S4** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
31 the genus *Ancylobacter* obtained through a similarity matrix built in Bioedit
32 version (v. 7.2.5). The heatmap were generated using the Matplotlib and Seaborn
33 libraries. NI values above 90% are represented by different shades of blue, yellow
34 and red, with blue being the values closest to 0.9 and red being the values closest
35 to 1.



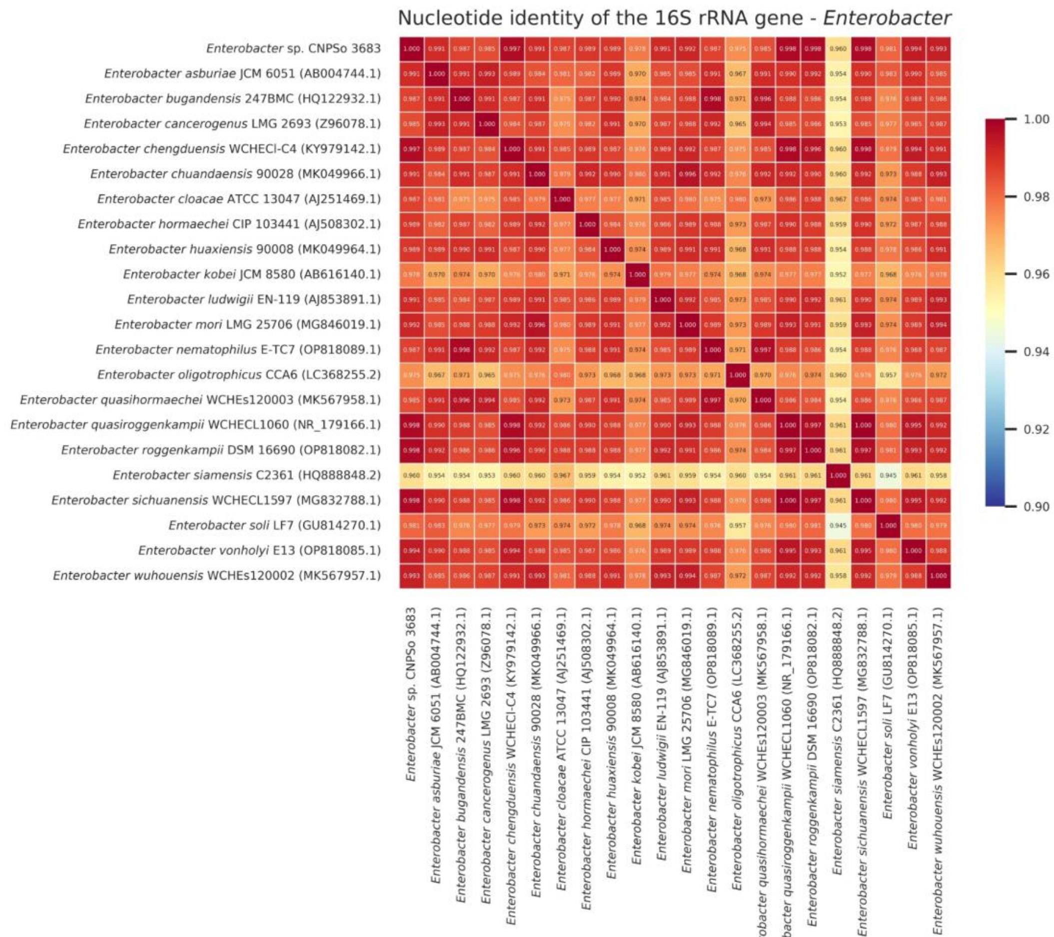
36

37 **Fig. S5** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
38 the genus *Paraburkholderia* obtained through a similarity matrix built in Bioedit
39 version (v. 7.2.5). The heatmap were generated using the Matplotlib and Seaborn
40 libraries. NI values above 90% are represented by different shades of blue, yellow
41 and red, with blue being the values closest to 0.9 and red being the values closest
42 to 1.



43

44 **Fig. S6** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
45 the genus *Burkholderia* obtained through a similarity matrix built in Bioedit version
46 (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn libraries.
47 NI values above 90% are represented by different shades of blue, yellow and red,
48 with blue being the values closest to 0.9 and red being the values closest to 1.
49



50

51 **Fig. S7** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of

52 the genus *Enterobacter* obtained through a similarity matrix built in Bioedit version

53 (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn libraries.

54 NI values above 90% are represented by different shades of blue, yellow and red,

55 with blue being the values closest to 0.9 and red being the values closest to 1.

56

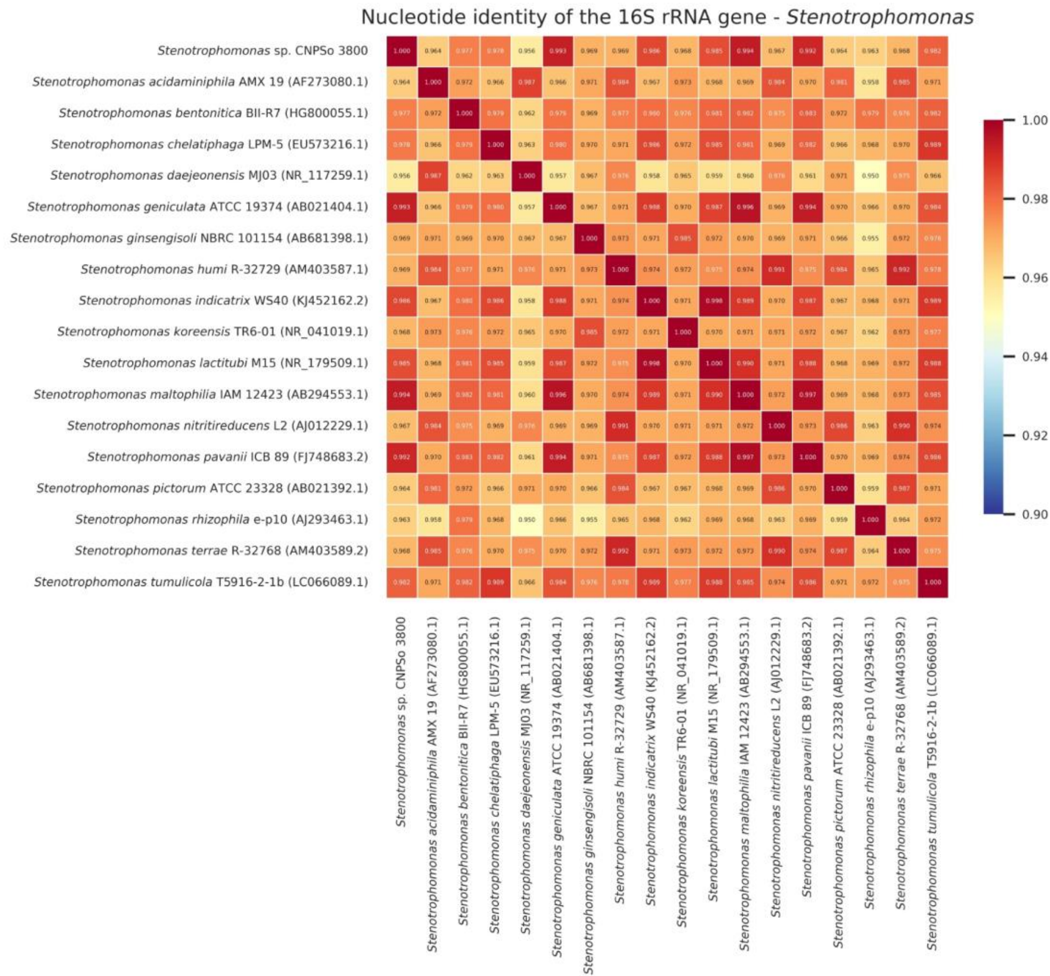
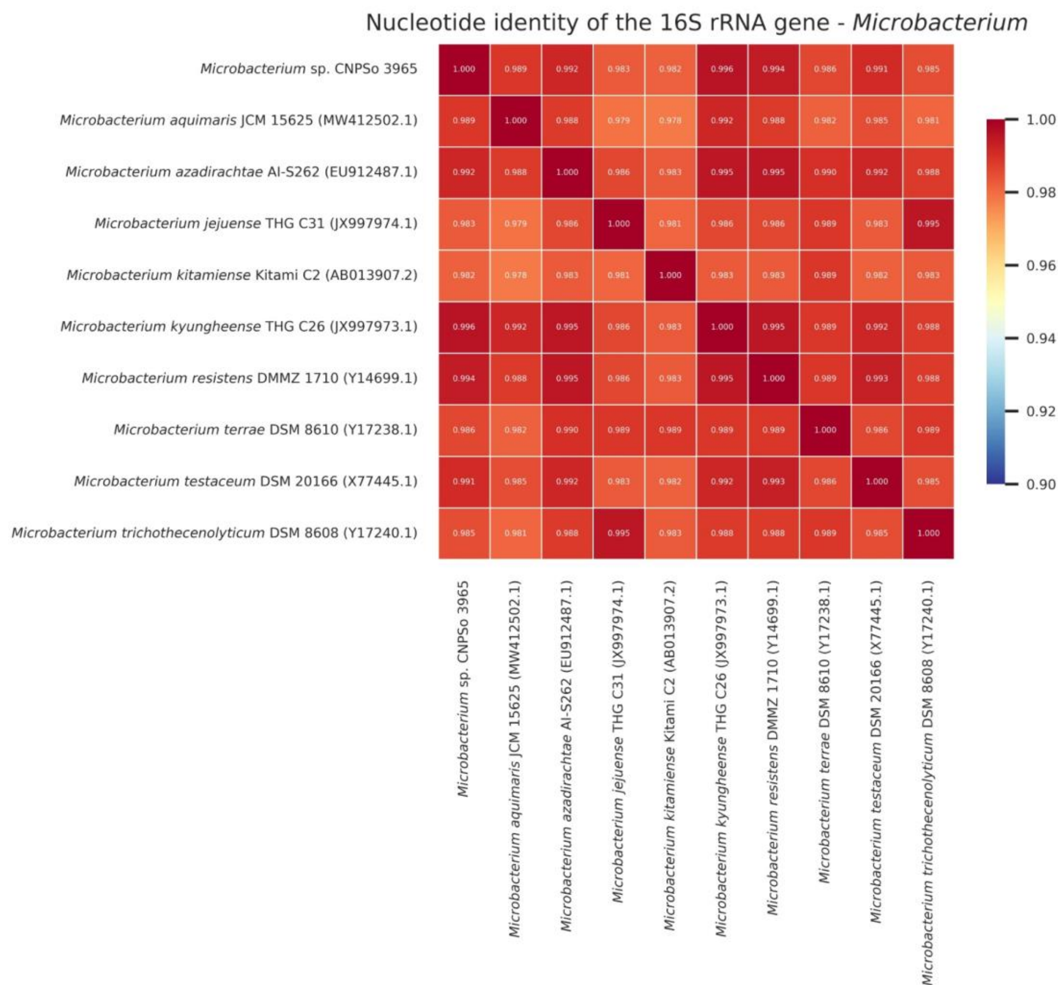


Fig. S8 Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of the genus *Stenotrophomonas* obtained through a similarity matrix built in Bioedit version (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn libraries. NI values above 90% are represented by different shades of blue, yellow and red, with blue being the values closest to 0.9 and red being the values closest to 1.



65

66 **Fig. S9** Heatmap of the Nucleotide Identity (NI) values of the 16S rRNA gene of
67 the genus *Microbacterium* obtained through a similarity matrix built in Bioedit
68 version (v. 7.2.5). The heatmap was generated using the Matplotlib and Seaborn
69 libraries. NI values above 90% are represented by different shades of blue, yellow
70 and red, with blue being the values closest to 0.9 and red being the values closest
71 to 1.

72

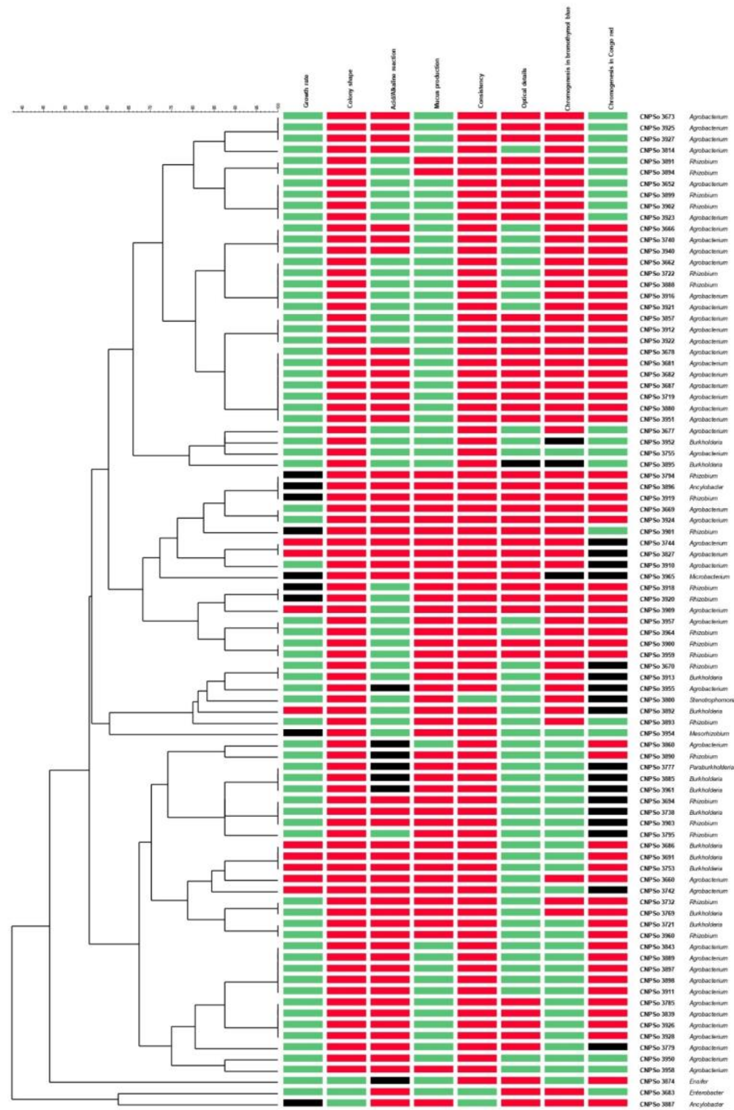


Fig. S10 Phenotypic dendrogram based on the morphophysiological characteristics of the 89 strains in study. Dendrogram was built on Bionumerics software (v. 7.6.3) using the UPGMA algorithm and similarity coefficient of categorical values).

6. CAPÍTULO II

A notável diversidade de microssimbiontes de rizóbios de feijão comum (*Phaseolus vulgaris* L.) no Mato Grosso do Sul, Região Centro-Oeste do Brasil, revelando novas espécies

https://doi.org/10.1007/s00203-023-03667-w

RESUMO

O feijão comum (*Phaseolus vulgaris*) é considerado uma leguminosa de grande importância socioeconômica, sendo capaz de estabelecer simbiose com uma grande variedade de espécies de rizóbios. No entanto, essa leguminosa também tem sido reconhecida por sua baixa eficiência na fixação biológica de nitrogênio. O Brasil é considerado pela sua alta biodiversidade e, em um estudo anterior, identificamos 13 estirpes isoladas de nódulos de feijão comum em três biomas do estado do Mato Grosso do Sul, região Centro-Oeste do Brasil, que podem representar novos grupos filogenéticos, merecendo melhor caracterização polifásica. A árvore filogenética do gene 16S RNAr dividiu as 13 estirpes em dois grandes clados, sete no *Rhizobium etli* e seis no *Rhizobium tropici*. A análise de sequência *multilocus* (*Multilocus Sequence Analysis* - MLSA) com quatro genes *housekeeping* (*glnII*, *gyrB*, *recA* e *rpoA*) confirmou a alocação filogenética das estirpes na árvore do gene 16S RNAr. Comparações genômicas indicaram oito linhagens em cinco prováveis novas espécies e as cinco restantes como *Rhizobium phaseoli*. A identidade média de nucleotídeos (*Average Nucleotide Identity* - ANI) e a hibridização DNA-DNA digital (HDDd) comparando as supostas novas espécies e os vizinhos mais próximos variaram de 81,84 a 92,50% e 24,0 a 50,7%, respectivamente. Outras características fenotípicas, genotípicas e simbióticas foram avaliadas. Outras características fenotípicas, genotípicas e simbióticas foram avaliadas. Além disso, algumas linhagens dos clados *R. etli* e *R. tropici* perderam sua capacidade de nodulação. Os resultados obtidos apoiam a descrição das novas espécies: “*Rhizobium cerradonense*” sp. nov. CNPSo 3464^{T}, “*Rhizobium atlanticum*” sp. nov. CNPSo 3490^{T}, “*Rhizobium*

aureum" sp. nov. CNPSo 3968^T, "*Rhizobium pantanalense*" sp. nov. CNPSo 4039^T e "*Rhizobium centroccidentale*" sp. nov. CNPSo 4062^T.

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ORIGINAL PAPER

The outstanding diversity of rhizobia microsymbionts of common bean (*Phaseolus vulgaris* L.) in Mato Grosso do Sul, central-western Brazil, revealing new *Rhizobium* species

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Abstract
Common bean is considered a legume of great socioeconomic importance, capable of establishing symbioses with a wide variety of rhizobial species. However, the legume has also been recognized for its low efficiency in fixing atmospheric nitrogen. Brazil is a hotspot of biodiversity, and in a previous study, we identified 13 strains isolated from common bean (*Phaseolus vulgaris*) nodules in three biomes of Mato Grosso do Sul state, central-western Brazil, that might represent new phylogenetic groups, deserving further polyphasic characterization. The phylogenetic tree of the 16S rRNA gene split the 13 strains into two large clades, seven in the *R. etli* and six in the *R. tropici* clade. The MLSA with four housekeeping genes (*glnII*, *gyrB*, *recA*, and *rpoA*) confirmed the phylogenetic allocation. Genomic comparisons indicated eight strains in five putative new species and the remaining five as *R. phaseoli.* The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) comparing the putative new species and the closest neighbors ranged from 81.84 to 92.50% and 24.0 to 50.7%, respectively. Other phenotypic, genotypic, and symbiotic features were evaluated. Interestingly, some strains of both *R. etli* and *R. tropici* clades lost their nodulation capacity. The data support the description of the new species *Rhizobium cerradonense* sp. nov. (CNPSo 3464^T), *Rhizobium atlanticum* sp. nov. (CNPSo 3490^T), *Rhizobium aureum* sp. nov. (CNPSo 3968^T), *Rhizobium pantanalense* sp. nov. (CNPSo 4039^T), and *Rhizobium centroccidentale* sp. nov. (CNPSo 4062^T).

Keywords *Rhizobium* · Biological nitrogen fixation · Diazotrophic bacteria · Polyphasic taxonomy · Housekeeping genes · MLSA

Communicated by Yusuf Akhter.

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Introduction

Several legume species belonging to the family Fabaceae (=Leguminosae) can fix atmospheric nitrogen (N_2) through the association with symbiotic diazotrophic bacteria collectively called "rhizobia" (de Bruijn 2015; Peix et al. 2015; Raza et al. 2020; Hungria and Nogueira 2023). The rhizobium–legume association promotes the formation of specific organs in plant roots and occasionally in stems, called nodules, where the biological nitrogen fixation (BNF) process takes place (de Bruijn 2015; Masson-Boivin and Sachs 2018; Wang et al. 2018; Hungria and Nogueira 2023). The nitrogen (N) fixed in an efficient association can meet the needs of several legumes of economic, social, and environmental importance, minimizing the use of chemical fertilizers (Telles et al. 2023). However, the symbioses vary in their effectiveness in fixing N_2, and common bean (*Phaseolus vulgaris* L.) has been long recognized for its low potential (Graham 1981; Peoples et al. 2021). Limitations may rely on several factors regarding the bacteria, the host plant, and the environment, for example, lower tolerance to abiotic stresses (Dwivedi et al. 2015; Shamseldin and Velázquez 2020). A main factor contributing to this low effectiveness is the high promiscuity of the common bean associated with a variety of indigenous strains (Ribeiro et al. 2009; Gomes et al. 2015) that are very competitive but, in general, with low capacity of BNF (Graham 1981; Gomes et al. 2015; Shamseldin and Velázquez 2020; Moura et al. 2022). Therefore, studies focused on the genetic diversity and efficiency of BNF of common bean rhizobia play an important role in establishing long-term strategies aimed at increasing crop's productivity based on BNF (Gomes et al. 2015; Santos et al. 2019; Goyal et al. 2021).

In a previous study, we performed an initial characterization of 82 isolates from common bean nodules collected in 14 municipalities of Mato Grosso do Sul state, central-western region of Brazil, comprising three biomes (Moura et al. 2022). Despite the high diversity pointed out in all 82 strains from the previous study (Moura et al. 2022), we identified and selected 13 *Rhizobium* strains that might represent new species to conduct a more robust analysis using a polyphasic approach. We emphasized genetic analyses and multilocus sequence analysis (MLSA), and those based on next-generation sequencing (NGS), average nucleotide identity (ANI), and digital DNA–DNA hybridization (dDDH), that should soon represent the basis of the taxonomy of prokaryotes (Chun et al. 2018; Helene et al. 2022). Although Brazil is not the genetic center of the common bean (Gepts and Debouck 1991), the legume has grown in the country for centuries and an amazingly high diversity of rhizobia microsymbionts was confirmed in this study, pointing out five new species.

Materials and methods

Strains and culture conditions

The study comprised 13 *Rhizobium* strains isolated from nodules of common bean (*Phaseolus vulgaris* L.) (Table 1), selected from 82 isolates from a previous study (Moura et al. 2022). The strains were chosen as representatives of the high genetic diversity described in the previous study, as they might represent putative new phylogenetic groups.

The 13 strains were isolated in seven different municipalities of Mato Grosso do Sul state, central-western region of Brazil, covering three biomes (Cerrado, Pantanal, and Mata Atlântica), as shown in Table 1. The main features of the three biomes were given by Kaschuk et al. (2011). Basically, the Cerrado occupies about 20% of the Brazilian lands and is characterized by a gradient of grassland savannah to forest, encompassing about 5% of the world diversity.

Table 1 Strains used in this study

Strains	Geographical origin	Brazilian biome
Rhizobium cerradense CNPSo 3464^T	Bataguassu, Brazil	Cerrado
Rhizobium atlanticum CNPSo 3490^T	Itaquiraí, Brazil	Mata Atlântica
Rhizobium aureum CNPSo 3968^T	Dourados, Brazil	Mata Atlântica
Rhizobium atlanticum CNPSo 3982	Itaporã, Brazil	Mata Atlântica
Rhizobium phaseoli CNPSo 3993	Maracajú, Brazil	Cerrado
Rhizobium phaseoli CNPSo 3997	Maracajú, Brazil	Cerrado
Rhizobium phaseoli CNPSo 4005	Rio Brilhante, Brazil	Cerrado
Rhizobium phaseoli CNPSo 4007	Maracajú, Brazil	Cerrado
Rhizobium centroccidentale CNPSo 4033	Rio Brilhante, Brazil	Cerrado
Rhizobium pantanalense CNPSo 4039^T	Aquidauana, Brazil	Pantanal
Rhizobium phaseoli CNPSo 4057	Dourados, Brazil	Mata Atlântica
Rhizobium centroccidentale CNPSo 4062^T	Dourados, Brazil	Mata Atlântica
Rhizobium centroccidentale CNPSo 4063	Dourados, Brazil	Mata Atlântica

Pantanal is the largest tropical wetland in the world, subject to seasonal inundation and desiccation and occupies 1.76% of the National territory. Mata Atlântica, which comprises 15% of the National territory, is composed of native forests and is characterized by high diversity (Brasil 2023).

All CNPSo (Centro Nacional de Pesquisa de Soja) strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection No. 1213, WDCM Collection No. 1054), Londrina, Paraná, Brazil. They are also deposited at the "Johanna Döbereiner Biological Resource Center" (CRB-JD) (WFCC Collection No. 364), Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil.

The strains were grown in a modified yeast extract–mannitol agar (YMA) medium (Hungria et al. 2016) at 28 °C for 3 days. Stock cultures were maintained in modified YMA at 4 °C, and for long-term storage, they were cryopreserved in liquid mannitol-modified yeast extract (YM) medium with 30% (v/v) glycerol at − 80 °C and − 150 °C and lyophilized.

Phylogeny

Partial sequences of the 16S rRNA and *glnII* genes were obtained from our previous study (Moura et al. 2022). In this study, we performed PCR amplification and sequencing of three housekeeping genes (*gyrB*, *recA*, and *rpoA*).

Total genomic DNA of the strains was extracted using the kit DNeasy Blood and Tissue (Qiagen), following the manufacturer's recommendations. Total DNA was used to perform PCR amplification, sequencing of the three housekeeping genes and the genomic analyses.

Amplification of the *gyrB*, *recA*, and *rpoA* genes was performed using specific primers and conditions shown in Table S1. The amplicons were purified with the PureLink kit (Invitrogen), following the manufacturer's instructions, and subsequently sequenced in an ABI 3500XL (Applied Biosystems) sequencer as described before (Delamuta et al. 2017). All sequences are deposited in the GenBank database (NCBI—National Center for Biotechnology Information), and the accession numbers are listed in parentheses in the phylogenetic trees and in Table S2.

Multiple sequence alignment was obtained with MUSCLE (Edgar 2004), the best evolutionary distance model was determined considering the lowest Bayesian information criterion score (BIC) (Schwarz 1978) for maximum likelihood (ML) (Felsenstein 1981), and the phylogenetic trees were built in the MEGA software (Molecular Evolutionary Genetics Analysis, version 7.0) (Kumar et al. 2016).

For 16S rRNA phylogeny, the Tamura 3-Parameter model (Tamura and Nei 1993) with gamma-distributed invariant sites (G+I) was used (Tamura 1992). MLSA (*glnII*, *gyrB*, *recA*, and *rpoA*) was constructed with the distance model General Time Reversible (Waddell and Steel 1997) and gamma-distributed invariant sites (G+I).

The statistical support for the phylogenetic trees was estimated by the bootstrap analysis (Felsenstein 1985) with 1000 replicates (Hedges 1992). *Bradyrhizobium japonicum* USDA 6^T was used as an outgroup. Nucleotide Identity (NI) was calculated with BioEdit Sequence Alignment Editor software (v.7.2.5) (Hall 1999).

For the MLSA, housekeeping gene sequences were manually concatenated. Accession numbers of gene sequences from this study and/or retrieved from the GenBank database are shown in the phylograms and in Table S2.

Genomes features

For genome analysis, total DNAs of strains CNPSo 3464^T, CNPSo 3490^T, CNPSo 3968^T, CNPSo 4007, CNPSo 4039^T, and CNPSo 4062^T were used to build libraries using the Nextera XT kit, following the manufacturer's instructions. Genome sequencing was performed on the NextSeq 1000 platform (Illumina) at Embrapa Soja. The assembly was performed with the pipeline A5-MiSeq (de novo) version 20140604. The pipeline consists of the steps of de read cleaning, contig assembly, crude scaffolding, misassembly correction, and final scaffolding (Coil et al. 2014); sequence adapters and low-quality regions are removed by Trimmomatic (Lohse et al. 2012), and errors in the reads are corrected with SGA's (String Graph Assembler) k-mer-based error correction algorithm (Simpson and Durbin 2012). Genome sizes were estimated with Rapid Annotation using Subsystem Technology (RAST v.2.0) (Aziz et al. 2008; Overbeek et al. 2014) using default parameters, and the sequences were deposited in the NCBI database. The genomes of the types strains *R. bangladeshense* BLR175^T (NZ_CP071612.1), *R. dioscoreae* S-93^T (NZ_BLAJ01000001.1), *R. esperanzae* CNPSo 668^T (NZ_MXPU01000001.1), *R. etli* CFN 42^T (NC_007761.1), *R. freirei* PFR 81^T (NZ_AQHN01000095.1), *R. hainanense* CCBAU 57015^T (NZ_FMAC01000030.1), *R. jaguaris* CCGE525^T (NZ_CP032694.1), *R. leguminosarum* USDA 2370^T (NZ_QBLB01000030.1), *R. leucaenae* CFN 299^T (NZ_LNCJ01000001.1), *R. miluonense* HAMBI 2971^T (NZ_FMAH01000103.1), *R. phaseoli* ATCC 14482^T (NZ_ML133565.1), and *R. tropici* CIAT 899^T (NC_020059.1) were retrieved from the GenBank database. As the genome of the type strain of *R. paranaense* PRF 35^T was not available, we used the genome of the *R. paranaense* strain SEMIA 4064 (NZ_JACHBI010000001.1) to perform the genomic comparisons. To confirm the taxonomic position of strain SEMIA 4064, we retrieved the *glnII*, *gyrB*, *recA*, and *rpoA* genes from its genome and performed an MLSA comparison with the genes of the type strain PRF 35^T; they share 99.8%

of identity, confirming that both belong to the *R. paranaense* species.

On each phylogenetic tree, the strains from this study were positioned close to *R. acidisoli* FH13^T, *R. calliandrae* CCGE524^T, and *R. mayense* CCGE526^T; therefore, the comparison was also made with these genomes. However, as the genome of the type strain FH13^T of *R. acidisoli* is not available, we used the genome of strain FH23 (NZ_CP034998.1). The genomes of *R. calliandrae* CCGE524^T (JARFYN000000000.1) and *R. mayense* CCGE526^T (JARFYM000000000.1) were sequenced for this study and were deposited at the NCBI (Moura et al. 2023).

ANI (Average Nucleotide Identity) analyses were evaluated with the ANI calculator (Rodriguez and Konstantinidis 2016) (available at: <http://enve-omics.ce.gatech.edu/ani/>). An in silico comparison for DDH was performed via digital DNA–DNA hybridization (dDDH) (Meier-Kolthoff et al. 2013, 2014) using Genome-to-Genome Distance Calculator (GGDC) version 3.0, and the 'formula 2' (identities/high-scoring pairs length), available at: <https://ggdc.dsmz.de/distcalc2.php>). The G+C DNA contents of CNPSo 3464^T, CNPSo 3490^T, CNPSo 3968^T, CNPSo 4007, CNPSo 4039^T, and CNPSo 4062^T strains were calculated using the SEED platform (Overbeek et al. 2014).

Phenotypic characterization in vitro

The morphophysiological characteristics of the 13 strains were accessed as described by Moura et al. (2022), and except where indicated, they were evaluated in a modified YMA medium (Hungria et al. 2016) at 28 °C. The characteristics evaluated included: growth at pH 4.0 and 8.0, growth at 37 °C and 40 °C, in 1% NaCl, the ability to grow on solid Luria–Bertani (LB) medium, and evaluation of urease activity using 2% urea and phenol red as an indicator (Hungria et al. 2016). All tests were performed in triplicate.

Carbohydrate metabolism was determined with the kit API 50CH (BioMérieux), using modified YM without mannitol with bromothymol blue as an indicator. Tolerance to antibiotics was performed by the disk-diffusion method (Bauer et al. 1966) with ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U), streptomycin (10 µg), and tetracycline (30 µg). Carbohydrate use and antibiotics were evaluated in duplicate for the type strains CNPSo 3464^T, CNPSo 3968^T, CNPSo 4039^T, and CNPSo 4062^T, and compared with the type strains of the phylogenetically closest species *R. dioscoreae* S-93^T, *R. esperanzae* CNPSo 668^T, *R. etli* CFN 42^T, *R. freirei* PFR 81^T, *R. hainanense* CCBAU 57015^T, *R. leucaenae* CFN 299^T, *R. paranaense* PRF 35^T, *R. phaseoli* ATCC 14482^T, and *R. tropici* CIAT 899^T.

Nitrogen fixation ability

Experiments to evaluate nodulation and nitrogen fixation efficiency were performed with the original host *Phaseolus vulgaris* and with *Macroptilium atropurpureum* (siratro), under controlled greenhouse conditions for 30 days. To improve germination, *M. atropurpureum* seeds were scarified with concentrated sulfuric acid for 5 min, and washed six times with sterile distilled water. Subsequently, the seeds of both legumes were superficially sterilized in 70% ethanol for 1 min, soaked in commercial sodium hypochlorite (0.4–0.6%) for 5 min, and washed six times in sterile distilled water. Common bean and siratro seeds were pre-germinated on germitest paper and 1% water agar, respectively, for 3 days at 28 °C. Tests were conducted in sterile polypropylene bags containing germitest paper (Moura et al. 2022) and in sterilized Leonard jars containing sand and charcoal (2:1, v:v), both with N-free nutrient solution (Yates et al. 2016). The 13 strains were grown in a modified YM medium (Hungria et al. 2016) and inoculated on seeds (1 mL per plant) after transplanting. The experiments were conducted with three replicates and included the negative control without inoculation and the positive control inoculated with *Rhizobium tropici* CIAT 899^T. The nitrogen fixation efficiency of the nodules was evaluated by the red or pink color in nodule cross sections, indicating active leghemoglobin, as well as the green (efficient) or yellow (inefficient) color of the shoots.

Results

Phylogenetic analysis of housekeeping genes

A 16S rRNA phylogenetic tree was built with all 109 validly named *Rhizobium* species according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (available at: http://www.bacterio.net) at the time of writing (06/2023) (Fig. S1). The 13 strains from our study were split in two major clades. The *R. etli/R. phaseoli/R. leguminosarum*, called as *R. etli* clade, clustered strains CNPSo 3490^T, CNPSo 3982, CNPSo 3993, CNPSo 3997, CNPSo 4005, CNPSo 4007, and CNPSo 4057 with 24 type strains, sharing Nucleotide Identity (NI) ranging from 98.7 to 100%. The second clade, known as *R. tropici*, clustered strains CNPSo 3464^T, CNPSo 3968^T, CNPSo 4033, CNPSo 4039^T, CNPSo 4062^T, and CNPSo 4063 sharing from 96 to 100% NI with 15 type strains. Based on 16S rRNA phylogeny, the closest described species were selected and used for further analyses.

Four single and concatenated housekeeping genes were analyzed to perform a more robust analysis and clarify the phylogenetic position of the strains at the species level.

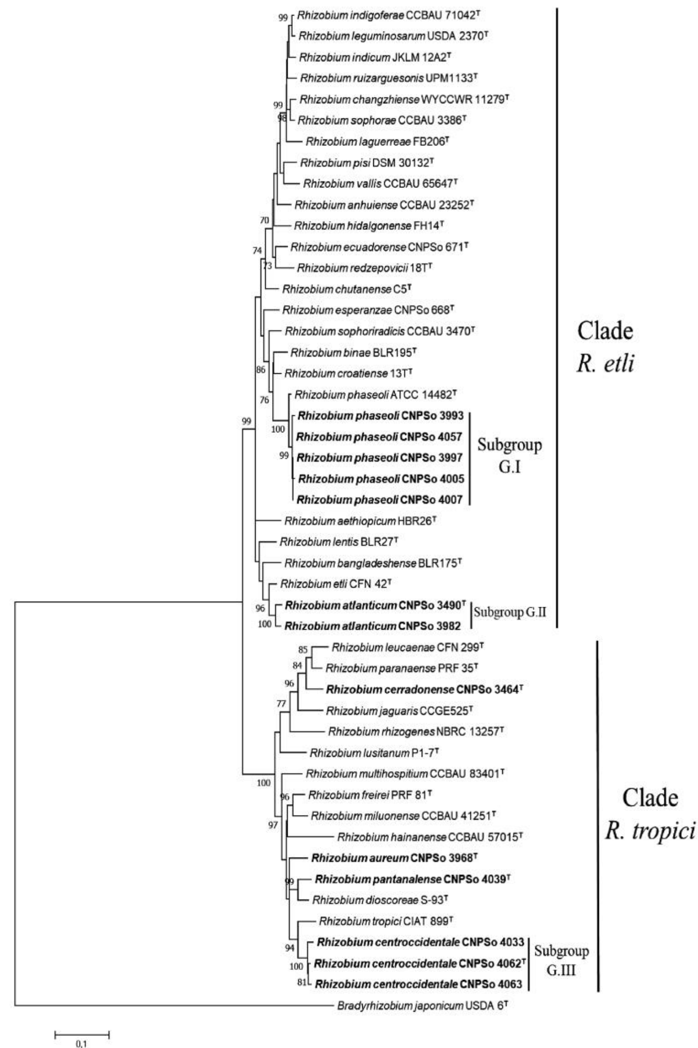

Fig. 1 Maximum Likelihood (ML) phylogeny based on the concatenated aligned sequences (1795 bp) of the *glnII* (343 bp) + *gyrB* (471 bp) + *recA* (382 bp) + *rpoA* (599 bp) genes using General Time Reversible + G + I model, showing the relationships of the strains in study (in bold) with related described *Rhizobium* species. Accession numbers are indicated in Table S2. Bootstrap values >70% are indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^T was used as outgroup. Bar indicates one substitution per 100 nucleotide positions

Single trees for the *glnII* (343 bp), *gyrB* (471 bp), *recA* (382 bp), and *rpoA* (599 bp) genes were constructed and are presented as supplementary material (Figs. S2–S5, respectively). In general, single trees were congruent with the 16S rRNA (Fig. S1), and with the concatenated analysis (Fig. 1). Only two discrepancies were observed, one in the *recA* phylogeny with the species *R. aquaticum* SA-276^T, and another in the *rpoA* phylogeny with *R. hainanense* CCBAU 57015^T, where both occupied isolated positions.

The MLSA phylogeny of the concatenated *glnII*, *gyrB*, *recA*, and *rpoA* housekeeping genes (1795 bp) was performed with the 13 strains and the closest *Rhizobium* species (Fig. 1) selected based on the 16S rRNA phylogeny (Fig. S1). Using the primers and amplification conditions

shown in Table S1, we were unable to amplify the *gyrB* gene of CNPSo 3490^{T} and CNPSo 3968^{T}, and the *rpoA* gene of CNPSo 3464^{T}; therefore, their sequences were retrieved from the genomes. The concatenated phylograms confirmed the phylogenetic position of the strains in the *R. etli* and *R.*

Table 2 Nucleotide sequence identity (% NI) between strains from this study and the closest *Rhizobium* type strains

Nucleotide identity (%)						
Strains	16S rRNA (930 bp)	*glnII* (343 bp)	*gyrB* (471 bp)	*recA* (382 bp)	*rpoA* (599 bp)	MLSA (1795 bp)
Rhizobium cerradonense CNPSo 3464^{T}						
Between strain and type strains						
R. leucaenae CFN 299^{T}	99.4	95.0	90.6	93.1	98.3	94.5
R. paranaense PRF 35^{T}	98.8	96.7	90.6	93.1	98.6	94.3
R. jaguaris CCGE525^{T}	99.5	90.6	90.0	92.9	98.4	93.5
R. rhizogenes NBRC 13257^{T}	99.6	90.6	86.6	92.3	96.6	91.9
Rhizobium atlanticum CNPSo 3490^{T} and CNPSo 3982						
Among strains	99.7	96.4	99.1	99.4	97.4	98.1
Between strains and type strains						
R. etli CFN 42^{T}	99.6	94.4–96.1	96.3–96.5	96.6	97.4–98.6	96.8–96.9
R. esperanzae CNPSo 668^{T}	97.5–97.5	94.4–97.3	92.2–93.1	91.9–92.7	97.1–98.6	94.6–94.9
R. bangladeshense BLR175^{T}	99.5	93.8–94.4	93.1–93.3	92.5	95.8–97.3	94.4–94.8
R. aethiopicum HBR26^{T}	99.7	92.3–93.5	93.3–93.5	92.9	96.9–97.1	94.4–94.5
Rhizobium aureum CNPSo 3968^{T}						
Between strains and type strains						
R. dioscoreae s-93^{T}	97.9	96.4	89.5	97.1	97.9	95.3
R. centroccidentale CNPSo 4033, 4062^{T} and CNPSo 4063	99.3	94.7–96.4	89.3–90.2	94.4–96.0	97.9–98.3	94.5–95.2
R. pantanalense CNPSo 4039^{T}	99.1	95.0	90.0	95.5	97.6	94.7
R. freirei PRF 81^{T}	99.3	95.6	90.4	94.7	97.1	94.5
R. miluonense CCBAU 41251^{T}	99.6	95.6	89.5	94.7	97.4	94.4
Rhizobium phaseoli CNPSo 3993, CNPSo 3997, CNPSo 4005, CNPSo 4007, and CNPSo 4057						
Among strains	99.5–100	100	98.9–100	98.1–100	100	99.3–100
Between strains and type strains						
R. phaseoli ATCC 14482^{T}	99.5–100	97.9	98.0–99.1	97.9–99.4	98.8	98.5–98.8
R. croatiense 13T^{T}	99.2–99.6	91.8	94.2–95.3	96.0–96.8	98.6	95.8–96.0
R. binae BLR195^{T}	99.3–99.5	92.6	93.8–94.6	93.4–94.2	97.8	95.0–95.2
Rhizobium pantanalense CNPSo 4039^{T}						
Between strains and type strains						
R. dioscoreae s-93	97.7	96.1	94.6	94.2	98.9	96.3
R. aureum CNPSo 3968^{T}	99.1	95.0	90.0	95.5	97.6	94.7
R. centroccidentale CNPSo 4033, 4062^{T} and CNPSo 4063	98.8	96.1–97.3	88.9–89.5	93.9–95.8	96.6–96.9	94.3–94.4
R. freirei PRF 81^{T}	98.8	95.0	91.2	94.2	95.8	94.1
R. miluonense CCBAU 41251^{T}	99.2	93.8	92.3	93.1	96.3	94.1
Rhizobium centroccidentale CNPSo 4033, 4062^{T} and CNPSo 4063						
Among strains	99.8–100	96.1–98.8	98.3–100	97.3–99.7	99.3–99.4	98.3–99
Between strains and type strains						
R. tropici CIAT 899^{T}	99.8–100	94.1–95.3	94.2–95.1	93.7–93.9	96.9–97.3	95.2–95.4
R. aureum CNPSo 3968^{T}	99.3	94.7–96.4	89.3–90.2	94.4–96.0	97.9–98.3	94.5–95.2
*R. freire*i PFR 81^{T}	99.8–100	95.3	90.6–91.5	92.9–94.2	97.4–97.6	94.5–94.7
R. pantanalense CNPSo 4039^{T}	98.8	96.1–97.3	88.9–89.5	93.9–95.8	96.6–96.9	94.3–94.4
R. hainanense CCBAU 57015^{T}	99.5	95.3–96.4	90.6–91.9	94.7–97.1	87.8–88.1	91.8–92.4

Similarities based on the sequence identity of the 16S rRNA, and of single and concatenated housekeeping genes (*glnII*, *gyrB*, *recA* and *rpoA*)

tropici clades (Fig. 1), as observed in the 16S rRNA phylogeny (Fig. S1).

Seven strains of our study were positioned in two subgroups of the *R. etli* clade. In subgroup G.I the strains CNPSo 3993, CNPSo 3997, CNPSo 4005, CNPSo 4007, and CNPSo 4057 were grouped with statistical support of 99% (Fig. 1), sharing from 99.3 to 100% NI among each other, having *R. phaseoli* ATCC14482^T as the closest species with statistical support of 100% and sharing from 98.5 to 98.8% NI (Table 2). The subgroup G.II clustered strains CNPSo 3490^T and CNPSo 3982 with statistical support of 100%, sharing among each other 98.1% NI, and 96.8% and 96.9%, respectively, with the closest species *R. etli* CFN 42^T.

Regarding the six strains positioned in the *R. tropici* clade, CNPSo 3464^T occupied an isolated position close to *R. leucaenae* CFN 299^T, *R. paranaense* PRF 35^T, and *R. jaguaris* CCGE525^T (Fig. 1) presenting high values of statistical support and NI of 94.5, 94.3 and 93.5%, respectively (Table 2). Strain CNPSo 3968^T occupied an isolated position, being closer to *R. dioscoreae* S-93^T (Fig. 1), sharing 95.3% of NI (Table 2). Strain CNPSo 4039^T also occupied an isolated position, closer to *R. dioscoreae* S-93^T, showing 96.3% of NI. Finally, the subgroup G.III included strains CNPSo 4033, CNPSo 4062^T, and CNPSo 4063 with statistical support of 100% (Fig. 1), sharing from 98.3 to 99% of NI among themselves, being closest to *R. tropici* CIAT 899^T, sharing from 95.2 to 95.4% of NI with statistical support of 94% (Table 2).

Genomes features

The genomes of the putative new type strains were sequenced and deposited at the NCBI with the accession numbers: CNPSo 3464^T (JAQJCK000000000), CNPSo 3490^T (JAQJCJ000000000), CNPSo 3968^T (JAQJCI000000000), CNPSo 4039^T (JAQJCH000000000), and CNPSo 4062^T (JAQJCG000000000); we also sequenced the genome of strain CNPSo 4007 (JAQJCF000000000), which was included in the analyses.

Details about the genome size, number of contigs, N_{50}, coverage, and G+C content are shown in Table 3. Genome sizes ranged from 6,404,365 to 7,104,401 bp, with G+C content ranging from 59.6 to 61.2 mol% (Table 3).

The closest species were selected for ANI and dDDH comparisons based on the MLSA analysis (Fig. 1, Table 2), in addition to four new rhizobial species recently described (Hsouna et al. 2023; Young et al. 2023). Genome comparison of strain CNPSo 3464^T and the closest species *R. leucaenae* CFN 299^T indicated 90.16% ANI and 41.3% dDDH (Table 4). Strain CNPSo 3490^T showed the highest genomic similarity (91.60% of ANI and 45.9% of dDDH) with *R. esperanzae* CNPSo 668^T. Strains CNPSo 3968^T and CNPSo 4039^T showed 88.68% of ANI and 36.5% of dDDH among themselves. With the closest species *R. dioscoreae* S-93^T, strains CNPSo 3968^T and CNPSo 4039^T presented 88.07 and 92.5% of ANI and 35.6% and 50.7% of dDDH, respectively. Strain CNPSo 4062^T shared highest values with CNPSo 4039^T (92.40% ANI and 50.5% dDDH) and 90.16% ANI and 41.8% dDDH with *R. tropici* CIAT 899^T. The genome of CNPSo 4007 was also sequenced and confirmed its relatedness with *R. phaseoli* ATCC 14482^T, showing 97.03% ANI and 74.2% dDDH (Table 4).

Phenotypic characterization in vitro

The 13 strains were positive or weak for growth under acidic conditions (pH 4.0), temperature of 37 °C, 2% urea, and 1% NaCl, except for CNPSo 4057, which did not grow in 1% NaCl (Table S3). All strains were able to grow at pH 8.0. Differential phenotypic features between the strains from this study and closer species are described in Table S3.

Concerning carbohydrate metabolism, all strains were able to use glycerol and d-mannitol as carbon sources (Table S4). Five strains, CNPSo 3464^T, CNPSo 3490^T, CNPSo 3968^T, CNPSo 4039^T, and CNPSo 4062^T were tolerant or sensitive to the antibiotics ampicillin, bacitracin, cefuroxime, chloramphenicol, erythromycin, nalidixic acid, penicillin G, and tetracycline, while for neomycin and streptomycin were moderately tolerant or sensitive. Comparisons of carbon source utilization and antibiotic tolerance with the closest species are shown in Table S4.

Table 3 Genomic parameters of the strains sequenced in this study, including putative new *Rhizobium* species

Strain	Size (bp)	Contigs (number)	N50 (bp)	Coverage (fold)	G+C content (mol%)
R. cerradonense CNPSo 3464^T	7,104,401	87	397,015	145	59.6
R. atlanticum CNPSo 3490^T	6,734,954	94	240,017	159	60.8
R. aureum CNPSo 3968^T	7,027,168	51	345,964	171	59.7
R. phaseoli CNPSo 4007	7,040,350	155	151,452	134	61.2
R. pantanalense CNPSo 4039^T	7,152,469	75	483,536	154	59.6
R. centroccidentale CNPSo 4062^T	6,404,365	77	204,568	150	59.6

Table 4 DNA–DNA hybridization (dDDH) values among the strains of study and close related *Rhizobium* species and four new recently described

Strain used as reference		
R. cerradonense CNPSo 3464^T		
	ANI (%)	dDDH (%)
R. calliandrae CCGE524^T	87.29	33.0
R. jaguaris CCGE525^T	87.40	34.8
R. leucaenae CFN 299^T	90.16	41.3
R. mayense CCGE526^T	86.57	34.3
R. paranaense SEMIA 4064	89.80	40.5
R. atlanticum CNPSo 3490^T		
	ANI (%)	dDDH (%)
R. acaciae 1AS11^T	86.42	32.3
R. acidisoli FH23	86.89	33.2
R. aethiopicum HBR26^T	89.68	40.1
R. beringeri SM51^T	86.48	32.4
R. bangladeshense BLR175^T	89.23	39.2
R. brockwellii CC275e^T	86.33	32.2
R. esperanzae CNPSo 668^T	91.60	45.9
R. etli CFN 42^T	91.18	44.2
R. johnstonii 3841^T	86.46	32.5
R. leguminosarum USDA 2370^T	86.33	32.2
R. aureum CNPSo 3968^T		
	ANI (%)	dDDH (%)
R. calliandrae CCGE524^T	82.59	24.5
R. dioscoreae S-93^T	88.07	35.6
R. freirei PFR 81^T	86.01	30.8
R. mayense CCGE526^T	82.16	25.1
R. miluonense HAMBI 2971^T	85.24	29.9
R. pantanalense CNPSo 4039^T	88.68	36.5
R. centroccidentale CNPSo 4062^T	87.57	34.6
R. phaseoli CNPSo 4007		
	ANI (%)	dDDH (%)
R. acaciae 1AS11^T	86.99	33.5
R. beringeri SM51^T	87.01	33.5
R. binae BLR195^T	91.69	45.7
R. brockwellii CC275e^T	86.89	33.3
R. croatiense 13T^T	91.96	47.1
R. johnstonii 3841^T	86.97	33.4
R. leguminosarum USDA 2370^T	86.92	33.2
R. phaseoli ATCC 14482^T	97.03	74.2
R. pantanalense CNPSo 4039^T		
	ANI (%)	dDDH (%)
R. calliandrae CCGE524^T	82.27	24.1
R. dioscoreae S-93^T	92.50	50.7
R. freirei PFR 81^T	85.76	30.5
R. miluonense HAMBI 2971^T	85.04	29.5

Table 4 (continued)

R. pantanalense CNPSo 4039^T		
	ANI (%)	dDDH (%)
R. mayense CCGE526^T	81.90	24.6
R. aureum CNPSo 3968^T	88.68	36.5
R. centroccidentale CNPSo 4062^T	92.40	50.5
R. centroccidentale CNPSo 4062^T		
	ANI (%)	dDDH (%)
R. calliandrae CCGE524^T	82.30	24.0
R. hainanense CCBAU 57015^T	88.53	38.0
R. mayense CCGE526^T	81.84	24.8
R. tropici CIAT 899^T	90.16	41.8
R. aureum CNPSo 3968^T	87.57	34.6
R. centroccidentale CNPSo 4039^T	92.40	50.5

Nitrogen fixation ability and symbiotic genes

Six out of the 13 strains studied (CNPSo 3464^T, 3490^T, 3982, 3993, 3997, and 4005) were able to reinfect and nodulate the original host plant *Phaseolus vulgaris* L. As for *Macroptilium atropurpureum*, four strains (CNPSo 3464^T, 3982, 3993, and CNPSo 4005) formed nodules. All these strains, except for CNPSo 4005, formed efficient nitrogen-fixing nodules with pink or red internal coloration in both common bean and siratro (Fig. S8). The strains showing efficient nitrogen-fixing nodules presented shoots with green color. The other strains (CNPSo 3968^T, 4007, 4033, 4039, 4057, 4062^T, and 4063) were not able to form nodules in any of the two tested plants and resulted in shoots with yellow color.

The results were similar to the RAST annotation, which showed that the strains CNPSo 3464^T and 3490^T had the *nodABCDJS* and *nolO* genes related to nodulation and *nifABEHNQSTUWXZ* and *fixABCJLX* genes related to nitrogen fixation. Strain CNPSo 3968^T presented the *nolR*, *nifU*, and *fixJL* genes. Strain CNPSo 4007 showed *nodADLX* and *nolR* genes related to nodulation and *nifABEHNUX* and *fixJL* genes related to nitrogen fixation. In strains CNPSo 4039^T and 4062^T, no nodulation or nitrogen fixation-related genes were found, only *nolR* and *nifU* genes.

The two strains of this study that had the identifiable *nodC* gene (CNPSo 3464^T and 3490^T), according to the RAST annotation, were positioned in two different groups in the *nodC* tree (363 bp) (Fig. S6), showing statistical support of 100 and 92%, respectively, and NI of 100%, as shown in Table S5.

The *nifH* genes identified in three strains (CNPSo 3464^T, 3490^T, and 4007) were positioned in two different groups in the *nifH* tree (181 bp) (Fig. S7), showing statistical support of 100 and 96%, respectively, and NI of 100% (Table S5).

Discussion

In a previous study, the characterization of 82 isolates from common bean nodules representatives of three biomes of Mato Grosso do Sul, central-western Brazil, indicated high inter- and intraspecies diversity in the analysis of the 16S rRNA and BOX-PCR profiles (Moura et al. 2022). Now we proceeded with a more robust polyphasic approach using phenotypic, genotypic, and phylogenetic techniques (Rosselló-Móra and Amann 2015; Raina et al. 2019) of 13 *Rhizobium* strains from the previous study that were the most genetically divergent when compared to the type species.

Several studies performed with *Rhizobium* species reported their classification in two large clades, *R. etli* and *R. tropici* (Ramírez-Bahena et al. 2008; Saïdi et al. 2014; Zhang et al. 2015; Jorrin et al. 2020). In our study, the phylogenetic tree built with the 16S rRNA also positioned the 13 strains within these two clades. However, due to the predominance of conserved sites of the 16S rRNA gene, closely related species cannot be distinguished, restricting identification and requiring other conserved protein-coding genes analyses to clarify the phylogenetic relationship among the group (Helene et al. 2022; Rajkumari et al. 2022). The MLSA analyses have been helpful in defining phylogenetic groups and improving taxonomic definition, and the analysis of at least three housekeeping genes helps to prevent misinterpretation caused by recombination events and Horizontal Gene Transfer (HGT) (de Vos 2011; Glaeser and Kämpfer 2015; Chidebe et al. 2018; Helene et al. 2022). In our study, a better phylogenetic resolution was achieved with the concatenated partial sequences of the *glnII*, *gyrB*, *recA*, and *rpoA* housekeeping genes and allowed the identification of five new putative species.

A cut-off limit of 95–96% for species delineation in the analysis of at least three concatenated sequences of

housekeeping genes has been applied to the *Rhizobium* genus (Dall'Agnol et al. 2013; Zhang et al. 2015; Yan et al. 2017; Cordeiro et al. 2017; Jorrin et al. 2020). In our study, considering four housekeeping genes, the subgroup G.II of the *R. etli* clade, with strains CNPSo 3490^T and 3982, showed NI of 96.8 and 96.9%, respectively, with the closest specie. In the *R. tropici* clade, subgroup G.III with the strains CNPSo 4033, 4062^T, and 4063 showed from 95.2 to 95.4% NI, and the strains CNPSo 3968^T and 4039^T, which occupied isolated positions, showed NI of 95 and 95.9%, respectively; these values are in accordance with the proposed cut-off for species delineation. It is worth mentioning that with this set of data, other described species also presented high values of similarity in the MLSA considering four housekeeping genes: *R. changzhiense* WYCCWR 11279^T with *R. sophorae* CCBAU 3386^T (98.2% NI), *R. ruizarguesonis* UPM1133^T with *R. leguminosarum* USDA 2370^T (97.2% NI), and *R. sophorae* CCBAU 3386^T (97.1%) (Jorrin et al. 2020; Zhang et al. 2021). These observations indicate that in these clades, it might be necessary to include more housekeeping genes in the MLSA analysis.

To confirm if the strains from our study represented new species, we followed with the genomic analyses of ANI and dDDH, nowadays considered the gold standard for species descriptions (Chun et al. 2018; Barco et al. 2020; Helene et al. 2022). Goris et al. (2007) indicated that 95–96% ANI corresponds to 70% dDDH, and these values were proposed as a reference for species delineation when analyzing genome relatedness. For phylogenetic and taxonomic purposes, it is not necessary to close the genomes, and the parameters obtained in the genome sequencing of all strains (Table 4) were adequate to evaluate the genomic parameters (Chun et al. 2018; Helene et al. 2022). The comparison of the genomes of the putative new species with the closest described species indicated values that were far below the cut-off established for species delineation, ranging from 81.84 to 92.50% for ANI and 24.0 to 50.7% for dDDH. More specifically, CNPSo 3464^T presented 90.16% ANI and 41.3% dDDH with *R. leucaenae* CFN 299^T, also isolated from *P. vulgaris* in Brazil (Ribeiro et al. 2012). The strain CNPSo 3490^T showed higher genomic similarity with *R. esperanzae* CNPSo 668^T (91.60% ANI and 45.9% dDDH), isolated from *P. vulgaris* in Mexico (Cordeiro et al. 2017). Strains CNPSo 3968^T and CNPSo 4039^T showed 88.07% and 92.50% ANI, respectively, and 35.6% and 50.70% dDDH, respectively, with *R. dioscoreae* S-93^T, an endophytic nitrogen-fixing bacterium isolated from yam (*Dioscorea esculenta* L.) in Japan (Ouyabe et al. 2020). Finally, strain CNPSo 4062^T shared 90.16% ANI and 41.8% dDDH with *R. tropici* CIAT 899^T, isolated from a common bean nodule in Colombia (Martínez-Romero et al. 1991). Therefore, the genomic results confirm that strains CNPSo 3464^T, 3490^T, 3968^T, 4039^T, and 4062^T represent new species.

The phenotypic characterization was performed with the type strains of the new species, and the results agree with those commonly found in *Rhizobium* (Jiao et al. 2015; Zhang et al. 2015; Román-Ponce et al. 2016). However, it is worth mentioning a distinct feature detected in this study: strains CNPSo 3490^T, CNPSo 3982, CNPSo 4005, and CNPSo 4007, belonging to the *R. etli* clade, grew weakly in LB medium, a feature generally found in only a few species of the *R. tropici* clade, such *R. lusitanum* and *R. tropici* (Martínez-Romero et al. 1991; Valverde et al. 2006). It was reported by Ormeño-Orrillo and Martínez-Romero (2013) that the loss and/or gain of plasmids or islands in the genomes can affect phenotypic characteristics, leading to strains differentiation within a species (Kumar et al. 2015).

Regarding the nitrogen fixation ability, five strains (CNPSo 3464^T, 3490^T, 3982, 3993, and 3997) were able to form red or pink nodules in common bean; three strains (CNPSo 3464^T, 3982, and 3993) were able to form red or pink nodules in siratro, and strain CNPSo 4005 formed inefficient nodules in both legumes. In our first study (Moura et al. 2022), we evaluated the nodulation and nitrogen fixation capacity on the host plant (*P. vulgaris*), and the results corroborated with those obtained in this study, where the five cited strains were able to form red or pink nodules, except for CNPSo 4005.

Remigi et al. (2016) emphasize that genes such as *nod*, *nif*, and *fix* are essential and required for nodulation and nitrogen fixation in rhizobia. In the genomes of strains CNPSo 3968^T, 4039^T, and 4062^T, no genes related to nodulation or fixation were found, and none of these strains were able to form nodules in *P. vulgaris*, its original host, or with *M. atropurpureum*. This might indicate loss of nodulation and fixation capacity of these strains, what could also indicate loss of the symbiotic plasmid. Noteworthy, all three strains are positioned in the *R. tropici* clade, until now known by higher stability of the symbiotic plasmid in comparison to species of the *R. etli* clade (Ormeño-Orrillo et al. 2012; Gomes et al. 2015). Strains that have acquired essential symbiosis genes by HGT, or that have these genes in plasmids are in competition with other strains for survival and host colonization, in addition to being subject to various stresses. In addition, metabolic and physiological costs associated with acquired plasmids may lead to loss of symbiosis traits (Remigi et al. 2016). As for strain CNPSo 4007, although not capable of reinfecting the original host *P. vulgaris*, or nodulating *M. atropurpureum*, we were able to find in the genome the *nifH* gene, but it was not possible to locate the *nodC* gene.

Similar reports were made for *R. hidalgonense* FH14^T, which could not reinfect the original host legume, although carrying the *nifH* gene in the genome (Yan et al. 2017). Also, *R. acidisoli,* isolated from the root nodules of *Phaseolus vulgaris* had the *nodC* and *nifH* genes amplified and sequenced,

but the strains were not able to form nodules in the host plant (Román-Ponce et al. 2016). For all these cases, we might suppose that the strains could be nitrogen-fixing endosymbionts, as for *R. dioscoreae* S-93^T isolated from yam in Japan (Ouyabe et al. 2020). In addition to symbiotic rhizobia, nodules can also be colonized by endophytic bacteria that are unable to induce nodule formation (Ibáñez et al. 2017); however, bacterial endophytes perform diverse functions such as promoting plant growth by acquiring nutrients, synthesizing phytohormones and siderophores, phosphate solubilization, and conferring resistance to abiotic and biotic stresses (Papik et al. 2020). It is worth mentioning that *R. hidalgonense*, *R. acidisoli*, and strain CNPSo 4007 belong to the clade *R. etli*, known to have symbiotic plasmids less stable than the species of the *R. tropici* clade (Ormeño-Orrillo et al. 2012; Gomes et al. 2015), although we have now shown that this statement must be reviewed, as CNPSo 3968^T, CNPSo 4039^T, and CNPSo 4062^T lost their nodulation capacity.

Brazil is recognized as a country with remarkable biological diversity, being among the 20 most megadiverse countries, with 70% of the cataloged species of animals and plants in the world (Pylro et al. 2014). However, Brazilian microbial diversity is still poorly known, considering that the number of species described is relatively low, despite several studies confirming high diversity, e.g., with the description of the common bean symbionts *R. freirei*, *R. leucaenae*, and *R. paranaense* (Ribeiro et al. 2012; Dall'Agnol et al. 2013, 2014).

Considering the biogeographic diversity, our study confirmed that Mato Grosso do Sul state is a hotspot of rhizobial diversity, as pointed out in previous studies that showed outstanding high genetic diversity in this region (Costa et al. 2018; Moura et al. 2022).

In conclusion, based on a polyphasic approach comprising phylogenetic, genotypic, and phenotypic analyses, our results indicate that strains CNPSo 3993, 3997, 4005, 4007, and 4057 belong to the species *R. phaseoli*. Additionally, our results allow the description of five new species, comprising *R. cerradonense* (CNPSo 3464^T), *R. atlanticum* (CNPSo 3490^T and 3982), *R. aureum* (CNPSo 3968^T), *R. pantanalense* (CNPSo 4039^T), and *R. centroccidentale* (CNPSo 4062^T, 4063 and 4033).

Description of *Rhizobium cerradonense* CNPSo 3464^T sp. nov.

Rhizobium cerradonense (cer.ra.do nen'se. N.L. neutr. adj. *cerradonense*, pertaining to the Brazilian Cerrado biome).

Cells are Gram-stain-negative, aerobic, and non-spore-forming rods. Colonies on modified YMA medium at pH 6.8–7.0 and Congo red are white, circular, opaque, exhibit moderate mucus production, with gummy consistency, and 3–4 mm in diameter after 3 days of growth at 28 °C. The strain shows a neutral reaction on modified YMA with bromothymol blue and positive urease activity. Strain CNPSo 3464^T shows weak growth at 37 °C, pH 4.0, and modified YMA containing 1% NaCl, and grows well at pH 8.0 after 3 days. The strain is unable to grow on solid LB medium and at 40 °C. Regarding carbon sources in the API test, strain is able to use glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, *N*-acetylglucosamine, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, and D-arabitol; it weakly uses dulcitol, D-sorbitol, methyl α-D-glucopyranoside and xylitol; and is unable to use L-sorbose, methyl α-D-mannopyranoside, amygdalin, inulin, D-melezitose, starch, glycogen, L-arabitol, potassium gluconate, potassium 2-keto-gluconate, and potassium 5-keto-gluconate. The strain is tolerant to bacitracin (10 U), chloramphenicol (30 µg), nalidixic acid (30 µg) and penicillin G (10 U), and sensitive to ampicillin (10 µg), cefuroxime (30 µg), erythromycin (15 µg), neomycin (30 µg), streptomycin (10 µg), and tetracycline (30 µg).

The type strain is CNPSo 3464^T (= BR15055^T), isolated from nodules of *Phaseolus vulgaris* in the Cerrado biome, Mato Grosso do Sul State, central-western Brazil. *Rhizobium cerradonense* CNPSo 3464^T is able to nodulate *P. vulgaris* and *Macroptilium atropurpureum*. The DNA G+C content of strain CNPSo 3464^T is 59.6 mol%.

Description of *Rhizobium atlanticum* CNPSo 3490^T sp. nov.

Rhizobium atlanticum (at.lan'ti.cum. N.L. neut. adj. *atlanticum*, pertaining to the Brazilian Atlantic Forest biome).

Cells are Gram-stain-negative, aerobic, and non-spore-forming rods. Colonies on modified YMA medium at pH 6.8–7.0 and Congo red are red, circular, translucent, exhibit low mucus production, with gummy consistency, and 1–2 mm in diameter after 3 days of growth at 28 °C. The strain shows a neutral reaction on modified YMA with bromothymol blue and positive urease activity. Strain CNPSo 3490^T shows weak growth at pH 4.0, on modified YMA containing 1% NaCl and solid LB medium, and grows well at 37 °C and pH 8.0 after 3 days. The strain is unable to grow at 40 °C. Regarding carbon sources in the API test, strains are able to use glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, *N*-acetylglucosamine, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, xylitol, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose,

D-arabitol, L-arabitol, and potassium 5-keto-gluconate; it weakly uses dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, and D-melezitose; and is unable to use erythritol, L-sorbose, amygdalin, inulin, starch, glycogen, potassium gluconate, and potassium 2-keto-gluconate. The strain is tolerant to bacitracin (10 U) and nalidixic acid (30 μg), moderately sensitive to neomycin (30 μg), and sensitive to ampicillin (10 μg), cefuroxime (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), penicillin G (10 U), streptomycin (10 μg), and tetracycline (30 μg).

The type strain is CNPSo 3490^T (=BR 15056^T), isolated from nodules of *Phaseolus vulgaris* in Atlantic Forest biome, Mato Grosso do Sul State, central-western Brazil. *Rhizobium atlanticum* CNPSo 3490^T is able to nodulate and form effective nodules in *P. vulgaris*, but does not nodulate *Macroptilium atropurpureum*. The DNA G+C content of the type strain is 60.8 mol%.

Description of *Rhizobium aureum* CNPSo 3968^T sp. nov.

Rhizobium aureum (au're.um. L. neut. adj. *aureum*; golden, referring to the Dourados municipality in Mato do Grosso do Sul State, central-western Brazil, city name attributed to a fish called golden).

Cells are Gram-stain-negative, aerobic, and non-spore-forming rods. Colonies on modified YMA medium at pH 6.8–7.0 and Congo red are red, circular, opaque, exhibit moderate mucus production, with gummy consistency, and 2–3 mm in diameter after 3 days of growth at 28 °C. The strain shows a neutral reaction on modified YMA with bromothymol blue and positive urease activity. CNPSo 3968^T grows well at 37 °C, 40 °C, pH 4.0, pH 8.0, on modified YMA containing 1% NaCl and solid LB medium after 3 days. Regarding carbon sources in the API test, strain is able to use glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D- xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, xylitol, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, and D-arabitol; it weakly uses dulcitol and D-sorbitol; and is unable to use L-sorbose, methyl α-D-mannopyranoside, amygdalin, inulin, D-melezitose, starch, glycogen, L-arabitol, potassium gluconate, potassium 2-keto-gluconate, and potassium 5-keto-gluconate. The strain is tolerant to ampicillin (10 μg), bacitracin (10 U), cefuroxime (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), penicillin G (10 U), and tetracycline (30 μg), and moderately sensitive to neomycin (30 μg) and streptomycin (10 μg).

The type strain is CNPSo 3968^T (=BR 15057^T), isolated from nodules of *Phaseolus vulgaris* in the municipality of Dourados, Atlantic Forest biome, Mato Grosso do Sul State, central-western Brazil. *Rhizobium aureum* CNPSo 3968^T lost the capacity to nodulate the original host *P. vulgaris*, is unable to nodulate *Macroptilium atropurpureum*, and does not carry *nodC* or *nifH* genes in its genome. The DNA G+C content of strain CNPSo 3968^T is 59.7 mol%.

Description of *Rhizobium pantanalense* CNPSo 4039^T sp. nov.

Rhizobium pantanalense (pan.ta.nal.en'se. N.L. neut. adj. *pantanalense*, pertaining to the Brazilian Pantanal biome).

Cells are Gram-stain-negative, aerobic, and non-spore-forming rods. Colonies on modified YMA medium at pH 6.8–7.0 and Congo red are red, circular, translucent, exhibit moderate mucus production, with gummy consistency, and 2–3 mm in diameter after 3 days of growth at 28 °C. The strain shows a neutral reaction on modified YMA with bromothymol blue and positive urease activity. CNPSo 4039^T grows well at 37 °C, 40 °C, pH 4.0, pH 8.0, modified YMA containing 1% NaCl and solid LB medium after 3 days. Regarding carbon sources in the API test, strain is able to use glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, xylitol, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, and D-arabitol; it weakly uses L-sorbose and methyl α-D-mannopyranoside; and is unable to use amygdalin, inulin, D-melezitose, starch, glycogen, L-arabitol, potassium gluconate, potassium 2-keto-gluconate, and potassium 5-keto-gluconate. The strain is tolerant to ampicillin (10 μg), bacitracin (10 U), cefuroxime (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), penicillin G (10 U) and tetracycline (30 μg), moderately sensitive to streptomycin (10 μg), and sensitive neomycin (30 μg).

The type strain is CNPSo 4039^T (=BR 15058^T), isolated from nodules of *Phaseolus vulgaris* in the Brazilian Pantanal biome, Mato Grosso do Sul State, central-western Brazil. *Rhizobium pantanalense* CNPSo 4039^T lost the capacity to nodulate the original host *P. vulgaris*, is unable to nodulate *Macroptilium atropurpureum*, and does not carry *nodC* or *nifH* genes in its genome. The DNA G+C content of strain CNPSo 4039^T is 59.6 mol%.

Description of *Rhizobium centroccidentale* CNPSo 4062^T sp. nov.

Rhizobium centroccidentale (centr.oc.ci.den.ta'le. L. neut. n. *centrum*, center; L. masc. adj. *occidentalis*, western; N.L. neut. adj. *centroccidentale*, pertaining to the central-western region of Brazil).

Cells are Gram-stain-negative, aerobic, and non-spore-forming rods. Colonies on modified YMA medium at pH 6.8–7.0 and Congo red are red, circular, opaque, exhibit moderate mucus production, with a gummy consistency, and 2–3 mm in diameter after 3 days of growth at 28 °C. The strain shows a neutral reaction on modified YMA with bromothymol blue and positive urease activity. Strain CNPSo 4062^T grows well at 37 °C, 40 °C, pH 4.0, pH 8.0, on modified YMA containing 1% NaCl and solid LB medium after 3 days. Regarding carbon sources in the API test, strain is able to use glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol; methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, xylitol, gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, and potassium 5-keto-gluconate; is unable to use L-sorbose, amygdalin, inulin, D-melezitose, starch, glycogen, L-arabitol, potassium gluconate, and potassium 2-keto-gluconate. The strain is tolerant to ampicillin (10 μg), bacitracin (10 U), cefuroxime (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), penicillin G (10 U) and tetracycline (30 μg), and moderately sensitive to neomycin (30 μg), and streptomycin (10 μg).

The type strain is CNPSo 4062^T (= BR 15059^T), isolated from nodules of *Phaseolus vulgaris* in Atlantic Forest biome, Mato Grosso do Sul State, central-western Brazil. *Rhizobium centroccidentale* CNPSo 4062^T lost the capacity to nodulate the original host *P. vulgaris*, is unable to nodulate *Macroptilium atropurpureum*, and does not carry *nodC* or *nifH* genes in its genome. The DNA G+C content of strain CNPSo 4062^T is 59.6 mol%.

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Author contributions FTM and MH conceived and designed the experiments. FTM, LCFH, and RAR performed the experiments and analyses. All authors prepared the manuscript and agreed with the final version. MH and MAN were responsible for funding.

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Data availability Data will be made available upon request.

Declarations

Conflict of interest Authors declare that they have no conflict of interests, or ethical conflicts.

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1 **SUPPLEMENTARY MATERIAL**

2

3 **Table S1**

4

5 Primers and amplification conditions used in this study.

6

Gene	Primer	Sequence (5`- 3`)	Size*	PCR cycling	References
gyrB	gyrB343F	TTCGACCAGAAYTCCTAYAAGG	562	5 min 95°C, 5 x (2 min 94°C, 2 min 58°C, 1 min 72°C)	Martens et al. (2008)
	gyrB1043R	AGCTTGTCCTTS GTCTGCG		28 x (30 s 94°C, 1 min 58°C, 1 min 72°C) e 5 min 72°C	
recA	recA6F	CGKCTSGTAGAGGAYAAATCGGTGGA	360	2 min 95°C, 35 x (45s 95°C, 30s 58°C, 1.5 min 72°C)	Gaunt et al. (2001); Stępkowski et al. (2005)
	recA555R	CGRATCTGGTTGATGAAGATCACCAT		e 7 min 72°C	
rpoA	RRrpoAf	GGAAATCGCCATCAAGATGG	371	2 min 95°C, 35 x (45s 94°C, 45s 55°C, 2 min 72° C)	Ribeiro et al. (2009)
	RRrpoAr	ACGCTTGGCGAGATCTTC		e 5 min 72°C	

7 *Size used in this study considering the complete aligned sequences.

8

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10

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31 **Table S2**

32

33 Accession numbers of the genomic and housekeeping genes sequences used in

34 this study. Sequences obtained in this study are shown in bold.

35

Strains	Genome	*glnII*	*gyrB*	*recA*	*rpoA*
***R. cerradonense* CNPSo 3464^T**	JAQJCK000000000	MZ595149.1	OQ123831	OQ095982	-
***R. atlanticum* CNPSo 3490^T**	JAQJCJ000000000	MZ595150.1	-	OQ095983	OQ095995
***R. aureum* CNPSo 3968^T**	JAQJCI000000000	MZ595157.1	-	OQ095984	OQ095996
***R. atlanticum* CNPSo 3982**	-	MZ595165.1	OQ123832	OQ095985	OQ095997
***R. phaseoli* CNPSo 3993**	-	MZ595166.1	OQ123833	OQ095986	OQ095998
***R. phaseoli* CNPSo 3997**	-	MZ595168.1	OQ123834	OQ095987	OQ095999
***R. phaseoli* CNPSo 4005**	-	MZ595170.1	OQ123835	OQ095988	OQ096000
***R. phaseoli* CNPSo 4007**	JAQJCF000000000	MZ595172.1	OQ123836	OQ095989	OQ096001
***R. centroccidentale* CNPSo 4033**	-	MZ595174.1	OQ123837	OQ095990	OQ096002
***R. pantanalense* CNPSo 4039^T**	JAQJCH000000000	MZ595177.1	OQ123838	OQ095991	OQ096003
***R. phaseoli* CNPSo 4057**	-	MZ595178.1	OQ123839	OQ095992	OQ096004
***R. centroccidentale* CNPSo 4062^T**	JAQJCG000000000	MZ595180.1	OQ123840	OQ095993	OQ096005
***R. centroccidentale* CNPSo 4063**	-	MZ595181.1	OQ123841	OQ095994	OQ096006
R. acidisoli FH13^T	-	KJ921080.1	-	KJ921098.1	-
R. aegyptiacum 1010^T	-	-	-	KU664569.1	-
R. aethiopicum HBR26^T	NZ_FMAJ01000062.1	JN580703.1	-	JN580642.1	-
R. anhuiense CCBAU 23252^T	NZ_ML133521.1	KF111913.1	KR183844.1	KF111980.1	-
R. aquaticum SA-276^T	-	KY947544.1	-	KY947545.1	-
R. bangladeshense BLR175^T	NZ_CP071612.1	JN648979.1	-	JN649057.1	-
R. binae BLR195^T	JABDXP000000000.1	-	-	JN649058.1	-
R. calliandrae CCGE524^T	JARFYN000000000	-	KF761514.1	JX855189.1	KF761517.1
R. changzhiense WYCCWR 11279^T	NZ_JABFCQ010000001.1	MT169727.1	-	-	-
R. chutanense C5^T	NZ_NWSV01000010.1	KJ438839.1	-	KJ438844.1	-
R. croatiense 13T^T	NZ_JAILYJ010000001.1	-	-	-	-
R. dioscoreae S-93^T	NZ_BLAJ01000001.1	-	-	-	-
R. ecuadorense CNPSo 671^T	-	JN129306.1	JN129336.1	JN129351.1	JN129366.1
R. esperanzae CNPSo 668^T	NZ_MXPU01000001.1	KC293515.1	KC293521.1	KC293529.1	KC293532.1
R. etli CFN 42^T	NC_007761.1	-	-	-	-
R. freirei PRF 81^T	NZ_AQHN01000095.1	EU488789.1	KJ603458.1	-	EU488836.1
R. hainanense CCBAU 57015^T	NZ_FMAC01000030.1	GU726294.1	HQ438236.1	HM047132.1	KF738132.1
R. hidalgonense FH14^T	NZ_LODW01000001.1	-	KJ921048.1	-	-
R. indicum JKLM 12A2^T	NZ_CP054021.1	-	-	-	-
R. indigoferae CCBAU 71042^T	NZ_JABFCO010000001.1	JN580717.1	-	EF027965.1	-
R. jaguaris CCGE525^T	NZ_CP032694.1	-	-	JX855192.1	-

R. laguerreae FB206^T	NZ_MRDM01000016.1	JN558671.1	-	JN558681.2	-
R. leguminosarum USDA 2370^T	NZ_QBLB01000030.1	AF169586.1	-	AM182125.1	-
R. lentis BLR27^T	NZ_CP071454.1	JN648976.1	-	JN649031.1	-
R. leucaenae CFN 299^T	NZ_LNCJ01000001.1	EU488777.1	KC293524.1	EU488817.1	EU488845.1
R. lusitanum P1-7^T	-	EF639841.1	KC293525.1	DQ431674.1	JF318205.1
R. mayense CCGE526^T	JARFYM000000000	-	-	JX855195.1	-
R. miluonense CCBAU 41251^T	NZ_FMAH01000103.1	HM047120.1	KC293527.1	HM047131.1	JF318206.1
R. multihospitium CCBAU 83401^T	-	EF490040.1	KC293528.1	EF490029.1	JF318207.1
R. paranaense PRF 35^T	-	EU488787.1	KF738135.1	EU488826.1	EU488842.1
R. phaseoli ATCC 14482^T	NZ_ML133565.1	JN580716.1	KC293518.1	EF113136.1	-
R. pisi DSM 30132^T	NZ_RJJT01000010.1	JN580715.1	JQ795183.1	EF113134.1	-
R. redzepovicii 18T^T	NZ_JAILYH010000001.1	-	-	-	-
R. rhizogenes NBRC 13257^T	NZ_BAYX01000053.1	-	-	-	-
R. ruizarguesonis UPM1133^T	NZ_PQIG01000001.1	MG927334.1	-	MG927330.1	-
R. sophorae CCBAU 3386^T	NZ_JABFCN010000001.1	KJ831241.1	-	-	-
R. sophoriradicis CCBAU 3470^T	NZ_RQIH01000010.1	KJ831237.1	-	-	-
R. tropici CIAT 899^T	NC_020059.1	EU488791.1	-	EU488815.1	EU488833.1
R. vallis CCBAU 65647^T	NZ_ML133686.1	GU211771.1	-	GU211770.1	-
B. japonicum USDA 6^T	NC_017249.1	HQ587875.1	-	AM168341.1	-

36

Table S3

Distinctive phenotypic features of *Rhizobium* strains in study and phylogenetically related *Rhizobium* species. Strains: 1, *R. cerradonense* CNPSo 3464^T; 2, *R. leucaenae* CFN 299^T; 3, *R. atlanticum* CNPSo 3490^T; 4, *R. atlanticum* CNPSo 3982; 5, *R. esperanzae* CNPSo 668^T; 6, *R. aureum* CNPSo 3968^T; 7, *R. pantanalense* CNPSo 4039^T; 8, *R. dioscoreae* S-93^T; 9, *R. phaseoli* CNPSo 3993; 10, *R. phaseoli* CNPSo 3997; 11, *R. phaseoli* CNPSo 4005; 12, *R. phaseoli* CNPSo 4007; 13, *R. phaseoli* CNPSo 4057; 14, *R. phaseoli* ATCC 14482^T; 15, *R. centroccidentale* CNPSo 4033; 16, *R. centroccidentale* CNPSo 4062^T; 17, *R. centroccidentale* CNPSo 4063; 18, *R. tropici* CIAT 899^T. Data are evaluated as: +, growth; W, weakly positive; −, no growth; ND, no data.

Characteristic	1	2[*]	3	4	5[#]	6	7	8[&]	9	10	11	12	13	14[#]	15	16	17	18[†*]
Growth at:																		
	w	+	+	+	-	+	+	ND	+	w	+	w	+	-	+	+	+	+
40 °C	-	w	-	w	-	+	+	ND	-	+	w	w	-	-	+	+	+	+
pH 4.0	w	w	w	w	-	+	+	-	w	w	w	w	w	-	+	+	+	+
NaCl 1%	w	-	w	w	-	+	+	ND	w	w	w	w	-	-	+	+	+	+
LB	-	-	-	-	-	+	+	ND	-	-	w	w	-	-	+	+	+	+
Urease activity	+	w	+	+	+	+	+	-	w	+	w	+	w	+	+	+	+	+

Data obtained from [†]Martínez-Romero et al. (1991); *Ribeiro et al. (2012); [#]Cordeiro et al. (2017); [&]Ouyabe et al. (2020)

Reference

Cordeiro AB, Ribeiro RA, Helene LCF et al (2017) *Rhizobium esperanzae* sp. nov., a N_2-fixing root symbiont of *Phaseolus vulgaris* from Mexican soils. International Journal of Systematic and Evolutionary Microbiology 67(10):3937-3945. https://doi.org/10.1099/ijsem.0.002225

Martínez-Romero E, Segovia L, Mercante FM et al (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. International Journal of Systematic and Evolutionary Microbiology 41(3):417-426. https://doi.org/10.1099/00207713-41-3-417

65 Ouyabe M, Tanaka N, Shiwa Y et al (2020) *Rhizobium dioscoreae* sp. nov., a
66 plant growth-promoting bacterium isolated from yam (*Dioscorea* species).
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71 *tropici* type A strains as *Rhizobium leucaenae* sp. nov. International Journal of
72 Systematic and Evolutionary Microbiology 62(Pt_5):1179-1184.
73 https://doi.org/10.1099/ijs.0.032912-0

Table S4

Carbon source utilization and tolerance to antibiotics of *Rhizobium* strains in study and phylogenetically related *Rhizobium* species. Strains: 1, *R. cerradonense* CNPSo 3464^{T}; 2, *R. leucaenae* CFN 299^{T}; 3, *R. atlanticum* CNPSo 3490^{T}; 4, *R. esperanzae* CNPSo 668^{T}; 5, *R. aureum* CNPSo 3968^{T}; 6, *R. pantanalense* CNPSo 4039^{T}; 7, *R. dioscoreae* S-93^{T}; 8, *R. centroccidentale* CNPSo 4062^{T}; 9, *R. tropici* CIAT 899^{T}. Data are evaluated as: +, growth; W, weakly positive; −, no growth; ND, no data.

Characteristic	1	2^{*}	3	$4^{\#}$	5	6	$7^{\&}$	8	$9^{\dagger *}$
Glycerol	+	+	+	+	+	+	-	+	+
Erythritol	+	-	-	-	+	+	+	+	+
D-arabinose	+	+	+	+	+	+	-	+	+
L-arabinose	+	+	+	+	+	+	+	+	+
D-ribose	+	+	+	w	+	+	-	+	+
D-xylose	+	+	+	w	+	+	w	+	+
L-xylose	+	+	+	w	+	+	-	+	+
D-adonitol	+	+	+	w	+	+	-	+	+
Methyl-β-D-xylopyranoside	+	+	+	w	+	+	-	+	+
D-galactose	+	+	+	w	+	+	+	+	+
D-glucose	+	+	+	w	+	+	+	+	+
D-fructose	+	+	+	w	+	+	w	+	+
D-mannose	+	+	+	w	+	+	+	+	+
L-sorbose	-	+	-	-	-	w	-	-	ND
L-rhamnose	+	+	+	w	+	+	w	+	+
Dulcitol	w	-	w	-	w	+	-	+	-
Inositol	+	+	+	w	+	+	w	+	+
D-mannitol	+	+	+	w	+	+	+	+	+
D-sorbitol	w	-	+	w	w	+	-	+	-
Methyl-αD-mannopyranoside	-	ND	w	-	-	w	-	+	ND
Methyl-αD-glucopyranoside	w	+	w	w	+	+	-	+	+
N-AcetylGlucosamine	+	-	+	-	+	+	+	+	-
Amygdalin	-	+	-	-	-	-	-	-	-
Arbutin	+	w	+	-	+	+	-	+	w
Aesculin	+	+	+	+	+	+	+	+	ND
Salicin	+	w	+	-	+	+	-	+	w
D-cellobiose	+	+	+	w	+	+	-	+	+
D-maltose	+	+	+	w	+	+	+	+	+
D-lactose	+	w	+	w	+	+	-	+	w
D-melibiose	+	w	+	w	+	+	-	+	w
D-saccharose (sucrose)	+	+	+	w	+	+	-	+	+

D-trehalose	+	+	+	w	+	+	-	+	+
Inulin	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	w	-	-	-	-	-	-
D-raffinose	+	w	+	w	+	+	-	+	w
AmiDon (starch)	-	ND	-	+	-	-	-	-	-
Glycogen	-	-	-	+	-	-	-	-	-
Xylitol	W	+	+	-	+	+	-	+	-
Gentiobiose	+	w	+	w	+	+	-	+	w
D-turanose	+	w	+	+	+	+	-	+	w
D-lyxose	+	+	+	w	+	+	w	+	+
D-tagatose	+	-	+	-	+	+	-	+	-
D-fucose	+	+	+	+	+	+	+	+	+
L-fucose	+	+	+	+	+	+	+	+	+
D-arabitol	+	+	+	+	+	+	-	+	+
L-arabitol	-	+	+	-	-	-	-	-	w
Potassium gluconate	-	+	-	-	-	-	-	-	ND
Potassium 2-keto-gluconate	-	ND	-	-	-	-	-	-	ND
Potassium 5-keto-gluconate	-	ND	+	-	-	-	-	+	ND
Tolerance (µg per disc):									
Ampicillin (10)	-	-	-	-	+	+	ND	+	+
Bacitracin (10 U)	+	+	+	+	+	+	ND	+	+
Cefuroxime (30)	-	-	-	-	+	+	ND	+	+
Chloramphenicol (30)	+	-	-	w	+	+	ND	+	+
Erythromycin (15)	-	+	-	w	+	+	ND	+	w
Nalidixic Acid (30)	+	+	+	+	+	+	ND	+	+
Neomycin (30)	-	-	w	w	w	-	ND	w	w
Penicillin G (10 U)	+	+	-	+	+	+	ND	+	+
Streptomycin (10)	-	-	-	-	w	w	ND	w	+
Tetracycline (30)	-	+	-	-	+	+	ND	+	-

Data obtained from †Martínez-Romero et al. (1991); *Ribeiro et al. (2012); #Cordeiro et al. (2017); &Ouyabe et al. (2020)

References

Cordeiro AB, Ribeiro RA, Helene LCF et al (2017) *Rhizobium esperanzae* sp. nov., a N_2-fixing root symbiont of *Phaseolus vulgaris* from Mexican soils. International Journal of Systematic and Evolutionary Microbiology 67(10):3937-3945. https://doi.org/10.1099/ijsem.0.002225

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99 Ouyabe M, Tanaka N, Shiwa Y et al (2020) *Rhizobium dioscoreae* sp. nov., a
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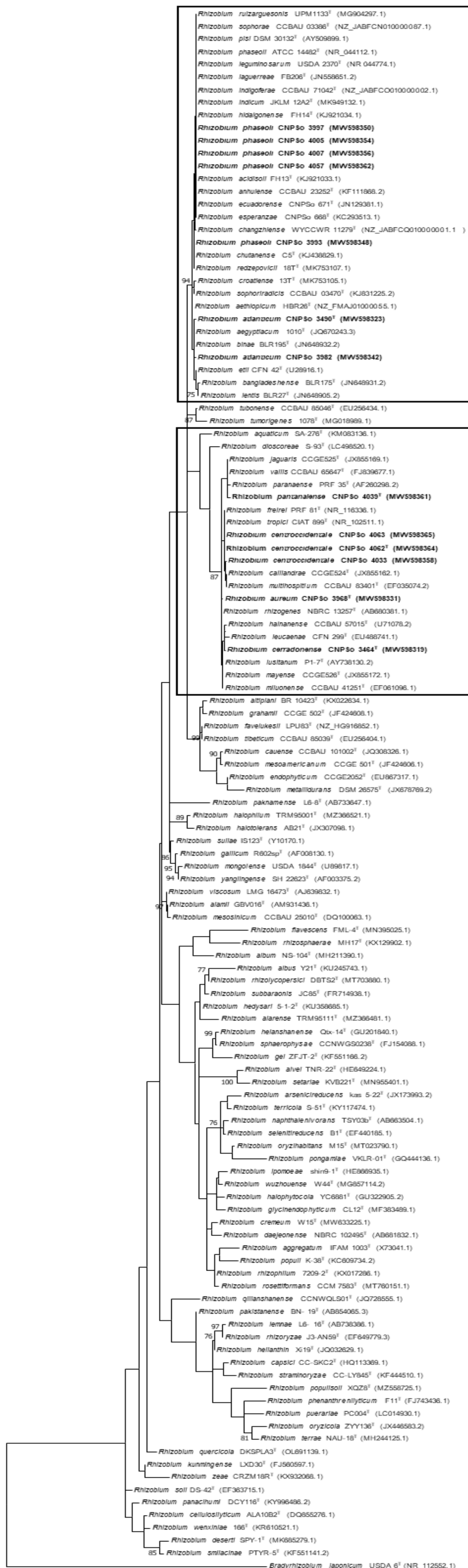
103
104 Ribeiro RA, Rogel MA, López-López A et al (2012) Reclassification of *Rhizobium*
105 *tropici* type A strains as *Rhizobium leucaenae* sp. nov. International Journal of
106 Systematic and Evolutionary Microbiology 62(Pt_5):1179-1184.
107 https://doi.org/10.1099/ijs.0.032912-0

Table S5

Nucleotide Identity (NI) between strains from this study and, *Rhizobium* type strains, based on the sequences of symbiotic genes (*nodC* and *nifH*).

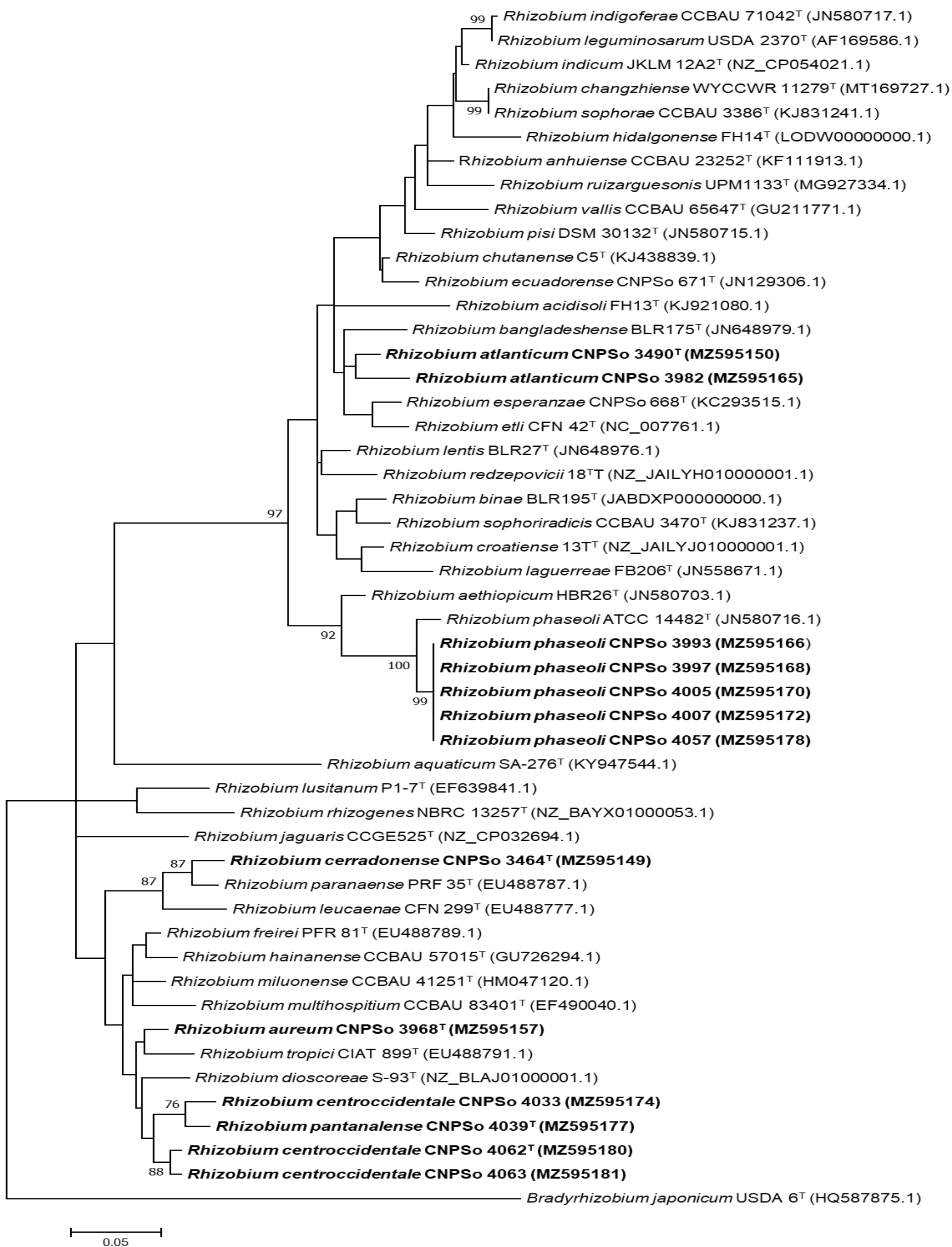
Nucleotide Identity (%)		
R. cerradonense CNPSo 3464^T		
	nodC (363 pb)	*nifH* (181 pb)
R. freirei PRF 81^T	100	100
R. leucaenae CFN 299^T	100	100
R. lusitanum P1-7^T	100	100
R. mesosinicum CCBAU 25010^T	100	-
R. miluonense CCBAU 41251^T	77.6	100
R. multihospitium CCBAU 83401^T	74.1	100
R. tropici CIAT 899^T	100	100
R. atlanticum CNPSo 3490^T		
	nodC	*nifH*
R. aethiopicum HBR26^T	99.1	100
R. chutanense C5^T	100	100
R. croatiense 13T^T	100	100
R. ecuadorense CNPSo 671^T	100	100
R. etli CFN 42^T	97.2	100
R. hidalgonense FH14^T	97.5	100
R. phaseoli ATCC 14482^T	97.5	100
R. phaseoli CNPSo 4007	-	100
R. redzepovicii 18T^T	100	100
R. sophoriradicis CCBAU 3470^T	99.1	100
R. vallis CCBAU 65647^T	100	100
R. phaseoli CNPSo 4007		
	nodC	*nifH*
R. aethiopicum HBR26^T	-	100
R. atlanticum CNPSo 3490^T	-	100
R. chutanense C5^T	-	100
R. croatiense 13T^T	-	100
R. ecuadorense CNPSo 671^T	-	100
R. etli CFN 42^T	-	100
R. hidalgonense FH14^T	-	100
R. phaseoli ATCC 14482^T	-	100
R. redzepovicii 18T^T	-	100
R. sophoriradicis CCBAU 3470^T	-	100
R. vallis CCBAU 65647^T	-	100



Clade
R. etli

Clade
R. tropici

114 **Fig. S1** - Maximum
115 likelihood (ML) phylogeny
116 based on the 16S rRNA
117 gene alignment sequences
118 (930 bp) using Tamura 3-
119 Parameter +G+I model,
120 showing the relationships of
121 the strains in study (in bold)
122 with described *Rhizobium*
123 species. Accession numbers
124 are indicated in
125 parentheses. Bootstrap
126 values >70% are indicated at
127 the nodes. *Bradyrhizobium*
128 *japonicum* USDA 6^T was
129 used as outgroup. Bar
130 indicates five substitutions
131 per 100 nucleotide positions.
132



133

134 **Fig. S2** - Maximum likelihood phylogeny based on the *glnII* housekeeping gene
135 alignment of the genus *Rhizobium* (343 bp), using Tamura Nei +G+I model show
136 the relationships of the strains in study (in bold) with related *Rhizobium* species.
137 Accession numbers are indicated in parentheses. Bootstrap values >70% are
138 indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^T was used as
139 outgroup. Bar indicates five substitutions per 100 nucleotide positions.

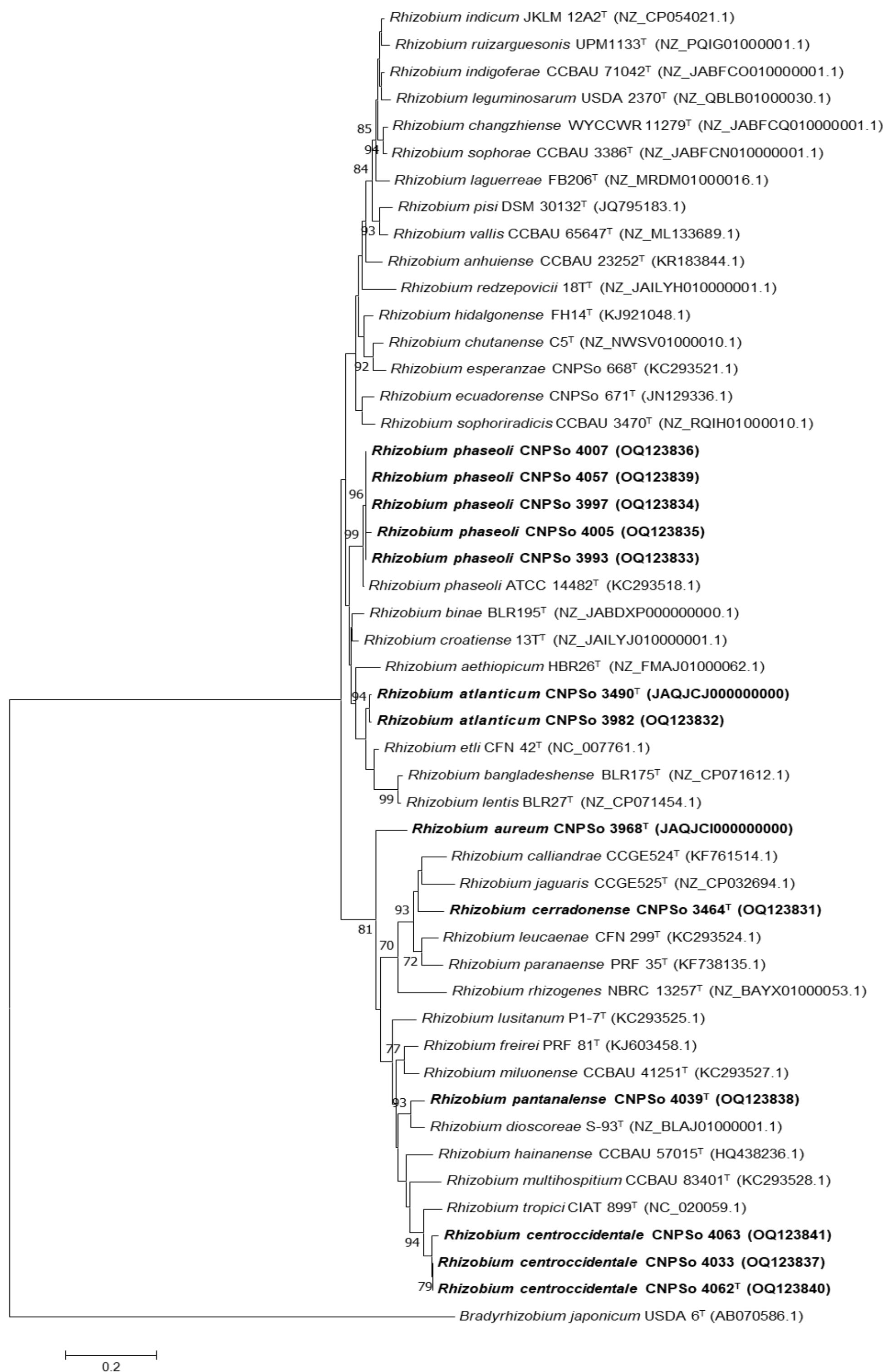
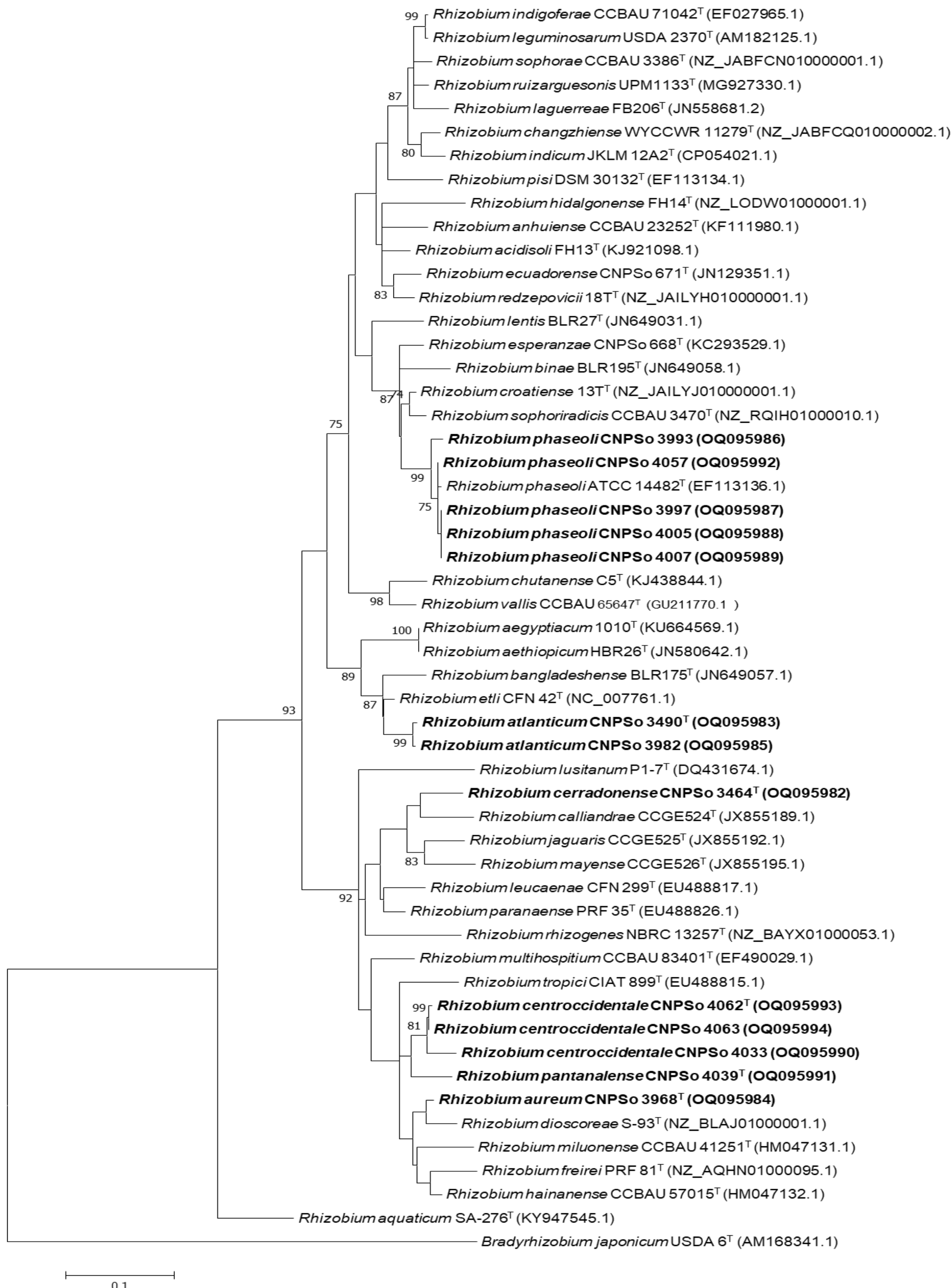
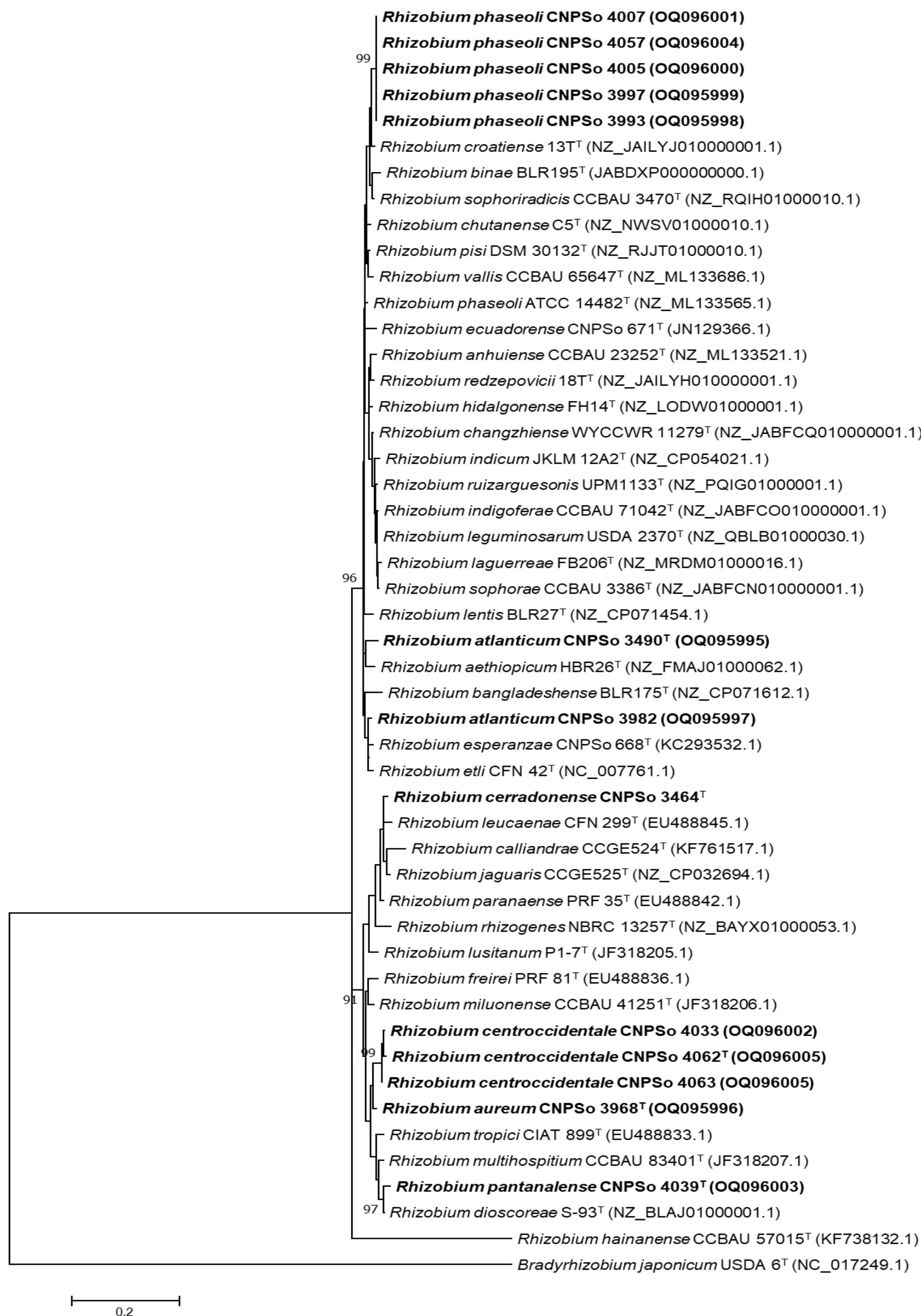


Fig. S3 – Maximum likelihood phylogeny based on the *gyrB* housekeeping gene alignment of the genus *Rhizobium* (471 bp), using Tamura 3-Parameter +G model show the relationships of the strains in study (in bold) with related *Rhizobium* species. Accession numbers are indicated in parentheses. Bootstrap values >70% are indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^{T} was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions.



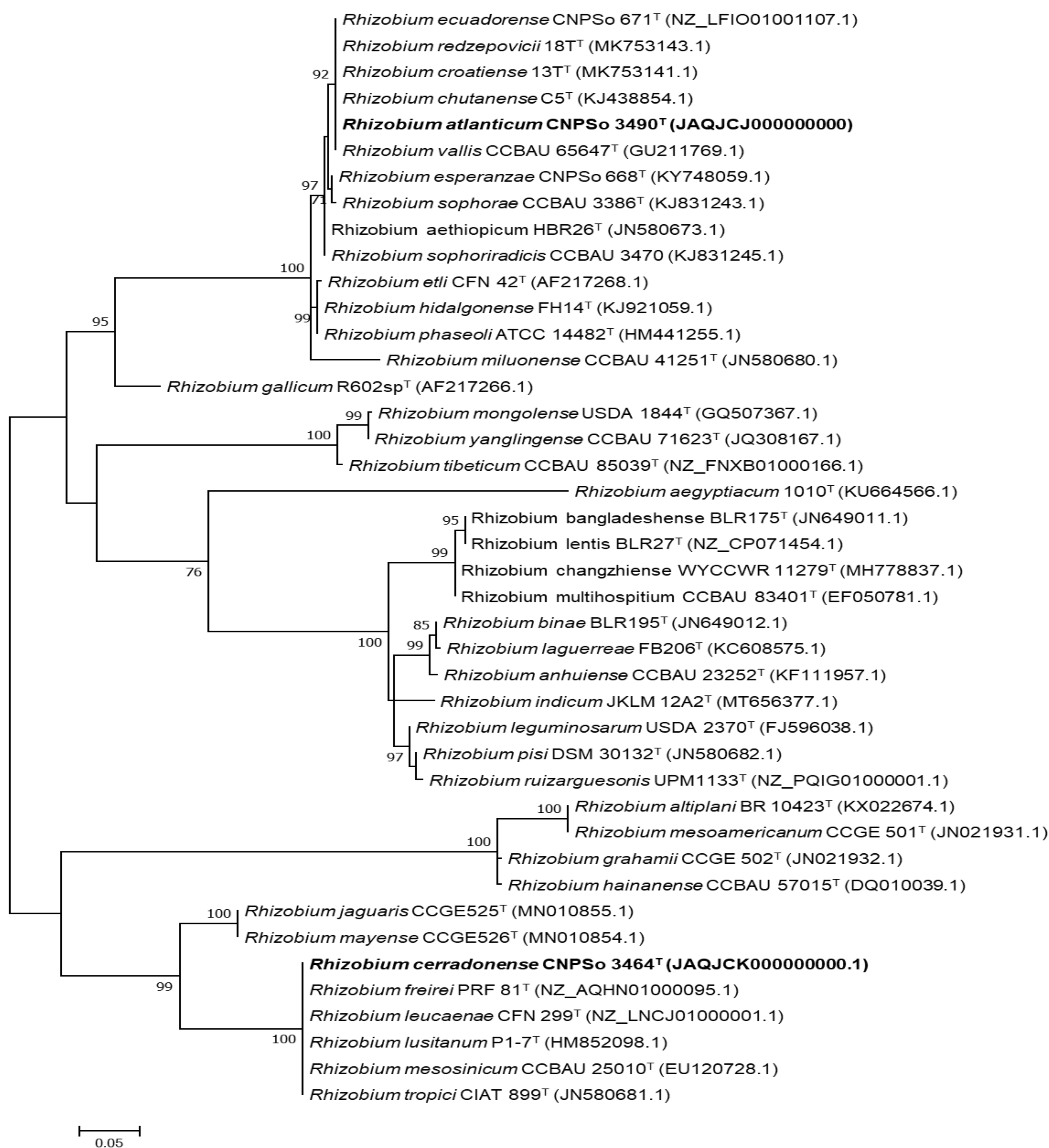
148

149 **Fig. S4** - Maximum likelihood phylogeny based on the *recA* housekeeping gene
150 alignment of the genus *Rhizobium* (382 bp), using Tamura Nei +G+I model show
151 the relationships of the strains in study (in bold) with related *Rhizobium* species.
152 Accession numbers are indicated in parentheses. Bootstrap values >70% are
153 indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^T was used as
154 outgroup. Bar indicates one substitution per 100 nucleotide positions.



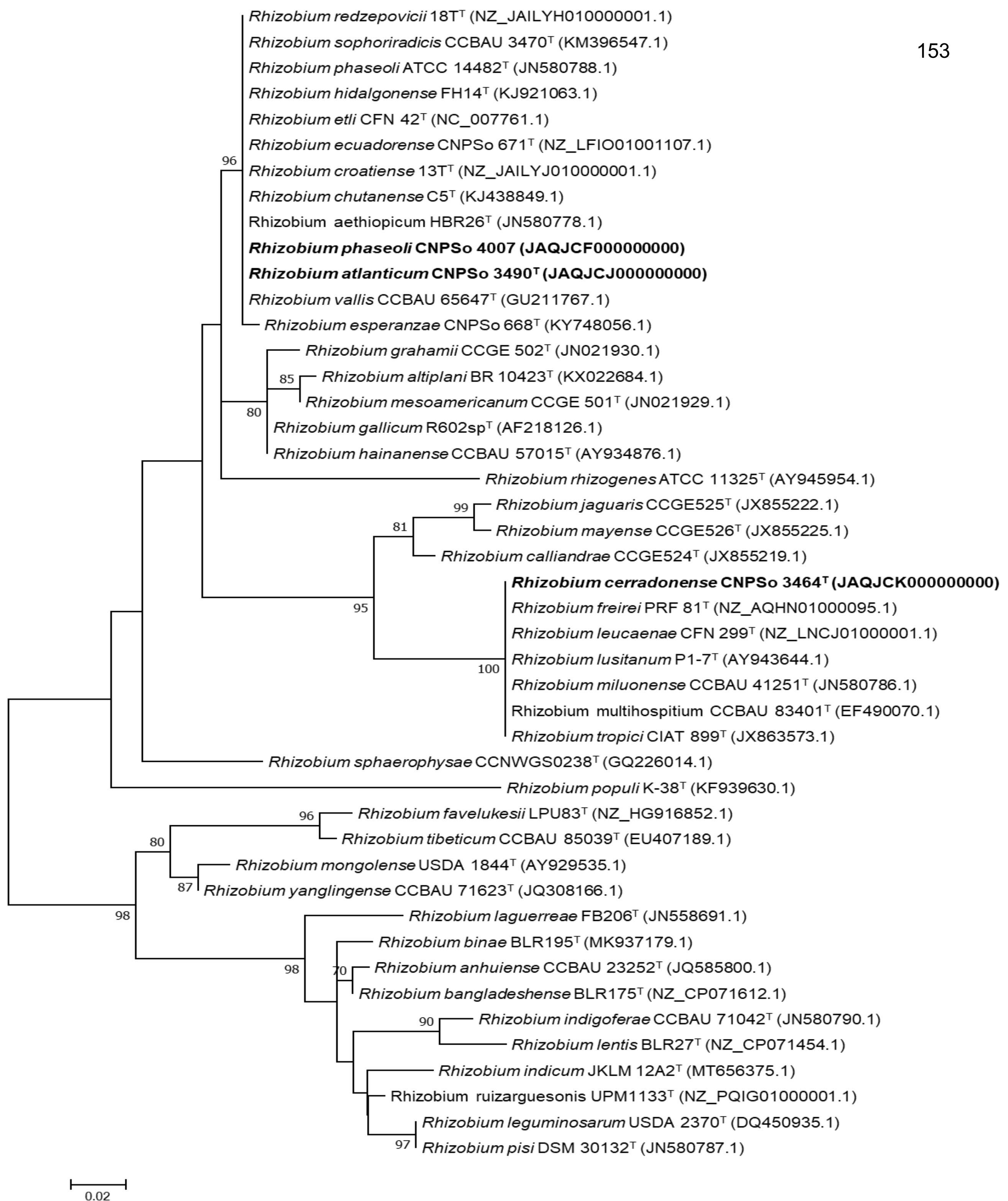
155

156 **Fig. S5** - Maximum likelihood phylogeny based on the *rpoA* housekeeping gene
157 alignment of the genus *Rhizobium* (599 bp), using Tamura Nei +G+I model show
158 the relationships of the strains in study (in bold) with related *Rhizobium* species.
159 Accession numbers are indicated in parentheses. Bootstrap values >70% are
160 indicated at the nodes. *Bradyrhizobium japonicum* USDA 6^{T} was used as
161 outgroup. Bar indicates two substitutions per 100 nucleotide positions.



162

163 **Fig. S6** - Maximum likelihood phylogeny based on *nodC* gene alignment (363
164 bp), using Tamura 3-Parameter +G model. Accession numbers are indicated in
165 parentheses. The strains in study are shown in bold. Bootstrap values >70% are
166 indicated at the nodes. Bar indicates five substitutions per 100 nucleotide
167 positions.



168

169 **Fig. S7** - Maximum likelihood phylogeny based on *nifH* gene alignment (181 bp),
170 using Tamura 3-Parameter +I model. Accession numbers are indicated in
171 parentheses. The strains in study are shown in bold. Bootstrap values >70% are
172 indicated at the nodes. Bar indicates two substitutions per 100 nucleotide
173 positions.

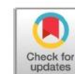
Fig. S8 – (A) *Phaseolus vulgaris*, the original host plant, inoculated with the CNPSo 3464^T strain; (B) Nodules obtained from the root *P. vulgaris* plant inoculated with the CNPSo 3464^T strain with a red or pink color inside, indicating efficient nodules; (C) Nodules obtained from the root *P. vulgaris* plant inoculated with the CNPSo 4005 strain with a white color inside, indicating inefficient nodules.

Genomas de duas estirpes tipo do grupo *Rhizobium tropici*: *R. calliandrae* CCGE524^T e *R. mayense* CCGE526^T

https://doi.org/10.1128/MRA.00472-23

RESUMO

As sequências genômicas de duas estirpes tipo fixadoras de nitrogênio do grupo *Rhizobium tropici* foram obtidas: *Rhizobium calliandrae* CCGE524^T e *R. mayense* CCGE526^T. As análises genômicas confirmaram a posição taxonômica dessas estirpes e identificaram três sequências completas dos genes *repABC*, indicativas de três plasmídeos, sendo um deles portador de genes simbióticos.



| Bacteriology | Announcement

Genomes of two type strains of the *Rhizobium tropici* group: *R. calliandrae* CCGE524^T and *R. mayense* CCGE526^T

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ABSTRACT The genome sequences of two nitrogen-fixing type strains of the *Rhizobium tropici* group were obtained: *Rhizobium calliandrae* CCGE524^T and *R. mayense* CCGE526^T. Genomic analyses confirmed their taxonomic position and identified three complete sequences of the *repABC* genes, indicative of three plasmids, one of them carrying symbiotic genes.

KEYWORDS *Rhizobium tropici*, *Rhizobium calliandrae*, *Rhizobium mayense*, biological nitrogen fixation, nodulation, plasmid, symbiosis

The *Rhizobium tropici* group encompasses species of nitrogen-fixing rhizobia that, in general, are very promiscuous in nodulating several legumes and may contribute with high rates of nitrogen fixation (1–3). *Rhizobium calliandrae* CCGE524^T and *R. mayense* CCGE526^T were isolated from an effective nodule of the medicinal legume *Calliandra grandiflora* in rainforests of Chiapas, Mexico (4). Genetic characterization based on 16S rRNA and housekeeping genes (*atpD*, *recA*, and *rpoB*) positioned the two species into the *R. tropici* clade (4).

Nowadays, the use of genomic parameters is highly encouraged in studies of taxonomy and phylogeny of bacteria, including rhizobia (5). As there were no genomes available for these two type strains, we performed next-generation sequencing to obtain high-quality genomes. The type strains were recovered from cryopreserved stocks at −80°C in modified yeast extract mannitol (YM) culture medium (6) with 30% glycerol (vol/vol) from the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja," in Londrina, Paraná, Brazil, grown in liquid modified YM medium, and incubated at 28°C for 3 days. Total DNAs were extracted with the DNeasy Blood and Tissue kit (Qiagen). Libraries were constructed with Nextera XT kit, and paired-end sequencing was performed on the NextSeq 1000 platform (Illumina) at Embrapa Soja. The reads were assembled with the A5-MiSeq pipeline (*de novo*) v.20140604 (7), with default quality control parameters, trimming sequence adapters, and low-quality regions with Trimmomatic (8), errors were corrected with String Graph Assembler's (SGA's) k-mer-based algorithm (9), and annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), version 6.4 (10). ANI (Average Nucleotide Identity) (11) and digital DNA-DNA hybridization (dDDH) (12, 13) were calculated with the species of the *R. tropici* clade.

The two strains present genomes of similar sizes, estimated at 7,547,379 bp for *R. calliandrae* CCGE524^T and 7,367,519 bp for *R. mayense* CCGE526^T. The genome coverages ranged from 140- to 143-fold, and the G + C contents were also similar for the two strains. These and other genomic parameters are shown in Table 1.

Three complete sequences of the *repABC* genes (plasmid replication genes) were detected in the two strains, that may indicate three plasmids, one of which carrying the nodulation and nitrogen fixation genes. We may suppose that the three plasmids

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TABLE 1 Statistical parameters and genomic comparisons for CCGE524ᵀ and CCGE526ᵀ

Statistical parameters	Data of the strains			
	CCGE524ᵀ		CCGE526ᵀ	
16S accession number	JX855162		JX855172	
Genome accession number	JARFYN000000000		JARFYM000000000	
Raw reads	SRR24766110		SRR24767366	
Genome size (bp)	7,547,379		7,367,519	
G + C content (%)	59.1		59.7	
N_{50} (bp)	97,471		156,426	
Read length (bp)	100		100	
Number of contigs	273		154	
No. of reads in total	11,243,138		10,565,130	
No. of coding sequences	7,364		6,948	
No. of RNAs	56		56	
Coverage (×)	143		140	
Species of the *R. tropici* clade	*R. calliandrae* CCGE524ᵀ		*R. mayense* CCGE526ᵀ	
	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)
R. calliandrae CCGE524ᵀ	100	100	91.24	45.6
R. dioscoreae S-93ᵀ	81.95	24.1	82.29	24.6
R. freirei PRF 81ᵀ	82.43	24.9	82.73	25.4
R. hainanense CCBAU 57015ᵀ	82.16	24.4	82.44	24.9
R. jaguaris CCGE525ᵀ	89.77	40.7	90.67	43.7
R. leucaenae CFN 299ᵀ	85.94	31.8	86.69	33.0
R. lusitanum P1-7ᵀ	82.70	25.5	83.06	26.0
R. mayense CCGE526ᵀ	91.24	45.6	100	100
R. miluonense HAMBI 2971ᵀ	82.15	24.6	82.60	25.2
R. multihospitium HAMBI 2975ᵀ	82.11	24.3	82.51	24.9
R. paranaense SEMIA 4064	86.29	32.5	87.02	33.8
R. rhizogenes NBRC 13257ᵀ	83.85	27.9	84.43	28.8
R. tropici CIAT 899ᵀ	82.47	24.7	82.59	24.9
R. vallis CCBAU 65647ᵀ	80.08	21.8	80.37	22.0

correspond to the same organization reported for *R. jaguaris* CCGE525ᵀ, another close species of the *R. tropici* group that carries a chromid, a symbiotic plasmid, and an additional plasmid (14). In addition, the genomes of *R. jaguaris* and of the two type strains CCGE524ᵀ and CCGE526ᵀ share the same organization of the genes *nodABCD* and *nifABEHNQSTUWXZ*.

The genome comparison of the species of the *R. tropici* clade indicated ANI values of 80.08%–91.24% for *R. calliandrae* CCGE524ᵀ and of 80.37%–91.24% to *R. mayense* CCGE526ᵀ, while the dDDH values ranged from 21.8% to 45.6%, and 22.0% to 45.6%, respectively (Table 1). Therefore, the values of ANI and dDDH are below the species delineation cutoffs established in literature, of 70% and 95%–96%, respectively (5, 15), confirming the taxonomic status of the two strains.

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Fernanda Terezinha Moura, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review and editing | Luisa Caroline Ferraz Helene, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review and editing | Milena Serenato Klepa, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review and editing, Validation | Renan Augusto Ribeiro, Data curation, Methodology, Writing – original draft, Writing – review and editing | Marco Antonio Nogueira, Writing – original draft, Writing – review and editing, Funding acquisition, Project administration.

DATA AVAILABILITY

The whole-genome shotgun project for *Rhizobium calliandrae* CCGE524^T has been deposited at DDBJ/EMBL/GenBank under the GenBank accession number JARFYN000000000 (accession numbers BioProject PRJNA940114, BioSample SAMN33553174). The version described in this paper is JARFYN000000000. The whole-genome shotgun project of *Rhizobium mayense* CCGE526^T has been deposited at DDBJ/EMBL/GenBank under the GenBank accession number JARFYM000000000 (accession number BioProject PRJNA940111, BioSample SAMN33553147). The version described in this paper is JARFYM000000000.

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7. CONSIDERAÇÕES FINAIS

Os biomas brasileiros abrigam uma ampla variedade de formas de vida, incluindo microrganismos como os rizóbios, cuja diversidade ainda permanece pouco explorada. Estudos sobre a diversidade microbiana são fundamentais para entender as interações entre organismos e seus ambientes, além de serem cruciais para o desenvolvimento de estratégias de conservação eficazes e a promoção da sustentabilidade agrícola em um contexto tão diverso e rico como o dos biomas brasileiros.

Os estudos apresentados nesta tese evidenciam não apenas a grande diversidade de rizóbios isolados em diferentes biomas brasileiros, mas também a importância da utilização da abordagem polifásica para a caracterização e descrição de novas espécies. A identificação de novas espécies, como *"Rhizobium atlanticum*", "*R. aureum*", "*R. centroccidentale*", "*R. cerradonense*" e "*R. pantanalense*", reforça a importância do Brasil na biodiversidade bacteriana.

Além disso, os avanços nas ferramentas genômicas e de bioinformática, aliados a estudos em regiões ainda pouco exploradas, como aldeias indígenas e os biomas brasileiros, têm o potencial de revelar novas espécies bacterianas com aplicabilidade para a agricultura. Este trabalho reafirma a necessidade de ampliar os esforços de pesquisa sobre a diversidade de rizóbios, visando maximizar os benefícios ecológicos e econômicos desses microrganismos.